

UNIVERSITY OF BIRMINGHAM



EPIGENETIC BIOMARKERS IN THE PROGRESSION OF BARRETT'S OESOPHAGUS TO OESOPHAGEAL ADENOCARCINOMA

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ABSTRACT

Introduction

Barrett's Oesophagus (BO) represents a benign condition with no life limiting consequences. 0.33% of BO patients progress to oesophageal adenocarcinoma (OADC) which is a potentially catastrophic illness with high associated morbidity and mortality. If patients at high risk of progression to cancer could be identified, they could potentially be treated at an earlier disease stage.

Aims

To assess existing epigenetic biomarkers predicting progression from BO to OADC and validate the novel methylation biomarker OR3A4. Explore the functional relevance of OR3A4 and assess the immunological landscape of BO which progresses to OADC.

Methods

Genome wide methylation analysis and validation pyrosequencing of OR3A4 in BO tissue samples was performed. An OR3A4 over-expressing vector was transfected into BO and OADC cell lines and cell functional assays, total RNA sequencing and real time PCR was performed. Multispectral immunohistochemistry was performed to investigate the immunological landscape of BO.

Results

OR3A4 is hypomethylated in patients that progress from BO to OADC and OR3A4 over-expression has a functional effect in BO in vitro models and may contribute to immunological changes in BO tissues.

Conclusion

Hypomethylation of OR3A4 may provide a mechanism to explain the progression of BO to OADC and predict progression of disease.

DEDICATION

This thesis is dedicated to Tilly.

You remind me every day of what is most important in life.

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ABBREVIATIONS

AP-1	Activator protein 1
APC	Argon plasma coagulation
AUOX	Area under receiver operating characteristic curve
BMI	Body mass index
BO	Barrett's Oesophagus
BSG	British Society of Gastroenterology
C value	Circumferential extent
CCK2R	Cholecystokinin receptors
CD4	Cluster of differentiation 4
CI	Confidence interval
CLE	Confocal laser endomicroscopy
CT	Computed tomography
Ct	Threshold cycle values
DAVID	Database for annotation, visualisation and integrated discovery
DCt	Delta Threshold cycle values
DDCt	Delta delta Ct
DNMT	DNA methyltransferase
DUSP	Dual specificity phosphatase
ECX	Epirubicin, cisplatin and capecitabine
EGFR	Epidermal growth factor receptors
EMR	Endoscopic mucosal resection
EMT	Epithelial-mesenchymal transition

ENDCaP-C	Enhanced Neoplasia Detection and Cancer Prevention in Chronic Colitis
EUS	Endoscopic ultrasound
FBS	Foetal Bovine Serum
FFPE	Formalin Fixed, Paraffin Embedded
FOXP3	Forkhead box P3
GFP	Green fluorescent protein reporter
GI	Gastro-intestinal
GOJ	Gastro-oesophageal junction
GPX7	Glutathione peroxidase 7
GWAS	Genome wide association study
H+	Hydrochloric acid
HBRC	Human Biomaterials Resource Centre
HER2	Human epidermal growth factor 2
HLA	Human leucocyte antigen
HOTAIR	HOX antisense intergenic RNA
HPV	Human papilloma virus
HR	Hazards ratio
HRE	High resolution endoscopy
HULC	Highly upregulated in liver cancer
IAP	Inhibitor of apoptosis protein
IF	Immunofluorescence
IM	Intestinal metaplasia
IQR	Interquartile Range

ITS	Insulin-Transferrin-Selenium
IWGCO	International Working Group for the Classification of Oesophagitis
JCGA	Japanese Gastric Cancer Association
JNK	c-Jun N-terminal Kinases
KEGG	Kyoto encyclopaedia of genes and genomes
LDA	Linear discriminant analysis
lncRNA	Long non-coding RNAs
LOOCV	Leave-one-out crossvalidation
M value	Maximal extent
MALAT2	Metastasis associated lung adenocarcinoma transcript 1
MAP2K3	MKK3, mitogen activated protein kinase kinase
MAPK	Mitogen activated protein kinase
MDT	Multidisciplinary team
MEG3	Maternally expressed gene 3
MHC	Major histocompatibility
MHC	Major histocompatibility
MIO	Minimally invasive oesophagectomy
miRNA	Micro RNA
MLE	Maximum likelihood estimates
mRNA	Messenger RNA
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NAC	Neo-adjuvant chemotherapy
nCRT	Neoadjuvant chemo-radiotherapy

NDBO	Non-dysplastic Barrett's Oesophagus
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells pathway
NIBR	Northern Ireland Barrett's Oesophagus Register
NMV	Normalised methylation value
NNT	Number needed to treat
NP	Non-progressive lesion
OADC	Oesophageal adenocarcinoma
OC	Oesophageal cancer
OGD	Oesophago-gastro-duodenoscopy
OR	Odds ratio
OR3A4	Pseudogene for the olfactory receptor (OR) molecule number 4 in OR subfamily 3A
OSCC	oesophageal squamous cell carcinoma
P	Progressor lesion
P:NP	Progressor:Non-progressor patient numbers
PDT	Photodynamic therapy
PET	Positron emission tomography
PG	Prostaglandins
PGE2	Prostaglandin E2
PKR	Protein kinase R
PMS	Phenazine methosulfate
PRC2	Polycomb repressive complex 2
PRKRIP1	PRKR Interacting Protein 1

PTEN	Phosphatase and tensin homolog
PTENP1	Phosphatase and tensin homolog pseudogene 1
QC	Quality control
RFA	Radiofrequency ablation
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
RTqPCR	Real time quantitative polymerase chain reaction
SCJ	Squamo-columnar junction
shRNA	Short hairpin RNA
SMAC	Second mitochondria-derived activator of caspases
SNVs	Single nucleotide variants
SWB	Sandwell and West Birmingham NHS Trust
TCR	T cell receptor
TNF	Tumour necrosis factor
TRIAP1	TP53-Regulated inhibitor of apoptosis 1
TSA	Tyramide Signal Amplification
UC	Ulcerative colitis
UHB	University Hospital Birmingham NHS Trust
UHS	University Hospital Southampton NHS Foundation Trust
UKFSST	UK Flexible Sigmoidoscopy Screening Trial
XIST	X-inactive-specific transcript
Z-Line	Squamo-columnar junction

1. INTRODUCTION

1.1 BARRETT'S OESOPHAGUS

Barrett's oesophagus is defined by the British Society of Gastroenterology (BSG) as "an oesophagus in which any portion of the normal distal squamous epithelium is replaced by metaplastic columnar epithelium which is clearly visible endoscopically more than one centimetre above the gastro-oesophageal junction and confirmed histopathologically from oesophageal biopsies"(1). Barrett's oesophagus is an eponymous syndrome named after Norman Barrett (1903-1979), a British-Australian thoracic surgeon. Responding to increasing confusion in the diagnosis of oesophagitis and chronic oesophageal ulceration, in his 1950 paper "Chronic peptic ulcer of the oesophagus and 'oesophagitis'"(2) Barrett notes that chronic ulceration of the oesophagus resembles that of chronic gastric ulcers and that the surrounding mucosa in the oesophagus closely resembles that of the stomach. From this evidence he arrives at two striking conclusions. Firstly, that the clinical and pathological definition of the oesophagus are at odds with one another, remarking that the clinical definition of the oesophagus should be "that part of the foregut, distal to the cricopharyngeal sphincter which is lined by squamous epithelium", thus disregarding classical anatomic landmarks which previously defined the oesophagus. Secondly, he concludes that peptic ulceration of the oesophagus is due to cases of "congenital short oesophagus" whereby part of the stomach extends upwards into the mediastinum. He reasoned that oesophageal strictures formed due to the secretion of gastric juices

into the oesophagus by ectopic islets of gastric mucosa or by “congenital short oesophagus” were a protective measure to stop further reflux of gastric juices into the squamous lined oesophagus. It is also noted that the squamo-columnar junction in these patients is often not a straight line and finger like projections of gastric mucosa can extend into the oesophagus. It may appear ironic that the condition is named after Barrett, as he initially did not interpret the pathological mechanism correctly. However, he recognised three important features of the disease – the columnar lined oesophagus, association with hiatal herniation and the association with symptoms of gastro-oesophageal reflux. In 1957 Barrett published “the lower oesophagus lined by columnar epithelium”(3) which concludes that columnar lined oesophagus differs from a sliding hiatus hernia and that metaplastic change was responsible.

There is controversy regarding the development of BO from normal squamous epithelium. The two prevailing theories are the trans-differentiation of mature cells and the stem cell origin of BO (4). The stem cell origin theory suggests that pluripotent cells from either bone marrow origin or within the oesophagus itself may develop to form columnar epithelium in islands in the presence of environmental factors such as reflux of gastrointestinal secretions, alcohol or cigarette smoking. These may have a specific metaplastic phenotype which self-perpetuates and forms BO. In the trans-differentiation theory, squamous cells with an established phenotype undergo change to columnar epithelium. Whilst there is evidence in murine gastric models to support the stem cell theory, there is no such evidence in BO at this time.

1.2 DIAGNOSIS OF BARRETT'S OESOPHAGUS

Diagnosis of Barrett's oesophagus in the modern era must meet the strict criteria outlined by the BSG and adhere to the minimum dataset(1). It requires examination by oesophago-gastro-duodenoscopy (OGD) using a flexible endoscope. Whilst macroscopic findings at endoscopy may be highly suggestive of a diagnosis of Barrett's, the diagnosis must be confirmed histologically with a tissue biopsy.

Table 1 BSG Minimum Endoscopic Dataset

Finding	Reporting System	Nomenclature
Barrett's oesophagus length	Prague Classification	C & M (see below)
Barrett's islands	Describe distance from the incisors and length in cm	Descriptive
Hiatus hernia	Distance between diaphragmatic pinch and GOJ	Yes/no – cm
Visible lesions	Number and distance from incisors	Yes/no – cm
Classification of visible lesions	Paris classification	0-Ip – 0-III (See below)
Biopsies	Number and location of samples taken	X cm, n cm.

1.3 PRAGUE C&M CRITERIA

The Prague C&M criteria(5) allows reliable estimation of the length of Barrett's segments. It was developed from a subgroup of the International Working Group for the Classification of Oesophagitis (IWGCO) and presented at the United European Gastroenterology Week in Prague 2004. 50 videos of endoscopic examination were disseminated to 29 independent endoscopy experts with a specialist interest in Barrett's oesophagus. They were asked to note the depths of standardised landmarks such as the diaphragmatic hiatus, the proximal margin of the gastric fold and a circumferential pinch point near the proximal end of the gastric folds. They also judged the length of any Barrett's segment which was present by giving circumferential extent (C value) and the maximal extent (M value) in centimetres. Reliability coefficients for the diaphragmatic hiatus, proximal margin of the gastric fold and the pinch at the distal oesophagus were 0.85, 0.88 and 0.78 respectively. The C and M values had reliability coefficients of 0.94 and 0.93 respectively demonstrating a rapid and reproducible method of estimating the extend of Barrett's oesophagus.

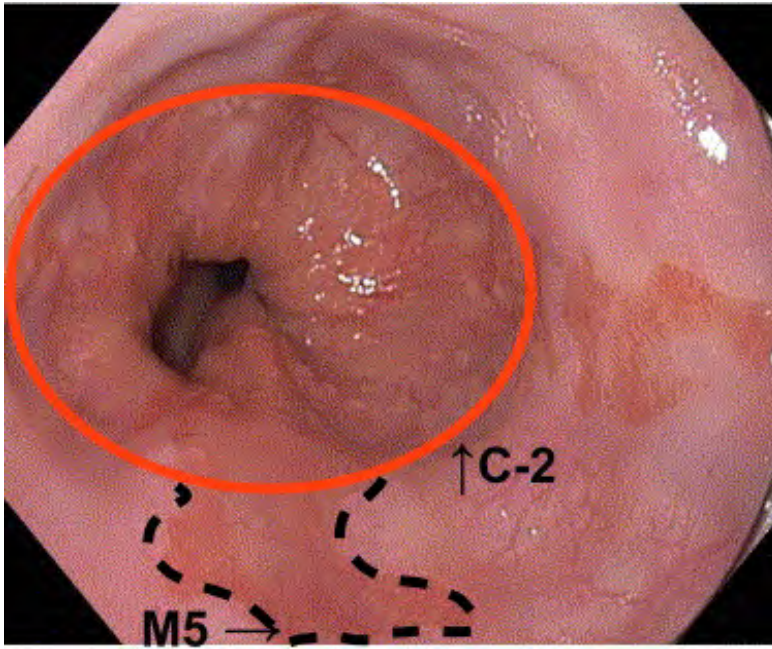


Figure 1 - Endoscopic image showing Barrett's Oesophagus. The maximum circumferential extent of Barrett's mucosa is denoted by "C2" circled in red, 2 cm above the GOJ. The maximum total extent of Barrett's mucosa is denoted by the black dashed line "M5", indicating that a tongue of metaplasia extends 5 cm above the GOJ. Image adapted from: p Sharma et al 2006 (5)

1.4 PARIS CLASSIFICATION

The Paris classification(6) was developed in 2002 to classify and categorise superficial or small neoplastic lesions of the GI tract. This is particularly relevant in the Barrett's oesophagus and oesophageal adenocarcinoma as it progresses along a spectrum of disease. It is important to classify early metaplastic and dysplastic lesions as this information is currently the only means of quantifying the risk of progression to oesophageal adenocarcinoma for patients with Barrett's oesophagus. Submucosal adenocarcinomas must also be classified and accurately differentiated. With improvements in endoscopic therapy these may be treated without the need for resectional surgery, however with increasing

depth of invasion comes increasing likelihood of lymph node and systemic metastases and the requirement for oesophagectomy.

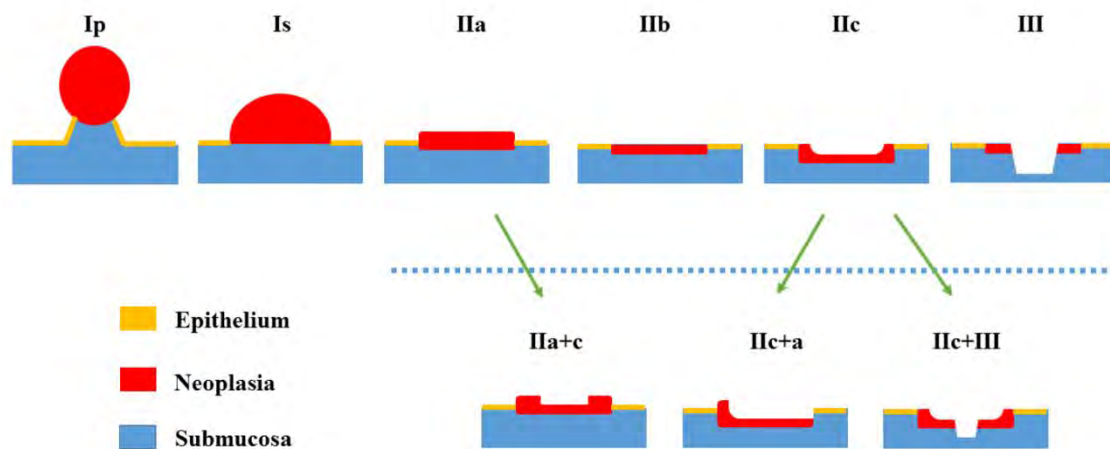


Figure 2 - The Paris Classification of polypoid lesions of the gastrointestinal tract. Image taken from <http://www.endoscopy-campus.com> (7). The classification's nomenclature is presented in

Table 2. Lesions are not always of a single type, and mixed forms are shown in addition. Precise description of GI tract lesions are important for therapeutic planning. Superficial and polypoid lesions are often amenable to endoscopic therapy rather than surgical resection.

Table 2 Paris Classification

Classification	Morphology
0-Ip	Protruded, pedunculated
0-Is	Protruded, sessile
0-IIa	Superficial, elevated
0-IIb	Flat
0-IIc	Superficial shallow, depressed
0-III	Excavated

The Paris classification takes into account the Japanese Gastric Cancer Association's (JCGA) classification of gastric carcinoma (8) which describes the degree of invasion of gastric cancers.

The exact diagnosis of Barrett's oesophagus has historically been debated due to difficulty defining the normal junction between squamous and columnar epithelium at the distal oesophagus (squamo-columnar junction (SCJ) or Z-line) and the exact point of the gastro-oesophageal junction (GOJ). Hayward concluded that it is anatomically normal for up to 2cm of the lower oesophagus to be lined with columnar epithelium, leading to early studies on progression of Barrett's oesophagus to OADC to discount patients with less than 5cm of columnar lined oesophagus. Additional factors such as hiatus hernia or inflammation can lead to difficulty accurately defining the GOJ and the Z-line respectively leading to inaccurate measurement of segment length.

The most up to date definition of the GOJ is the point at which the palisade vessels of the oesophageal mucosa penetrate into the gastric submucosa, visible as a distal end or apparent termination of the vessels on endoscopy. Alternatively, the proximal end of the gastric folds may be used as a landmark.

The Barrett's segment length is defined as the distance of the Z-line (squamo-columnar junction) from the GOJ on OGD. An irregular Z-line has tongues of columnar epithelium 1cm or less in length which is not circumferential. Whilst this is associated with GORD, it cannot be called Barrett's oesophagus.

Table 3 Classification of macroscopic gastric tumour types

Macroscopic Type	Degree of invasion	Description
Type 0	Superficial	Not invading the muscularis propria
Type 1	Mass	Polypoid tumour sharply demarcated from surrounding mucosa
Type 2	Ulcerative	Ulcerated tumours with raised margins surrounded by a thickened gastric wall with clear margins
Type 3	Infiltrative ulcerative	Ulcerated tumours with raised margins surrounded by a thickened gastric wall without clear margins
Type 4	Diffuse infiltrative	Tumours without marked ulceration or raised margins, the gastric wall is thickened and indurated and the margin is unclear.
Type 5	Unclassifiable	Tumours which cannot be classified into the above categories

The segment length is of clinical importance. The Rotterdam Esophageal Tumour Study Group (9) revealed that doubling the length of Barrett's segment increases the chance of developing OADC by 1.7 times.

Obviously, it is impossible to histologically examine the entire oesophagus, or even the entirety of a Barrett's segment in a patient unless they have undergone

surgical resection of the oesophagus. The only current marker of progression from NDBO to OADC is the development of dysplasia within the Barrett's segment diagnosed on an oesophageal biopsy. It is impossible to capture every potential island of dysplastic Barrett's, however a systematic and reproducible biopsy protocol may aid in minimising the risk of missing both the initial diagnosis of Barrett's and potential dysplasia. The Seattle Protocol(10) provides a rigorous, systematic and safe method of reducing sampling error when taking biopsies of Barrett's oesophagus segments. Using endoscopic biopsy forceps, it describes sampling by quadrantic biopsy of the Barrett's segment at 2 cm intervals from the GOJ to the SCJ. If high grade dysplasia was previously diagnosed, this is reduced to 1cm. Any distinct lesions within the Barrett's segment can be biopsied up to 10 times. The safety of this protocol was validated on a cohort of 705 patients who underwent 1458 OGDs over a 14 year period, with over 50,000 biopsies taken(10). No deaths, perforations, strictures, aspiration or cases of gastrointestinal bleeding were directly attributable to endoscopic biopsy in this cohort. They observed a 1.6% overall complication rate. A US study of 211,410 diagnostic OGDs(11) reported an overall complication rate of 0.13%. The increased complication rate of the Seattle protocol was attributed to increased procedure time, increased dose of sedative medication and an increased number of biopsies. A diagnostic OGD with minimal biopsies does not usually require sedation, just local anaesthetic spray to numb the oropharynx, however with an extended and systematic biopsy protocol, patients were administered sedation to make the procedure more tolerable. Five significant events requiring hospital admission were recorded: 2 episodes of upper GI bleeding following

simultaneous dilatation of oesophageal stricture and biopsy, 2 cases of cardiac dysrhythmia and one respiratory arrest.

1.5 HISTOLOGICAL EXAMINATION

Histological examination requires an experienced GI pathologist. The BSG recommend that the diagnosis of dysplasia in BO be made by 2 specialist GI pathologists, as there is inter operator variability when reporting and calling the degree of dysplasia(12, 13). In Curvers et al's study (13), 147 patients in 6 Danish hospitals that had been diagnosed with LGD in BO had their cases re-examined by a panel of expert GI pathologists. 85% of those patients were downstaged to NDBO or indeterminate for dysplasia after specialist panel review. Ensuring the correct initial diagnosis is paramount as those patients whose diagnosis was confirmed as LGD had an 85% cumulative risk of progressing to HGC or OADC in 9 years, compared to 4.6% in those that were downstaged. There is also argument between UK and US pathologists about the importance of specialised intestinal metaplasia (IM) within histological specimens and whether this must be present to make the diagnosis of Barrett's oesophagus. The BSG argue that IM is not a mandatory requirement in the diagnosis of Barrett's oesophagus due to sampling error which can miss these features. Harrison et al(14) concluded that a minimum of 8 random biopsies would be required to diagnose benign IM. The American College of Gastroenterology guidelines(15) consider intestinal metaplasia (IM) to be a mandatory histological finding to diagnose Barrett's oesophagus. Evidence has suggested that IM has the highest neoplastic potential, Bhat et al reported in their 2011 study (16) that the incidence of

oesophageal adenocarcinoma in patients with IM was 0.38% per year compared to 0.07% per year in patients without. This represents a hazard ratio of 3.54 (CI 2.09 – 6.00 $p < 0.001$). In light of this study the BSG conclude that the presence or absence of IM should guide clinicians regarding their surveillance frequency, increasing it if IM is present.

There is no clinical test other than OGD and biopsy which can reliably detect the presence of Barrett's mucosa. There is no definitive way to predict which patients will progress from non-dysplastic BO to OADC. Due to limitations in current diagnostic methods it is impossible to definitively identify a high risk phenotype before dysplasia has developed, and it can only be inferred by studies with long follow up that not all patients with BO will progress to dysplasia or OADC(16-19).

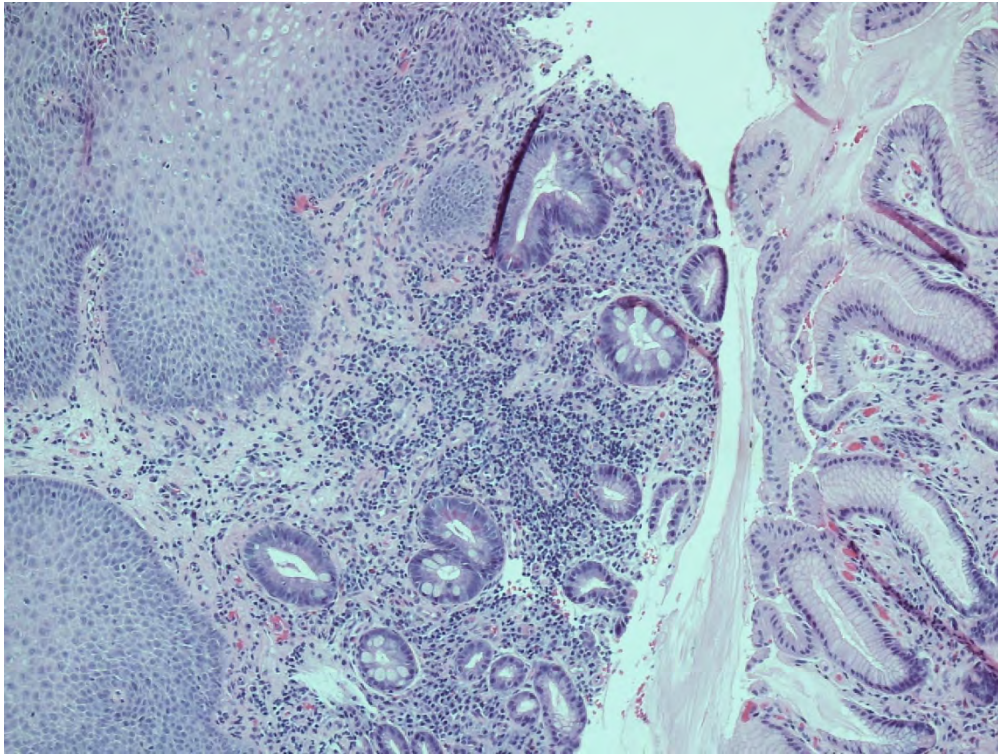


Figure 3 – Low power view with light microscopy of an oesophageal biopsy. Normal oesophageal squamous epithelium is visible on the left of the image. BO mucosa is visible on the right of the image, with typical columnar epithelium and IM (clear, goblet cells). The central portion of BO demonstrates an inflammatory lymphocytic infiltrate. Pictures of BO and OADC courtesy of Dr Rahul Hejmadi.

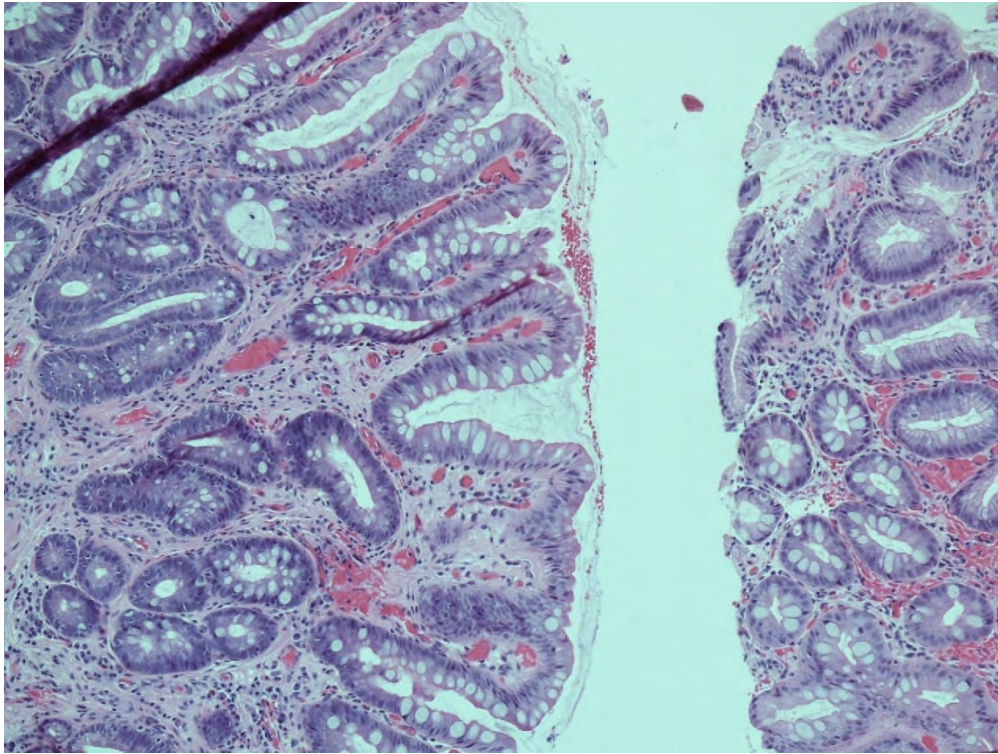


Figure 4 – High power view with light microscopy of an oesophageal biopsy showing BO with IM.

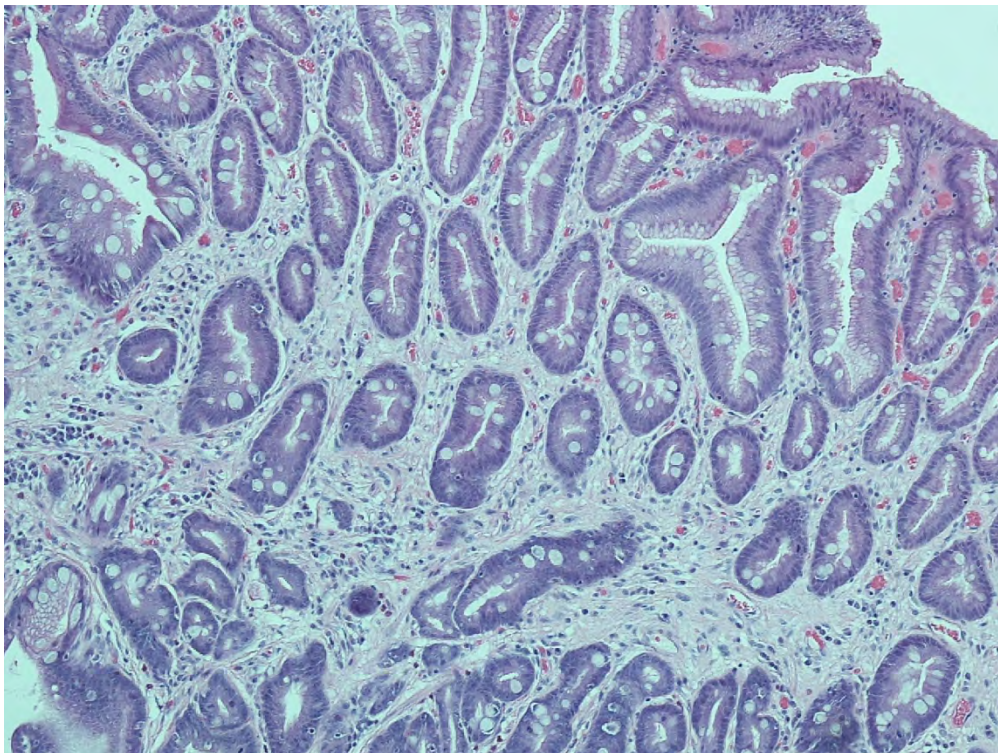


Figure 5 – High power view with light microscopy of an oesophageal biopsy showing islands of columnar epithelium with diffuse IM, diagnostic of BO.

1.6 EPIDEMIOLOGY

The incidence of BO in the UK is 27.7 new cases per 100,000 patient years and 31.9 new cases per 1000 OGDs(19). The true incidence of BO is unclear as the lack of concerning patient symptoms associated with NDBO result in many cases not being investigated with OGD. The incidence of BO in clinically indicated OGD is 26.1 per 100,000 for males and 8.1 per 100,000 for females(20) in the USA. A recent population study has shown that only 7.3% of OADC cases had a prior diagnosis of BO (21). With updated surveillance guidelines and awareness, this number is likely to rise. An important observation of this study was that diagnosis of BO prior to the diagnosis of OADC was associated with a significantly better survival.

1.7 PROGRESSION FROM NDBO TO OADC

From the early 1970's the connection between GORD and the development of columnar lined oesophagus was confirmed and it became increasingly clear that OADC was associated with Barrett's oesophagus. Resectional specimens demonstrated specialised intestinal metaplasia with dysplastic change surrounding the tumours. Like other tumours of inflammatory origin such as colonic adenocarcinoma arising in ulcerative colitis, OADC progresses along the metaplasia, dysplasia, adenocarcinoma pathway. Barrett's oesophagus is a precursor lesion to OADC.

1.7.1 RATE OF PROGRESSION FROM NDBO TO OADC

The rate of progression from NDBO to OADC has been reported by Masclee et al (19) as 0.3% from a UK wide primary care database analysis. 12,312 incident cases of BO were identified from the database and 40 of these subsequently developed OADC (63% male, 37% female). They also analysed a similar database from The Netherlands. 1383 incident BO cases were identified and 5 subsequently developed OADC (0.4% incidence). In Desai et al's meta-analysis of 57 studies, comprising 11,434 patients and 58,547 patient year follow up, the pooled annual incidence of OADC in patients with NDBO was found to be 0.33% (95% CI 0.28-0.38%) (18). In a Danish population wide study by Hvid-Jensen et al (17), 11,028 cases of BO were identified (66.8% male, 33.2% female). 66 patients went on to develop OADC after the first year giving an incidence rate of 1.2 cases per 1000 person years (95% CI 0.9-1.5). 7.6% of incident OADC diagnoses in the Danish population studied had a preceding BO diagnosis. When compared to the incidence rate of OADC in the general population the relative risk of developing OADC with a prior diagnosis of BO was 11.3 (95% CI 8.8-14.4).

The annual risk of developing OADC with pre-existing BO was 0.12% (95% CI, 0.09 to 0.15), or 1 case of adenocarcinoma per 860 patient-years. In Bhat et al's 2011 study of 8522 patients on the Northern Ireland Barrett's Oesophagus Register (NIBR) (16) with a mean follow up of 7 years (58.2% male, 41.8% female, mean age 60.1 years) revealed a 0.22% per year incidence of OADC or HGD in patients with BO.

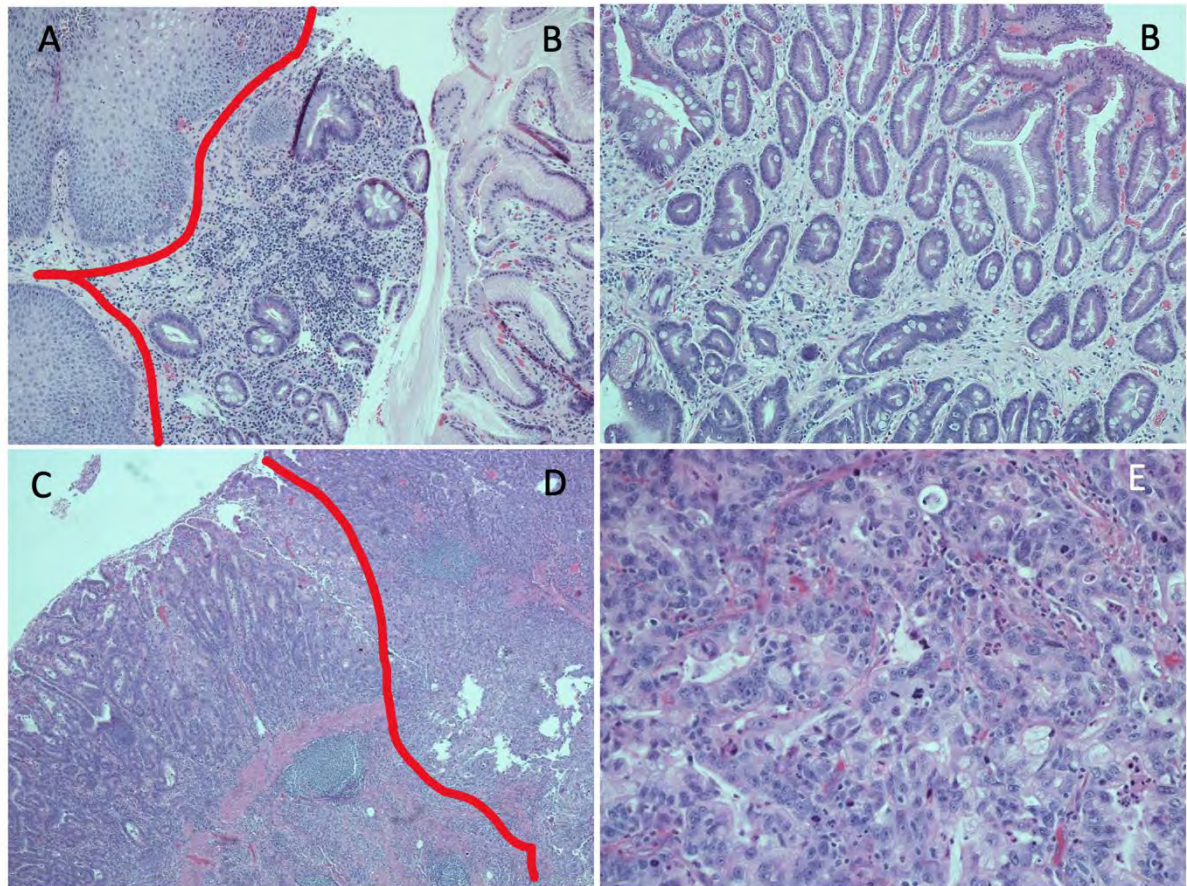


Figure 6 – Progression of NDBO to OADC. Low power light microscopy of oesophageal biopsies. A: Normal oesophageal squamous epithelium. B: NDBO with IM. C: BO with HGD with the muscularis mucosa clearly visible at the base of the dysplastic columnar epithelium. D: OADC which has breached the muscularis mucosa. All of the architecture of the columnar lined epithelium is lost with OADC. E: high power view of OADC. There are a number of mucin containing cells and mitotic figures are visible centrally. Images courtesy of Dr Rahul Hejmadi.

1.7.2 MECHANISMS RESPONSIBLE FOR BARRETT'S CARCINOGENESIS

There are several mechanisms which are likely to be responsible for carcinogenesis in Barretts. Firstly, chronic injury and inflammation of oesophageal mucosa occurs due to increased acidity in the oesophagus from gastric acid reflux, however controlling reflux symptoms either pharmacologically or surgically with fundoplication does not prevent OADC. Parrilla et al (22) performed a RCT of 101 patients randomised to medical therapy with PPI or H₂ receptor antagonists (n=43) versus surgery with gastric fundoplication (n=58) and found no difference in progression to HGD between the groups. This may be because bile salts continue to reflux and are known to cause mucosal injury over a range of pH environments dependant on their composition.

The arachidonic acid pathway, which controls inflammation, can be activated by gastric acid and bile salts, increasing expression of COX-2. COX-2 catalyses arachidonic acid to prostaglandins including Prostaglandin E₂ (PGE₂), which binds to EP2 receptors. Jimenez et al (23) showed that COX-2 protein expression increased in parallel with progression along the metaplasia-dysplasia-adenocarcinoma sequence, with the highest grade lesions exhibiting the highest levels of expression. Similarly EP2 receptor expression increases along with increasing dysplasia in BO. In vitro PGE₂ upregulation results in increased cell migration, angiogenesis and inhibition of apoptosis (24).

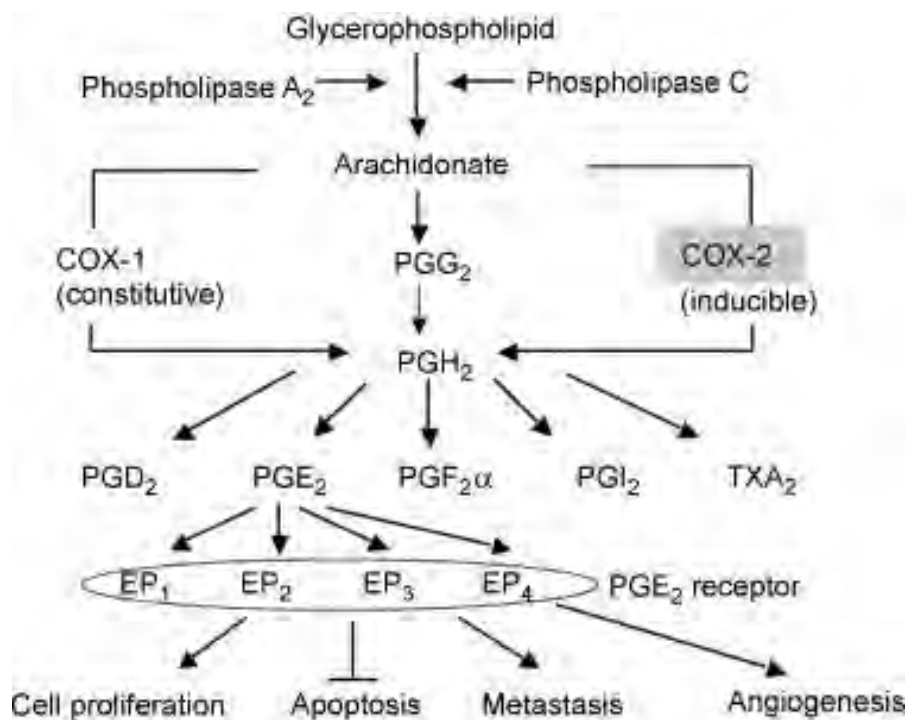


Figure 7 - The Arachidonic acid pathway. Gastric acid and bile salts can stimulate the production of prostaglandins (PG) via the arachidonic acid pathway. PGE₂ stimulates cell proliferation, angiogenesis and metastasis and is an inhibitor of apoptosis. (24)

Secondly, oxidative stress resulting from chronic inflammation due to gastric acid and bile salt reflux into the oesophagus produces reactive oxygen species (ROS) within epithelial cells. Vitamin C acts as a scavenger of ROS. In Fountoulakis et al's study of 96 patients (48 BO, 48 squamous epithelium), metaplastic Barrett's epithelium was shown to contain reduced levels of vitamin C when compared with normal squamous oesophageal epithelium (25). A possible explanation for this reduction in vitamin C is that continuing acid reflux and subsequent ROS production uses up the mucosal vitamin C stores in redox reactions when scavenging ROS. This leaves the mucosa deplete of vitamin C and vulnerable to the effects of increased ROS which results in DNA damage and mutation in cell

regulatory genes. Pro-inflammatory NF- κ B signalling is activated by bile salts and acid in vitro. In their in vitro experiment, Peng et al (26) demonstrated that restoring expression of the antioxidant enzyme glutathione peroxidase 7 (GPX7) in oesophageal cancer cell lines can reverse activation of NF- κ B signalling and reduce downstream synthesis of inflammatory cytokines. The *GPX7* promoter region is hypermethylated in oesophageal adenocarcinoma resulting in its downregulation, Peng et al have demonstrated that *GPX7* has a potential tumour suppressor function in vitro and in murine xenograft models (27).

Thirdly, longstanding PPI usage leads to hypergastrinaemia (28). Gastrin binds to cholecystinin receptors (CCK2R) to stimulate gastric acid production. CCK2R are upregulated during ulcer healing and inhibition impairs mucosal repair. Gastrin binding to CCK2R can induce expression of epidermal growth factor and trefoil peptide which can lead to COX-2 expression, leading to further prostaglandin production. Increased gastrin has been shown to promote carcinogenesis in cell culture models(29). Lee et al's murine model (29) suggests that PPI induced hypergastrinaemia may promote metaplasia and dysplasia in BO through increased stimulation of CCK2R in the mucosa. This is important as chronic GORD is often treated with *H. pylori* eradication therapy and PPI. This has the potential to increase the risk of developing OADC. Furthermore, overgrowth of nitrogen reducing bacteria associated with PPI use increase nitrite concentration in the lower oesophagus(30). Nitrites react with gastro-oesophageal reflux content to form nitrous acid which decomposes to nitric oxide. Nitric oxide is implicated in chronic inflammatory states and early stages of carcinogenesis of all tissue types(30).

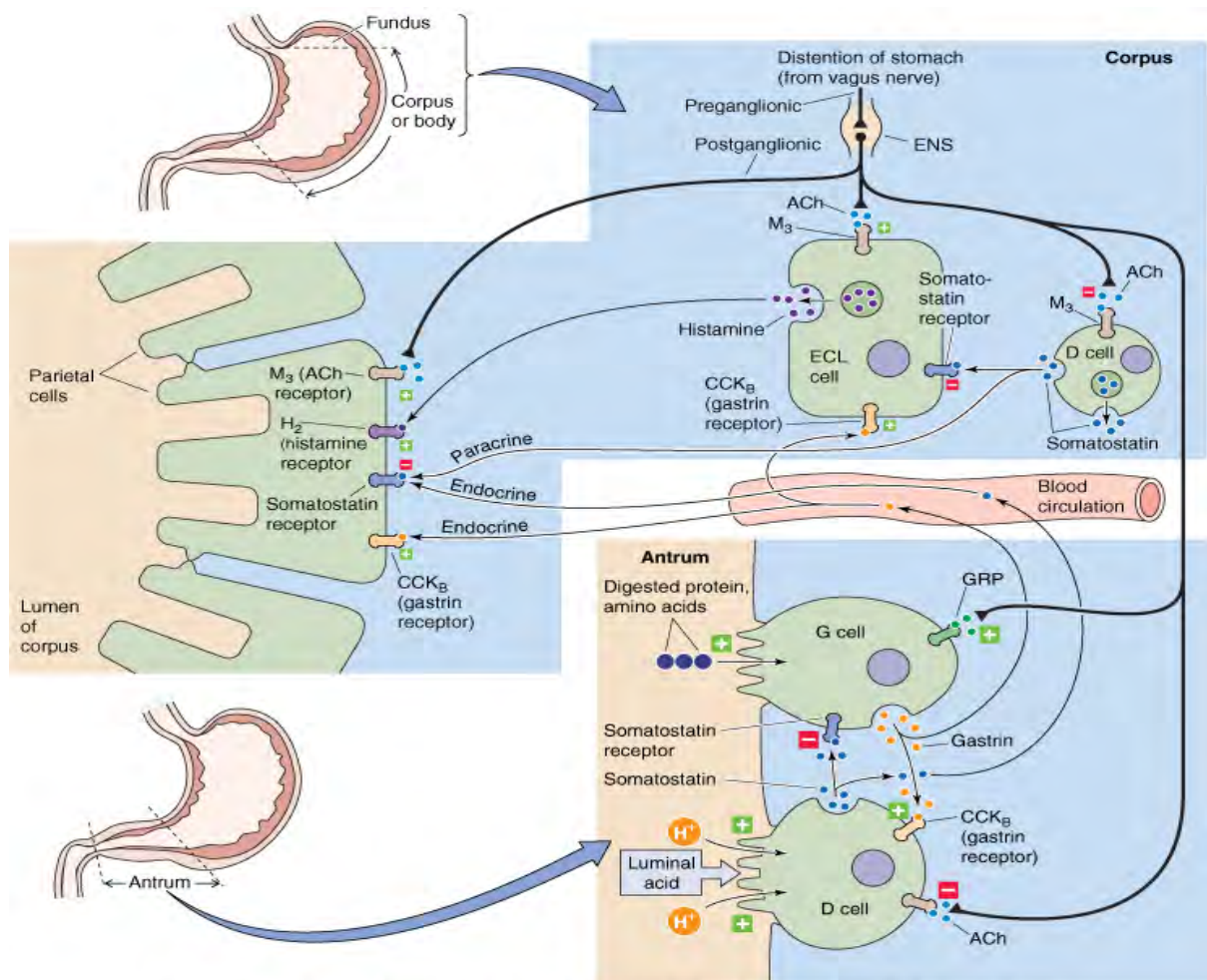


Figure 8 - Diagram detailing endocrine regulation of gastric acid production in the stomach. Gastrin is a hormone which stimulates production of gastric acid by triggering the proton pump in parietal cells of the stomach. High H^+ (acid) concentration in the gastric lumen causes D cells to secrete somatostatin which inhibits stomach acid production as a negative feedback loop. Direct proton pump inhibition reduces the luminal H^+ which will in turn reduce somatostatin secretion and cause increased production of gastrin from G cells to try to decrease luminal pH. Diagram taken from Medical Physiology by W Boron and E Boulpaep.

Helicobacter pylori is a major risk factor for developing non-cardia gastric cancer, gastric and duodenal ulceration and gastric MALTomas, however *H.pylori* infection rates in patients with GORD, BO and OADC are lower than in control

groups (31, 32). The negative association of *H.pylori* and risk of BO and OADC suggests that *H.pylori* infection may be protective against GORD and therefore OADC. *H.pylori* infection decreases the secretion of gastric acid and increases the risk of gastric atrophy(32). Fischbach et al (33) concluded from their study of 657 patients that *H.pylori* infection decreased the risk of developing BO, with the most significant effect in patients where gastric acid secretion is suppressed either by medication or atrophic gastritis. Further studies have shown that rather than *H.pylori* itself affecting carcinogenesis in the oesophagus directly, it may be the pattern of reflux and gastritis that is the more significant factor. *H.pylori* positive patients with antral predominant, non-atrophic gastritis have a higher risk of developing OADC(34) and *H.pylori* positive patients with atrophic gastritis have a higher risk of OSCC (35).

Fourthly, obesity is associated with GORD(36) which increases the risk of developing OADC. Individuals with a BMI greater than 30 have a relative risk of 3 for developing OADC compared to those with a BMI between 18 and 25(37). Obesity in itself is multifactorial in the development of OADC. Not only are there mechanical, pressure effects of increased abdominal fat causing GORD, resulting in direct chemical irritation of the lower oesophagus as discussed previously, but increased visceral fat has a metabolic effect which generates a low grade pro-inflammatory environment which is thought to be tumourigenic(38). Yeung et al(39) demonstrated an increase in circulating pro-inflammatory mediators in those with central obesity. Patients with central obesity from the study cohort of 2893 patients from the Hong Kong Cardiovascular Risk Factors Prevalence Study (CRISPS) demonstrated increased circulating $\text{TNF } \alpha$, $\text{IL-1 } \beta$,

IL-6, IL-8, interferon- γ , monocyte chemoattractant protein 1 and vascular endothelial growth factor (VEGF). Of the 205 patients that developed cancer, 36.6% had central obesity versus 24.5% of the non-cancer group.

In Doyle et al's study (40) OADC patients had higher levels of serum insulin like growth factor 1 (IGF-1) than controls and patients with NDBO and OSCC. OADC patients with a visceral fat volume greater than 130 cm² had higher levels of serum IGF-1 than those with lower visceral fat. Increased expression of IGF-1 receptors and circulating levels of IGF-1 is associated with progression of Barretts oesophagus to neoplasia(40). This would suggest that increased visceral fat increases the likelihood of a patient to progress to OADC through increased expression of IGF-1.

Thus, obesity may contribute to Barrett's carcinogenesis by increasing GORD and chemical irritation, generation of a pro-inflammatory environment and an increase in IGF-1.

Finally, patients with GORD alone are 1.7 times as likely as the normal population to develop OADC. This rises to 10.6 times with a diagnosis of Barrett's oesophagus(41).

1.8 OESOPHAGEAL CANCER

There are two major histological types of oesophageal cancer: oesophageal adenocarcinoma (OADC) and oesophageal squamous cell carcinoma (OSCC)

which represent over 90% of oesophageal cancer diagnoses. Less commonly, small cell carcinoma and sarcomas, carcinoids, melanomas and lymphomas can occur in the oesophagus(42). OADC is a tumour arising from columnar, glandular epithelium within the oesophagus which replaces stratified squamous epithelium (Figure 10). OSCC is a tumour arising directly from the stratified squamous epithelium lining the oesophagus. OSCC is the most common subtype of oesophageal cancer worldwide, and although not investigated in this thesis, its presenting symptoms, diagnosis and often resulting treatment is similar, with current gold standard curative treatment being resectional surgery. Where OADC and OSCC differ is their pathogenesis and developmental risk factors which are summarised in table 4.

Table 4 – Risk factors for oesophageal carcinoma (adapted from Penathur et al 2013)(43)

Risk Factors for Oesophageal Adenocarcinoma	Risk Factors for Oesophageal Squamous Cell Carcinoma
Tobacco use	Tobacco use
Diet deficient in fruit and vegetables	Nutritional deficiencies
History of thoracic irradiation	History of thoracic irradiation
Symptomatic GORD	Achalasia
Barrett's Oesophagus	Caustic oesophageal injury
Obesity	Mutations of enzymes which metabolise alcohol
Medications which relax the lower oesophageal sphincter	Alcohol consumption
Increased age	Low socio-economic status
Male sex	Poor oral hygiene
Family history	Non-epidermolytic palmoplantar keratoderma

1.9 EPIDEMIOLOGY

Oesophageal cancer is the 8th most prevalent cancer worldwide making up 3% of the total global cancer burden (456,000 cases estimated)(44). There is a male predominance with a male:female ratio of 2.4:1. There are large differences worldwide in incidence with 0.8 per 100,000 in Western Africa to 17.0 per 100,000 in Eastern Asia in men and 0.2 per 100,000 in Micronesia/Polynesia to 7.8 per 100,000 in Eastern Africa in women(44). The global distribution of these cancers is distinct with Far Eastern and developing countries having a greater burden of OSCC than Western and developed countries. The incidence of OADC has been increasing both in developed countries and worldwide in parallel with rising

obesity and Westernised diets. With regard to anatomical distribution of oesophageal tumours, Siewert et al reported a case series of 1,552 oesophageal cancers (OADC n= 539, OSCC n= 1,013). OADC was most commonly located in the distal third of the oesophagus (97.1%), with no OADC found in the cervical oesophagus and 2.9% in the middle third. OSCC was distributed more evenly in the middle (42.3%) and distal two thirds (47.9%), with sporadic occurrence in the cervical oesophagus (9.8%)(45).

In the UK, the incidence and prevalence of oesophageal cancer is increasing with 9,000 new cases diagnosed per year(46). Statistics gathered by Cancer Research UK reveal that oesophageal cancer accounts for 3% of cancer diagnoses in the UK and is the 9th most common cancer in males and 15th most common in females. OSCC accounted for 28% of oesophageal tumours and OADC 55%(46). Lepage et al (47) reviewed over 43,000 cases of OADC in England and Wales between 1971 and 2001. This revealed a 10 fold increase in cumulative risk of developing OADC in men aged between 15 and 74 from 0.1% to 10%.

Table 5 Estimated number (x1000) of oesophageal cancer cases worldwide 2012 (44)

	Male		Female	
	Cases	Deaths	Cases	Deaths
Developing Countries	255	225	114	104
Developed Countries	68	56	18	15

1.10 JUNCTIONAL TUMOURS

Junctional tumours are adenocarcinomas at the gastro-oesophageal junction, or the cardia. Siewert et al described a classification system to differentiate between these tumours and aid clinicians to treat them appropriately(48). This is an important distinction as the lymphatic drainage of tumours situated above the GOJ mandate that they are treated as OADC whereas those at or below the cardia may be treated as gastric adenocarcinomas and do not require oesophagectomy for curative treatment.

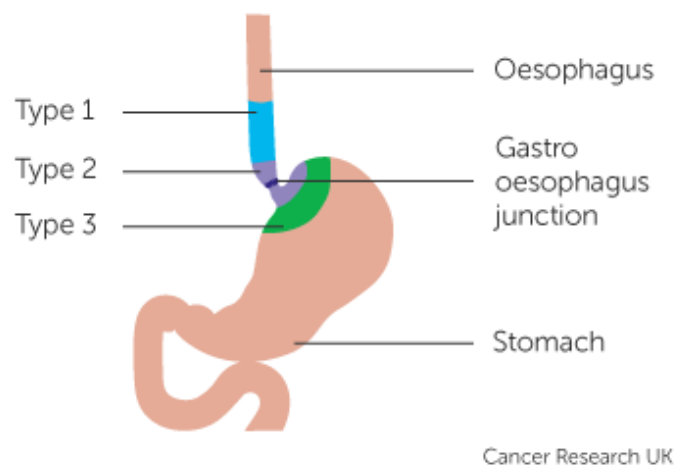


Figure 9 - Illustration of the Siewert Classification of Adenocarcinoma of the Gastro Oesophageal Junction. The Siewert Classification is shown in more detail in Table 6. Image Taken from Cancer Research UK (49)

Table 6 - Siewert Classification of Junctional Adenocarcinoma

Classification	Definition	Location in relation to the GOJ
Type I	Distal oesophageal adenocarcinoma	1-5 cm above
Type II	Adenocarcinoma of the true gastric cardia	1 cm above or 2 cm below
Type III	Adenocarcinoma of the stomach below the cardia	2-5 cm below

1.11 DIAGNOSIS OF OESOPHAGEAL CANCER

Oesophageal cancer commonly presents at a late stage with symptoms of weight loss, anaemia, dysphagia and odynophagia. New symptoms of GORD may be neoplastic in origin. Public Health England launched a UK wide patient awareness campaign “Be Clear on Cancer” in January 2015 which encourages patients with heartburn on most days over a period of three weeks to present to their GP for further investigation(50).

As with BO, the gold standard investigation for oesophageal cancer is OGD which allows direct visualisation and biopsy of the lesion. Histological analysis of the lesion from tissue biopsies allows the cancer subtype to be determined. Technologies such as the Cytosponge(51) are in development which could provide a valuable screening tool for BO and OADC without the need for formal endoscopy using oesophageal brushings. This technology would require robust tissue biomarkers to be validated before it could be brought into widespread clinical practice. The BEST3 trial (52) is investigating whether diagnosis of BO can be increased in primary care settings using Trefoil Factor 3 (*TFF3*) as a tissue

biomarker in conjunction with the cytosponge. OGD can only assess the intraluminal component of the tumour and cannot accurately stage lesions as an isolated investigation. Endoscopic ultrasound (EUS) is used to assess the depth of invasion and computed tomography (CT) is performed to search out metastatic lesions with positron emission tomography (PET) used to differentiate between metastatic and benign lymph nodes. Prior to definitive surgical resection, laparoscopy is performed to assess for omental and peritoneal spread within the abdomen.

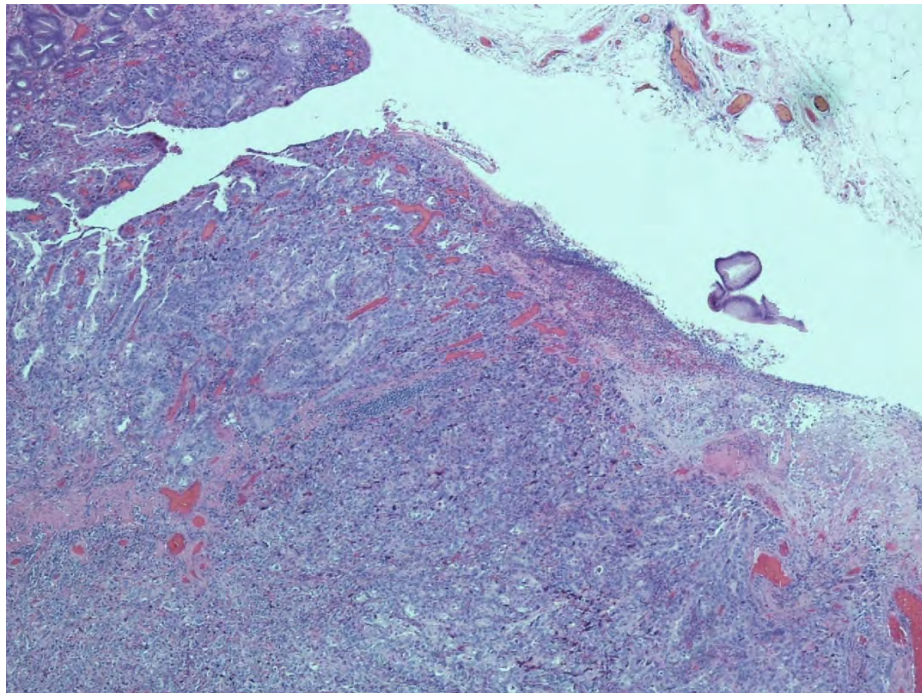


Figure 10 - Low power light microscopy of a tissue section of OADC with superficial ulceration. None of the usual columnar epithelial architecture of BO can be seen within the lesion and the normal oesophageal squamous epithelium is obliterated.

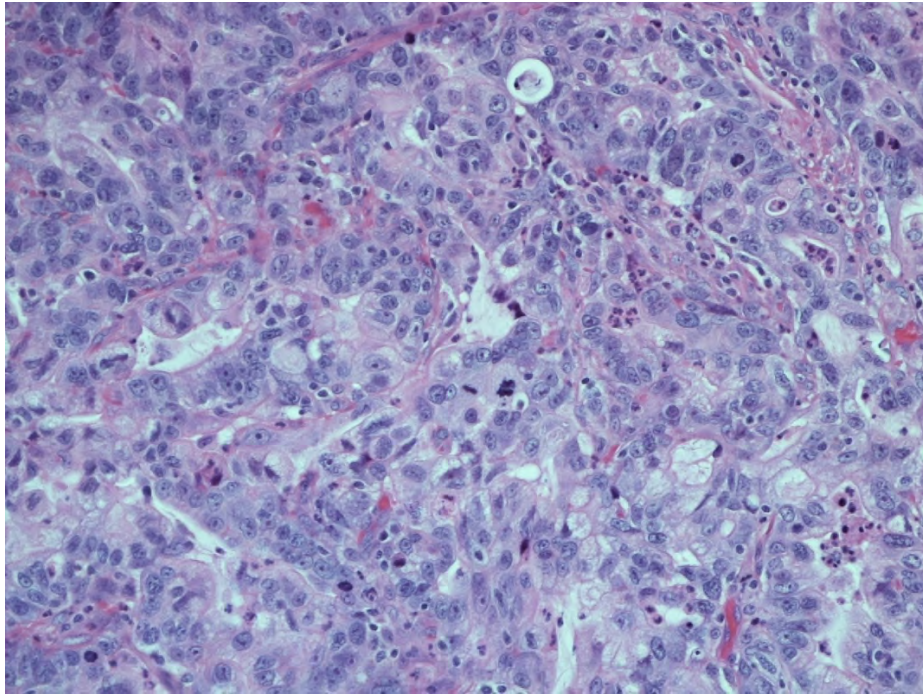


Figure 11 - High power light microscopy of an oesophageal biopsy showing OADC. There are a number of mucin containing cells and mitotic figures are visible centrally. These features are typical for OADC.

1.12 TNM STAGING

TNM staging provides a universal language to stage all types of cancer. This provides clinicians with information regarding the suitability for treatment and an indication of prognosis for a particular lesion. In oesophageal cancer, "T" describes the tumour itself and takes into account tumour size and degree of invasion into the submucosa, adventitia and adjacent structures. "N" describes nodal involvement and "M" describes the presence or absence of distant metastases. Histological tumour grade is denoted "G". The prefix "p" indicates that staging has been performed by the pathologist on a resection specimen. The prefix "y" indicates the use of pre-operative neoadjuvant chemo-radiotherapy. Anatomical location of the tumour is defined by the distance from the incisor teeth to the upper margin of the lesion. The anatomical location can be subdivided into

cervical (15-20 cm), upper thoracic (20-25 cm), middle thoracic (25-30 cm) and lower thoracic (30-40cm). The full TNM staging for OADC is given in Table 7.

Table 7 - TNM Staging for OADC

Tumour Grade	Description
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	High grade dysplasia
T1	Tumour invades lamina propria, muscularis mucosae or submucosa
T1a	Tumour invades lamina propria, muscularis mucosae
T1b	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4	Tumour invades adjacent structures
T4a	Resectable tumour involving pleura, pericardium or diaphragm
T4b	Unresectable tumour invading other adjacent structures (aorta, vertebral body or trachea)

Nodal Staging	Regional Lymph Node Involvement
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-2 regional lymph nodes
N2	Metastasis in 3-6 regional lymph nodes
N3	Metastasis on >7 regional lymph nodes

Distant Metastasis	
M0	No distant metastasis
M1	Distant metastasis

Histological Grade	Description
GX	Grade cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

1.13 TREATMENT OF OADC

The treatment of oesophageal adenocarcinoma is performed in a multidisciplinary setting and all treatment decisions should be undertaken with a multidisciplinary team (MDT) of an Upper GI surgeon, a specialist GI endoscopist, a GI radiologist and a histopathologist. After careful evaluation of the tumour's TNM stage by the MDT, those suitable for curative therapy will be treated as described below. Unfortunately, OADC often presents at a late stage when it has already invaded local structures or developed lymph node or distant metastases rendering it impossible to resect the tumour with curative intent. Neoadjuvant therapy can downstage tumours with a high T and is discussed in section 1.15. In cases invading adjacent structures such as the trachea and aorta, or where distant metastases are present, palliative therapy is offered. Palliative chemotherapy can be offered to delay disease progression and improve survival time and quality of life. Platinum containing chemotherapeutic agents in combination with 5-fluorouracil (5-FU) or capecitabine have been shown to convey quality and quantity of life benefits over symptomatic control alone(53). In older or comorbid patients, chemotherapy may not be possible due to toxic side effects. Oesophageal stenting to widen the narrowed gullet to allow food and liquid to pass, improves quality of life and also allows nutrition and hydration to continue. If stenting is not possible, a percutaneous gastrostomy can be placed for this function. If neither of these procedures are possible, best supportive care with analgesic and antiemetic medication is the only remaining option.

The treatment for BO should also be considered under the umbrella of OADC treatment as a pre-cancerous lesion. The metaplasia-dysplasia-adenocarcinoma

pathway (Figure 6) is well documented and recent evidence suggests that even BO with LGD should be treated with Ablative therapy. In a multicentre randomised clinical trial, 136 patients diagnosed with LGD in BO were randomised into either endoscopic surveillance or radiofrequency ablation (RFA) of their BO segment. The risk of progression to HGD or OADC over 3 years of follow up in the control arm was 26.5% compared to 1.5% in those receiving RFA(54). Whilst this seems to provide clear evidence, diagnostic challenges remain for histopathologists in calling the degree of dysplasia in tissue samples. Firstly, the biopsy sample is taken to be representative of the entire BO segment. Secondly, the interpretation of BO biopsies is subject to inter-observer variability. Duits et al (55) gathered a panel of expert pathologists to retrospectively review the histology of 293 cases of BO with LGD gathered over an 11 year period. 73% of these cases were subsequently downgraded to NDBO or indeterminate for dysplasia, leaving 27% as confirmed LGD. For those patients with confirmed LGD, the risk of progression to HGD or OADC was 9.1% per patient per year compared to 0.6% and 0.9% for the NDBO and indeterminate groups respectively. Thus, it is possible that LGD may be over treated if the histological grade is over-called.

If HGD is detected on biopsy samples, it is recommended that the patient undergoes high resolution endoscopy (HRE) to fully assess the BO segment and establish if there are lesions suitable for endoscopic mucosal resection (EMR). Sessile or depressed lesions as characterized by the Paris classification described above are more likely to contain invasive cancer than flat or pedunculated lesions.

1.14 ENDOSCOPIC THERAPY FOR HIGH GRADE DYSPLASIA AND EARLY STAGE CANCER

Endoscopic therapy is rapidly becoming the preferred method for treating dysplastic BO and early stage OADC, with the resection of visible lesions and ablation of the remainder of the BO mucosa (56). Endoscopic therapy for HGD and selected early stage OADC can provide comparable disease free survival rates to oesophagectomy. Ell et al reported a calculated 98% 5 year survival when superficial T1 lesions up to 20mm in size were resected by EMR, achieving complete local remission in 99 of 100 patients(57). Pennathur et al (58) studied 100 oesophageal resections performed for T1 disease. They reported an estimated 5 year survival of 70% and 35% for N0 and N1 disease respectively, although this data would appear to favour endoscopic therapy, Ell et al's cohort was very carefully selected whereas Pennathur's selection criteria was less stringent. The post procedure morbidity and mortality of endoscopic therapy is significantly lower than that of oesophagectomy. A mortality from endoscopically treated HGD of 0.04% (2831 patient studied) is reported by Menon et al compared to 1.2% of patients treated surgically(59). A consensus group set up by Bennett et al concluded that HGD is preferentially treated endoscopically rather than with resectional surgery (60) and furthermore, HGD and early OADC should be treated endoscopically rather than subject to endoscopic surveillance. Endoscopic therapy carries the risk of bleeding, infection, stricture, perforation and disease recurrence. Complications of oesophagectomy are detailed later in this section.

Due to the nature of lymphatic drainage of the oesophagus, even early stage lesions can result in nodal involvement. In T1a lesions the risk of lymph node metastases is 1.3% whereas this increased to 22% in T1b lesions(61). Thus, not all lesions are suitable for endoscopic therapy, as lymph node clearance is required for curative therapy and the gold standard for curative treatment of T1b lesions or above remains resectional surgery. This demonstrates that in carefully selected cases, excellent outcomes can be achieved using endoscopic therapy rather than resectional surgery for HGD and early stage OADC.

1.14.1 ABLATIVE THERAPY

Numerous endoscopic techniques for ablating BO are available: photodynamic therapy (PDT), argon plasma coagulation (APC), cryotherapy, RFA or radical stepwise EMR. There is little evidence directly comparing these techniques to one another, however ablative therapy for HGD (active treatment) provides better outcomes than surveillance for disease eradication (conservative treatment). In a randomised controlled trial of 127 patients by Shaheen et al, 1.2% of patients who received RFA of their BO with dysplasia went on to develop OADC compared to 9.3% in the surveillance control group (62). In a similar study of 208 patients by Overholt et al using PDT and omeprazole compared to omeprazole alone, 13% of the PDT group went on to develop OADC compared to 205 in the control group(63). Stepwise EMR can achieve 97-100% eradication of dysplasia. RFA, cryotherapy, APC and PDT achieve up to 98, 95, 78 (eradication of IM) and 80 percent clearance respectively (56). Ablative therapy is only indicated in cases of “flat” dysplasia (Paris IIb). Endoscopic ablation therapy must be followed up with surveillance endoscopy, as by its very nature endoscopic therapy does not

remove the oesophagus and disease may recur at a later stage. If further dysplasia occurs the ablative therapy can easily be repeated. Ablation of the mucosa has a good risk profile compared to other therapies, but it carries the risk of bleeding, infection, perforation and oesophageal stricture. EMR specimens can be sent for histology, however the other ablative therapies destroy the mucosa rather than excising it and this cannot be analysed histologically. The added disadvantage of PDT and APC is that glands may become buried under the ablated mucosa and progress to invasive cancer without becoming visible endoscopically.

1.14.2 ENDOSCOPIC MUCOSAL RESECTION

Endoscopic mucosal resection (EMR) is an endoscopic procedure to remove the oesophageal mucosa. This allows superficial, visible lesions to be excised completely using a minimally invasive technique. This carries the advantage over ablative therapy that raised lesions can be excised. In resection specimens, the highest grade of dysplasia or histological abnormality corresponds to visible lesions in the oesophagus(64). This finding has been replicated in step wise EMR of complete BO segments. Diathermy is used to mark the visible lesion. EMR relies on raising the mucosa to be excised. This can be performed either by suction, rubber band application and snaring of the pseudopolyp, or by injecting the submucosa and then suctioning the mucosa into a cap device and applying a snare(56). This can be performed in a stepwise fashion along the BO segment to completely excise the diseased area. Large areas of mucosal excision can increase the risk of oesophageal strictures, and the BSG guidelines

recommended that raised lesions are treated by EMR and remaining flat sections of BO are ablated using RFA(1).

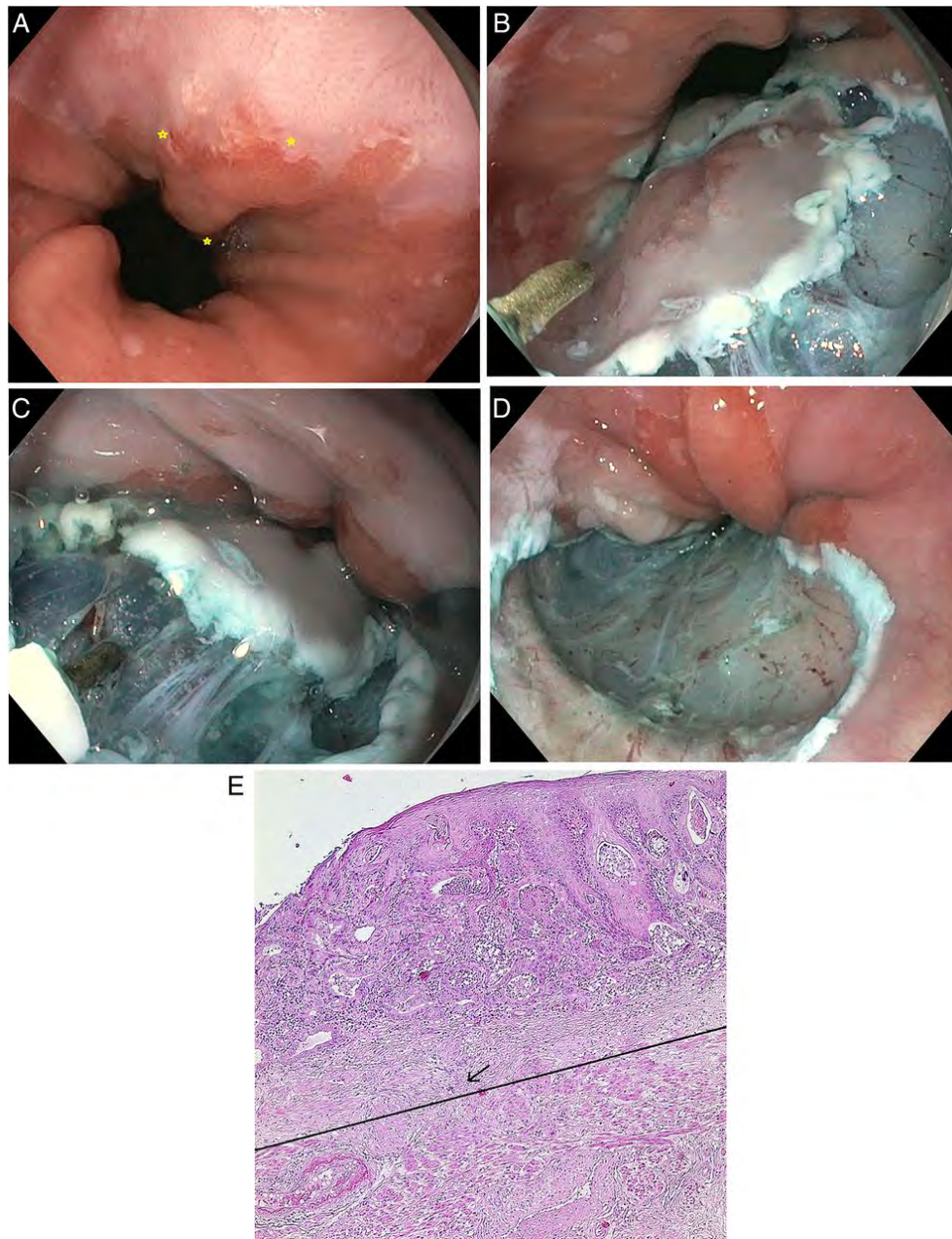


Figure 12 - A) Early OADC (Paris IIc) shown between yellow markers. B) Endoscopic Mucosal Resection (EMR) with circumferential incision. C) Hydrodissection of the submucosa with saline injection D) Completed EMR. E) Histology of resection specimen showing pT1a OADC. Arrow detailing tumour invading top layer of muscularis mucosae. Image taken from Terheggen et al, Gut 2017 (65)

1.15 NEOADJUVANT THERAPY

The aim of neoadjuvant chemo-radiotherapy (nCRT) is to downstage tumours to allow complete surgical resection, to control systemic disease and eliminate micrometastases. A meta-analysis of six RCTs by Fiorica et al (66) revealed a statistically significant pooled treatment effect on overall survival when nCRT was used versus surgery alone (OR 0.53 (95% CI 0.31–0.92); $z=-2.23$; $p=0.03$) (NNT= 10)). Similarly, when four RCTs were pooled to assess the effect of nCRT, it showed that resection specimens from patients receiving nCRT prior to surgery were more likely to be a lower pathological grade than those that did not receive nCRT (OR = 0.43 (CI 0.26–0.72, $z=-3.26$, $p=0.001$) (NNT=5))(66).

The MRC OEO2 trial (67) demonstrated a survival advantage at 2 years when patients were treated with neo-adjuvant chemotherapy (NAC) consisting of two, 4 day cycles of cisplatin and 5-FU prior to surgical resection compared to surgical resection alone (43% vs 34%). Investigators were free to give patients additional neo-adjuvant radiotherapy. 9% of patients split equally between the control and experimental groups received radiotherapy. There was no effect in overall survival when radiotherapy was added. The trial authors concluded that neo-adjuvant chemotherapy alone was sufficient to provide an overall survival benefit. The OEO5 trial (68) investigated whether a survival benefit could be achieved with four cycles of neoadjuvant epirubicin, cisplatin and capecitabine (ECX) over standard two cycles of cisplatin and 5-FU (CF). 897 patients with OADC were studied. No overall or disease-free survival advantages were conveyed by undergoing the more intensive NAC regime. There were higher rates of chemotherapy related adverse events related to toxicity and a higher rate of

patient drop out before completing the full course of NAC in the ECX group compared to the CF group.

Shapiro et al's CROSS study (69) randomised 366 patients with OSCC (n=84) and OADC (n=275) into surgery alone versus nCRT and surgery. Median follow up for surviving patients at the time of analysis was 84.1 months (61.1-116.8, IQR 70.7-96.6). For OADC treated in the nCRT and surgery group, median overall survival was 43.2 months (95% CI 24.9-61.4) and 27.1 months in the surgery alone group (95% CI 13.0-41.2), (HR 0.73 [95% CI 0.55–0.98]; log-rank $p=0.038$). As a result of the trial, they concluded that nCRT with surgery provided improved locoregional and distant disease control.

1.16 DEFINITIVE CHEMORADIOTHERAPY

Chemo-radiotherapy has been used for definitive treatment of oesophageal cancer owing to the highly morbid nature of oesophagectomy. Patients who are unfit for resectional surgery or choose not to undergo major surgery may be offered definitive chemo-radiotherapy as an alternative. The trial RTOG 85-01 conducted by Cooper et al demonstrated an improvement in median survival (14 vs 9 months) and 5 year overall survival (27% vs 0%) in patients treated with combination chemo-radiotherapy versus those treated with radiotherapy alone (70). The trial INT 0123 conducted by Minsky et al demonstrated no survival benefit between high dose radiotherapy at 64.8 Gy versus standard dose 50.4 Gy (71). These two randomised controlled trials have established the standard of

care for definitive oncological treatment of OADC and OSCC as 4 cycles of 5-FU and cisplatin with a radiotherapy dose of 50.4 Gy.

Patients with locally advanced oesophageal cancer may also benefit from definitive treatment with chemo-radiotherapy, although the majority of patients studied to date have OSCC rather than OADC, overall response rate in patients with OSCC was up to 98% using docetaxel and cisplatin regimes (72).

Human epidermal growth factor 2 (HER2) positivity has been reported in up to 30% of OADC and appears to correlate with poor prognosis. The ToGA trial (73) assessed the effect of trastuzumab, a monoclonal antibody against HER2, in combination with cisplatin or 5-FU and cisplatin versus chemotherapy alone. This demonstrated a small but significant median overall survival benefit (13.8 vs 11.1 months) in gastric and GOJ tumours.

1.17 OESOPHAGECTOMY

Oesophagectomy is a surgical procedure to remove the gullet. It is major surgery entering both the thoracic and abdominal cavities and carries a high risk of both peri and post-operative morbidity and mortality. Open surgery involves laparotomy and right sided thoracotomy. There are various methods of minimally invasive oesophagectomy (MIO) which have been developed aiming to reduce the trauma of open surgery. These involve either a totally minimally invasive approach using laparoscopy and thoracoscopy or hybrid approaches with a single stage of the procedure performed using a minimally invasive approach. Uttley et al (74) performed a narrative review of 28 studies comparing MIO with open surgery and concluded that MIO had comparable levels of safety and efficacy to open surgery. Intra-operatively the entire oesophagus is removed

along with corresponding lymph nodes. Continuity of the gastrointestinal tract can be achieved by using either a gastric or colonic conduit. During the intra-abdominal stage, the gastric conduit is fashioned by mobilising the stomach from all its attachments intra-abdominally. The lesser curve of the stomach is excised with the resection specimen and a gastric conduit fashioned from the remnant greater curve. In open surgery, a right postero-lateral thoracotomy is performed in the 5th intercostal space and the right lung collapsed using a double lumen endobronchial tube. The azygous vein is divided and the oesophagus and associated lymphatic tissue is dissected free from the mediastinum. The oesophagus is divided and removed. The gastric conduit is moved into the chest and anastomosed to the proximal oesophageal remnant.

1.17.1 COMPLICATIONS OF OESOPHAGECTOMY

Oesophagectomy is a highly morbid procedure which requires a patient to have a good level of pre-operative function and fitness to proceed. Care in the immediate post operative period is undertaken on the intensive care unit. Complications of oesophagectomy are summarised in Table 8. The UK National Oesophago-Gastric Cancer Audit 2018 (75) reports that in hospital mortality following oesophagectomy in the UK between 2015 and 2017 was 2.7%, with 30 and 90 day mortality at 2.4% and 3.9% respectively. Median length of hospital stay post operatively was 9 days.

Table 8 - Complications of Oesophagectomy. Adapted from: Minimally invasive oesophagectomy versus open surgery: is there an advantage? Uttley et al, Surgical endoscopy, 2013 (74)

Outcome	Mean MIO	Mean open
Mortality	2.4 %	3.8 %
Conversion to open surgery	5.3 %	n/a
Recurrence	23.4 %	24.8 %
Length of hospital stay	14.0 days	16.6 days
Tracheal perforation	2.3 %	6.5 %
Damage to adjacent structures	4.9 %	6.5 %
Anastomotic leak	10.1 %	10.7 %
Chyle leak	4.4 %	2.9 %
Infection	12.9 %	18.2 %
Laryngeal/vocal cord damage	12.7 %	9.6 %
Pulmonary complications	9.6 %	6.5 %

1.18 SURVIVAL

A diagnosis of OADC carries a poor prognosis. Oesophageal cancer net survival in the UK (all histological types and TNM grades) at 1, 5 and 10 years are 41.9%,

15.1% and 12.3% respectively(76). In OADC with no metastatic spread, 80% will be alive at 1 year, falling to 50% at 3 years(77). The 5 year survival rate in the United States for OADC was reported at 13.6%(78).

1.19 SURVEILLANCE OF BO

The well described progression from non-dysplastic Barrett's oesophagus to OADC and the poor outcome associated with late diagnosis of OADC has led both the BSG and the ACG to recommend regular surveillance of patients with BO. To facilitate early diagnosis of OADC and the precursor lesions of dysplastic BO. The BSG recommend that patients with a Barrett's segment of less than 3 cm should have a repeat endoscopy every 5 years. Those with a Barrett's segment greater than 3 cm should have 2 yearly surveillance.

Unlike colorectal, breast and cervical cancer, oesophageal adenocarcinoma has no UK screening programme.

The World Health Organisation's principles for screening for disease(79) are:

- The condition should be an important health problem
- There should be an accepted treatment for patients with recognised disease
- Facilities for diagnosis and treatment should be available
- There should be a recognisable latent or early symptomatic stage

- There should be a suitable test or examination
- The test should be acceptable to the population
- The natural history of the condition, including development from latent to declared disease, should be adequately understood
- There should be an agreed policy on who to treat as patients
- The cost of case finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible medical care as a whole.
- Case finding should be an ongoing process rather than a “once and for all” project.

Oesophageal adenocarcinoma is an important health problem. Oesophageal cancer has been identified as a cancer of unmet need by Cancer Research UK. The incidence of oesophageal adenocarcinoma is increasing in parallel with increasing obesity and western diets, however the net survival trend over the last decade has not improved despite improvement in peri-operative care and surgical technique including minimally invasive surgery.

The “accepted treatment” of oesophageal adenocarcinoma is resectional surgery. This is a high risk surgical procedure which cannot cure those with advanced disease. The accepted treatment of this disease is shifting towards early detection and ablation or resection of pre-malignant lesions or early stage submucosal cancer, yielding much improved survival rates. The metaplasia → dysplasia → adenocarcinoma pathway is well understood in Barrett’s carcinogenesis and dysplasia provides a recognisable latent stage which

provides an opportunity for early intervention. The NHS is fortunate to be equipped with excellent endoscopy and histology services which can identify patients with dysplastic Barrett's or early oesophageal adenocarcinomas and the facilities to treat these early lesions.

Patients diagnosed with Barrett's oesophagus are offered surveillance on an individual basis depending on the extent of their disease. Despite a greatly increased risk of developing oesophageal adenocarcinoma in these patients, the yearly incidence is less than one percent and the vast majority will never develop cancer. Therefore, there are a large number of patients who will be subjected to invasive and uncomfortable endoscopic examinations with their associated risks with no long term benefit. Endoscopic surveillance is extremely time consuming and expensive for the NHS.

The key to implementing a successful national screening programme for oesophageal adenocarcinoma will be identification of patients who are at significantly higher risk of progression to cancer than the general population. If these patients could be identified using a tissue, blood or other marker on index endoscopy, those at low risk can be reassured and spared repeated endoscopic surveillance. Those at high risk may benefit from an increased frequency of endoscopic surveillance to diagnose and treat dysplastic Barrett's or early adenocarcinomas before the need for resectional surgery.

1.20 GENETIC CHANGES IN BARRETT'S OESOPHAGUS

The molecular landscape of OADC is complex, spanning both germline and somatic genetic and epigenetic changes. It is only since the advent of so-called 'omics' technologies there has been a better understanding of the genetic change in BO develops. A number of studies have indicated that BO is highly mutated even in non-dysplastic tissues and contains a high mutational rate even compared to certain human neoplasms including prostate and breast cancer. Chalmers et al(80) analysed the tumour mutation burden in 100,000 cancer specimens from The Cancer Genome Atlas (TCGA). They found the highest mutational burden occurred in skin basal cell carcinoma and squamous cell carcinoma with median rates of 47.3 and 45.2 mutations per megabase. The lowest were found in bone marrow myeloproliferative neoplasm and bone marrow myelodysplastic syndrome, both with a median mutational rate of 0.8 mutations per megabase. The rate of mutation in NDBO is reported ranging between 1.3-6.8 single nucleotide variations per megabase (81). Stachler et al report the somatic mutation rate of NDBO ranging from 1.3-5.4 mutations per megabase(82) and Ross-Inness et al report an average of 6.76 mutations per megabase(81). In Stachler et al's study, as NDBO progressed to dysplasia the mutational frequency increased to 4.9 mutations per megabase which is similar to the 4.1 mutations per megabase of OADC. The mean number of focal deletions and amplifications were seen to increase progressively during progression from NDBO to OADC suggesting that these rather than point mutations may be the trigger for neoplastic progression in BO. These results have been generated from paired samples which are taken from patients who have developed OADC and

subsequently had their tumour resected. The BO tissues have been harvested from adjacent areas in the resected oesophagus, thus it is impossible to accurately analyse true genetic change over time in the progression of BO tissues from these samples as there is no way to know the true timescale of the mutations.

Maley et al performed a prospective cohort study on 268 patients with BO and monitored them with surveillance endoscopies to determine their cancer outcome (83). Using a method adapted from ecological and evolutionary studies they aimed to quantify clonal diversity in BO. They discovered that increasing clonal diversity in BO tissues corresponds to increased likelihood of progression to OADC, possibly arising from underlying genetic instability within the tissues. This appears to correspond with findings of increased mutational frequency as BO progresses towards dysplasia.

The tumour suppressor gene *CDKN2A* is lost regardless of progression status in BO, whereas *TP53* is lost at a later stage of progression(84), with p53 immunohistochemistry offering an aid to histological diagnosis of dysplasia in BO (1).

Palles et al (85) performed a genome wide association study (GWAS) study on 10,158 patients with BO compared to 21,062 controls. They identified 2 novel SNPs pre-disposing to BO. The first is situated on 2p24 in close proximity to *GDF7*. *GDF7* encodes BMP12 which is part of the bone morphogenic protein signalling pathway. Dysregulation of this pathway has been implicated in

metaplastic transformation of Barrett's epithelium(86). The second is on 12q24 which is close to *TBX5*. Deficiency of *TBX5* can cause abnormalities in the diaphragmatic musculature and predispose to hiatus hernia and acid reflux, important factors in BO and subsequent progression to OADC. A further 4 SNPs, *CRTC1*, *BARX1*, *FOXP1*, and *ALDH1A2*, were identified which were associated with both BO and OADC in the study. Su et al (87) performed a GWAS study of BO with 1,852 cases and 5,172 controls which demonstrated 2 SNPs associated with BO tissues. The first is situated on 6p21 at the edge of the major histocompatibility (MHC) locus. The human leucocyte antigen (HLA) or MHC complex is hypervariable as this determines host versus foreign tissue for the individual's immune system. Due to the complexity of this region, it is difficult to make a robust connection between this SNP and OADC in all individuals. The second SNP on 16q24 lies adjacent to the *FOXF1* gene which is associated with oesophageal development. They also found evidence that the predisposition to BO was made up of multiple common variants of small effects rather than having a single genetic driver and SNPs associated with predisposition to obesity also carry increased risk for BO.

A subsequent meta-analysis of all genome wide association studies of BO and OADC was performed by Gharahkhani et al in 2016 (88). Four GWAS studies were included. The resultant study population included 6,167 cases of BO, 4,112 cases of OADC and 17,159 normal controls. Over 11 million SNPs for BO and 13 million SNPs for OADC were analysed. It identified 16 independent risk loci for BO and OADC. The SNPs reaching significance in the meta-analysis are detailed in Table 9.

Table 9 - Top SNPs from loci meeting the threshold for genome-wide significance in the combined Barrett's oesophagus and oesophageal adenocarcinoma meta-analysis. Table adapted from Gharahkhani et al 2016 (88)

SNP ID	Chr	Position*	Nearest gene or region	OR (95% CI)	p
rs7255	2	20878820	<i>GDF7 and LDAH</i>	1.14 (1.09–1.18)	9.1×10^{-11}
rs2464469	15	58362025	<i>ALDH1A2</i>	0.89 (0.85–0.92)	4.6×10^{-10}
rs17451754	7	117256712	<i>CFTR</i>	0.84 (0.80–0.89)	4.8×10^{-10}
rs17749155	8	10068073	<i>MSRA</i>	1.18 (1.12–1.24)	5.2×10^{-10}
rs10108511	8	11435516	<i>LINC00208 and BLK</i>	1.12 (1.08–1.16)	2.1×10^{-9}
rs2687202	3	70929983	<i>FOXP1</i>	1.13 (1.08–1.17)	2.3×10^{-9}
rs1247942	12	114673723	<i>LOC105369996 and TBX5</i>	0.89 (0.86–0.92)	2.3×10^{-9}
rs62423175	6	62195368	<i>KHDRBS2 and MTRNR2L9</i>	1.17 (1.11–1.23)	3.0×10^{-9}
rs9918259	5	663092	<i>TPPP and CEP72</i>	1.20 (1.13–1.27)	3.2×10^{-9}
rs9257809	6	29356331	<i>MHC region</i>	1.23 (1.14–1.31)	5.9×10^{-9}
rs7852462	9	100310501	<i>TMOD1</i>	0.89 (0.86–0.93)	1.5×10^{-8}
rs139606545	2	200045039	<i>SATB2</i>	0.90 (0.86–0.93)	2.0×10^{-8}
rs1979654	16	86396835	<i>LOC732275 and FOXF1</i>	0.90 (0.86–0.93)	3.3×10^{-8}
rs199620551	19	18804294	<i>CRTC1</i>	0.90 (0.87–0.93)	4.7×10^{-8}

The OCCAMS group have undertaken a study whereby whole genome sequencing of OADC was carried out as well as targeted sequencing of “never-dysplastic” BO i.e. that which did not progress to malignancy as well as high grade dysplasia(89). The aim was to investigate whether malignant progression potential could be identified using genetic biomarkers. A panel of 26 genes found to be mutated in OADC were selected following stringent filtering criteria to ensure functionally relevant mutations were targeted. 66 samples (n=40) which never progressed beyond NDBO were analysed using targeted amplicon sequencing. The median follow up time was 58 months (range: 4-132). 21 out of 40 patients were found to have mutations in one or more of the 26 pre-selected genes in their BO segment. These included mutations in *SMARCA4*, *ARID1A*,

and *CNTNAP5* which are known driver mutations in OADC. BO with HGD was examined in 43 samples (n=39) using the same genetic panel. 39 of 43 samples contained one or more mutations in the 26 candidate genes. The majority of the 26 genes did not demonstrate a significantly different mutational frequency between NDBO, HGD and OADC. The only two genes which were differentially mutated in the varying disease stages were *TP53* and *SMAD4*. *TP53* was found to be mutated in only one NDBO sample (2.5%), 72% of BO with HGD and 69% of OADC samples. *SMAD4* was only mutated in 13% of OADC cases, but uniquely in invasive OADC cases with no mutations found in BO with or without dysplasia. There is no data provided in the NDBO cohort as to the length of follow up in individual cases. It may be that those cases which have been followed up for a shorter period of time have not had the opportunity to develop dysplasia.

Given the OCCAMS findings above, it is difficult to use *TP53* mutation as a sole genetic biomarker of progression to adenocarcinoma as it has also been identified in the “never-dysplastic” group of patients who should not progress to adenocarcinoma. This would require further validation studies to prove efficacy. As a precursor to the OCCAMS study, the same group studied gene expression of tumour:normal pairs using microarray experiments (90). They found a pattern of copy number alterations and associated expression change that could identify poor prognosis OADC. Using RNA microarray experiments, the OCCAMS group also demonstrated that a 4 gene expression panel of *DCK*, *PAPSS2*, *SIRT2* and *TRIM44* were independently predictive of survival from oesophageal carcinoma (91).

1.21 GENETIC CHANGE IN OESOPHAGEAL ADENOCARCINOMA

Weaver et al (89) performed whole genome sequencing of 22 tumour/normal pairs, of which 17 had evidence of background BO in the resection specimen. The normal tissues were derived either from patient blood or normal squamous tissue from the oesophagus. They identified a median of 16,994 somatic SNVs (4,518-56,528) and 994 small indels per sample (262-3,573). 1,086 identified coding region mutations were subjected to ultra deep targeted amplicon resequencing, confirming that 1,081 (99.5%) were somatic. 23 of 25 indels were confirmed as somatic using Sanger sequencing. There is a predominance for T:A to G:C transversions in OADC in this study which differentiated the tumour from breast cancer and colorectal cancer which show a predominance for C to T mutations(92, 93). A panel of 26 genes were selected for targeted amplicon resequencing on the basis that they were mutated significantly above the background rate. These were also tested on a further validation cohort of 90 OADCs. *TP53* was the dominant mutation seen in OADC, present in 77 of 112 samples. 15 gene mutations were identified in 4 or more samples, these were *TP53* (n=77), *SMAD4* (n=13), *ARID1A* (n=13), *MYO18B* (n=13), *SEMA5A* (n=9), *CDKN2A* (n=9), *TRIM 58* (n=7), *CNTNAP5* (n=7), *ABCB1* (n=7), *PCDH9* (n=7), *SMARCA4* (n=7), *PNLIPRP3* (n=5), *UNC13C* (n=5), *TLR4* (n=4) and *CCDC102B* (n=4). Clonal analysis in the discovery cohort of 22 OADCs was undertaken in 15 of the genes which were found to be mutated in 4 or more samples. This revealed that *SMAD4*, *TP53*, *ARID1A*, *SMARCA4*, *TLR4*, *CDKN2A* and *PNLIPRP3* mutations were present in all cells of the tumours and as such, occur at the very early stage of neoplastic change. 8 other genes, *MYO18B*, *SEMASA*, *TRIM58*, *CNTNAP5*, *ABCB1*, *PCDH9*,

UNC13C and *CCDC102B* were found not to be present in the dominant clone and may have occurred later on in the progression of disease.

Both the Broad Institute and the OCCAMS group have studied OADC. The Broad Institute project undertook whole exome sequencing and whole genome sequencing in 149 tumour-normal pairs verifying previously identified mutations in *TP53*, *CDKN2A*, *SMAD4*, *ARID1A* and *PIK3CA* (94). They also found previously unidentified mutations in *SPG20*, *TLR4*, *ELMO1* and *DOCK2*, identifying a possible role for the RAC1 pathway (a modulator of epithelial-mesenchymal transition).

1.22 EPIGENETICS

Epigenetics is an emerging field which describes the alteration of gene regulation and expression without altering the underlying genetic code(95). These mechanisms are important in both the normal functioning of the transcriptome and in disease states and has not been previously extensively explored in Barrett's Oesophagus

1.23 MECHANISMS OF EPIGENETIC CHANGE AND REGULATION

1.23.1 CPG METHYLATION.

In mammalian DNA, “islands” rich in cytosine and guanine signal the start of genes and are referred to as promoter regions(96). The term CpG refers to a cytosine base followed immediately by a guanine base along the 5' to 3' single strand sequence, and can be used to distinguish between the CG base pairing across double stranded DNA. The “p” refers to the phosphate which links two nucleotides together along a single strand of DNA. DNA methylation occurs when DNA methyltransferase (DNMT) adds a methyl group (CH₃) to a DNA base. In humans the only known location where this is possible is a cytosine base creating 5-methylcytosine. This is thought to interfere with DNA transcription by projecting a methyl group into the DNA groove and physically blocking transcription factors from binding to the promoter region(97, 98). Thus, relative hypermethylation of a CpG region leads to reduced expression of that gene whereas relative hypomethylation leads to increased expression. TET enzymes act to demethylate 5-methylcytosine by sequentially oxidising the methyl group, eventually allowing 5-carboxylcytosine to be replaced by base excision-repair mechanisms(99). Mutations of the TET coding region and loss of function of TET proteins may be responsible for unique patterns of differential methylation in cancer genomes, and may also contribute deficiencies in genome repair mechanisms and increase genomic instability(99).

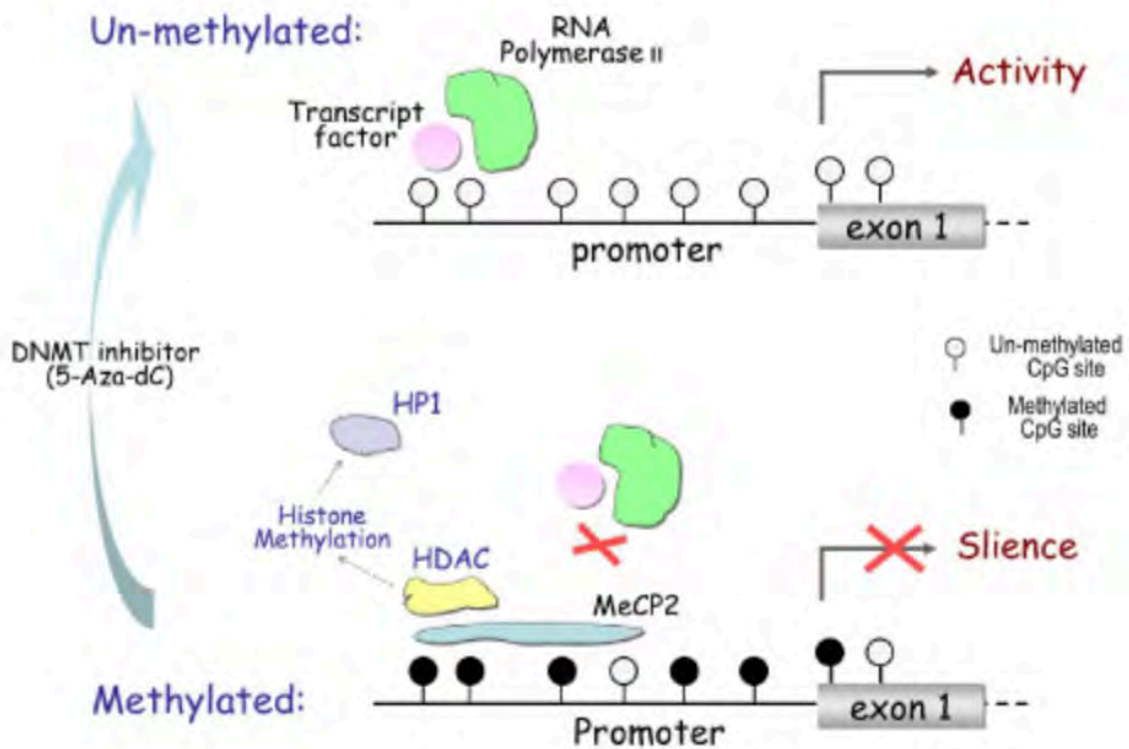


Figure 13 - Hypermethylation at the CpG promoter site blocks transcription factor binding by recruiting Methyl-CpG-binding protein 2 (MeCP2). It also recruits histone deacetylase (HDAC) which further recruits heterochromatin protein 1 (HP1) through histone methylation resulting in histone modification. Image taken from Zhu et al 2014 (98)

1.23.2 HISTONE MODIFICATION

Histone modification is a post translational alteration to histone proteins which package DNA into nucleosomes and eventually chromosomes by winding DNA around them. If the histone structure is altered, the DNA cannot be correctly unravelled and cannot be correctly transcribed.

1.23.3 NON-CODING RNAs

Many different types of non-coding RNAs have been discovered to alter gene expression by targeting coding messenger RNA (mRNA) after its transcription from DNA. Both micro RNAs (miRNA) and long non-coding RNAs have been implicated in gene regulation. These bind to mRNA molecules and cause them to be denatured and halt protein translation and cause genetic silencing(100).

1.24 CLINICAL SIGNIFICANCE OF EPIGENETIC REGULATION

These regulatory mechanisms are important in normal human development, for example silencing of one of the X-chromosomes in females by the X-inactive-specific transcript (XIST). This inactivates the duplicate X-linked genes by binding to the chromosome and blocking DNA transcription(101). Epigenetic change is important in numerous pathological processes both acquired and inherited. Epigenetic change should be erased during the production of specialised reproductive cells by “reprogramming” during meiosis, however some changes escape this and the residual change is passed transgenerationally, called genomic imprinting(102). Transgenerational epigenetic inheritance has been demonstrated in Angelman’s syndrome, Prader-Willi syndrome(103) and Beckwith-Wiedemann syndrome (associated with Wilm’s tumour of the kidney)(104) as a result of deregulation of imprinted genes. These conditions are particularly interesting as there is no deleterious effect due to the epigenetic

change in the affected parent, but the genomic imprinting results in a pathological state in the offspring.

Epigenetic change has been widely shown to have an acquired component much like genetic mutation and is effected by a number of environmental factors, some of which represent a modifiable risk to patients.

Cigarette smoking in particular has been shown to cause widespread change in DNA methylation. Besingi et al(105) demonstrated 95 differentially methylated sites between smokers and non-smokers using Illumina Humanmethylation 450K arrays. Nine of their sites also corresponded to differentially expressed genes in neonatal cord blood from children born to maternal smokers. Of these differentially methylated CpG sites, seven were located at 2q37.1 which is associated with increased breast cancer risk. In this gene locus, a number of alkaline phosphatase genes are clustered including ALPPL-2 which is over-expressed in pancreatic ductal adenocarcinoma, ovarian and testicular cancers (106). Shenker et al(107) also used Illumina Humanmethylation 450K arrays to demonstrate differential methylation in breast (n=92/92) and colon cancer (n=95/95) patients who smoked compared to non-smoking controls. 17 loci in breast cancer and 19 loci in colon cancer were hypomethylated in smokers when compared to non-smoking controls. Their study showed that hypomethylation at 2q37.1 was associated with developing breast cancer and estrogen receptor status. Their data supports earlier findings from Breitling et al (108) demonstrating hypomethylation at *F2RL3* in smokers. They were also able to correlate smoking intensity with methylation levels at *AHRR*. Current smokers

were hypomethylated at this locus. *AHRR* methylation in former smokers appeared to return to the level of patients who had never smoked. The study was unable to find an independent epigenetic risk factor in smoking in colon cancer patients due to inadequate study power.

Smoking is a well known risk factor in OSCC and head and neck squamous cell carcinoma (HNSCC) and results in both genome wide hypomethylation and histone-chromatin modification. The human papilloma virus (HPV) is a risk factor for HNSCC and infection with HPV appears to have an effect on tumour epigenetics. HPV positive cases display genome wide hypomethylation whilst HPV negative cases tend towards CpG promoter hyper-methylation(109)

Acquired epigenetic change has been implicated in numerous human cancers with a pattern of global hypomethylation demonstrated in ovarian, prostate, hepatocellular, cervical and colon cancer. It is also present in Wilms tumours and leukocytes from B cell chronic lymphocytic leukaemia (110). This global pattern of hypomethylation mirrors the findings of Agarwal et al which demonstrate this phenomenon in BO that progresses to OADC (111).

Epigenetic change drives carcinogenesis in ulcerative colitis (UC), an inflammatory condition of the colon which progresses through the metaplasia, dysplasia, carcinoma sequence in a similar fashion to oesophageal adenocarcinoma. Epigenetic change has been demonstrated in UC early in this process before neoplasia has occurred leading to the intriguing possibility of an epigenetic biomarker which can predict progression to cancer in these

patients(112). The Enhanced Neoplasia Detection and Cancer Prevention in Chronic Colitis (ENDCaP-C, ISRCTN81826545 (113)) trial has demonstrated that a panel of 11 epigenetic biomarkers increases accuracy in the detection of UC associated dysplasia and neoplasia in endoscopic biopsy samples(112). A prospective clinical trial is underway to validate whether it can be used in conjunction with screening colonoscopy to help risk stratify patients who are at a higher risk of progressing to adenocarcinoma.

Methylation biomarkers offer a number of advantages – they are stable in archival tissue, easy to analyse and are usually homogenous across a gene rather than being a spectrum across a gene, as in *TP53* mutations. Methylation microarrays provide a highly reproducible and quantitative analysis of the entire epigenome and will be discussed in more detail in the materials and methods section. Epigenetic change in Barrett's carcinogenesis could provide a method of risk stratifying patients who are at risk of progression to OADC.

2. THESIS AIMS

The aims of this thesis are:

1. To explore and summarize the literature on epigenetic change in BO that progresses to OADC to investigate whether there are viable epigenetic biomarkers that can risk stratify patients.
2. Validate hypomethylation at the OR3A4 CpG locus, as a novel biomarker to predict progression of BO to OADC.
3. Explore the functional relevance of differential expression of OR3A4 in vitro using cell models and the pathways by which it may affect carcinogenesis.
4. Investigate the expression of OR3A4 in tissue samples of patients who have progressed from BO to OADC using immunohistochemical staining as potential biomarker.
5. Assess the immunological landscape of BO which progresses to OADC using multispectral IHC.

Combining these modalities, the thesis aims to generate novel insight into the potential mechanisms of Barrett's carcinogenesis and develop a potential tissue biomarker for BO with a high risk of progression to OADC.

3. RESEARCH HYPOTHESIS

Previous research performed at the University of Birmingham demonstrates that hypomethylation of OR3A4 offers a potential epigenetic biomarker to predict which patients are likely to progress from NDBO to OADC(114). There is a clinical need for enhanced risk stratification of BO to facilitate intervention at an earlier disease stage and minimise the use of resectional surgery for the treatment of OADC. Diagnosis and treatment of OADC at an earlier disease stage confers improved patient outcomes in both survival benefit and reduction of peri-operative morbidity.

Hypomethylation of CpG promoter sites causes increased expression of the relevant gene. Hypomethylation at the OR3A4 CpG site should increase expression of the lncRNA OR3A4 in tissues.

I hypothesise that:

1. Hypomethylation at the OR3A4 CpG site in BO tissues can predict which patients are at high risk of progression from NDBO to OADC.
2. Hypomethylation at the CpG site of OR3A4 will cause increased expression in BO tissues.
3. BO tissue samples from patients that progressed to OADC will be differentiable from non-progressors by OR3A4 staining.
4. Due to the inflammatory aetiology of Barrett's carcinogenesis, progressor patient tissue samples will have a different immune microenvironment within the tissues.

5. OR3A4 over-expression will cause functional changes in Barrett's oesophagus cell lines in vitro and may affect downstream regulatory pathways controlling cell viability.

4. MATERIALS AND METHODS

4.1 PATIENT SELECTION

99 patients were identified for inclusion in the epigenetic study. 37 from Sandwell and West Birmingham NHS Trust (SWB), 30 from University Hospital Birmingham NHS Trust (UHB) and 32 from University Hospital Southampton NHS Foundation Trust (UHS). Of these, 38/99 progressed from non-dysplastic Barrett's oesophagus and 61/99 did not. The age range was between 42-60 years with a median age of 56 years of which (89.6%) were of male gender. The median time to diagnosis of OADC in "progressor" patients was 114 months, with a range of 14-162 months. In order for patients to be considered non-progressors they must have had NDBO biopsies at least 10 years apart with no reported dysplasia or OADC. Epigenetic analysis of the SWB patients was undertaken by MP Dilworth and presented in his MD Thesis(115). Of the patients recruited, 24 were taken forward for whole genome methylation array analysis. In the SWB group there were 6 progressors and 6 non-progressors and in the UHB group there were 6 progressors and 6 non-progressors, giving a total of 12 progressors and 12 non-progressors. All 32 patients from the UHS external cohort (14 non-progressors and 18 progressors) and 34 samples from the UHB cohort (21 non-progressor, 13 progressor) underwent validation pyrosequencing using the Q48 instrument. 40 Samples identified from Aberdeen Royal Infirmary (27 non-progressor and 13 progressor) could not be used for epigenetic analysis due to poor or unrecordable DNA yields from the FFPE samples. 20 tissue samples (14

progressor, 6 non-progressor) from 12 patients were taken forward from the UHB cohort for IHC staining for OR3A4.

4.2 SAMPLE HANDLING AND LAB SAFETY

Standard laboratory safety procedures were adhered to and personal protective equipment was used whenever experiments were ongoing including a lab coat, single use gloves and eye protection. All sample Eppendorf tubes were autoclave sterilised to avoid external DNA and RNA contamination. The laboratory bench was decontaminated with 70% ethanol between each sample to avoid cross contamination.

4.3 FFPE SAMPLE PREPARATION

Formalin fixed, paraffin embedded tissue samples were sectioned to 8 micron thickness using a microtome and mounted on to glass microscope slides for macrodissection. Macrodissection was performed using a sterile scalpel blade and areas of histological interest were transferred to an Eppendorf tube. When macrodissection was not required blocks were sectioned into tissue scrolls and placed directly into an Eppendorf tube.

4.4 DNA EXTRACTION METHODS

4.4.1 MAXWELL RSC INSTRUMENT (PROMEGA)

The Maxwell RSC Instrument is a magnetic liquid handling robot which provides a semi-automated method for DNA extraction of up to 16 samples simultaneously. It uses paramagnetic beads in a pre-prepared cartridge to bind the DNA. Numerous kits for differing sample types are available to laboratory researchers. DNA was extracted for this thesis from FFPE samples and blood using the kits Maxwell RSC DNA FFPE Kit (AS1450) and Maxwell RSC Blood DNA Kit (AS1400). In addition to the instrument, DNA extraction required a microcentrifuge, vortexer, pipettes and tips and heat blocks set to 56°C and 80°C.

4.4.2 MAXWELL RSC DNA FFPE KIT (AS1450)

300 µL mineral oil was added to FFPE Tissues for extraction and vortexed for 10 seconds and the samples subsequently heated to 80°C for 2 minutes to melt the paraffin. A master mix of lysis buffer (224 µL x (n + 2)), proteinase K (25 µL x (n + 2)) and blue dye (1 µL x (n + 2)) was prepared during the sample heating. 250 µL of the master mix was added to each sample and vortexed for 5 seconds and centrifuged at 10,000 x g for 20 seconds to separate the layers. If a pellet was present in the lower blue aqueous layer it was pipette mixed, taking care not to disrupt the layers. The samples were then incubated at 56 °C for 30 minutes for proteinase K digestion and then at 80 °C for 4 hours. After the samples had cooled for 5 minutes, 10 µL RNase A was added to the blue aqueous layer and pipette mixed. This was left to incubate at room temperature for 5 minutes while the instrument cartridges were prepared. The deck tray was removed from the

instrument and one cartridge for each sample clicked into the deck tray. The foil seal was removed, and a plunger placed into well number 8 and a sterile 0.5 ml elution tube placed in the corresponding deck tray hole. 50 μ L nuclease free water was added to each elution tube. Following the room temperature incubation, the samples were centrifuged at full speed for 5 minutes to ensure separation of the paraffin and aqueous layer containing lysed tissue. The blue aqueous layer containing the sample was transferred to well number 1 of the corresponding cartridge and the deck tray replaced in the instrument. The relevant protocol was selected and run. The program was approximately 40 minutes in duration.

4.4.3 MAXWELL RSC BLOOD DNA KIT (AS1400)

The same additional lab equipment as above is required for extracting DNA from whole blood samples. Blood samples at room temperature in citrate tubes were mixed by inverting the tubes for 5 minutes to avoid any separation of blood components. 30 μ L Proteinase K was added to an Eppendorf tube for each sample and 300 μ L liquid blood for DNA extraction was added to the Eppendorfs. 300 μ L lysis buffer was added to each sample and vortexed. The samples were subsequently incubated at 56 °C for 20 minutes. The cartridges were prepared as described above for FFPE extraction. Each blood lysate sample was transferred into well number 1 of the corresponding cartridge and the deck tray replaced in the instrument and the relevant protocol selected and run.

4.4.4 QIAGEN DNEASY (69504)

The Qiagen DNeasy spin column DNA extraction method is a simple and relatively cheap method of extracting DNA from organic material using a silica based membrane. The manufacturer's protocol was modified to allow extraction of FFPE samples. In addition to the supplied kit, a microcentrifuge, 100% ethanol, a heat block or incubator set to 56 °C and pipettes and tips were required for this protocol.

180 µL Buffer ATL was added to each FFPE sample for extraction. 360 µL was used if tissue scrolls were used as the samples were not completely immersed with the smaller volume. 20 µL proteinase K was added and the samples were briefly vortexed and centrifuged. The samples were subsequently incubated at 56 °C for 24 hours with intermittent vortexing. Once the proteinase K digestion was complete the samples were vortexed and immediately centrifuged at 10,000 rpm for one minute to separate the paraffin from the lysed tissue sample. The sample was incubated at room temperature for 5 minutes or until a wax plug had formed. This was then pierced with a sterile pipette tip and a fresh pipette tip used to aspirate the liquid sample. The manufacturer's protocol was followed from this point onwards. 200 µL Buffer AL and 200 µL 100% ethanol were added to each sample and vortexed thoroughly. The mixture was transferred to a DNeasy spin column mounted in a collection tube and centrifuged for 1 minute at 8,000 rpm. The collection tube was replaced and the flow through discarded. The DNA remains bound to the spin column membrane until the elution step. 500 µL Buffer AW1 was added to the spin column and centrifuged for 1 minute at 8,000 rpm. The collection tube was again replaced and 500 µL buffer AW2 was added to the

spin column and centrifuged for 4 minutes at maximum speed. This step dries the membrane and avoids ethanol carry over to the elution tube. The spin column was then mounted in a sterile 1.5 ml Eppendorf tube for elution and 100 μ L Buffer AE added and centrifuged for 1 minute at 8,000 rpm. The elution step was repeated once more to increase total DNA yield.

4.4.5 QIAGEN QIAAMP

This kit uses a similar silica based spin column to the Qiagen DNeasy kit, but it includes a manufacturer supported protocol specifically for FFPE samples. In addition to the supplied kit, xylene, phosphate buffered saline, 100% ethanol, 1.5ml Eppendorf tubes, pipettes and tips, a microcentrifuge, vortexer, and 2 heat blocks set at 56°C and 90 °C were required. Steps involving xylene handling were performed in a fume hood.

1ml xylene was added to each sample, vortexed and centrifuged for 5 minutes at maximum speed to dissolve the paraffin and pellet the sample material. The supernatant was removed with a pipette taking care not to disturb the pellet. 1ml 100% ethanol was then added, the samples vortexed and centrifuged for 2 minutes at maximum speed. The residual ethanol and xylene were aspirated from the sample and the incubated at 37 °C with the lids open to allow the remaining ethanol to evaporate. The dry sample pellets were resuspended in 180 μ L Buffer ATL, 20 μ L Proteinase K was added and vortexed. The samples were incubated at 56 °C for 90 minutes to allow proteinase K digestion and then incubated at 90 °C for 60 minutes. Following this incubation the samples were briefly centrifuged and 200 μ L Buffer AL and 200 μ L 100% ethanol were added and the samples

vortexed thoroughly. The mixture was transferred to a QiaAMP spin column mounted in a 2 ml collection tube and centrifuged for 2 minutes at 8,000 rpm. The collection tube was replaced and the flow through discarded. 500 μ L Buffer AW1 was added to the column and centrifuged for 2 minutes at 8,000 rpm and the flow through discarded. 500 μ L Buffer AW2 was added and centrifuged for 2 minutes at 8,000 rpm and the flow through discarded. The membrane was thoroughly dried by centrifuging at maximum speed for 3 minutes. The spin column was placed in a sterile 1.5 ml Eppendorf tube and 100 μ L Buffer ATE added to the spin column. The columns were incubated at room temperature for 5 minutes and then centrifuged at maximum speed for 1 minute. The elution step can be repeated to increase DNA yields but it was not required for these purposes.

4.5 RNA EXTRACTION METHODS

4.5.1 QIAGEN RNEASY

Cellular RNA was extracted using the Qiagen RNeasy Mini Kit (74104). In addition to the supplied kit, a microcentrifuge, 70% ethanol, RNase free water, pipettes and tips, β -Mercaptoethanol, Qiagen QiaShredder (79654) cell and tissue homogeniser kits and Qiagen RNase-Free DNase (79254) were required for this protocol. The maximum input is 1×10^7 cells to avoid overloading the spin columns. The cells were counted using the BioRad TC20 automated cell counter to ensure the maximum number of cells was not exceeded. The cells in

suspension were centrifuged for 5 minutes at 200 x g to form a pellet. The old media was removed using a pipette. 600 µL Buffer RLT with 10 µL/ml β-Mercaptoethanol was added to each sample. The lysate was transferred into a QiaShredder cell and tissue homogeniser spin column mounted in a collection tube. The QiaShredder spin column is then discarded. This was centrifuged for 2 minutes at maximum speed. Add 600 µL of 70% ethanol to the homogenized lysate and pipette mix. 700 µL of the sample was then transferred into a RNeasy spin column mounted in a fresh collection tube and centrifuged at maximum speed for 15 seconds. The flow through was discarded. The remaining sample was then added to the spin column and the centrifugation step repeated. DNase treatment on the spin column was performed with Qiagen RNase-Free DNase (79254). 350 µL of buffer RW1 was added to the spin column, centrifuged at maximum speed for 15 seconds and the flow through discarded. For each sample a mixture of 10 µL DNase I stock solution was added to buffer RDD and the enzyme mixture added to the spin column membrane and incubated at room temperature for 15 minutes. 350 µL of buffer RW1 was added to the spin column and centrifuged at maximum speed for 15 seconds. The flow through was then discarded. 500 µL of buffer RPE was added to the spin column and centrifuged at maximum speed for 15 seconds. The flow through was discarded and a further 500 µL buffer RPE added to the column and repeat centrifuged, discarding the flow through. A further centrifugation of the samples with a fresh collection tube and no additional reagents was performed to ensure the spin column membrane was dry. RNA was eluted using 50 µL RNase free water and transferred

immediately to a -80 °C freezer for storage. The elution step was repeated to maximize RNA yield.

4.5.2 INVITROGEN PURELINK FFPE RNA EXTRACTION KIT (K156002)

The Invitrogen Purelink FFPE RNA Extraction Kit (K156002) was used to extract RNA from FFPE samples. In addition to the supplied kit, RNase free water, 100 % ethanol, heat blocks set to 60 °C and 72 °C, 1.5 mL Eppendorf tubes, pipettes and tips, a microcentrifuge and vortexer were required. 300 µL of melting buffer was added to each sample in an Eppendorf tube and briefly pulse centrifuged to ensure the sample was covered. The samples were incubated for 10 minutes at 72 °C with intermittent flicking to mix. Following the incubation, 20 µL proteinase K was added to the sample, pipette mixed and incubated for 60 minutes at 60 °C with intermittent mixing. After the incubation, the samples were immediately centrifuged for 1 minute at full speed to separate the paraffin. The paraffin layer was pierced with a pipette tip and the lysate transferred into a fresh Eppendorf tube. 400 µL Binding Buffer L3 and 800 µL 100 % ethanol was added to each sample and vortex mixed. The sample was transferred 700 µL at a time into the provided spin column mounted in a collection tube and centrifuged for 1 minute at 800 x g. If any sample remained in the spin column, the centrifugation step was repeated. The flow through was discarded. 500 µL wash buffer W5 was added to the spin column and centrifuged for 1 minute at maximum speed. The flow through was discarded. A total of 3 washes were undertaken and a final centrifugation step was performed to dry the membrane with no wash buffer added to the column. The spin column was then mounted in a 1.5 mL Eppendorf

tube for elution. RNA was eluted by adding 50 μ L RNase free H₂O which had been pre-heated to 65 °C and centrifuged at maximum speed for 1 minute. The elution step was repeated to maximize RNA yield and the RNA transferred immediately to a -80 °C freezer for storage.

4.6 DNA METHYLATION METHODS

4.6.1 CPG METHYLTRANSFERASE (M.SssI - NEW ENGLAND BIOLABS M0226S)

Methylation of Genomic DNA was undertaken using CpG Methyltransferase (M.SssI - New England BioLabs M0226S). Blood DNA with a concentration of 244 ng/ μ L was used to create 100% methylated control DNA. In addition to the supplied kit, pipettes and tips, a vortexer and 2 heat blocks set to 37 °C and 65 °C were required. Up to 4 μ g DNA can be methylated in a 20 μ L reaction, but for the more dilute DNA used in this experiment, a 50 μ L reaction was required. For a 50 μ L reaction volume, the SAM was diluted to 1600 μ M (kit supplied at 32mM) by adding 2 μ L SAM to 38 μ L of nuclease free water. The following were added in order to an Eppendorf tube: 35 μ L nuclease free water, 5 μ L 10X NE Buffer 2, 5 μ L SAM 1600 μ M and 4 μ L Genomic DNA (976 ng). This mixture was pipette mixed and incubated at 37 °C for 2 hours. The reaction was stopped by heating the mixture to 65 °C for 20 minutes.

4.6.2 QIAGEN REPLI-G MINI KIT (150023)

The Qiagen Repli-g Mini Kit (150023). This kit performs whole genome amplification from genomic DNA. This was used to produce de-methylated genomic DNA. In addition to the supplied kit, a microcentrifuge, vortexer, pipettes and tips, Eppendorf tubes, heat block set at 30 °C, ice and nuclease free water were required. The reaction requires greater than 10 ng genomic DNA input. Extracted blood DNA with a starting concentration of 300 ng/μL was diluted with nuclease free water to a final concentration of 30 ng/μL. The DNA polymerase was thawed on ice. In the meantime, Buffer DLB was prepared by adding 500 μL nuclease free water to the tube and mixed thoroughly. Buffer D1 (denaturation) was prepared by adding 9 μL of reconstituted Buffer DLB to 32 μL nuclease free water. Buffer N1 (neutralization) was reconstituted by adding 12 μL stop solution to 68 μL nuclease free water. This gave enough buffer to perform 15 reactions. 2.5 μL buffer D1 was added to 2.5 μL genomic DNA, vortexed and pulse centrifuged and the mixture incubated at room temperature for 3 minutes. 5 μL Buffer N1 was added and the mixture vortexed and pulse centrifuged. The PCR master mix was prepared by adding 10 μL nuclease free water, 29 μL reaction buffer and 1 μL DNA polymerase. 10 μL of denatured DNA was added to this 40 μL master mix. The PCR was incubated at 30 °C for 12 hours and the reaction inactivated by heating to 65 °C for 3 minutes.

4.7 DNA AND RNA QUANTIFICATION

4.7.1 QUaBIT

The QuBit Fluorometer 2.0 and 3.0 instruments (ThermoFisher) were used to quantify DNA and RNA. The QuBit fluorometer utilises fluorescent dyes which are specific to the target of interest and only emit when they are bound to the target. The dsDNA BR and RNA BR kits were used to quantify DNA and RNA. Prior to running the quantification assay 1:200 solutions of assay specific reagents were made up to create a working solution. All reactions were performed in 0.5 ml PCR tubes. 10 μ L of the standards provided with the kit (positive and negative control) were added to 190 μ L of the working solution and read on the fluorometer. Once completed 1 μ L of the sample was added to 199 μ L working solution and read by the instrument in triplicate. This provided a ng/ μ L sample concentration.

4.7.2 AGILENT TAPESTATION 2200

The Agilent TapeStation 2200 is an automated DNA and RNA electrophoresis instrument which can provide rapid sample quality control including DNA fragment length (Figure 14). The D1000 ScreenTape (5067-5582) was used for DNA analysis RNA ScreenTape (5067-5576) was used for RNA analysis. The associated reagents, D1000 sample buffer (5067-5602) and D1000 ladder (5067-5586) and RNA ScreenTape Ladder (5067-5578) and RNA ScreenTape Sample

Buffer (5067-5577) were used. All reagents were allowed to equilibrate to room temperature prior to analysis. For DNA QC, 3 uL of D1000 sample buffer and 1 uL DNA sample were added to a sample tube and vortexed for 1 minute and spun down. The samples were transferred to the Tapestation instrument and software analysis started. For RNA QC, 5 uL RNA sample buffer and 1 uL RNA were added to a sample tube, vortexed for 1 minute and spun down. The sample and buffer mix was heated for 3 minutes at 72°C and then placed on ice for 2 minutes. The samples were then transferred to the Tapestation instrument for analysis.

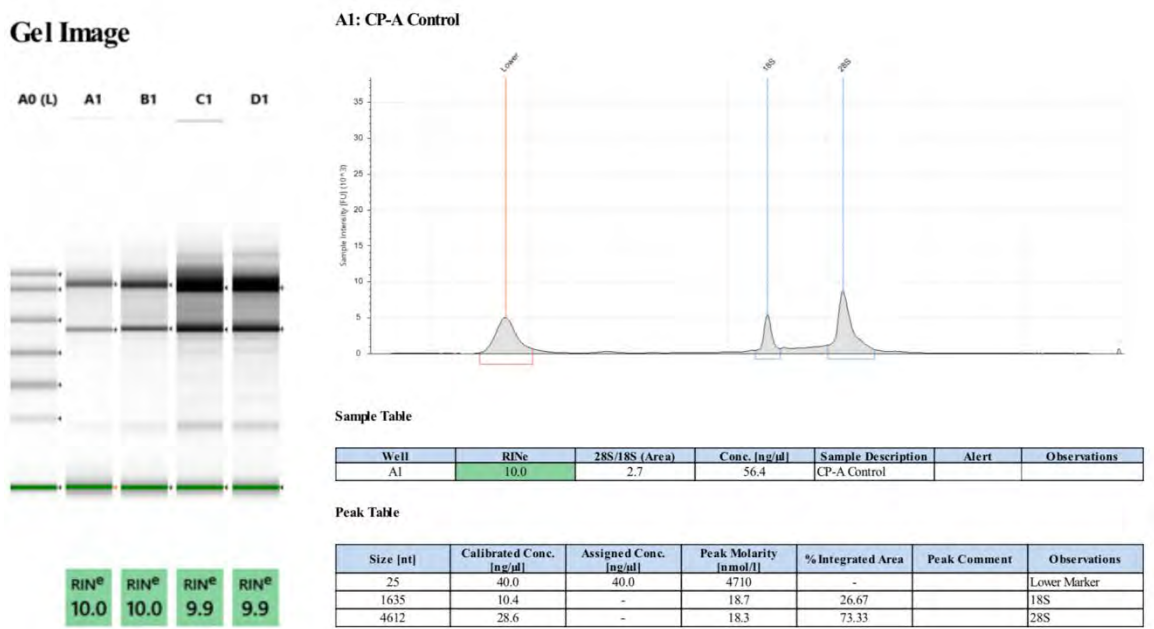


Figure 14 – Tapestation RNA screen tape quality control for RNA from CP-A cells in culture. RNA extracted with Qiagen RNeasy kit.

4.7.3 PCR AND GEL ELECTROPHORESIS

DNA was extracted form biopsy samples from Aberdeen as described above. Due to unreadable concentrations of DNA on the QuBit instrument, a PCR of the APOE 2 gene was performed to amplify any extracted DNA which may have been

present. 10 samples were processed including positive and negative controls. The master mix was prepared by combining 20 μL Buffer and MgCl_2 , 4 μL dNTP, 134 μL nuclease free water, 2 μL Taq polymerase, 10 μL forward and 10 μL reverse primers and 10 μL DMSO to aid DNA strand separation in G-C rich areas (Table 10). 19 μL master mix and 1 μL sample was added to a 96 well PCR plate and cycled according to the conditions listed in Table 11.

Table 10 PCR conditions for validation of DNA extraction

Reagent	Volume (μL)
<i>Buffer and MgCl_2</i>	20
<i>dNTP</i>	4
<i>Nuclease Free H_2O</i>	134
<i>Taq Polymerase</i>	2
<i>Forward Primer</i>	10
<i>Reverse Primer</i>	10
<i>DMSO</i>	10

Table 11 Thermal Cycling Conditions for APOE-2 PCR

Step	Temperature (°C)	Time (minutes:seconds)
Hot Start	95	15:00
Denaturation	95	00:30
Annealing	60	00:30
Extension	72	00:30
Cycle Repeats	29x	
Final Extension	72	5:00
Hold	4	Infinite

4.7.4 GEL ELECTROPHORESIS

A 1% agarose gel was made by adding 50 mL 1 x TBE Buffer, 0.5 g gel agarose and 2.5 µL gel red and heated in the microwave to dissolve the agarose. Care was taken not to allow the mixture to boil over as the lid was left loose to avoid the beaker cracking. Once the mixture was fully dissolved, it was cooled under cold running water to avoid the hot gel cracking the gel mould. The gel was poured into the gel mould with the sample comb in place and allowed to set for 20 minutes. The comb was removed and the tank filled with 1 x TBE buffer to cover the gel entirely. A genomic ladder was loaded into the first well as a reference. A small dot of loading dye, roughly 1 µL, for each sample was pipetted on to a clean piece of parafilm. 4 µL of the sample was added to the loading dye, pipette mixed and immediately loaded into the corresponding well on the gel. The

cover was attached and the electrodes attached. The gel was run at 120 V for 30 minutes. The gel was imaged on the gel dock UV imaging system.

4.8 PYROSEQUENCING

Pyrosequencing is a semi quantitative method for determining a genetic sequence using the sequencing by synthesis principle. The pyrosequencing instrument introduces and removes individual nucleotides sequentially into the reaction mixture containing single stranded sample DNA in the presence of the enzymes DNA polymerase, ATP sulfurylase, firefly luciferase and apyrase. When the correct nucleotide is introduced for DNA sequence extension, DNA polymerase attaches the new nucleotide base to the complementary DNA strand and releases pyrophosphate. This is then converted to ATP by ATP sulfurylase. ATP is used as a substrate to convert luciferin to oxyluciferin which generates visible light which can be detected by a camera on the pyrosequencing instrument. The amount of light generated is proportional to the amount of ATP, and the intensity of light can determine whether one or more nucleotides have been added to the DNA strand. The instrument generates a pyrogram (Figure 15) for analysis.

When sample DNA is bisulphite converted, as described in section 4.8.1, differences in the genetic sequence arise, and the intensity of light generated when specific nucleotides are introduced into the reaction mixture differ and can give a methylation percentage at a particular CpG site of interest when methylation specific primer sequences are used in conjunction with the Qiagen Pyromark software.

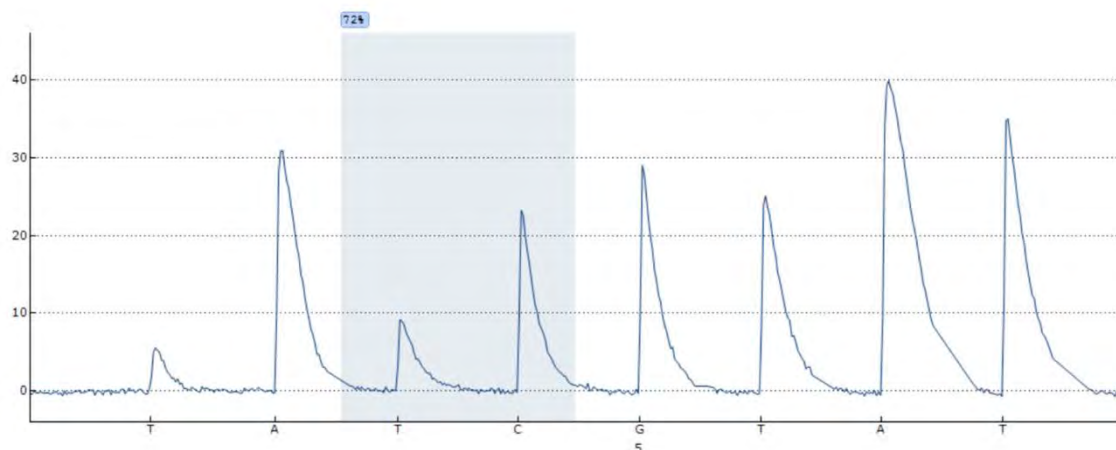


Figure 15 - Pyrogram Generated by the Qiagen Q48 Instrument Showing 72% Methylation at OR3A4 cg07863524

4.8.1 BISULPHITE CONVERSION

Analysis of CpG methylation as an epigenetic change relies on bisulphite conversion. During bisulphite conversion, unmethylated cytosine bases are converted to uracil, whereas methylated cytosine (5-methylcytosine) is protected from the conversion process and will remain unchanged. For genetic sequencing purposes uracil functions as a thymine base and complementary binds adenine. It is this change in the genetic sequence of interest by the bisulphite conversion reaction that allows methylation percentage to be analysed.

The Zymo EZ DNA and EZ-96 DNA Methylation Kits (D5001, D5004) were used to bisulphite convert sample DNA. The experimental protocol is the same for both kits, the only difference is that the EZ-96 is in 96 well plate format. The protocol has been optimized for use with Illumina Infinium Humanmethylation whole genome Bead Chip Arrays.

The CT conversion reagent was prepared by adding 7.5 mL of nuclease free water and 1.85 mL M-dilution buffer to the CT conversion reagent powder. The CT conversion reagent was mixed thoroughly and incubated for 10 minutes at room temperature with frequent vortexing. All samples were normalized to give 500 ng DNA in 42.5 μ L volume by diluting them with nuclease free water. Any sample with less than 500 ng DNA was not diluted. 7.5 μ L M-Dilution buffer was added to each sample and pipette mixed. The samples were incubated at 42 °C for 30 minutes. Following the incubation, 97.5 μ L of the prepared CT conversion reagent was added to the samples and pipette mixed. The samples were pulse centrifuged and incubated in the dark on a thermal cycler overnight with 16 cycles of 95 °C for 30 seconds and 50 °C for 60 minutes. The samples were then placed on infinite hold at 4 °C. Once the thermal cycling was complete 400 μ L M-Binding Buffer was added to the spin column (EZ DNA D5001) or binding plate (EZ-96 DNA, D5004). The samples were loaded into the plate and pipette mixed. The samples on the plate were centrifuged for 5 minutes at 3,000 g and the flow through discarded. 500 μ L M-wash buffer was added to the plate and centrifuged at 3,000 x g and the flow through discarded. 200 μ L M-Desulphonation Buffer was added to the plate and incubated at room temperature for 20 minutes. It was important not to leave the buffer in contact with the samples for more than 20 minutes and extra care was required when handling this hazardous reagent. Following the incubation the samples were centrifuged at 3,000 g for 5 minutes and the flow through discarded. 2 further washes were performed by adding 500 μ L M-wash buffer to the samples and centrifuging at 3,000 g. The first wash should be centrifuged for 5 minutes and the second for 10 minutes, with the flow

through discarded after each centrifugation. The binding plate was then placed on an elution plate and 15 μ L M-Elution Buffer added to each sample taking care to add the elution buffer directly to the membrane in the binding plate. The samples were centrifuged at 3,000 g for 3 minutes and the elution stored at -20 °C for downstream applications.

4.8.2 BISULPHITE PCR

Bisulphite PCR was used to amplify bisulphite converted regions of interest for use in downstream bisulphite pyrosequencing. Bisulphite conversion of sample DNA was performed as previously described in section 4.8.1.

Two regions of the OR3A4 gene were selected (cg 09890332 and cg07863524) for amplification. Methylation insensitive primers were designed to optimise amplicons for FFPE pyrosequencing at 80-150 base pairs in length. Primer design was performed using Qiagen PyroMark Primer Design software v2.0. Primers were designed to flank CpG sites of interest. All other settings were as per the standard Qiagen design parameters. Primers were ordered from Sigma Aldrich Oligos, with the biotinylated pyrosequencing primer being purified by high performance liquid chromatography. PCR was performed using the Qiagen Pyromark PCR kit. 50 μ L PCR reaction volumes were made up as shown in Table 12 and placed on a thermal cycler with the conditions detailed in Table 13

Table 12 Qiagen Pyromark PCR Kit Reaction

Reagent	Volume (μL)
Master Mix	25
Coral Load Dye	5
MgCl ₂	3
Q Solution	10
Forward Primer (100 mM)	2.5
Reverse Primer (100 mM)	2.5
DNA	2
Total Volume	50

Table 13 Qiagen Pyromark PCR Thermal Cycling Conditions

Step	Temperature (°C)	Time (minutes:seconds)
Hot Start	95	15:00
Denaturation	94	00:30
Annealing	56	00:30
Extension	72	00:30
Cycle Repeats	45x	
Final Extension	72	10:00
Hold	4	Infinite

4.8.3 QIAGEN Q48 AUTOPREP

The Qiagen Q48 Autoprep is a pyrosequencing instrument which automatically anneals biotinylated PCR products to magnetic beads without the need for complex library preparation workflows. It uses pyrosequencing which allows sequencing by synthesis for quantitative analysis of DNA strands, and with bisulphite PCR it can calculate CpG methylation percentage. The instrument is largely automated with easy to follow on screen instructions. Briefly, the instrument was switched on and the pre-programmed CpG assay software with the sequence of interest to be analysed was loaded. Following the on-screen instructions, an absorber strip and dNTPs, enzyme and substrate were added to the corresponding cartridge wells according to the volumes instructed on the instrument screen. The injector covers were closed and locked. Qiagen magnetic beads were thoroughly mixed by vortexing and 3 μ L added to each well on the Q48 sample disc to be analysed. 10 μ L biotinylated PCR product was added to each well containing beads. If there was not enough product available, the total volume was made up to 13 μ L with nuclease free H₂O. The run was then started by following the on screen instructions.

4.8.4 QIAGEN Q96

The Qiagen Q96 is a pyrosequencing instrument which can analyse up to 96 samples on a single plate. It does not possess the automatic sample preparation qualities of the Q48 instrument and requires larger sample loading volumes.

40 μ L Bisulphite PCR Product was loaded on to 96 well sample plate and 3 μ L Streptavidin beads and 37 μ L binding buffer added to each well for analysis. The plate was vortex mixed for 5 minutes at 1,300 rpm. Using a pyrosequencing plate, 1.5 μ L Sequencing Primer and 38.5 μ L annealing buffer was added to each well. The beads which the samples were annealed to were prepared using the Qiagen hedgehog vacuum device. Firstly, the prongs were washed in H₂O and lowered into the 96 well sample plate to capture the beads using vacuum suction. In sequence, with the vacuum on, the beads were washed for 5 seconds in 70% Ethanol, 0.2 M NaOH for denaturation and finally Qiagen Pyromark wash buffer (979008). The beads on the tips of the hedgehog prongs were lowered carefully into the pyrosequencing plate, taking care not to submerge the prongs into the sequencing solution with the vacuum on. Slight agitation was required to encourage the beads to fall into the sequencing solution. The plate was heated to 80 °C for 2 minutes using a heat block. Once the plate had cooled to room temperature, the plate was transferred to the Q96 instrument and the run performed. The pre-determined sequence for analysis was loaded into the software. The dispensing cartridge was placed into the instrument and charged with the volumes of enzyme, substrate and dNTPs (Pyromark Gold Q24 Reagents (970802)) as instructed by the Pyromark software. Once the pyrosequencing plate and reagents were loaded, the sequencing run was commenced.

4.9 ILLUMINA INFINIUM HUMAN METHYLATION 450K ARRAYS

The Illumina Infinium Humanmethylation 450K bead chip array is read on the Illumina iScan instrument. This platform is capable of performing whole genome methylation analysis on bisulphite converted genomic DNA. DNA fragments anneal to CpG locus specific beads mounted on the chip. The chip contains two beads for each CpG locus, one methylated, one unmethylated. Single nucleotide extension of the oligonucleotides bound to the beads generate fluorescence which is translated into a methylation percentage for each CpG site. The 450K arrays contain 450,000 unique CpG loci for genome wide methylation analysis. Genomic DNA extracted from FFPE samples was bisulphite converted using the Zymo EZ DNA Methylation Kit as described in section 4.8.1. Ideally 500 ng of input DNA is used, however due to the nature of the FFPE samples used for analysis, often this was not achievable, however lower DNA input amounts are feasible if the FFPE restore kit was used. Samples were optimised for array analysis using the Illumina Infinium FFPE Restore Kit (WG-321-1002) which uses enzymatic reactions to repair some of the nucleotide degradation and base alterations which occur with FFPE tissue samples. Once restored, the Infinium protocol was followed, samples were pre-amplified and then denatured to give single stranded DNA ready for isothermal DNA amplification for 24 hours at 37°C. The amplified DNA was fragmented to allow hybridisation of the sample to the corresponding bead on the array. The DNA was precipitated in isopropanol, centrifuged to form a pellet and re-suspended in hybridisation buffer. The samples were loaded on to the bead chip and hybridised at 48 °C for 20 hours. The bead chips were mounted in a flow through chamber for ease of washing.

The arrays were washed with RA1 reagent to remove unhybridised and non-specifically hybridised DNA from the array. The array underwent single base extension using TEM reagent which incorporates labelled nucleotides into the extended primers. Formamide/EDTA was used to remove the hybridised DNA and the arrays neutralised using XC3 reagent. Multi-layer staining was performed using Illumina reagents using a detectable red (un-methylated) or green (methylated) fluorescent dye label. The bead chip was read on the iScan device to give a methylation percentage for each CpG site. Methylation data was analysed using Illumina BaseSpace software.

4.10 CELL CULTURE

Cell culture was performed to analyse cell models and response to OR3A4 over-expression. HEK 293T, CP-A, OE-19, OE-21, OE-33 and MFD-1 cells were cultured during the period of study. More detail of individual cell lines and media requirements are shown in Table 14. All cells lines were incubated at 37°C with 5% injected CO₂. The cell lines were split twice weekly at 90% confluence which provided an opportunity to refresh the media. All cell culture work was performed using aseptic technique in a sterile cell culture hood with sterile pipettes and flasks. All media was supplemented with 1 % penicillin/streptomycin antibiotic.

4.10.1 TECHNIQUE FOR SPLITTING ADHERENT CELL LINES

Adherent cell lines were split by first removing the old culture medium and the confluent cell monolayer washed with sterile PBS to inactivate any remaining serum in the media. For T75 flasks, 2 ml of 1x Trypsin EDTA was added to dissociate the cells and the flasks were returned to the incubator for approximately 10 minutes until the cells had completely detached from the flask. This was confirmed using light microscopy. A trypsin wash was performed by adding 5 ml fresh media to the flask to inactivate the trypsin and the cell suspension was centrifuged for 5 minutes at 200 x g at room temperature to form a cell pellet. The supernatant was carefully aspirated taking care not to disturb the cell pellet. Fresh media was added to re-suspend the pellet, if a 1:3 split was required 3 ml media was added to re-suspend and 1 ml of this fresh suspension was added to a new culture flask. The flask was then topped up with fresh media (12 ml for a T75) and returned to the incubator.

4.10.2 TECHNIQUE FOR SPLITTING NON-ADHERENT CELL LINES

Cell culture flasks were removed from the incubator and transferred to a tissue culture hood. The cells were re-suspended by pipette mixing the media and then transferred to a sterile centrifuge tube. The cells were counted on the BioRad TC20 as described below. The cells were centrifuged for 5 minutes at 400 x g to form a pellet. The supernatant was aspirated taking care not to disturb the cell pellet. For a 1:3 split, the pellet was re-suspended in 3 ml of fresh media. 1 ml of the new cell suspension was transferred to a new, sterile culture flask. The flask

was topped up with fresh media according to its size (5-10 ml T25, 12-24 ml T75). The cells were returned to the incubator.

4.10.3 EZ PCR MYCOPLASMA TEST

To ensure cell lines for experimental use were free of mycoplasma, the EZ-PCR kit was used (Biological Industries 20-700-20). 1 mL cell supernatant was placed into a microcentrifuge tube and cellular debris pelleted by spinning at 250 g for 1 minute. The cell debris was discarded and the resultant supernatant was transferred into a fresh tube and spun at maximum speed for 10 minutes to pellet mycoplasma. The supernatant was discarded and the pellet re-suspended with 50 uL supplied buffer solution and heated to 95°C for 3 minutes. 5 uL of the test sample was added to 35 uL nuclease free water and 10 uL of the supplied reaction mix. Positive and negative controls were run simultaneously to the test samples. These were placed in a thermal cycler and cycled as per Table 15. 20 uL of the PCR product was added to a 2% agarose gel and gel electrophoresis performed. An example of the gel is shown in Figure 16. The cell line MFD-1 was contaminated with mycoplasma and was not taken forward for lentiviral transfection and discarded.

Table 14 Cell Lines, required media and subculture ratio

Cell Line	Subculture Ratio	Base Media	Media Additives
HEK 293 T Human embryonic kidney	1:4	EMEM	+ 10% Foetal Bovine Serum (FBS) + 1% Pen/Strep
CP-A (ATCC CRL-4027) Non-dysplastic Barrett's Oesophagus	1:3	MCDB-153	+ 0.4µg/ml hydrocortisone – add 200 µL of 100 mg/mL stock solution. + 20ng/mL Recombinant human epidermal growth factor – add 100 µL of 100µg/mL stock solution. + 8.4 µg/L Cholera toxin – add 42 µL of 100 µg/mL stock solution + 20 mg/L Adenine – add 10 ml of 1 mg/mL stock solution + 140 µg/ml Bovine Pituitary Extract – add 5 mL + 1 x Insulin-Transferrin-Selenium (ITS) Supplement + 4 mM L-Glutamine + 5% FBS + 1% Pen/Strep
OE-19 Oesophageal Adenocarcinoma	1:3	RPMI 1640	+ 2 mM L-glutamine + 10 % FBS + 1% Pen/Strep
OE-21 Oesophageal Squamous Cell Carcinoma	1:3	RPMI 1640	+ 2 mM L-glutamine + 10 % FBS + 1% Pen/Strep
OE-33 Oesophageal Adenocarcinoma	1:3	RPMI 1640	+ 2 mM L-glutamine + 10 % FBS + 1% Pen/Strep
MFD-1 Oesophageal Adenocarcinoma	1:3	DMEM	+ 10% FBS + 1% Pen/Strep

Table 15 - EZ-PCR Thermal Cycler Conditions

Step	Temperature (°C)	Time (minutes:seconds)
Hot Start	94	00:30
Denaturation	94	00:30
Annealing	60	2:00
Extension	72	1:00
Cycle Repeats	35x	
Final Extension	72	5:00
Hold	4	Infinite

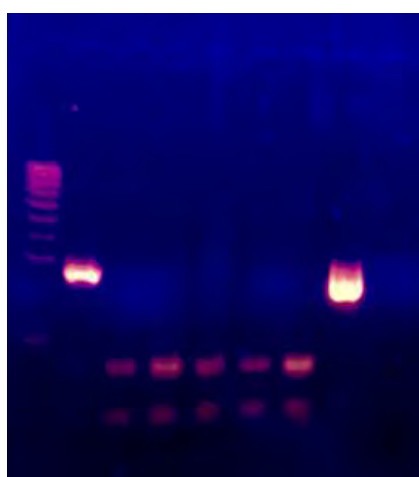


Figure 16 - EZ Mycoplasma Test agarose gel. Left to Right: Positive Control, Negative Control, OE-19, OE-33, CP-A, HEK-293T, MFD-1. MFD-1 demonstrates a positive result for mycoplasma contamination.

4.10.4 BioRAD TC20 AUTOMATED CELL COUNTER

The BioRad TC20 instrument generates an automated cell count and percentage of viable cells using trypan blue dye. Viable cells have an intact cell membrane

and so do not take up the dye. 50 μ L of trypan blue was pipetted into a sterile 1.5 ml Eppendorf tube. 50 μ L of cells in suspension were added and pipette mixed. The cells and trypan blue mixture was subsequently loaded on to the chamber in the BioRad slides (1450011) and inserted into the automated cell counter. On screen instructions were followed and cell counts obtained.

4.10.5 EVOS FL CELL IMAGING SYSTEM

Cells in culture were imaged using the EVOS FL Cell Imaging System (ThermoFisher). This allowed digital image capture to be performed and all plain light microscopy of cells in culture presented in this thesis was taken using this imaging system.

4.11 PUROMYCIN KILL CURVES

The lentiviral vector used to transduce the OR3A4 over-expressing gene also incorporates a puromycin resistance gene to allow selection of cells which are successfully transduced. Cells which do not contain this gene, and thus have not been successfully transduced, are killed by adding puromycin to the culture media. To find the minimum concentration for this purpose, puromycin kill curve experiments were performed. 5×10^5 Cells were seeded in each well of a 6 well culture plate. A graded concentration of puromycin was added at 0, 0.25, 0.5, 1, 2 and 4 μ g/ml to the wells. Pictures of each well were taken at 24, 48 and 72 hour intervals to assess cell death rates. Cells were counted using ImageJ using an

automated algorithm to identify the maxima within the image. This corresponded to the nuclei and the cell count. This experiment was performed in triplicate.

4.12 P-GIPZ LENTIVIRAL TRANSDUCTION OF CELL LINES

The cell lines CP-A, OE-19, OE-21, OE-33 and HEK-293T were transduced with an OR3A4 over-expressing gene using a lentiviral p-GIPZ vector. The design of the viral vector and supervision of transfection was kindly carried out by Dr Mat Coleman. The Dharmacon pGIPZ lentiviral vector (116) was modified by removing the green fluorescent protein reporter (GFP) and replacing it with a small multiple cloning site, into which the lncRNA OR3A4 was cloned. There was no short hairpin RNA (shRNA) cloned or expressed in the vector used.

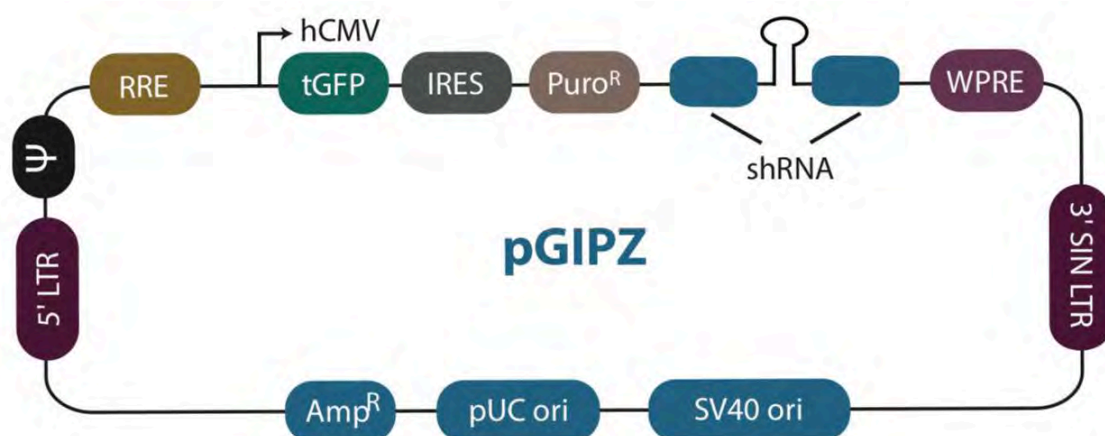


Figure 17 – Dharmacon p-GIPZ Lentiviral vector. The GFP site was removed and replaced with a multiple cloning site into which OR3A4 lncRNA was cloned. No shRNA was expressed in the experimental vector.

HEK-293T cells are used as a packaging cell line so that the target gene sequence (OR3A4) can be integrated into the lentiviral plasmid using the host cell's machinery.

On day 1 HEK-293T cells were seeded at 2.5×10^5 cells/ml into a 6 well plate. The following day lentivirus plasmids pMD2.G (150ng), psPAX2 (350ng) and gene of interest (OR3A4) (500ng), were transfected in the presence of Optimem media and Fugene transfection reagent.

On day 3 the host cells (CP-A, OE-19, OE-21, OE-33 and HEK-293T) were seeded at 1×10^5 cells/ml in a 6 well plate. The following day viral media was removed from transfected HEK-293T cells and mixed with an equal volume of with fresh host cell culture media, filtered through a PES 0.45 μ M filter, and placed on recipient cells.

On day 4, viral media was replaced with fresh host cell culture media containing 1 μ g/ml puromycin. Control (non-infected) cell death was used as a marker of successful transduction.

4.13 MTS ASSAY

The MTS assay is a colourimetric assay used for measuring cell viability and metabolic activity. It uses a yellow tetrazolium dye (MTS) which is reduced to formazan by metabolically active cells. In the presence of phenazine methosulfate (PMS), MTS reduces to a formazan product which absorbs light at a wavelength of 490 nm. Absorbance was measured using a PerkinElmer EnSpire plate reader. Promega CellTitre96 Aqueous one solution cell proliferation assay (G3582) was used to perform the MTS cell viability

experiments detailed in this thesis. Cells were counted using the BioRad TC20 instrument as detailed previously. 10^3 Cells were seeded into each well of a clear bottomed 96 well culture plate and media added to make up the total volume in each well to 100 μ L. Blank wells containing media alone were also included on the plates to allow background absorbance to be corrected. The plates were incubated for 24 hours to allow the cells to adhere to the culture plate. Enough wells were seeded to allow MTS assays to be run over the course of 5 days. At 24, 48, 72 and 96 hours post seeding, 20 μ L of the CellTitre96 MTS reagent was added to each well to be studied including the media blanks. The plate was returned to the incubator and read on the plate reader at 1, 2, 3 and 4 hours after adding MTS reagent. The absorbance of the control blank wells was subtracted from the absorbance of the experimental wells to give a change in absorbance. Each experimental plate was seeded with 4 wells of the same cell type and the experiment was repeated on 3 separate occasions.

4.14 SCRATCH WOUND HEALING ASSAY

Ibidi culture plates with 2 well inserts (81176) were used for scratch wound healing assays. Specialised culture plates with inserts were used following many aborted attempts to generate a regular scratch wound in a cultured monolayer of cells by hand. Adherent cells were trypsinised when they reached 90% confluence and re-suspended in culture media. 70 μ L of the cell suspension was pipetted into each of the wells on the plate insert. Once the cells had re-attached and reached 100% confluence, the plate inserts were removed to create a 500 μ m cell free gap. The culture plates were imaged at regular time intervals until

the cell free gap was completely closed. The cell free gap was measured using ImageJ software. The experiment was performed in triplicate.

4.15 DRUG TREATMENT OF CELL LINES

The cell lines CP-A, CP-A PIPZ control and CP-A PIPZ OR3A4 were selected for drug treatment with the p38 MAPK inhibitor Doramapimod (Selleckchem S1574) and Aspirin (Acetylsalicylic acid, Sigma-Aldrich A2093). In order to assess their viability following drug treatment they were subjected to MTS assays as described in section 4.13. The cells were cultured as detailed in section 4.10. 1000 cells were seeded in each well of a white walled 96 well plate containing 100 μ L media. After 24 hours each well was dosed with either aspirin, doramapimod or a combination of agents with increasing concentrations. Aspirin was dosed from 0-100 mM concentration. Doramapimod was dosed initially from 0-10 nM, then subsequently from 0-10 μ M. This was taken as day 1, and an immediate MTS assay performed. MTS reagent was added after a further 24, 48, 72 and 96 hours and the plate re-read. The experiment was performed in duplicate, once as a single experiment by myself and kindly repeated once by Y Sinha. No further repeats were performed due to time constraints.

4.16 RNA SEQUENCING

Total RNA from CPA, CPA PIPZ Control, CPA PIPZ OR3A4, OE33, OE33 PIPZ Control and OE33 PIPZ OR3A4 cells was sequenced using the Illumina NextSeq 500 platform.

RNA was extracted using the Qiagen RNeasy kit and QC'd using the Qubit fluorometer and Agilent Tapestation 2200. It was then subject to automated library preparation using the Illumina NeoPrep instrument and following the TruSeq Stranded mRNA Library Prep for NeoPrep protocol(117).

25 ng total RNA from each sample was transferred into a PCR plate and mixed with nuclease free H₂O to a final volume of 12.5 µL in each sample well and pipette mixed. RPB2 was vortexed to resuspend the reagent and 12.5 µL was added to each well and pipette mixed. The plate was sealed with a film and placed on a thermal cycler to denature the RNA. The lid was pre-heated to 100 °C and the samples heated to 65 °C for 5 minutes followed by 25 °C for 5 minutes and held at 25 °C.

The library card was loaded as follows. The card guide and reagent card can be seen in Figure 18. The card guide was placed on to the library card and placed on to the loading stage of the NeoPrep instrument. On screen instructions were followed to verify the card. The card was filled with oil using the oil funnel. 125 µL of the large reagents i-vii were transferred from the reagent plate into the library

card. 80 μ L pre-vortexed DMB was added to well viii. 15 μ L of the small reagents 1-8 were transferred to the library card. A fresh 8 tube strip was used to pierce the foil covering the small reagents 9-12 and then 15 μ L was transferred to the library card. This step was repeated for small reagents 13-16. 5 μ L of the small reagents a-h were then transferred from the reagent card to the library card. The samples were pipette mixed once and then 25 μ L was transferred to the corresponding wells. As fewer than 16 samples were used, 25 μ L RSB was transferred to the blank sample wells. 3 μ L of adapters A-H was transferred from reagent to library card. The library card guide was removed and the door of the instrument was closed and the run started.

Once the run was finished, the samples were unloaded by first adding 10 μ L RSB to a new PCR plate. A 200 μ L pipette was used to transfer 20 μ L from the library card collection wells and transferred to the corresponding wells on the PCR plate. A blue dot should appear in the pipette as shown in Figure 19. The library and RSB were pipette mixed in the PCR plate and then centrifuged briefly. The libraries were transferred into library separation tubes to remove the oil and left to stand for 10 minutes. Once separated, the libraries were transferred to a fresh PCR plate ready for running on the NextSeq 500. At this point, the prepared libraries can be sealed and stored at -20°C for up to 2 months.

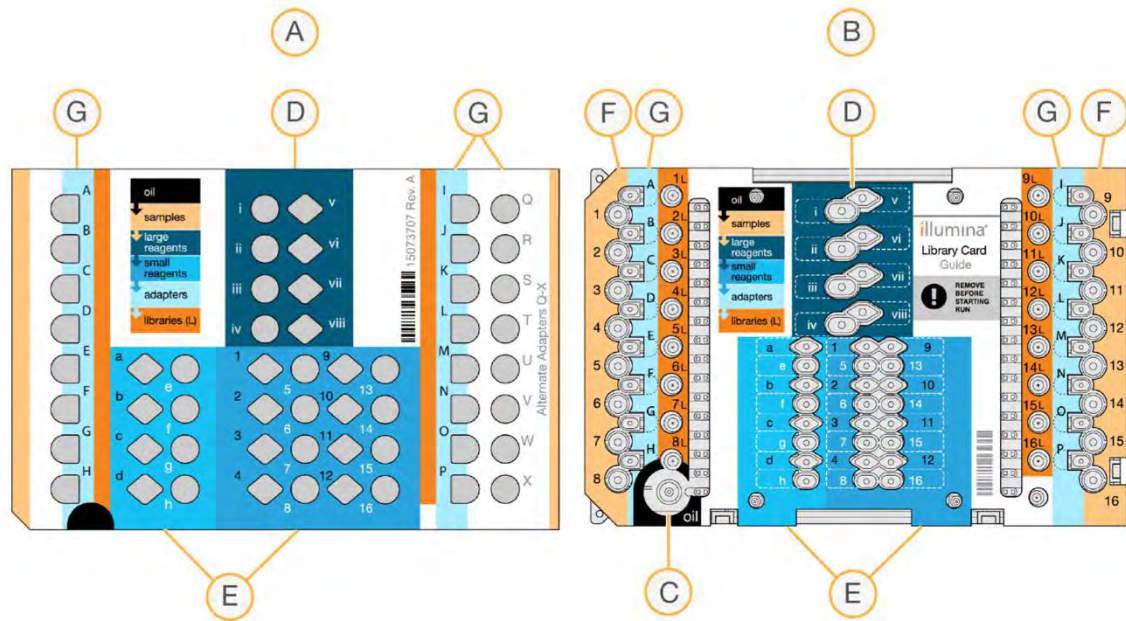


Figure 18 - NeoPrep Reagent and library cards. A: Reagent card, B: Library card, C: Oil reservoir, D: Large reagents, E: Small reagents, F: Samples, G: Adapters

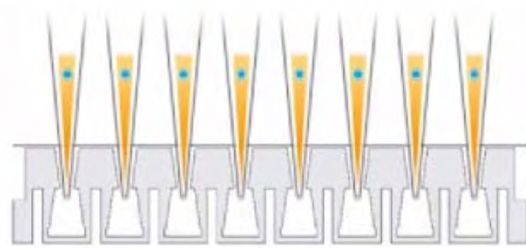


Figure 19 - Retrieval of libraries (blue dot) from the NeoPrep Library Card.

The experiment used a NextSeq 500/550 High Output, 150 cycle flow cell (#20024907) for RNA sequencing on the NextSeq 500 instrument. The instrument can be seen in Figure 20. The NextSeq 500 utilises pre-loaded reagent and buffer cartridges. The foil seal of reservoir number 10 on the reagent cartridge was first pierced with a sterile pipette tip. The libraries were loaded into the reagent cartridge at a volume of 1.3 mL with a 1.8 pM concentration. The flow cell was loaded into the imaging compartment (Figure 20 label F) and the spent

reagents container emptied and replaced (Figure 20 label C). A fresh buffer cartridge was inserted into the instrument (Figure 20 label C). The reagent cartridge containing the libraries was then loaded (Figure 20 label D). On screen instructions are followed and the run progress monitored on Illumina BaseSpace software.



Figure 20 – Illumina NextSeq 500 instrument. Image taken from NextSeq 500 user guide (118). A: Touch screen monitor, B: Status bar, C: Buffer compartment, D: Reagent compartment, E: Power button, F: Imaging compartment, G: Air filter compartment.

Bioinformatic analysis was kindly performed by Mr AD Beggs using Partek Flow software.

4.16.1 DAVID ANALYSIS

The database for annotation, visualisation and integrated discovery (DAVID) (119) allows functional annotation of a gene list to be undertaken. Functional annotation allows groups of differentially expressed genes to be mapped from long lists of genes created from total RNA sequencing. This allows biological relevance to be inferred using gene-annotation enrichment analysis and pathway analysis. DAVID Bioinformatics is an online tool provided by the Laboratory of Human Retrovirology and Immunoinformatics (LHRI)(120). The output list of genes of the RNA sequencing of CPA control and CPA OR3A4 over-expressing cells was inputted to the online tool. The list was pre-filtered for zero counts and sorted by fold change. The identifier “ENTREZ_GENE_ID” was selected and the list submitted. The output of enriched genes was outputted in table format.

4.16.2 KEGG PATHWAY ANALYSIS

The Kyoto encyclopaedia of genes and genomes (KEGG)(121) is a database which can be interrogated and analysed to provide information about biological function from molecular level information such as lists of genes generated by RNA sequencing. It consists of a pathway database that consist of graphical diagrams of biochemical pathways including metabolic and regulatory pathways. Using DAVID bioinformatics resources 6.8(120) KEGG pathway analysis was performed. The analysis used the same gene list as for the DAVID analysis in section 4.16.1 and was uploaded in the same session using the same gene identifier. The pathways of interest were outputted in table format.

4.17 REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION

The real time quantitative polymerase chain reaction (RTqPCR) measures the amplification of PCR products in real time and gives a quantitative measure of relative gene expression. Total RNA must first be reverse transcribed using a reverse transcriptase enzyme to produce cDNA. The cDNA is subjected to a PCR reaction with specific primers according to the gene of interest. As PCR product is produced, it is detected using a fluorescent dye in the qPCR instrument. The rate of PCR product production is compared to a highly expressed control “housekeeping gene” to give a measure of relative gene expression compared to the control gene. The number of cycles taken to reach a PCR product detection threshold is used to calculate fold change in gene expression.

RNA was extracted using the Qiagen RNeasy kit as described above. This was then converted to cDNA with the Applied Biosystems High Capacity RNA to cDNA Kit (4387406). An input RNA weight of 484ng was chosen to allow the maximum amount of the lowest concentration of RNA extracted from the cells to undergo reverse transcription. All RNA was handled on ice during preparation of the reaction mixture. It was important to standardise the input RNA weight across all the different reactions in order to maintain the same concentration in the output and for ease of setting up downstream RTqPCR reactions. The RNA concentrations are shown in Table 16 and the final reaction volumes are detailed in Table 17. The reaction mixture was incubated at 37°C for 60 minutes and then at 95°C for 5 minutes.

Table 16 - RNA Concentration (ng/uL)

OE33 Control	OE33 OR3A4	PIPZ	CP-A Control	CP-A OR3A4	PIPZ
259.3		370.0	53.8		212.0

Table 17 - RNA to cDNA Reaction Volumes (uL)

	OE33 Control	OE33 PIPZ OR3A4	CP-A Control	CP-A PIPZ OR3A4	OE33 Control RT-ve
2x RT Buffer	10	10	10	10	10
20x RT Enzyme	1	1	1	1	0
RNA Sample	1.87	1.31	9	2.28	1.87
Nuclease Free H ₂ O	7.13	7.69	0	6.72	8.13
Reaction Volume	20	20	20	20	20

For each RTqPCR reaction using individual probes, the PCR mix was made up as per Table 18. Details of individual probes are found in Table 19.

Table 18 - TaqMan RTqPCR Reaction Mix Components

RTqPCR Mix Component	Volume (uL)
20X TaqMan Gene Expression Assay	1
2x TaqMan Gene Expression Master Mix	10
cDNA template (25ng)	4
RNase free H ₂ O	5

Table 19 - TaqMan RTqPCR Probes

TaqMan Assay	Gene	Expression	Description	Catalogue Number (ThermoFisher)
Hs99999905_m1			GAPDH	4331182
Hs01661539_s1			OR3A4	4331182
Hs00429934_g1			TRIAP1	4331182
Hs00398895_m1			PRELID3A	4331182

Table 20 - RTqPCR Thermal Cycling Conditions

Step	Temperature	Time (standard cycling mode)	Cycles
UNG incubation	50 °C	2 minutes	1
Enzyme activation	95 °C	10 minutes	1
Denature	95 °C	15 seconds	40
Anneal / Extend	60 °C	1 minute	

The RTqPCR reaction mix was made up in a 96 well plate and read on the Quantstudio 7 Flex Real-Time PCR System (ThermoFisher) using the thermal cycling conditions in Table 20. Threshold cycle values (Ct) were calculated and delta Ct (DCt) values calculated by subtracting the threshold value of GAPDH from the threshold value of the gene of interest. Delta delta Ct (DDCt) values were generated from the data by subtracting threshold values of the experimental cell line cDNA from the control cell line cDNA threshold values. Fold change in target gene expression between the cell lines was generated using the formula $FC = 2^{-DDCt}$.

A cDNA input test was performed using the GAPDH Taqman gene expression assay using a serial dilution of cDNA from 1-100 ng input to gauge the minimum reliable input weight for analysis. This was performed using RNA from OE-33

cells with the GAPDH Taqman expression assay. An input weight of 25 ng generated reliable amplification and was used for further experiments.

OR3A4 over-expression was validated using RTqPCR and the individual probes TRIAP1 and PRELID3A were targeted using the same reaction conditions as described in Table 18 and Table 20. All experiments using individual probes were performed in triplicate.

TaqMan gene expression array plates were used to explore the effects on apoptosis and MAPK pathways in CPA cells when OR3A4 was over-expressed. The human MAPK pathway 96 well plate (ThermoFisher 4414093) and human apoptosis pathway 96 well plate (ThermoFisher 4414072) were used. The plate layouts for the MAPK and apoptosis arrays are shown in Figure 21 and Figure 22 respectively. A stock solution of nuclease free water and experimental cDNA was made to a concentration of 2.5 ng/uL. 1080 uL of stock cDNA and 1080 uL of 2X TaqMan master mix was prepared and 20 uL added to each well on the pre-prepared 96 well array plate. This was read on the Quantstudio 7 Flex Real-Time PCR System (ThermoFisher) using the thermal cycling conditions in Table 20 and fold change calculated as described above. Due to time and funding constraints, experiments using the MAPK and apoptosis gene array plates were performed once for each cell line.

	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	GUSB	ARAF	ATF2	BRAF	CCND1	CCNE1	CDC42	CDKN1B	CDKN1C
B	CHUK	COL1A1	CREB1	CREBBP	DUSP1	DUSP10	DUSP16	DUSP2	DUSP4	DUSP5	DUSP6	DUSP7
C	DUSP8	DUSP9	FOS	GRB2	HRAS	IGF1	KRAS	MADD	MAP2K1	MAP2K1IP1	MAP2K2	MAP2K3
D	MAP2K4	MAP2K5	MAP2K6	MAP2K7	MAP3K1	MAP3K10	MAP3K11	MAP3K2	MAP3K3	MAP3K4	MAP3K5	MAP3K6
E	MAP3K7	MAP3K8	MAP3K9	MAP4K1	MAP4K2	MAPK1	MAPK10	MAPK11	MAPK12	MAPK13	MAPK14	MAPK15
F	MAPK3	MAPK4	MAPK6	MAPK7	MAPK8	MAPK8IP1	MAPK8IP2	MAPK9	MAPKAPK1	MAPKAPK2	MAPKAPK3	MEF2A
G	MEF2C	MKNK1	MKNK2	MYC	NLK	NRAS	PAK1	PAK2	PAK3	PAK4	PTK2B	RAC1
H	RAF1	RELA	RPS6KA1	RPS6KA2	RPS6KA3	RPS6KA4	RPS6KA5	RPS6KB1	RPS6KB2	SHC1	TP53	ZAK

Figure 21 - TaqMan Human MAPK RTqPCR 96 well plate layout (ThermoFisher 4414093)

	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	GUSB	BIRC2	APAF1	BAD	BAK1	BAX	BBC3	BCAP31	BCL10
B	BCL2	BCL2A1	BCL2L1	BCL2L10	BCL2L11	BCL2L13	BCL2L14	BCL2L2	BCL3	BID	BIK	NAIP
C	BIRC3	XIAP	BIRC5,EPR	BIRC6	BIRC7	BIRC8	BNIP3	BNIP3L	BOK	NOD2	NOD1	CARD6
D	CARD9	CASP1	CASP10	CASP14	CASP2	CASP3	CASP4	CASP5	CASP6	CASP7	CASP8	CASP8AP2
E	CASP9	CFLAR	CHUK	CRADD	DAPK1	DEDD	DEDD2	DIABLO	IFT57	FADD	FAS	FASLG
F	HIP1	HRK	HTRA2	ICEBERG	IKBKB	IKBKE	IKBKG	LRDD	LTA	LTB	MCL1	NLRP1
G	NFKB1	NFKB2	NFKBIA	NFKBIB	NFKBIE	NFKBIZ	PEA15	PMAIP1	PYCARD	REL	RELA	RELB
H	RIPK1	RIPK2	TBK1	TNF	TNFRSF10A	TNFRSF10B	TNFRSF10C	TNFRSF10D	TNFRSF10E	TNFRSF10F	TNFRSF10G	TRADD

Figure 22 - TaqMan Human Apoptosis RTqPCR 96 well plate layout (ThermoFisher 4414072)

4.18 IMMUNOFLUORESCENCE

Immunofluorescence is an imaging technique which uses fluorescently labelled antibodies to target and image specific molecules or architecture within cells. Phalloidin, Caspase 3 and Hoescht (nuclear counter stain) stains were used. An indirect staining method with primary and secondary antibodies was performed.

To prepare the slides, cells were grown in a monolayer on sterile coverslips in media. Once the cells had adhered and reached the desired levels of confluence, the coverslips were removed from the media and placed in a clean dish for the staining process. The cells were washed twice with PBS at 37 °C taking care not to dislodge the adherent cells from the coverslip. The cells were then fixed with 4% Paraformaldehyde in PBS for 10 minutes. The cells were then washed with PBS 3 times for 5 minutes. To allow the stain to penetrate the cells, the membrane was permeabilised with 0.1% Triton diluted in PBS for 2 minutes. The cells were again washed with PBS 3 times for 5 minutes. Non-specific antibody binding was blocked by adding a solution of 10% heat inactivated goat serum for 40 minutes at room temperature.

Rabbit primary monoclonal antibody was diluted to a 1:400 with PBS (6 µL antibody to 2.4 mL PBS). Parafilm was stretched over the lid of a 6 well culture plate. A 200 µL globule of the antibody solution was pipetted on to the parafilm. The coverslip was carefully transferred on to the antibody solution with the cells in contact with the antibody. This was transferred to the fridge for overnight incubation.

The coverslip was washed three times with PBS to remove the primary antibody solution. The secondary antibodies were prepared with a 1:400 dilution (4 mL PBS, 10 μ L secondary anti-rabbit, 100 μ L phalloidin/caspase 3). Cell Signalling Technologies Cleaved Caspase 3 (ASP175) and Alexa Fluor 594 Phalloidin (Invitrogen) were used, along with secondary Goat Anti-rabbit Alexa Fluor 488 (Invitrogen). 300 μ L of the secondary antibody solution was placed on each coverslip and incubated for 40 minutes at room temperature. Following the incubation the coverslips were washed three times with PBS.

Hoechst DNA counter stain was prepared with a 1:1000 dilution and applied to the coverslips for 5 minutes. The coverslips were then mounted on slides and allowed to air dry. Once dry, they were sealed with cyto seal. The slides were then imaged using the Vectra confocal microscope.

4.19 IMMUNOHISTOCHEMISTRY

4.19.1 OR3A4 STAINING IN BO TISSUES

A subset of 12 patients from UHB were stained for OR3A4 expression in BO tissue samples (6 non-progressor, 6 progressor). H&E slides from NDBO tissues were reviewed by a specialist GI Histopathologist (R Hejmadi) to ensure that no dysplasia was present. The pathologist was blinded to the progressor status of each tissue sample at all stages. The selected tissue blocks were then re-cut, mounted on slides and stained using the Leica Bond Rx system using a mouse

polyclonal OR3A4 antibody (Abcam ab67107). This antibody has been subject to validation by the supplier (Abcam). No further validation was undertaken for the purpose of this experiment. The antibody was diluted to 1:100 with a primary incubation time of 15 minutes. Staining was carried out by the Human Biomaterials Resource Centre (HBRC) at the University of Birmingham (C Bagnall). The stained tissue sections were reviewed again by the pathologist and Mr AD Beggs and staining intensity (0-10) was scored in stromal and epithelial components of the tissue biopsies.

4.19.2 MULTISPECTRAL IHC

Sections of NDBO tissue from samples pre-dating any dysplastic change were taken from 12 BO progressor patients and sections of NDBO from 8 non-progressor patients. These sections were kindly stained and scanned by C Bagnall at HBRC. A panel of CD4, CD8, CC20, CD68 and FOXP3 was used for multispectral IHC. All sections were reported as non-dysplastic on histological review (R Hejmadi).

The antibodies used are detailed in Table 21. The manufacturers recommended dilution was used on each antibody and a two-fold increased and decreased dilution was used to find the optimal condition with DAB staining. The optimal dilutions are detailed in Table 21. The optimal dilution for each antibody was paired with each of the opal dyes (OPAL 520, OPAL 540, OPAL 570, OPAL 620, OPAL 650 and OPAL 690), to find out which dye was compatible. Single

antibody/opal dye combinations were used for the spectral library and the best combination of antibody/opal dye was combined in sequential staining. The order of antibodies was switched to find the optimal panel combination. The optimal panel combination was achieved when the multiplex image was comparable to that of the single IHC.

Table 21 - Antibodies used for multispectral IHC including staining time, optimal dilutions, TSA OPAL marker dyes and secondary antibodies. The antibodies were stained in sequence one through five.

Antibody	Stain Order	Clone	Ab time	Ab dilution	TSA OPAL	TSA dilution	TSA time	PRE-TREAT	Secondary
CD4	1	4B12	30	1:50	520	1:400	10	Ph9 20	mouse 1:100 (dako RABBIT ANTI MOUSE)
CD8	2	4B11	30	1:400	570	1:400	10	pH6 10	mouse 1:100 (dako RABBIT ANTI MOUSE)
CD20	3	L26	30	1:200	620	1:200	10	pH6 10	mouse 1:100 (dako RABBIT ANTI MOUSE)
FOXP3	4	236A/E7	30	1:400	650	1:400	10	pH6 10	mouse 1:100 (dako RABBIT ANTI MOUSE)
CD68	5	KDI	30	1:400	690	1:200	10	pH6 10	mouse 1:100 (dako RABBIT ANTI MOUSE)

Once the optimal antibody dilutions and conditions were finalised, the FFPE sections were heated to 60 °C to de-wax. The stains were added in the sequence shown in Table 21. The sections were re-hydrated and subjected to heat induced epitope retrieval (HIER) which can recover antigen reactivity in FFPE tissues. The CD4 antibody underwent pre-staining HEIR in BOND Epitope Retrieval Solution 2, the other antibodies used BOND Epitope Retrieval Solution 1. The sections were heated to 100 °C for 20 minutes in the Epitope Retrieval Solution. Following HEIR, the primary antibody was added. Secondary rabbit, anti-mouse antibodies along with the corresponding Tyramide Signal Amplification (TSA)

OPAL dye were then added. The sections were stained in the sequence: CD4, CD8, CD20, FOXP3, CD68. Finally DAPI nuclear counter stain was used.

4.19.3 VECTRA

The VECTRA 3 automated quantitative pathology imaging system was used to scan the slides. To create a spectral library, single stained slides, OPAL 520, OPAL 570, OPAL 620, OPAL 650, OPAL 690, DAPI and an auto fluorescence slide were scanned. All filters were used at x20 magnification. A spectral library was created in the InForm software from the images.

For multiplex scanning, whole slides were scanned at x10 magnification and at x20 for multispectral imaging. Each multiplex slide was exposed with each of the five available filters: DAPI, FITC, CY3, Texas Red and CY5, ensuring the slide was in focus with each of the images.

The multispectral IHC images were analysed using the Perkin Elmer inForm Cell Analysis Software. This is a software package with advanced image processing capabilities which utilises a machine learning algorithm to recognise different cell and tissue types within a histopathology slide image. Once the images are loaded on to the software, the different cell types are manually selected by the user. At least 8 of each differentially stained cell type must be identified for the algorithm to accurately detect them in new, previously “unseen” images. Once the cell types are identified, different zones within the tissue section are marked. Background, stroma and epithelium is manually traced by the user using the computer mouse.

The software algorithm processes the information and attempts to train and then delineate the different zones on unseen images. Although the tissue sections were all from BO, stained in a homogenous manner, the algorithm required training on numerous different sections in order to begin automated recognition of tissue zones.

Once the software reliably de-lineated epithelium and stroma, the finalised algorithm was run with all sections of all samples to generate a count of immune cells and their distribution across the tissue sections. Microsoft Excel pivot tables were used to collate the data and determine the distribution of cells.

5. A SYSTEMATIC REVIEW OF EPIGENETIC BIOMARKERS IN PROGRESSION FROM NON-DYSPLASTIC BARRETT'S OESOPHAGUS TO OESOPHAGEAL ADENOCARCINOMA

5.1 INTRODUCTION

A systematic review of the literature was undertaken to assess the current status of epigenetic biomarkers in BO which progresses to OADC. The potential of epigenetic tissue markers to make up part of a risk stratification model for BO is intriguing, as epigenetic change has been demonstrated in colorectal cancer associated with UC even before dysplasia has occurred. If patients at high risk of progression to OADC could be identified before dysplastic change occurred, they could undergo enhanced surveillance with more regular OGD with the aim of performing endoscopic resection at an early disease stage rather than undergoing oesophagectomy. Although studies have been performed previously investigating epigenetic change in BO and OADC, these were often performed in a cross sectional fashion on different patients rather than using longitudinal follow up in the same patient cohort. Another common design was to take tumour and normal paired samples from an OADC resection specimen. Whilst these studies are valid ways to investigate the mechanisms of Barrett's carcinogenesis, there is no means to predict which individual patients are likely to progress from BO to OADC. The specific aim of the systematic review was to consolidate the literature and inform our research as to which epigenetic markers could predict which patients progressed from NDBO to OADC.

5.2 LITERATURE REVIEW

A scoping search was performed using MEDLINE, the Cochrane Library and internet sources to identify any systematic reviews or meta-analyses on epigenetic biomarkers in BO and oesophageal cancer (OC). It revealed in excess of 2000 primary studies which are relevant for inclusion into the proposed systematic review. No systematic reviews which draw together all aspects of epigenetic change within the field of Barrett's carcinogenesis were identified. Nine systematic reviews and meta-analyses were identified(122-130) which included mixed patient populations with OADC and oesophageal squamous cell carcinoma (OSCC) with only 3 reviews incorporating patients with BO(125, 126, 130). These studies concentrated on a single type of epigenetic alteration with 4 investigating DNA methylation(122-124) and 3 looking at miRNA expression(125-128). The remaining 2 studies investigated genetic alterations in progression of BO to OADC(129, 130). Based on these results it was clear that no systematic review had been performed to include all aspects of epigenetic change in the progression of Barrett's oesophagus to OADC.

5.3 RESEARCH AIMS

The systematic review aimed to identify and summarise studies which examine epigenetic biomarkers in BO and their association with progression to OADC with the aim of consolidating the literature and informing future laboratory work.

5.4 METHODS AND ANALYSIS

The systematic review protocol has been reported in accordance with PRISMA-P guidelines (131).

5.5 SELECTION CRITERIA

5.5.1 POPULATION: All patients over the age of 18 with BO, BO with dysplasia, OADC or unspecified oesophageal cancer were included.

5.5.2 PROGNOSTIC MARKERS: Epigenetic markers including DNA methylation, histone modification, chromatin remodelling, micro and non-coding RNAs of all types were included.

5.5.3 OUTCOME: Progression from non-dysplastic BO with or without intestinal metaplasia to BO with LGD, HGD or OADC.

5.5.4 STUDY DESIGN: All prospective and retrospective primary studies, and systematic reviews were included.

5.5.5 PUBLICATION TYPE: Abstracts and full texts were included with letters and editorials excluded from the final data synthesis.

5.5.6 EXCLUSION CRITERIA: Oesophageal squamous cell carcinoma (OSCC) and established oesophageal cancers with no evidence of a pre-existing BO diagnosis were excluded. Case reports, narrative reviews, in vitro studies (e.g.

cell lines), studies of genetic mutations, studies using biomarkers to predict a response to treatment (e.g. chemotherapy) were excluded. A decision was made to exclude animal studies, as scoping searches indicated that there were comparatively few (compared to human studies), and therefore were likely to add heterogeneity to an already heterogeneous evidence base. In addition, we concluded that issues relating to transferability of experimental findings from animal models to a clinical setting would occur.

5.6 SEARCH STRATEGY

The following electronic bibliographic databases were searched from inception: EMBASE, MEDLINE, MEDLINE in Process, DARE, CDSR, Cochrane Central. Conference (Conference Proceeding Citation Index, Zetoc) and registers of clinical trials (ClinicalTrials.gov and ICTRP) were also searched. Reference lists of identified studies and systematic reviews were screened for any relevant primary studies that were not retrieved from the database searches. Date or language restrictions were not placed on searches. A search strategy was developed using combinations of text and index words relating to the population, exposure and outcome, such as: “Barrett’s Oesophagus”, “epigenetic”, “DNA methylation”, “marker” and “oesophageal adenocarcinoma”. A sample search strategy for MEDLINE is shown below.

5.7 SAMPLE SEARCH STRATEGY

Database: Ovid MEDLINE(R) <1946 to February Week 1 2018>

- 1 Barrett Esophagus/
- 2 ((esophageal or oesophageal) adj3 (cancer or adenocarcinoma or carcinoma or neoplasm\$)).ti,ab.
- 3 (metaplas\$ adj (intestinal or columnar or dysplas\$ or adenocarcinoma\$)).ti,ab.
- 4 (Barrett\$ adj1 (esophagus or oesophagus)).ti,ab.
- 5 (Barrett\$ adj3 (adenocarcinoma\$ or intra-epitheli\$ or epitheli\$ or dysplasia or carcinoma\$ or cancer or neoplas\$)).ti,ab.
- 6 or/1-5
- 7 (methylation or hypermethylation).mp. or hypomethylation.ti,ab. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
- 8 DNA methylation/
- 9 (histone adj2 (methylation or modification or acetylation)).ti,ab.
- 10 chromatin remodel\$.ti,ab.
- 11 ((non-coding or untranslated) adj1 RNA).ti,ab.
- 12 (microRNA or siRNA or piRNA or sncRNA or lncRNA).ti,ab.
- 13 ((biomarker\$ or marker\$) adj2 (progress\$ or predict\$ or prognos\$)).ti,ab.
- 14 (epigen\$ adj2 (alteration\$ or change or changes or marker\$ or biomarker\$)).ti,ab.
- 15 or/7-14
- 16 6 and 14

5.8 STUDY SELECTION

Study selection was a two-step process. Titles and abstracts identified in our literature search were screened independently by two reviewers using pre-specified screening criteria. These were broadly based on whether the studies firstly included measuring epigenetic markers in patients with OADC and secondly whether the patient cohort studied had progressed from BO to OADC with non-dysplastic samples included in experimental analysis. Full texts of any potentially relevant articles were obtained and subjected to the full inclusion criteria. Any discrepancies found were referred to a third reviewer. The study selection process was documented using the PRISMA flow diagram. Endnote X7 was used as reference management software and decisions on inclusion or exclusion were recorded using a Microsoft Excel spreadsheet.

5.9 DATA EXTRACTION

Data was extracted by two independent reviewers using an agreed, standard data extraction form generated in Microsoft Excel. Any disagreements which could not be resolved by discussion were referred to a third reviewer who acted as arbitrator.

Data was extracted on the following study characteristics:

Study design characteristics – for example, prospective or retrospective and length of follow up. Confounding factors, study blinding and independent external validation were recorded.

Population – for example, tissue samples from patients with BO or patients with OADC looking retrospectively at BO samples, patient demographics.

Prognostic markers – Epigenetic markers including DNA methylation, histone modification, chromatin remodelling, micro and non-coding RNAs of all types.

Outcomes – Progression from non-dysplastic BO with or without intestinal metaplasia to BO with LGD, HGD or OADC.

5.10 ASSESSMENT OF STUDY QUALITY

The quality and risk of bias of individual studies was assessed in duplicate by two reviewers using a modified Quality in Prognostic Studies (QUIPS) tool(132). This tool reviewed each individual study in six criteria: study participation, study attrition, prognostic factor measurement, outcome measurement (e.g. undertaken in duplicate), study confounding factors, and statistical analysis and reporting. We anticipated that due to the difficulty obtaining samples and the length of follow up required to assess progression from BO to OADC there would be significant sample selection bias. Eligible studies were likely to be subject to confounding, with main confounding factors relating to age, obesity, smoking and alcohol intake(1). The risk of bias assessment therefore included an assessment of which confounding factors (if any) had been measured and whether they were adjusted for in the design or analysis of the study. There may have been differences in how robust the methods were for measuring the prognostic markers in each study and the outcome reported; for example, published guidelines recommend confirmation of high grade dysplasia by two independent pathologists(1). These factors were assessed carefully for each study so that a

judgement could be made on whether epigenetic changes seen in these studies were truly reflective of Barrett's carcinogenesis on a population level and whether they could be reproduced easily and accurately for screening purposes. Selected elements from the PROBAST tool (133) were used to assess the methodological quality of prognostic models in a similar fashion to Ensor et al's 2016 systematic review of prognostic models of venous thromboembolism(134) including aspects on patient selection, statistical models used and model validation.

5.11 EVIDENCE SYNTHESIS

Synthesis was narrative, with the main findings tabulated. Studies were grouped by individual epigenetic marker, or panel of markers. A lack of consistency in reported outcome metrics, and heterogeneity relating to study design, length of follow up, frequency of endoscopy and biopsy and experimental technique, precluded any quantitative synthesis. Meta-analysis could not be performed as multiple studies reported on individual biomarker types such as DNA methylation and micro RNA but investigated different biomarkers using different experimental techniques and reported different outcome metrics. Most studies presented results as percentage methylation or a ratio of differential methylation. Formal assessment of publication bias was not possible.

5.12 REPORTING

The systematic review was reported according to the PRISMA(135) guidelines.

5.13 SYSTEMATIC REVIEW RESULTS

2,965 records were screened and 14 studies met the inclusion criteria (see Figure 23 for study selection process and reasons for exclusion). The most common reasons for exclusion were lack of progression in the same population from NDBO to HGD or OADC, in vitro experimentation or no epigenetic change analysed in patient samples.

5.14 STUDY CHARACTERISTICS

Table 22 shows the main study characteristics. All included studies were of a similar retrospective cohort design. 12 of 14 studies reported patient numbers. A total of 404 patients were included. 2 studies reported total lesions only (n=223). CpG promoter methylation was investigated in 13 studies and miRNA expression in 1 study(136). 42 unique epigenetic markers were reported. No studies investigated histone modification. 13 included studies analysed 38 differentially methylated CpG promoter sites, 9 used methylation specific polymerase chain reaction (PCR) (137-145), 2 used methylight methylation specific PCR(146, 147), one used bisulphite pyrosequencing(148) and one used methylation microarray techniques (111).



PRISMA 2009 Flow Diagram

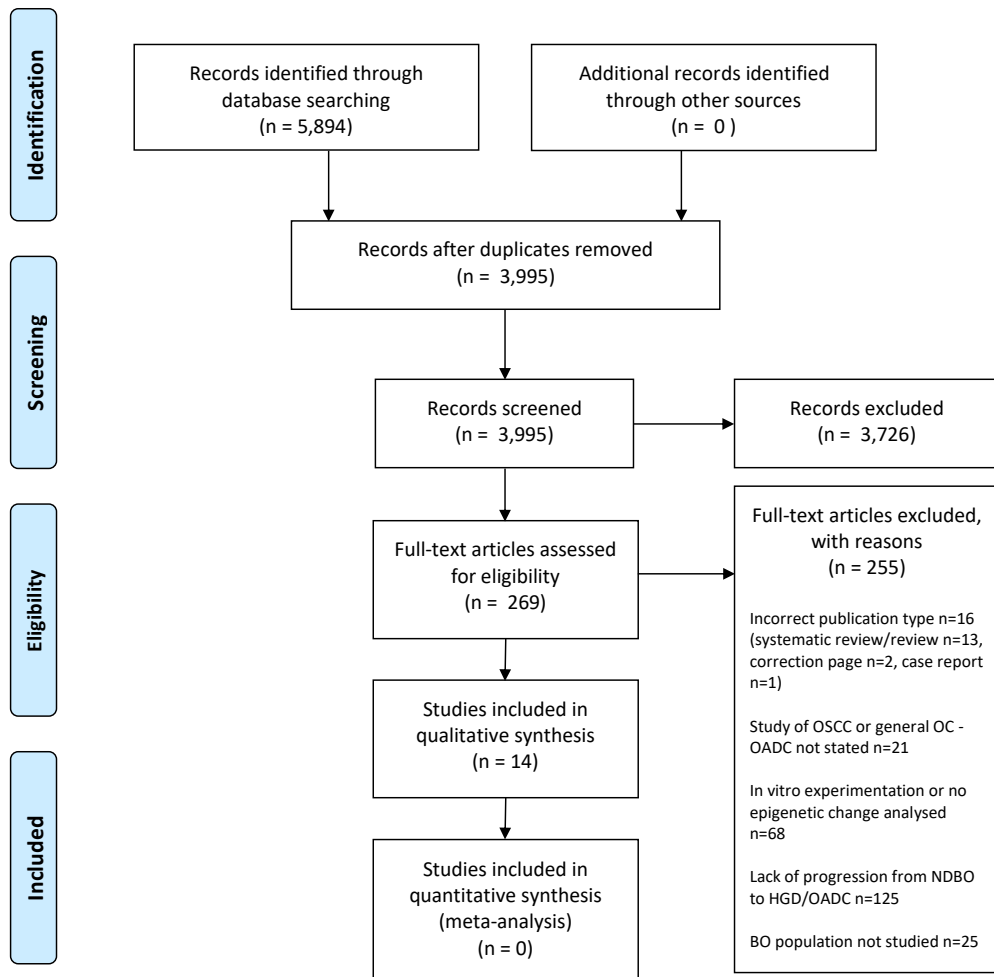


Figure 23 - PRISMA 2009 Flow Diagram

						Pro_ZBTB7B	0.56
						Pro_ZNF358	1.25
Barrett 1999 [37]	Retrospective cohort	Fred Hutchinson Cancer Research Centre + University of Washington	31 (25:6)	Not stated	CpG Methylation (Methylation Specific PCR)	CDKN2A	Hypermethylation in pre-malignant samples
Boerwinkel 2014 [43]	Retrospective cohort	AMC BO Surveillance	44 (48:10)	Mean age = 66 32M : 12F	CpG Methylation (Methylight Methylation Specific PCR)	HPP1	NS
						p16	P Hypermethylated
						RUNX3	NS
Clement 2006 [35]	Retrospective cohort	Lausanne Switzerland	28* (12:16)	Not stated	CpG Methylation (Methylation Specific PCR)	APC	P Hypermethylated
						CDKN2A	NS
						SFRP1	Hypermethylated in all samples P & NP
						TIMP3	P Hypermethylated
						TERT	P Hypermethylated
Clement 2008 [34]	Retrospective cohort	Lausanne Switzerland	31 (16:15)	Not stated	CpG Methylation (Methylation Specific PCR)	WIF-1	P Hypermethylated
Eads 2001 [44]	Retrospective cohort	Norris Comprehensive Cancer Centre	20 (12:8)	Not stated	CpG Methylation (Methylight Methylation Specific PCR)	CALCA	P Hypermethylated
						CDKN2A	P Hypermethylated
						ESR1	P Hypermethylated
						MGMT	P Hypermethylated
						MYOD1	P Hypermethylated
						TIMP3	P Hypermethylated
Jin 2009 [41]	Case Control	5 participating US clinics	195* (50:145)	P significantly older than NP	CpG Methylation (Methylation Specific PCR)	HPP1	P Hypermethylated
						p16	P Hypermethylated
						RUNX3	P Hypermethylated
						CDH13	NS
						TAC1	NS

							NELL1	NS
							AKAP12	NS
							SST	NS
Klump 1998 [36]	Retrospective cohort	Under routine Barrett's Surveillance	14 (10:4)	P mean age 57.4 8M :2F	NP mean age 55 3M :1F	CpG Methylation (Methylation Specific PCR)	p16	P Hypermethylated
Moinova 2012 [42]	Retrospective cohort	Retrospective collection from pathology database	5 (2:3)	Not stated		CpG Methylation (Methylation Specific PCR)	VIM	VIM methylation present in NDBO, not relevant in progression
Puertas Canteria 2012 [45]	Retrospective cohort	No details	55 (6:14)	Not stated		CpG Methylation (Bisulphite Pyrosequencing)	p16	Methylation % P 12.04 % NP 6.53%
Revilla-Nuin 2013 [33]	Retrospective cohort	1982 trial assessing medical vs surgical therapy for prevention of progression of BO. Randomly selected.	5 (7:17)	Not stated		miRNA Expression Analysis	miR-192	Overexpressed AUROC 0.61
							miR-194	Overexpressed AUROC 0.70
							miR-196a	Overexpressed AUROC 0.80
							miR-196b	Overexpressed AUROC 0.74
Sato 2008 [40]	Retrospective cohort	MAYO and UMD mixed cohort	62 (28:34)	Not stated		CpG Methylation (Methylation Specific PCR)	HPP1	P Hypermethylated
							p16	P Hypermethylated
							RUNX3	P Hypermethylated
Schulmann 2005 [39]	Retrospective cohort	Baltimore Veteran's Affairs hospitals and University of Maryland Hospitals	53 (8:45)	P mean age 63.3 4M : 0F	NP mean age 62.2 35M : 3F Segment length 5.8 cm	CpG Methylation (Methylation Specific PCR)	RUNX3	P Hypermethylated
							HPP1	P Hypermethylated
							p16	P Hypermethylated
							CRBP1	Hypermethylation not independently associated with P

				Segment Length 10.0 cm			TIMP3	Hypermethylation not independently associated with P
							APC	Hypermethylation not independently associated with P
Wang 2009 [38]	Retrospective cohort	Johns Hopkins under surveillance for GORD/BO	57 (7:50)	No significant differences in N vs NP. Data not provided.	CpG Methylation (Methylation Specific PCR)	APC	p16	P Hypermethylated
								P Hypermethylated

*Number of lesions, no patient numbers described in study; Number of progressor lesions (P), number of non-progressor lesions (NP)

M:F – Male to female ratio

ROC – Receiver operator curve

AUROC – Area under receiver operator curve

OR – Odds ratio

HR – Hazard ratio

Fisher's PLSD – Fisher's protected least significant difference

OADC – Oesophageal adenocarcinoma

BO – Barrett's Oesophagus

NDBO – Non-dysplastic BO

GORD – Gastro-oesophageal reflux disease

P – Progressing patients

NP – Non progressing patients

NS – Not statistically significant

PCR – Polymerase Chain Reaction

5.15 PROGNOSTIC MODELS

Table 23 – Models aiming to predict progression to OADC

Author/Year	Study type	Patient number (P:NP)	Model used	Result
Clement 2006 [35]	Retrospective cohort	28* (12:16)	APC+TIMP3+TERT	Hypermethylation in P vs NP (P 81% vs NP 26% p<0.0001)
Jin 2009 [41]	Retrospective cohort	195* (50:145)	Biomarker panel (p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12, SST)	AUROC 0.72
			Biomarker panel + Age (p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12, SST)	AUROC 0.85
Sato 2008 [40]	Retrospective cohort	62 (28:34)	Methylation index (p16, HPP1, RUNX3)	Hypermethylation in P vs NP AUROC 0.75 (No CI stated)
			Methylation index (p16, HPP1, RUNX3), Segment Length, pathology	AUROC 0.79 (95% CI 0.6968-0.8853) Sensitivity 91.4 Specificity 51.8
Schulmann 2005 [39]	Retrospective cohort	53 (8:45)	Age, Segment Length, HPP1, TIMP3, APC, p16, CRBP1, RUNX3	HPP1, p16, RUNX3 independent risk factors in multivariate analyses. Model combined HR index >5 leads to an increased likelihood of progression within 2 years.
Wang 2009 [38]	Retrospective cohort	57 (7:50)	p16 + APC	Hypermethylation of both APC and p16 OR = 14.97 (CI = 1.73-∞, p=0.012) for neoplastic progression.

*Number of lesions, no patient numbers described in study; Number of progressor lesions (P), number of non-progressor lesions (NP)

5 studies developed models aiming to predict progression to OADC (Table 23) (138, 141, 142) (143) (144). Three models included both epigenetic markers and clinical parameters.(142) (143) (144)

Schulmann et al (142) performed a retrospective longitudinal analysis of 53 patients contributing 106 specimens enrolled in a BO surveillance programme using Cox proportional hazards regression. Initially, ten candidate prognostic marker genes were assessed using cross sectional data. Six of the genes (*HPP1*, *TIMP3*, *APC*, *p16*, *CRBP1*, *RUNX3*) which demonstrated hypermethylation in OADC patients and relative hypomethylation in normal oesophageal tissues were investigated in the longitudinal study. All except *APC* were associated with progression (HGD and OADC combined) in univariate analysis. A multivariate model including all six genes, age, and segment length found evidence that *HPP1*, *p16* and *RUNX3* were independently prognostic. The performance of the full model (all 8 covariates) was assessed by calculating the exponentiated multiplier of baseline hazard for covariates for each specimen (HR index). For specimens taken within 2-years of progression the HR index was >5 in progressors compared to <5 in non-progressors. The authors report the HR index was not predictive for specimens taken over two years before progression. The study reports odds ratios (OR) but doesn't explain how as the Cox model produces hazard ratios (HR). The authors note that the sample size is small, the study is retrospective, other potential clinical predictors were unavailable, and the model uses a combined end point of HGD and OADC. In univariate analysis *TIMP3* had an OR for progression of 1.68 (95% CI 1.14-2.38) but this flipped to 0.5 (95% CI 0.22-1.04) in the multivariate analysis. The authors conclude that

further validation of the markers (*HPP1*, *p16*, and *RUNX3*), ideally in prospective multi-centre trials is required.

Sato et al (143) performed a retrospective cohort study on 62 patients providing a total of 118 specimens. They developed a model using linear discriminant analysis incorporating both clinical and epigenetic markers. Sex, BO segment length and histological diagnosis were combined with *p16*, *HPP1* and *RUNX3* CpG methylation status. This was further combined with a methylation index with a score of 0-3. The best receiver operating characteristic curve (ROC) for 4 year follow up in this cohort was generated using segment length, pathology and methylation index with an area under ROC (AUROC) of 0.7910 (95% CI 0.6968-0.8853) with a specificity and sensitivity of 91.4% and 51.8% respectively. Change in AUROC between this model and a model including predictive markers alone was not assessed. Patient selection criteria were not reported, and there was a small sample size (62 patients) with a large number of possible parameter combinations (n=127). Cut-points for variables were chosen to optimise performance in this dataset. These factors result in a high level of uncertainty about the optimal parameter set. No external validation was performed.

Jin et al (144) performed a double blind multicentre case control study of 195 tissue specimens using an 8 methylation biomarker panel of *p16*, *HPP1*, *RUNX3*, *CDH13*, *TAC1*, *NELL1*, *AKAP12*, and *SST* combined with patient age to predict which patients will progress to OADC. When assessed individually *p16*, *HPP1* and *RUNX3* were associated with progression to OADC ($P < 0.05$). When the panel of all 8 markers was used compared with age alone as a predictor of

progression, the increment in AUROC was 0.114 in a 4 year follow up (0.630 age alone, 0.753 age + markers), demonstrating a clinically important improvement in the predictive power of the model within the dataset. This study compared clinical factors between progressor and non-progressor groups and found no significant difference in gender, body mass index (BMI), BO segment length, smoking status or alcohol consumption. External validation was not performed.

Clement et al (138) performed a retrospective cohort study of 28 tissue specimens. 81% of patients (n=12) with combined hypermethylation of *APC*, *TIMP3* and *TERT* progressed to OADC compared with 26% of non-progressors (n=16) ($p<0.0001$). It was suggested that in combination these three markers could be used to predict which patients are at higher risk of progression. No sensitivities or specificities were reported and there was no ROC analysis. There is no indication of independent prognostic value and no internal or external validation was performed using this model.

Wang et al (141) performed a retrospective cohort study on 7 progressor and 50 non-progressor patients. They reported that hypermethylation in both *p16* and *APC* was a strong predictor of progression to dysplastic BO or OADC. Patients who were negative for both *p16* and *APC* hypermethylation did not progress. Hypermethylation of both *APC* and *p16* yielded an odds ratio of 14.97 (CI = 1.73- ∞ , $p=0.012$) for subsequent progression to HGD or OADC. A limitation of this study is the short follow up time of 4.1 years for the non-progressor group which may not be sufficient time for dysplasia or neoplasia to develop. The reported

confidence interval is extremely wide making it difficult to accurately interpret the odds ratio.

5.16 INDIVIDUAL MARKERS ANALYSED

42 individual epigenetic markers were analysed in the 14 included studies. Ten studies investigated one or more of the following 5 individual markers: - *p16* (*CDKN2A*), *RUNX3*, *TIMP3*, *HPP1*, and *APC*. Details of markers, assessment methods and findings can be found in tables 24-28. All 5 markers demonstrated CpG hypermethylation in patients who progressed from NDBO to OADC in at least one of the included studies.

p16 (a tumour suppressor protein encoded by the *CDKN2A* gene) offered the most experimental data with 10 studies reporting on 220 progressor samples compared to 332 non-progressor samples. 8 of the 10 studies demonstrated a statistically significant difference in hypermethylation at p16. (139, 141-144, 146-148). Barrett et al (140) demonstrated CpG hypermethylation of *CDKN2A* in 7 progressor samples of a specific genetic clonality, however no control group was analysed in parallel for this specific marker in this study.

Table 24 - P16/CDKN2A

Author/Year	Patient number (P:NP)	Experimental Technique Used	Methylation threshold definition	Statistical Analysis	Result
Barrett 1999 [37]	49 (39:6)	Methylation Specific PCR	Positive or negative PCR on agarose gel	Not stated	7 progressor patients with specific genetic abnormality (LOH at 17p and 9p) displayed hypermethylation at CDKN2A No numerical value offered
Boerwinkel 2014 [43]	44 (48:10)	Methylight Methylation Specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation: p16 cutoff = 0.02	Mann-Whitey test	Raw MSP values showed Hypermethylated in P $p < 0.05$
Clement 2006 [35]	28* (12:16)	Methylation Specific PCR	Intensity of methylation specific dot-blot assay compared	Not stated	0% P vs 12% NP methylated. No significant difference between P and NP.
Eads 2001 [44]	20 (12:8)	Methylight Methylation Specific PCR	Intensity of methylated genes compared to controls. "PMR" value of 4 used as cutoff to indicate methylated gene.	Fisher's PLSD	Intensity of PCR band used for quantitative analysis Hypermethylated in P $p = 0.0048$
Jin 2009 [41]	195* (50:145)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Student's T Test and Chi Squared Test AUROC	Normalised methylation values P:NP 0.138:0.069 AUROC = 0.628 (0.534, 0.722) 90% sensitivity 90% specificity Hypermethylated in P $p = 0.0066$
Klump 1998 [36]	14 (10:4)	Methylation Specific PCR	Yes/No detection of PCR product	Chi squared test	Hypermethylated in P 8% P vs 0% NP $p = 0.0001$

Puertas Canterina 2012 [45]	35 (6:14)	Bisulphite Pyrosequencing	Quantitative CpG methylation technique	No statistical analysis	Hypermethylated in P Methylation Grade 12.04% P : 6.53% NP
Sato 2008 [40]	62 (28:34)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Linear discriminant analysis (LDA), Leave- one-out crossvalidation (LOOCV), AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index = 0.0335 90% sensitivity 90% specificity p=0.00576
Schulmann 2005 [39]	53 (8:45)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Cox proportional hazards ratio	Hypermethylated in P OR 1.74 p=0.0005
Wang 2009 [38]	57 (7:50)	Methylation Specific PCR	No threshold described	Odds ratio using univariate logistic regression	Hypermethylated in P OR 10.02 p=0.034

*Number of lesions, no patient numbers described in study; Number of progressor lesions (P), number of non-progressor lesions (NP)

RUNX3 and ***HPP1*** were analysed in 4 studies(142-144, 146) as individual markers. 134 progressors and 234 non-progressors were studied overall. Three studies (142-144) demonstrated significant hypermethylation in both *RUNX3* and *HPP1* CpG sites in progressor samples, however one study (146) found no difference for either *HPP1* or *RUNX3*.

Table 25 – RUNX3

Author/Year	Patient number (P:NP)	Methods	Methylation threshold definition	Statistical Analysis	Result
Boerwinkel 2014 [43]	44 (48:10)	Methylight Methylation Specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation. RUNX3 cutoff = 0.02	Mann-Whitey test	Raw MSP values compared but no numerical value offered P vs NP not significantly differentially methylated
Jin 2009 [41]	195* (50:145)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Student's T Test and Chi Squared Test AUROC	Hypermethylated in P NMV 0.104:0.063 AUROC = 0.671 (0.586, 0.756) 90% sensitivity 90% specificity p=0.0002
Sato 2008 [40]	62 (28:34)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Linear discriminant analysis (LDA), Leave-one-out crossvalidation (LOOCV), AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index = 0.0216 90% sensitivity 90% specificity p=0.016
Schulmann 2005 [39]	53 (8:45)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Cox proportional hazards ratio	Hypermethylated in P OR = 1.80 p=0.0267

*Number of lesions, no patient numbers described in study; Number of progressor lesions (P), number of non-progressor lesions (NP)

Table 26 – HPP1

Author/Year	Patient number (P:NP)	Methods	Methylation threshold definition	Statistical Analysis	Result
Boerwinkel 2014 [43]	44 (48:10)	Methylight Methylation Specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation. HPP1 cutoff = 0.05	Mann-Whitey test	P vs NP not significantly differentially methylated
Jin 2009 [41]	195* (50:145)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Student's T Test and Chi Squared Test AUROC	Hypermethylated in P AUROC = 0.647 (0.556, 0.739) p=0.0025
Sato 2008 [40]	62 (28:34)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Linear discriminant analysis (LDA), Leave-one-out crossvalidation (LOOCV), AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index 0.028 90% sensitivity 90% specificity p=0.018
Schulmann 2005 [39]	53 (8:45)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Cox proportional hazards ratio	Hypermethylated in P OR = 1.77 p=0.0311

*Number of lesions, no patient numbers described in study; Number of progressor lesions (P), number of non-progressor lesions (NP)

Three studies investigated **TIMP3** CpG methylation (138, 142, 147) (32 progressor and 69 non-progressor). One study showed a statistically significant difference in CpG hypermethylation between progressors and non-progressors(138). One study showed significant hypermethylation and calculated an odds ratio of 1.68 (CI 1.14-2.38) for progression on univariate analysis (142); however **TIMP3** could not be regarded as an independent risk factor as the OR

dropped to 0.5 (CI = 0.22-1.04) on multivariate analysis. The final study (147) study showed significantly increased *TIMP3* methylation in non-dysplastic tissues of patients with associated dysplasia elsewhere in the oesophagus, however there was no difference between progressors and non-progressors.

Table 27 – TIMP3

Author/Year	Patient number (P:NP)	Methods	Methylation threshold definition	Statistical Analysis	Result
Clement 2006 [35]	28* (12:16)	Methylation Specific PCR	Intensity of methylation specific dot-blot assay compared	Not Stated	Hypermethylated in P % Samples Methylated P=91% : NP=23% p<0.0001
Eads 2001 [44]	20 (12:8)	Methylight Methylation Specific PCR	Intensity of methylated genes compared to controls. "PMR" value of 4 used as cutoff to indicate methylated gene.	Fisher's PLSD	Hypermethylated in non-dysplastic tissue in patients with associated dysplasia, however not statistically significant when comparing P vs NP p=0.13
Schulmann 2005 [39]	53 (8:45)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Cox proportional hazards ratio	Hypermethylated in P, but not independent risk factor in multivariate analysis OR = 1.68 univariate OR = 0.50 multivariate p=0.0109

*Number of lesions, no patient numbers described in study; Number of progressor lesions (P), number of non-progressor lesions (NP)

Three studies analysed **APC** (138, 141, 142) (26 progressor, 116 non-progressor), Wang et al (141) demonstrated hypermethylation in progressors. Clement et al(138) reported hypermethylation but did not provide any statistical analyses. Wang et al calculated an OR of 9 (CI 1.01 – 88.52) for hypermethylation of *APC* in progressors. Conversely Schulmann et al (142) found no evidence of a difference (OR=1.00 (CI 0.59 – 1.33)) in progression with or without hypermethylation of *APC*. All three studies have similar sample size, study design

and experimental techniques. These contradictory findings with limited statistical validation and wide confidence intervals call into question the validity of *APC* as an epigenetic marker of BO progression.

Table 28 – APC

Author/Year	Patient number (P:NP)	Methods	Methylation threshold definition	Statistical Analysis	Result
Clement 2006 [35]	28* (12:16)	Methylation Specific PCR	Intensity of methylation specific dot-blot assay compared	No statistical analysis offered	Hypermethylated in P Methylation: P=100% vs NP=36%
Schulmann 2005 [39]	53 (8:45)	Methylation Specific PCR	Normalised methylation value (NMV) = Amount of methylated DNA compared to control Beta-Actin DNA generated by PCR	Cox proportional hazards ratio	Hypermethylation not an independent risk factor for P OR = 1.00 (CI 0.59 – 1.33) p=0.99
Wang 2009 [38]	57 (7:50)	Methylation Specific PCR	No threshold described	Odds ratio using univariate logistic regression	Hypermethylated in P Methylation: P=86% vs NP=40% p=0.02 OR = 9 (1.01-80.52) p=0.049

**Number of lesions, no patient numbers described in study; Number of progressor lesions (P), number of non-progressor lesions (NP)*

The main methodological limitations across the studies were poor reporting of patient characteristics and study populations, including patient selection. There was little information about loss to follow up and it was often unclear exactly which samples were used and at which time-points in longitudinal analyses. There was limited blinding of researchers to the histology and progressor status of the samples was often not described. Confounding factors were described in 6 of the studies (111, 136, 141-144), but only Sato et al (143) adjusted for BO segment length and patient sex in their predictive model.

Given these methodological uncertainties, the findings need to be viewed with caution.

5.17 DISCUSSION

This systematic review of the literature of epigenetic markers and their role in predicting progression of BO to HGD and OADC has revealed a heterogeneous and disparate dataset. Fourteen studies were identified, with five incorporating prognostic models. This is the first systematic review to examine all evidence on epigenetic change and its role in Barrett's carcinogenesis. It suggests a role for *p16* hypermethylation as an individual epigenetic biomarker in predicting progression from BO to OADC (138-144, 146-148). However, a paucity of evidence for other epigenetic markers (and combinations), poor reporting of patient characteristics and methods employed, and lack of external model validation limit the conclusions that can be drawn.

Only 14 studies were identified with small patient numbers (median 31 (5- 195)). The extent of loss to follow-up was usually poorly described. All studies suffered from a paucity of clinical information and a lack of reporting of study patient demographics. Those studies which did report co-morbidities and potential confounding factors such as BMI, smoking status, age, histological diagnosis and BO segment length rarely adjusted for these in their analysis. Without adjusting for these factors, it is difficult to assess the incremental predictive clinical value of any epigenetic changes. Future models should explore the predictive ability of epigenetic changes in the context of clinical variables. In their study Riley et

al(149) concluded that provision of individual patient data could have overcome the majority of the reporting issues including poorly reported summary statistics, adjustment factors and outcome measures used.

It is challenging to compare data from the different studies due to variable measurement of epigenetic change. Five different experimental techniques were utilised: miRNA analysis (n=1)(136) and CpG methylation including, methylation specific PCR (n=9) (150), methylight methylation specific PCR (n=2) (151), bisulphite pyrosequencing (n=1) (148, 152), and methylation microarray technology (n=1) (111). Early methods of measuring differential DNA methylation involve semi-quantitative reporting of results including analysis of intensity of PCR product bands on agarose gel. More advanced techniques utilise computerised photometry, although differing PCR conditions and equipment may result in variability between laboratories. More advanced techniques such as bisulphite pyrosequencing and methylation microarrays can provide an accurate methylation percentage at individual CpG sites which are suitable for quantitative analysis. The 12 studies using methylation specific PCR revealed a global pattern of hypermethylation of progressor patients in the CpG regions of interest(137-147), however, each used differing methylation thresholds and outcome measures for reporting positive results. One study(148) utilised bisulphite pyrosequencing to analyse *p16*, but no further markers were analysed on more than one platform. The different methods used, as well as the different outcome metrics reported and lack of clearly reported clinical information meant that there was insufficient clinical and methodological homogeneity. Meta-analysis was thus

not appropriate, despite individual epigenetic markers being reported in up to 10 independent studies.

Five models looked at various combinations of epigenetic markers and clinical risk factors. Findings from four of the models suggest a potential role for p16 in combination with *HPP1*, *RUNX3* and/or *APC* and clinical factors of segment length or age. All models used slightly different combinations of model parameters and reported analyses differently, thus making it difficult to compare findings between models. All suffered from a lack of external validation and there were uncertainties around the representativeness of the included populations. More research is needed to validate and further explore these initial findings.

p16, a tumour suppressor protein, is the most consistently hypermethylated CpG site in progressors. Hypermethylation leads to reduced *p16* production allowing neoplastic cells to progress from G1 to S phase of cell cycle and replicate uncontrollably. *p16* deletions are observed in melanoma, oesophageal, lung, pancreatic, mesothelioma, bladder, head and neck squamous cell carcinoma, breast, lymphocyte, brain, ovarian, osteosarcoma and renal cancer cell lines (153, 154). In BO, *p16* genetic mutations have been shown to occur early in tumorigenesis and may appear before dysplasia has occurred(155). Timmer et al(156) demonstrated the presence of *p16*, *MYC* and aneusomy in a model with age and segment length determined whether patients were at low or high risk of progression from NDBO to OADC. Without further in depth temporal analysis it remains unclear whether the index abnormality of Barrett's carcinogenesis is epigenetic or genetic in origin.

The pattern of hypermethylation in *RUNX3* and *HPP1* in progressors was less clear with not all studies demonstrating a statistically significant difference between progressor and non-progressor patients. *TIMP3*, and *APC* provide variable results in the literature and much smaller patient groups are analysed. Agarwal et al reported a predominance of hypomethylation in progressors (16 of 19 CpG sites) with only three CpG sites (Pro_MMD2, Pro_ZNF358 and Intra_F10) hypermethylated. The methylation microarray used in this study can analyse 28,700 unique CpG sites, whilst the latest technology offers up to 850,000 sites on a single chip(157). With only a single study using an outdated technique, it is not possible to say whether CpG hypomethylation is a driver in progressive BO. Prior to this technology being available researchers had to carefully select CpG sites of interest for analysis; selection was based on previous research and literature review giving rise to a positive selection and publication bias. This is a well recognised phenomenon in prognostic factor research. Sekula et al (158) reviewed published and unpublished work into *p53*'s role in bladder cancer and discovered that 31% of observational studies were unpublished in a 15 year period. Whilst Sekula's review is concerned with a different pathology, the type of included studies are of a similar design to those included in this review. A combination of positive publication bias and marker selection bias raises questions as to the validity of the selected markers in studies demonstrating CpG hypermethylation. Selective reporting may also erroneously inflate the importance of individual prognostic markers. Kyzas et al(159) analysed published and unpublished data sought directly from researchers investigating the importance of *TP53*'s role in head and neck squamous cell carcinoma on patient mortality. They found that if all published and unpublished data was

included in their meta-analysis the risk ratio decreased from 1.38 to 1.16 (95% CI 0.99 – 1.35 $p = 0.06$). They also had difficulty conducting their analysis due to non-standardised definitions and reporting. Heterogeneity in definitions and reporting were also identified within our dataset, and it must also be considered whether selective reporting of results is over stating the importance of individual markers such as *p16* in the progression of BO. Similarly if ever increasing numbers of CpG sites are analysed with microarray technology there will be an increasing number of negative results which are likely to be under reported in the literature. As illustrated by Kyzas and Sekula (158, 159) despite a large volume of literature, without standardisation of reporting and outcome measures the research is often not suitable for translation into clinical use.

The variable quality of prognostic studies has been previously reported in systematic reviews (160, 161), and improvements suggested for collaboration and standardisation especially with regards to statistical analysis and outcome reporting. (162-164) Poor quality of reporting was also a feature in this systematic review. For example, only 7 of 14 included studies documented both the number of patients and samples analysed in each group. Improved standardisation would make meta-analyses more feasible and lead to more informative systematic review results. This in turn would aid the translation of laboratory work into clinical trials.

5.18 CONCLUSION

OADC represents a large global burden of disease which is increasing in incidence and carries a poor prognosis. The metaplasia-dysplasia-adenocarcinoma sequence is well understood and the pre-cancerous lesion BO is well recognised. There is currently no robust predictive biomarker which can predict which patients with BO will develop OADC and rigorous surveillance using repeated, invasive OGD represents the only way to capture progressive disease and initiate treatment at an early stage. The evidence from the above systematic review is suggestive of a role for *p16* as an individual epigenetic biomarker in predicting progression from BO to OADC. Prognostic models incorporating this and other markers also suggest a role for *p16* in combination with *HPP1*, *RUNX3* and/or clinical markers. These markers were not examined further in this thesis as current genome wide methylation techniques provide better markers with which to inform future cohort studies. Further large primary studies using current epigenetic techniques and standardised reporting are required to inform future models to further explore the role of epigenetics in progression to HGD and OADC.

6. VALIDATION OF OR3A4 AS AN EPIGENETIC BIOMARKER OF “HIGH RISK” BARRETT’S OESOPHAGUS

6.1 INTRODUCTION

There is no current single diagnostic test which can identify which patients will progress from NDBO to OADC. Patients with BO must first be diagnosed using OGD and biopsy and then followed up at regular intervals with surveillance endoscopies. This allows direct visualisation and monitoring of the diseased segment of the lower oesophagus. As discussed in the introduction, this is an imperfect system which not only subjects the patient to multiple invasive surveillance endoscopies, but also to much inter and intra-observer variability, even in the hand of the most well trained clinicians. Epigenetic biomarkers are stable in archival tissue, easy to analyse and give a quantitative result. In UC, epigenetic change can be detected in the early stages of the metaplasia-dysplasia-adenocarcinoma sequence in those patients who go on to develop UC associated neoplasia. These changes are apparent even before dysplasia is visible on histological analysis of the tissues. The ENDCAP-C trial is assessing the utility of epigenetic biomarkers in risk stratification in UC patients which will allow more targeted surveillance in higher risk groups (112). A similar approach could be applicable in Barrett’s associated OADC. A systematic review of the literature has revealed numerous epigenetic changes which are present prior to development of OADC in BO tissues, however only p16 appears to be a robust epigenetic marker in its own right. Predictive models using combinations of

epigenetic biomarkers and clinical factors appear to be the most robust method of risk stratification, however the studies were lacking in external validation and used small patient numbers. They also did not utilise the latest genome wide methylation array technologies and as a result had to be very selective over the epigenetic targets analysed, a possible source of bias and leaving many CpG sites unexplored. If a robust epigenetic biomarker were found which could reliably predict patients who are at high risk of progressing from NDBO to OADC, these patients could undergo enhanced surveillance with the aim of diagnosing and treating BO with dysplasia using minimally invasive techniques before it progressed to OADC. Those at lower risk of progression (99.64% of patients) could be reassured and spared regular invasive surveillance OGDs. A preliminary epigenetic biomarker discovery experiment at the University of Birmingham using the Illumina Humanmethylation 450K arrays (115) showed that the long non-coding RNA OR3A4 CpG promoter site was hypomethylated in patients that progressed from NDBO to OADC. This could provide a novel epigenetic biomarker for progression in BO.

Epigenetic regulation of gene expression by CpG promoter methylation results in a methyl group being projected into the groove between the strands of the DNA double helix. This physically blocks RNA polymerase from transcribing a gene. A CpG site which is proportionally hypermethylated will result in a lower rate of gene expression than one which is hypomethylated. Hypomethylation at OR3A4 in BO tissues which progress to OADC suggest that OR3A4 will be over-expressed in these tissues compared to non-progressing samples.

6.2 Aim

The aim of this experiment was to determine whether OR3A4 methylation status in archival BO tissue samples could differentiate between patients who had progressed to OADC and those that had remained with NDBO. The experiment also aimed to investigate if OR3A4 was over-expressed in NDBO tissue samples from patients that subsequently progressed to OADC compared to those which did not progress.

6.3 METHODS

The experiment was conducted in two phases. The first stage was to perform genome wide methylation analysis using the Illumina Humanmethylation 450K arrays to identify differentially methylated CpG sites between progressor and non-progressor BO patients. The second stage was targeted bisulphite pyrosequencing of any significantly differentially methylated CpG sites using the Qiagen Q48 instrument to validate the methylation array findings.

The experiment was performed in duplicate. The previous clinical research fellow in the Surgical Research Laboratory, Mr MP Dilworth, performed initial epigenetic analysis using a cohort of patients from SWB and presented the findings in his MD Thesis(115). He performed whole genome methylation analysis using Illumina Humanmethylation 450K arrays on a cohort of 12 patients (6 progressors and 6 non-progressors) from SWB and then performed validation bisulfite pyrosequencing on 25 progressing samples and 37 non-progressing samples from SWB.

I then undertook validation work using external cohorts of patient samples from UHB and UHS. I performed whole genome methylation analysis using Illumina Humanmethylation 450K arrays on 6 progressing and 6 non-progressing patients from UHB as described in the section 4.9. The results were then combined to give a total of 12 progressor and 12 non-progressor patients. I performed validation bisulphite pyrosequencing on 32 patients from the UHS external cohort (14 non-progressors and 18 progressors) and 16 patients from the UHB cohort (34 samples, 21 non-progressor, 13 progressor) as described in section 4.8. Pyrosequencing results from 67 patients from SWB, UHB and UHS were combined in the final analysis.

6.3.1 COMPARISON OF DNA EXTRACTION METHODS

DNA is a stable molecule which can preserve genetic information for tens of thousands of years in the correct conditions. It is not immune to degradation, and over time, information is lost. The techniques used for DNA extraction and library preparation differ depending on the intended downstream application. When performing next generation sequencing and epigenetic analysis, single nucleotide resolution is required to make a call and generate accurate results when determining genetic mutations. When first devising optimal tissue preservation and fixing solutions in the late 19th century histopathologists were unaware of DNA and were concerned only with the preservation of cellular and tissue architecture which remains the gold standard for clinical diagnosis from tissue samples(165). Previous fixing with alcohol solutions caused a great deal of tissue distortion and shrinkage leading to diagnostic difficulties. Formaldehyde minimised these architectural changes and was also an antibacterial agent. Tissues which are suspended in formaldehyde undergo hardening, or “fixing”, which preserves the tissue appearances on both a macroscopic and microscopic scale allowing long term storage and serial examinations. These tissues can then be embedded in paraffin for ease of sectioning and then mounted on slides for microscopic examination – Formalin Fixed, Paraffin Embedded (FFPE). Despite adequate tissue fixation and storage conditions, FFPE tissues suffer degradation over time and with it the amount of useful DNA and RNA which can be extracted.

Formaldehyde fixation causes a number of different DNA sequence artefacts(166) including formaldehyde induced DNA crosslinks, DNA fragmentation, Abasic sites and de-amination of cytosine bases. Increasing the amount of input DNA for downstream applications can increase read depth and accuracy of sequence analysis. With more limited amounts of DNA, limitation in sequencing read depths casts doubt over results generated as these may be artefact from the fixation process or produced by chance.

To determine whether hypomethylation of OR3A4 was an epigenetic marker for progression of NDBO to OADC it was necessary to collect samples pre-dating any dysplasia, it was often more than 15 years since the initial samples were processed. Since diagnosis of NDBO only requires endoscopic biopsy, there was also limited tissue available from these samples which measured 2-3 mm in their maximal dimension. Large volumes of tissue were only present in oesophageal resection specimens. It was paramount to ensure the most efficient DNA extraction methods were used for the samples.

12 FFPE samples from oesophageal resection specimens were extracted in duplicate to compare the Maxwell RSC semi-automated DNA extraction and Qiagen DNEasy kit. The protocols are detailed in section 4.4.2 (Maxwell RSC) and 4.4.4 (Qiagen DNeasy).

6.3.2 ILLUMINA HUMANMETHYLATION 450K ARRAY

DNA was extracted from FFPE tissue samples using the Qiagen DNEasy kit and subject to quality control as previously described. DNA was bisulphite converted using the Zymo EZ DNA methylation kits and then restored using Illumina Restore kits. The restored, bisulphite converted DNA was hybridised to the Humanmethylation 450K arrays and read on the Illumina iScan instrument. Methylation data was kindly analysed by Mr AD Beggs using the ChAMP package in R studio. DNA copy number analysis was performed using the DNACopy module of ChAMP.

6.3.3 QIAGEN Q48 BISULPHITE PYROSEQUENCING

Extracted DNA was subject to bisulphite conversion and subsequent targeted bisulphite PCR. The Q48 instrument disc was loaded with biotinylated bisulphite PCR product and magnetic beads and the instrument injectors charged as per the software instruction. Results were analysed using the Pyromark Q48 software.

6.3.4 OR3A4 EXPRESSION IN TISSUE SAMPLES

A subset of 12 patients from UHB were selected for analysis (6 non-progressor, 6 progressor). H&E slides from NDBO tissues were reviewed by a specialist GI Histopathologist (R Hejmadi) to ensure that no dysplasia was present. There was no positive control available for OR3A4 tissue staining. The pathologist was blinded to the progressor status of each tissue sample at all stages. The selected tissue blocks were then re-cut, mounted on slides and stained using OR3A4

antibody (Abcam ab67107) by the Human Biomaterials Resource Centre (HBRC) at the University of Birmingham (C Bagnall) as detailed in section 4.19. The stained tissue sections were reviewed again by the pathologist and by Mr AD Beggs and staining intensity (0-10) was scored in stromal and epithelial components of the tissue biopsies.

6.4 RESULTS

6.4.1 COMPARISON OF DNA EXTRACTION METHODS

The Qiagen kit consistently outperformed the semi-automated instrument in eluted DNA concentration as shown in

Table 29, and thus this technique was used for all experimental samples.

Table 29 - Comparison of Maxwell RSC and Qiagen DNEasy DNA Extraction Techniques

Sample Year	Sample Number	Maxwell DNA Concentration (ng/uL)	Qiagen Dneasy ng/uL
2007	4	6.04	13.3
2007	5	0	7.78
2007	6	5.56	12.6
2007	20	6.04	13.3
2007	21	0	7.78
2007	22	5.56	12.6
2013	9	5.76	10.3
2013	10	28.6	37.6
2013	24	5.76	10.3
2013	25	28.6	37.6
2014	12	39.8	97.8
2014	26	39.8	97.8

6.4.2 ILLUMINA HUMANMETHYLATION 450K ARRAY

24 patient samples (12 progressor, 12 non-progressor) were hybridised successfully to the Illumina Humanmethylation 450K arrays. These demonstrated 44 significantly differentially methylated CpG sites between the cohorts.

Table 30 – Top 20 array identified differentially methylated CpG sites in progressor vs non-progressor NDBO samples

Probe ID	t	P.Value	Adj.P.Value	Gene Name
cg09890332	-12.26	2.02E-08	0.0031	OR3A4
cg24007926	-11.05	6.84E-08	0.0032	chr2:206842761– 206842761, hg19 coordinates
cg17337672	-10.95	7.54E-08	0.0032	FGFR2
cg02226469	-10.88	8.17E-08	0.0032	NA
cg17433294	-10.51	1.22E-07	0.0038	NMUR2
cg18479711	-10.31	1.52E-07	0.0039	HDAC4
cg09011162	-10.15	1.82E-07	0.0040	LMF1
cg19733463	-9.83	2.61E-07	0.0045	NMUR1
cg16150571	-9.64	3.26E-07	0.0045	SNORD116-22
cg24424217	-9.62	3.35E-07	0.0045	ZNF511
cg13164993	-9.57	3.52E-07	0.0045	RBP3
cg14019464	-9.53	3.70E-07	0.0045	TRIB3
cg24581378	-9.53	3.71E-07	0.0045	ZAP70
cg05230642	-9.36	4.54E-07	0.0051	SNORD115-14
cg12297814	-9.26	5.08E-07	0.0052	IGFN1
cg11231240	-9.23	5.33E-07	0.0052	NA
cg11864327	-8.96	7.40E-07	0.0063	ZFP2
cg16771467	-8.92	7.78E-07	0.0063	ATP8B1
cg17304276	-8.88	8.14E-07	0.0063	CUX2
cg11443888	-8.86	8.36E-07	0.0063	TMEM151B

Table 30. There was a global tendency towards hypomethylation discovered in progressor patients compared to non-progressors as demonstrated in the volcano plot (Figure 24).

The top ranked differentially methylated CpG site was OR3A4 cg09890332 (chr17:3212495 – 3212495, hg19 coordinates) which was found to be hypomethylated in patients who had progressed to OADC from NDBO compared to those who had not progressed from NDBO (median methylation in progressors = 67.8% vs. 96.7% in non-progressors, $p=0.0001$, $z = 3.85$, Wilcoxon rank sum test).

6.4.3 VALIDATION PYROSEQUENCING

Samples from 67 patients were subject to validation bisulphite pyrosequencing. MP Dilworth performed bisulphite pyrosequencing on 25 progressing samples and 37 non-progressing samples from SWB. I performed bisulphite pyrosequencing on all 32 patients from the UHS external cohort (14 non-progressors and 18 progressors) and 34 samples from the UHB cohort (21 non-progressor, 13 progressor) using the Q48 instrument.

Targeted bisulphite pyrosequencing validated the methylation array findings of hypomethylation at OR3A4 cg09890332. The assay incorporated a further 2 CpG sites within the design at 4 and 10 base pairs downstream of the index CpG cg09890332.

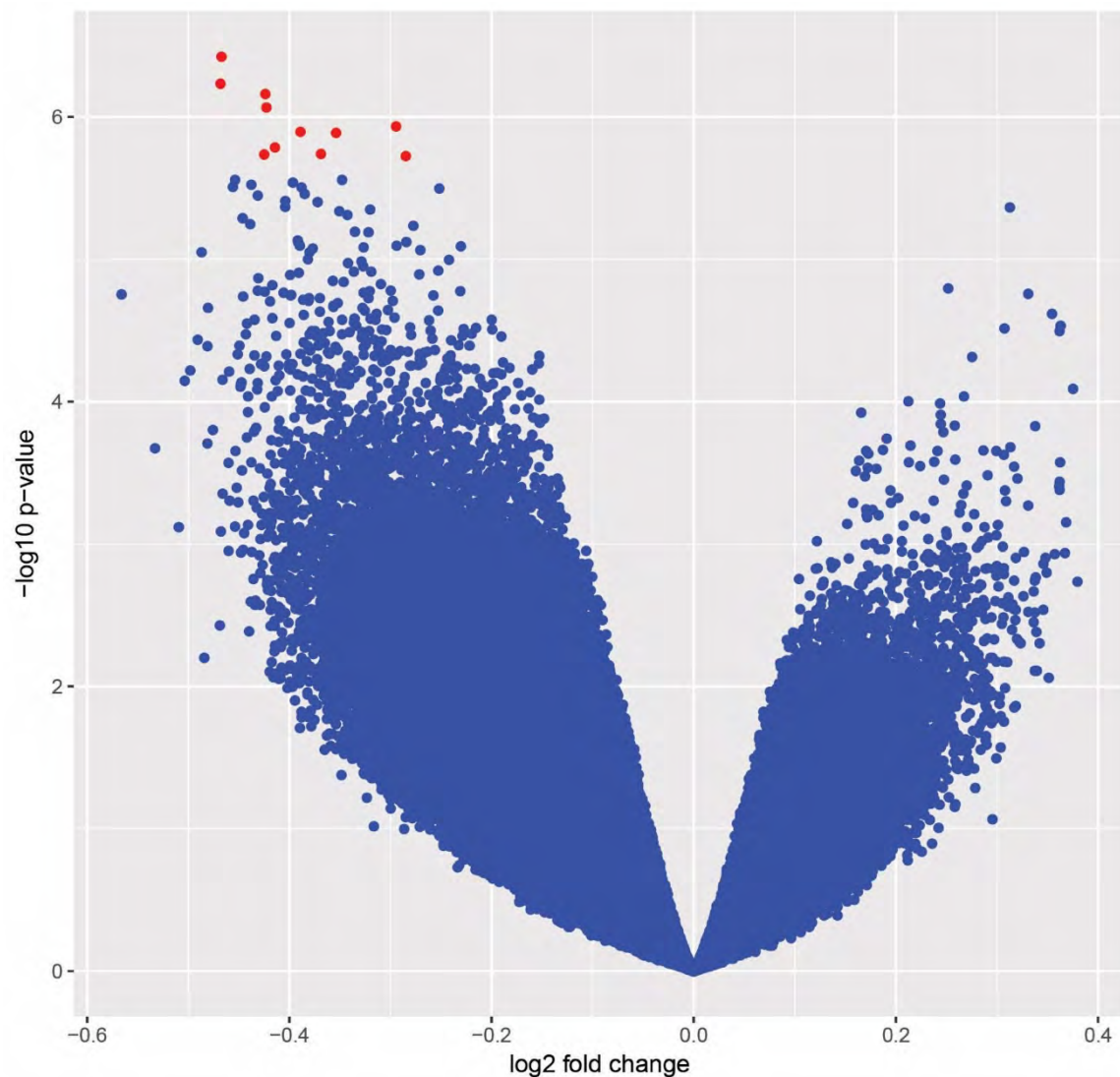


Figure 24 - Volcano plot of probe-level methylation in progressors vs. non-progressors. Blue points = Bayes factor < 5; Red points = Bayes factor > 5. The Bayes Factor is a means of ranking differential methylation by statistical significance in genome wide studies. A Bayes Factor of 5 is equivalent to a p value of 1×10^{-6} .

Median methylation for progressor patients at cg09890332 was 67.8% (Interquartile range 12.1) and 96.7% in non-progressors (IQR 16.1) ($p = 0.0001$, $z = 5.158$; Wilcoxon rank-sum test). The second CpG site showed median methylation of 66.8% in progressors and 75.0% in non-progressors ($p=0.0280$,

Wilcoxon rank sum $z = 2.197$) and the third, 59.7% in progressors and 68.1% in non-progressors ($p=0.0368$, Wilcoxon rank sum $z = 2.088$). An additional pyrosequencing assay was designed to investigate whether this was a localised effect. cg07863524 is located 976 base pairs downstream of cg09890332, 68 base pairs upstream of the OR3A4 transcription start site. Targeted pyrosequencing revealed median methylation of 62.2% in progressors and 56.7% in non-progressors which was not a statistically significant change ($P = 0.600$, $z = -0.524$; Wilcoxon rank-sum test).

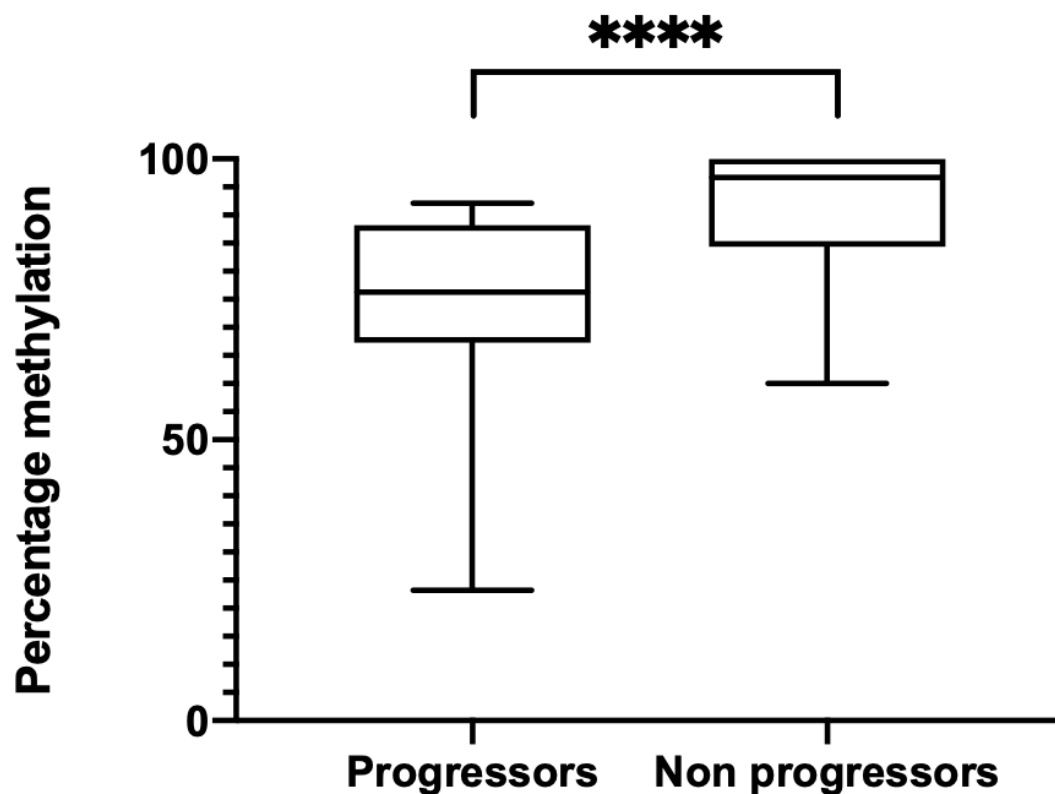


Figure 25 - Box plot of Methylation Percentage at OR3A4 cg09890332 in progressor vs non-progressor patients from targeted bisulphite pyrosequencing. Median methylation for progressor patients at cg09890332 was 67.8% (Interquartile range 12.1) and 96.7% in non-progressors (IQR 16.1) ($p = 0.0001$, $z = 5.158$; Wilcoxon rank-sum test)

6.4.4 EXPRESSION OF OR3A4 IN PROGRESSORS VS. NON-PROGRESSORS

Immunohistochemical assessment of expression of OR3A4 was assessed in a subset of 12 patients. For the stromal compartment, a median staining intensity of 6 (IQR 4-7) was seen in progressors and 2 (IQR 1-3) in non-progressors (Wilcoxon rank sum $p=0.0308$, $z=-2.160$). An example OR3A4 staining in a non-progressing patient is shown in Figure 26 at low power and Figure 27 at high power. An example of a progressing patient is shown in

Figure 28 at low power and Figure 29 at high power. For the epithelial compartment, a median staining intensity of 8 (IQR 8-10) was seen in progressors and 5.5 (IQR 3-10) in non-progressors (Wilcoxon rank sum $p=0.4587$, $z=-0.741$). Percentage methylation at OR3A4 and stromal expression was strongly negatively correlated (Pearson correlation coefficient = -0.85 , $p=0.014$) and a similar, but non-significant correlation was observed with epithelial expression and methylation at OR3A4 (Pearson correlation coefficient = -0.40 , $p=0.373$).

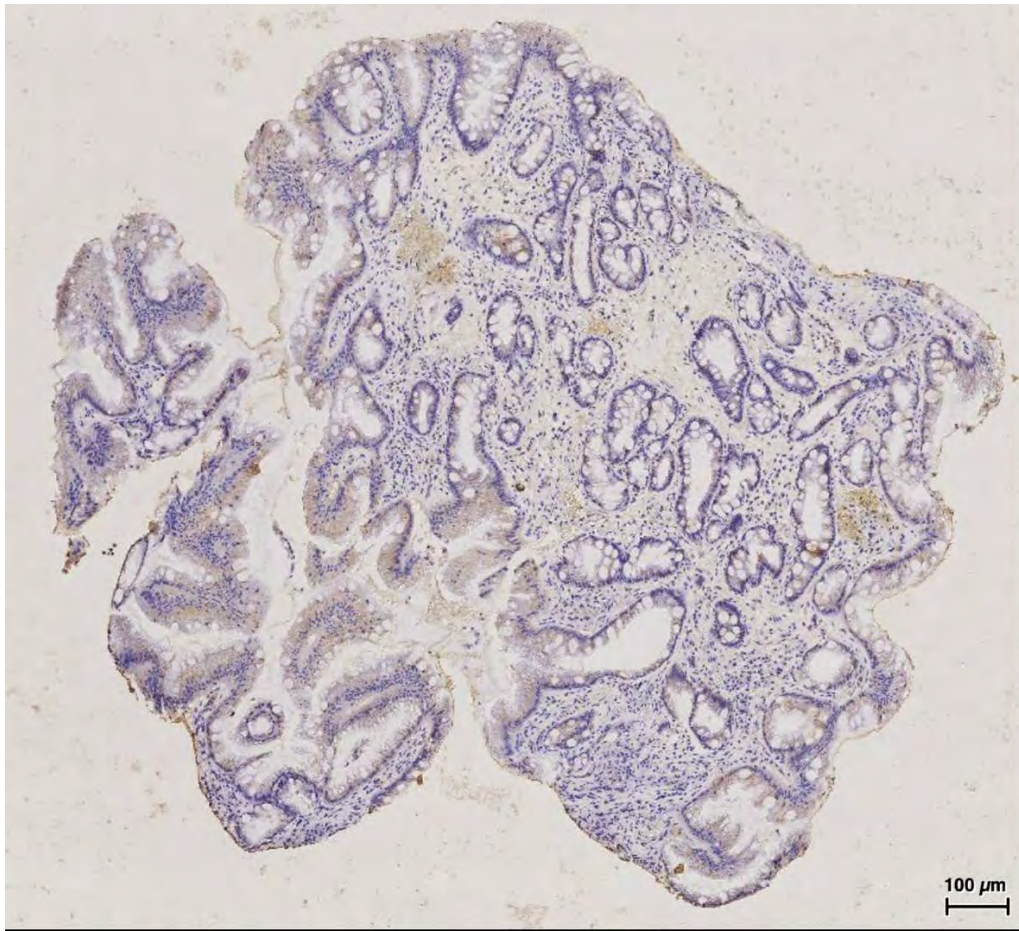


Figure 26 - NDBO from non-progressor patient with OR3A4 staining (4x). OR3A4 is stained brown. In these sections from a patient which did not progress to OADC there is weak OR3A4 staining in both stromal and epithelial components.

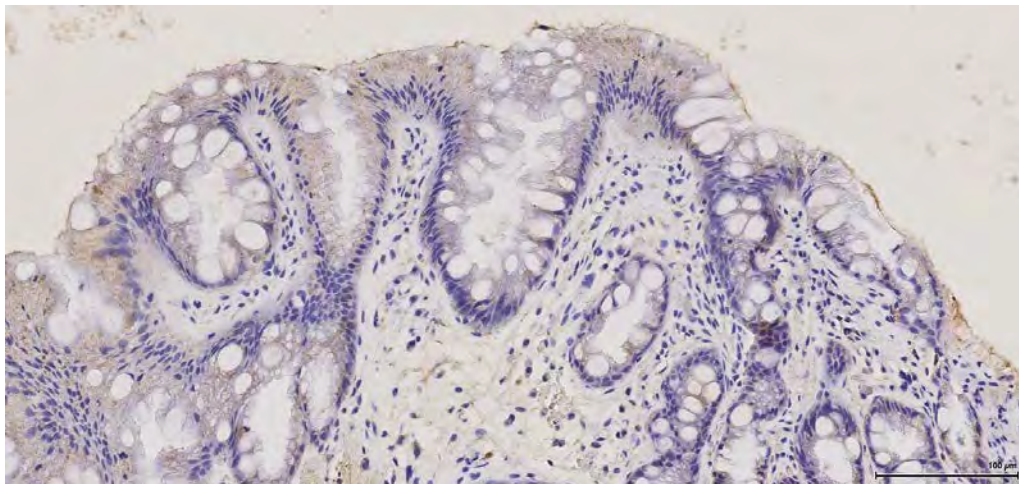


Figure 27 - NDBO from non-progressor patient with OR3A4 IHC staining (20X)



Figure 28 - NDBO tissue section from a patient that progressed to OADC, stained with OR3A4 (4X). OR3A4 is stained strongly in these tissue sections from a patient that subsequently progressed to OADC. In this section both stroma and epithelial components stain strongly indicating upregulation of OR3A4.

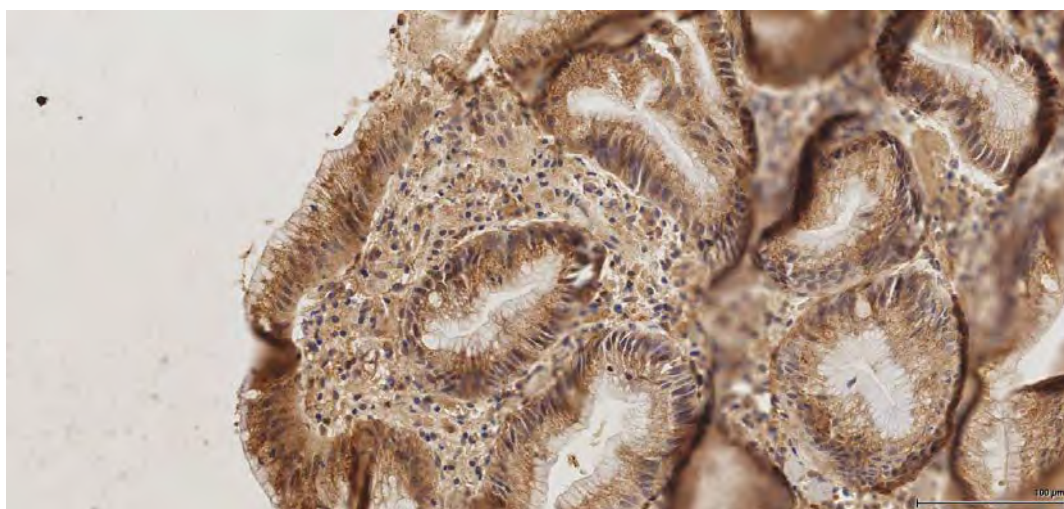


Figure 29 - NDBO from progressor patient with OR3A4 staining (20X)

6.5 DISCUSSION

Epigenetic analysis of tissue samples from patients who have progressed from NDBO to OADC have shown significant hypomethylation at OR3A4 cg09890332 when compared to non-progressing patients. This appears to be a very localised effect at this CpG locus in OR3A4. Cg07863524, which is located closer to the transcription start site of OR3A4 than cg09890332, did not demonstrate significantly different methylation percentages between progressor and non-progressor patients.

OR3A4 is a long non-coding RNA, over-expression of which has been implicated in tumorigenesis in breast(167) and gastric cancer(168), but never before in oesophageal adenocarcinoma. This is particularly interesting as hypomethylation of a CpG gene promoter site should lead to increased expression of that gene. In previous studies citing OR3A4 as a pro-oncogene, there is no suggestion that over-expression is epigenetically modulated. These results suggest that OR3A4 should show increased expression in BO tissue samples in patients who progress to OADC.

The global pattern of hypomethylation seen in progressing patient samples is echoed in Agarwal et al's study using Agilent 244K Human CpG Island microarrays(111) which contain 244,000 probes which correspond to 27,800 CpG sites. In the systematic review earlier in this thesis, most of the studies analysed the epigenetics of targeted regions of interest rather than shotgun approaches which can give a broader view of methylation within the genome. These targeted studies reported hypermethylation of the CpG loci of interest, but due to the

limited nature of the epigenetic analysis offered have not reported on the global pattern of hypomethylation seen in the whole genome methylation analysis reported above. Agarwal et al's study is noteworthy as they also report on progressing BO samples and use a similar board spectrum approach to our experiment. Global hypomethylation suggests an upregulation of genes leading to development of OADC. This pattern of hypomethylation in pre-malignant lesions is also seen in Beggs et al's study (169) which compared normal colorectal tissue to adenomatous polyps and colorectal cancer. This showed that pre-cancerous adenomas demonstrated global hypomethylation when compared to normal tissue samples whereas carcinomas were globally hypermethylated in comparison to adenomas and normal tissues.

The disadvantage of our study model was the long follow up required to demonstrate "never dysplastic" BO and the small endoscopic biopsy samples used for surveillance meant that there was limited and degraded tissue for analysis. These issues caused challenges with DNA extraction and as a result an entire cohort of external samples were rendered unsuitable for taking forward to methylation array analysis due to limited DNA weight and quality. Power calculations for whole genome methylation arrays from Tsai et al (170) were used. A sample size of 24 patients allows differential methylation of 9% to be detected with a power of 80% at $p < 0.05$ in an EWAS case control study. For a more stringent statistical significance ($p < 1 \times 10^{-6}$) a sample size of 25 would allow differential methylation of 30% to be detected with a power of 90%, thus the effect seen in this study has sufficient power to detect differential methylation of this magnitude in OR3A4.

DNA methylation is an ideal candidate as a tissue biomarker as methylation remains stable in archival tissue samples. This would allow patients who have historical endoscopic oesophageal tissue biopsies to undergo risk stratification using OR3A4 methylation status in conjunction with other clinical and biochemical parameters. Those who are at high risk of progression could be entered into enhanced surveillance programmes. Targeted bisulphite pyrosequencing can reduce the amount of DNA required for epigenetic analysis as a PCR step is incorporated to amplify the sample DNA. Devices such as the Cytosponge(171) could be used to gather cytological samples in BO screening and potentially eliminate the need for an index tissue biopsy for screening purposes. The cytosponge is a device in which a dissolvable capsule containing a sponge on a long tether is ingested orally and passed into the stomach. Once the capsule dissolves, the sponge expands. When the sponge is retrieved from the stomach, the sponge scrapes the surface of the oesophagus and collects cytological samples of the entire length of the oesophagus. If epigenetic change at OR3A4 could be analysed in these cytological specimens the patients could be advised whether they are at low or high risk of developing Barrett's associated neoplasia in the future and could undergo an OGD. This technique could further minimise the use of endoscopy in the identification of high risk BO and low risk patients may be able to avoid endoscopy entirely. The advantage of the Cytosponge is that it does not require specialist endoscopic facilities to perform the test and could be performed in primary care or even at patients' homes much like the faecal occult blood test for colon cancer screening currently being offered to the over 60's in the UK(172). It is unrealistic to expect that the presence of

hypomethylation at OR3A4 on oesophageal cytology from Cytosponge examination will be the sole determinant in OADC screening, however it may provide a useful, minimally invasive initial triage.

The effect of hypomethylation on the OR3A4 gene seems to be functional, in that immunohistochemistry reveals an increase in OR3A4 expression in samples with hypomethylation.

The finding that the stromal expression in progressor patients is increased is of interest given the known effect of “pathological” stroma in the pathogenesis of oesophageal cancer(173). Underwood et al’s study focuses on cancer associated fibroblasts rather than other tissue biomarkers. It describes how a stromal component of OADC which is rich in cancer associated fibroblasts with α -SMA positivity conveys a worse prognosis. This is also a more strongly prognostic factor than traditional histopathological criteria. Additional staining of tissue biopsy samples could be used in conjunction with OR3A4 methylation status, histological assessment using H&E slides and clinical parameters to aid risk stratification of BO and guide further surveillance on an individual patient basis.

6.6 CONCLUSION

Hypomethylation at the CpG gene promoter site of OR3A4 in BO tissues can identify patients at high risk of progression to OADC before dysplastic change has occurred. This effect is seen across multiple cohorts and methylation array findings have been validated with targeted bisulphite pyrosequencing at

cg09890332. IHC staining of OR3A4 in NDBO tissues reveals increased expression of OR3A4 in the stroma of patients who progress to OADC compared to non-progressors. This suggests that OR3A4 may possess a functional effect in Barrett's carcinogenesis and may provide an adjunct to standard histological analysis for risk stratification of progression to OADC. Further prospective clinical trials are required to validate OR3A4 hypomethylation as a tissue biomarker.

7. FUNCTIONAL ANALYSIS OF OR3A4

7.1 INTRODUCTION

Patients who progress from NDBO to OADC have relative hypomethylation at the OR3A4 CpG site. Functionally, hypomethylation of a gene should cause upregulation in its expression and its downstream pathways. Thus, patients who progress to OADC from BO should over-express OR3A4 when compared to non-progressors. Tissue sections of NDBO from patients that progress to OADC demonstrate more intense staining of OR3A4 in the stroma than non-progressors which appears to validate this hypothesis.

7.1.1 FUNCTIONAL RELEVANCE OF LNCRNAs IN HUMAN CARCINOGENESIS

The human genome contains roughly 20,000 protein coding regions. This represents less than 2% of the total genetic sequence. Transcriptomics has revealed that 90% of the genome is transcribed, and although initially much of this activity was thought to be noise generated from the transcription of proteins with no functional relevance, there is mounting evidence that dysregulation of these non-protein coding regions may have significant effects on the pathogenesis of human disease, especially cancer (174).

Long non-coding RNAs (lncRNA) are defined as RNA molecules greater than 200 nucleotides in length which carry no clear protein coding capacity. lncRNAs are of increasing interest as they appear to directly affect the transcriptome. For example, the lncRNAs HULC (highly upregulated in liver cancer) (175) and pseudogene pair “phosphatase and tensin homolog” (PTEN) and “phosphatase and tensin homolog pseudogene 1” (PTENP1)(176) act upon the transcriptome by blocking miRNA function. PTENP1 is extremely similar to PTEN and contains only 18 mismatches compared to the coding gene with a single missense mutation stopping translation(177). These lncRNAs appear to bind miRNAs directly and in the case of PTENP1, miRNAs which would usually downregulate PTEN, a tumour suppressor gene, bind instead to PTENP1 which allows normal expression of this gene to occur(176). Alimonti et al demonstrated that increasing levels of PTEN expression in murine models resulted in decreased cancer incidence, with even small decreases in expression resulting in increased cancer incidence between cohorts (178). This suggests that even minute changes in the pseudogene may reduce the miRNA binding efficacy and increase an individual’s susceptibility to develop cancer.

Dysregulation of lncRNAs have been associated with numerous human cancers(174, 179). The HOX antisense intergenic RNA (HOTAIR) was found to be upregulated in breast cancer and especially in metastatic breast cancer (180). Over-expression of HOTAIR was a significant predictor of breast cancer metastasis and death resulting from breast cancer and is thought to act by binding and remodelling chromatin at the polycomb repressive complex 2 (PRC2) and downregulating its target genes. Metastasis associated lung adenocarcinoma

transcript 1 (MALAT1) is a lncRNA described by Ji et al (181). It is significantly associated with lung metastasis in non-small cell lung cancer and could be used as a prognostic factor in stage I disease. MALAT1 is also upregulated in breast cancer(182), endometrial stromal sarcoma(183) and hepatocellular carcinoma(184, 185). Upregulation of the lncRNA HULC was initially associated with hepatocellular cancer and colorectal liver metastases, but it has also been implicated in other cancers including gastric cancer, pancreatic cancer and osteosarcoma (186). The lncRNA BC200 was primarily of interest as it was selectively expressed in neural tissues when it was first isolated, although its functional significance remained unknown at the time (187). Subsequent studies have demonstrated that differential expression of BC200 occurs in breast, cervical, oesophageal, lung, ovarian, parotid and tongue cancers(188).

lncRNAs can also act as tumour suppressors as is the case with maternally expressed gene 3 (MEG3). Over-expression of MEG3 appears to activate p53 tumour suppressing pathways and increase p53 levels in vitro (189). In HeLa (cervical cancer), MCF-7 (breast cancer) and H4 (neuroglioma) cell lines, over-expression of MEG3 inhibits growth(190).

7.1.2 OR3A4 FUNCTIONAL RELEVANCE IN OTHER TUMOUR TYPES

Initially, the long non-coding RNA OR3A4 pseudogene was thought to have no functional relevance. It is a pseudogene for the olfactory receptor (OR) molecule number 4 in OR subfamily 3A. However, there is increasing evidence that over-expression of OR3A4 may cause increasingly aggressive behaviours in tumour

cells. Guo et al (168) investigated the biological relevance of OR3A4 in gastric cancer. They have demonstrated that OR3A4 expression is upregulated in metastatic gastric cancer tissues compared to normal gastric and gastric cancer tissues. OR3A4 expression in 130 paired tumour/normal gastric cancer tissue samples correlated with lymph node metastasis, depth of tumour invasion and distal metastasis. Plasma DNA from 130 gastric cancer patients and healthy controls was taken and examined for OR3A4 expression levels. In blood the relative expression levels of OR3A4 were 56.95 ± 49.01 for healthy controls and 155.83 ± 73.65 for gastric cancer patients. ROC analysis showed an AUC of 0.852 ± 0.025 with a sensitivity of 86.94% and a specificity of 91.27%. This suggests that plasma levels of OR3A4 could act as a molecular marker for gastric cancer. High OR3A4 expression levels also correlated with poorer survival and a shorter time to recurrence of gastric cancer, although it is not clear from their manuscript whether this is blood or tissue expression levels. It is also not clear what threshold values for high and low expression of OR3A4 are used.

When investigating the functional relevance of OR3A4 in gastric cancer, RTqPCR was used to assess expression levels in the gastric cancer cell lines SNU-16, AGS, SNU-1, KATOIII, MKN45, NCI-N87, and SGC7901 and the immortalised gastric mucosal line GES-1. This revealed that OR3A4 was highly expressed in these lines, particularly NCI-N87, SUN-16 and AGS where expression increased 39.88 fold, 25.22 fold and 12.87 fold respectively. In vitro models showed increased proliferation of SGC7901 cells when they were transfected with an OR3A4 over expressing vector and decreased proliferation of NCI-N87 cells when siRNA knockdown of OR3A4 was performed. OR3A4 over expression also promoted cell migration and invasion on scratch wound healing assays, whereas

the reverse was observed with OR3A4 siRNA knockdown. In murine models, similar effects were demonstrated when gastric cancer cells were injected into nude mice. OR3A4 over-expressing gastric cancer cells developed more rapidly and spread more readily within the peritoneal cavity. When siRNA knockdown of OR3A4 over-expressing cells was performed, the tumours reverted to the original behaviour of the parent cell line, suggesting the effect is mediated by OR3A4 over-expression.

Pathway analysis to investigate OR3A4 target genes by RTqPCR and subsequent RNA immunoprecipitation demonstrated significant enrichment of PDLIM2, MACC1, NTN4 and GNB2L1 with OR3A4 RNA which suggests that these genes are a direct target of OR3A4 and may contribute to tumourigenesis and metastasis in gastric cancer. The function of PDLIM2 was further investigated by transfecting a PDLIM2 over-expressing vector into MKN-45 and NCI-N87 gastric cancer cells which revealed a reduction in tumourigenicity by reduction in cell growth and invasion not seen in the control cells. It would appear that the molecular mechanism for OR3A4 increasing tumourigenicity involves downregulation of PDLIM2.

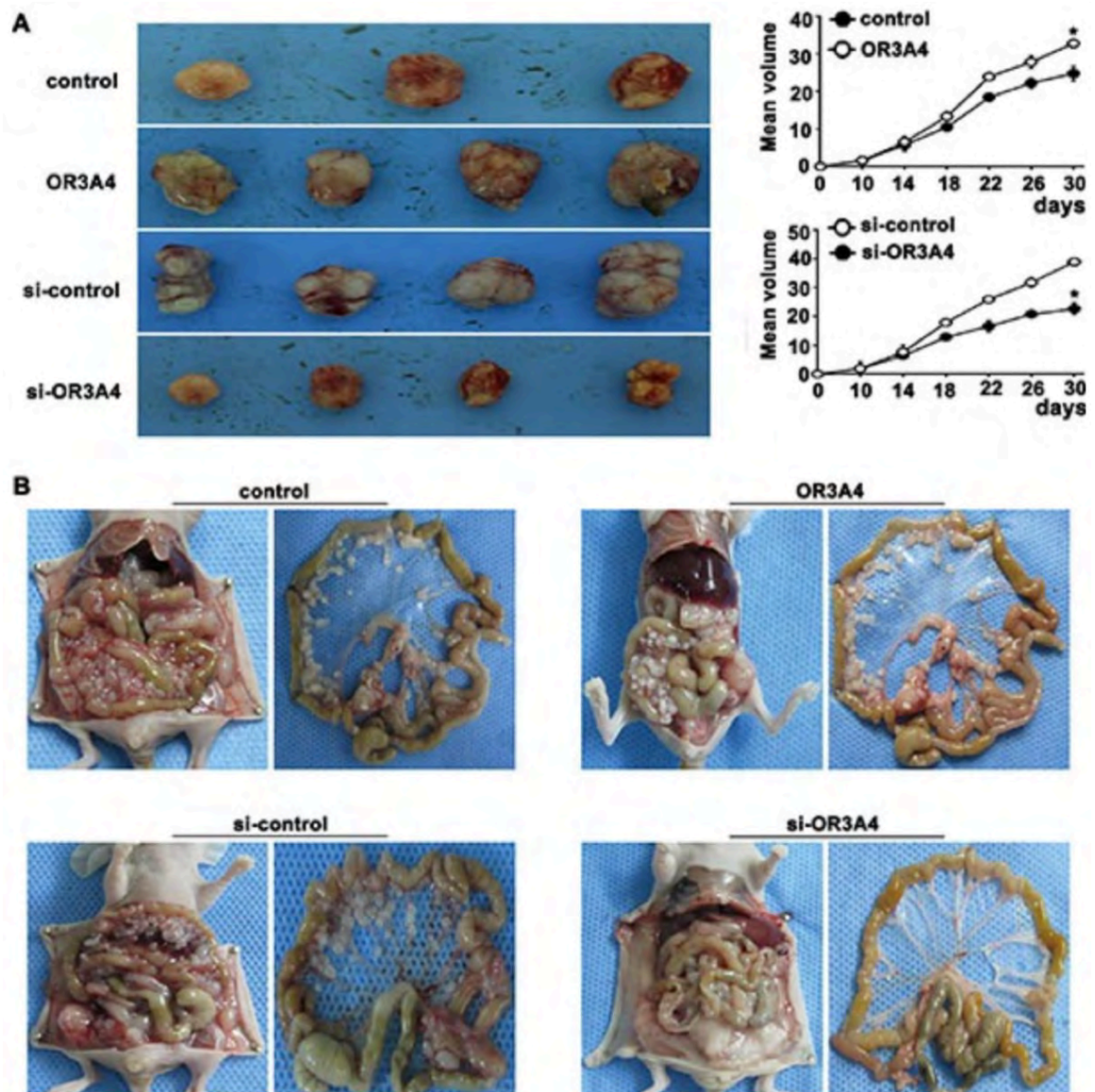


Figure 30 – Tumourigenicity of gastric cancer cells (SGC-7901) when inoculated in nude mice. A: Subcutaneous inoculation of gastric cancer cells. OR3A4 over-expressing cells demonstrated increased tumour volume compared to the parental cell line B: Peritoneal inoculation showed similar results with increased tumour spread and volume in OR3A4 over-expressing cells. Knockdown of OR3A4 reverts the cell behaviour to that of the parent cell line. Image from Guo et al 2016 (168)

Liu et al(167) report that OR3A4 is upregulated in breast cancer tissues and cell lines in comparison to adjacent normal tissue and cell lines. Interestingly it also appears that high OR3A4 expression levels in breast cancer is a poor prognostic indicator. It correlated with lymph node status, differentiation, ER status and TNM grading. However when Cox's regression analysis was performed, high OR3A4 expression levels were independently prognostic. When siRNA knockdown of OR3A4 was performed on two breast cancer lines MDA-MB-468 and MCF-7 it cut down the migratory and invasive cell numbers. This is thought to be due to the disruption of the epithelial-mesenchymal transition (EMT) process. This is the process where epithelial cells lose their cell-cell adhesion and polarity and gain migratory and invasive capabilities to become mesenchymal stem cells. When OR3A4 was knocked out, levels of the mesenchymal marker N-cadherin were downregulated and the epithelial protein marker E-cadherin were upregulated. In OR3A4 over-expressing cell lines this pattern of N-cadherin and E-cadherin expression was reversed illustrating OR3A4's ability to modulate the EMT process. In over-expressing lines apoptosis rates were lower and cells were preferentially found to be in S phase rather than G0/1. Cell proliferation assays showed that OR3A4 over-expressing cells had increased viability compared to normal controls.

OR3A4 upregulation has also been implicated in a more aggressive phenotype of non-small cell lung cancer (NSCLC). Zhong et al (191) demonstrated that NSCLC tissues demonstrate OR3A4 upregulation compared to adjacent normal tissues. They also suggest that a higher OR3A4 expression conveys a worse prognosis from the disease. (192). Shang et al used in vitro models using

A549/DDP cells which demonstrated a high native OR3A4 expression in cisplatin resistant lines. They suggested that OR3A4 could be a therapeutic target for NSCLC, as they were able to demonstrate a reversibility of cisplatin resistance when OR3A4 was knocked down.

Guo et al (193) demonstrated that cell migration and invasion were enhanced in ovarian cancer cell lines when OR3A4 was upregulated. This effect was seen to be reversible in vitro when OR3A4 was knocked down. A similar effect in colorectal cancer cell lines was demonstrated by Sun et al (194). They conclude that OR3A4 enhances migration and invasion by activating the Wnt/ β -catenin signaling pathway. Similar effects in vitro have been observed in osteosarcoma cell lines(195, 196), thought to be mediated by a miRNA sponge effect of OR3A4. Li et al have implicated OR3A4 in angiogenesis in hepatocellular carcinoma in vitro (197).

There would appear to be mounting evidence that OR3A4 may possess a pivotal role in carcinogenesis in numerous tumour types and often demonstrates reversibility in vitro raising the intriguing prospect of a therapeutic target.

7.2 AIM

The aim of this experiment was to investigate the functional effect of OR3A4 over-expression in BO and OADC cell lines in vitro by creating stable cell lines which over-expressed OR3A4.

7.3 METHODS

7.3.1 PUROMYCIN KILL CURVES

To ensure that only successfully transfected cells were represented in downstream experiments, un-transfected cells (CP-A, OE-19 and OE-33) were subjected to increasing doses of puromycin at 0, 0.25, 0.5, 1, 2 and 4 $\mu\text{g/ml}$ in otherwise standard culture conditions as previously described in section 4.11.

7.3.2 TRANSFECTION OF OR3A4 OVER-EXPRESSING VIRAL VECTOR

The cell lines CP-A, OE-19, OE-33 and HEK-293T were transfected with a PIPZ lentiviral vector (Figure 17) containing an OR3A4 over-expressing gene as detailed in methods section 4.12. Due to mycoplasma infection the line MFD-1 was not transfected. This vector also contained a puromycin resistance gene to allow selection of successfully transfected cells. As a control, an “empty” vector was also transfected which still contained the puromycin resistance gene, but no OR3A4 over-expressing gene to ensure no adverse results were generated as a result of the transfection process.

7.3.3 CELL MORPHOLOGY

Cell morphology was assessed using light microscopy and IF as described in methods section 4.18. Low and high power views were obtained of control and OR3A4 over-expressing cell lines CP-A, OE-19 and OE-33 and digital photographs taken using the EVOS FL Cell Imaging System (ThermoFisher). For

IF images, cells were cultured on slides, fixed and stained with phalloidin, DAPI counter nuclear stain and cleaved caspase 3 using the method detailed in section 4.18. IF images were obtained using a Vectra confocal microscope.

7.3.4 CELL FUNCTION ASSAYS

To assess the effect of OR3A4 expression on cell viability and growth rates, MTS and scratch wound healing assays were performed. The experiments were carried out as detailed in section 4.13 for the MTS assay and 4.14 for the scratch wound healing assay.

7.3.5 RNA SEQUENCING AND PATHWAY ANALYSIS

Total RNA sequencing was performed on RNA extracted from OR3A4 over-expressing cells and the control cell line to assess the functional effect of OR3A4 on the transcriptome and confirm OR3A4 over-expression. Sequencing of the RNA library was kindly performed by Ms C Whalley using the Illumina NextSeq 500 instrument as detailed in methods section 4.16. Functional analysis was performed using the online database for annotation, visualisation and integrated discovery (DAVID). This platform also allowed simultaneous KEGG analysis to be performed.

7.3.6 REAL TIME QUANTITATIVE PCR

RTqPCR was performed on cDNA generated from RNA extracted from CP-A, CP-A PIPZ Control, CP-A PIPZ OR3A4, OE-33, OE-33 PIPZ Control, OE-33

PIPZ OR3A4, OE-19 and MFD-1 cell lines in the manner previously described in methods sections 4.5 and 4.16. This was analysed on the Quantstudio 7 Flex Real-Time PCR System (ThermoFisher) using the thermal cycling conditions in Table 20.

7.3.7 DRUG TREATMENT OF CELL LINES

CP-A, CP-A PIPZ control and CP-A PIPZ OR3A4 cell lines were treated with the p38 MAPK inhibitor Doramapimod and Aspirin in increasing doses and MTS assays performed daily up to 5 days incubation to assess cell viability. The methodology is detailed in methods section 4.15.

7.4 RESULTS

7.4.1 PUROMYCIN KILL CURVES

Puromycin was added to culture media to ensure that only transfected cells would remain viable. Parental cell lines were treated with puromycin to assess the required media concentration to achieve this.

Cell counts and viability dramatically decreased when using a puromycin dose greater than 1 µg/ml (Figure 31). In both oesophageal cancer cell lines the cells became rounded and non-viable above 0.5 µg/ml (Table 32 and Table 33), and the Barrett's cells became rounded and non-viable above 1 µg/ml (Table 31). The increased counts of CP-A cells at higher doses of puromycin represent adherent, non-viable cells which have not undergone apoptosis. Non-viability was confirmed by disassociating the cells from the culture flasks and re-

suspending them in fresh media. No new cell growth was observed following dissociation in the higher puromycin concentrations.

Table 31 – CP-A Cell Counts when Treated with Puromycin at 24 and 120 hours

Puromycin Dose (0.5 µg/ml)	0	0.25	0.5	1	2	4
24 h	513	541	415	256	688	1338
120 h	4963	1478	487	385	2005	2790

Table 32 - OE-19 cell counts when treated with Puromycin at 24 and 120 hours

Puromycin Dose (0.5 µg/ml)	0	0.25	0.5	1	2	4
24 h	929	1259	1750	1461	1541	1572
120 h	3918	1543	842	298	129	60

Table 33 - OE-33 cell counts when treated with Puromycin at 24 and 120 hours

Puromycin Dose (0.5 µg/ml)	0	0.25	0.5	1	2	4
24 h	1670	1967	1609	1562	795	1370
120 h	4830	5702	3041	224	259	85

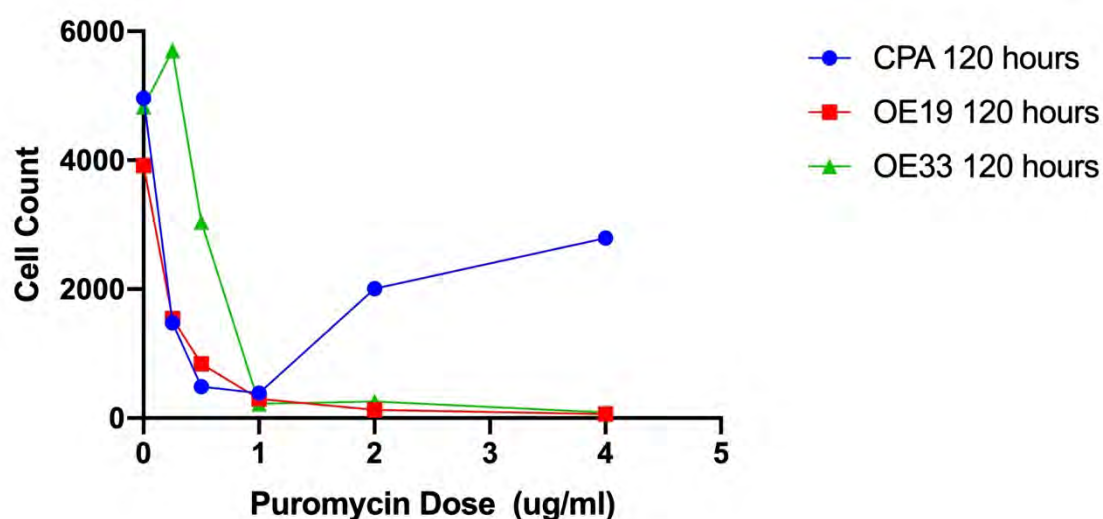


Figure 31 - Puromycin kill curves. Cell counts at 120 hours following variable dosing of puromycin.

7.4.2 TRANSFECTION OF OR3A4 OVER-EXPRESSING VIRAL VECTOR

In order to understand the functional relevance of OR3A4 hypomethylation and resultant over-expression in the progression of BO to OADC, OR3A4 was over-expressed to simulate the effect of OR3A4 promoter hypomethylation in target cell lines. The NDBO cell line CP-A and OADC lines OE-19, OE-33 and MFD-1 were selected as experimental lines. The SCC cell line OE-21 and non-neoplastic HEK-293T line were used as controls.

The transfection resulted in 4 stable cell lines, CP-A PIPZ control, CP-A PIPZ OR3A4, OE-33 PIPZ Control and OE-33 PIPZ OR3A4. HEK-293T did not survive two attempted transfections and OE-19 were unstable and were only viable to passage 2 and as a result no cell culture assays could be performed.

7.4.3 LIGHT MICROSCOPY

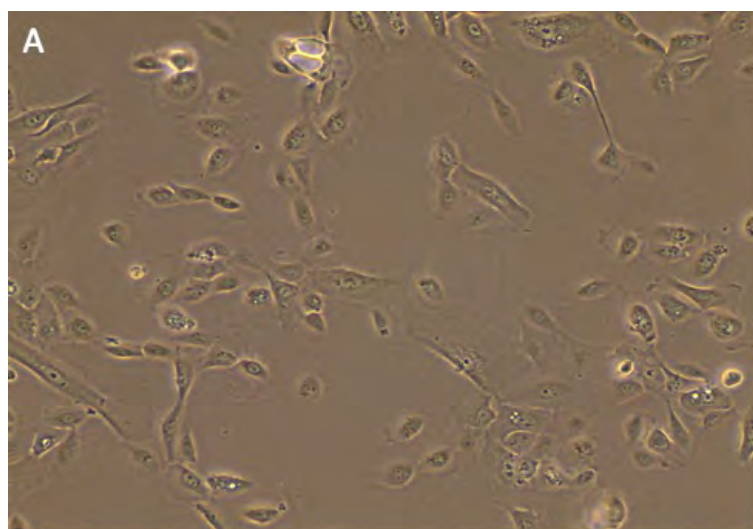
Once established in stable culture, the transfected control and experimental lines were observed using standard light microscopy to investigate any macroscopic, morphological changes that were present between the lines as a result of OR3A4 over-expression.

7.4.3.1 CP-A CELL LINES

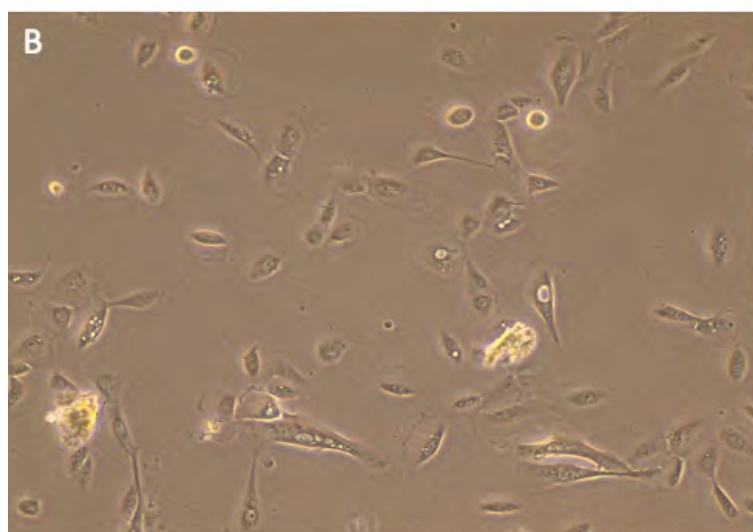
When viewed at high power at 50% confluence, CP-A cells are relatively homogenous with a spindle like appearance (Figure 32 (A)). The empty PIPZ vector did not appear to change the cell morphology when it was transfected (Figure 32 (B)). When OR3A4 was over-expressed in the CP-A line, the cells became larger and less homogenous in appearance. The nuclei of OR3A4 over-expressing cells also appeared enlarged compared to the controls (Figure 32(C)).

7.4.3.2 OE33 CELL LINES

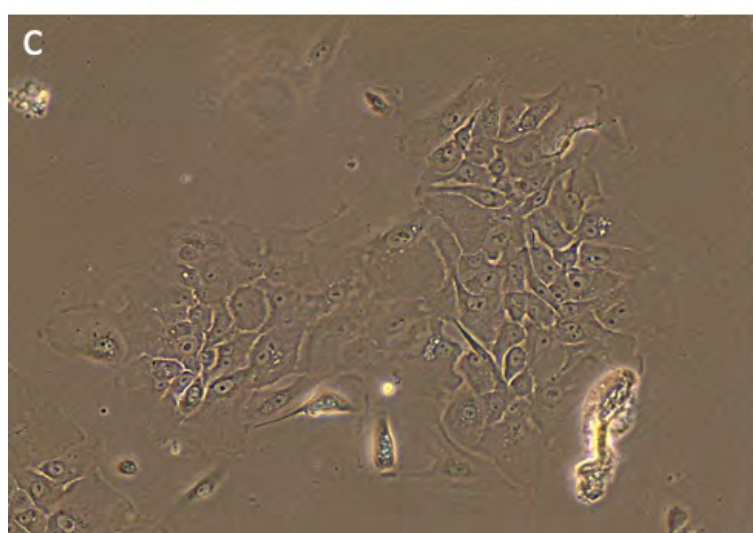
When viewed under light microscopy, the morphological differences between the OE-33 control and experimental cell lines is less pronounced than between the BO cell lines. The cells demonstrate an epithelioid morphology. The OR3A4 over-expressing OE-33 cells (Figure 33 (C)) maintain a similar nuclear to cytoplasmic ratio and outline when compared with both PIPZ control transfected (Figure 33 (B)) and non-transfected OE-33 cells (Figure 33 (A)). The nuclei also retain a typical granular appearance across the experimental and control lines. Interestingly, both the control transfected OE-33 cell line and the OR3A4 over-expressing cell line began to form domes when they reached 100% confluence, a phenomenon which was not observed in the un-transfected line. This may be an effect of viral infection in these lines as it cannot be explained by OR3A4 over-expression.



A) CPA un-transfected control (40X)

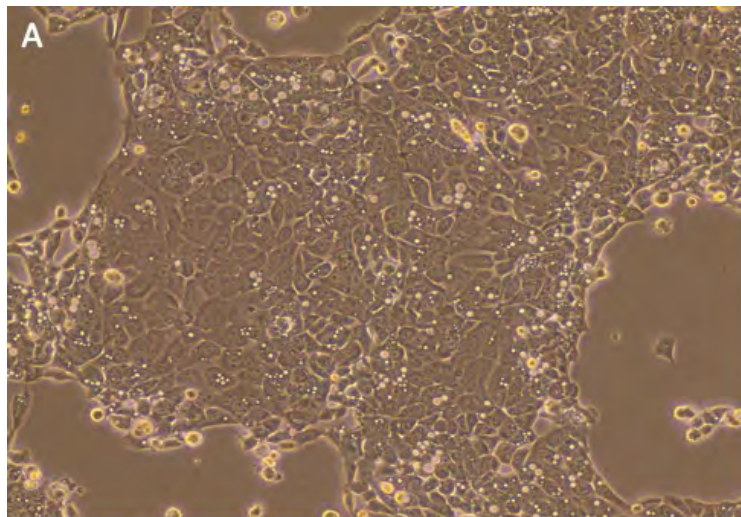


B) CPA PIPZ control (40X)

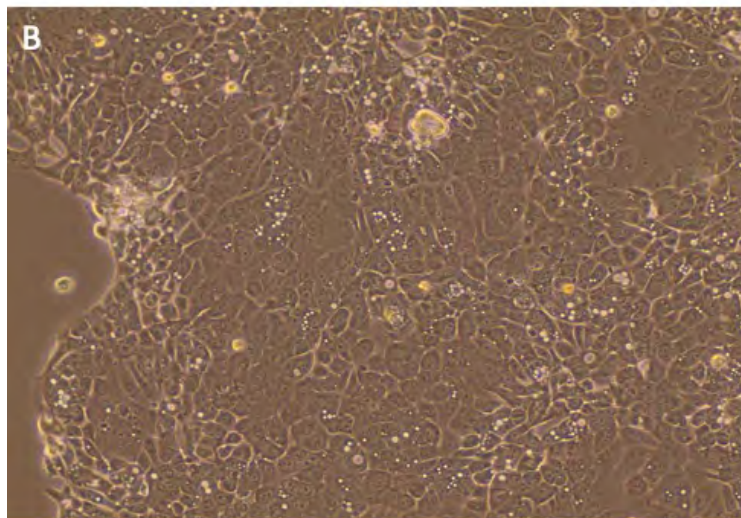


C) CPA PIPZ OR3A4 over-expressing cells (40X)

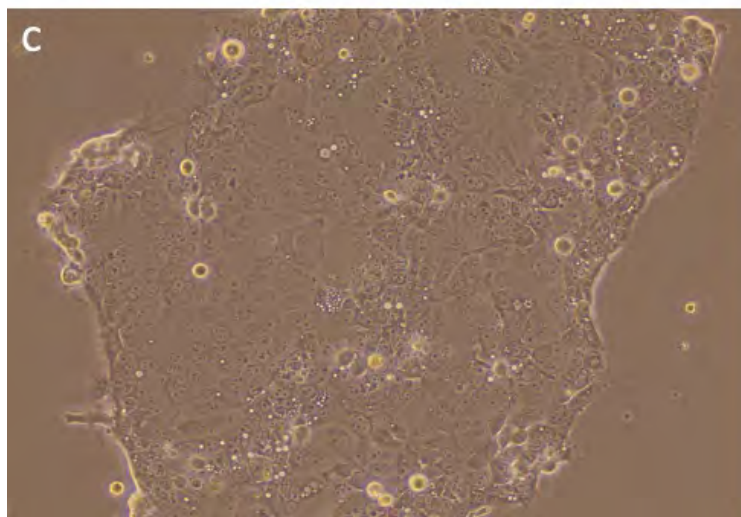
Figure 32 - CP-A control and experimental cells viewed under light microscopy



A) OE-33 untransfected control (40X)

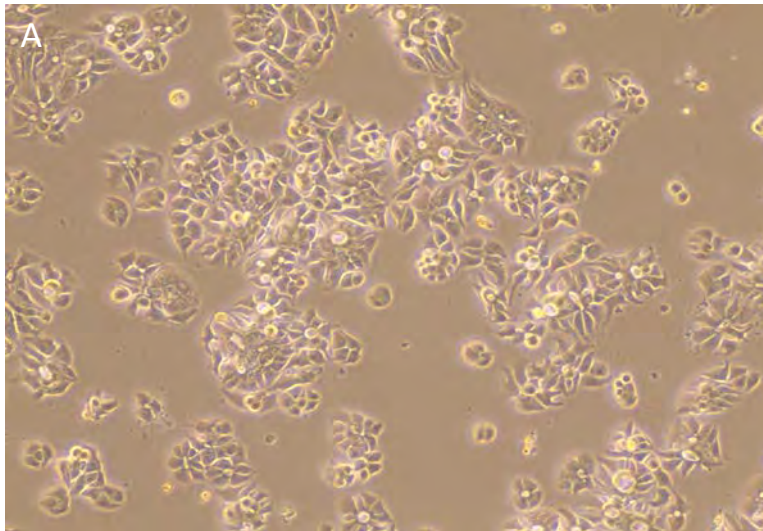


B) OE-33 PIPZ control (40X)

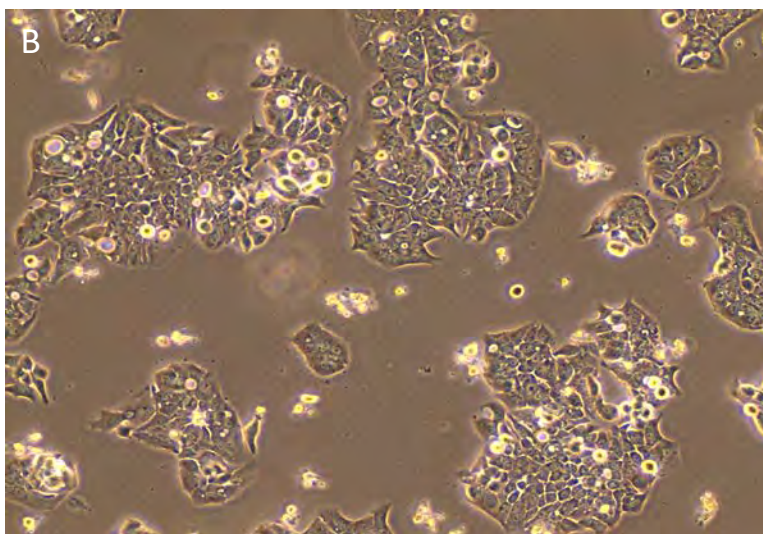


C) OE-33 PIPZ OR3A4 over-expressing cells

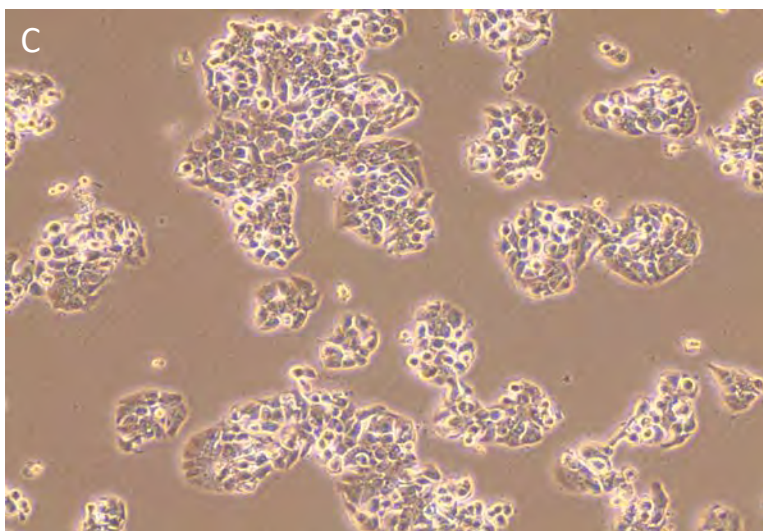
Figure 33 - OE-33 control and experimental cell lines viewed under light microscopy



A) OE-19 untransfected control (40X)



B) OE-19 PIPZ control (40X)



C) OE-19 PIPZ OR3A4 over-expressing cells

Figure 34 - A: OE-19 parental cell line (40X), B: OE-19 PIPZ control (40X), C: OE-19 PIPZ OR3A4 over-expressing cells (40X). Transfected cells did not grow to confluence.

7.4.3.3 OE19 CELL LINES

OE-19 cells demonstrate an epithelial morphology with pleomorphism and occasional multinucleate giant cells (Figure 34). The OE-19 cell line transfected with an OR3A4 over-expressing vector was unstable and did not survive past passage 2. The experimental cell line over-expressing OR3A4 never achieved 100% confluence. Morphological differences were unable to be confidently described due to the unstable nature of the cell line, and early passages did not appear to be morphologically different (Figure 34 (B and C)). The control vector was successfully transfected into the OE-19 cell line and the cells reached confluence, but as the experimental vector transfection resulted in an unstable line, no further experiments were performed on this cell type. The cell line OE19 was derived from a Siewart type III gastric cancer at the cardia(198). The biology of this cell line may be more comparable to gastric adenocarcinoma and thus the oncogenic driver may differ from OR3A4.

7.4.4 IMMUNOFLUORESCENCE

To further characterise and highlight morphological differences and investigate changes in the caspase apoptosis pathway, immunofluorescence (IF) was performed in CP-A and OE-33 cell lines using both control transfected and OR3A4 over-expressing lines. The IF images enhance the morphological differences between CP-A and CP-A PIPZ OR3A4 with an increased nuclear:cytoplasmic ratio clearly evident and a dysplastic appearance of the cells with irregular cell wall outline and nuclei. The CP-A control cells display a homogenous, spindle like appearance (Figure 35) whereas the OR3A4 over-expressing line appear to lose this morphology with increased cellular heterogeneity (Figure 36). There was no difference observed between caspase 3 staining in the different cell lines.

As seen under light microscopy, the morphology of the OE-33 cell lines was not appreciably different when enhanced by IF imaging. The control transfected (Figure 37) and OR3A4 over-expressing (Figure 38) cell lines both retained their homogenous epithelioid appearance and did not show changes in caspase 3 staining.

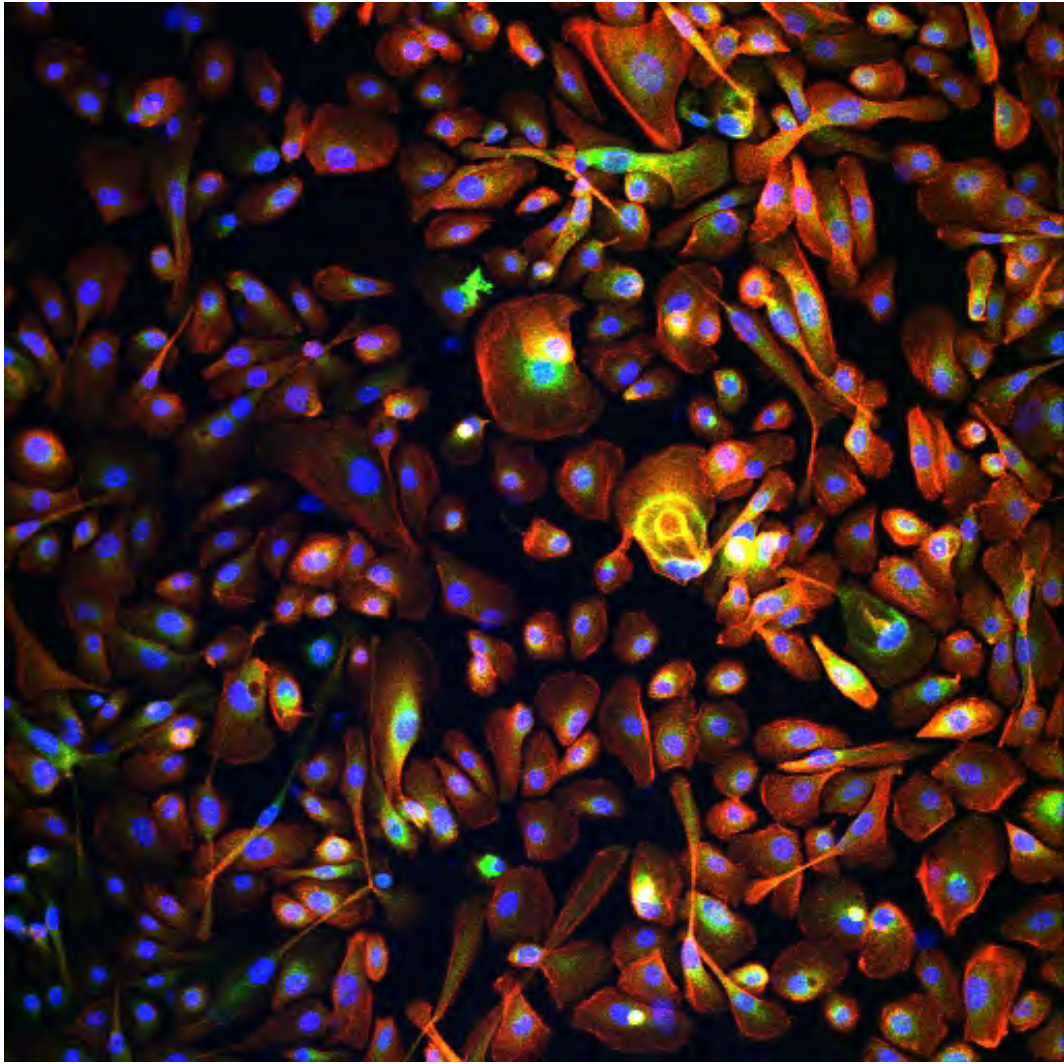


Figure 35 - IF Image of CPA Control Cells x10. Cells stained with DAPI nuclear counter stain (blue), Phalloidin (red) and Caspase 3 (yellow).

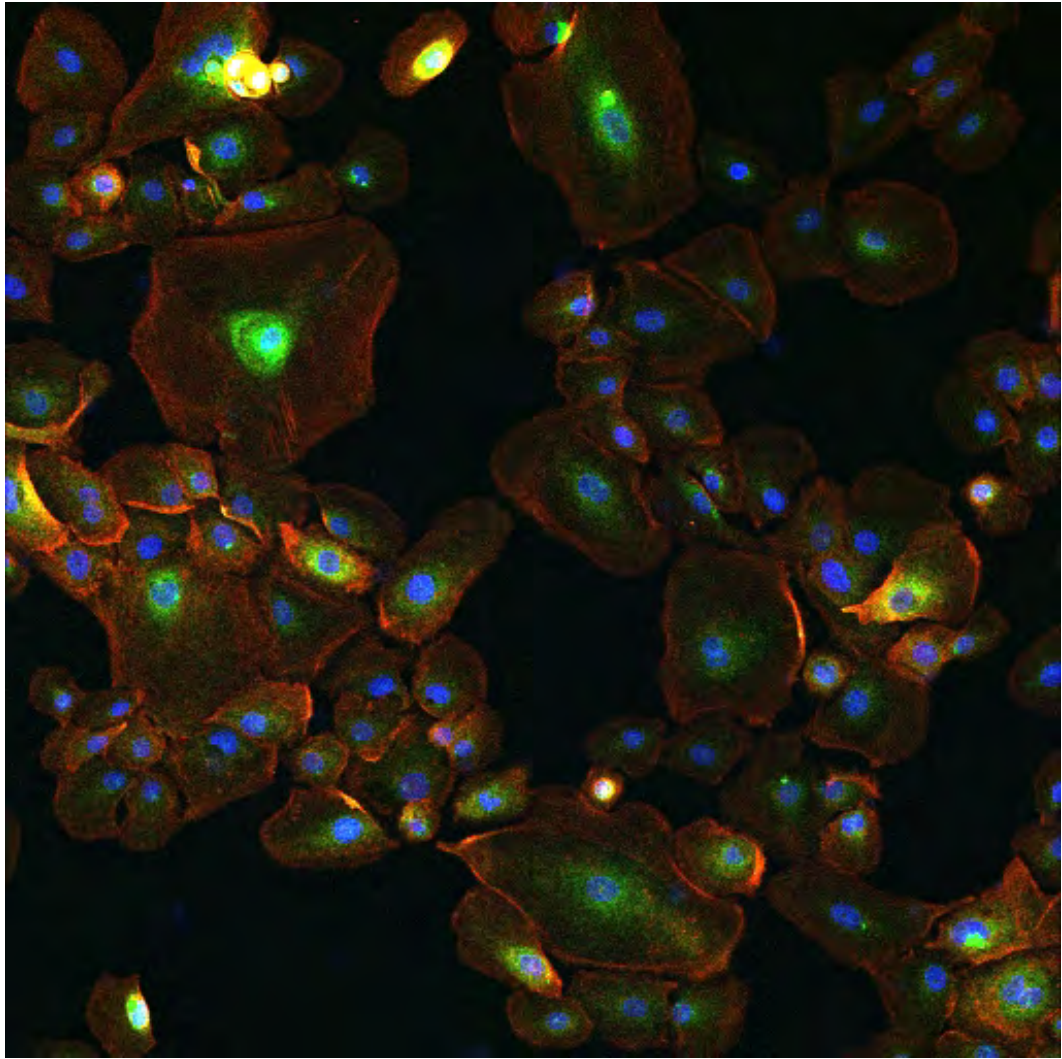


Figure 36 - IF Image of CPA OR3A4 Over-Expressing Cells x10. Cells stained with DAPI nuclear counter stain (blue), Phalloidin (red) and Caspase 3 (yellow).

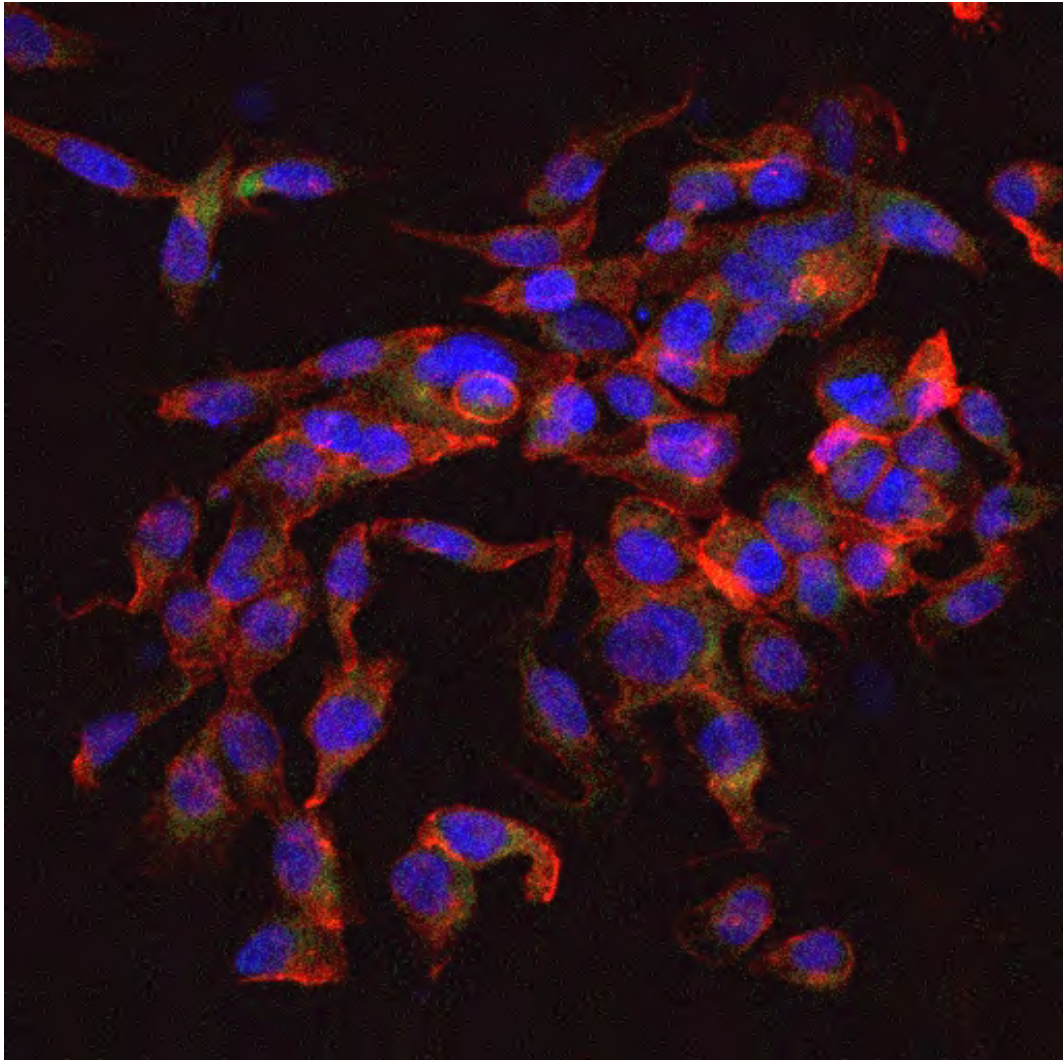


Figure 37 - IF Image of OE-33 Control Transfected Cell Line x10. Cells stained with DAPI nuclear counter stain (blue), Phalloidin (red) and Caspase 3 (yellow)

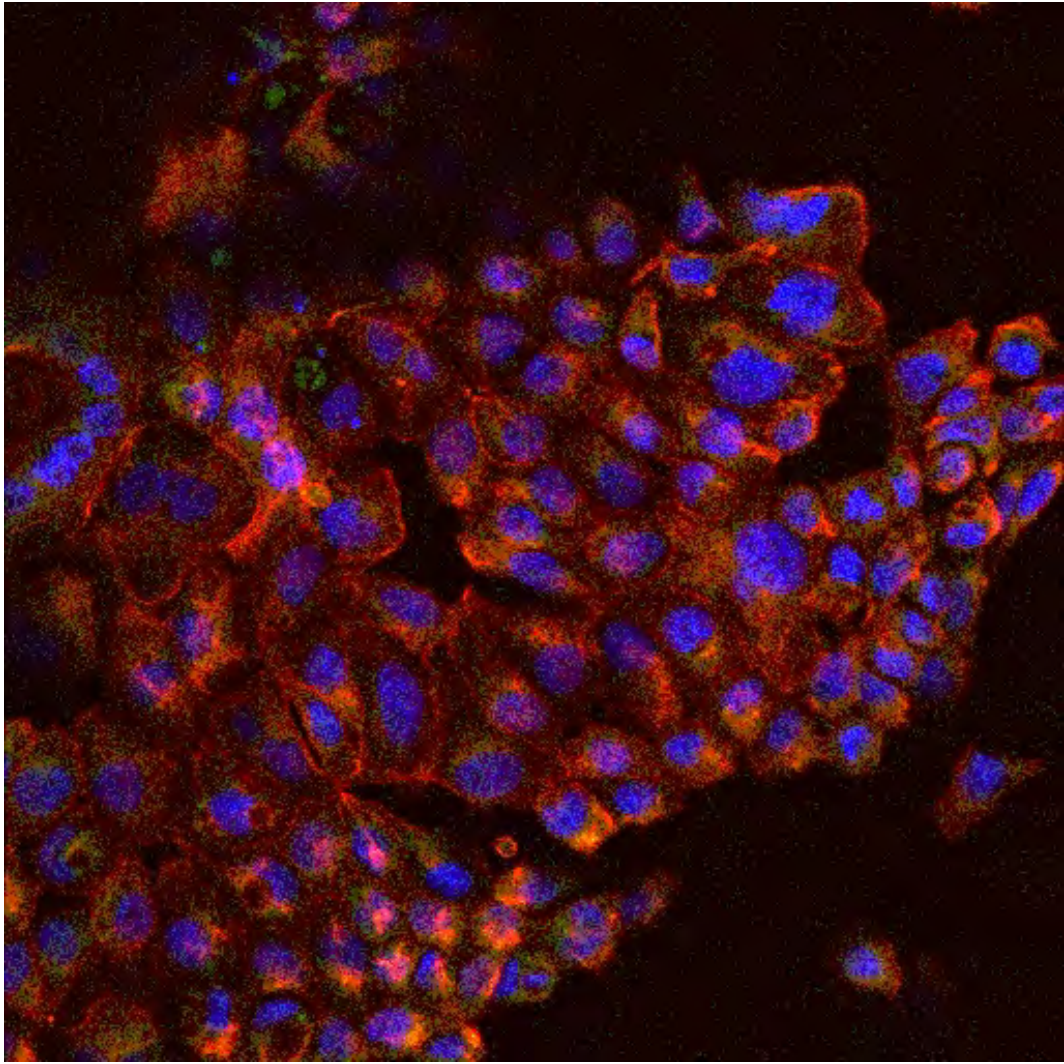


Figure 38 – IF image of OE-33 Cell Line Over-Expressing OR3A4 x10. Cells stained with DAPI nuclear counter stain (blue), Phalloidin (red) and Caspase 3 (yellow).

7.4.5 FUNCTIONAL ASSAYS OF CELLS IN VITRO

7.4.5.1 MTS ASSAY

Once visual, morphological changes were demonstrated between the cell lines, differences in behaviour between the control and OR3A4 over-expressing cell lines was assessed. To assess the effect of OR3A4 expression on cell viability and growth rates, an MTS assay was performed as described in section 4.13. This is a measure of NAD(P)H-dependent cellular oxidoreductase enzymes within cultured cells.

When CP-A, CP-A PIPZ Control and CP-A PIPZ OR3A4 were analysed it demonstrated increased cell viability in the over-expressing cell lines compared to the control lines. Absorbance in the OR3A4 over-expressing line plateaued at 72 hours, whilst the control lines plateaued at 96 hours (Figure 39). This indicates that the over-expressing cell lines reached confluence more rapidly than the controls ($p=0.024$).

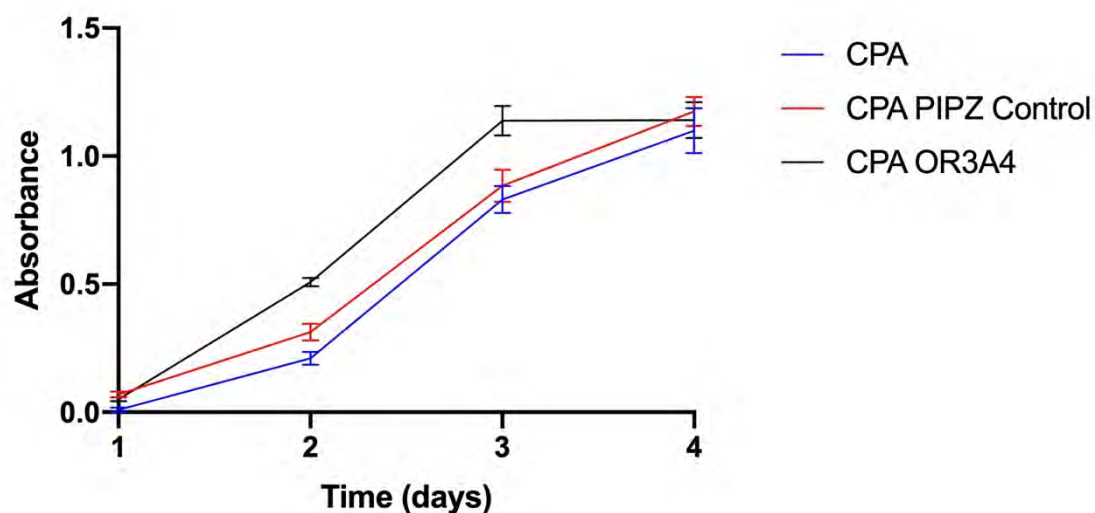


Figure 39 - MTS Assay: CP-A, CP-A PIPZ Control and CP-A PIPZ OR3A4. Absorbance readings taken 4 hours after MTS reagent added. $p = 0.002$ (day 2), $p = 0.024$ (day 3).

Similar results were found in the OADC line OE-33 with OE-33 PIPZ OR3A4 reaching confluence more rapidly ($p=0.026$, Figure 40).

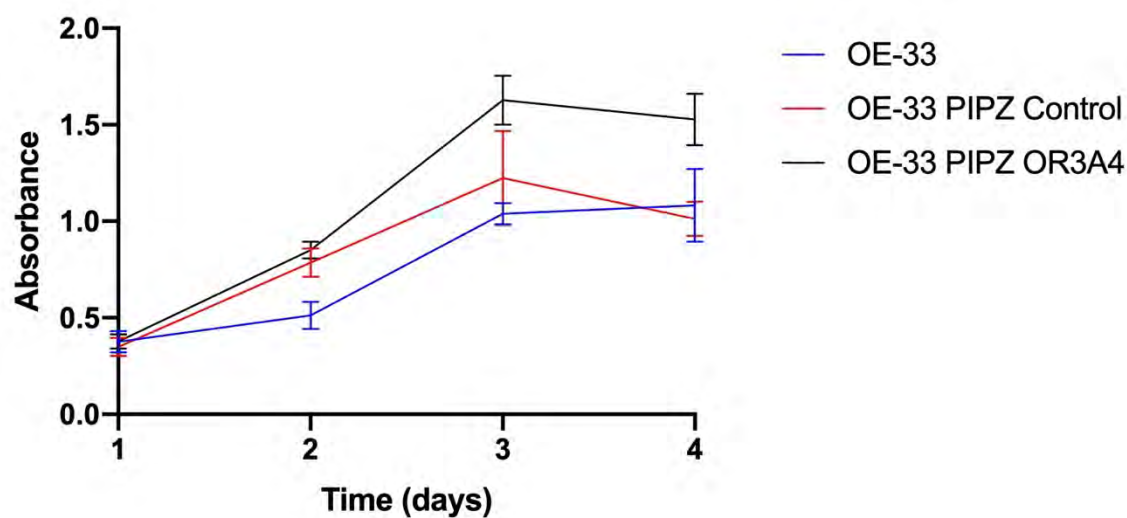


Figure 40 - MTS assay: OE-33, OE-33 PIPZ control AND OE-33 PIPZ OR3A4. Absorbance readings taken 4 hours after MTS reagent added. $p = 0.180$ (day 2), $p = 0.026$ (day 3).

7.4.5.2 SCRATCH WOUND HEALING ASSAY

The difference in cell proliferation and migration was assessed using a scratch wound healing assay using the protocol described in the materials and methods section 4.14. This assay assesses how rapidly cells migrate across and close a defined cell-free gap on a culture plate. Over a period of 24 hours, CPA cells which over-expressed OR3A4 closed the cell free gap more rapidly than both the transfected and normal control cell lines ($p=0.0018$). When the control cell lines were compared there was no significant difference between them. Cell free gap is presented in graphical form in Figure 41

Table 34 CP-A scratch wound healing assay results

Hours	Average Cell Free Gap (nm)	Standard Deviation	Standard Error
0	528	25.80	8.60
1	529	39.07	13.02
2	521	22.14	7.38
4	517	35.51	11.84
6	486	52.91	17.64
9	376	93.85	31.28
12	312	111.56	37.19
24	33	65.25	21.75

Table 35 CP-A PIPZ Control scratch wound healing assay results

Hours	Average Cell Free Gap (nm)	Standard Deviation	Standard Error
0	514	18.32	6.11
1	521	28.35	9.45
2	508	19.34	6.45
4	488	25.03	8.34
6	470	26.94	8.98
9	355	65.39	21.80
12	323	74.83	24.94
24	54	81.39	27.13

Table 36 CP-A PIPZ OR3A4 scratch wound healing assay results

Hours	Average Cell Free Gap (nm)	Standard Deviation	Standard Error
0	490	27.44	9.15
1	466	21.23	7.08
2	449	20.69	6.90
4	436	14.98	4.99
6	395	27.06	9.02
9	316	34.07	11.36
12	159	39.65	13.22
24	21	41.50	13.83

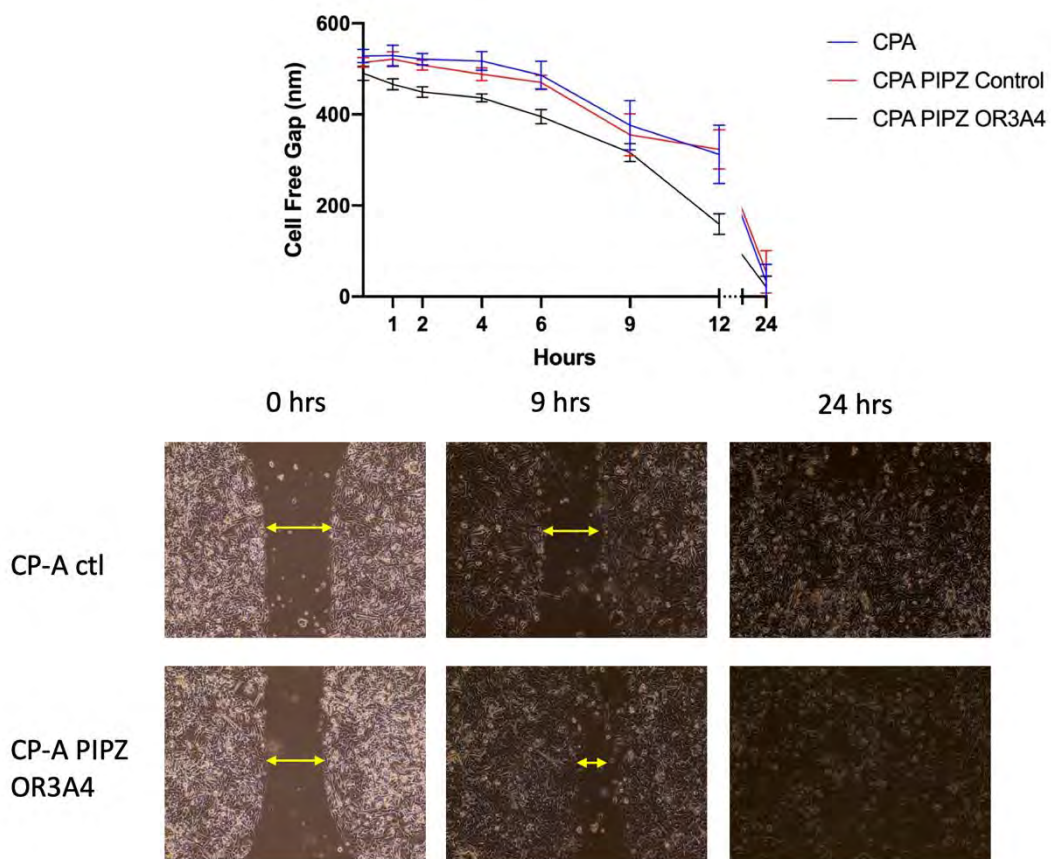


Figure 41: Scratch Wound Healing Assay. Cell free gap for CP-A, CP-A PIP.Z control and CP-A PIPZ OR3A4 over closing time. The cells over-expressing OR3A4 closed the cell free gap and reached confluence more rapidly than the control lines ($p=0.0018$).

7.4.6 RNA SEQUENCING

Having demonstrated altered morphology and cell behaviour between control and experimental CP-A cell lines, the underlying mechanisms responsible for these behaviours were investigated by total RNA sequencing. The lack of morphological change in OE-33 when OR3A4 was over-expressed coupled with limited time and resource meant that we focused our efforts on the CP-A cell line alone. RNA sequencing allowed the downstream effects of OR3A4 over-expression on the transcriptome to be measured, and pathway analysis was performed. The top 20 results are presented in order of statistical significance in Table 37 with total counts per million reads. The data was filtered for zero counts. A volcano plot excluding OR3A4 expression is presented in Figure 43. OR3A4, also known as OR3A4P, is highly upregulated in the experimental cell line and skews the data plot, thus it has been excluded. There were 495 genes significantly under-expressed ($p < 0.05$ cut-off) by the control cell line compared to the experimental line and 618 were over expressed ($p < 0.05$ cut-off). A further 89342 transcripts were investigated by total RNA sequencing, but were not significantly differentially expressed between the two cell lines ($p > 0.05$). The false discovery rate (FDR) step up was persistently 1. This is likely due to small sample size. The relatively large over-expression of OR3A4 compared to other transcripts could skew the analysis, however, filtering OR3A4 from the analysis did not alter the FDR. The two most significantly upregulated genes were PRKRIP1 and SLMO1 with increased fold change of 28.04 ($p = 0.0002$) and 12.89 ($p = 0.0004$) respectively.

Table 37 - Top 20 Differentially Expressed Genes of CPA Control Vs CPA OR3A4 Over-Expressing Cell Lines on RNA Sequencing

Gene ID	Total counts (Counts per million)	P-value (Control OR3A4) vs.	FDR step up (Control OR3A4) vs.	Fold change (Control OR3A4) vs.
OR3A4P	571.20	<0.00001	0.00427	-17170.12
PRKRIP1	3.25	0.00022	1.00	28.04
PRELID3A	3.49	0.00042	1.00	12.89
NCOA7	1.26	0.00042	1.00	-34.69
SMCO4	0.86	0.00060	1.00	-23.37
PCMTD2	6.96	0.00061	1.00	9.80
TXNL4A	0.76	0.00072	1.00	-20.93
UBLCP1	1.54	0.00072	1.00	16.35
SETD5- AS1	0.98	0.00079	1.00	-20.98
FBXO36	0.85	0.00081	1.00	-18.93
MRPL30	1.00	0.00083	1.00	-20.78
CRYZL1	2.41	0.00086	1.00	23.03
ATRIP	0.69	0.00087	1.00	-18.21
TSEN2	1.39	0.00096	1.00	-17.08
GSR	0.84	0.00101	1.00	-22.97
FAM86C1	0.70	0.00132	1.00	-18.09
HMCES	0.58	0.00132	1.00	-15.37
CEP41	0.60	0.00136	1.00	-15.88
ATXN1	0.65	0.00145	1.00	-14.45
SLC22A23	0.59	0.00150	1.00	-15.71

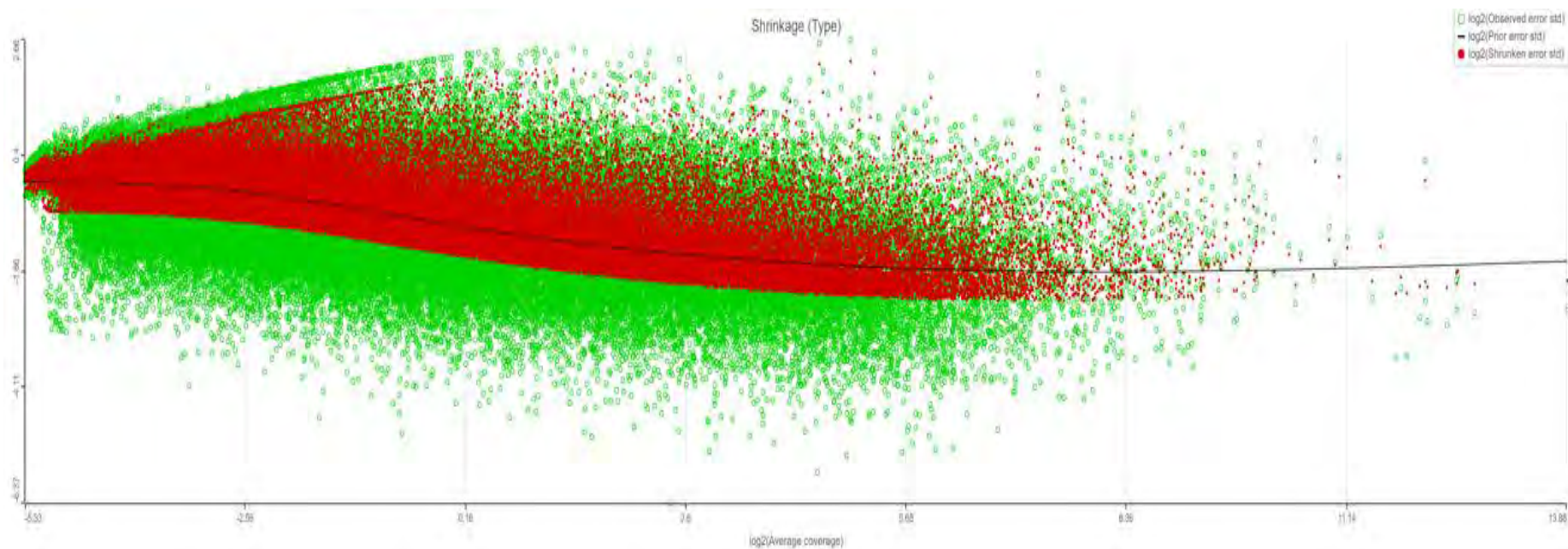


Figure 42 - The RNAseq shrinkage plot demonstrates variability of each probe within the sample. Bayesian methods were used to reduce the sample variability and correct for low counts. This method allows error to be reduced when small sample sizes are used. The green plot shows sample variability prior to Bayesian shrinkage. The red plot shows sample variability after Bayesian shrinkage. Maximum likelihood estimates (MLE) are calculated for each gene individually from the log fold change. A curve is then fit to each of these data points to provide the prior mean. A second estimation is then used which provides a shrinkage towards the consensus prior mean line. This allows differentially expressed genes to be ranked by counts and by statistical significance when variability is low.

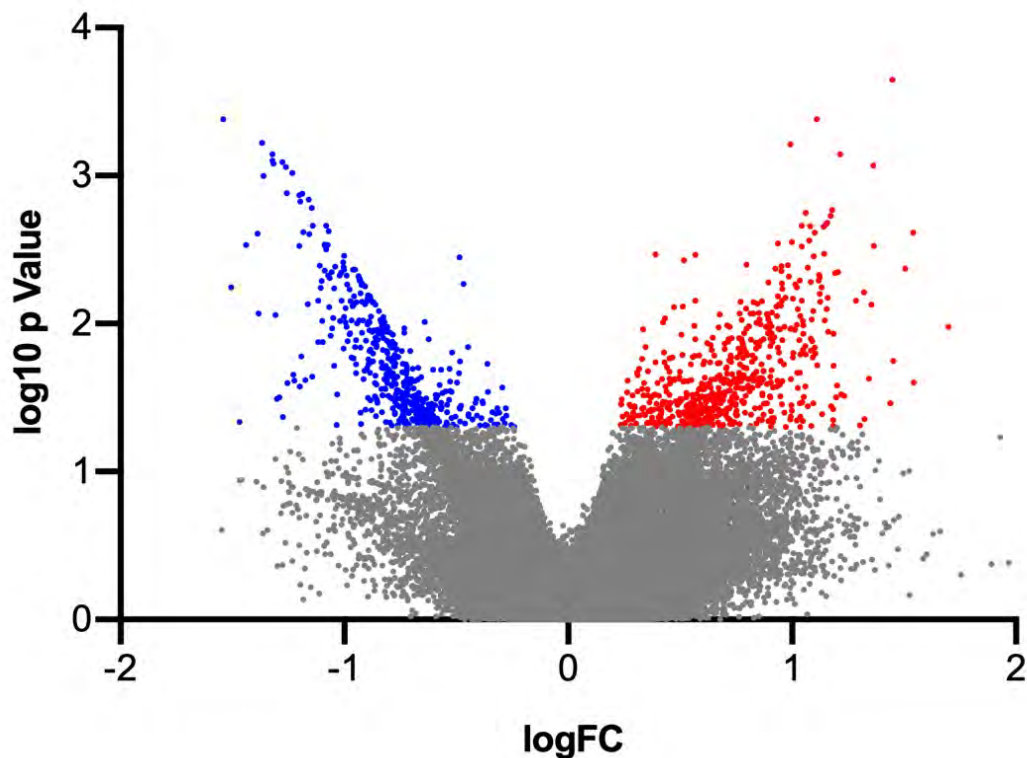


Figure 43 - Volcano Plot of Differential Gene Expression in OR3A4 Over-Expressing CPA Cells Vs CPA Control Cells. $p < 0.05$ Highlighted in Colour. OR3A4 Excluded.

7.4.6.1 DAVID ANALYSIS

To better understand the context of the differentially expressed genes discovered on total RNA sequencing, a pathway based analysis using the database for annotation, visualisation and integrated discovery (DAVID) (119) was carried out as described in section 4.16.1. Functional annotation allows groups of differentially expressed genes to be mapped from long lists of genes created from total RNA sequencing. This allows biological relevance to be inferred using gene-annotation enrichment analysis and pathway analysis.

Functional annotation clustering revealed enrichment in protein kinase like domains and serine/threonine protein kinase activity with an enrichment score of 5.16. This includes genes such as PRKRIP1. DNA damage and repair genes were enriched at 3.48.

7.4.6.2 KEGG PATHWAY

The Kyoto encyclopaedia of genes and genomes (KEGG) is a database which can be interrogated and analysed to provide information about biological function from molecular level information such as lists of genes generated by RNA sequencing. Using DAVID bioinformatics resources 6.8 KEGG pathway analysis was performed as described in section 4.16.2, with the results detailed in Figure 44. The analysis reveals high gene counts in the endocytosis and MAPK pathways.

Table 38 – DAVID analysis: Annotation Cluster 1 Enrichment Score = 5.16

Category	Term	Fold Enrichment	Count	PValue	List Total	FDR
INTERPRO	IPR011009:Protein kinase-like domain	2.21	55	7.47E-08	871	0.0001
INTERPRO	IPR000719:Protein kinase, catalytic domain	2.23	51	1.71E-07	871	0.0003
SMART	SM00220:S_TKc	2.41	42	2.34E-07	488	0.0003
INTERPRO	IPR008271:Serine/threonine-protein kinase, active site	2.60	38	2.13E-07	871	0.0004
UP_KEYWORDS	Serine/threonine-protein kinase	2.42	42	3.17E-07	909	0.0004
UP_SEQ_FEATURE	domain:Protein kinase	2.26	48	2.98E-07	891	0.0005
INTERPRO	IPR017441:Protein kinase, ATP binding site	2.35	42	6.60E-07	871	0.0011
UP_SEQ_FEATURE	nucleotide phosphate-binding region:ATP	1.77	78	1.28E-06	891	0.0022
UP_SEQ_FEATURE	active site:Proton acceptor	1.94	58	2.17E-06	891	0.0038
UP_KEYWORDS	Transferase	1.54	116	2.86E-06	909	0.0040
GOTERM_MF_DIRECT	GO:0004672~protein kinase activity	2.23	39	6.10E-06	824	0.0096
UP_KEYWORDS	Kinase	1.85	60	6.90E-06	909	0.0097
UP_SEQ_FEATURE	binding site:ATP	1.98	49	9.58E-06	891	0.0167
GOTERM_MF_DIRECT	GO:0004674~protein serine/threonine kinase activity	2.12	39	1.79E-05	824	0.0280
UP_KEYWORDS	Nucleotide-binding	1.39	110	3.27E-04	909	0.4569
GOTERM_BP_DIRECT	GO:0046777~protein autophosphorylation	2.50	21	2.84E-04	821	0.5070
UP_KEYWORDS	ATP-binding	1.45	89	3.85E-04	909	0.5389
GOTERM_BP_DIRECT	GO:0018105~peptidyl-serine phosphorylation	2.78	17	3.85E-04	821	0.6870
GOTERM_MF_DIRECT	GO:0005524~ATP binding	1.37	100	9.91E-04	824	1.5424
GOTERM_BP_DIRECT	GO:0006468~protein phosphorylation	1.70	38	1.73E-03	821	3.0501

Table 39 - DAVID analysis: Annotation Cluster 2, Enrichment Score 3.48

Category	Term	Fold Enrichment	Count	PValue	List Total	FDR
UP_KEYWORDS	DNA damage	2.32	36	6.72E-06	909	9.43E-03
UP_KEYWORDS	DNA repair	2.16	28	2.59E-04	909	3.63E-01
GOTERM_BP_DIRECT	GO:0006281~DNA repair	1.74	20	2.15E-02	821	3.22E+01

Table 40 - DAVID analysis: Annotation Cluster 3, Enrichment Score 2.51

Category	Term	Fold Enrichment	Count	PValue	List Total	FDR
UP_KEYWORDS	Cell cycle	1.88	54	1.29E-05	909	1.81E-02
UP_KEYWORDS	Cell division	1.81	31	2.16E-03	909	2.99E+00
GOTERM_BP_DIRECT	GO:0007067~mitotic nuclear division	1.73	21	1.91E-02	821	2.92E+01
GOTERM_BP_DIRECT	GO:0051301~cell division	1.58	27	2.23E-02	821	3.32E+01
UP_KEYWORDS	Mitosis	1.73	20	2.35E-02	909	2.83E+01

Term	Count	P-Value	Fold Enrichment	FDR
hsa04144:Endocytosis	21	8.89E-03	1.86	11.02
hsa04010:MAPK signaling pathway	19	4.95E-02	1.60	48.54
hsa04120:Ubiquitin mediated proteolysis	16	1.77E-03	2.49	2.29
hsa04142:Lysosome	15	1.47E-03	2.64	1.91
hsa04110:Cell cycle	12	3.02E-02	2.06	33.07
hsa04068:FoxO signaling pathway	12	4.88E-02	1.91	48.07
hsa04724:Glutamatergic synapse	11	4.03E-02	2.05	41.65
hsa04919:Thyroid hormone signaling pathway	11	4.24E-02	2.04	43.29
hsa04115:p53 signaling pathway	9	1.24E-02	2.86	15.06
hsa03320:PPAR signaling pathway	9	1.24E-02	2.86	15.06
hsa04146:Peroxisome	9	3.96E-02	2.31	41.05
hsa03460:Fanconi anemia pathway	8	1.11E-02	3.21	13.64
hsa00071:Fatty acid degradation	7	1.27E-02	3.55	15.37
hsa03420:Nucleotide excision repair	7	2.14E-02	3.17	24.62
hsa01212:Fatty acid metabolism	7	2.35E-02	3.11	26.73
hsa05416:Viral myocarditis	7	4.91E-02	2.62	48.27

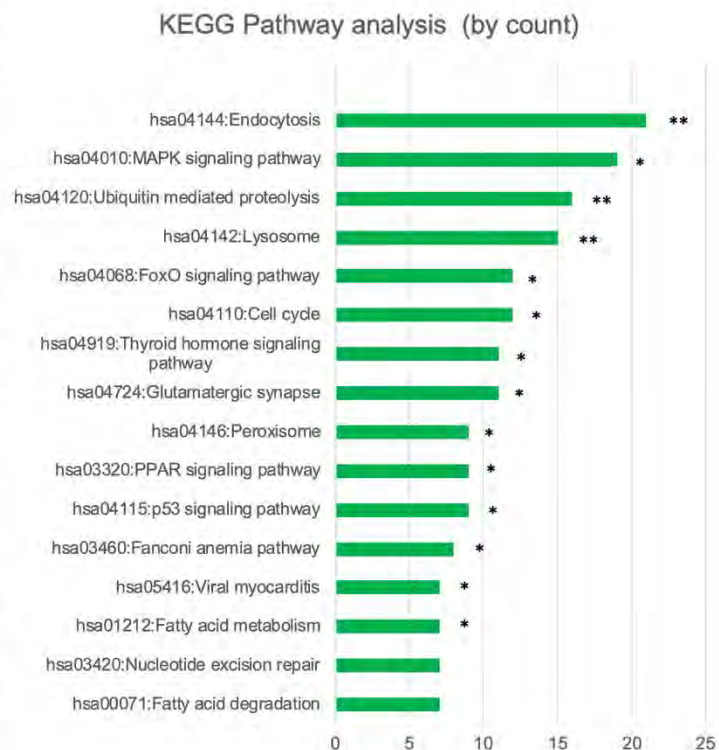


Figure 44 - KEGG Pathway Analysis

7.4.7 REAL TIME QUANTITATIVE PCR

In order to validate the results of total RNA sequencing, real time quantitative PCR (RTqPCR) was performed to demonstrate differential gene expression.

RTqPCR was used to confirm OR3A4 over-expression in the transfected cell lines in comparison to the controls. DAVID and KEGG pathway analysis showed enrichment in both MAPK and apoptosis pathways, and this was used to inform subsequent RTqPCR targets. The pre-loaded Taqman array plates provided ideal tools for investigating these pathways further. Targeted RTqPCR of the TRIAP1/PRELID3A genes was performed as this was a significantly upregulated in total RNA sequencing when OR3A4 was over-expressed and was not included in the pre-loaded Taqman apoptosis array plate.

Prior to evaluating experimental cell lines, an input test of cDNA was performed to ensure reliable PCR amplification of the target cDNA.

RNA was extracted from cell lines CP-A, CP-A PIPZ Control, CP-A PIPZ OR3A4, OE-33, OE-33 PIPZ Control and OE-33 PIPZ OR3A4 in the manner previously described in section 4.5. This was converted to cDNA and then the RTqPCR reaction mixture was made up using GAPDH (control gene) and OR3A4 TaqMan primers. This was read on the Quantstudio 7 Flex Real-Time PCR System (ThermoFisher). Threshold cycle values (Ct) were calculated and delta delta Ct values generated from the data to indicate fold change in target gene expression between the cell lines.

Successful transfection of a functional over-expressing OR3A4 gene into the cell lines CP-A and OE-33 was achieved. CP-A PIPZ OR3A4 cells exhibited a 14,198 (95%CI +/- 6840) fold increase in expression of OR3A4 compared to CP-A normal controls, whereas the CP-A PIPZ control cells showed a 1.28 (95%CI +/- 0.53) fold increase in OR3A4 expression. Similarly, the OE-33 PIPZ OR3A4 cell line exhibits a 14,518 (95%CI +/- 9090) fold increase in expression of OR3A4 when compared to the OE-33 normal controls, whereas the OE-33 PIPZ controls show a 0.58 (95%CI +/- 0.51) fold increase.

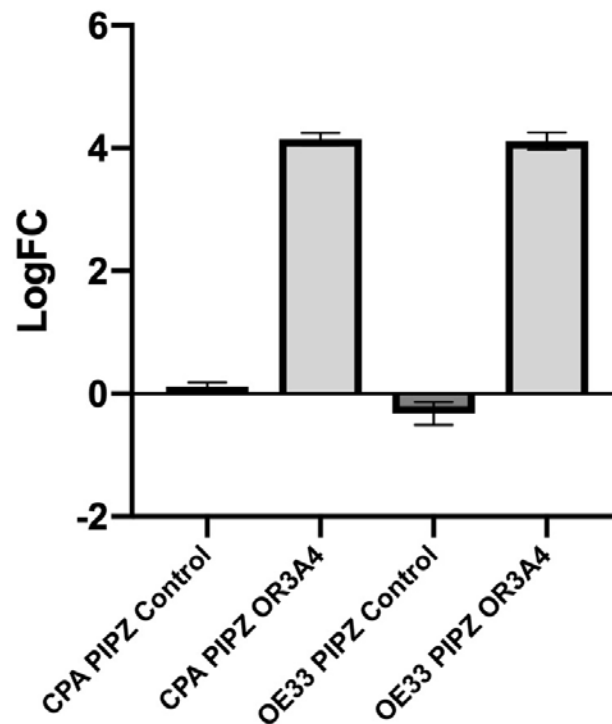


Figure 45 - OR3A4 over-expression in experimental cell lines compared to parental cell lines. Log fold change: CPA PIPZ Control 0.10 (95%CI +/- 0.18), CPA PIPZ OR3A4 4.15 (95% CI +/- 0.19), OE33 PIPZ control -0.33 (95%CI +/- 0.37), OE33 PIPZ Or3A4 4.12 (95%CI +/-0.28).

To investigate differential OR3A4 expression between oesophageal cancer cell lines, RNA from cell lines OE-19, OE-21, OE-33 and MFD-1 was converted to cDNA and RTqPCR performed using OR3A4 and GAPDH probes. Fold change in OR3A4 expression was calculated in comparison to control CP-A cells. OE-33 cells demonstrated a 1.45 fold increase in OR3A4 expression when compared to CP-A cells. OE-19 increased 1.72 times (95%CI +/- 0.556), OE-21 1.69 times (95% CI +/- 0.418) and MFD-1 exhibited a 5.09 fold change (95%CI +/- 0.935). MFD-1 exhibits a higher native OR3A4 expression than OE-33 (5.09 vs 1p=0.0095). OE-19 and OE-21 cell lines did not demonstrate significantly different OR3A4 expression to OE-33 cells (p=0.443 and p=0.371 respectively). The differential expression of MFD-1 should be discounted however, as the cell line was subsequently found to be infected with mycoplasma.

7.4.7.1 RTqPCR PROBE SELECTION

RNA sequencing data was used to highlight further genes and pathways for validation with RTqPCR. PRELID3A and TRIAP1 were selected to investigate whether the TRIAP1:PRELID3A complex was upregulated in OR3A4 over-expressing cells. Furthermore, pre-loaded Taqman plate assays containing multiple probes to investigate the human mitogen activated protein kinase (MAPK) and apoptosis pathways were used. These pathways were identified as potentially of interest following the gene ontology and pathway analysis of RNA sequencing data. MAPK pathways were of particular interest as these were enriched on pathway analysis in whole genome methylation analysis of BO tissue samples which subsequently progressed to OADC.

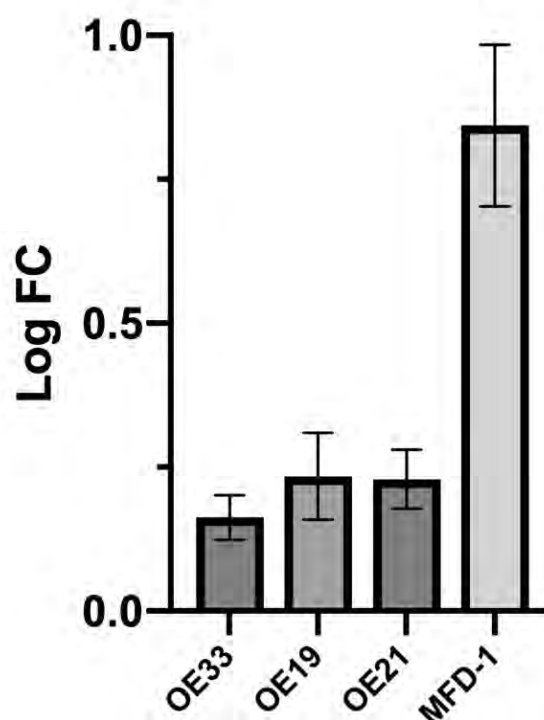


Figure 46 – Differential OR3A4 over-expression in oesophageal cancer cell lines compared to CPA Cells. Log fold change in OR3A4 expression in OE33, OE19, OE21 and MFD-1 cell lines was 0.16 (95%CI +/- 0.076), 0.23 (95%CI +/- 0.15), 0.23 (95%CI +/- 0.10) and 0.70 (95%CI +/- 0.080) respectively.

7.4.7.2 TRIAP1:PRELID3A

Expression of PRELID3A and TRIAP1 were analysed by RTqPCR in CP-A, CP-A PIPZ Control, CPA PIPZ OR3A4, OE-33, OE-33 PIPZ Control and OE-33 PIPZ OR3A4 cell lines. These genes were selected based upon RNA sequencing data. This was performed in parallel with a validation run of OR3A4 expression. This showed no significant increase in expression of TRIAP1 or PRELID3A in the control or experimental cell lines using this method (Figure 47).

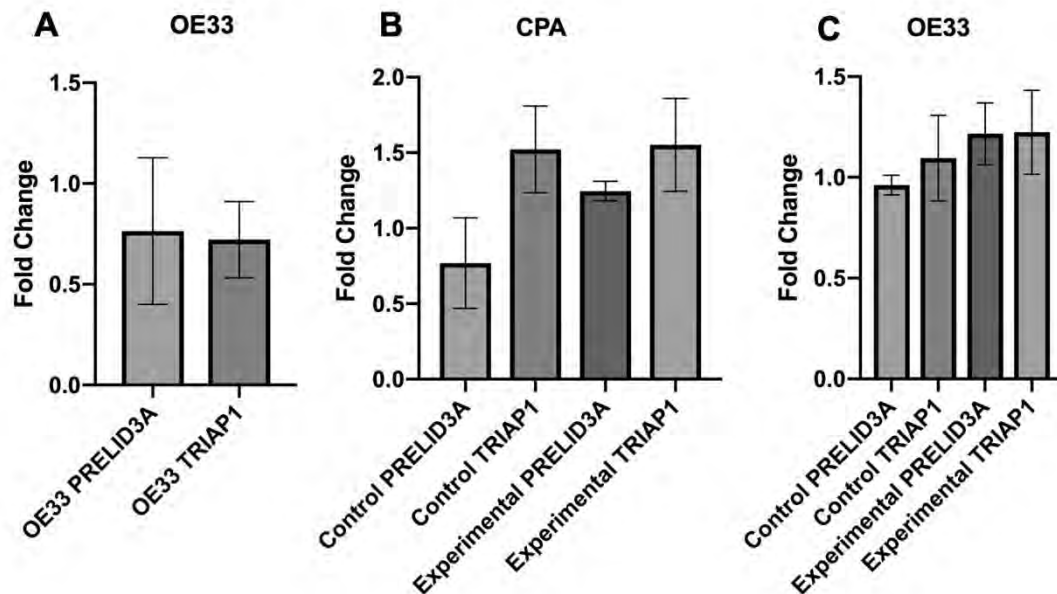


Figure 47 – RTqPCR analysis of differential expression A: PRELID3A and TRIAP1 in control OE-33 vs CP-A cells. B: PRELID3A and TRIAP1 in transfected CPA cell lines (PIPZ Control and PIPZ OR3A4) compared to parental CPA Cells. C: PRELID3A and TRIAP1 in Transfected OE-33 cell lines (PIPZ control and PIPZ OR3A4) compared to parental OE-33 Cells.

7.4.7.3 TAQMAN RTQPCR PLATE ASSAYS

Pre-loaded TaqMan RTqPCR gene expression array plates (ThermoFisher) were used to investigate the differences in gene expression in human apoptosis and MAPK pathways between control CP-A cells and OR3A4 over-expressing CP-A cells. These were chosen because functional annotation analysis of RNA sequencing data revealed significant dysregulation of these pathways in the CP-A cells which over-expressed OR3A4 compared to the control lines. These plates are designed to offer a broad screening tool for further validation experiments. The full plate layouts detailing target genes of interest for MAPK and apoptosis pathways can be seen in Figure 21 and Figure 22 respectively.

7.4.7.4 HUMAN APOPTOSIS PATHWAYS

The human apoptosis pathway array revealed differences between the two cell lines. When OR3A4 is over expressed, there is a 31.38 fold increase in DIABLO which is also known as second mitochondria-derived activator of caspases (SMAC). Caspase 14 is over-expressed by 5.48 fold and DEDD2 by 2.00 fold. In the CP-A control cells lymphotoxin alpha (LT- α) has a 51.22 fold upregulation in comparison to the OR3A4 over-expressing cells.

7.4.7.5 HUMAN MITOGEN ACTIVATED PROTEIN KINASE (MAPK) PATHWAYS

Using RTqPCR, the differences in MAPK pathway gene expression were investigated between CP-A and CP-A PIPZ OR3A4.

There were significant changes in regulation of the MAPK pathway between the two cell lines. In the control cell line, DUSP4 shows a 7.48 fold increase in expression when compared to the OR3A4 over-expressing line.

MAP2K3 demonstrated a 19.71 fold increase in expression in the OR3A4 over-expressing CP-A cell line compared to the normal control cells. OR3A4 over-expressors demonstrated an 11.23 fold change in MAPK15.

Table 41 - Top 10 differentially expressed Human Apoptosis genes from RTqPCR plate array analysis. CPA OR3A4 vs CPA control

Sample Name	Detector	DDCt	Fold Change
CPA OR3A4	DIABLO-Hs00219876_m1	4.9720	31.38
CPA OR3A4	CASP14-Hs00201637_m1	2.4549	5.48
CPA OR3A4	DEDD2-Hs00370206_m1	0.9967	2.00
CPA OR3A4	NOD2-Hs00223394_m1	0.8650	1.82
CPA OR3A4	BCL2L11-Hs00708019_s1	0.8288	1.78
CPA OR3A4	BID-Hs00609632_m1	0.7610	1.69
CPA OR3A4	HIP1-Hs00193477_m1	0.5946	1.51
CPA OR3A4	RIPK2-Hs01572688_m1	0.5358	1.45
CPA OR3A4	HTRA2-Hs00376860_g1	0.5204	1.43
CPA OR3A4	DAPK1-Hs00234480_m1	0.4595	1.38

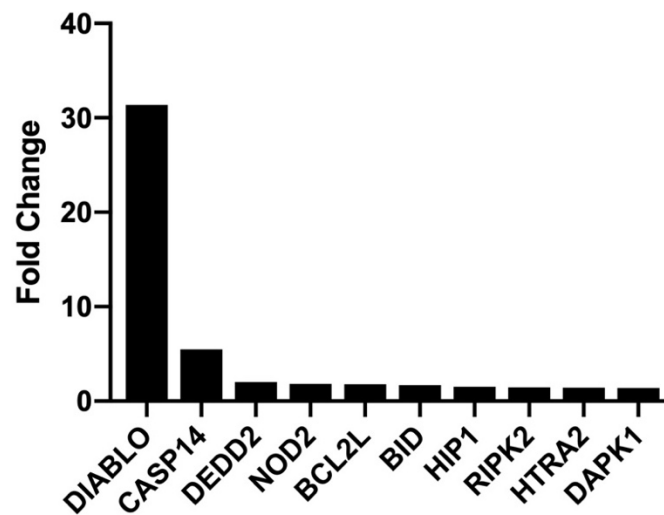


Figure 48 - Top 10 over-expressed genes in CPA PIPZ OR3A4 transfected cells compared to CPA Control in Human Apoptosis Pathways. Data Generated by RTqPCR

Table 42 – Top 10 differentially expressed genes in the human apoptosis pathway. CP-A control vs CP-A OR3A4

Sample Name	Detector	DDCt	Fold Change
CPA	LTA-Hs99999086_m1	5.6785	51.22
CPA	BIRC5;EPR1-Hs00977611_g1	3.1174	8.68
CPA	NAIP-Hs01847653_s1	2.6039	6.08
CPA	LTB-Hs00242739_m1	1.8757	3.67
CPA	TNF-Hs00174128_m1	1.8139	3.52
CPA	TNFSF10-Hs00234355_m1	1.5211	2.87
CPA	TNFRSF1B-Hs00153550_m1	1.2850	2.44
CPA	NLRP1-Hs00248187_m1	1.2696	2.41
CPA	TRADD-Hs00182558_m1	1.1942	2.29
CPA	CFLAR-Hs00153439_m1	1.1408	2.21

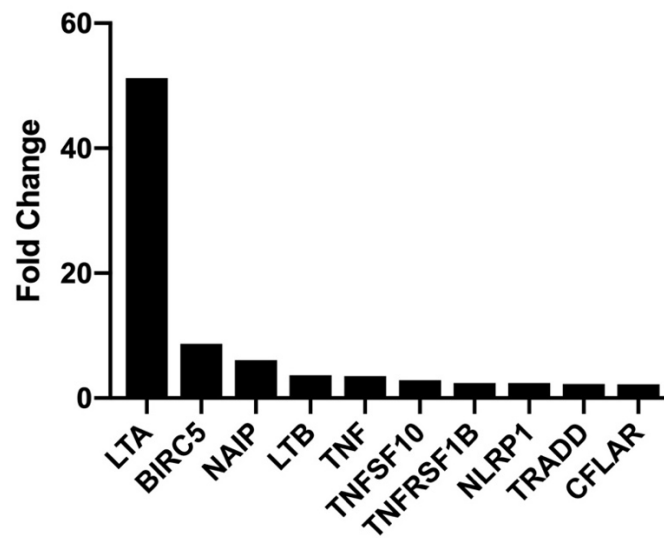


Figure 49 - Top 10 over-expressed genes in CPA control cells compared to CPA PIPZ OR3A4 transfected cells in Human Apoptosis Pathways. Data Generated by RTqPCR

Signaling Pathways Activating p38 MAP Kinase

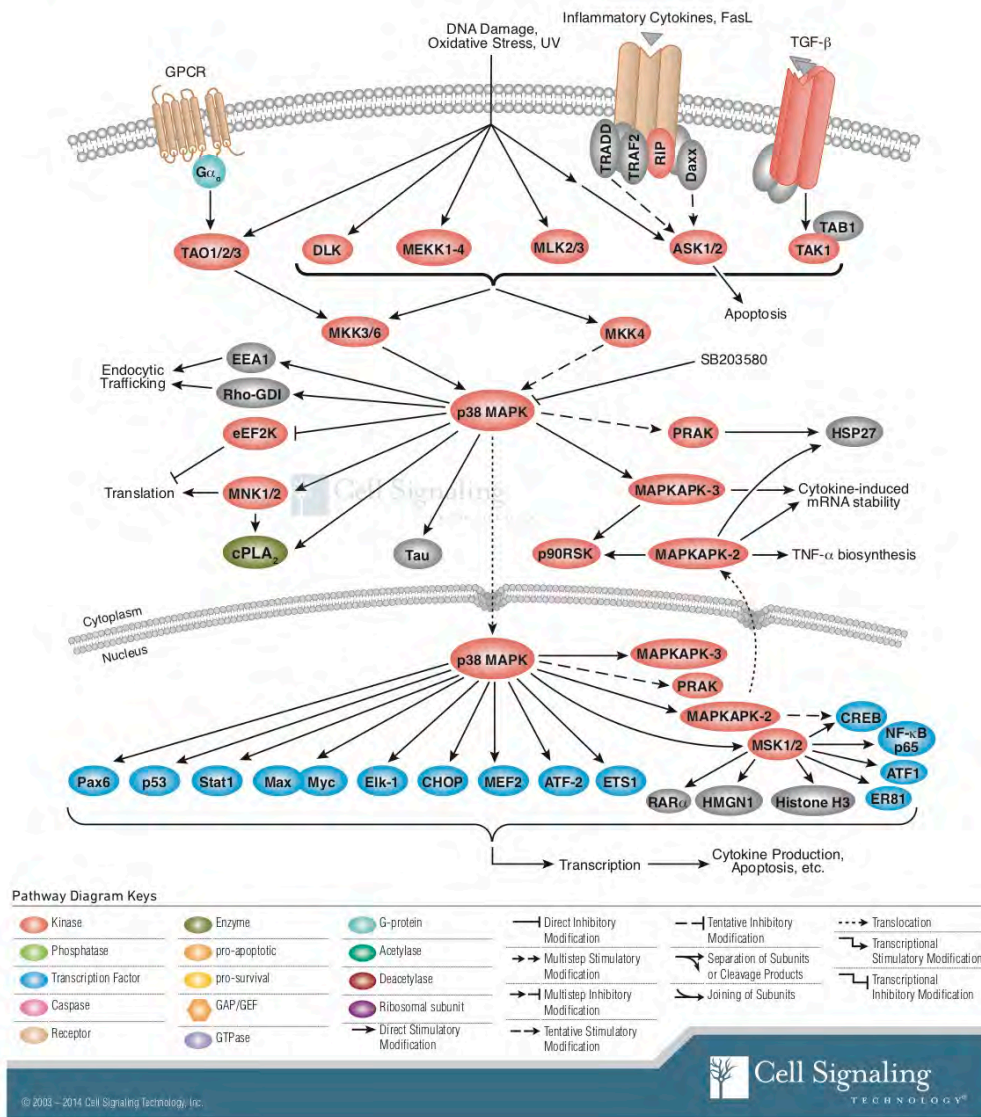


Figure 50 - p38 MAPK Pathway. Diagram reproduced with permission from Cell Signalling Technology.

Table 43 Top 10 differentially expressed Human MAPK Pathway genes, CP-A OR3A4 vs CP-A control

Sample Name	Detector	DDCt	Fold Change
CPA OR3A4	MAP2K3-Hs00177127_m1	-4.3006	19.71
CPA OR3A4	MAPK15-Hs00371187_m1	-3.4889	11.23
CPA OR3A4	DUSP2-Hs00358879_m1	-3.3471	10.18
CPA OR3A4	DUSP9-Hs00154830_m1	-3.2127	9.27
CPA OR3A4	MYC-Hs00153408_m1	-0.8421	1.79
CPA OR3A4	FOS-Hs00170630_m1	-0.6008	1.52
CPA OR3A4	MAP2K6-Hs00177150_m1	-0.5979	1.51
CPA OR3A4	IGF1-Hs00153126_m1	-0.5244	1.44
CPA OR3A4	MAP4K1-Hs00179345_m1	-0.4998	1.41
CPA OR3A4	DUSP1-Hs00610256_g1	-0.4476	1.36

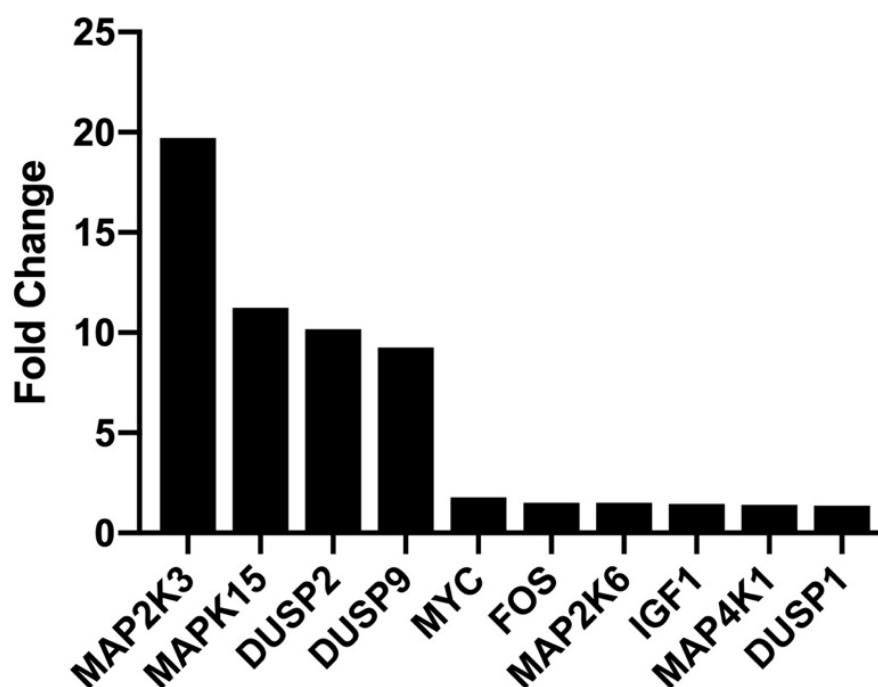


Figure 51 - Top 10 over-expressed genes in CPA PIPZ OR3A4 transfected cells compared to CPA Control cells in Human MAPK Pathways. Data Generated by RTqPCR

Table 44 - Top 10 differentially expressed Human MAPK Pathway genes, CP-A control vs CP-A PIPZ OR3A4

Sample Name	Detector	DDCt	Fold Change
CPA	DUSP4-Hs00365085_m1	-2.9039	7.48
CPA	MAP3K5-Hs00178726_m1	-2.1283	4.37
CPA	PAK2-Hs00605586_m1	-1.8456	3.59
CPA	DUSP10-Hs00200527_m1	-1.8296	3.55
CPA	MAPK13-Hs00234085_m1	-1.5029	2.83
CPA	MAPK10-Hs00373461_m1	-1.4226	2.68
CPA	MAPK7-Hs00177079_m1	-1.3965	2.63
CPA	PAK3-Hs00176828_m1	-1.3767	2.60
CPA	ZAK-Hs00370447_m1	-1.2664	2.41
CPA	PAK1-Hs00176815_m1	-1.2562	2.39

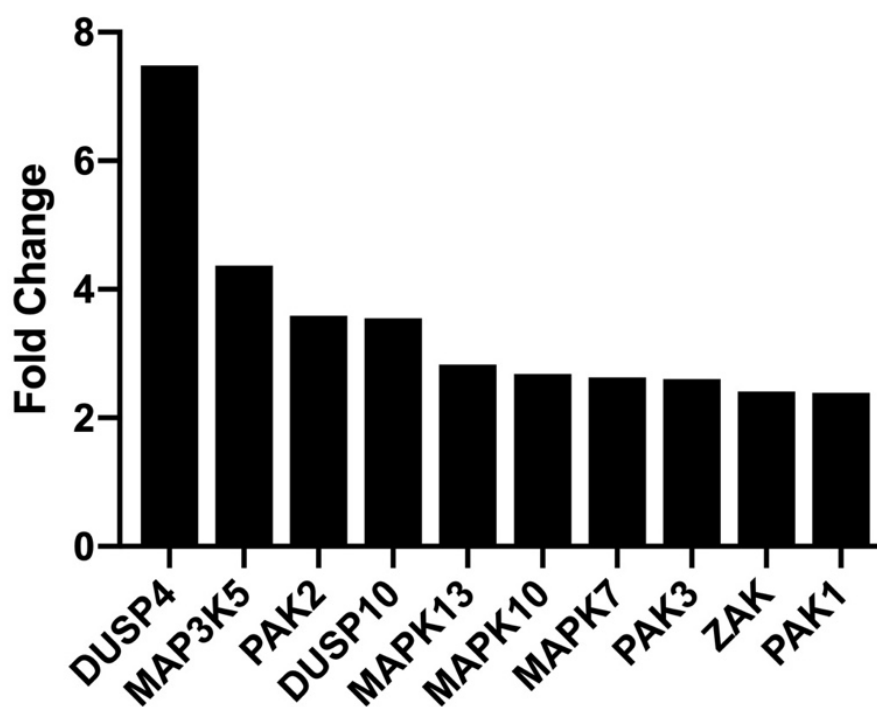


Figure 52 - Top 10 over-expressed genes in CPA control cells compared to CPA PIPZ OR3A4 transfected cells in Human MAPK Pathways. Data Generated by RTqPCR

7.4.8 DORAMAPIMOD KILL CURVES

To further investigate the functional relevance of the altered MAPK pathway, and a potential pharmacological target for high risk BO, CP-A cells were treated with the p38 MAPK inhibitor Doramapimod (BIRB 796). CP-A, CP-A transfected control and CP-A OR3A4 cells were cultured and treated with increasing concentrations of Doramapimod from 0 to 10 nM. Their viability was then assessed with the MTS assay over the course of 5 days.

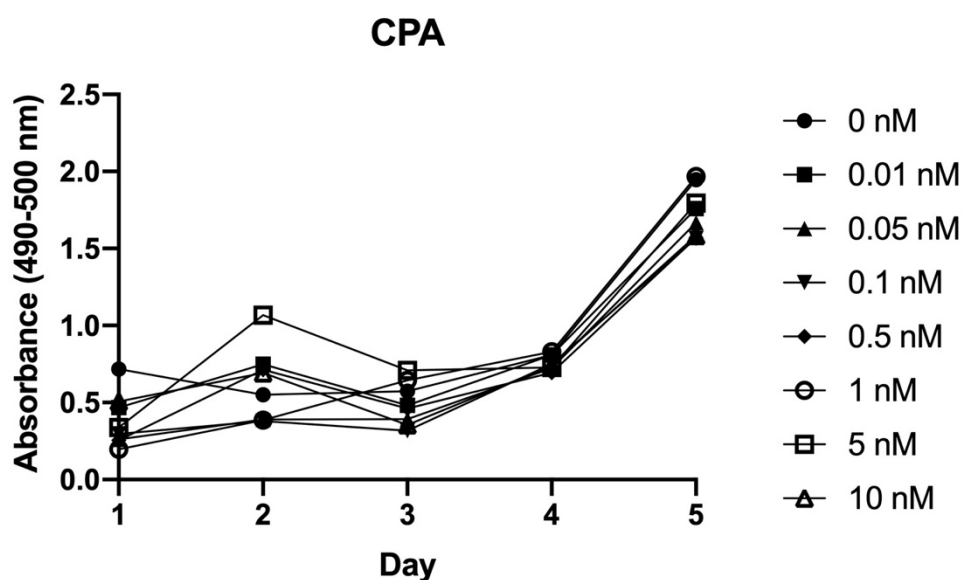


Figure 53 – MTS Assay. Viability of CPA cells cultured with variable doses of doramapimod over 5 days.

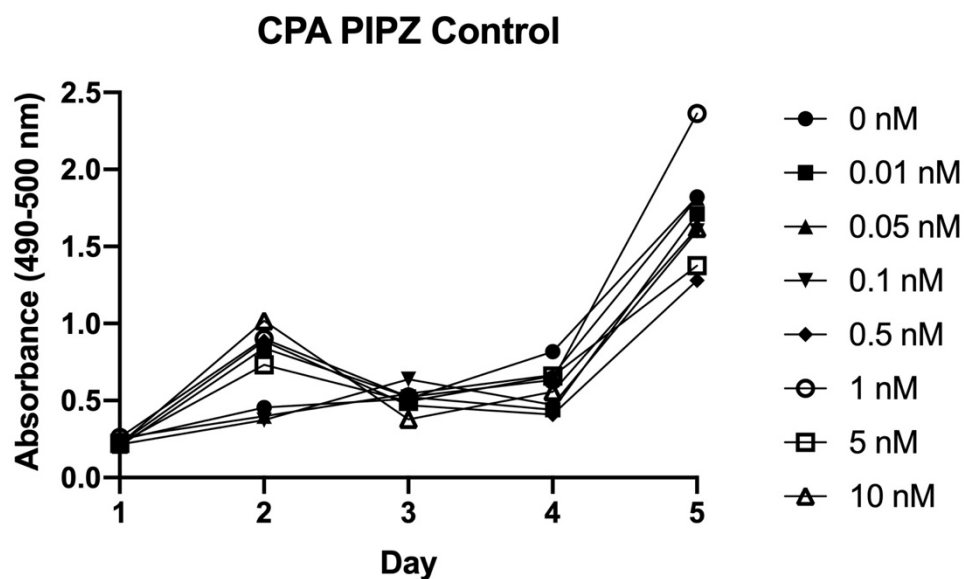


Figure 54 - MTS Assay. Viability of CPA PIPZ control cells cultured with variable doses of doramapimod over 5 days.

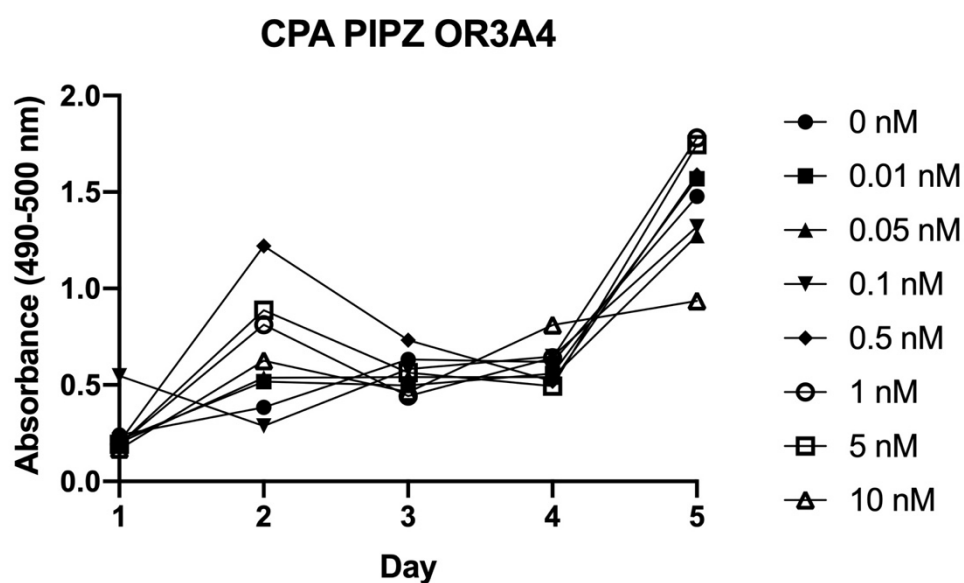


Figure 55 - MTS Assay. Viability of CPA PIPZ OR3A4 cells cultured with variable doses of doramapimod over 5 days.

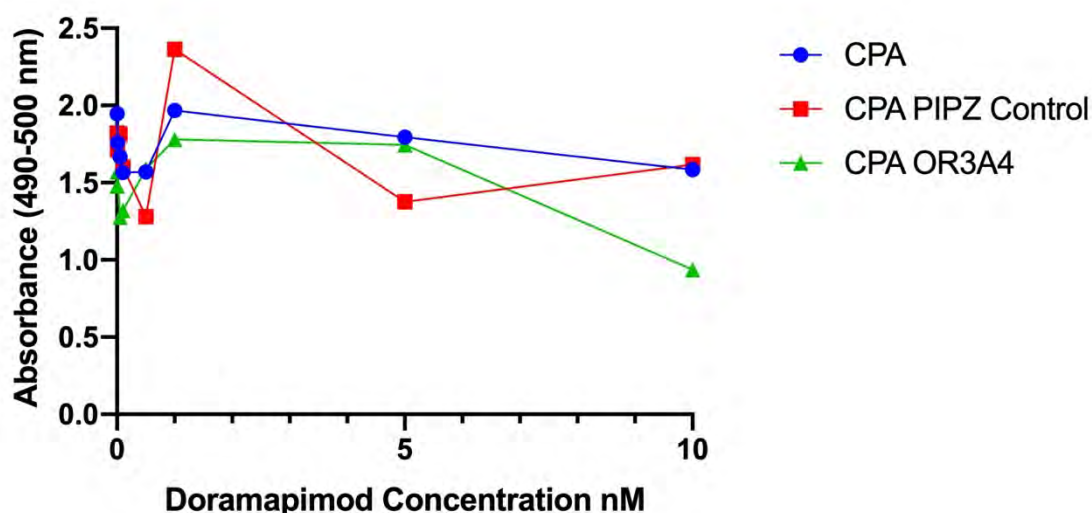


Figure 56 – MTS Assay. Graph showing cell viability after 5 days following variable dosing of doramapimod in CPA, CPA PIPZ Control and CPA PIPZ OR3A4 cell lines.

After 5 days there appeared to be no effect on cell viability under these experimental conditions. The dose range was selected in response to the Selleckchem product literature which quoted Regan et al's study (199) that calculated the IC₅₀ for inhibition of c-Raf-1 and JNK2 α 2 at 1.4 and 0.1 nM respectively. On review of the original published literature on which the product information was based, the IC₅₀ for inhibition of c-Raf-1 and JNK2 α 2 cells were 1.4 and 0.1 μ M, thus the concentration of doramapimod is likely to have been too low to have had an effect in these experimental conditions.

The experiment was therefore kindly repeated by Y Sinha using μ M rather than nM concentrations. This revealed that OR3A4 over-expressing cells required higher concentrations of doramapimod to reduce viability compared to control cell

lines and would suggest that the product literature had misprinted the IC50 as nanomolar rather than the correct micromolar concentrations.

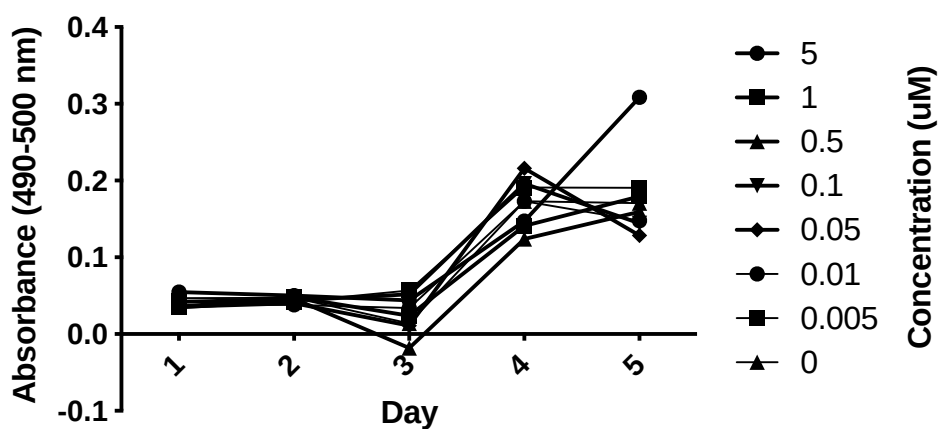


Figure 57 - MTS Assay. CPA cell viability over time when treated with doramapimod

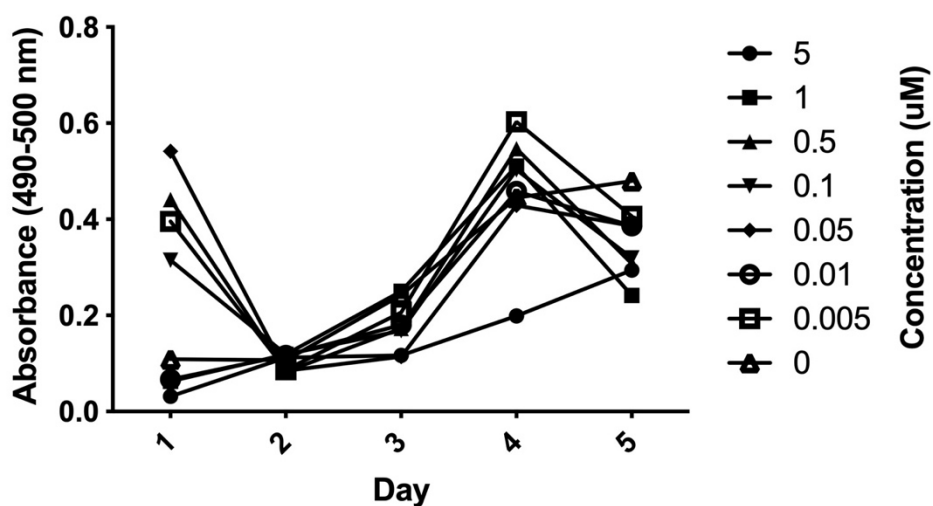


Figure 58 – MTS Assay. CPA PIPZ Control cell viability over time when treated with doramapimod

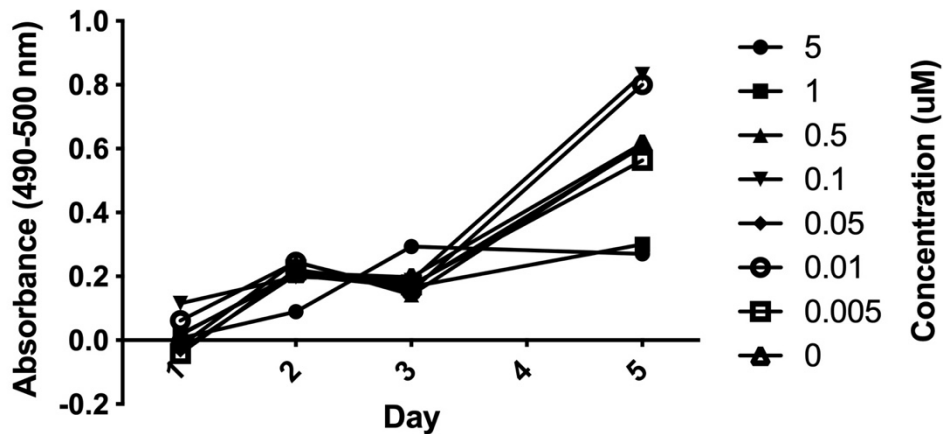


Figure 59 – MTS Assay. CPA PIPZ OR3A4 cell viability over time when treated with doramapimod

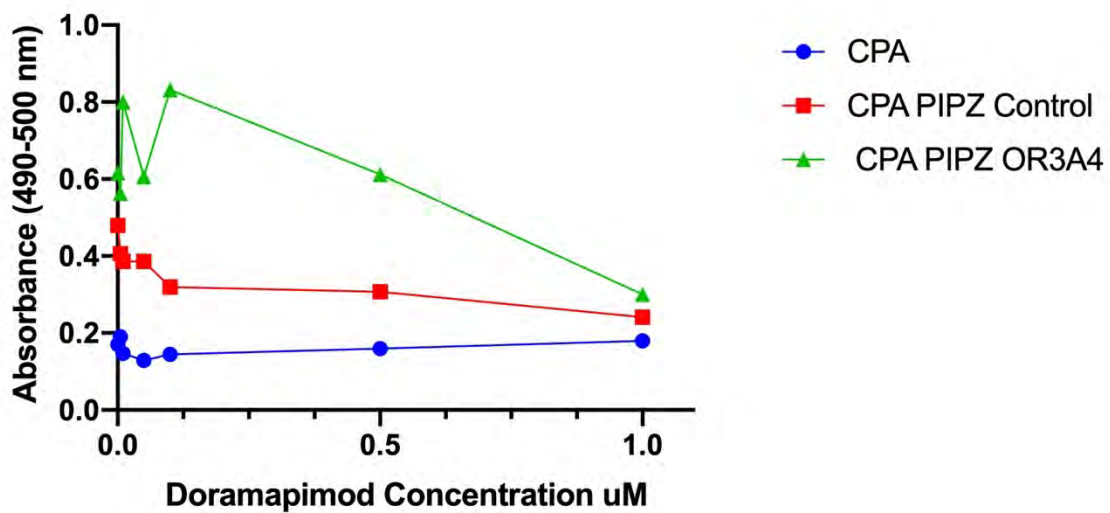


Figure 60 - MTS Assay. Cell viability after 5 days of incubation with increasing concentration of doramapimod

7.4.9 ASPIRIN KILL CURVES

The AspECT trial (200) evaluated the effect of high dose proton pump inhibitor (PPI) with aspirin for improving outcomes in patients with BO. It demonstrated a protective effect against progression of BO when aspirin was taken both in isolation and in combination with PPI. The strongest effect was in combination

with PPI, where patients took 1.67 times longer to develop BO with HGD or progress to OADC. In order to explore the potential mechanism of this effect, CPA, CPA PIPZ Control and CPA PIPZ OR3A4 cells were assessed for viability following administration of increasing doses of aspirin.

Treatment with aspirin in isolation appeared to have no effect on cell viability. In combination with doramapimod, cell viability of OR3A4 over-expressing CPA cells is preserved even at 5 uM concentration, whereas treatment with doramapimod alone appeared to reduce the viability of these cells at higher concentrations. In Din et al's study (201), they demonstrated a mean IC₅₀ of 2.86 mM aspirin in colorectal cancer cell lines (1.48-3.21, n=6). In their experiment, non-colorectal cancer cell lines (3 breast, 1 ovarian, 1 endometrial) were far less susceptible to apoptosis following aspirin treatment. It may be that there is similarly no effect on BO cells in vitro with aspirin treatment, and the observed effect from aspirin in the AspECT trial results from another mechanism. The apparent interaction between doramapimod and aspirin is more difficult to explain as they should both act to suppress p38 MAPK. Zhang et al (202) demonstrated that aspirin treatment of RAW264.7 murine macrophage cells resulted in suppression of NF- κ B and the downstream phosphorylation of all MAPK subtypes including ERK 1 and 2, p38 and c-Jun N-terminal Kinases (JNK). Huo et al (203) demonstrated that the NF- κ B pathway is more readily activated in response to bile acids in BO cell lines compared to normal oesophageal squamous epithelial cells. They have demonstrated that this pathway is suppressed by treatment with aspirin. A pharmacological reaction between the

two drugs may be responsible, rendering doramapimod inactive. These results require further validation to reach a firm conclusion.

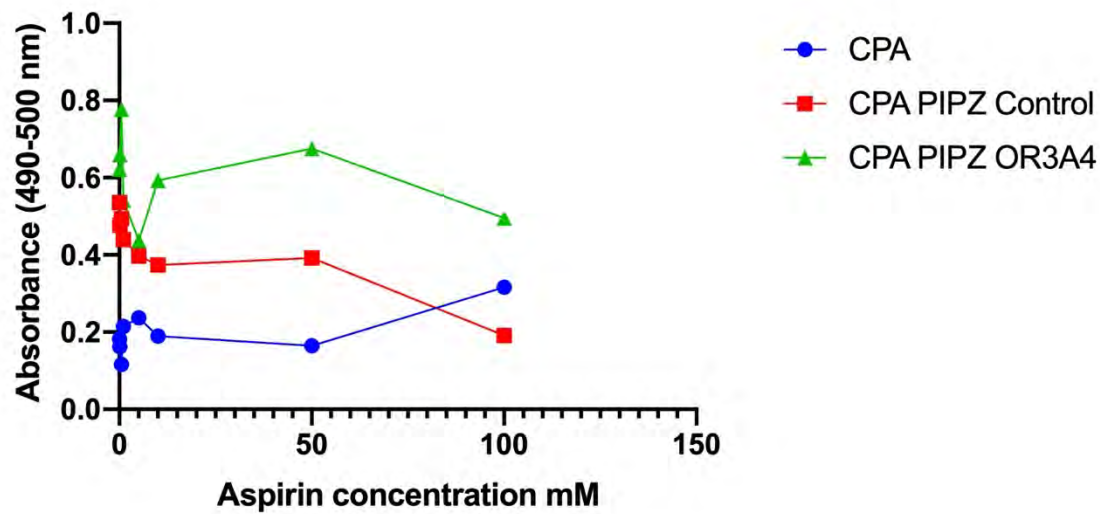


Figure 61 - MTS assay. Cell viability after 5 days of incubation with increasing concentration of Aspirin

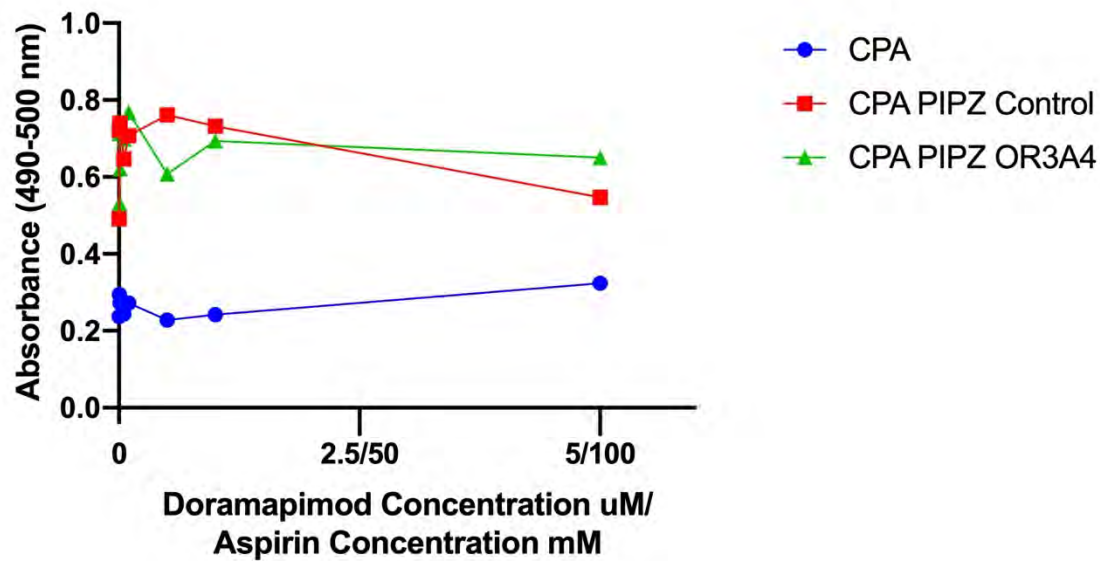


Figure 62 - Cell viability after 5 days of incubation with increasing concentrations of doramapimod and aspirin.

7.5 DISCUSSION

The results presented in this chapter suggest that OR3A4 may not only represent a methylation biomarker to predict progression in BO, but may form an integral part of Barrett's carcinogenesis.

When an OR3A4 over-expressing vector was transfected into the non-dysplastic Barrett's cell line CP-A, clear morphological changes could be seen under light microscopy which were further highlighted with immunofluorescence. CP-A cells over-expressing OR3A4 exhibited an increased nuclear:cytoplasmic ratio and cellular heterogeneity. The cells also exhibited functional differences. Over-expression of OR3A4 resulted in increased viability, proliferation and migration of CP-A cells. This mirrors work by Guo et al (168) and Liu et al (167) in gastric and breast cancer cells in vitro which display increased proliferation and migration when OR3A4 was over-expressed.

The top two ranked differentially expressed genes from the RNAseq data show two different mechanisms by which BO may progress to dysplasia and subsequent OADC. PRKRIP1 blocks the action of PKR which results in inhibition of apoptosis. Defective PKR pathways are also implicated in malignant transformation of cells in vitro and tumorigenesis in murine models. SLMO1 upregulation acts as a protective mechanism against apoptosis, and thus cells which over-express this may be resistant to the induction of apoptosis.

PRKRIP1

Protein kinase R (PKR, also known as PRKR) is a serine/threonine kinase which is active in pro-inflammatory signalling pathways (Figure 63). PKR autophosphorylates and also phosphorylates a number of downstream pathways, including eIF-2 α which acts as an inhibitor of protein synthesis. PKR is activated by binding dsRNA in response to extracellular stresses such as viral infection, acting to stop viral replication in infected cells. The control “empty” vector was transfected into experimental cell lines in addition to the OR3A4 over-expressing vector to exclude the role of viral infection in gene expression changes.

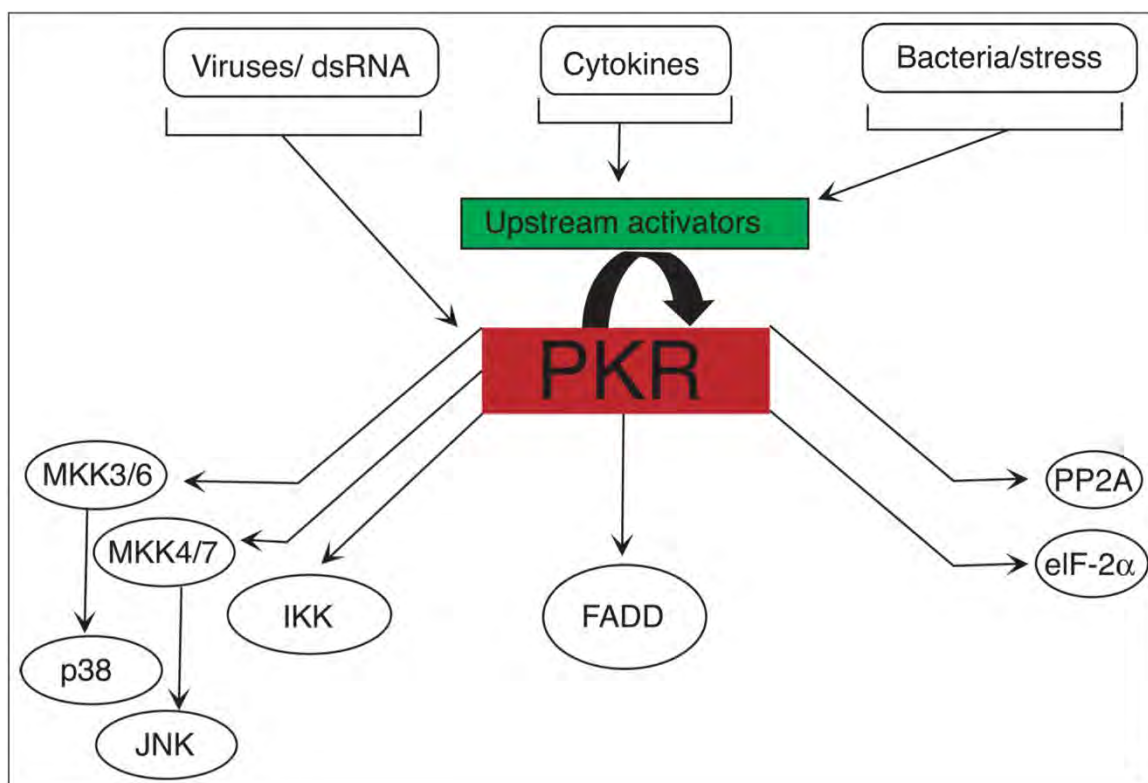


Figure 63 - PKR activation by viral dsDNA, cytokines or environmental stressors leads to downstream activation of MAPK pathways including p38 and JNK. Diagram taken from Williams et al 2001 (204)

PKR signalling can regulate the NF- κ B pathway and is also active in the stress activated protein kinase p38 pathway, increasing levels of PKR resulted in amplified p38 activation(204). PKR is also a key component of TNF- α induced apoptosis(205). PRKRIP1 (PRKR Interacting Protein 1) blocks the action of PKR. In murine embryonic fibroblast cells, reduction of PKR activity resulted in tumour like cellular morphology and increased cell density(206) and in murine models, mice inoculated with NIH-3T3 cells which expressed mutant inactive PKR developed large tumours at the inoculation site whereas the controls with normally expressed PKR did not display this malignant phenotype(207).

PRELID3A (SLMO1)

Cardiolipin is integral to apoptosis. Accumulation of cardiolipin within the mitochondria is protective against apoptosis as it binds cytochrome C. If cytochrome C is presented to the outer mitochondrial membrane, caspase 8 activation and subsequent apoptosis results. Phosphatidic acid import is required for cardiolipin synthesis in the mitochondrial inner membrane. The TRIAP1:PRELID3A complex mediates the transfer of phosphatidic acid between liposomes and facilitates this process across the mitochondrial membrane(208). TP53-Regulated inhibitor of apoptosis 1 (TRIAP1) in isolation mediates cell survival by inhibiting the activation of caspase 9, thereby preventing the induction of apoptosis(209). The interplay between extrinsic and intrinsic caspase mediated apoptosis is detailed in Figure 64. If the TRIAP1:PRELID3A complex is upregulated, cardiolipin is transported preferentially into the mitochondria and prevents induction of apoptosis.

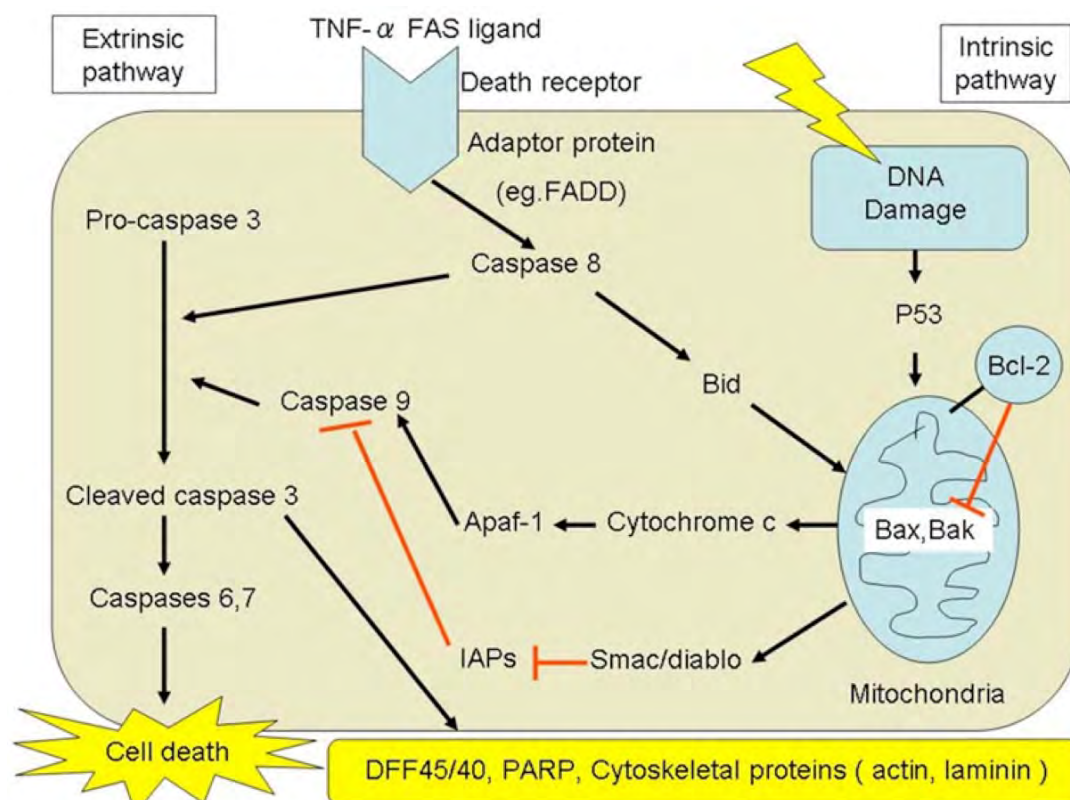


Figure 64 – Diagram of Intrinsic and Extrinsic apoptotic pathways, image taken from Yokobori et al 2014 (210). The Inhibitor of apoptosis protein (IAP) TRIAP-1 is upregulated in OR3a4 over-expressing CPA cells. This directly inhibits the action of caspase 9 and prevents cell death. The upstream inhibitor of IAPs SMAC/DIABLO is highly upregulated in OR3A4 over-expressing cells which raises the possibility of an interaction between OR3A4 and SMAC/DIABLO, inhibiting its action upon IAPs and thus preventing apoptosis.

Further investigation of the human apoptosis pathway by RTqPCR revealed upregulation of DIABLO, also known as SMAC (second mitochondria-derived activator of caspases). DIABLO is a gene that encodes an inhibitor of apoptosis protein (IAP) binding protein. This allows cells to undergo apoptosis by binding and thus inhibiting the inhibitor of apoptosis proteins when they enter the cytosol. This process promotes caspase activation and apoptosis. DIABLO has been shown to translocate from mitochondria into the cytosol in response to gamma

radiation, UV radiation and in the presence of glucocorticoids (important in the stress response) and promotes apoptosis of cells which may have been damaged by such stimuli. This ties in with over-activation of the p38 MAPK pathway which is activated in response to external stressful stimuli. The release of DIABLO requires activated caspases in order to perform its pro-apoptotic function. This suggests that there is increased activation of apoptotic pathways in the OR3A4 over-expressing cells.

LT- α is over-expressed in control cells compared to OR3A4 over-expressing cells. LT- α is a member of the tumour necrosis factor (TNF) family and is otherwise known as TNF- β . It is a cytokine produced by lymphocytes 24-48 hours following mitogenic induction(211). LT- α forms a complex with LT- β , allowing binding to LT- β receptors (LT β R). These receptors activate the nuclear factor kappa light chain enhancer of activated B cells pathway (NF- κ B). This promotes immune regulation through the innate immune response(212). Activation of LT β R causes increased cellular expression of adhesion molecules which aid lymphocyte migration to tumour cells and combat tumour growth. This is especially interesting in the context of progression of BO to OADC as the loss of this pathway may contribute to the blinding of the immune system to prevent destruction of dysplastic cells and subsequent neoplastic transformation. The anti-cancer effects of LT β R have been demonstrated in vitro in numerous human epithelial cancer cell lines and have been shown to arrest tumour growth in mouse models with colorectal cancer xenografts(213).

In vitro modelling shows that there is a 51.22 fold increase in TNF- β in the control CP-A cell line compared to cells over-expressing OR3A4. If OR3A4 acts to inhibit the LT β R signalling mechanism it would result in reduced CD4 lymphocyte recruitment where OR3A4 is over-expressed in the tissues. When DIABLO binds to inhibitor of apoptosis proteins it promotes caspase mediated apoptosis. It appears that DIABLO is recruited to LT β R and is a promoter of apoptosis(214). In OR3A4 over-expressing cells, DIABLO is considerably upregulated, but without corresponding upregulation of TNF- β . Without the corresponding activation of LT β R, DIABLO could be rendered ineffective in the promotion of apoptosis.

The relative downregulation of LT- α and upregulation of DIABLO in the OR3A4 over-expressing cells may provide an explanation as to why seemingly pro-apoptotic genes are upregulated, yet the cells are more viable. A relative lack of LT- α may impinge on the proper functioning of caspase mediated apoptosis in OR3A4 over-expressing cells causing increased viability. The upregulation may be a compensatory cellular mechanism to try to drive apoptosis in these cells.

MAPKs are serine/threonine protein kinases which can phosphorylate cytoplasmic and nuclear targets. MAPK pathways are activated in response to stressful stimuli such as pro-inflammatory cytokines, ultraviolet radiation and heat shock. These stimuli cause stimulation of receptor tyrosine kinases such as epidermal growth factor receptors (EGFR) on the cell surface and autophosphorylation of tyrosine residues. This subsequently causes downstream

activation of Ras by the recruitment of adaptor proteins to the tyrosine kinase receptors which in turn stimulates MAPK activity(215). p38 MAPK in particular is activated by environmental stress. This triggers an inflammatory response which is a key process in the normal response to disease. Over-activation of the p38 MAPK pathway is implicated in a number of inflammatory conditions such as Rheumatoid Arthritis, Psoriatic Arthritis, Asthma and Crohn's Disease(216). MAP2K3 (MKK3, mitogen activated protein kinase kinase) is a specific activator of the p38 MAPK pathway (217) and does not activate ERK or JNK. It is activated by environmental stresses to phosphorylate p38 MAPK and leads to downstream activation of the p38 MAPK pathway. Activation of the p38 MAPK pathway has shown both proliferative and inhibitory effect depending on cell types. 3T3 murine embryonic fibroblasts, and haemopoietic stem cells proliferate in response to p38 stimulation, but in CCL and NIH fibroblasts it exhibits an inhibitory role. p38 also appears to have different effects upon apoptosis depending on cell type independent of stressful environmental stimuli(218).

MAPK15, otherwise known as ERK8 phosphorylates JNK and results in increased activator protein 1 (AP-1) complex activity. JNK is known to possess a pro-proliferative function(219). In vitro modelling of JB6 mouse epidermal cells over-expressing this MAPK showed a greater malignant transformation potential. Knockdown of ERK8 can reduce the tumourigenic properties of HCT15 human colorectal cells resulting in slower growth. Nude, athymic mice injected with HCT15 cells all developed tumours, however when ERK8 was knocked down in these cells and injected into the mice, only 3 of 7 mice formed tumours (220). In liver tumours, c-Jun is required for tumour development, is protective against

apoptosis and is required for tumour cell survival after initiation. Interestingly once tumours are established, inactivating JNK does not alter their progression (221). This may represent a triggering factor for dysplastic change in BO cells.

The dual specificity phosphatase (DUSP) family act as an inhibitor of the p38 MAPK pathway by causing sequestration of MAPKs in the cytoplasm or nucleus and preventing them reaching their intended targets(222). This causes downregulation of the p38 MAPK pathway. The p38 MAPK pathway appears to be abnormally stimulated by MAP2K3 with a 19.71 fold increase in expression of this gene in the OR3A4 over-expressing CP-A cell line. DUSP4 exhibits a 7.48 fold increase in expression in the CP-A control line which should inhibit the p38 MAPK pathway. In this context, abnormal over-stimulation of the p38 MAPK pathway could be responsible for the increased proliferation demonstrated in the OR3A4 over-expressing CP-A cell line when compared to the normal control cells in MTS and scratch wound healing assays. The relative upregulation of DUSP4 in the control cell line may act to regulate cellular proliferation in the control cells.

Over-expression of MAPK15 by 11.23 fold may also provide an explanation for the behaviour of the OR3A4 over-expressing cells through the activation of c-Jun (JNK), a positive regulator of cell proliferation. This is concordant with observations in colorectal cancer cell lines(220).

Doramapimod is a p38 MAPK inhibitor which has been the subject of a number of different Phase II clinical trials by Boehringer Ingelheim investigating Rheumatoid Arthritis in patients who have failed at least one DMARD (1175.18),

patients suffering with severe psoriasis (1175.10) and Crohn's disease (1175.12). At lower doses, there seemed to be no beneficial clinical effect in any of the patient groups. When higher doses were administered patients experienced hepatotoxicity and the trials were abandoned on safety grounds. Iwano et al (223) have suggested that a reactive intermediate of Doramapimod which is produced in the liver as part of the response to oxidative stress may be responsible for hepatotoxicity when this drug is administered to patients. Targeting the underlying cellular drivers responsible for progression of BO to OADC may be possible with drugs such as Doramapimod, however it may be difficult to titrate this drug due to reported toxic side effects. The ability to target the underlying mechanism of disease progression in BO would be beneficial due to challenges with endoscopic diagnosis and treatment and the possibility of disease recurrence.

A limitation of the results presented in this chapter is the lack of replicates in both RTqPCR and drug treatment experiments. These results require further validation to cement the pathway by which OR3A4 over-expression causes morphological and functional changes to Barrett's cells in vitro. Validation in the protein phase using western blots would also add certainty to the proposed pathway. A more comprehensive drug screening experiment may more accurately demonstrate potential therapeutic targets for high risk BO.

7.6 CONCLUSION

The long non-coding RNA OR3A4 appears to possess a functional role in BO in vitro. CP-A cells over-expressing OR3A4 appear morphologically different compared to controls with features of cellular atypia which could potentially represent dysplastic change in histological samples. OR3A4 over-expressers show increased viability and cell migration compared to control cell lines. The mechanism responsible for these changes appears to be dysregulation of p38 MAPK, MAPK15 and apoptosis pathways. While the overall role for p38 MAPK pathway in cell proliferation is unclear, in the case of OR3A4 over-expression in CP-A cells, it would appear that its over-stimulation favours increased cellular proliferation. MAPK15 stimulation of c-Jun (JNK) may be responsible for cellular atypia or the dysplastic change seen in this cell line. The dysregulation in apoptosis pathways would appear to centre on altered lymphocyte recruitment in cells which over-express OR3A4 by relative downregulation of LT- α . The upregulation of SMAC/DIABLO in vitro would usually drive apoptosis. It would appear that in OR3A4 over-expressing cells that in spite of this, there is downstream inhibition or dysregulation of apoptosis, possibly also explained by downregulation of LT- α . Downregulation of LT- α in dysplastic Barrett's may also contribute to blinding of the immune system and favour progression to OADC, however further validation is required at a protein level.

8. IMMUNOLOGICAL LANDSCAPE OF BARRETT'S OESOPHAGUS PROGRESSING TO OESOPHAGEAL ADENOCARCINOMA

8.1 INTRODUCTION

BO progresses along an inflammatory path to dysplasia and then adenocarcinoma. Using multispectral IHC we aim to investigate the differential immune response in BO progressors versus non-progressors. From the previous chapter, a mechanism of lymphocyte recruitment may be potentially impaired via LT β R dysregulation as a result of OR3A4 over-expression.

The human immune system's role is to recognise and eliminate potentially harmful entities within the body. The immune system is not only responsible for eliminating foreign threats such as bacterial or viral infection, but also cells within the body which have become dysfunctional and could potentially develop into tumours. Antigens are the molecules responsible for eliciting an immune response and bind to antibodies. There are two major mechanisms of neutralising such threats, humoral and cell-mediated immunity. Humoral immunity is mediated by antibodies, complement and other extra-cellular molecules. Antibodies are used by the acquired immune system to disable pathogens either by directly blocking pathogenic receptors or by binding to their specific antigen. Antibody-antigen binding causes agglutination and precipitation of immune complexes which are then targeted by neutrophils and tissue macrophages for

phagocytosis. Cell mediated immunity is effected directly by cells such as phagocytes and antigen-specific cytotoxic T cells which directly destroy the threat. Immunity is also subdivided into innate and adaptive systems. The innate immune system works on a pattern recognition basis using pattern recognition receptors, toll like receptors and cytosolic receptors to deliver a short term, reactive response to a pathogen. The adaptive immune system utilises antigen presenting cells in conjunction with T and B lymphocytes which are able to not only respond to a specific threat, but also memorise and react more rapidly to that threat if it is encountered in the future. In the adaptive immune system, T cells effect a cell mediated response (Th1), whereas B cells act via the humoral immune system (Th2). A panel of CD4, CD8, CC20, CD68 and FOXP3 was chosen, as it represents key components of each mechanism of immune response. These are described in more detail below.

CD4 (cluster of differentiation 4) is an immunoglobulin which is expressed on the surface of CD4⁺ T helper lymphocytes. CD4⁺ cells are important in the adaptive immune system and recognise new foreign material which is presented to them by antigen presenting cells (APC) in the lymph nodes. APCs present foreign antigens bound to major histocompatibility (MHC) class II molecules, which CD4 has a high affinity to. CD4 acts as a co-receptor of the T cell receptor (TCR) which, when CD4⁺ cells are activated release interleukin 2, activating CD8⁺ cytotoxic T cells and macrophages. In cancer, CD4⁺ T cells effect an anti-tumour response by activating tumour specific cytotoxic T cells (CD8⁺) and tissue macrophages to clear tumour cells(224).

CD8, like CD4 is a co-receptor for TCR, but the key difference is that it is specific to class I MHC. It is primarily expressed on the surface of CD8+ cytotoxic T cells. It is also present on natural killer T cells and dendritic cells. An important function of CD8+ cytotoxic T cells is to destroy damaged or cancerous cells. When CD8+ cells are exposed to such cells they release cytotoxins which activate the caspase cascade in the target cell causing apoptosis.

CD20 is a phosphoprotein which is expressed on the surface of B lymphocytes which are the primary cell type involved in antibody mediated or humoral immunity. They act as antibody secreting and antigen presenting cells.

CD68 is expressed by monocytes and both circulating and tissue macrophages. CD68 binds to tissue and organ specific molecules called selectins which allow macrophages to target specific cells or cell debris for phagocytosis.

FOXP3 (forkhead box P3) acts as a tumour suppressor by regulating the action of regulatory T cells (TREGs). Regulatory T cells reduce immune response and in certain cancers an excess of regulatory T cells can stop the destruction of tumour cells by the immune system.

The immune response to neoplasia has been associated with prognosis in numerous different tumour types. It would appear that an increased immune response in the form of tumour infiltrating lymphocytes (TILs) can decrease tumour progression and improve prognosis in melanoma, colorectal cancer and ovarian cancer (224, 225).

In colorectal cancer, an increased Th1 immune response, activating cell mediated immunity by the production of interferon gamma, confers a better clinical outcome. Galon et al (225) demonstrated that colorectal tumours which elicited a weak immune response resulted in a poorer patient survival than those displaying a strong immune response. Tumours with higher immune cell densities were less likely to recur, an effect mediated by adaptive immune system (225).

In OADC, Noble et al (226) demonstrated that decreased TIL numbers were associated with an increasing tumour stage. They also showed that tumours with high CD3, CD4, CD8 and FOXP3 immune cell infiltrates were associated with improved patient survival. A low proportion of patients with OADC had high levels of TILs in their study, and poor immune infiltration of OADC carried a worse prognosis.

In NAC for OADC, high CD4+ infiltrates were associated with better tumour response to NAC and high CD8+ infiltrates improved lymph node response(226). Schumacher et al (227) demonstrated that high CD8+ infiltration was an independent prognostic factor for favourable outcome in OADC and OSCC. Cho et al (228) showed that simultaneous CD4+ and CD8+ infiltration was associated with an improved prognosis in OSCC.

Overall it would appear that increased inflammatory infiltration in GI tumours confers a better prognosis and prevents disease recurrence.

8.2 Aim

The aim of this experiment was to investigate the differential immune response in BO progressors versus non-progressors using multispectral IHC. The IHC panel used could investigate both the numbers of immune cells and their distribution within the tissues and differential immune cell type.

8.3 METHODS

Sections of NDBO tissue from samples pre-dating any dysplastic change were taken from 12 BO progressor patients and sections of NDBO from 9 non-progressor patients were stained using immunohistochemistry with a panel of CD4, CD8, CC20, CD68 and FOXP3 as detailed in methods section 4.18. All sections were reported as non-dysplastic on histological review (R Hejmadi). These were scanned using the Vectra automated quantitative pathology imaging system and analysed with inForm software. A multiplex IHC image is displayed in Figure 65. The software can segment tissue subtypes and differentiate between regions of interest within a sample using a machine learning algorithm. The software algorithm was trained to differentiate between the stroma, epithelium and background as shown in Figure 66. The multiplex staining generates a different light wavelength for each IHC marker. Each immune cell expressing the marker of interest can be identified by a different colour on the image. The software is trained to identify each individual cell subtype by the staining as shown in Figure 67. Once the algorithm had been trained, images were batch processed and cells were identified and counted in the regions of

interest using the InForm software. Data was exported to Microsoft Excel and pivot tables were used to generate total counts.

8.3 RESULTS

One non-progressor sample was excluded from analysis as the total cell count was below 1000 (average total count = 20,300). Cell counts of each inflammatory cell type were compared to total cell counts in each tissue section to give a percentage value in progressor and non-progressor samples. CD68 (progressor = 2.168% vs non-progressor 4.755%. $p = 0.002$, 95% CI = 1.134 to 4.038) and CD8 (progressor = 0.132% vs non-progressor = 0.721%. $p = 0.041$, 95% CI - 0.02820 to 1.150) showed an increased global infiltration in non-progressors compared to progressors. No further significant difference in immune cell infiltration was seen by individual type, and the overall inflammatory infiltrate when all subtypes were taken into account did not reach statistical significance (progressor = 9.909%, non-progressor 12.822%, $p = 0.316$, 95% CI = -4.011 to 11.74).

Differential percentages of inflammatory cell infiltrates were compared between progressors and non-progressors in the epithelium of BO tissue sections. None of the cell types analysed showed a significant difference when an un-paired T-test was applied. When the stromal or epithelial inflammatory infiltrates of the two groups were analysed in isolation there was no significant difference between them.

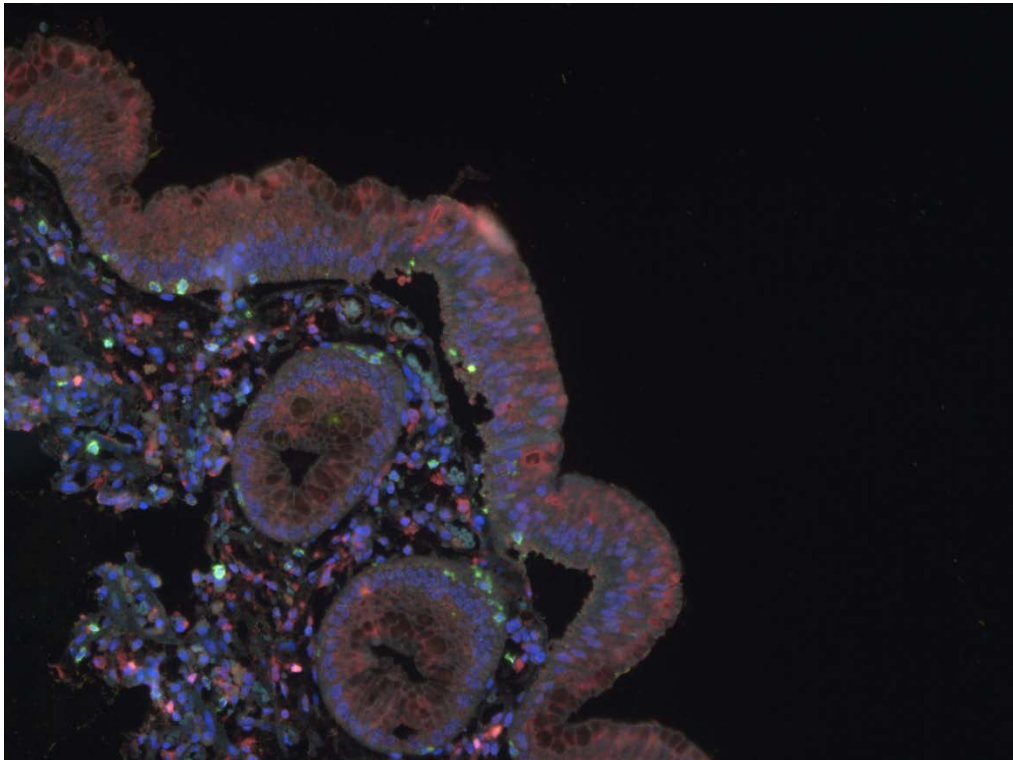


Figure 65 - Image of NDBO tissue section following multiplex IHC staining taken with Vectra Imaging System. This image has the background fluorescence subtracted and leaves only the cellular staining of interest. This allows the InForm software to identify and differentiate between different cell types. The software algorithm was initially trained by manually selecting each individual cell type. Each cell was stained with a different OPAL dye corresponding to the light emission wavelength. CD68 690nm Red, FOXP3 650nm orange, CD20 620nm yellow, CD8 570nm green, CD4 520nm Cyan, DAPI nuclear Counterstain 460nm Blue.

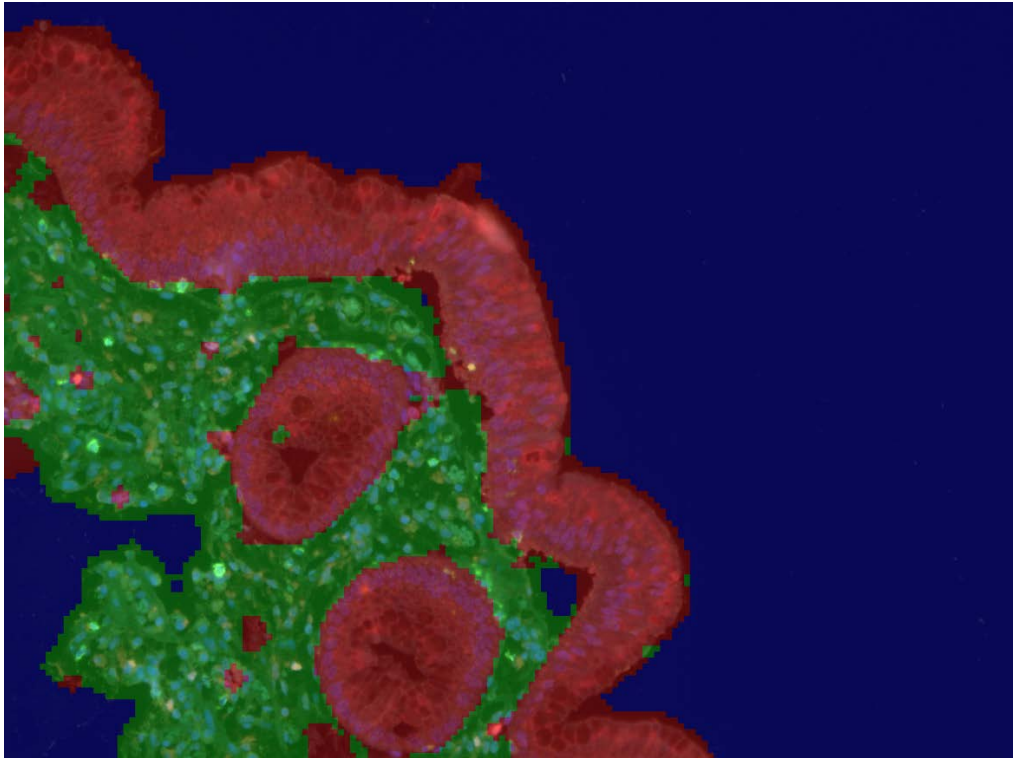


Figure 66 - InForm Software tissue segmentation - The same tissue section is then segmented according to histological areas of interest. The epithelium is highlighted in red, stroma in green, background in blue. Areas are first highlighted manually, and the algorithm trained until it is capable of autonomously segmenting previously unseen images. This required manually segmenting multiple images to ensure accuracy of the final image processing.

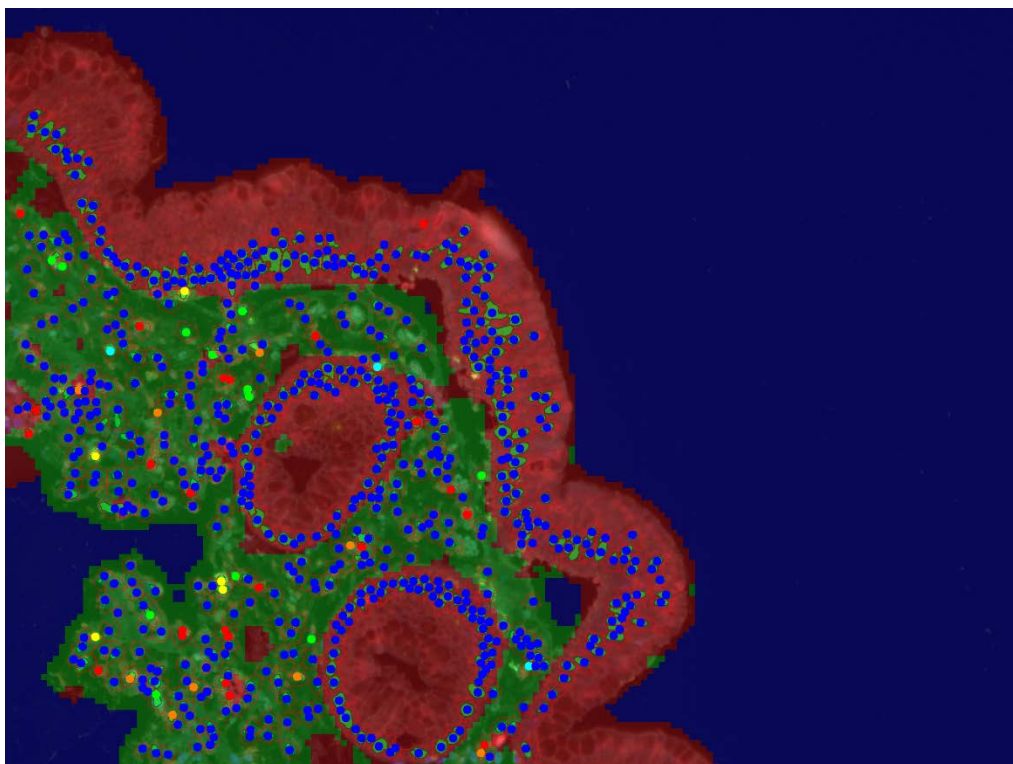


Figure 67 - InForm Software Image with tissue segmentation and Cellular Identification. This image is the final result of the automated image processing. This allows the software to count the number and type of cells and give their distribution between the stroma and cytoplasm.

Table 45 - Inflammatory Infiltration of Immune Cells into BO Tissues. Average number of immune cells as a percentage of total cells.

Inflammatory Cell Type	Progressor	Non-Progressor	P Value
CD20	4.067	4.978	0.701
CD4	1.106	2.408	0.064
CD68	2.168	4.755	0.002 **
CD8	0.132	0.721	0.041 *
FOXP3	2.435	0.911	0.309
All Types	9.909	12.822	0.316

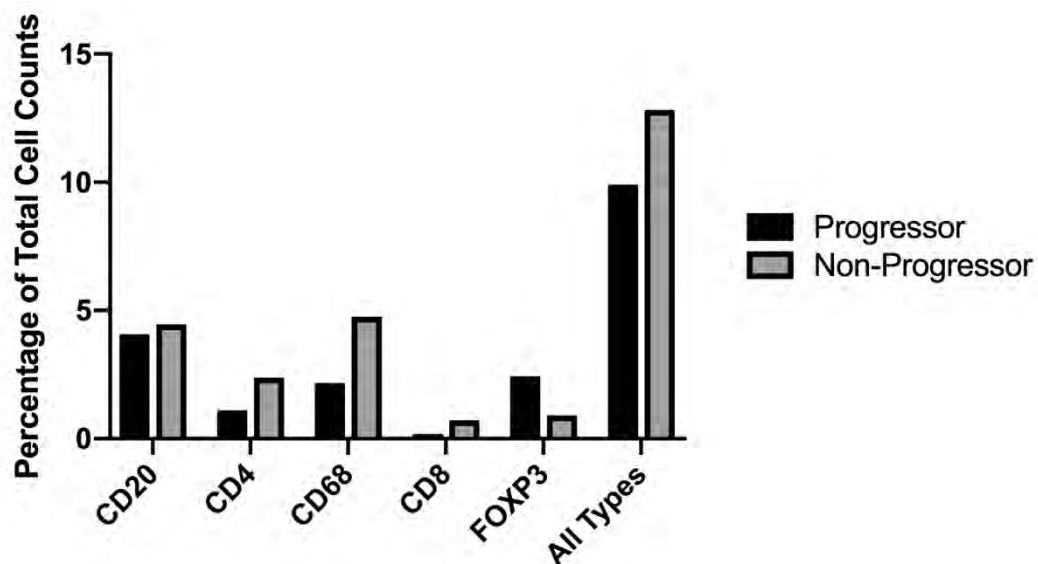


Figure 68 - Immune Cell Infiltration as a Percentage of Total Cell Counts in BO Progressors and Non-Progressors. CD68 ($p = 0.002$, 95% CI = 1.134 to 4.038) and CD8 ($p = 0.041$, 95% CI - 0.02820 to 1.150) showed significantly higher infiltration in non-progressors then progressors.

Table 46 – Table showing the percentage of immune cell types in the stroma and epithelium of progressor vs non-progressor BO samples. There was no significant difference between progressors and non-progressors when stroma and epithelium were compared between the groups using an un-paired T-test.

Inflammatory Cell Type	Non-progressor mean epithelial percentage	Progressor mean epithelial percentage	Non-progressor mean stromal percentage	Progressor mean stromal percentage	P Value
CD20	55.55	56.46	46.73	43.33	0.714
CD4	35.11	45.70	66.24	54.30	0.294
CD68	51.28	57.41	50.69	42.58	0.524
CD8	22.02	23.55	80.73	76.45	0.850
FOXP3	66.62	69.67	36.24	30.26	0.981
Other	69.29	75.34	31.66	24.58	

When differential immune cell distribution between stroma and epithelium was analysed together in each sample (Figure 69) there was a non-significant difference between CD20 and CD68 cells which showed a relatively even distribution between stroma and epithelium in both groups. CD8 cells showed a stromal predominance whereas FOXP3 showed an increased epithelial distribution in both groups. When CD4 was compared, non-progressor samples showed a significant increase in stromal distribution of these cells ($p=0.0014$), whereas progressor samples showed that CD4 cells are more evenly distributed across the stroma and epithelium ($p=0.37$).

Table 47 – Average Percentage Distribution of Immune Cells in BO

	Progressor Epithelial	Progressor Stromal	p value	Non-Progressor Epithelial	Non-Progressor Stromal	p value
CD20	56.46	43.33	0.123	53.09	46.73	0.523
CD4	45.70	54.30	0.370	33.76	66.24	0.001 **
CD68	54.29	42.58	0.171	49.29	50.69	0.840
CD8	17.05	66.28	0.001 **	19.27	80.73	<0.001 **
FOXP3	63.53	36.47	0.007 **	63.76	36.24	0.010 *

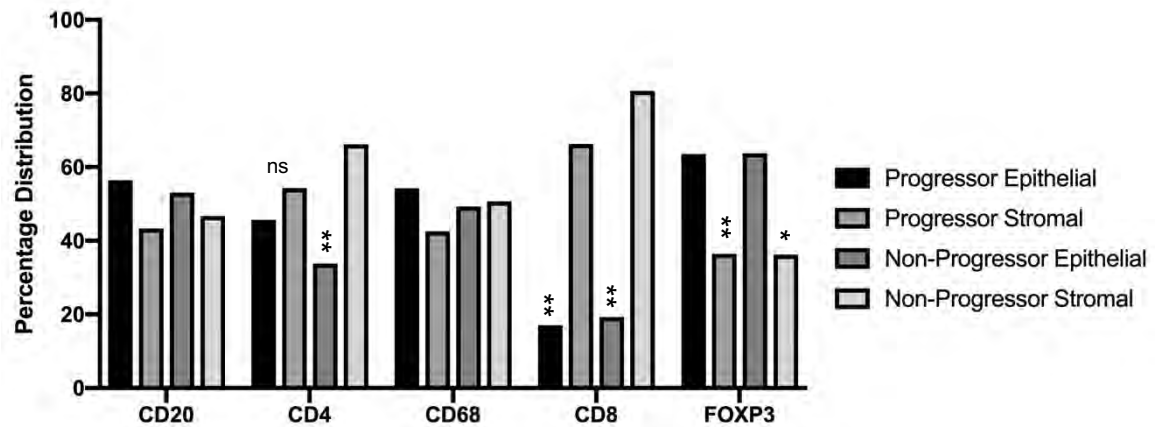


Figure 69 - Percentage distribution of Immune Cells in Barrett's Oesophagus. CD4 cells demonstrate a more even percentage distribution between stroma and epithelium in patients that progressed from NDBO to OADC than non-progressors (stroma/epithelium percentage, progressor = 54.30/45.70 ($p=0.370$), non-progressor = 66.24/33.76 ($p=0.001$)).

8.4 DISCUSSION

The mechanism of progression of BO to OADC cannot be explained by epithelial genetic mutations alone. The immune system may play a key role in Barrett's carcinogenesis.

Multispectral IHC demonstrated an increase in the total numbers of CD8+ cytotoxic T cells and CD68+ macrophages in BO which does not progress to OADC. It would appear that a decreased inflammatory infiltrate confers a worse prognosis in GI tumours (224-228). In the context of BO, the results presented above would support the hypothesis that an increased inflammatory infiltrate may be protective against progression of NDBO to dysplasia and OADC, as increased numbers of cytotoxic T cells and macrophages would be able to target and destroy dysplastic cells more readily, effecting the anti-tumour properties of CD4+ cells.

There was also a change in the distribution of CD4+ T helper lymphocytes, with decreased stromal infiltration in BO progressing to OADC. The overall numbers of CD4+ cells were not different between progressor and non-progressor samples. However, the distribution change could prove significant as CD4+ cells are essential for the activation of CD8+ tumour specific cytotoxic lymphocytes. If CD8+ lymphocytes are not activated, the proliferation of dysplastic and neoplastic cells could be allowed to continue unchecked. Underwood et al have suggested that tumour stroma in OADC plays an important role in progression of OADC and pathological stroma subtypes can negatively affect patient survival(173).

Specifically, increased numbers of cancer associated fibroblasts (CAF) expressing α -SMA and periostin results in worse survival following oesophageal resection for OADC. These studies were confirmed in vitro with CAFs promoting the invasion and proliferation of OADC cells in culture(173). If CD8+ cells are not activated as readily due to a relative loss of CD4+ cells in the stroma, it may result in a proliferation of stromal CAFs. This may represent a further mechanism of progression to dysplasia and OADC.

The results presented in this thesis suggest that there is a stromal change in NDBO prior to progression to dysplasia. There appears to be increased OR3A4 expression in the stroma of BO which progresses to OADC, demonstrated by IHC and presented in section 6.4.5. This is also further supported by the differential distribution of CD4+ lymphocytes in the tissue specimens of progressing and non-progressing patients. There appears to be a more even distribution of CD4+ cells between the stroma and epithelium in progressor patients compared to a larger proportion of this cell type in the stroma of non-progressors. This in combination with the RTqPCR data in section 7.4.7.4 supports the dysregulation of lymphocyte recruitment in progressive BO due to altered LT β R signalling which is discussed in detail in section 7.5. A relative lack of CD4+ cells would reduce the magnitude of cytotoxic T cell activation and tissue macrophage recruitment. This may result in a reduced immune response to abnormal stromal cells and allow BO to progress to dysplasia and OADC.

Zingg et al (224) found that tumour infiltration of TREGs (FOXP3+) was not an independent prognostic factor in OADC. Similarly, Noble et al (226) did not find that TREG infiltration was independently prognostic in OADC or an independent

marker for response to NAC. In the BO specimens examined in this experiment, there was no difference in number or distribution of TREG (FOXP3) cells between progressors and non-progressors. This, along with previous studies in OADC (224, 226), suggests that TREGs may not play an active role in Barrett's carcinogenesis.

Reduction of the immune response to BO may be achieved by a number of mechanisms. The hypermutated genetic landscape of BO includes SNPs in the HLA region (87). This is important as the immune system relies on MHC class I (CD8+) and II (CD4+) for identification of foreign material for eradication. If this process is affected due to mutations in the MHC causing improper antigen presentation, cytotoxic T cells are unable to effectively target dysplastic or tumour cells for destruction, thus allowing the abnormal tissue to progress along the metaplasia-dysplasia-adenocarcinoma sequence. Reduced CD4+ lymphocyte recruitment in BO progressors may be as a result of hypomethylation at the OR3A4 CpG site. Dysregulation of the LT β R signalling mechanism could result from a relative downregulation of LT- α that was seen in CP-A cells over-expressing OR3A4 in vitro (section 7.4.7.4). Increased OR3A4 staining in the stroma of BO progressor samples and a relative reduction of stromal CD4+ cells in this group suggests the effect could be mediated by OR3A4.

8.5 CONCLUSION

An increased inflammatory infiltrate of CD8+ and CD68+ cells may be protective against progression from NDBO to OADC as a result of direct cytotoxic effects against dysplastic cells. The differential infiltration of CD4+ lymphocytes suggests a role in the adaptive immune system for progressive BO, with a diminished stromal distribution in progressing versus non-progressing tissue samples leading to diminished lymphocyte recruitment and may contribute to progression by reducing the immune response to pathological stromal changes. This in conjunction with increased OR3A4 expression in the stroma seen in IHC sections presented in chapter 6 suggests that OR3A4 plays a key role in this process.

9. DISCUSSION

A systematic review of the existing literature was undertaken to consolidate the available evidence and investigate any potential candidate markers for further analysis. The review did not reveal any validated epigenetic biomarkers which could predict progression of BO to OADC. It revealed a heterogeneous and disparate dataset with limited crossover of epigenetic analysis techniques, semi quantitative methodology and potential selection and publication bias of candidate markers. Hypermethylation of p16 may offer one such marker in combination with clinical risk scoring, but this has not been validated on a large scale, nor has p16 hypermethylation been demonstrated in genome wide methylation studies of progression in BO in both the experiments presented in this thesis or in previously published literature identified in the systematic review. Of particular interest, the genome wide methylation studies to date report a picture of global hypomethylation in those patients which progress to OADC rather than hypermethylation of individual CpG loci. Genome wide methylation studies are of particular importance in biomarker discovery experiments, as they do not suffer as readily with selection bias of candidate markers and provide a much wider range of candidate markers than methylation specific PCR arrays.

Epigenetic analysis of tissue samples from patients who have progressed from NDBO to OADC have shown significant hypomethylation at OR3A4 cg09890332 when compared to non-progressing patients. This appears to be a very localised effect at this CpG locus in OR3A4. Cg07863524, which is located closer to the transcription start site of OR3A4 than cg09890332, did not demonstrate

significantly different methylation percentages between progressor and non-progressor patients. It is unclear as to why this particular CpG site is so specifically hypomethylated, but the effect has been replicated in independent patient cohorts. Environmental factors are known to contribute to epigenetic change, and smoking in particular has been shown to cause widespread hypomethylation (105-108). A limitation with the retrospective design of the study is access to a full complement of clinical and environmental factors for each case, especially as samples pre-dating dysplasia were often over 15 years old.

Hypomethylation at OR3A4 cg09890332 has been observed before dysplasia has occurred and may provide an epigenetic biomarker to risk stratify patients with BO. OR3A4 hypomethylation may serve as an epigenetic biomarker to predict patients who are at high risk of progression from NDBO to OADC and could be used in conjunction with clinical parameters to risk stratify and perform enhanced surveillance on patients at high risk of developing OADC.

Hypomethylation of a gene promoter CpG site allows upregulation of that gene. In this case hypomethylation of OR3A4 should result in increased production of this long non-coding RNA. Historically the biological function of OR3A4 has not been well understood as it was initially thought to be a pseudogene. Recent work on OR3A4 as a potential oncogene demonstrated that increased expression of the lncRNA OR3A4 promotes the progression of neoplastic lesions. This has been observed in both patient tissue samples and in vitro models in breast(167) and gastric cancer(168).

Not only could OR3A4 methylation status in patients act as a marker for progression, OR3A4 appears to possess a functional role in BO carcinogenesis. In vitro, OR3A4 over-expression in non-dysplastic CP-A cells promotes cell proliferation and migration and appears to trigger morphological, dysplastic changes in the cells.

The RTqPCR analysis presented in this thesis suggests that the morphological and viability changes in BO cells in vitro as a result of OR3A4 over-expression are a result of dysregulation of MAPK and apoptosis pathways. OR3A4 over-expression drives a significant increase in MAP2K3 and MAPK15 expression when compared to control cells. When control cells are compared, there is an upregulation of DUSP4. DUSPs act as an inhibitor of MAPK pathways.

The p38 MAPK pathway is activated in response to extracellular stressors such as UV light, heat, growth factors and inflammatory cytokines.(229) It plays an important role in human tumorigenesis, as when the p38 MAPK pathway is activated, it usually functions to promote cell cycle arrest and cell death. Epigenetic change seen in BO may be as a result of direct tissue injury by stomach and bile acid reflux. It is possible that hypomethylation at OR3A4 is the primary phenomenon which triggers subsequent p38 MAPK pathway activation following this environmental stimulus. Dysregulation of the p38 MAPK pathway may result in a loss of tumour senescence and increased proliferation and increased likelihood of tumorigenic conversion.

Over-activation of the JNK pathway may result in increased cell proliferation in vitro (219) It may be protective against apoptosis and is required for tumour cell survival after initiation. Interestingly once tumours are established, inactivating JNK does not alter their progression (221). This may represent a triggering factor for dysplastic change in BO cells.

In the apoptosis pathways studied, the reduction of LT- α and the associated downregulation of downstream pathways may result in blinding of the immune system to abnormal cells and allow proliferation of dysplastic cells which may subsequently undergo neoplastic change. Over-expression of OR3A4 may interfere with the LT β R signalling mechanism which recruits lymphocytes and acts to slow tumour growth by direct destruction of tumour cells. This is supported in the IHC models which demonstrate increased OR3A4 expression in the stroma of patients that progress to OADC.

In support of this, differential distribution of CD4⁺ lymphocytes has been demonstrated using multispectral IHC between progressor and non-progressor tissue samples. In non-progressor BO tissues there is a larger proportion of CD4 lymphocytes within the stroma, whereas in progressing samples there is a more even distribution of these immune cells. The relative lack of TNF- β as a result of increased OR3A4 expression may block apoptosis in BO and allow the disease to progress through the metaplasia-dysplasia-adenocarcinoma pathway. There is also an increase in number of CD8⁺ and CD68⁺ cells in non-progressors which would suggest that the immune system plays a vital role in keeping NDBO from progressing to dysplasia.

Hypomethylation at the OR3A4 promoter region appears to be a significant epigenetic driver for progression from NDBO to OADC. This epigenetic change, resulting in an over-expression of the non-coding RNA OR3A4, affects downstream pathways involved in cell cycle control and apoptosis.

In retrospective tissue analysis, OR3A4 over-expression was epigenetically mediated by hypomethylation of the OR3A4 CpG site. This epigenetic change is likely to occur as a result of environmental or acquired factors, and so it is possible that established columnar epithelium at the Z line may undergo epigenetic change in patients which increases cellular viability and reduces apoptosis, increasing the dysplastic potential of this tissue. This would tally with the increasing incidence of BO with increasing exposure to environmental factors such as increasing BMI, GORD and Westernised diets.

Figure 70 illustrates a proposed mechanism for the progression of BO to OADC. Environmental factors, leading to epigenetic change at the Z line results in hypomethylation within the Barrett's mucosa. Specific hypomethylation of OR3A4 results in increased OR3A4 expression in these tissues. This results in upregulation of the p38 MAPK and C-Jun pathways and a downregulation of LT β R which in results in dysregulation of apoptosis pathways and may contribute to blinding of the immune system by reducing lymphocyte recruitment. These factors in combination would allow dysplastic BO cells to proliferate and eventually progress to OADC.

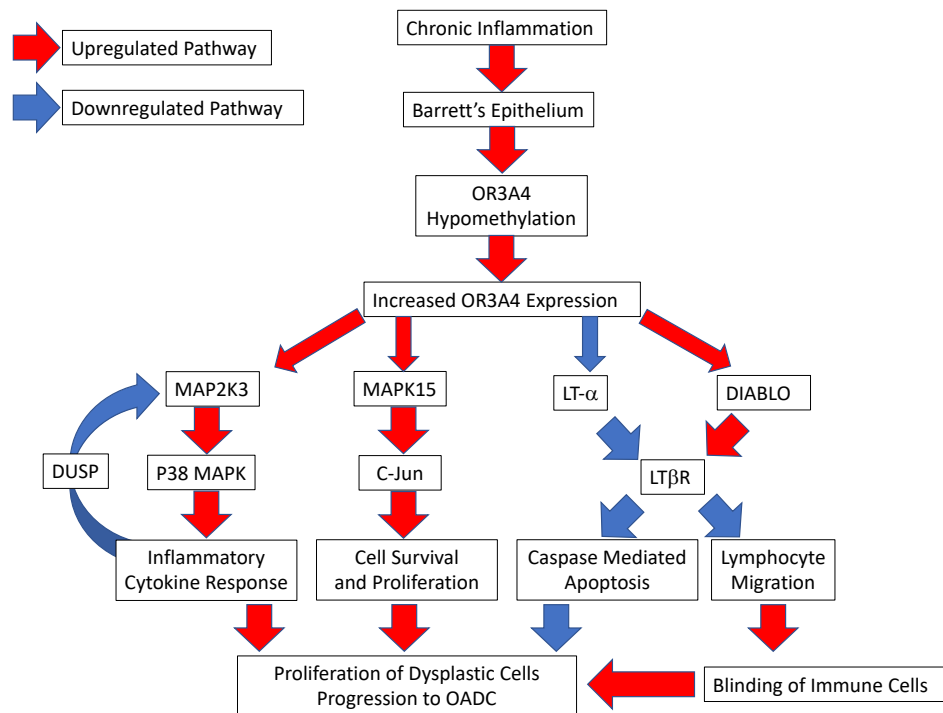


Figure 70 - Proposed mechanism for Barrett's Carcinogenesis as a result of OR3A4 over-expression secondary to hypermethylation of the OR3A4 CpG Site

If a single screening OGD examination could identify patients at high risk of progression to OADC, and reassure those who are at lower risk, those at low risk could be invited to return only if their symptoms change. Patients are currently advised to undergo repeat OGD only if they develop new symptoms. In the high risk group, protocolised biopsy regimes and advanced endoscopic surveillance could be undertaken to facilitate early diagnosis of dysplastic BO or early OADC. Another intriguing potential screening tool is the Cytosponge (51) which could potentially negate the use of a screening OGD entirely, as the proposed model for risk stratification does not require a preceding histological or endoscopic diagnosis of BO.

Understanding the underlying mechanism of Barrett's neoplasia could lead to more targeted treatments being offered to patients. Currently the treatment of choice for dysplastic BO is minimally invasive endoscopic mucosal ablation. This has the benefit of treating the pathology without the need for oesophageal resection. Mucosal ablation is dependant on a highly skilled operator performing the procedure and is limited to treating lesions which can be visualised on endoscopy. This can result in areas being sub-optimally treated or missed entirely. There is also the potential for submucosal disease recurrence under the ablated area which is clinically extremely difficult to detect. Targeting the mechanism of disease could ensure that even the tiniest focus of Barrett's dysplasia is treated and could minimise the risk of disease recurrence. Drugs targeting the p38 MAPK or the JNK MAPK pathways could provide such a targeted therapy.

The major limitation of the work to date is the small numbers of patients and lack of validation of OR3A4 methylation and expression levels in a prospective clinical trial environment. Identifying patients who have truly non-dysplastic BO prior to developing OADC is challenging with incomplete patient clinical information and samples stretching back many years limiting the quantity and quality of DNA which can be analysed. With contemporaneous sample collection and OR3A4 analysis these problems can be avoided. Risk stratification of patients with BO is an important area of unmet clinical need.

10. FURTHER EXPERIMENTAL WORK

Further experimental work is required in order to validate the findings presented in this thesis and potentially translate the findings into clinical practice.

10.1 IN VITRO EXPERIMENTS

The dysplastic BO cell line CP-B (ATCC CRL-4028) would be studied in conjunction with the NDBO cell lines to investigate their viability and migration in comparison to non-dysplastic BO cell lines and CP-A PIPZ OR3A4 using MTS and scratch wound healing assays. The dysplastic CP-B cell line was isolated from primary cultures of BO from areas of confirmed HGD by Palanca-Wessels et al (230), although it is not known whether the patient subsequently progressed to OADC.

Reversibility of OR3A4 over-expression on cell morphology and behaviour in vitro could be studied by using rescue SiRNA knockdown on existing OR3A4 over-expressing CP-A cells. CP-A, CP-B, CP-A PIPZ control and CP-A PIPZ OR3A4 cell lines would be subject to a SiRNA or CRISPR mediated knockdown of the OR3A4 gene. The cell lines would then be subject to MTS viability and scratch wound healing assays as well as imaging under light microscopy to investigate resultant morphological changes. The hypothesis is that SiRNA mediated knockdown of OR3A4 in CP-A PIPZ OR3A4 and CP-B cell lines would revert their behaviour to that of the non-dysplastic CP-A line. In addition, knockdown of OR3A4 in non-dysplastic control lines would not affect cell morphology and

behaviour in vitro. An Annexin assay could also assess rates of apoptosis in a quantitative fashion and may act to validate the findings of RNAseq pathway analysis and RTqPCR experiments.

10.2 RTqPCR

RNA would be extracted from CP-A, CP-B, CP-A PIPZ control, CP-A OR3A4 and the same lines but subject to SiRNA mediated knockdown of OR3A4. This would be converted to cDNA and subject to RTqPCR. OR3A4 differential expression would be analysed across all 8 lines. Subsequently, the significantly differentially expressed genes found in the MAPK and Human Apoptosis pathway pre-loaded plates would be investigated with particular interest in DIABLO, LT- α , MAK2K3, MAPK15 and DUSP4.

10.3 WESTERN BLOT ANALYSIS

Western blot analysis would give confirmation of differential protein expression between the cell lines and could be used to investigate changes in both MAPK and apoptosis pathways. In particular, Hsp27 seems to be able to inhibit the release of Smac/Diablo from mitochondria(231) and by this means can also induce inhibition of caspases, preventing apoptosis.

10.4 DRUG SCREENING IN HIGH RISK BARRETT'S OESOPHAGUS

The effect of Doramapimod on dysplastic CP-B cells in vitro would be intriguing, as it may act to reduce viability and migration. It has the potential to directly affect one of the underlying mechanisms for Barrett's carcinogenesis and could potentially eliminate small foci of disease that would otherwise be missed using endoscopy. Other drugs acting on both the MAPK15 pathway and TNF- β function should also be incorporated into future experiments.

10.5 NATIONAL MULTICENTRE VALIDATION COHORT

OR3A4 hypomethylation in tissues requires large scale validation if it is to be used as a biomarker clinically. The BOSS Trial (232) is a large, national multi-centre trial to investigate the efficacy of routine endoscopic screening versus "at need" endoscopy in BO. This trial benefits from a large patient cohort (3,400), but also from standardised patient data recording, biopsy protocols and long term follow up (10 years). It would be ideal to use patient samples from this study to create a large cohort of progressors and non-progressors upon which to investigate OR3A4 hypomethylation. Standardisation of patient demographics and comorbidity is paramount to avoid confounding clinical factors. These could be adjusted for in subsequent analysis, and utilised in creating a risk stratification model. A proposed risk stratification model would include patient factors such as age, sex, smoking and BMI and methylation status of OR3A4 in tissue samples.

10.6 PROSPECTIVE CLINICAL TRIAL OF ENHANCED SURVEILLANCE IN BARRETT'S OESOPHAGUS

A prospective clinical trial investigating risk stratification of BO patients should be performed with the use of OR3A4 as a biomarker for high risk BO in conjunction with patient clinical factors if a model is successfully validated. This trial would help to target BO surveillance to those groups at the highest risk of progression whilst minimising the number of OGDs for those at low risk. The ENDCaP-C trial is an excellent model for a risk stratification trial and focuses on early detection of neoplasia in UC to improve outcomes. The screening pathway is shown in Figure 71. Patients who have biopsy proven dysplasia are offered a resection as per standard practice. The trial of methylation biomarkers allows patients who have no dysplasia detected on colonic biopsy, but have tissue hypermethylation to undergo enhanced colonoscopic surveillance after 6-9 months, whereas those who are negative on the epigenetic biomarker panel and have no dysplasia detected on histological examination of colonic biopsies are randomised into 6-9 month follow up colonoscopy or no further surveillance. This allows false negatives to be captured and a false negative rate of the methylation marker test to be accurately described giving a diagnostic value and prognostic value of the index test. This is transferrable to OGD surveillance for BO as there are already guidelines in place for timing of repeat endoscopy for patients with a diagnosis of BO. This is important as it minimises risk to patients of developing an undetected OADC in the “low risk” arm of the trial who do not have hypomethylation at OR3A4.

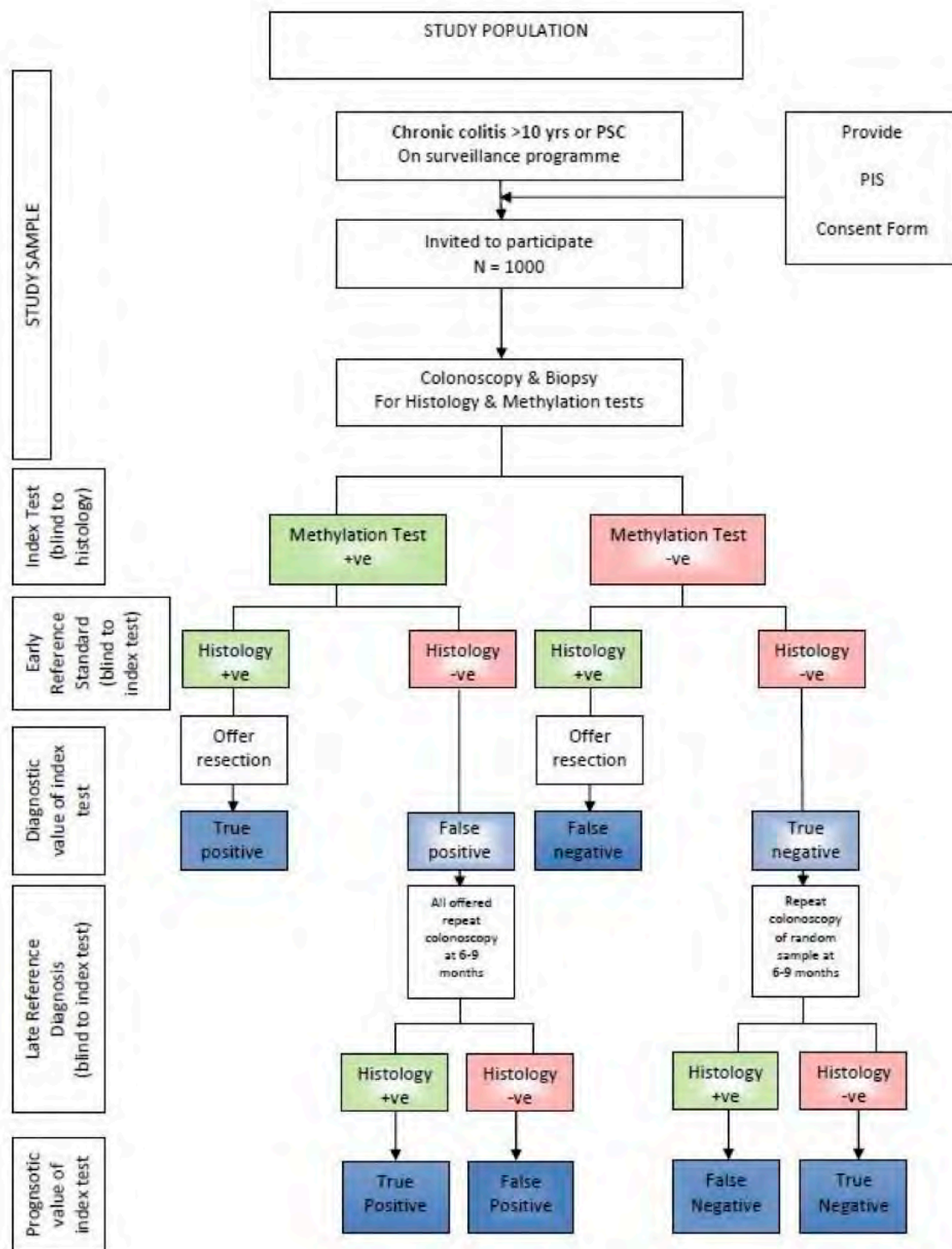


Figure 71 - EndCAP-C Enhanced Endoscopic Screening Pathway

Small, difficult to detect foci of HGD and early OADC remain a major risk for patients to progress to invasive OADC. Advanced diagnostic endoscopic

techniques are available to aid detection of small foci of advanced disease (233). These include high definition white light endoscopy which allows for more detailed inspection of BO mucosa and increased accuracy when mapping BO and targeting lesions for biopsy. In addition, narrow band imaging using specific blue-green light wavelengths can be used to enhance and highlight the characteristic vascular pattern of BO and dysplastic mucosa. This allows tissue sampling of areas which may have been missed on conventional endoscopy. Chemical agents(234) and confocal laser endomicroscopy (CLE) (235) may also be used to enhance endoscopy.

These novel endoscopic imaging techniques should act to reduce sampling error and the incidence of missed neoplasia, however they remain invasive and expensive. It is unrealistic to expect a national OADC screening programme to incorporate advanced endoscopic imaging techniques into every case. Advanced imaging techniques may be more appropriately reserved for patients who are at a demonstrably higher risk of progression from NDBO to OADC than those in the low risk group and could be incorporated into the trial design as a separate arm of the “high risk” surveillance group.

10.7 INTRODUCTION OF A NATIONAL SCREENING PROGRAMME FOR BARRETT’S OESOPHAGUS

A similar model to the UK Flexible Sigmoidoscopy Screening Trial (UKFSST) (236) could be introduced for BO if a robust risk stratification model could be validated and introduced into clinical practice. The UKFSST is investigating the

effect of a single flexible sigmoidoscopy screening test in those aged 55-64 in the UK. It randomised patients who had expressed an interest in endoscopic screening for colorectal cancer into two groups, a control (n = 113,195) and intervention group (n = 57,237) and has followed them up for 17 years. The intervention group received a single flexible sigmoidoscopy followed by a colonoscopy if the initial sigmoidoscopy discovered high risk polyps. Surveillance of colonic polyps relies on classification of patients into low, intermediate and high risk groups. In colorectal cancer surveillance, the presence of three or more adenomatous polyps with one at least 1cm in size, 20 or more hyperplastic colonic polyps or histological features such as villous, tubulovillous, or dysplastic change significantly increase the individual risk of developing colorectal neoplasia and these "high risk" patients are placed in an enhanced surveillance group and advised to undergo a further surveillance colonoscopy in 12 months . The UKFSST has demonstrated that the incidence of all site colorectal cancer in screened patients was reduced by 35% and the incidence of distal colorectal cancer (present in the part of the colon directly visualised by the screening endoscopy) was reduced by 56%. Of 40,674 patients who underwent a screening sigmoidoscopy and polypectomy, 2,131 were referred for colonoscopy due to the presence of high risk polyps. The study comments that despite the significant reduction of all site colonic neoplasia, the incidence of proximal colonic neoplasms was unaffected as these could not be screened using flexible sigmoidoscopy. The difficulty facing colorectal cancer screening is that a full endoscopic examination of the colon is a time consuming and painful procedure requiring patient sedation and full bowel preparation with powerful laxatives. There is a 0.06% - 2.0% risk of perforation of the colon and a 0.4% - 2.7% risk of

major haemorrhage following polypectomy(237). A left sided colonic endoscopy usually requires no sedation and less intensive bowel preparation, and Gatto et al report that sigmoidoscopy carries approximately half the perforation risk of colonoscopy (238). However, by definition it cannot visualise the entire colon. There is a risk of perforation with OGD. In a study of 217,507 patients undergoing OGD Merchea et al report 72 iatrogenic perforations yielding a rate of 0.033%(239).

If a low risk group of patients could be identified with a one off OGD, they could be reassured and discharged as their lifetime risk of developing OADC would be so low that further surveillance would not be beneficial. The small proportion of individuals who are at high risk of progression to OADC would be invited for regular surveillance with the aim of intervention prior to malignant transformation using endoscopic therapy.

11. THESIS CONCLUSION

NDBO represents a benign condition with no life limiting consequences in the vast majority of patients who are diagnosed with the disease. It is likely to be present undetected in many more of the general population due to the lack of symptoms. In 0.33% of patients who do undergo dysplastic and subsequent neoplastic change from NDBO to OADC it represents a potentially catastrophic illness with high associated morbidity and mortality. However, if OADC can be detected in its early stages (T1 lesions), EMR can be attempted with curative intent. Due to the lymphatic drainage of the oesophagus, even early lesions risk lymph node metastasis if they have invaded the submucosa. There is no risk of lymphatic invasion with HGD associated with BO. The appropriate timing for curative therapy for OADC should therefore be at the pre-cancerous stage of BO rather than once OADC has developed.

Hypomethylation of the OR3A4 CpG locus appears to be present before dysplasia has occurred in patients which have progressed to OADC and could provide a tissue biomarker to identify patients at high risk of progression and reassure those with a lower risk. Using OR3A4 hypomethylation as a tissue epigenetic biomarker in combination with clinical risk factors could allow targeted, enhanced endoscopic surveillance of patients at high risk of progression of BO.

Epigenetic change in BO resulting in hypomethylation of the OR3A4 CpG locus and subsequent over-expression of OR3A4 may provide a mechanism to explain the progression of NDBO to OADC. Understanding the exact mechanism of

Barrett's carcinogenesis may provide options for targeted therapies, avoiding or potentially even reversing dysplastic change.

Development of a clinical and epigenetic model for risk stratification of patients with BO could allow integration of advanced imaging and screening techniques into patient follow up care. The aim is to provide earlier intervention in dysplastic BO and improve survival for patients with OADC.

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APPENDIX

PUBLISHED WORKS RESULTING FROM THIS THESIS

1. Epigenetic biomarkers in progression from non-dysplastic Barrett's oesophagus to oesophageal adenocarcinoma: A systematic review protocol

Nieto T, Tomlinson C, Dretzke J, Bayliss S, Dilworth M, Beggs A, Tucker O

BMJ Open 2016;6:e013361. doi:10.1136/bmjopen-2016-013361

Author Involvement: Using the PRISMA-P guidelines I formulated the protocol for the systematic review including key methodological steps, I undertook and reviewed the scoping searches and wrote the manuscript for publication.

BMJ Open Epigenetic biomarkers in progression from non-dysplastic Barrett's oesophagus to oesophageal adenocarcinoma: a systematic review protocol

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ABSTRACT

Introduction: Barrett's oesophagus (BO), a metaplastic condition affecting the lower oesophagus due to long-standing gastro-oesophageal reflux and chronic inflammation, is a precursor lesion for oesophageal adenocarcinoma (OADC). There is no clinical test to predict which patients with BO will progress to OADC. The British Society of Gastroenterology recommends endoscopic surveillance of patients with BO. Epigenetic changes have been well characterised in the neoplastic progression of ulcerative colitis to colonic carcinoma, another gastrointestinal cancer associated with chronic inflammation. This systematic review protocol aims to identify and evaluate studies which examine epigenetic biomarkers in BO and their association with progression to OADC.

Methods and analysis: All prospective and retrospective primary studies, and existing systematic reviews investigating epigenetic markers including DNA methylation, histone modification, chromatin remodelling, micro and non-coding RNAs of all types will be eligible for inclusion. Eligible patients are those over the age of 18 with BO, BO with dysplasia, OADC or unspecified oesophageal cancer. A comprehensive search of bibliographic databases using combinations of text and index words relating to the population, prognostic markers and outcome will be undertaken with no language restrictions. Results will be screened by 2 independent reviewers and data extracted using a standardised proforma. The quality and risk of bias of individual studies will be assessed using the Quality in Prognostic Studies (QUIPS) tool. A narrative synthesis of all evidence will be performed with key findings tabulated. Meta-analysis will be considered where studies and reported outcomes are considered sufficiently homogeneous, both clinically and methodologically. Findings will be interpreted in the context of the quality of included studies. The systematic review will be reported according to PRISMA guidelines.

Ethics and dissemination: This is a systematic review of completed studies and no ethical approval is required. Findings from the full systematic review will

Strengths and limitations of this study

- Systematic review protocol following PRISMA-P guidelines, including description of key methodological steps.
- Rationale for a new systematic review in this area based on scoping searches.
- Exhaustive search strategy likely to capture all relevant published literature on epigenetic markers for progression of Barrett's oesophagus and oesophageal adenocarcinoma.
- Heterogeneity of published research anticipated (differing epigenetic biomarkers studied, variation of study design, sampling methods and follow-up length).
- Above may limit certain epigenetic markers to narrative evidence synthesis

be submitted for publication and presentation at national and international conferences which will inform future research on risk stratification in patients with BO.

Review registration number: CRD42016038654.

INTRODUCTION

The incidence of oesophageal adenocarcinoma (OADC) has dramatically increased in recent years to 5.7 per 100 000 for women and 14.1 per 100 000 for men in the UK.^{1 2} Unfortunately the majority of patients present with advanced unresectable disease with an overall 5-year survival of <13%.³ The five-year survival rates improve considerably to 39% with localised disease.⁴ Barrett's oesophagus (BO), is defined as an oesophagus in which any portion of the normal distal squamous epithelial lining is replaced by metaplastic columnar epithelium which is

clearly visible endoscopically (≥ 1 cm) above the gastro-oesophageal junction (GOJ) and confirmed histopathologically from oesophageal biopsies.⁵ BO arises due to long-standing gastro-oesophageal reflux disease (GORD) and chronic inflammation and is a precursor lesion for OADC with progression through the metaplasia-dysplasia-carcinoma sequence.⁶ The likelihood of developing OADC is increased 1.7 times in patients with GORD, increasing to 10.6 times with BO.⁷ The incidence of OADC has risen in parallel with increasing obesity and GORD in Western populations.¹ With rising rates of obesity the incidence of OADC is predicted to further increase.⁸ Currently there is no robust way of predicting which patients with BO will progress to OADC. The current clinical biomarker for the progression of BO is the presence of worsening cellular dysplasia, also known as intraepithelial neoplasia, on histological examination of serial oesophageal biopsies.⁵ The presence of high-grade dysplasia (HGD), and recently low-grade dysplasia (LGD), triggers intervention.⁹ As a result, the British Society of Gastroenterology recommends endoscopic surveillance of patients with BO and the American College of Gastroenterology endorses screening of high-risk patients for BO.^{5 10} Endoscopic surveillance is invasive, expensive and despite rigorous biopsy protocols, dysplasia and early cancers can be missed. Importantly a meta-analysis published in 2012 demonstrated lower risk for progression of non-dysplastic BO than previously reported with a pooled 0.33% (95% CI 0.28% to 0.38%) annual incidence of OADC.¹¹ The annual incidence rate of OADC for patients with BO with HGD is 7–19%.^{12–14}

Epigenetics is an emerging field which describes mechanisms of alteration of gene regulation and expression without changing the genetic code.¹⁵ These regulatory mechanisms are important in normal human development, for example, silencing of the X-chromosome in females.¹⁶ Epigenetic changes may be inherited but can also be acquired through environmental factors such as cigarette smoking.¹⁷ Epigenetic change can occur through various methods. The most recognised are covalent modifications including DNA methylation, histone modification and altered gene expression by non-coding RNAs.¹⁵ DNA methylation occurs when DNA methyltransferase adds a methyl group (CH_3) to a DNA base. In humans this is most commonly a cytosine base creating 5-methylcytosine.¹⁵ Methylation, which occurs at gene promoter (CpG) sites causes downregulation of these genes. It is thought that the mechanism responsible is the projection of a methyl group into the DNA groove which physically blocks transcription.¹⁸ Histone modification is a post-translational alteration to histone proteins which package DNA into nucleosomes and eventually chromosomes by winding DNA around them. If the histone structure is altered, the DNA cannot be correctly unravelled and cannot be correctly transcribed. The above modifications are carried over when a cell divides and can be inherited.¹⁹ Many different types

of non-coding RNAs have been discovered to alter gene expression by targeting coding messenger RNA (mRNA) after its transcription from DNA. Both micro RNAs (miRNA) and long non-coding RNAs have been implicated in gene regulation. These bind to mRNA molecules and cause them to be denatured and halt protein translation and cause genetic silencing.²⁰

Abnormal silencing of a tumour suppressor or a DNA repair gene through hypermethylation of their CpG promoter sites may cause the cells to grow uncontrollably and lead to tumourigenesis. Epigenetic changes have been well characterised in the neoplastic progression of ulcerative colitis,^{21–24} another tumour arising as a result of chronic inflammation progressing through dysplasia and resulting in colonic carcinoma.²⁵ Intriguingly epigenetic change has been shown to occur early in this process before neoplasia has developed.²⁶ The Enhanced Neoplasia Detection and Cancer Prevention in Chronic Colitis (ENDCaP-C) trial is investigating whether a panel of methylated biomarkers detected in endoscopic biopsy samples can be used as a tool in conjunction with screening colonoscopy to help risk stratify patients who are at higher risk of progressing to carcinoma.²⁷ With the latest next generation sequencing and methylation microchip arrays, it is possible to detect epigenetic changes accurately and reproducibly even in archival tissue samples. In light of this there is a need to consolidate the literature on epigenetic changes in Barrett's carcinogenesis to determine if such changes provide a method of risk stratifying patients who are at risk of progression to OADC.

A scoping search was performed using MEDLINE, the Cochrane Library and internet sources to identify any systematic reviews or meta-analyses on epigenetic biomarkers in BO and oesophageal cancer (OC). It revealed in excess of 2000 primary studies which are relevant for inclusion into the proposed systematic review. No systematic reviews which draw together all aspects of epigenetic change within the field of Barrett's carcinogenesis were identified. Nine systematic reviews and meta-analyses were identified^{28–36} which included mixed patient populations with OADC and oesophageal squamous cell carcinoma (OSCC) with only three reviews incorporating patients with BO.^{31 32 36} These studies concentrated on a single type of epigenetic alteration with four investigating DNA methylation^{28–30} and three looking at miRNA expression.^{31–34} The remaining two studies investigated genetic alterations in progression of BO to OADC.^{35 36} Based on these results, we believe that a systematic review on this topic is both timely and required.

Research aims

This systematic review will identify and summarise studies which examine epigenetic biomarkers in BO and their association with progression to OADC with the aim of consolidating the literature and informing future laboratory work.

METHODS AND ANALYSIS

This systematic review protocol has been reported in accordance with PRISMA-P guidelines.

Selection criteria

Population: All patients over the age of 18 with BO, BO with dysplasia, OADC or unspecified OC will be included.

Prognostic markers: Epigenetic markers including DNA methylation, histone modification, chromatin remodelling, miRNAs and non-coding RNAs of all types will be included.

Outcome: Progression from non-dysplastic BO with or without intestinal metaplasia to BO with LGD, HGD or OADC.

Study design: All prospective and retrospective primary studies, and systematic reviews will be included.

Publication type: Abstract and full texts will be included with exclusion of letters and editorials

Exclusion criteria: OSCC and established OCs with no evidence of a pre-existing BO diagnosis will be excluded. Case reports, narrative reviews, in vitro studies (eg, cell lines), studies of genetic mutations, studies using biomarkers to predict a response to treatment (eg, chemotherapy) will be excluded. A decision was made to exclude animal studies, as scoping searches indicated that there were comparatively few (compared with human studies), and therefore were likely to add heterogeneity to an already heterogeneous evidence base. In addition, we concluded that issues relating to transferability of experimental findings from animal models to a clinical setting would occur.

Search strategy

The following electronic bibliographic databases will be searched from inception: EMBASE, MEDLINE, MEDLINE in Process, DARE, CDSR, Cochrane Central, Conference (Conference Proceeding Citation Index, Zetoc) and registers of clinical trials (ClinicalTrials.gov and ICTRP) will also be searched. Reference lists of identified studies and systematic reviews will be screened for any relevant primary studies that were not retrieved from the database searches. Date or language restrictions will not be placed on searches. A search strategy will be developed using combinations of text and index words relating to the population, exposure and outcome, such as: 'Barrett's Oesophagus', 'epigenetic', 'DNA methylation', 'marker' and 'oesophageal adenocarcinoma'. A sample search strategy for MEDLINE is shown in online supplementary appendix 1.

Study selection

This will be a two-step process. Titles and abstracts identified in our literature search will be screened independently by two reviewers using prespecified screening criteria. These are broadly based on whether the studies first include measuring epigenetic markers in patients with OADC and second whether these patients have

progressed from BO to OADC. Full texts of any potentially relevant articles will be obtained and subjected to the full inclusion criteria. Any discrepancies found will be referred to a third reviewer. The study selection process will be documented using the PRISMA flow diagram. Endnote X7 will be used as reference management software and decisions on inclusion or exclusion will be recorded.

Data extraction

Data will be extracted by two independent reviewers using an agreed, standard data extraction form. Any disagreements which cannot be resolved by discussion will be referred to a third reviewer who will act as an arbitrator.

Data will be extracted on the following study characteristics:

1. Study design characteristics—for example, prospective or retrospective and length of follow-up.
2. Population—for example, tissue samples from patients with BO or patients with OADC looking retrospectively at BO samples, patient demographics.
3. Prognostic markers—epigenetic markers including DNA methylation, histone modification, chromatin remodelling, miRNAs and non-coding RNAs of all types.
4. Outcomes—progression from non-dysplastic BO with or without intestinal metaplasia to BO with LGD, HGD or OADC.

Assessment of study quality

The quality and risk of bias of individual studies will be assessed using the Quality in Prognostic Studies (QUIPS) tool.³⁷ This tool will review each individual study in six criteria: study participation, study attrition, prognostic factor measurement, outcome measurement, study confounding factors, and statistical analysis and reporting. We anticipate that due to the difficulty in obtaining samples and the length of follow-up required to assess progression from BO to OADC, there may be significant sample selection bias. Eligible studies are likely to be subject to confounding, with main confounding factors relating to age, obesity, smoking and alcohol intake. The risk of bias assessment will therefore include an assessment of which confounding factors (if any) have been measured and whether they were adjusted for in the design or analysis of the study. There may be differences in how robust the methods are for measuring the prognostic markers and the outcome; for example, published guidelines recommend confirmation of HGD by two independent pathologists.⁵ These factors need to be assessed carefully for each study so that a judgement can be made on whether epigenetic changes seen in these studies are truly reflective of Barrett's carcinogenesis on a population level and whether they can be reproduced easily and accurately for screening purposes. We do not anticipate finding any studies that test models

predicting progression based on patient factors and panels of epigenetic markers.

Evidence synthesis

A narrative synthesis of all evidence will be performed with key findings tabulated. An assessment of clinical and methodological heterogeneity will be undertaken in order to determine the feasibility of meta-analysis. The main sources of heterogeneity are likely to be subtype of biomarker, study design, length of follow-up, sampling interval and experimental technique and equipment used to demonstrate epigenetic change. Meta-analysis may be performed if there are multiple studies reporting on individual biomarker types such as DNA methylation, histone methylation, histone acetylation, miRNA and non-coding RNA providing the same outcomes (and outcome statistic) are reported. Results will most likely be presented as different risks of progression, for example, relative risk (RR) of progression with and without the prognostic marker. Where studies have reported time to progression, HRs will be extracted where possible.

Studies of different study design and those reporting adjusted or unadjusted results will be analysed separately. RR of progression from non-dysplastic BO to BO with LGD, HGD or OADC will be calculated where possible. Adjusted results, for example, from multivariate analyses, are likely to be more informative in terms of the prognostic ability of a given marker in the context of other potential prognostic factors (such as clinical and lifestyle factors). Where meta-analyses are performed, a random-effects model will be more appropriate to account for between-study heterogeneity. Heterogeneity will also be measured statistically using the I^2 statistics and the χ^2 test. Publication bias will be assessed (by generating Funnel plots) only if more than 10 studies are present in each meta-analysis. The strength of the overall body of evidence generated by the systematic review will be assessed using the GRADE approach (Grades of Recommendation, Assessment, Development and Evaluation Working Group).³⁸ The full systematic review will be reported according to PRISMA guidelines.³⁹

DISCUSSION

This systematic review will aim to comprehensively identify studies reporting on epigenetic changes in progressive BO. The results will help to inform future research on risk stratification and a personalised approach to endoscopic surveillance in patients with BO. The findings may inform future research into the optimisation of the Barrett's surveillance programmes using epigenetic markers as part of a multimodal screening tool.

ETHICS AND DISSEMINATION

This is a systematic review of completed studies and no ethical approval is required. Findings from the full

systematic review will be submitted for publication and presentation at national and international conferences which will inform future research on risk stratification in patients with BO.

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Contributors TN, CLT, JD and OT conceived the systematic review protocol.

TN, CLT, SB and JD undertook and reviewed scoping searches and contributed to the methodological development of the protocol with input from OT. TN drafted the initial manuscript and all authors (TN, CLT, JD, SB, MD, ADB and OT) were involved in its critical revision. All authors have given approval of the final version to be published. OT is the review guarantor.

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Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All data and information generated by this protocol will be shared by the publication and dissemination of our manuscript.

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2. Whole genome methylation analysis of non-dysplastic Barrett's oesophagus that progresses to invasive cancer

Dilworth MP, Nieto T*, Stockton JD, Whalley C, Tee L, James JD, Noble F, Underwood TJ, Hallissey MT, Hejmadi R, Trudgill N, Tucker O, Beggs AD*

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Author Involvement: I performed whole genome methylation analysis using Illumina Humanmethylation 450K arrays on 6 progressing and 6 non-progressing patients from UHB as described in the section 4.9. The results were then combined to give a total of 12 progressor and 12 non-progressor patients. I performed validation bisulphite pyrosequencing on 32 patients from the UHS external cohort (14 non-progressors and 18 progressors) and 16 patients from the UHB cohort (34 samples, 21 non-progressor, 13 progressor) as described in section 4.8. Pyrosequencing results from 67 patients from SWB, UHB and UHS were combined in the final analysis. The previous clinical research fellow in the Surgical Research Laboratory, Mr MP Dilworth, performed initial epigenetic analysis using a cohort of patients from SWB and presented the findings in his MD Thesis(115). He performed whole genome methylation analysis using Illumina Humanmethylation 450K arrays on a cohort of 12 patients (6 progressors and 6 non-progressors) from SWB and then performed validation bisulfite pyrosequencing on 25 progressing samples and 37 non-progressing samples from SWB.

3. A systematic review of epigenetic biomarkers in progression from non-dysplastic Barrett's oesophagus to oesophageal adenocarcinoma.

Nieto T, Tomlinson CL, Dretzke J, Bayliss S, Price MJ, Dilworth M, Beggs AD, Tucker O.

BMJ Open. 2018 Jun 30;8(6):e020427. doi: 10.1136/bmjopen-2017-020427.

Author Involvement: Using the PRISMA-P guidelines I formulated the protocol for the systematic review including key methodological steps, I undertook and reviewed the literature searches, performing screening and selecting manuscripts for inclusion in the study. I synthesised the data from the individual markers in the studies and wrote the manuscript for publication.

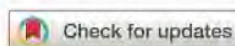
BMJ Open A systematic review of epigenetic biomarkers in progression from non-dysplastic Barrett's oesophagus to oesophageal adenocarcinoma

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ABSTRACT

Objectives The objective of this systematic review is to identify and summarise studies which examine epigenetic biomarkers in patients with Barrett's oesophagus (BO) and their association with progression to oesophageal adenocarcinoma (OADC). BO is a precursor lesion for OADC. There is no clinical test to predict patients who are likely to progress to OADC. An epigenetic biomarker could predict patients who are at high risk of progression from BO to OADC which could facilitate earlier diagnosis and spare those unlikely to develop cancer from regular invasive surveillance endoscopy.

Setting A systematic search was conducted of the following databases: MEDLINE, MEDLINE in Process, EMBASE, Cochrane Central, ISI Conference Proceedings Citation Index and the British Library's ZETOC. Studies were conducted in secondary and tertiary care settings.

Participants All studies measuring epigenetic change in patients over 18 years old who progressed from non-dysplastic BO to OADC were included. Genetic, in vitro and studies which did not measure progression in the same patient cohort were excluded. Study inclusion and risk of bias of individual eligible studies were assessed in duplicate by two reviewers using a modified Quality in Prognostic Studies tool.

Results 14 studies met the inclusion criteria. 42 epigenetic markers were identified, and 5 studies developed models aiming to predict progression to OADC.

Conclusions The evidence from this systematic review is suggestive of a role for p16 as an epigenetic biomarker for the progression of BO to OADC.

Prospero number CRD42016038654.

INTRODUCTION

Barrett's oesophagus (BO) is defined as an oesophagus in which any portion of the normal distal squamous epithelial lining is replaced by metaplastic columnar epithelium which is clearly visible endoscopically (≥ 1 cm) above the gastro-oesophageal junction and confirmed histopathologically from oesophageal biopsies.¹ BO arises due to long-standing gastro-oesophageal reflux

Strengths and limitations of this study

- Systematic review conducted following strict Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.
- Systematic and reproducible methodology using two independent reviewers.
- All mechanisms of epigenetic change included.
- Limited meta-analysis resulting from lack of standardisation between studies.
- Small patient numbers in the included studies.

disease (GORD) and chronic inflammation and is a precursor lesion for oesophageal adenocarcinoma (OADC) with progression through the metaplasia-dysplasia-carcinoma sequence.² The likelihood of developing OADC is increased 1.7 times in patients with GORD, increasing to 10.6 times with BO.³ The incidence of OADC has risen in parallel with increasing obesity and GORD in Western populations.⁴ Patients with OADC who are diagnosed at an early disease stage benefit from much improved 5-year survival rates of up to 39%⁵ in comparison to less than 13% with invasive late stage lesions,⁶ highlighting the importance of early diagnosis and treatment.

Currently, there is no robust way of predicting which patients with BO will progress to OADC. Clinical and histological information is currently the only tools at the clinician's disposal to aid early detection of OADC. The British Society of Gastroenterology recommends endoscopic surveillance of patients with BO, and the American College of Gastroenterology endorses screening of high-risk patients for BO.^{1 7} Endoscopic surveillance is invasive and expensive, and despite rigorous biopsy protocols, dysplasia and early cancers can be missed. A recent meta-analysis published in 2012 demonstrated lower risk for progression

of non-dysplastic BO (NDBO) to OADC than previously reported with a pooled 0.33% (95% CI 0.28% to 0.38%) annual incidence of OADC in patients with NDBO.⁸ The annual incidence rate of OADC for patients with BO with high-grade dysplasia (HGD) is 7%–19%.^{9–11}

Epigenetics is an emerging field which describes mechanisms of alteration of gene regulation and expression without changing the genetic code.¹² The most widely recognised mechanisms of epigenetic change are covalent modifications and altered gene expression by non-coding RNAs. Covalent modifications alter the structure of DNA and include DNA methylation and histone modification.¹² Mechanisms of epigenetic change are discussed in more detail in the protocol for this systematic review.¹³

Epigenetic changes in ulcerative colitis (UC) are well described^{14–17} and have been shown to occur before neoplasia occurs at an early stage of UC-associated carcinogenesis.¹⁸ UC-associated carcinomas progress in a similar fashion to OADC as a result of chronic inflammation through the metaplasia-dysplasia-adenocarcinoma sequence.¹⁹ The Enhanced Neoplasia Detection and Cancer Prevention in Chronic Colitis trial is investigating whether a panel of methylated biomarkers detected in endoscopic biopsy samples can be used as a tool in conjunction with screening colonoscopy to help risk stratify patients who are at higher risk of progressing to carcinoma.²⁰ In light of this, there is a need to consolidate the literature on epigenetic changes in Barrett's carcinogenesis to determine if such changes provide a method of risk stratifying patients who are at risk of progression to OADC.

A scoping search was performed using MEDLINE, the Cochrane Library and internet sources to identify any systematic reviews or meta-analyses on epigenetic biomarkers in BO and oesophageal cancer (OC). Nine systematic reviews and meta-analyses were identified^{21–29} which included mixed patient populations with OADC and oesophageal squamous cell carcinoma with only three reviews incorporating patients with BO.^{24 25 29} Seven reviews concentrated on a single type of epigenetic alteration with four investigating DNA methylation^{21–23} and three looking at micro RNA (miRNA) expression.^{25–27} The remaining two reviews investigated genetic alterations in progression of BO to OADC.^{28 29} No systematic reviews drawing together all aspects of epigenetic change within the field of Barrett's carcinogenesis were identified.

AIM

To identify and summarise studies which examine epigenetic biomarkers in patients with BO and their association with progression to OADC.

METHODS

Details of the methodology were registered on PROSPERO (CRD42016038654) and have also been published.¹³ A summary is reported here.

Patient and public involvement

This research question was developed to address the issue of BO surveillance. The priority for patients is early diagnosis of OADC and accurate surveillance. In order to achieve this, it is imperative that the correct patient group, that is, those who are at highest risk of progression to OADC, is placed under the most intensive of surveillance, and those at a lower risk can be spared such frequent invasive investigation. Epigenetic biomarkers may provide a robust way of risk stratifying patients for BO surveillance.

Patients and the public were not involved in the development of this systematic review.

Eligibility criteria

Any prospective and retrospective primary studies were eligible for inclusion provided they measured epigenetic markers in patients over the age of 18 years with BO. To be included, the study must have reported on progression from NDBO to BO with HGD or OADC in the same patient cohort. Relevant epigenetic markers are DNA methylation, histone modification, chromatin remodelling and micro and non-coding RNAs. Studies were excluded if they were case reports, narrative reviews, in vitro studies (eg, using cell lines), studies of genetic (rather than epigenetic) mutations, studies using biomarkers to predict a response to treatment (eg, chemotherapy) or animal studies.

Search

A systematic search of the literature to the end of February 2018 was undertaken. Text and index terms relating to the population (BO), the prognostic marker (epigenetic change) and the outcome (BO with HGD or OADC) were combined (see online supplementary appendix 1 for sample search strategy in MEDLINE). No study design, date or language restrictions were applied. MEDLINE, MEDLINE in Process, EMBASE, Cochrane Central, ISI Conference Proceedings Citation Index and the British Library's ZETOC were searched from inception. Reference lists of identified studies and systematic reviews were screened for any additional relevant primary studies. Registers of clinical trials (ClinicalTrials.gov and ICTRP) were searched for ongoing studies.

Study selection

Two reviewers (TN and CLT) independently screened all titles and abstracts to identify potentially relevant studies using prespecified screening criteria. Full texts of potentially relevant articles were assessed against prespecified eligibility criteria. Eligibility was determined by two reviewers (TN and CLT) independently with any discrepancies resolved by discussion or referral to a third reviewer (OT).

Data extraction

Data extraction of the included studies was carried out by one reviewer (TN) using a standardised data extraction form and checked independently by a second reviewer

(OT). Discrepancies were resolved by discussion or referral to a third reviewer (CLT and JD). Data were extracted on study design characteristics, patient characteristics, prognostic marker and outcomes.

Quality assessment

The risk of bias of individual eligible studies was assessed in duplicate by two reviewers (TN and OT) using a modified Quality in Prognostic Studies tool.³⁰ Risk of bias criteria was related to study participation (eg, method of sampling), study attrition, prognostic factor measurement and selection (eg, reliability of epigenetic technique and publication bias), outcome assessment (eg, undertaken in duplicate) and study confounding factors (measured and adjusted for). The main confounders are considered to be age, obesity, smoking and alcohol intake.¹ Selected elements from prediction study risk of bias assessment tool (PROBAST) which is currently under development (Wolff R) were used to assess the methodological quality of prognostic models in a similar fashion to Ensor *et al*'s 2016 systematic review of prognostic models of venous thromboembolism³¹ including aspects on patient selection, statistical models used and model validation.

Synthesis

Synthesis was narrative, with main findings tabulated. Studies were grouped by individual epigenetic marker

or panel of markers. A lack of consistency in reported outcome metrics and heterogeneity relating to study design, length of follow-up, frequency of endoscopy and biopsy and experimental technique precluded any quantitative synthesis. Most studies presented results as percentage methylation or a ratio of differential methylation. Formal assessment of publication bias was not possible.

Reporting

Reporting of this systematic review was according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses³² guidelines (online supplementary appendix 2)

SYSTEMATIC REVIEW RESULTS

Overall, 3995 records were screened, and 14 studies met the inclusion criteria (see figure 1 for study selection process and reasons for exclusion). The most common reasons for exclusion were lack of progression in the same population from NDBO to HGD or OADC, in vitro experimentation or no epigenetic change analysed in patient samples.

Study characteristics

Table 1 shows the main study characteristics. All included studies were of a similar retrospective cohort design. In

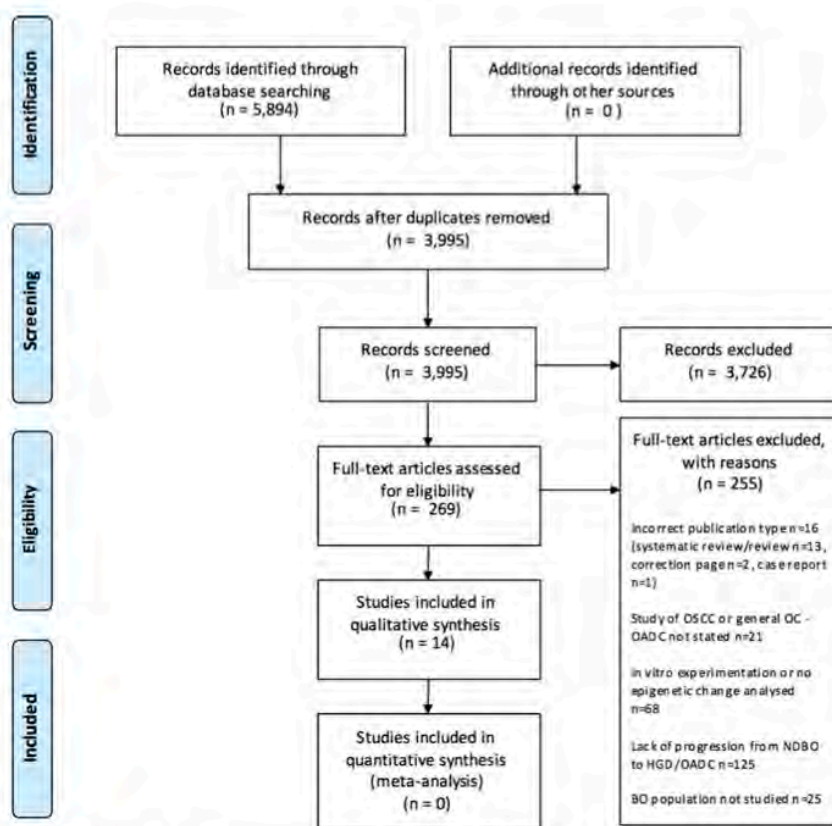


Figure 1 PRISMA 2009 flow diagram with reasons for exclusion. BO, Barrett's oesophagus; HGD, high-grade dysplasia; NDBO, non-dysplastic Barrett's oesophagus; OADC, oesophageal adenocarcinoma; OC, oesophageal cancer; OSCC, oesophageal squamous cell carcinoma; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

Table 1 Summary table of included studies

Author/year	Study type	Study population	Patient no (P:NP)	Patient demographics (mean age, M:F ratio)	Epigenetic analysis	Markers analysed	Result
Agarwal <i>et al</i> 2012 ¹⁶	Retrospective cohort	Pathology archives at Baltimore Veterans Affairs Medical Centre and Johns Hopkins University School of Medicine	9 (5:4)	Not stated	CpG methylation (244K human CpG microarray)	Extragenic x 3	0.45 – relative CpG methylation
						Intra_BCL11B	0.17
						Intra_CDC57	0.23
						Intra_F10	1.86
						Pro_CKB	0.83
						Pro_ELAVL3-ZNF553	0.6
						Pro_GPR177	0.7
						Pro_HOXB7	0.67
						Pro_IGF1R	0.58
						Pro_ITGB8	0.55
						Pro_JARID 1B	0.35
						Pro_ILUND	0.54
						Pro_LAMA5	0.45
						Pro_LOCS55565	0.76
Barrett <i>et al</i> 1999 ³⁷	Retrospective cohort	Fred Hutchinson Cancer Research Centre+University of Washington	31 (25:6)	Not stated	CpG methylation (methylation-specific PCR)	Pro_MGC35308	0.52
						Pro_MMD2	1.34
						Pro_TAF10	0.43
						Pro_TLX3	0.31
						Pro_UBP1	0.71
						Pro_WNK4	0.76
						Pro_WWC1	0.68
						Pro_ZBTB7B	0.56
						Pro_ZNF358	1.25
						CDKN2A	Hypermethylation in premalignant samples
Boerwinkel <i>et al</i> 2014 ⁴³	Retrospective cohort	AMC BO Surveillance	44 (48:10)	Mean age=6632M:12F	CpG methylation (methylight methylation-specific PCR)	HPP1	NS
						p16	P Hypermethylated
						RUNX3	NS
Clément <i>et al</i> 2006 ³⁵	Retrospective cohort	Lausanne, Switzerland	28* (12:16)	Not stated	CpG methylation (methylation-specific PCR)	APC	P Hypermethylated
						CDKN2A	NS
						SFRP1	Hypermethylated in all samples P and NP
						TIMP3	P Hypermethylated
						TERT	P Hypermethylated
Clément <i>et al</i> 2008 ³⁴	Retrospective cohort	Lausanne, Switzerland	31 (16:15)	Not stated	CpG methylation (methylation-specific PCR)	WIF-1	P Hypermethylated

Continued

Table 1 Continued

Author/year	Study type	Study population	Patient no (P:NP)	Patient demographics (mean age, M:F ratio)	Epigenetic analysis	Markers analysed	Result
Eads <i>et al</i> 2001 ⁴⁴	Retrospective cohort	Norris Comprehensive Cancer Centre	20 (12:8)	Not stated	CpG methylation (methylight methylation-specific PCR)	CALCA CDKN2A ESR1 MGMT MYOD1 TIMP3	P hypermethylated P hypermethylated P hypermethylated P hypermethylated P hypermethylated P hypermethylated
Jin <i>et al</i> 2009 ⁴¹	Case control	Five participating US clinics	195* (50:145)	P significantly older than NP	CpG methylation (methylation-specific PCR)	HPP1 p16 RUNX3 CDH13 TAC1 NELL1 AKAP12 SST	P hypermethylated P hypermethylated P hypermethylated NS NS NS NS NS
Klump <i>et al</i> 1999 ³⁶	Retrospective cohort	Under routine Barrett's surveillance	14 (10:4)	P mean age 57.48M:2F	CpG methylation (methylation-specific PCR)	p16	P hypermethylated
Moinova <i>et al</i> 2012 ⁴⁵	Retrospective cohort	Retrospective collection from pathology database	5 (2:3)	Not stated	CpG methylation (methylation-specific PCR)	VIM	VIM methylation present in NDBO, not relevant in progression
Puertas Canteria 2012 ⁴⁵	Retrospective cohort	No details	55 (6:14)	Not stated	CpG methylation (bisulfite pyrosequencing)	p16	Methylation P 12.04% versus NP 6.53%
Revilla-Nuin <i>et al</i> 2013 ³³	Retrospective cohort	1982 trial assessing medical versus surgical therapy for prevention of progression of BO. Randomly selected	5 (7:17)	Not stated	miRNA expression analysis	miR-192 miR-194 miR-195a miR-198b	Overexpressed AUROC 0.61 Overexpressed AUROC 0.70 Overexpressed AUROC 0.80 Overexpressed AUROC 0.74
Sato <i>et al</i> 2008 ⁴⁰	Retrospective cohort	MAYO and UMD mixed cohort	62 (28:34)	Not stated	CpG methylation (methylation-specific PCR)	HPP1 p16 RUNX3	P hypermethylated P hypermethylated P hypermethylated
Schulmann <i>et al</i> 2005 ³⁹	Retrospective cohort	Baltimore Veteran's Affairs hospitals and University of Maryland Hospitals	53 (8:45)	P mean age 83.34M:9F segment length 10.0 cm NP mean age 62.23M:3F segment length 5.8 cm	CpG methylation (methylation-specific PCR)	RUNX3 HPP1 p16 CREP1 TIMP3 APC	P hypermethylated P hypermethylated P hypermethylated P hypermethylated Hypermethylation not independently associated with P Hypermethylation not independently associated with P Hypermethylation not independently associated with P
Wang <i>et al</i> 2009 ³⁸	Retrospective cohort	Johns Hopkins under surveillance for GORD/BO	57 (7:50)	No significant differences in N versus NP. Data not provided	CpG methylation (methylation-specific PCR)	APC p16	P hypermethylated P hypermethylated

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; BO, Barrett's Oesophagus; GORD, gastro-oesophageal reflux disease; M:F, male-to-female ratio; miRNA, micro RNA; NDBO, non-dysplastic BO; NP, non-progressing patients; NS, not statistically significant; P, progressing patients; PLSD, protected least significant difference; ROC, receiver operating characteristic.

Table 2 Models aiming to predict progression to OADC

Author/year	Study type	Patient no (P:NP)	Model used	Result
Clément <i>et al</i> 2006 ³⁵	Retrospective cohort	28* (12:16)	APC+TIMP3+TERT	Hypermethylation in P versus NP (P 81% vs NP 26% P<0.0001)
Jin <i>et al</i> 2009 ⁴¹	Retrospective cohort	195* (50:145)	Biomarker panel (p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12, SST)	AUROC 0.72
			Biomarker panel+age (p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12, SST)	AUROC 0.85
Sato <i>et al</i> 2008 ⁴⁰	Retrospective cohort	62 (28:34)	Methylation index (p16, HPP1, RUNX3)	Hypermethylation in P versus NP AUROC 0.75 (no CI stated)
			Methylation index (p16, HPP1, RUNX3), segment length, pathology	AUROC 0.79 (95% CI 0.6968 to 0.8853) Sensitivity 91.4 Specificity 51.8
Schulmann <i>et al</i> 2005 ³⁹	Retrospective cohort	53 (8:45)	Age, segment length, HPP1, TIMP3, APC, p16, CRBP1, RUNX3	HPP1, p16, RUNX3 independent risk factors in multivariate analyses Model combined HR index >5 leads to an increased likelihood of progression within 2 years
Wang <i>et al</i> 2009 ³⁸	Retrospective cohort	57 (7:50)	P16+APC	Hypermethylation of both APC and p16 OR 14.97 (95% CI 1.73 to ∞, P=0.012) for neoplastic progression

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; NP, non-progressing patients; OADC, oesophageal adenocarcinoma; P, progressing patients.

total, 12 of 14 studies reported patient numbers. A total of 404 patients were included. Two studies reported total lesions only (n=223). CpG promoter methylation was investigated in 13 studies and miRNA expression in one study.³⁵ Forty-two unique epigenetic markers were reported. No studies investigated histone modification. Thirteen included studies analysed 38 differentially methylated CpG promoter sites, nine used methylation-specific PCR,^{34–42} two used methylight methylation-specific PCR,^{43–44} one used bisulfite pyrosequencing⁴⁵ and one used methylation microarray techniques.⁴⁶

Prognostic models

Five studies developed models aiming to predict progression to OADC (table 2).^{35–38–41} Three models included both epigenetic markers and clinical parameters.^{39–41}

Schulmann *et al*³⁹ performed a retrospective longitudinal analysis of 53 patients contributing 106 specimens enrolled in a BO surveillance programme using Cox proportional hazards regression. Initially, 10 candidate prognostic marker genes were assessed using cross-sectional data. Six of the genes (HPP1, TIMP3, APC, p16, CRBP1 and RUNX3) which demonstrated hypermethylation in patients with OADC and relative hypomethylation in normal oesophageal tissues were investigated in the longitudinal study. All except APC were associated with progression (HGD and OADC combined) in univariate analysis. A multivariate model including all six genes, age and segment length found evidence that HPP1, p16

and RUNX3 were independently prognostic. The performance of the full model (all eight covariates) was assessed by calculating the exponentiated multiplier of baseline hazard for covariates for each specimen (HR index). For specimens taken within 2 years of progression, the HR index was >5 in progressors compared with <5 in non-progressors. The authors report that the HR index was not predictive for specimens taken over 2 years before progression. The study reports OR but doesn't explain how as the Cox model produces HRs. The authors note that the sample size is small, the study is retrospective, other potential clinical predictors were unavailable and the model uses a combined end point of HGD and OADC. In univariate analysis, TIMP3 had an OR for progression of 1.68 (95% CI 1.14 to 2.38) but this flipped to 0.5 (95% CI 0.22 to 1.04) in the multivariate analysis. The authors conclude that further validation of the markers (HPP1, p16 and RUNX3), ideally in prospective multicentre trials, is required.

Sato *et al*⁴⁰ performed a retrospective cohort study on 62 patients providing a total of 118 specimens. They developed a model using linear discriminant analysis incorporating both clinical and epigenetic markers. Sex, BO segment length and histological diagnosis were combined with p16, HPP1 and RUNX3 CpG methylation status as well as a methylation index with a score of 0–3 was considered. The best receiver operating characteristic (ROC) curve for a 4-year follow-up in this cohort was

generated using segment length, pathology and methylation index with an area under ROC (AUROC) of 0.7910 (95% CI 0.6968 to 0.8853) with a specificity and sensitivity of 91.4% and 51.8%, respectively. Change in AUROC between this model and a model including predictive markers alone was not assessed. Patient selection criteria were not reported, and the small sample size (62 patients), the large number of possible parameter combinations ($n=127$), and the cut-points for variables were chosen to optimise performance in this dataset results in a high level of uncertainty about the optimal parameter set. No external validation was performed.

Jin *et al*⁴¹ performed a double-blind multicentre case-control study of 195 tissue specimens using an eight methylation biomarker panel of p16, HPP1, RUNX3, CDH13, TAC1, NELL1, AKAP12 and SST combined with patient age to predict which patients will progress to OADC. When assessed individually, p16, HPP1 and RUNX3 were associated with progression to OADC ($P<0.05$). When the panel of all eight markers was used compared with age alone as a predictor of progression, the increment in AUROC was 0.114 in a 4-year follow-up (0.630 age alone and 0.753 age+markers), demonstrating a clinically important improvement in the predictive power of the model within the dataset. This study compared clinical factors between progressor and non-progressor groups and found no significant difference in gender, body mass index (BMI), BO segment length, smoking status or alcohol consumption. External validation was not performed.

Clément *et al*⁸⁵ performed a retrospective cohort study of 28 tissue specimens. Eighty-one per cent of patients ($n=12$) with combined hypermethylation of APC, TIMP3 and TERT progressed to OADC compared with 26% of non-progressors ($n=16$) ($P<0.0001$). It was suggested that in combination, these three markers could be used to predict which patients are at higher risk of progression. No sensitivities or specificities were reported, and there was no ROC analysis. There is no indication of independent prognostic value, and no internal or external validation was performed using this model.

Wang *et al*⁸⁸ performed a retrospective cohort study on 7 progressor and 50 non-progressor patients. They reported that hypermethylation in both p16 and APC was a strong predictor of progression to dysplastic BO or OADC. Patients who were negative for both p16 and APC hypermethylation did not progress. Hypermethylation of both APC and p16 yielded an OR of 14.97 (95% CI 1.73 to ∞ , $P=0.012$) for subsequent progression to HGD or OADC. A limitation of this study is the short follow-up time of 4.1 years for the non-progressor group which may not be sufficient time for dysplasia or neoplasia to develop. The reported CI is extremely wide, making it difficult to accurately interpret the OR.

Individual markers analysed

Forty-two individual epigenetic markers were analysed in the 14 included studies. Ten studies investigated one

or more of the following five individual markers: p16 (CDKN2A), RUNX3, TIMP3, HPP1 and APC. Details of markers, assessment methods and findings can be found in tables 3–7. All five markers demonstrated CpG hypermethylation in patients who progressed from NDBO to OADC in at least one of the included studies. p16 (a tumour suppressor protein encoded by the *CDKN2A* gene) offered the most experimental data with 10 studies reporting on 220 progressor samples compared with 332 non-progressor samples. Eight of the 10 studies demonstrated a statistically significant difference in hypermethylation at p16.^{36 38–41 43–45} Barrett *et al*³⁷ demonstrated CpG hypermethylation of CDKN2A in seven progressor samples of a specific genetic clonality; however, no control group was analysed in parallel for this specific marker in this study.

RUNX3 and HPP1 were analysed in four studies^{39–41 43} as individual markers. Overall, 134 progressors and 234 non-progressors were studied. Three studies^{39–41} demonstrated statistically significant hypermethylation in both RUNX3 and HPP1 CpG sites in progressor samples; however, one study⁴³ found no difference for either HPP1 or RUNX3.

Three studies investigated TIMP3 CpG methylation^{35 39 44} (32 progressors and 69 non-progressors). One study showed a statistically significant difference in CpG hypermethylation between progressors and non-progressors.³⁵ One study showed significant hypermethylation and calculated an OR of 1.68 (95% CI 1.14 to 2.38) for progression on univariate analysis³⁹; however, TIMP3 could not be regarded as an independent risk factor as the OR dropped to 0.5 (95% CI 0.22 to 1.04) on multivariate analysis. The final study⁴⁴ showed significantly increased TIMP3 methylation in non-dysplastic tissues of patients with associated dysplasia elsewhere in the oesophagus; however, there was no difference between progressors and non-progressors.

Three studies analysed APC^{35 38 39} (26 progressors and 116 non-progressors). Wang *et al*⁸⁸ demonstrated hypermethylation in progressors. Clément *et al*⁸⁵ reported hypermethylation but did not provide any statistical analyses. Wang *et al* calculated an OR of 9 (95% CI 1.01 to 88.52) for hypermethylation of APC in progressors. Conversely, Schulmann *et al*³⁹ found no evidence of a difference (OR=1.00 (95% CI 0.59 to 1.33)) in progression with or without hypermethylation of APC. All three studies have similar sample size, study design and experimental techniques. These contradictory findings with limited statistical validation and wide CIs call into question the validity of APC as an epigenetic marker of BO progression.

The main methodological limitations across the studies were poor reporting of patient characteristics and study populations, including patient selection. There was little information about loss to follow-up, and it was often unclear exactly which samples were used and at which time-points in longitudinal analyses. There was limited blinding of researchers to the histology, and progressor status of the

Table 3 P16/CDKN2A

Author/year	Patient no (P:NP)	Experimental technique used	Methylation threshold definition	Statistical analysis	Result
Barrett <i>et al</i> 1999 ³⁷	49 (39:6)	Methylation-specific PCR	Positive or negative PCR on agarose gel	Not stated	Seven progressor patients with specific genetic abnormality (LOH at 17p and 9p) displayed hypermethylation at CDKN2A. No numerical value offered
Boerwinkel <i>et al</i> 2014 ⁴³	44 (48:10)	Methylight methylation-specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation: p16 cut-off=0.02	Mann-Whitney test	Raw MSP values showed Hypermethylated in P P<0.05
Clément <i>et al</i> 2006 ³⁵	28* (12:16)	Methylation-specific PCR	Intensity of methylation-specific dot-blot assay compared	Not stated	0% P versus 12% NP methylated No significant difference between P and NP
Eads <i>et al</i> 2001 ⁴⁴	20 (12:8)	Methylight methylation-specific PCR	Intensity of methylated genes compared with controls. 'PMR' value of 4 used as cut-off to indicate methylated gene	Fisher's PLSD	Intensity of PCR band used for quantitative analysis Hypermethylated in P P=0.0048
Jin <i>et al</i> 2009 ⁴¹	195* (50:145)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Student's t test and chi-squared test AUROC	NMVs P:NP 0.138:0.069 AUROC=0.628 (0.534, 0.722) 90% sensitivity 90% specificity Hypermethylated in P P=0.0066
Klump <i>et al</i> 1998 ³⁶	14 (10:4)	Methylation-specific PCR	Yes/no detection of PCR product	Chi-squared test	Hypermethylated in P 8% P versus 0% NP P=0.0001
Puertas Cantera 2012 ⁴⁵	35 (6:14)	Bisulfite pyrosequencing	Quantitative CpG methylation technique	No statistical analysis	Hypermethylated in P Methylation grade 12.04% P versus 6.53% NP
Sato <i>et al</i> 2008 ⁴⁰	62 (28:34)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	LDA, LOOCV, AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index=0.0335 90% sensitivity 90% specificity P=0.00576
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P OR 1.74 P=0.0005
Wang <i>et al</i> 2009 ³⁸	57 (7:50)	Methylation-specific PCR	No threshold described	OR using univariate logistic regression	Hypermethylated in P OR 10.02 P=0.034

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; LDA, linear discriminant analysis; LOH, Loss of Heterozygosity; LOOCV, leave-one-out cross-validation; MSP, methylation specific PCR; NMV, normalised methylation value; NP, non-progressing patients; P, progressing patients; PLSD, protected least significant difference; PMR, percent methylated reference.

Table 4 RUNX3

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Boerwinkel <i>et al</i> 2014 ⁴³	44 (48:10)	Methylight methylation-specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation. RUNX3 cut-off=0.02	Mann-Whitney test	Raw MSP values compared but no numerical value offered P versus NP not significantly differentially methylated
Jin <i>et al</i> 2009 ⁴¹	195* (50:145)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Student's t test and chi-squared test AUROC	Hypermethylated in P NMV 0.104:0.063 AUROC=0.671 (0.586, 0.756) 90% sensitivity 90% specificity P=0.0002
Sato <i>et al</i> 2008 ⁴⁰	62 (28:34)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	LDA, LOOCV, AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index=0.0216 90% sensitivity 90% specificity P=0.016
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P OR=1.80 P=0.0267

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; LDA, linear discriminant analysis; LOOCV, leave-one-out cross-validation; MSP, methylation specific PCR; NMV, normalised methylation value; NP, non-progressing patients; P, progressing patients.

samples was often not described. Confounding factors were described in six of the studies,^{33 38–41 46} but only Sato *et al*⁴⁰ adjusted for BO segment length and patient sex in their predictive model. Full details of quality assessment can be found in table 8.

Given these methodological uncertainties, the findings need to be viewed with caution.

DISCUSSION

This systematic review of the literature of epigenetic markers and their role in predicting progression of BO to HGD and OADC has revealed a heterogeneous and disparate dataset. Fourteen studies were identified, with five incorporating prognostic models. This is the first systematic review to examine all evidence on epigenetic change and its role in Barrett's carcinogenesis. It suggests a role for p16 hypermethylation as an individual epigenetic biomarker in predicting progression from BO to OADC.^{35–41 43–45} However, a paucity of evidence for other epigenetic markers (and combinations), poor reporting of patient characteristics and methods employed and lack of external model validation limit the conclusions that can be drawn.

Only 14 studies were identified with small patient numbers (median 31 (5–195)). The extent of loss to follow-up was usually poorly described. All studies suffered from a paucity of clinical information and a

lack of reporting of study patient demographics. Those studies which did report comorbidities and potential confounding factors such as BMI, smoking status, age, histological diagnosis and BO segment length rarely adjusted for these in their analysis. Without adjusting for these factors, it is difficult to assess the incremental predictive clinical value of any epigenetic changes. Future models should explore the predictive ability of epigenetic changes in the context of clinical variables. In their study, Riley *et al*⁴⁷ concluded that provision of individual patient data could have overcome the majority of the reporting issues including poorly reported summary statistics, adjustment factors and outcome measures used.

It is challenging to compare data from different studies due to variable measurement of epigenetic change. Five different experimental techniques were used: miRNA analysis (n=1),³³ and CpG methylation including, methylation-specific PCR (n=9),⁴⁸ methylight methylation-specific PCR (n=2),⁴⁹ bisulfite pyrosequencing (n=1)^{45 50} and methylation microarray technology (n=1).⁴⁶ Early methods of measuring differential DNA methylation involve semiquantitative reporting of results including analysis of intensity of PCR product bands on agarose gel. More advanced techniques use computerised photometry, although differing PCR conditions and equipment may result in variability

Table 5 HPP1

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Boerwinkel <i>et al</i> 2014 ⁴³	44 (48:10)	Methylight methylation-specific PCR	Previously published cut-off values applied to raw MSP data to calculate frequency of hypermethylation. HPP1 cut-off=0.05	Mann-Whitney test	P versus NP not significantly differentially methylated
Jin <i>et al</i> 2009 ⁴¹	195* (50:145)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Student's t test and chi-squared test AUROC	Hypermethylated in P AUROC=0.647 (0.556, 0.739) P=0.0025
Sato <i>et al</i> 2008 ⁴⁰	62 (28:34)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	LDA, LOOCV, AUROC	Hypermethylated in P AUROC increment when added to segment length, histology and global methylation index 0.028 90% sensitivity 90% specificity P=0.018
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P OR=1.77 P=0.0311

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). AUROC, area under receiver operating characteristic; LDA, linear discriminant analysis; LOOCV, leave-one-out cross-validation; MSP, Methylation Specific PCR; NP, non-progressing patients; P, progressing patients.

between laboratories. More advanced techniques such as bisulfite pyrosequencing and methylation microarrays can provide an accurate methylation percentage at individual CpG sites which are suitable for quantitative analysis. The 12 studies using methylation-specific PCR revealed a global pattern of hypermethylation of progressor patients in the CpG regions of interest³⁴⁻⁴⁴;

however, each used differing methylation thresholds and outcome measures for reporting positive results. One study⁴⁵ used bisulfite pyrosequencing to analyse p16, but no further markers were analysed on more than one platform. The different methods used, as well as the different outcome metrics reported and lack of clearly reported clinical information, meant that there

Table 6 TIMP3

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Clément <i>et al</i> 2006 ³⁵	28* (12:16)	Methylation-specific PCR	Intensity of methylation-specific dot-blot assay compared	Not stated	Hypermethylated in P % samples methylated P=91%: NP=23% P<0.0001
Eads <i>et al</i> 2001 ⁴⁴	20 (12:8)	Methylight methylation-specific PCR	Intensity of methylated genes compared with controls. 'PMR' value of 4 used as cut-off to indicate methylated gene	Fisher's PLSD	Hypermethylated in non-dysplastic tissue in patients with associated dysplasia; however, not statistically significant when comparing P versus NP P=0.13
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylated in P, but not independent risk factor in multivariate analysis OR=1.68 univariate OR=0.50 multivariate P=0.0109

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). NP, non-progressing patients; P, progressing patients; PLSD, protected least significant difference; PMR, percent methylated reference.

Table 7 APC

Author/year	Patient no (P:NP)	Methods	Methylation threshold definition	Statistical analysis	Result
Clément <i>et al</i> 2006 ³⁵	28* (12:16)	Methylation-specific PCR	Intensity of methylation-specific dot-blot assay compared	No statistical analysis offered	Hypermethylated in P methylation: P=100% versus NP=36%
Schulmann <i>et al</i> 2005 ³⁹	53 (8:45)	Methylation-specific PCR	NMV=amount of methylated DNA compared with control beta-actin DNA generated by PCR	Cox proportional HRs	Hypermethylation not an independent risk factor for P OR=1.00 (95% CI 0.59 to 1.33) P=0.99
Wang <i>et al</i> 2009 ³⁸	57 (7:50)	Methylation-specific PCR	No threshold described	OR using univariate logistic regression	Hypermethylated in P methylation: P=86% versus NP=40% P=0.02 OR=9 (1.01–80.52) P=0.049

*Number of lesions, no patient numbers described in the study; number of progressor lesions (P), number of non-progressor lesions (NP). NP, non-progressing patients; P, progressing patients.

was insufficient clinical and methodological homogeneity. Meta-analysis was, thus, not appropriate, despite individual epigenetic markers being reported in up to 10 independent studies.

Five models looked at various combinations of epigenetic markers and clinical risk factors. Findings from four of the models suggest a potential role for p16 in combination with HPP1, RUNX3 and/or APC and clinical factors of segment length or age. All models used slightly different combinations of model parameters and reported analyses differently, thus making it difficult to compare findings between models. All suffered from a lack of external validation, and there were uncertainties around the representativeness of the included populations. More research is needed to validate and further explore these initial findings.

P16, a tumour suppressor protein, is the most consistently hypermethylated CpG site in progressors. Hypermethylation leads to reduced p16 production, allowing neoplastic cells to progress from G1 to S phase of cell cycle and replicate uncontrollably. p16 deletions are observed in melanoma, oesophageal, lung, pancreatic, mesothelioma, bladder, head and neck squamous cell carcinoma, breast, lymphocyte, brain, ovarian, osteosarcoma and renal cancer cell lines.^{51–52} In BO, p16 genetic mutations have been shown to occur early in tumorigenesis and may appear before dysplasia has occurred.⁵³ Timmer *et al*⁵⁴ demonstrated the presence of p16, MYC and aneusomy in a model with age and segment length determined whether patients were at low or high risk of progression from NDBO to OADC. Without further in-depth temporal analysis, it remains unclear whether the index abnormality of Barrett's carcinogenesis is epigenetic or genetic in origin.

The pattern of hypermethylation in RUNX3 and HPP1 in progressors was less clear with not all studies demonstrating a statistically significant difference between progressor and non-progressor patients. TIMP3 and APC provide variable results in the literature, and much

smaller patient groups are analysed. Agarwal *et al* reported a predominance of hypomethylation in progressors (16 of 19 CpG sites) with only three CpG sites (Pro_MMD2, Pro_ZNF358 and Intra_F10) hypermethylated. The methylation microarray used in this study can analyse 28 700 unique CpG sites, whereas the latest technology offers up to 850 000 sites on a single chip.⁵⁵ With only a single study using an outdated technique, it is not possible to say whether CpG hypomethylation is a driver in progressive BO. Prior to this technology being available, researchers had to carefully select CpG sites of interest for analysis; selection was based on previous research and literature review giving rise to a positive selection and publication bias. This is a well-recognised phenomenon in prognostic factor research. Sekula *et al*⁵⁶ reviewed published and unpublished work into p53's role in bladder cancer and discovered that 31% of observational studies were unpublished in a 15-year period. While Sekula's review is concerned with a different pathology, the type of included studies is of a similar design to those included in this review. A combination of positive publication bias and marker selection bias raises questions as to the validity of the selected markers in studies demonstrating CpG hypermethylation. Selective reporting may also erroneously inflate the importance of individual prognostic markers. Kyzas *et al*⁵⁷ analysed published and unpublished data sought directly from researchers investigating the importance of TP53's role in head and neck squamous cell carcinoma on patient mortality. They found that if all published and unpublished data were included in their meta-analysis, the risk ratio decreased from 1.38 to 1.16 (95% CI 0.99 to 1.35 P=0.06). They also had difficulty conducting their analysis due to non-standardised definitions and reporting. Heterogeneity in definitions and reporting was also identified within our dataset, and it must also be considered whether selective reporting of results is over stating the importance of individual markers such as p16 in the progression of BO. Similarly, if ever increasing numbers of CpG sites

Table 8 Quality assessment for prognostic factor studies including prognostic models

Study	Representative patient sample?	Loss to follow-up equal in both arms?	Study conducted prospectively (PRO) or retrospectively (RET)?	Samples collected prospectively (PRO) or retrospectively (RET)?	Sampling method: quadrant biopsy used?	Validated epigenetic technique used?	Epigenetic analysis undertaken blindly? (without knowledge of histology)	Epigenetic analysis undertaken in duplicate?	Was the threshold defined?	Histology undertaken blindly? (without knowledge of epigenetic age, smoking, alcohol)	Were confounding factors adjusted for in design or analysis?	Which confounding factors were adjusted for?
Agarwal <i>et al</i> 2012 ⁴⁶	Unclear	Unclear	PRO	RET	No	Unclear	Unclear	Unclear	Yes	No	No	N/A
Barrett <i>et al</i> 1999 ⁴⁷	Yes	Unclear	PRO	RET	No	Yes	No	Unclear	Unclear	Unclear	No	N/A
Boerwinkel <i>et al</i> 2014 ⁴⁸	Yes	Unclear	PRO	RET	Yes	Yes	Unclear	Unclear	Yes	Yes	No	N/A
Clément <i>et al</i> 2006 ⁴⁹	Unclear	Unclear	PRO	RET	No	Yes	No	Unclear	Unclear	Unclear	No	N/A
Clément <i>et al</i> 2008 ³⁴	Unclear	Unclear	PRO	RET	No	Yes	No	Unclear	Unclear	Unclear	No	N/A
Eads <i>et al</i> 2001 ⁴⁴	Unclear	Unclear	PRO	RET	No	Yes	Unclear	Unclear	Yes	Unclear	No	N/A
Jin <i>et al</i> 2009 ⁴¹	Unclear	Unclear	RET	PRO	Yes	Yes	No	Unclear	Yes	Yes	No	N/A
Klump <i>et al</i> 1998 ⁴⁶	Unclear	Yes	RET	RET	No	Yes	No	Yes	Yes	No	No	N/A
Moinova <i>et al</i> 2012 ⁴²	Unclear	Unclear	Unclear	Unclear	No	Yes	No	Unclear	Yes	Unclear	No	N/A
Revilla-Núñez <i>et al</i> 2013 ⁴³	Yes	Unclear	PRO	Unclear	Yes	Yes	No	Unclear	Yes	Yes	Unclear	N/A
Puertas Canteria 2012 ⁴⁵	Unclear	Unclear	Unclear	Unclear	No details	Yes	No	Unclear	Unclear	No	No	N/A
Sato <i>et al</i> 2008 ⁴⁰	Unclear	Unclear	Unclear	Unclear	Yes	Yes	No	Unclear	Yes	Yes	Yes	BO segment length
Schulmann <i>et al</i> 2003 ³⁹	Unclear	Unclear	RET	Unclear	Yes	Yes	No	Unclear	Yes	Yes	No	N/A
Wang <i>et al</i> 2005 ³⁸	Yes	Unclear	PRO	RET	Yes	Yes	No	Unclear	Yes	Yes	No	N/A

BO, Barrett's oesophagus; FFPE, formalin-fixed paraffin-embedded.

are analysed with microarray technology, there will be an increasing number of negative results which are likely to be under-reported in the literature. As illustrated by Kyzas *et al* and Sekula *et al*,^{56 57} despite a large volume of literature, without standardisation of reporting and outcome measures, the research is often not suitable for translation into clinical use.

The variable quality of prognostic studies has been previously reported in systematic reviews^{58 59} and improvements suggested for collaboration and standardisation especially with regard to statistical analysis and outcome reporting.^{60–62} Poor quality of reporting was also a feature in this systematic review. For example, only 7 of 14 included studies documented both the number of patients and samples analysed in each group. Standardised reporting of epigenetic studies in BO should provide basic clinical details for each patient including age, sex, smoking status, BMI and previous OC diagnosis and treatment. Each patient used in the study should have temporal information regarding OGD and biopsies taken, the number of biopsies, BO length, BO islands, hiatus hernia, visible lesions and classification of visible lesions documented according to the British Society of Gastroenterology (BSG) minimum endoscopic reporting dataset.¹ In resection specimens, full histological reports should be available or at minimum staging tumour stage, nodal involvement, metastasis (TNM). It should be reported which patient samples have been used to perform epigenetic analysis and which methods of laboratory analysis have been used. Clear reporting of statistical analysis between cohorts must be provided to allow future meta-analysis of studies. Improved standardisation would make meta-analyses more feasible and lead to more informative systematic review results. This in turn would aid the translation of laboratory work into clinical trials.

CONCLUSION

The evidence from this systematic review is suggestive of a role for p16 as an individual epigenetic biomarker in predicting progression from BO to OADC. Prognostic models incorporating this and other markers also suggest a role for p16 in combination with HPP1, RUNX3 and/or clinical markers. Further large primary studies using current epigenetic techniques and standardised reporting are required to inform future models to further explore the role of epigenetics in progression to HGD and OADC.

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