The Clinical and Molecular Genetic Investigation of Genetic Conditions Predisposing to Kidney Cancers

by

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ABSTRACT

Beckwith Wiedemann syndrome (BWS) and Birt-Hogg-Dube syndrome (BHD) are two examples of genetic conditions that are associated with an increased risk of renal neoplasia (Wilms tumour and renal cell carcinoma (RCC) respectively). BWS is a model imprinting disorder characterised by overgrowth, developmental defects, predisposition to embryonal tumours and results from disordered expression of imprinted genes on chromosome 11p15.5. There is an association between the use of assisted reproductive technologies (ART) and BWS. BHD is an autosomal dominantly inherited condition characterised by cutaneous fibrofolliculomas, lung cysts predisposing to spontaneous pneumothorax and an increased lifetime risk of RCC and is caused by germline mutations in the *FLCN* gene. Both BWS and BHD show phenotypic variation in their manifestation. The clinical and molecular genetic investigations described in this thesis aimed to uncover factors influencing variation in phenotypic expression in these two conditions. Conclusions: Phenotypic variation in BWS can result from locus heterogeneity, epigenetic and environmental modifiers. Phenotypic variation in BHD may reflect allelic heterogeneity and the presence of genetic modifiers.

DEDICATION

I would like to dedicate this work to my family: My wife Pek Keng and son Brandon who are my daily rays of sunshine and hope; and my parents and siblings for their continuous support throughout life.

This work is also dedicated to all who work for the National Health Service (NHS). At times of need by my family and me, the NHS was there to provide exceptional care and treatment. Long may it continue to be a publicly funded health care system that provides medical care to all who require it.

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LIST OF ABBREVIATIONS

ART	Assisted reproductive technologies
AS	Angelman syndrome
BHD	Birt-Hogg-Dube syndrome
BWS	Beckwith Wiedemann syndrome
CCTF	CCCTC-binding factor
cDNA	Complementary DNA
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CRC	Colorectal cancer
CRUK	Cancer Research UK
DDS	Denys-Drash syndrome
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
FcRCC	Familial/inherited non-syndromic clear cell renal cell carcinoma
FF	Fibrofolliculomas
FLCN	Folliculin
HIF	Hypoxia-induced factor
HIL	Hypomethylation of multiple imprinted loci
HGVS	Human Genome Variation Society
IC	Imprinting centre

ICSI	Intra-cytoplasmic sperm injection
IVF	*In vitro* fertilisation
LOM	Loss of methylation
LOVD	Leiden Open Variation Database
LSDB	Locus specific database
MBP	Methyl-CpG-binding proteins
MINAS	Multiple inherited neoplasia allele syndrome
MLID	Multi locus imprinting disturbance
MLPA	Multiplex ligation-dependant probe amplification
MS-MLPA	Methylation-specific multiplex ligation-dependant probe amplification
MS-PCR	Methylation specific polymerase chain reaction
mTOR	Mammalian target of rapamycin
PCR	Polymerase chain reaction
PTX	Pneumothorax
PWS	Prader-Willi syndrome
PSP	Primary spontaneous pneumothorax
RCC	Renal cell carcinoma
REC	Reseach ethics committee
RNA	Ribonucleic acid
TNDM	Transient neonatal diabetes mellitus
UPD	Uniparental disomy
VHL	von Hippel-Lindau disease
WAGR	Wilms tumour-Aniridia-Genital anomalies-Retardation syndrome
WT	Wilms tumour

CHAPTER 1. GENERAL INTRODUCTION

For the purpose of this thesis I will give a brief general introduction in this section on the epidemiology of renal cancer followed by an introduction to renal cell carcinomas and Wilms tumour, the two renal tumour types that are associated with Birt-Hogg-Dube syndrome and Beckwith-Wiedemann syndrome respectively. As I am using the two conditions to investigate factors that affect variability of phenotypic expression, I will present my investigation of the conditions in separate chapters for each syndrome. Therefore for each chapter, I will give a detailed introduction to the syndrome, the sub-aims of the chapter, the material and methods of investigation, the results and discussion. At the end of the thesis, I will present the overall conclusions and future directions with this research.

1.1 RENAL CANCER

1.1.1 EPIDEMIOLOGY OF RENAL CANCER

In the UK, renal cancer is now the 8th commonest cancer in men and the 9th commonest in women(Cancer Research UK, http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/kidney-cancer, Accessed May 2016). Renal cancer affecting adults includes malignant tumours arising from the renal parenchyma and renal pelvis. The predominant renal cancer arises from the renal parenchyma and is mainly adenocarcinoma or better known as renal cell carcinoma. Renal cell carcinoma (RCC) accounts for around 80% of all renal cancers in the UK. Renal pelvis cancers are mostly

transitional cell cancers. It is rare for people under the age of 40 years to develop renal cancer. However, Wilms tumour (nephroblastoma), an embryonal malignant tumour usually occurs in children (Curado *et al.*, 2007).

1.1.2 AETIOLOGY OF RENAL CANCER

Risk factors for the development of renal cancer include

- Smoking
- Obesity
- Hypertension
- Genetic predisposition or Family history
- Predisposing renal disease that requires dialysis
- Hepatitis C infection
- Previous treatment for testicular or cervical cancer

There is evidence that cigarette smoking increases the risk of renal cell carcinoma by 50% and may account for about one third of cases of renal cancer (Chow *et al.*, 2010). The risk increases in a dose-dependent manner with the amount of cigarette smoked. Cessation of smoking reduces this risk only in quitters of ten years or more (Hunt *et al.*, 2005). Cigarette smoking is postulated to increase the risk of renal cancer by chronic tissue hypoxia as a result of carbon monoxide exposure and smoking related disease such as chronic obstructive pulmonary disease (COPD) (Sharifi *et al.*, 2006). In addition, there are higher levels of DNA damage in the peripheral blood lymphocytes due to tobacco-specific N-nitrosamine in renal cell cancer patients compared to normal controls (Clague *et al.*, 2009).

Obesity is another risk factor for the development of renal cancers with a linear relationship with increasing body weight. This effect is seen more prominently in women (Renehan *et al.*, 2008). Although several mechanisms to explain how obesity is associated with this increased risk have been postulated, direct evidence in humans is lacking. These mechanisms include insulin resistance and compensatory hyperinsulinaemia, tissue hypoxia, hormonal imbalance, obesity related inflammatory response and oxidative stress (Klinghoffer *et al.*, 2009).

Other risk factors that are associated with an increased risk of renal cancers include hypertension, pre-existing renal disease, previous chemotherapy for treatment for other cancers, and other medications such as phenacetin containing analgesics (Chow *et al.*, 2010). I will be discussing the genetic factors that can increase the risk of renal cancers in relation to renal cell carcinomas and Wilms tumour below.

1.2 RENAL CELL CARCINOMA

1.2.1 EPIDEMIOLOGY OF RENAL CELL CARCINOMA

RCCs are heterogeneous tumours of the renal tubular epithelium. The highest incidence of RCC occurs between the ages of 50 and 70 years.

1.2.2 SUB CLASSIFICATION OF RENAL CELL CARCINOMA

RCC can be subclassified depending on their location within the nephron and the cell types from which the tumour originates.

Clear cell RCC and papillary RCC originate from the epithelial cells of the proximal tubules whereas chromophobe and collecting duct tumours originate from the collecting duct (Thoenes *et al.*, 1986).

Histologically, there are 5 major subtypes of RCC: Clear cell (conventional) RCC (accounts for 70-80%), papillary (chromophile) (10-15%), chromophobe (3-5%), collecting duct (1%) and unclassified (1%).

The Heidelberg classification subdivides renal cell tumours into benign or malignant tumours and where possible, each category is linked to the most commonly documented genetic abnormalities (Kovacs *et al.*, 1997). This is represented schematically in Figure 1-1.

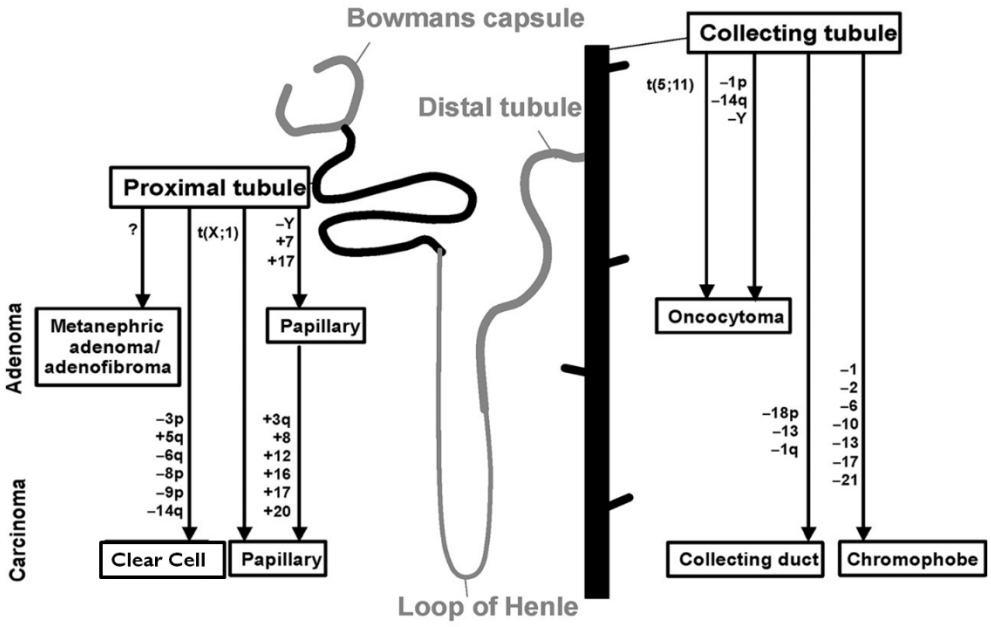


Figure 1-1 Schematic representation of the different subtypes of RCC according to the 'Heidelberg' classification in relation to their positions within the nephron and collecting tubule of the kidney (Bodmer *et al.*, 2002) by permission of Oxford University Press

1.2.3 INHERITED FORMS OF RCC

Characteristic features of inherited forms of RCC include a younger age of diagnosis, tumours occurring in both kidneys (bilateral) or multiple tumours in the kidneys at the same time (multifocal). Inherited forms of RCC account for about 3-4% of all RCC. The majority of the inherited RCC cancer predisposition syndromes are inherited in an autosomal dominant manner. The histology of the tumour can often provide clues into the likely inherited cause. Table 1 summarises the main RCC predisposing genetic conditions.

The studies of inherited RCC predisposition syndromes have provided important insights into the molecular basis of tumourigenesis in renal cancer. An example of this is the study of von Hippel-Lindau disease (VHL) which is the commonest inherited renal cancer predisposition syndrome. The *VHL* gene was identified in 1993 and researchers were then able to study the VHL protein that functions as a tumour suppressor and its involvement in the regulation of hypoxia-response genes in the HIF (hypoxia-induced factor) pathway that affects proliferation and angiogenesis (Kondo *et al.*, 2002; Latif *et al.*, 1993; Maxwell *et al.*, 1999). The finding that sporadic clear cell RCC also frequently showed acquired somatic mutations in *VHL* meant that the understanding of the pathways involved in rare cancer predisposition syndromes have implications on more common sporadic tumours (Maher, 2004). An example is the development of novel drugs to target the downstream targets of the HIF pathway now in use for the treatment of metastatic RCC (Motzer *et al.*, 2006).

Table 1. Inherited disorders predisposing to RCC

Disorder	MIM	Type of RCC	Associated tumours and other features	Gene and location
von Hippel-Lindau Disease	193300	Clear cell	Retinal and CNS haemangioblastoma, phaeochromocytoma, pancreatic tumour, visceral cysts	*VHL* 3p25
Birt-Hogg-Dube syndrome	135150	variable	Fibrofolliculomas, lung cysts and pneumothorax and colorectal polyps	*FLCN* 17p11.2
Familial non-syndromic clear cell RCC	144700	Clear cell	-	?
Hereditary papillary renal cancer	605074	Papillary type 1	-	*MET* 7q31
Hereditary leiomyomatosis and renal cancer	605839	Papillary type 2	Cutaneous and uterine leiomyomas, leomyosarcoma	*FH* 1q25-32
Succinate dehydrogenase subunit mutations		variable	Extra-adrenal and adrenal phaeochromocytoma, head and neck paraganglioma	*SDHB* 1p36-p35 *SDHD* 11q23
Hyperparthyroidism-jaw tumour	145001	Papillary RCC, renal hamartoma, Wilms tumour	Parathyroid tumours, fibro-osseus mandibular and maxillary tumours , renal cysts	*CDC73*/*HRPT2* 1q25
Chromosome 3 translocation		Clear cell	-	Various
Lynch syndrome		Transitional Cell carcinomas of the renal pelvis and ureter	Colorectal cancer, endometrial cancer	*MSH2* 2p22-p21 *MLH1* 3p21.3 *PMS22* 7p22.2 *PMS1* 2q31.1 *MSH6* 2p16

Reproduced from (Maher, 2010) with permission from Karger AG, Basel.

1.3 WILMS TUMOUR

1.3.1 EPIDEMIOLOGY OF WILMS TUMOUR

Wilms tumour or nephroblastoma is the most common renal tumour of childhood and accounts for 6.3% of malignancies in children. Wilms tumour affects approximately 1 in 8000-10000 children. It is named eponymously after Dr. Max Wilms who first described it. Around 5-10% of children who develop Wilms tumor develop bilateral or multifocal tumours. Although the prevalence of bilateral Wilms tumour is higher in children with a Wilms tumour genetic predisposition syndrome, around 85% of children with Beckwith-Wiedemann syndrome (BWS) or Wilms tumour-Aniridia-Genital anomalies-Retardation (WAGR) syndrome have only unilateral tumours (Huff, 1998; Porteus *et al.*, 2000).

The average age of presentation in children with unilateral tumour is 42-47 months and for bilateral tumours, the average age is 30-33 months (Breslow *et al.*, 1993). The male to female ratio is 0.92 to 1.0 with a slightly increased risk in girls. Occasionally, Wilms tumour can also occur in adulthood.

1.3.2 AETIOLOGY OF WILMS TUMOUR

Wilms tumour is an embryonal malignancy that consists of blastermal, epithelial or stromal cells. Wilms tumour is thought to develop due to genetic mutations that predispose to nephrogenic rests. Nephrogenic rests are benign foci of embryonal renal cells that persist abnormally into postnatal life. They are found in around 1% of newborn kidneys and should regress or differentiate by early childhood (Beckwith *et al.*, 1990). The rests are considered to be precursors to Wilms tumours and those that accumulate additional mutations

transform into a Wilms tumour (Dome *et al.*, 2002; Gylys-Morin *et al.*, 1993). The presence of multiple nephrogenic rests in the kidneys is termed nephroblastomatosis. It may present as a diffuse overgrowth of rests, forming a rim that enlarges the size of the kidney, or as multiple distinct rests (Perlman *et al.*, 2006).

Nephrogenic rests can be classified into intralobar or perilobar rests. Intralobar rests are usually solitary and randomly distributed throughout the kidney. However, they are usually located centrally in the renal lobe. Perilobar rests are located at the periphery of the kidney and are often multiple. WAGR and Denys-Drash syndrome are usually associated with intralobar rests, and Beckwith-Wiedemann syndrome is usually associated with perilobar rests (Breslow *et al.*, 2006). However, this association is not absolute.

1.3.3 FAMILIAL WILMS TUMOUR

A hereditary cause for Wilms tumour is thought to account for 10-15% of individuals who develop Wilms tumour. This hereditary cause can be syndromic or non-syndromic.

1.3.3.1 Syndromic predisposition to Wilms Tumour

Syndromic predisposition to Wilms tumour can generally be subdivided according to syndromes that involve the Wilms tumour gene *WT1* or involve the chromosome 11p15.5 imprinted locus. Table 2 summarises the features of the common syndromic causes of Wilms tumour.

Table 2. Syndromic causes of Wilms tumour

Syndrome	MIM	Associated features	Gene/Chromosome locus
WAGR	194072	Aniridia, Genital anomalies, mental retardation	11p13 deletion involving *WT1* & *PAX6*
Denys-Drash syndrome (DDS)	194080	Male genital anomalies, microscopic renal anomalies	*WT1* mutations
Frasier syndrome	136680	Male genital anomalies, microscopic renal anomalies, gonadoblastoma	*WT1* intron 9 splice site mutation
Beckwith-Wiedemann syndrome (BWS)	130650	Overgrowth, macroglossia, anterior abdominal wall defects hemihypertrophy, embryonal tumours	Chromosome 11p15.5 abnormalities due to copy number changes, mutations or loss of imprinting

1.3.3.2 Familial Non-syndromic Wilms Tumour

1-2% of individuals who develop Wilms tumour have a family history of another affected relative, usually a sibling, parent, aunt, uncle or close cousin (Breslow *et al.*, 1996).

1.3.4 Sporadic Wilms Tumour

Somatic mutations can also be found in sporadic cases of Wilms tumour. *WT1* mutations have been identified in 10-20% (of which >70% are somatic) of individuals who develop Wilms tumour without genitourinary anomalies or DDS (Dome *et al.*, 1993).

Somatic mutations involving *WTX1* (20-30% of tumours), *CTNNB1* (15%), *TP53* (5%) and *FBXW7* (4%) have been reported (Dome *et al.*, 1993).

Somatic defects affecting the BWS critical region at 11p15.5 have been discovered in Wilms tumour tissue and in some cases in the surrounding normal renal tissue (Ogawa *et al.*, 1993; Okamoto *et al.*, 1997). Subsequently, Scott and colleagues studied the genomic DNA from lymphoctyes of 437 cases of non-syndromic Wilms tumour patients and detected constitutional 11p15.5 defects in 3% of cases (and 12% of bilateral cases) (Scott *et al.*, 2008a).

1.4 AIMS OF THE THESIS

The aim of this thesis is to explain the range of phenotypic variation in the kidney cancer predisposition disorders and to define potential candidates of this variability.

Beckwith-Wiedemann syndrome is a disorder of genomic imprinting resulting in overgrowth, malformations and an increased risk of embryonal tumours including Wilms tumour. Birt-Hogg-Dube syndrome is an autosomal dominant disorder which results in a predisposition to benign skin tumours, lung cysts with or without spontaneous pneumothorax and an increased lifetime risk of renal cell carcinoma. Both conditions show variability in their clinical manifestations.

Therefore these two familial kidney cancer predisposition syndromes will serve as ideal models to explore the genetic and epigenetic factors that might influence phenotypic expression.

The phenotypic variability studied in this thesis is not just limited to kidney cancer but covered the whole spectrum of clinical features associated with BWS and BHD. Although the particular subgroup of BWS caused by imprinting centre 2 (IC2) epimutations have a

relatively lower risk of Wilms tumour than some other subgroups, renal tumours can occur and it was of interest to investigate whether there were relationships between both this and other aspects of BWS phenotypic variability with factors such as assisted reproductive technologies (ART) and the emerging entity of Muti-locus imprinting disturbance (MLID) in BWS.

CHAPTER 2. THE CLINICAL, MOLECULAR AND EPIGENETIC INVESTIGATION OF BECKWITH-WIEDEMANN SYNDROME

DECLARATION:

The results of this BWS study have been published in the journal Human Reproduction (Lim *et al.*, 2009a). Sections of the introduction to this chapter have been published as review articles in Epigenomics (Lim *et al.*, 2009b) and The Obstetrician & Gynaecologist (Lim *et al.*, 2010b).

2.1 INTRODUCTION

2.1.1 GENOMIC IMPRINTING

Genomic imprinting is a form of epigenetic regulation of gene expression resulting in preferential expression of one allele of a gene according to the parent-of-origin of the allele. Imprinting can be incomplete and/or tissue or stage-specific, but when imprinting is complete, only one allele is expressed. Some imprinted genes are expressed from the paternally inherited allele and others from the maternally inherited allele (Reik *et al.*, 2001).

2.1.1.1 The Importance of Biparental Contribution To the Mammalian Genome

The maternal and paternal contribution to the mammalian embryonic genomes are not equivalent. This was first demonstrated in experimental nuclear transfer in mouse embryos, which indicated that both a paternal and maternal contribution is required for complete embryogenesis. Diploid gynogenetic (only maternal contribution) and androgenetic (only

paternal contribution) embryos cannot develop normally. (McGrath *et al.*, 1984; Surani *et al.*, 1984)

The importance of this biparental contribution to the human genome was demonstrated by abnormal pregnancies resulting in hydatidiform moles and ovarian teratomas. Hydatidiform moles can result from a conception lacking maternal contribution to the genome (androgenetic conceptus) (Kajii *et al.*, 1977; Ohama *et al.*, 1981). On the other hand, ovarian teratomas can occur as a result of a spontaneous activation of an ovarian oocyte, resulting in a duplication of the maternal genome (gynogenetic conceptus)(Ohama *et al.*, 1985).

Subsequently, it was demonstrated that the requirement for biparental inheritance was restricted to specific regions of the genomes. This was done by experiments with mice carrying balanced translocations that generated mouse embryos containing small genomic regions that were derived only from the mother or father (uniparental disomies)(Cattanach *et al.*, 1985). These specific regions of the genomes were then found to harbour imprinted genes and the first imprinted genes were reported in 1991 (Barlow *et al.*, 1991).

There are currently approximately 100 imprinted genes identified in the human and/or mouse genomes (www.mgh.har.mrc.ac.uk/imprinting.impstables.html). Most imprinted genes reside in clusters and this clustering enables co-ordinated regulation of their expression by imprinting control elements or imprinting centres (IC) (Reik *et al.*, 2001). These clusters are rich in cytosine-phosphoguanine dinucleotide sequences (CpG) islands (Neumann *et al.*, 1995).

Genomic imprinting in mammals has also been shown to prevent parthenogenetic development in mice. Parthenogenesis refers to a form of asexual reproduction where growth and development of the embryo occurs without fertilisation by a male. Parthenogenetic mice embryos usually die by 10 days of gestation. The successful survival to adulthood in parthenogenetic mice from two maternal genomes following the deletion of an imprinted cluster in mice oocyte resulting in expression of paternally expressed genes provides further proof of the importance of biparental contribution in normal development (Kono *et al.*, 2004).

2.1.2 MECHANISMS INVOLVED IN GENOMIC IMPRINTING

The establishment and maintenance of genomic imprinting are regulated by a number of mechanisms including DNA methylation, chromatin modification and expression of large noncoding RNAs. DNA methylation plays a crucial role in the establishment and maintenance of genomic imprinting (Jaenisch *et al.*, 2003). The covalent addition of a methyl group to the cytosine in the unmethylated CpG dinulceotide (methylation of CpG dinucleotides) can affect the expression of genes in a number of ways.

1.) Methylated CpGs in the promoter region of a gene can result in gene silencing by preventing the binding of transcription factors (Figure 2-1).

2.) Methylation of CpGs can also prevent the binding of insulator proteins such as CTCF to methylated differentially methylated regions (DMRs) (see later).

3.) DNA methylation can also induce changes in chromatin structure by attracting methyl-CpG-binding proteins (MBPs) that bind to methylated CpGs and attract proteins such as

chromatin remodelling proteins and histone deacetylases (HDACs) resulting in modification of histones to produce a more compact structure (heterochromatin), which prevents gene transcription (Dennis, 2003).

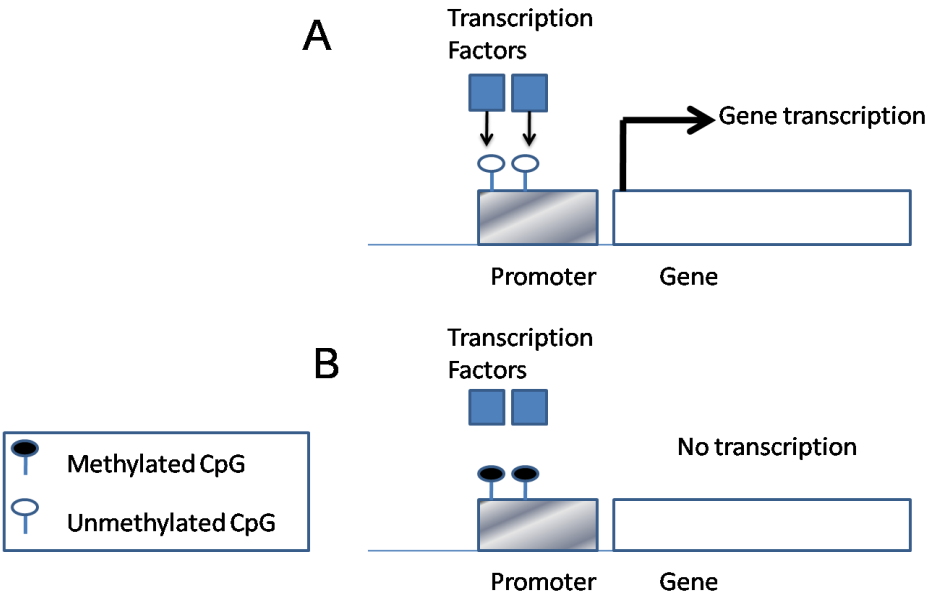


Figure 2-1 DNA methylation regulates gene expression.

(A) The CpG island promoter is unmethylated and allows binding of transcription factors, which is required for transcription initiation. (B) The CpG Island promoter methylation prevents binding of transcription factors and results in gene silencing. (Lim *et al.*, 2010b) by permission of John Wiley and Sons

The epigenetic marking of the human genome by DNA methylation is heritable (from one cell to another during cell division), stable and allows a form of "cellular memory". The process of DNA methylation is catalysed by the DNA methyltransferases. DNMT3a and DNMT3b are involved in the establishment of methlyation and DNMT1 is involved in the maintenance of methylation. This allows control of expression of developmental genes at specific times of embryonic development in specific tissues and also ensures maintenance of

the correct methylation marking of imprinted genes (Bestor *et al.*, 1983; Okano *et al.*, 1999; Pradhan *et al.*, 1999).

CpG islands are a common occurrence in imprinted gene domains. At imprinting centre associated differentially methylated regions (DMRs), the methylation status of the CpG islands differs according to the parent of origin (i.e., one parental allele is methylated and the opposite parental allele is unmethylated) (Reik *et al.*, 2001). A key feature of parent-specific imprinting marks in DMRs is that they are erased and then reset (established) in the primordial germ cells during gametogenesis to reflect the sex of the parent for the next generation (Lee *et al.*, 2002; Szabo *et al.*, 2002).

DMRs mark imprinting control centres which can act as insulator elements regulating the expression of imprinted genes. One example of this is at the 11p15.5 imprinted region involving the *IGF2/H19* locus. The *H19* DMR maps between *H19* and *IGF2* and contains binding sites for a zinc finger protein, CCCTC-binding factor (CTCF). On the maternal allele the CTCF protein binds to the unmethylated *H19* DMR and insulates the *IGF2* promoter region from an enhancer downstream of *H19*. This causes the enhancer to preferentially interact with the *H19* promoter and the untranslated *H19* RNA is expressed from the maternal allele whereas *IGF2* expression is silenced. In contrast the *H19* DMR is methylated on the paternal allele and CTCF is unable to bind. This allows the downstream enhancer to preferentially interact with the *IGF2* promoter and *IGF2*, but not *H19*, is expressed from the paternal allele (Bell *et al.*, 2000).

Imprinted noncoding RNAs (e.g. *H19*, *KCNQ1OT1*) are also a frequent feature of imprinted gene clusters and are often in close proximity to oppositely imprinted protein coding genes. For examples, *H19* and *IGF2*, *KCNQ1O1T* and *CDKN1C*, and *Igf2r* and *Airn*. The mechanisms by which how these noncoding RNAs affect imprinting establishment and maintenance is currently an area of active research. In addition, the role of chromatin modifications in the establishment and maintenance of imprinting is another area of active research (Kacem *et al.*, 2009).

2.1.3 MECHANISMS LEADING TO DISORDERS OF IMPRINTING

Mutations or epimutations (pathogenic alterations in DNA methylation or chromatin structure without a change in DNA sequence) affecting the function of the expressed imprinted gene allele (but not the silenced allele) can result in an imprinting disorder. In addition, loss of imprinting (i.e. both alleles are expressed) can also cause disease. Common mechanisms that give rise to an imprinting disorder include (a) uniparental disomy (upd), (b) intragenic mutations or copy number alterations that directly alter the function of an imprinted gene and (c) mutations or epimutations in imprinting control centres that result in altered imprinting/expression of imprinted gene(s) (Table 3). In imprinting disorders such as Beckwith-Wiedemann syndrome and Silver-Russell syndrome (Abu-Amero *et al.*, 2008; Cooper *et al.*, 2005) altered gene expression from aberrant loss or gain of methylation at an imprinting centre DMR is the most frequent cause of disease. These epimutations result from a primary alteration in a DMR (*in cis*), but in rare cases may be secondary to alterations in remote (*in trans*) factors (Reik *et al.*, 2001).

Table 3. Examples of mechanisms leading to imprinting disorders

Mechanism	Definition/explanation
Uniparental disomy	Both chromosomes in a cell are derived from a single parent (and there is no chromosomal material from the other parent). Uniparental disomy may affect the whole chromosome (complete) or part of a chromosome. If the chromosomal region contains an imprinted gene then uniparental disomy will be associated with alterations in imprinted gene expression.
Deletion/duplication	Deletions involving imprinted regions can affect imprinting by either: • deletion of an expressed gene on that allele (so abolishing gene expression); or • deletion of the imprinting control centre, which results in loss of regulatory control of imprinting. Duplications involving the imprinted regions can double the expression of imprinted genes expressed from that allele.
Mutation	Loss of function mutations in a gene on the expressed allele will impair gene function whereas a mutation in the silenced allele will have no apparent effect. Hence mutations in imprinted genes are associated with parent-of-origin effects on clinical phenotype.
Epimutation	An epimutation is a specific loss of methylation (hypomethylation) or gain of methylation (hypermethylation) at an imprinting control centre without any change in DNA sequence. This alters expression of imprinted genes on the same allele.

(Lim *et al.*, 2010b) by permission of John Wiley and Sons

As an inherited mutation in an imprinted gene will only be of functional significance if it is in the expressed allele, imprinting disorders are notable for unusual inheritance patterns with parent-of-origin effects on penetrance and expression (Figure 2-2). In some cases, members of the same family may have divergent phenotypes that result from a similar genomic abnormality. For example, Prader Willi Syndrome and Angelman Syndrome may both result from deletions of chromosome 15q11-q13 and the phenotype depends on which parent the deletion is inherited from.

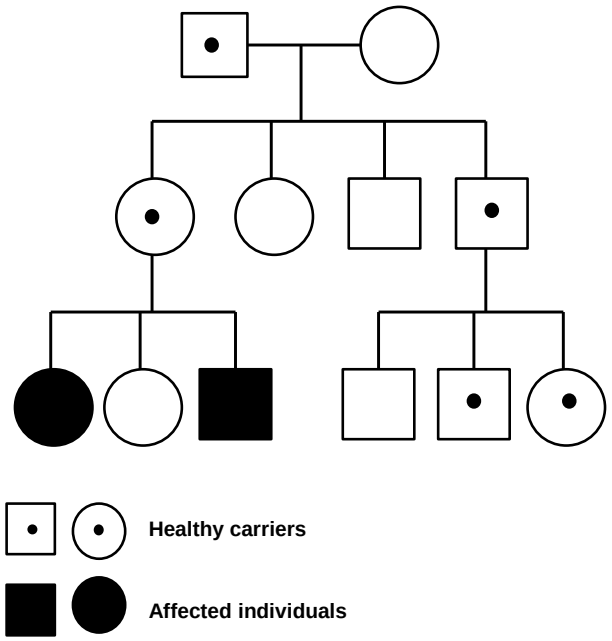


Figure 2-2 Example of parent-of-origin effects in Beckwith-Wiedemann syndrome (BWS)

Pedigree demonstrating the manifestation of BWS in this family only when the familial *CDKN1C* mutation is inherited from the mother.

2.1.4 HUMAN IMPRINTING SYNDROMES

Imprinting disorders are often associated with abnormalities of growth and development and there may be an increased risk of malignancy, most notably in Beckwith-Wiedemann Syndrome, a disease that is associated with the 11p15.5 imprinted gene cluster. The human imprinting syndromes are summarised in Table 4 below. The imprinted loci involved in the imprinting syndromes summarised are presented in Figure 2-3 and the reported mechanisms involved at each loci are summarised in Table 5. For this thesis, I will concentrate on Beckwith-Wiedemann syndrome.

Table 4. Summary of Human Imprinting syndromes

Human Imprinting Syndromes
The 11p15.5 imprinted region: Beckwith-Wiedemann Syndrome (BWS) & Silver-Russell Syndrome (SRS):   ***Beckwith-Wiedemann Syndrome (BWS)***   • **Congenital overgrowth disorder associated with developmental anomalies and susceptibility to embryonal tumours**   • **Results from alteration in expression (or function) of 11p15.5 genes *IGF2/H19* and/or *CDKN1C/KCNQ1OT1***   • **Genotype-phenotype correlations exist in BWS**   • **There is an increased risk of BWS in children born following assisted reproductive technologies (ART)**   • **In IC2 epimutation cases, loss of methylation at other imprinted loci have been reported**   ***Silver-Russell Syndrome (SRS)***   • **Congenital growth disorder characterised by growth retardation and facial dysmorphology.**   • **Most cases are associated with an epimutation at IC1 causing loss of expression of *IGF2* (opposite to that seen in BWS)**   • **5-10% of cases are due to upd(7)mat**

The 15q11-q13 imprinted region: Angelman syndrome & Prader-Willi syndrome

Angelman syndrome (AS)

- **Neurodevelopmental disorder characterised with severe learning disability, microcephaly, characteristic seizure patterns, speech difficulties, ataxia and generally happy demeanour**
- **Caused by inactivation of maternally expressed genes on 15q11-q13 with *UBE3A* being the key gene involved**
- **Reported increased risk of AS following ART**

Prader-Willi syndrome (PWS)

- **Neurodevelopmental disorder characterised by hypotonia and poor feeding in early life followed by excessive eating and obesity together with hypogonadotrophic hypogonadism and typical physical characteristics**
- **Caused by inactivation of paternally expressed genes on 15q11.q13 (opposite to that seen in AS)**

6q24 imprinted region: Transient Neonatal Diabetes Mellitus

- **Diabetes occurs in the first 6 weeks of life which resolves by 18months of age but affected individuals are at risk of developing diabetes in later life**
- **Associated with intrauterine growth retardation**
- **Caused by biallelic expression of *PLAGL1* and *HYMA1* either by upd(6)pat, paternal duplications or epimutation at the maternal allele at the TND DMR**
- **In some epimutation cases, hypomethylation of multiple imprinted loci (HIL now known as multilocus imprinting disturbance (MLID)) are reported**
- **Homozygous mutations in *ZFP57* identified in some affected individuals with HIL/MLID**

The 14q32 imprinted region: Temple syndrome and Kagami-Ogata syndrome

Temple syndrome

- **Phenotype of initial growth retardation and hypotonia followed by precocious puberty, short stature and facial dysmorphisms**
- **Physical characteristics overlap with PWS but mental capacity is usually normal in 70% and severe mental retardation is rare**
- **Caused by loss of functional paternally expressed genes *DLK1* and *RTL1* due to upd(14)mat, paternal deletions or epimutation (loss of methylation) at the IG-DMR**

Kagami-Ogata syndrome

- **More severe phenotype with severe growth retardation, skeletal abnormalities, facial dysmorphism and abdominal muscular defects**

- **Characteristic "coat hanger rib" appearance on x-ray**
- **Caused by overexpression of the paternally expressed genes especially *RTL1* as a result of upd(14)pat, maternal deletions, and epimutation (gain of methylation) of the IG-DMR**

The GNAS imprinting cluster at 20q13: Pseudohypoparathyrodism:

- **The 20q13 imprinting cluster is complex with *GNAS* coding for multiple gene products via alternative first exons**
- **The main transcript Gsα is required for hormone stimulated cyclic AMP formation involved with calcium homeostasis in the renal tubules**
- **Pseudohypoparathyrodism refers to end organ resistance to parathyroid hormone (PTH) with a biochemical features of hypocalcaemia, hyperphosphataemia and elevated PTH levels**
- ***Pseudohypoprathyroidism type 1A (PHP-1A)* is due to maternally transmitted heterozygous inactivating mutations in *GNAS* coding sequences and is associated with the end organ resistance to PTH, TSH and gonadotrophins with typical biochemical features of pseudohypoparathyroidism and physical features of Albright Hereditary Osteodystrophy (AHO)**
- **Paternally transmitted heterozygous mutations in *GNAS* coding sequences result in *pseudopseudohypoparathyroidism (PHPP)* with AHO without the hormone resistance and biochemical features of pseudohypoparathyroidism**
- ***Pseudohypoparathyrodism type 1B (PHP-1B)* have the biochemical features of pseudohypoprathyroidism without AHO features**
- **Familial cases of PHP-1B are due to maternally inherited heterozygous *STX16* deletions leading to isolated exon 1A DMR loss of methylation and sporadic cases are due to epimutations at the exon 1A DMR with or without methylation changes at the other 3 DMRs in the cluster**

NLRP7 mutations and familial biparental complete hydatidiform moles:

- **Biparental complete hydatidiform moles are due to conceptions with both paternal and maternal chromosomes but there is loss of maternal methylation marks at imprinted loci across the genome**
- **Familial cases can result from homozygous *NLRP7* mutations in the mother**
- **Homozygote females have normal methylation patterns in somatic cells but imprinting of their germ cells is defective leading to molar pregnancies and reproductive wastage**
- **Homozygote males have normal methylation levels and normal fertility**

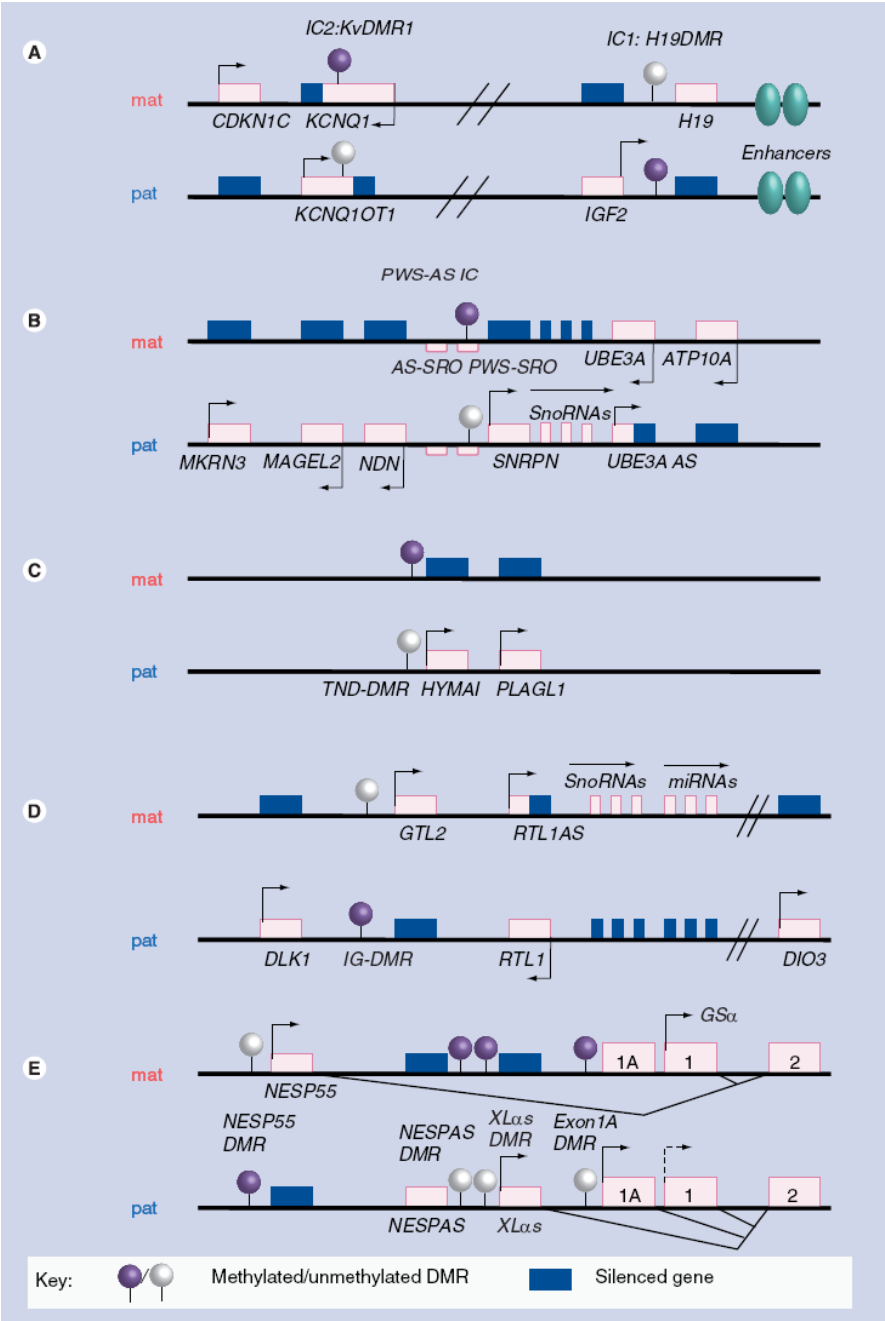


Figure 2-3 The imprinted gene clusters involved in human imprinting syndromes.

(a) 11p15.5 in Beckwith-Wiedemann syndrome and Russell-Silver syndrome (b) 15q11-q13 in Angelman syndrome and Prader-Willi syndrome (c)6q24 in Transient Neonatal Diabetes Mellitus (d) 14q32 in Temple syndrome and Kagami Ogata syndrome (e) 20q13 in Pseudohypoparathyroidism. Arrows represent direction of transcription of active genes. Diagonal lines represent alternate splicing of the alternative 1st exons to downstream exons in the GNAS cluster. Only relevant genes at each imprinted cluster are shown. Figure is not to scale. Republished with permission of Future Medicine Ltd, from (Lim *et al.*, 2009b); permission conveyed through Copyright Clearance Centre, Inc

Table 5. Human Imprinting syndromes and their reported mechanisms

Domain	Disease	Cytogenetic abnormality	Uniparental Disomy	Gene Mutation	Imprinting centre epimutation	Deletions/duplications involving imprinting centre
11p15.5	**BWS**	Chromosomal rearrangement (1-2%)	upd(11)pat (20%)	*CDKN1C* on maternal allele (5% sporadic, 50% in familial cases)	IC1 epimutation (*H19* DMR hypermethylation (5%) IC2 epimutation (KvDMR1 hypomethylation) (50%)	IC1 deletion on maternal allele (<5%) IC2 deletion on maternal allele (<2%)
11p15.5	**SRS**	Maternal duplication of 11p15 (<2%)	upd(11)mat (1 case)	*IGF2* mutation on paternal allele (1 family) *CDKN1C* gain of function mutation on maternal allele (1 family)	IC1 epimutation (*H19* DMR hypomethylation) (up to 70%)	IC2 duplication on maternal allele (1 case)
7p12.2, 7q32.2	**SRS**	Chromosomal rearrangement (<2%) Maternal duplication of 7p	upd(7)mat (5-10%%)	-	-	-
6q24	**TNDM**	Visible paternal duplication of 6q24 (2%)	upd(6)mat (35-40%)	-	Hypomethylation of the TNDM (*PLAGL1*) DMR (20%)	Submicroscopic duplication of the 6q24 region on the paternal allele (35-40%)
14q32.2	**Temple**	Chromosomal	upd(14)mat	-	Hypomethylation	Deletion involving

	Syndrome	rearrangement (rare)	(72-80%)		of the IG-DMR on the paternal allele (12-19%)	the 14q32 imprinted region on the paternal allele (9%)
14q32.2	**Kagami Ogata syndrome**	Chromosomal rearrangement (22%)	upd(14)pat (67%)	-	Hypermethylation of the IG DMR on the maternal allele (19%)	Deletion involving the 14q32 imprinted region on the maternal allele (17%)
15q11-q13	**AS**	5-7mb visible deletion on maternal allele at 15q11-q13 (65-75%) Chromosomal rearrangement (<1%)	upd(15)pat (7%)	*UBE3A* on maternal allele (11%)	Hypomethylation of the SNRPN DMR on the maternal allele (80-90% of imprinting defect cases) *2-5% of AS have imprinting defects	Deletion involving the AS-SRO on the maternal allele (10-20% of imprinting defect cases) *2-5% of AS have imprinting defects
15q11-q13	**PWS**	5-7mb visible deletion on paternal allele at 15q11-q13 (65-75%) Chromosomal rearrangement (<1%)	upd(15)mat (25-30%)	-	Hypermethlyation of the *SNRPN* DMR on the paternal allele (75-80% of imprinting defects cases) *1% of PWS are due to imprinting defects	Deletion involving the PWS-SRO on the paternal allele (15% of imprinting defect cases) *1% of PWS are due to imprinting defects
20q13	**PHP1b**	-	upd(20)pat (1 report in a sporadic case)	Familial cases: *STX16* mutation on maternal allele leads to isolated	Sporadic cases: Hypomethylation of the Exon1A DMR on the	Deletion involving the GNAS imprinting cluster on the maternal

				exon 1A DMR hypomethylation (Most familial cases)	maternal allele +/- epimutation involving *NESPAS*. *XLαS* & *NESP55* DMRS (Most sporadic cases)	allele (Rare – 2 familial cases reported without *STX16* mutation)
20q13	**PHP1a**	Deletion involving 20q13 on the maternal allele (1 case reported)	-	*GNAS* mutation (coding for Gsα) on maternal allele (Most cases)	-	-
20q13	**PPHP**	Deletion involving 20q13 on the paternal allele (1 case reported)	-	*GNAS* mutation (coding for Gsα) on paternal allele (Most cases)	-	-

AS: Angelman syndrome; BWS: Beckwith-Wiedemann syndrome; DMR: Differentially methylated region; IC: Imprinting centre; IG-DMR: Intergenic differentially methylated region; PHP1a: Pseudohypoparathyroidism type 1A; PHP1b: Pseudohypoparathyroidism type 1B; PPHP: Pseudo-pseudohypoparathyroidism; PWS: Prader-Willi Syndrome; SRO: Shortest region of overlap – region identified by deletion mapping in AS/PWS that is required for correct methylation pattern and gene expression of the imprinted region; SRS: Silver-Russell syndrome; TNDM: Transient Neonatal Diabetes Mellitus; UPD: Uniparental Disomy. Adapted with permission of Future Medicine Ltd, from (Lim *et al.*, 2009b); permission conveyed through Copyright Clearance Centre, Inc

2.1.5 THE 11P15.5 IMPRINTED REGION

The 11p15.5 imprinted region contains a cluster of imprinted genes including *IGF2*, *H19*, *CDKN1C* and *KCNQ1OT1* (Figure 2-3(a)). The cluster can be subdivided into two domains containing imprinting centres which regulate the expression of these genes in *cis*. Imprinting centre 1 (IC1) located more telomerically controls the imprinting/expression of *IGF2* and *H19*. Imprinting centre 2 (IC2) is located more centromerically and controls the imprinting of a number of genes including *CDKN1C*, *KCNQ1* and *KCNQ1OT1*.

2.1.5.1 Normal Imprinting at 11p15.5

IC1 is epigenetically marked by the *H19* DMR which is located between *H19* and *IGF2* and is normally methylated on the paternal allele (Bell *et al.*, 2000). As described earlier, the insulator protein CTCF binds to the unmethylated *H19* DMR on the maternal allele resulting in silencing of *IGF2* and preferential expression of *H19* from the maternal allele. On the other hand, the *H19* DMR is methylated on the paternal allele and hence CTCF is unable to bind and there is expression of *IGF2* and silencing of *H19* from the paternal allele (Hark *et al.*, 2000)(Figure 2-4).

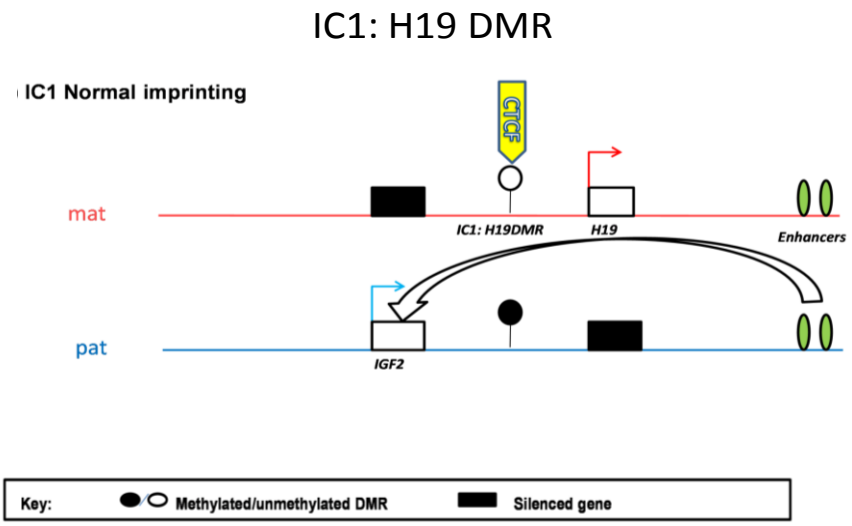


Figure 2-4 Normal imprinting at IC1 at 11p15.5.

On the maternal allele, the *H19*DMR is unmethylated allowing CTCF to bind to the CTCF sites at the *H19*DMR. This acts as a boundary element preventing downstream enhancers access to the *IGF2* promoter resulting in preferential expression of *H19* and silencing of *IGF2* on the paternal allele. On the maternal allele, the *H19*DMR is methylated preventing CTCF from binding to the DMR allowing the downstream enhancers access to the *IGF2* promoter. This results in preferential expression of *IGF2* from the paternal allele.

Figure 2-5 Normal imprinting at IC2.

KvDMR1 is methylated on the maternal allele resulting in expression of *CDKN1C* and *KCNQ1* from the maternal allele. On the paternal allele, KvDMR1 is unmethylated resulting in preferential expression of *KCNQ1OT1* (the antisense transcript RNA of *KCNQ1*) resulting in silencing of *CDKN1C* and *KCNQ1*

IC2 regulates a number of imprinted genes including the candidate tumour suppressor gene *CDKN1C*, *KCNQ1* and an antisense transcript *KCNQ1OT1* (a noncoding RNA previously known as LIT1). The DMR at IC2 is called the KvDMR1 which is located within intron 10 of *KCNQ1*, coincident with the transcriptional start site for *KCNQ1OT1 (Weksberg et al., 2005)*. KvDMR1 is normally methylated on the maternal allele. This results in preferential expression of both *KCNQ1* and *CDKN1C* from the maternal allele. On the paternal allele, KvDMR1 is unmethylated and this allows preferential expression of *KCNQ1OT1* and silencing of *CDKN1C* and *KCNQ1* from the paternal allele (Figure 2-5)(Diaz-Meyer *et al.*, 2003; Lam *et al.*, 1999;

Smilinich *et al.*, 1999). The mechanism by which this noncoding RNA causes silencing is still not understood. It remains unclear whether it causes silencing directly or as a secondary effect from its transcription.

The paternally expressed *IGF2* is a fetal growth promoter whereas the maternally expressed gene *CDKN1C* is a growth suppressor. As a result of genomic imprinting, there is "balance" between the expression of these two genes resulting in normal growth. This reflects Hague's parental conflict theory of imprinting whereby it is in the interest of the father to derive more maternal resources to his offspring which will lead to a greater survival fitness hence promoting the propagation of his genes to successive generations. In contrast, the mother's interest is to produce the maximum number of viable embryos in addition to ensuring her own nutritive needs for survival and future reproduction. Therefore embryo growth will be influenced by the expression of paternally derived growth promoters and maternally derived growth suppressors (Moore *et al.*, 1991).

2.1.5.2 Disordered imprinting at 11p15.5

Disruption of imprinting at the 11p15.5 imprinted region can be caused by a number of mechanisms and is associated with abnormal growth, developmental defects and tumour susceptibility but neurological development is mostly unaffected. The two human imprinting syndromes, Beckwith-Wiedemann syndrome (an overgrowth disorder) and Silver-Russell syndrome (a disorder of growth restriction) are associated with abnormal imprinting at 11p15.5.

2.1.6 BECKWITH-WIEDEMANN SYNDROME

Beckwith-Wiedemann syndrome (BWS) is a congenital overgrowth disorder and model human imprinting syndrome with an estimated incidence of 1/13700 (Weksberg *et al.*, 2009). BWS is characterised by features of prenatal or postnatal overgrowth, macroglossia (enlarged tongue) and anterior abdominal wall defects. The abdominal wall defects range from mild divarification of the abdominal recti muscles to more severe exomphalos requiring surgical correction. Additionally, other clinical features that are often variable in presentation include facial naevus flammeus, ear lobe creases, neonatal hypoglycaemia, organomegaly, hemihypertrophy, genitourinary anomalies and most significantly embryonal tumours (Cooper *et al.*, 2005; Elliott *et al.*, 1994; Reik *et al.*, 1997).

Complications can occur due to the various features associated with BWS. Polyhydramnios may complicate the pregnancy and an overgrown baby may pose difficulties in delivery at birth. Neonatal hypoglycaemia can be severe and require treatment with intravenous glucose. Untreated severe hypoglycaemia may present as seizures and if prolonged may affect neurodevelopment. An enlarged tongue may cause feeding difficulties and surgical correction is needed in some cases. Learning difficulties can occur as a result of untreated hypoglycaemia or if associated with a chromosomal abnormality. Overall, the overgrowth tends to become less prominent with increasing age and the prognosis is good if exomphalos is treated successfully and embryonal tumours do not occur (Elliott *et al.*, 1994).

2.1.6.1 Risk of tumours in BWS

Around 5-10% of BWS patients develop tumours which include Wilms tumour (WT; most common), hepatoblastoma, rhabdomyosarcoma, adrenocortical carcinomas and neuroblastomas. Most of the tumours occur in childhood, in the first 8 years of life with only a few cases being reported after this. Children with BWS are usually offered surveillance due to the increased risk of childhood tumours by serial abdominal ultrasound scans and monitoring of serum alpha-fetoprotein (Beckwith, 1998; Everman *et al.*, 2000). Certain clinical features in BWS that are associated with a higher risk of developing tumours include hemihypertrophy, nephromegaly and nephrogenic rests (Coppes *et al.*, 1999; DeBaun *et al.*, 1998).

2.1.6.2 Molecular mechanisms affecting imprinting in BWS

Most cases of BWS are sporadic. Familial cases of BWS account for around 15%. BWS is a heterogenous disorder and a wide variety of molecular mechanisms can cause disordered imprinting/function of *IGF2* and/or *CDKN1C* and result in BWS. In essence, BWS results from the over expression of *IGF2* and/or underexpression of *CDKN1C* (Cooper *et al.*, 2005). The molecular mechanisms disrupting genomic imprinting at 11p15.5 resulting in BWS reported to date include:

1. upd(11)pat (paternal uniparental disomy of chromosome 11)
2. IC1 epimutation
3. IC2 epimutation
4. Intragenic *CDKN1C* mutation
5. IC1 or IC2 deletion/mutation
6. Cytogenetic abnormalities affecting 11p15.5

20% of BWS cases are caused by upd(11)pat (mosaic isodisomy resulting from mitotic recombination). upd(11)pat result in two paternal alleles producing biallelic expression of *IGF2* and reduced expression of *CDKN1C* (Figure 2-6).

IC1 epimutations with gain of methylation of the maternal allele of the *H19*DMR result in biallelic expression of *IGF2* and silencing of *H19* (Figure 2-7). This epimutation accounts for 5% of BWS cases.

IC2 epimutations with loss of methylation (LOM) at KvDMR1 on the maternal allele cause biallelic *KCNQ1OT1* expression and loss of *CDKN1C* expression from the maternal allele (Figure 2-8). This is the commonest molecular mechanism which accounts for 50% of sporadic cases of BWS.

Germline mutations in *CDKN1C* on the maternally inherited allele occur (Figure 2-9) in 5% of sporadic cases of BWS cases but account for around 50% of familial cases (Hatada *et al.*, 1996; Lam *et al.*, 1999). Familial cases of BWS with *CDKN1C* mutations show autosomal dominant inheritance with parent-of-origin effects such that BWS is only seen with maternally transmitted mutations (see Figure 2-2 above).

Although IC1 and IC2 epimutations usually occur sporadically, IC1 and IC2 deletions can also occur and lead to familial disease. IC1 deletions on the maternal allele affecting the CTCF binding sites are associated with apparent *H19* DMR hypermethylation and biallelic expression of *IGF2*. An IC2 deletion is associated with silencing of *CDKN1C*. IC1 deletions occur more commonly than IC2 deletions (Niemitz *et al.*, 2004; Sparago *et al.*, 2004; Sparago *et al.*, 2007).

Finally, cytogenetic abnormalities (chromosomal rearrangements/ translocations) leading to copy number changes at 11p15.5 account for under 2% of BWS cases.

Molecular testing for BWS using methylation-specific multiplex ligation-dependant probe amplification (MS-MLPA) is widely used to both determine IC1 and IC2 methylation status and copy number (Scott *et al.*, 2008a; Scott *et al.*, 2008b).

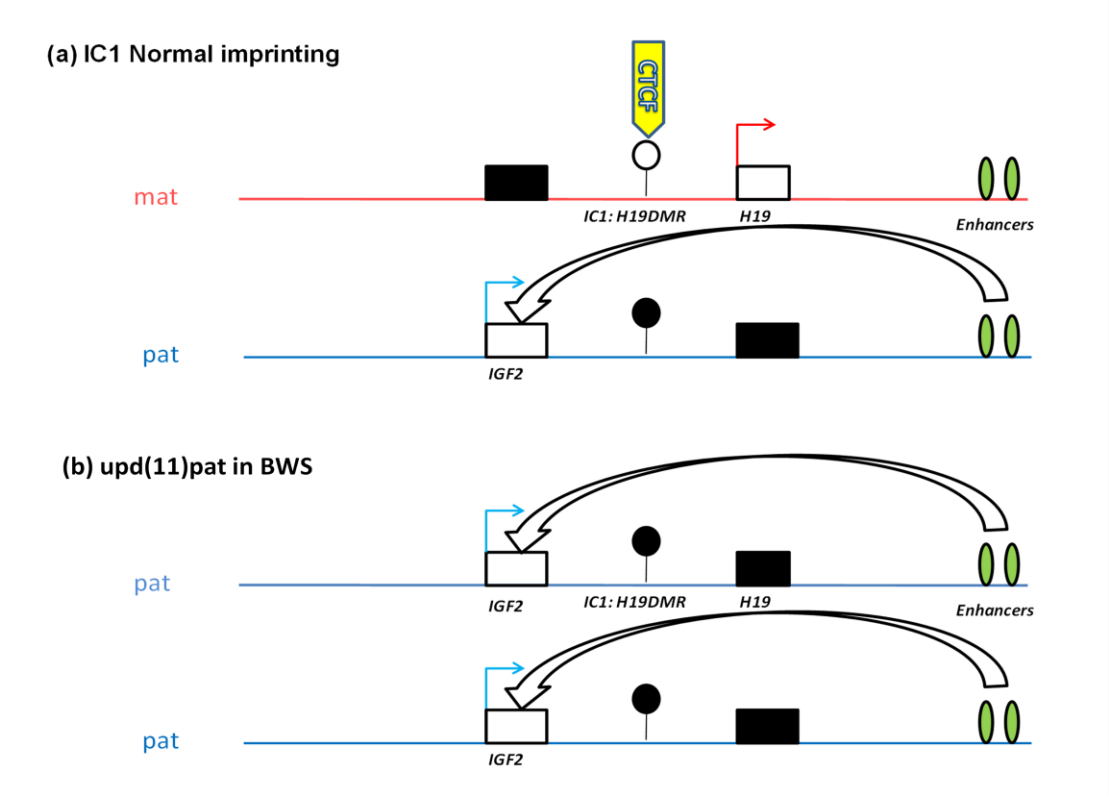


Figure 2-6 upd(11)pat causing BWS

(a) Normal imprinting at IC1. (b) upd(11)pat in BWS. 2 copies of the paternal allele result in biallelic expression of *IGF2*

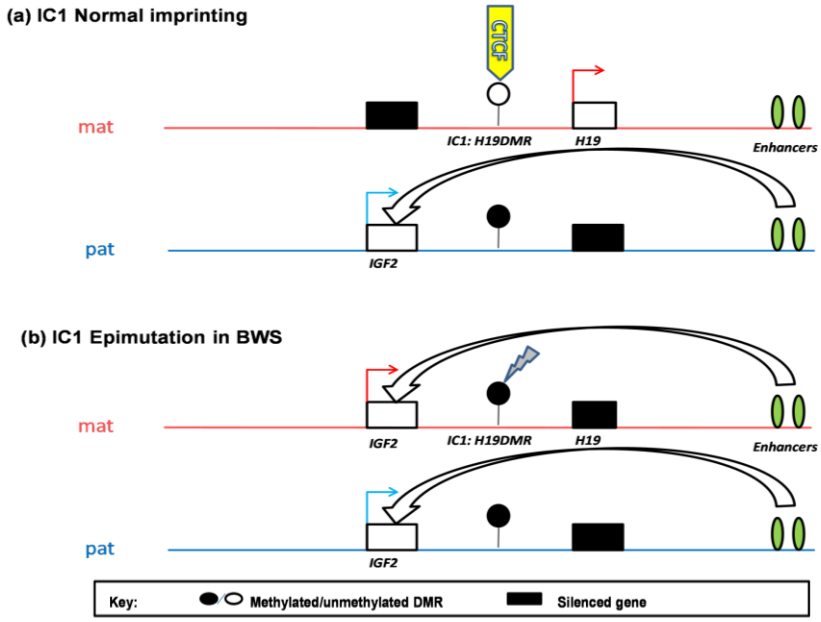


Figure 2-7 IC1 epimutation causing BWS

(a) Normal imprinting at IC1. (b) IC1 epimutation in BWS. There is gain of methylation at the *H19*DMR on the maternal allele. This results in biallelic expression of *IGF2*.

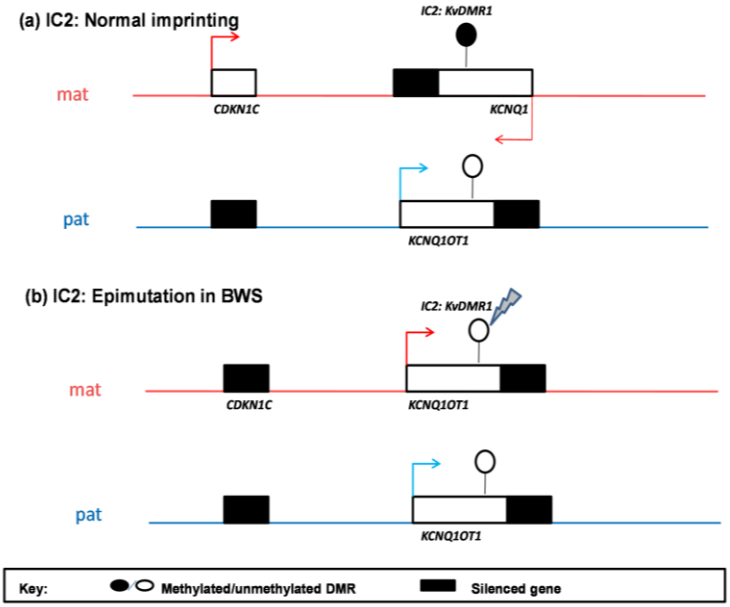


Figure 2-8 IC2 epimutation causing BWS

(a) Normal imprinting at IC2. (b) IC2 epimutation in BWS. There is loss of methylation of KvDMR1 on the maternal allele resulting in biallelic expression of *KCNQ1OT1* and silencing of *CDKN1C* and *KCNQ1* on both alleles.

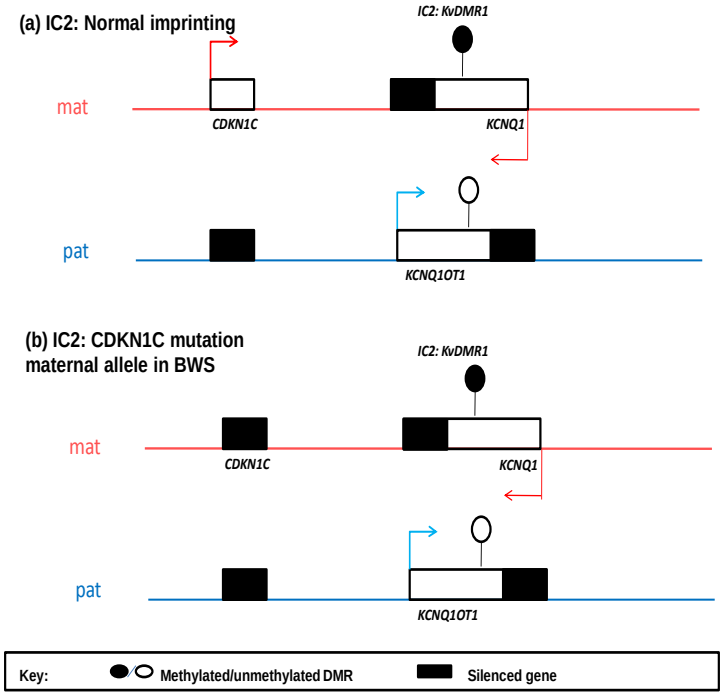


Figure 2-9 *CDKN1C* mutation on maternal allele causing BWS

(a) Normal imprinting at IC2. (b) *CDKN1C* mutation in maternal allele in BWS. There is biallelic silencing of *CDKN1C* due to an intragenic mutation on the maternal copy of *CDKN1C*.

2.1.7 GENOTYPE (& EPIGENOTYPE)-PHENOTYPE CORRELATIONS IN BWS

Analysis of genotype-phenotype correlations in BWS have revealed key associations that provide important insights into the pathogenesis of specific developmental abnormalities which can aid clinical management (Cooper *et al.*, 2005).

Although various non-tumour related clinical features associated with BWS can be seen across all BWS molecular subtypes, genotype (& epigenotype) phenotype- correlations have been identified in various studies. Hemihypertrophy is seen more commonly in upd(11)pat (Cooper *et al.*, 2005; Ibrahim *et al.*, 2014; Mussa *et al.*, 2015). Exomphalos is more common in IC2 epimutation and *CDKN1C* mutations (Brioude *et al.*, 2013; Cooper *et al.*, 2005; Ibrahim

et al., 2014; Mussa *et al.*, 2015). Cleft palate is reported more commonly in mutations in *CDKN1C* (Mussa et al., 2015). Organomegaly is found more commonly in the IC1 epimutation group compared to other molecular subgroups (Mussa *et al.*, 2015).

The overall risk of tumours in BWS as a whole is between 5-10% (Brioude *et al.*, 2013; Ibrahim *et al.*, 2014; Mussa *et al.*, 2015). However looking at the specific subgroups of molecular causes in BWS reveal differences in the incidence of tumours. IC1 epimutation have an overall tumour risk of 28%, upd(11)pat was 16%, *CDKN1C* mutation was 6.7% and IC2 epimutation was 2.6% (Maas *et al.*, 2016).

Together, upd(11)pat and IC1 epimutation are associated with the highest risk of WT and hepatoblastoma. IC1 epimutation cases have the highest risk of WT (24%) and accounts for 95% of malignancies in this subgroup (Mussa *et al.*, 2016). The incidence of WT in the *CDKN1C* mutation and IC2 epimutation cases is very rare but not zero (Ibrahim *et al.*, 2014; Maas *et al.*, 2016). Increased expression of *IGF2* has been shown to be linked to an increased WT risk in both BWS and in sporadic nonsyndromic WT cases. In BWS this is reflected in the increased incidence of WT in the upd(11)pat and IC1 epimutation subgroups as these both result in loss of imprinting of *IGF2* leading to biallelic expression of *IGF2*. IC1 epimutations also occur as a somatic event in many sporadic WTs (Scott *et al.*, 2008a; Scott *et al.*, 2008b).

Hepatoblastoma is the commonest tumour in the upd(11)pat patients occurring in 5% of upd(11)pat patients and accounts for 25% of all tumours in this molecular subgroup (Mussa *et al.*, 2016). Hepatoblastoma occurred rarely in other molecular subgroups (0.6% overall).

Neuroblastoma was most frequent in the *CDKN1C* mutation subgroup with an incidence of 4.3% representing more than 50% of tumours reported in this molecular subgroup (Maas *et al.*, 2016; Mussa *et al.*, 2016).

2.1.7.1 Tumour surveillance recommendation in BWS

In the United Kingdom WT screening/surveillance has been recommended in individuals with a >5% risk of developing WT (Scott *et al.*, 2006). In BWS this can be targeted to BWS cases resulting from increased expression of *IGF2* (upd(11)pat, IC1 epimutation, paternal duplication) and in cases of clinically diagnosed BWS without a known molecular cause. Surveillance is offered by 3-4 monthly abdominal ultrasound scans until the age of 7 years as the risk of developing WT following this age decreased to general population figures (Scott *et al.*, 2006).

The use of serial serum alpha-fetoprotein (aFP) measurement every 3 months until the age of 4 years as surveillance for hepatoblastoma have been recommended in BWS. However, the use of aFP as a surveillance method is currently debated due to the poor evidence on effectiveness, the invasiveness of regular phlebotomy in early childhood, low incidence of hepatoblastoma and the complexity of interpretation of aFP levels in childhood (Mussa *et al.*, 2015). As a result, some centres do not currently recommend routine aFP measurements for hepatoblastoma.

There is no current international consensus for tumour surveillance in BWS and practice may vary from not only country to county but between different clinical centres within a country. In the UK, the Clinical Genetics Society (CGS), a component society of the British

Society of Genetic Medicine, agreed WT screening should be recommended for BWS patients due to upd(11)pat, IC1 epimutations and those with an unknown molecular aetiology and not recommended in IC2 epimutation patients. However, no current guidance has been provided for hepatoblastoma. Therefore there is a need for an international consensus working group to discuss evidence based and best practice recommendations for surveillance and management in BWS.

2.1.8 ASSISTED REPRODUCTIVE TECHNOLOGIES AND BWS AND OTHER IMPRINTING DISORDERS

Assisted reproductive technologies (ART) such as in vitro fertilisation (IVF) and intra-cytoplasmic sperm injection (ICSI) are associated with an increased risk of prematurity, low birth weight, imprinting disorders and congenital malformations (mainly male urogenital tract anomalies) (Hyrapetian *et al.*, 2014). ART is associated with a ninefold increased risk of BWS (Halliday *et al.*, 2004). However, the absolute risk is low, in the region of less than 0.1%. The risk of BWS following ART appears to be associated with IC2 epimutations (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Halliday *et al.*, 2004; Maher *et al.*, 2003).

Around a similar time when these observations in BWS were made, there were 2 reports suggesting a link between Angelman syndrome and ICSI (Cox *et al.*, 2002; Orstavik *et al.*, 2003). Following these, a number of cases of Silver-Russell syndrome (SRS) were reported in the medical literature following the use of ART (Bliek *et al.*, 2006; Douzgou *et al.*, 2008; Kallen *et al.*, 2005; Svensson *et al.*, 2005).

Several papers have since reported correlation of ART with Angelman syndrome and Silver-Russell syndrome(Doornbos *et al.*, 2007; Hiura *et al.*, 2012; Lammers *et al.*, 2012; Sutcliffe *et al.*, 2006; Vermeiden *et al.*, 2013). Although, no correlation was found between ART and Prader-Willi syndrome, two studies found an increased incidence of fertility problems in parents of affected PWS children (Doornbos *et al.*, 2007; Sutcliffe *et al.*, 2006). The highest risks appear to be associated with BWS and SRS where a Japanese nationwide study found that the frequency of ART in BWS and SRS was almost 10-fold higher than anticipated compared to AS and PWS (Hiura *et al.*, 2012).

A number of studies have investigated whether ART-associated variables such as ovarian hyperstimulation, the timing of embryo transfer, culture, and other factors might influence the risk of imprinting disorders (Amor *et al.*, 2008; Manipalviratn *et al.*, 2009). However, currently, the relative importance of infertility (or treatment of infertility) and epigenetic changes during *in vitro* embryo culture is still disputed.

2.1.9 BWS AND LOSS OF METHYLATION AT MULTIPLE IMPRINTED LOCI

A subgroup of BWS patients have been discovered to have MLID i.e. altered methylation at DMRs in other imprinted regions in addition to 11p15.5 (Bliek *et al.*, 2009; Rossignol *et al.*, 2006).

In addition, patients with another imprinting disorder Transient Neonatal Diabetes Mellitus (TNDM) which are due to imprinting defects at 6q24 (See Figure 2-3, Table 4 and Table 5) have also been discovered to have loss of methylation at multiple imprinted loci including

11p15.5 (Mackay *et al.*, 2006a; Mackay *et al.*, 2006b). In addition to the typical clinical features of TNDM, these patients can have overlapping clinical features of other imprinting syndromes including BWS (Mackay *et al.*, 2006b). Mutations in a zinc finger protein gene *ZFP57* was identified as a cause of TNDM with MLID (Mackay *et al.*, 2008). Autosomal recessive mutations in *ZFP57* predispose these individuals to develop imprinting defects in 6q24 and other imprinted loci. Therefore *ZFP57* is a gene that affect imprinting in *trans*. *ZFP57* is implicated in the maintenance of maternal and paternal DNA methylation at DMRs (Li *et al.*, 2008).

Homozygous mutations in the gene *NLRP2* were identified in a mother of 2 children with BWS due to IC2 epimutations (Meyer *et al.*, 2009). In contrast to *ZFP57*, mutations in *NLRP2* did not predispose the homozygous/compound heterozygous mutation carrier to develop imprinting defects. The mother with the *NLRP2* mutations had normal methylation at imprinted loci but it was the offspring that had imprinting defects. One of her BWS child also had loss of methylation at a non-11p15.5 loci. This is similar to females with recessive *NLRP7* mutations (a paralogous gene to *NLRP2*) who have normal methylation levels themselves, but their conceptions are at risk of severe loss of methylation at imprinted loci across the genome resulting in familial hydatidiform molar pregnancies (Murdoch *et al.*, 2006). More recently autosomal recessive mutations in *NLRP5* have also been reported to cause affected women to have pregnancies at risk of methylation abnormalities resulting in imprinting syndromes with MLID (Docherty *et al.*, 2015).

2.1.10 SUMMARY

I have given an overview of genomic imprinting and how disruption of imprinting by a number of mechanisms can result in human imprinting syndromes. BWS is a model imprinting disorder and studies into BWS have provided novel insights into the mechanisms of genomic imprinting in human and pathways in growth and development. The identification of genotype-phenotype correlations in BWS have provided an important framework for diagnostic testing and surveillance in children with BWS in view of the increased embryonal tumour risk in childhood. The link between ART use and BWS provides further avenues for studying the aetiology of imprinting disorders. The identification of a subgroup of BWS patients with loss of imprinting at other imprinted loci (MLID) is an important finding which may explain phenotypic variability in BWS patients and overlapping phenotypes with other imprinting disorders.

2.1.11 AIMS OF PROJECT

The aim of the BWS project is to investigate the possible causes of phenotypic variability in BWS by elucidating the aetiology and the clinical and molecular characterisation of epigenetic changes in BWS.

1. To detect the prevalence of epigenetic changes at non-11p15.5 loci in BWS patients

It will be important to calculate the prevalence of additional epigenetic changes in a large cohort of BWS patients. I investigated BWS patients with IC2 epimutations for two reasons: 1) IC2 epimutation is the predominant molecular subgroup in BWS who develop loss of

methylation at other imprinted loci. 2) The epigenetic alterations in non-11p15.5 in BWS have all affected loss of methylation at normally methylated maternal imprinted loci thus far.

2. To clinically characterise the cohort of patients with additional epigenetic changes identified in our study and compare their phenotype with BWS IC2 epimutation cases without additional epigenetic changes.

It will be important to further study the clinical features/phenotype in this subgroup of patients to identify further epigenotype-phenotype correlations which may aid diagnostic testing, surveillance and prognosis.

3. To determine the molecular genetic defect in ART conceived BWS cases.

Studies to date have detected that the majority of ART conceived BWS cases have IC2 epimutations. I investigated the cause of BWS in the ART conceived BWS cases in our cohort to confirm this observation.

4. To study the prevalence of additional epigenetic changes in BWS patients who were conceived with ART

As ART causes an increased risk of BWS, it will be important to clarify if ART conceived BWS patients have additional epigenetic changes affecting other imprinted loci. As the exact cause of how ART causes an increased risk of BWS is still not understood, the study of BWS patients conceived with ART may provide novel insights into the understanding of the aetiology and may have implications to other children conceived by ART.

5. To compare the clinical features of BWS patients naturally conceived with ART conceived BWS patients

I compared the clinical features of ART conceived BWS cases with a control group of naturally conceived BWS patients with the same molecular aetiology of BWS to generate comparisons between the two groups of patients.

6. To study the phenotype of BWS patients with epigenetic changes at multiple imprinted loci

I studied the clinical features/phenotype of the BWS cases (naturally conceived and ART conceived) with additional epigenetic changes to determine the phenotypic differences.

2.2 METHODS

2.2.1 ACQUISITION AND PATIENT RECRUITMENT

Molecular diagnostic testing for Beckwith-Wiedemann syndrome was performed in the West Midlands Regional Genetics Laboratory in Birmingham Women's Hospital. Recruitment letters, patient information leaflets, consent forms and a clinical questionnaire for the study were sent to the referring clinicians of the patients tested positive for BWS. The clinicians would then consent the families for the research study and complete a standard clinical questionnaire.

2.2.2 PATIENT ASSESSMENT

Clinical information was obtained from the returned standard clinical questionnaire (see Appendix A), hospital medical notes or direct examination.

2.2.3 CONSENT AND ETHICS APPROVAL

Informed consent was obtained from all participants. All clinical research adhered to principles outlined by the Declaration of Helsinki. The BWS study is a sub-study of the Molecular Pathology of Human Genetic Disease study which has received ethical approval by the South Birmingham Research Ethics Committee (REC) (REC reference 5175) prior to my involvement in the project. However upon completion of my project, I have been involved in revising the information leaflet and consent form to an updated version that was submitted for an amendment in 2011 which received a favourable ethical opinion of the amendment by the South Birmingham REC. These are included in Appendix F.

2.2.4 ACQUISITION OF BLOOD OR DNA SAMPLES

After consent was obtained, DNA samples were obtained from the stored DNA samples used for molecular diagnostic testing. If further DNA samples were needed, letters were sent to the patient's clinicians for a repeat blood sample or an aliquot of DNA to be sent to the research team.

2.2.5 MATERIALS

2.2.5.1 Chemical reagents

Agarose *Bioline*

dNTPS (diluted from 100mM to a working stock of 2mM) *Bioline*

EDTA (ethylenediaminetetraacetic avid) *Sigma*

Ethanol *Fisher Scientific*

Ethidium bromide *Sigma*

Genescan-500 LIZ Size Standard *Applied Biosystems*

Hi-Di Formamide *Applied Biosystems*

Hyperladder TM I (Separation range 200- 10000bp) *Bioline*

Loading buffer

Primers *Sigma*

10X TBE Electrophoresis Buffer (diluted to 1X) *Geneflow*

Water (distilled-RNase/DNase free, dH20) *Invitrogen*

Big Dye *Applied Biosystems*

Streptavidin -coated Sepharose beads (Biotage)

Denaturation Solution 0.2M NaOH

Washing buffer (1x) *Biotage*

2.2.5.2 Kits

EZ DNA Methylation Gold Kit – *Zymo Research*

Pyro Gold Reagents – *Qiagen/Biotage*

PyroMark Prader-Willi/Angelman Kit *Biotage*

2.2.6 MOLECULAR GENETIC INVESTIGATION

2.2.6.1 DNA extraction

DNA extraction was performed by the West Midlands Regional Genetics Laboratory or by other NHS diagnostic laboratories in the UK. In the West Midlands Regional Genetics laboratory, DNA was extracted from peripheral lymphocytes in blood using the Pregene Genomic DNA Purification kit according to the manufacturer's instructions. Extracted genomic samples were then quantified by using nanodrop (ND-1000) by measuring absorbance at 260nM.

2.2.6.2 Bisulphite modification of DNA for methylation analysis

The analysis of DNA methylation status at CpG sites can be performed by various techniques using bisulphite modified DNA. Following the process of bisulphite modification, unmethylated Cytosines are converted to Uracil. Methylated Cytosines on the other hand remained unchanged by the process of bisulphite modification. This principle can then be used for analysis for DNA methylation changes (Figure 2-10).

Genomic DNA derived from peripheral blood lymphocytes was bisulphite modified using the EZ DNA Methylation Gold kit (Zymo Research) according to the manufacturer's protocol.

2.2.6.2.1 EZ DNA Methylation Gold Kit Protocol

Preparation of CT Converstion Reagent:

900µl water, 50ul of M-Dissolving Buffer and 300µl of M-Dilution Buffer was added to one tube of CT Conversion Reagent and mixed for 10 minutes

Preparation of M-Wash Buffer:

24ml of 100% ethanol was added to the 6ml M-Wash Buffer concentrate before use.

Protocol:

1. 130µl of the prepared CT Conversion Reagent was added to 20µl of DNA Sample (500 nanogram of DNA in 1ul added to 19µl of dH20) and mixed.

2. The following temperature steps was performed: 98°C for 10 minutes, 64°C for 2.5 hours, then held at 4°C.

3. 600µl of M-Binding Buffer was added to a Zymo-Spin IC Column, followed by the addition of the sample. The cap was then closed and mixed by inverting several times.

4. The column was then centrifuged at full speed (≥10,000 x g) for 30 seconds. The follow through was then discarded.

5. 100µl of M-Wash Buffer was added to the column and spun 30 seconds.

6. 200µl of M-Desulphonation Buffer was added to the column and left to stand for 15-20minutes.

7. The column was then spun at full speed for 30 seconds.

8. 200µl of M-Wash Buffer was then added to the column. This wash step was repeated.

9. 10µl of M-Elution Buffer was added directly to the column matrix and placed into a 1.5ml tube. The tube was then spun briefly to elute the DNA.

10. The DNA is then ready for immediate analysis or stored at or below -20°C for later use.

2.2.6.3 Bisulphite Sequencing of *PEG1* DMR at 7q32

Bisulphite sequencing refers to the process of conventional DNA sequencing following bisulphite modification of genomic DNA (Figure 2-10). Bisulphite sequencing was undertaken for analysis of the methylation status at the CpG sites at the *PEG1* DMR at 7q32. Primers were designed using the Methyl Primer Express software supplied by Applied Biosystems (sense primer 5'- AGTTGGGGTTGTTTTTGG-3') and 3' anti-sense primer 5'-TACCAAAATCTAAAAATCCCATT-3'). This amplified a 264bp fragment which contains 15 CpGs.

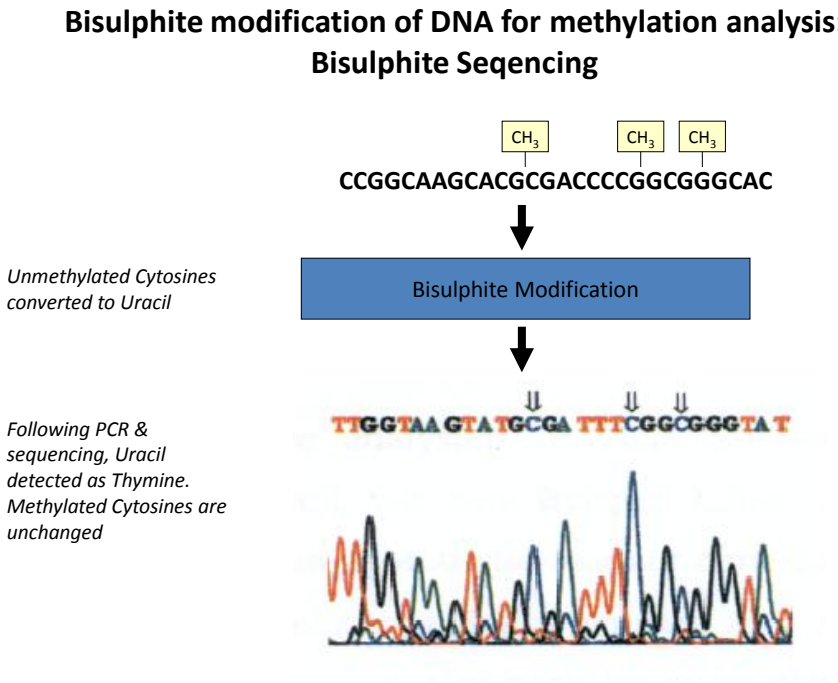


Figure 2-10 Bisulphite modification of DNA

Bisulphite modification of DNA for methylation analysis (e.g. bisulphite sequencing)

In normal controls, due to genomic imprinting, the bisulphite sequencing of the *PEG1* DMR will reveal cytosine and thymine peaks at CpG sites representing methylated and unmethylated alleles respectively, with taller cytosine peaks. In patients with loss of methylation or hypomethylation of this DMR, there will be a reversal of the peaks with taller thymine peaks at these CpG sites representing loss of methylation.

Protocol:

2.2.6.3.1 PCR amplification for sequencing

Standard conditions for PCR amplification were used. PCR amplification was performed in 20µl reactions as follows:

- 2µl of Bisulphite Modified DNA
- 2µl dNTP (0.2mM)
- 1.6µl MgCl2 (2mM)
- 0.2µl Hot Star *Taq*
- 2µl Buffer (qiagen)
- 0.2µl Forward Primer (0.2uM)
- 0.2µl Reverse Primer (0.2uM)
- 11.8µl dH20

PCR conditions were an initial denaturation of 95°C for 15 minutes followed by 35 cycles of 20 seconds denaturation at 95°C, 10 seconds annealing at 56°C and 10 seconds extension at 72°C. This was followed by a final extension step at 72°C for 5 minutes. Each set of PCR reactions included a negative control (in which dH20 was added instead of DNA) to check for contamination.

2.2.6.3.2 Agarose gel electrophoresis

PCR products were checked on 1.5% horizontal agarose gels to separate the PCR products and to ensure that the PCR reaction had worked without contamination. The agarose gels were made by melting agarose with 1X TBE in a domestic 600W microwave oven, then cooling the mixture before adding ethidium bromide (0.5g/ml final concentration) and casting the gel in a gel casting tray. 5ul of PCR products was mixed with loading buffer. A DNA sizing ladder (Hyperladder I) was added to lane 1 to check for correct PCR product size. Separation of DNA was achieved by electrophoresis at 60-140V for 30-120 minutes. The

bands were visualised using Ethidium Bromide and a UV transillumination 254nm wavelength).

2.2.6.3.3 PCR product clean up

The PCR product was cleaned up (to remove unwanted dNTPs and primer) by using Exosap with 9µl reaction comprising 2µl of Exosap, 2µl of dH20 and 5µl of PCR products with the following cycling conditions: 37°C for 30 min followed by 80°C for 15 min.

2.2.6.3.4 Sequencing reaction

The Exosaped PCR product was then sequenced in both forward and reverse direction using relevant primers. Each 10µl reaction was set up as follows:

- 4.5µl exosaped PCR products
- 0.5µl BigDye Reaction Mix
- 2.0µl 5X Sequencing buffer
- 1.0µL dH20
- 2.0µl forward or reverse primer (2µM)

The cycling conditions were 96°C for 3 minutes followed by 30 cycles of 96°C (30s), 50°C (15s) and 60°C (4 min).

2.2.6.3.5 Sequencing reaction clean-up preparation

Sequencing reactions were cleaned up to remove any unincorporated dye terminators. The EDTA method of precipitation was used. To the 10µl of sequencing reaction, 1µl EDTA (250nM) was added and mixed before adding 30µl of absolute ethanol. This was incubated

for 5-10min at room temperature and then centrifuged for 20min at 2000rpm at 20°C. The supernatant was removed by briefly spinning the plate upside down to a maximum of 400rpm. 90µl of 70% ethanol was then added, mixed and the plate centrifuged again for a further 10min at 2000rpm at 20°C. The supernatant was discarded by inverting the plate and spinning to 500rpm. The pellet was then left to air dry or place on heated plate for 2 minutes.

2.2.6.3.6 Preparation and analysis of sequencing reactions

Pellets were resuspended in 10µl Hi-Di Formamide and then denatured for 5 minutes before snap-cooling on ice. Sequencing reactions were run on the ABI 3730 DNA Analyzer. Analysed sequences were then analysed using Chromas software, printed and relative peak heights ratios at CpG sites were assessed manually.

2.2.6.4 Pyrosequencing of the *SNRPN* DMR at 15q13

The methylation status of the *SNRPN* DMR at 15q13 was performed by pyrosequencing the using the commercially available PyroMark Prader-Willi/Angelman kit by Biotage according to the manufacturer's protocol and using the Pyromark ID system.

Pyrosequencing is a method of sequencing DNA based on the "sequencing by synthesis" principle. It uses the detection of pyrophosphate release on nucleotide incorporation, rather than traditional Sanger sequencing which uses chain termination with dideoxynucleotides. It offers reliable quantification of allele representation and CpG methylation status with inbuilt control for bisulphite modification.

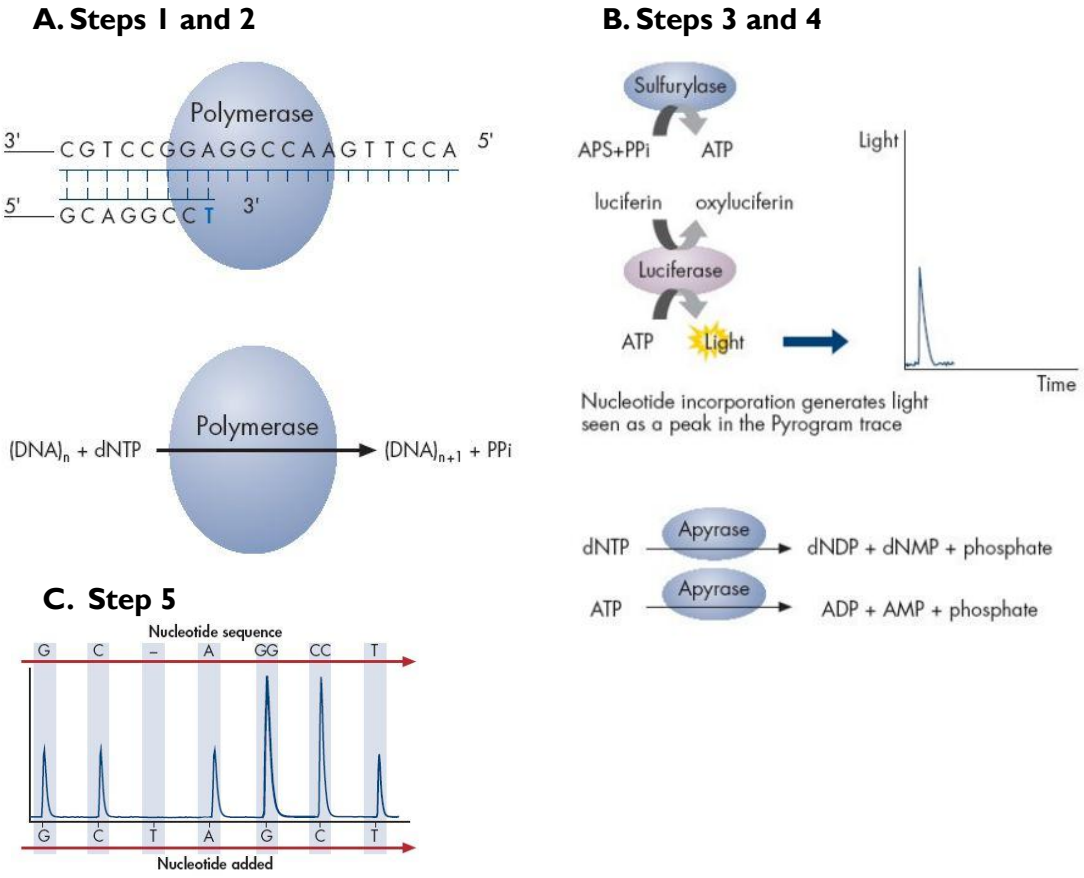


Figure 2-11 Principles of Pyrosequencing

A. Steps 1& 2 B. Steps 3 & 4 C. Step 5

The Pyrosequencing reaction involves five steps (www.qiagen.com):

Step 1

A sequencing primer is hybridized to a single-stranded PCR amplicon that serves as a template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase as well as the substrates, adenosine 5' phosphosulfate (APS), and luciferin (Figure 2-11. A).

Step 2

The first deoxribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribo-nucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide (Figure 2-11. A).

Step 3

ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) chip and seen as a peak in the raw data output (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated (Figure 2-11. B).

Step 4

Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added (Figure 2-11. B).

Step 5

Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alfa-thio triphosphate (dATP·S) is used as a substitute for the natural deoxyadenosine

triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace (Figure 2-11. C).

2.2.6.4.1 Preparation of reagents

Each PCR primer was dissolved in 120µl high purity water to give a concentration of 10uM. The sequencing primer was dissolved in 180µl Annealing Buffer to give a concentration of 10µM.

PCR amplification for pyrosequencing protocol

50µl PCR reactions were set up using Hot Star Taq DNA polymerase (Qiagen) with the following:

- 1µl of bisulphite modified DNA
- 1µl Forward primer (10uM)
- 1µl Reverse primer (10uM)
- dNTPs 5ul (2uM)
- 5µl 10X Buffer
- 0.4µl Hot Star Taq DNA polymerase
- 36.6µl dH20

PCR conditions were an initial denaturation of 95°C for 5 minutes followed by 50 cycles of 20 seconds denaturation at 95°C, 20 seconds annealing at 55°C and 20 seconds extension at 72°C. This was followed by a final extension step at 72°C for 5 minutes. Each set of PCR reactions included a negative control (in which dH20 was added instead of DNA) to check for contamination. PCR products were checked on 1.5% horizontal agarose gels to separate the PCR products and to ensure that the PCR reaction had worked without contamination (as described above).

2.2.6.4.2 Sample preparation

40µl of the biotinylated PCR product was immobilised on streptavidin-coated Sepharose beads (Streptavidin Sepharose™ High Performance, GE healthcare):

1. 40µl of PCR products was added onto a normal 96 well plate. 3µl of Sepharose beads and 37µl of Binding buffer were added to each well with the PCR products and the plate was sealed. The plate was incubated at room temperature on a mixer/shaker for 5 mintues to keep the beads dispersed.

2. 1.6µl of sequencing buffer (10µM) and 38.4µl of Annealing buffer were added to each well on a separate PSQ flat plate (PSQ96 Plate Low).

2.2.6.4.3 Pyrosequencer protocol

The pyrosequencer was switched on 1 hour before use. The Pyromark ID software system was launched on the attached computer and the following sequence to analyse was entered into Pyromark ID:

G/ACTCCATCG/ACG/ATCACG/AACCG/ACT

Boxed nucleotides indicate control for completion of bisulphite treatment.

The following nucleotide dispensation order was used:

TGAGCTCATCGATCGAGTCACTGATCGAGC

This setting was saved as a SNP run (Saved as PWS/AS).

Figure 2-12: Vacuum Prep Worktable layout.

2.2.6.4.4 Vacuum machine stand preparation protocol

1. Five of the plastic troughs were placed onto the Vacuum Prep Worktable (Figure 2-12).

2. The troughs were filled with approximately 180ml of their respective solution (High purity H20, 70% Ethanol, 1X Washing Buffer), except for the trough for Denaturation solution (0.2mMNaOH) which was filled with approximately 120ml.

3. The flat well PSQ plate with sequencing primer and buffer was placed in its position on the vacuum prep worktable (labelled in Figure 2-12) with well A1 to the front left at the designated position on the machine.

4. The 96 well plate with PCR products and beads was placed onto the designated slot on the Vacuum Prep Woktable with well A1 to the front and left.

5. The vacuum switch was closed (Off) and the vacuum pump was started.

6. The vacuum was applied to the Vacuum Prep Tool by operating the vacuum switch (On).

7. The probes on the Vacuum Prep Tool were washed by lowering the tool into high purity water (Parking position) for approximately 20 seconds ensuring that proper vacuum has been attained by checking that the arm of the vacuum gauge has moved beyond the red zone.

8. The beads containing immobilised templates were captured on the filter probes by slowly lowering the Vacuum Prep toll into the PCR plate. (Capturing of the beads

must take place within three minutes of the agitation being terminated as sepharose beads sediment quickly)

9. I ensured that the liquid has been aspirated evenly from all wells and that all beads have been captured onto the probe tips. The surface of the remaining solution should be level with or lower than the filter probe tips, when they are resting on the bottom of the well.

10. The Vacuum Prep Tool was moved to the trough containing 70% ethanol (trough1) and the solution was allowed to flush through the filters for 5 seconds.

11. The tool was then moved to Denaturation solution (trough2) and allowed to flush through the filters for 5 seconds.

12. The tool was then moved to Washing buffer (trough 3) and allowed to flush through the filters for 5 seconds.

13. The tool was picked up and moved beyond 90° vertical and held for a few seconds before returning to the horizontal position to allow the liquid to completely drain from the probe.

14. The vacuum was closed (Off) to release the vacuum when the tool is above the flat well PSQ96 plate.

15. The beads were released into the flat well PSQ96 plate. The Vacuum Prep tool was shaken while allowing the filter probed to rest on the bottom of the wells.

16. The Vacuum Prep tool was removed from the plate and the pump switched on again and placed into High Purity Water (trough 4) for 5 seconds then lifted to beyond 90° degree vertical then replaced into the parking position and the both the vacuum and pump switched off.

The steps above are summarized in Figure 2-13 below

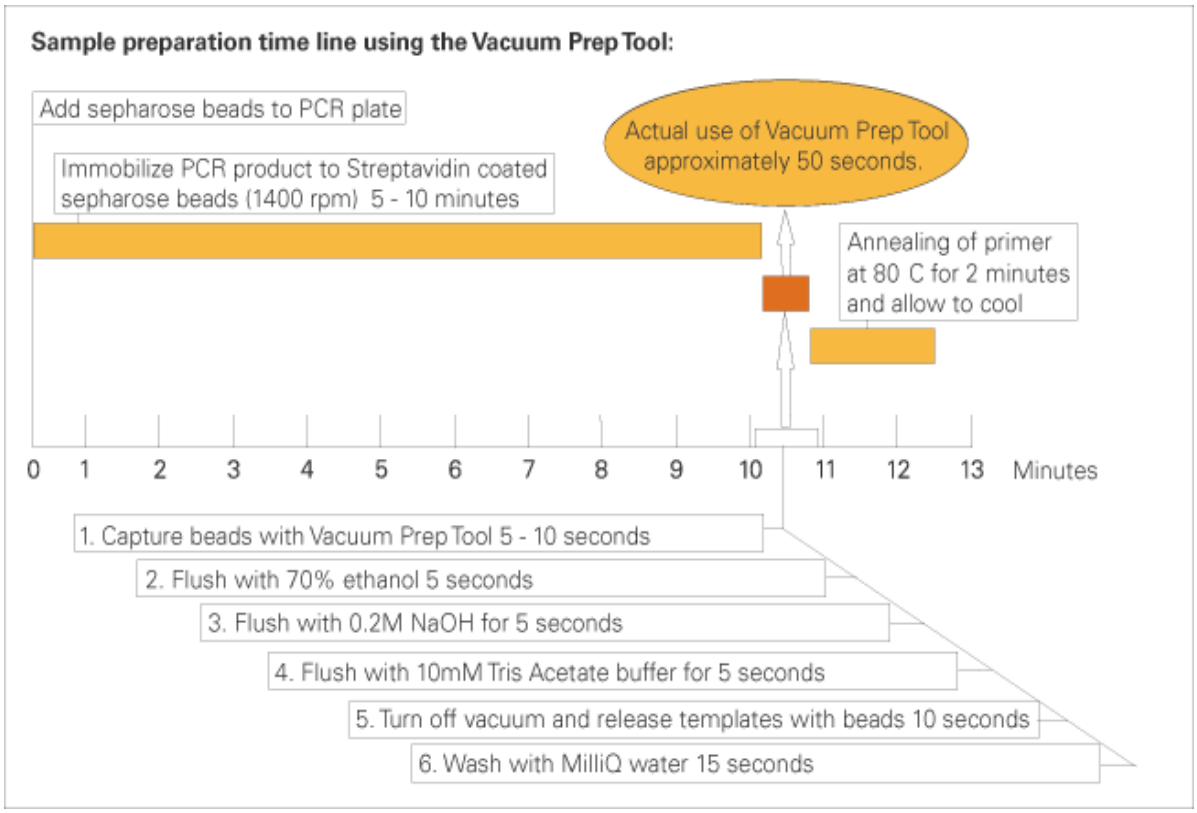


Figure 2-13 Sample preparation time line using the Vacuum Prep Tool

2.2.6.4.5 Primer Annealing

1. The plate with the samples was heated at 80°C for 2 minutes using the PSQ 96 Sample Prep Thermoplate Low.

2. The plate was removed from the heating block and then left to cool to room temperature.

2.2.6.4.6 Pyrosequencing Reaction

1. SNP runs was selected from the General Tab. In the Setup menu, the wells used on the 96well plate were selected for analysis. The selection Entry was selected and the saved PWS/AS assay was chosen followed by Sample ID (which will print the well number of the report) followed by Save.

2. The Pyromark Q96 Cartridge was loaded with Enzyme, Substrate, A, C, G, T (dNTPs) (Figure 2-14) from the Pyromark Gold Reagents Kit with volumes indicated on the computer during run Setup (Click View on General Tab).

3. The PSQ96 Plate with the samples and the filled Pyromark Q96 Cartridge was loaded for analysis in the PSQ 96 instrument.

4. The run was started by clicking Run.

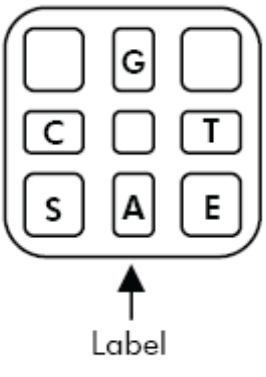


Figure 2-14 Illustration of the Pyromark Q96 Cartridge seen from above

Add enzyme mixture (E), substrate mixture (S), and nucleotides (A,T,C,G) according to the volume information provided by the Pyromark ID software during run setup

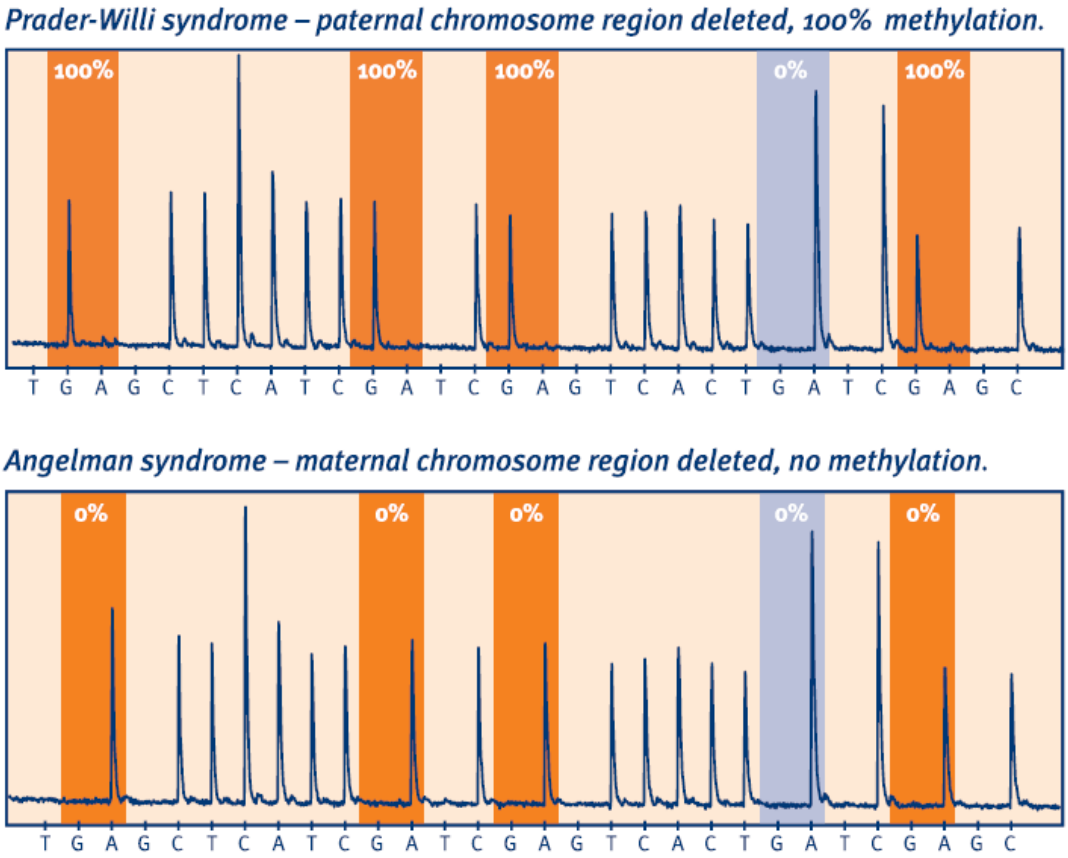


Figure 2-15 Illustration of PyroMark Prader-Willi/Angelman Syndroms assay results

Orange columns indicate quantified CpG sites. Blue columns indicate QC for completion of bisulfite treatment (a C followed by an A in the original sequence). The pyogram shows the reverse assay of the top strand (adapted from www.biotage.com)

2.2.6.4.7 Pyrosequencing results

Once run is complete, results were viewed using the Pyromark ID software according to the manufactures instructions. An example of analysis from the manufacturer for the 15q region (PWS and AS) is shown in Figure 2-15.

2.2.6.5 Methylation Specific Polymerase Chain Reaction (MS-PCR) of *PLAGL1* DMR at 6q24 and *DLK1* IG-DMR at 14q32

Methylation status at the *PLAGL1* CpG island at 6q24 and *DLK1* IG-DMR at 14q32 were analyzed using methylation-specific PCR (MS-PCR) using published primers (Mackay *et al.*, 2005; Temple *et al.*, 2007)

MS-PCR utilises the divergent sequence changes as a result of bisulphite modification of differentially methylated DNA, yielding different size product with a ratio that reflects that of the initial starting material. The reaction contained a forward primer and divergent reverse primers.

2.2.6.5.1 PLAGL1 DMR

Forward *PLAGL1*–fam CACRACATCTACCATTTATCATTCAACC [R=A/G]

Reverse *PLAGL1* –unmeth GGTTATGATGGTGATTTGGGGAAGTGTTTTG

Reverse *PLAGL1*-meth TTCGGGGAAGCGTTTCGCGCGTTAAGGTT

The amplification reaction with a final volume of 10µl with Hot Star Taq was set up using:

- 0.2µl Forward primer (20µM *PLAGL1*-fam)
- 0.2µl Reverse Methylated primer (20µM *PLAGL1*-meth)
- 0.2µl Reverse Unmethylated primer (25µM *PLAGL1*-unmeth)
- 1µl dNTPs (2µM)
- 0.8µl MgCL2
- 1µl 10X buffer
- 0.2ul Hot Star Taq
- 5.4ul dH20

- 1ul Bisulphite modified DNA

The following cycling conditions were used: Initial denaturation 95°C for 15min followed by 29 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30s, extension at 72°C for 30s. This was then followed by a final extension at 72°C for 5min.

2.2.6.5.2 *DLK1* IG DMR

GTL2- fam CTCCAACAACAAAACCCAAAATCAAACAAACTCTC.

*GTL2*b-unmeth GTGTAGATGGTGGAGAGTAGAGAGGGAGTGT

*GTL2*b-meth CGCGTTTTGGTTCGTTGGTTTTGGCGGCG.

The amplification reaction with a final volume of 10µl with Hot Star Taq were set up using:

- 0.2µl Forward primer (25uM GTL2-fam)
- 0.2µl Reverse Methylated primer (25uM GTL2b-meth)
- 0.2µl Reverse Unmethylated primer (25uM GTL2b-unmeth)
- 1µl dNTPs (2uM)
- 0.8µl MgCL2
- 1µl 10X buffer
- 0.2µl Hot Star Taq
- 5.4µl dH20
- 1µl Bisulphite modified DNA

The following cycling conditions were used: Initial denaturation 95°C for 15min followed by 28 cycles of denaturation at 95°C for 20s, annealing at 60°C for 20s, extension at72°C for 20s. This was then followed by a final extension at 72°C for 5min.

2.2.6.5.3 Genescan of both *PLAGL1* and *DLK1*

The following steps were used

1. 2.5µl of Genescan LIZ was added to 500µl of Hi-Di followed by a quick mix.
2. 140µl of dH20 is added to the PCR products.
3. Using a new plate, 10µl of Hi-Di/LIZ was added to each well.
4. 1µl of diluted PCR products was added to the wells in the plate.
5. The plate and samples was then denatured at 90°C for 5 minutes then snap cooled in ice.

Methylated and unmethylated product sizes were visualised on a ABI PRISM GeneMapper software version 3.0 according to the manufacturer's instruction using size standard GS500LIZ. Peaks were inspected and peak heights <100 or >8000 pixels were discarded.

2.2.7 CALCULATION OF METHYLATION INDICES

All experiments (bisulphite sequencing, MS-PCR, Pyrosequencing) were run in triplicate on at least 2 different bisulphite modified DNA for each sample. Each experiment included a normal control (to validate results) and a negative control (to rule out contamination).

Quantitative measurement of methylation levels was not possible for the *PEG1* DMR due to the technique of bisulphite sequencing which only generates qualitative data. Quantitative measurement of methylation levels using MS-PCR and Pyrosequencing was possible. For each DMR analysed, samples from 20 normal controls were used to determine the normal distribution of methylation indices (methylated:unmethylated).

For MS-PCR of *PLAGL1* DMR and *DLK1* IG-DMR, the methylation index is defined as the ratio of the area under the curve of the methylated peak to the area under the curve of the unmethylated peak.

For Pyrosequencing of the *SNRPN* DMR, the methylation index is defined as the ratio of quantified G:A alleles at each CpG on the reverse strand.

For both techniques, hypomethylation (or loss of methylation) is defined as a ratio of more than two standard deviations below the mean value.

2.2.8 STATISTICAL ANALYSIS

Fisher Exact testing, Wilcoxon-Rank sum, t-testing and Kaplan-Meier analysis were used as appropriate. Statistical significance was taken at the 5% level.

2.3 RESULTS

2.3.1 PATIENT DEMOGRAPHICS

2.3.1.1 Naturally conceived BWS IC2 epimutation patients

87 BWS children without a history of ART with an IC2 epimutation (KvDMR1 LOM of the maternal allele) tested at the West Midlands Regional Genetics laboratory were identified. The mean age of this cohort is 6.0 years. There were 42 males and 45 females in this group. The 87 children were from 87 pregnancies. There were 2 twin and 1 triplet pregnancies (all co-twins and co-triplets were unaffected).

2.3.1.2 ART conceived BWS patients

There were 25 affected BWS patients that were conceived with ART (12 IVF and 13 ICSI). The mean age of the group was 3.4 years. There were a total of 14 males and 11 females BWS patients in this group. The 25 patients were from 23 pregnancies with 10 twins (two affected twin pairs and six twins with no clinical evidence of BWS in their co-twins).

2.3.2 MOLECULAR ANALYSIS OF ART CONCEIVED BWS PATIENTS

IC2 epimutation is the predominant molecular cause of BWS in post-ART BWS patients

24/25 (96%) post-ART patients were found to have a molecular genetic diagnosis of BWS with IC2 epimutation (LOM of maternal KVDMR1) found in all these 24 patients. In addition they had at least 2 or more clinical features. The one patient without a molecular diagnosis had clinical features of macroglossia, macrosomia, umbilical hernia, ear lobe creases and mild speech and language delay, thus fitting the clinical diagnosis of BWS.

2.3.3 COMPARISON OF POST-ART AND NATURALLY CONCEIVED BWS WITH IC2 EPIMUTATIONS

The phenotype of the 24 post ART BWS cases with IC2 epimutations was compared with the 87 naturally conceived BWS IC2 epimutation cases.

2.3.3.1 Methylation Index

The calculated mean methylation index for KvDMR1 in the post-ART group and naturally conceived group were 4.6% (range 0-18) and 7.6% (range 0-13) respectively. There was no statistically significant difference (P=0.6) between the two groups. The operational diagnostic threshold for KvDMR1 LOM in the West Midlands Regional Genetics Laboratory is a methylation index of <20% following robust validation comparing normal controls and known positive controls.

2.3.3.2 Clinical Features: Differences in the frequencies of exomphalos and facial naevus flammeus

Table 6 summarises the frequencies of clinical features in the 2 groups.

There was no statistically significant difference in the frequencies of the occurrence of neonatal hypoglycaemia, macroglossia, ear lobe creases, intellectual disability and hemihypertrophy in both groups.

Facial naevus flammeus occurred more frequently in the ART conceived BWS cases. By contrast, exomphalos occurred less frequently in the ART conceived BWS cases.

Table 6. Frequency of clinical features in ART conceived BWS patients (ART Group) and naturally conceived BWS patients (Non-ART group)

	ART Group	Non-ART Group	
Clinical Feature	**Frequency (%)**	**Frequency (%)**	**P Value**
Neonatal Hypoglycaemia	50	50	NS
Macroglossia	89	87	NS
Ear Creases	56	65	NS
Hemihypertrophy	15	16	NS
Intellectual Disability	4	4	NS
Facial Naevus Flammeus	88	46	P=0.002
Exomphalos	39	69	P=0.015

Exomphalos occurred less frequently in the ART group. Facial naevus flammeus occurred more commonly in the ART group. Statistical significance was taken at the 5% level.

2.3.3.3 Embryonal Tumours: Increased risk in ART conceived BWS

2/24 (8.3%) of the ART conceived BWS patients developed tumours whereas no tumours occurred in the naturally conceived BWS group. One post IVF child developed a hepatoblastoma at the age of 8 months and one post ICSI child developed a rhabdomyosarcoma at the age of 9 months. No Wilms tumour developed in both groups.

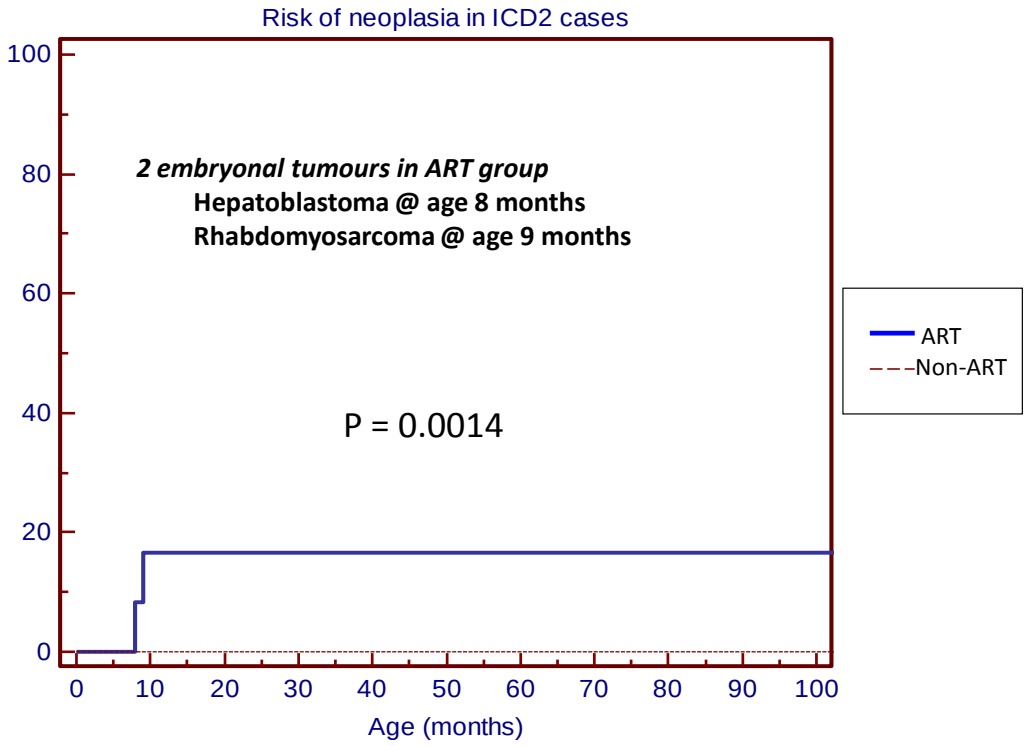


Figure 2-16 Kaplan-Meier curve comparing risk of embryonal tumours in IC2 epimutation BWS cases

A statistically significant increased risk of embryonal tumours was demonstrated in the ART group (Log rank X^2 = 10.18, P=0.0014)

Kaplan-Meier analysis (Figure 2-16) of tumour risk in the 24 ART conceived BWS cases (mean age 3.4 years) and the 87 naturally conceived BWS cases (mean age 6.0 years) demonstrated a significantly increased risk in the ART cases (log rank X^2 = 10.18, P=0.0014).

2.3.4 METHYLATION PROFILING OF BWS IC2 EPIMUTATION CASES

2.3.4.1 Analysis of maternally methylated DMRs

A cohort of 55 BWS IC2 epimutation cases (8 ART conceived and 47 naturally conceived BWS) in whom sufficient DNA was available, were analyzed to look at the methylation status at the following imprinted DMRs:

1. *PEG1* at 7q32
2. *PLAGL1* at 6q24
3. *SNRPN* at 15q13

Unfortunately, DNA from the two children with tumours was not available for analysis.

A total of 6 BWS IC2 epimutation patients were found to have LOM at additional DMRs. 3 were from the post-ART group and 3 from the naturally conceived group. Figure 2-17, Figure 2-18, and Figure 2-19, show examples of the results of the methylation analysis at the 3 DMRs affected. Figure 2-20 is a diagrammatic representation of the methylation analysis results.

In the ART group the prevalence of additional LOM is 37.5% (3/8). One post ICSI child demonstrated additional LOM at 2 DMRs; the *PEG1* and *SNRPN* DMRs. Another ICSI child had additional LOM at the *PLAGL1* DMR. An IVF child had additional LOM at *PEG1* DMR. In the naturally conceived group the prevalence of additional LOM is 6.4% (3/47). Two children had additional LOM at the *PEG1* DMR and one child had additional LOM at the *PLAGL1* DMR.

The frequency of additional LOM was found to be significantly higher in the ART conceived cases compared to naturally conceived cases (37.5% vs 6.4% P=0.034).

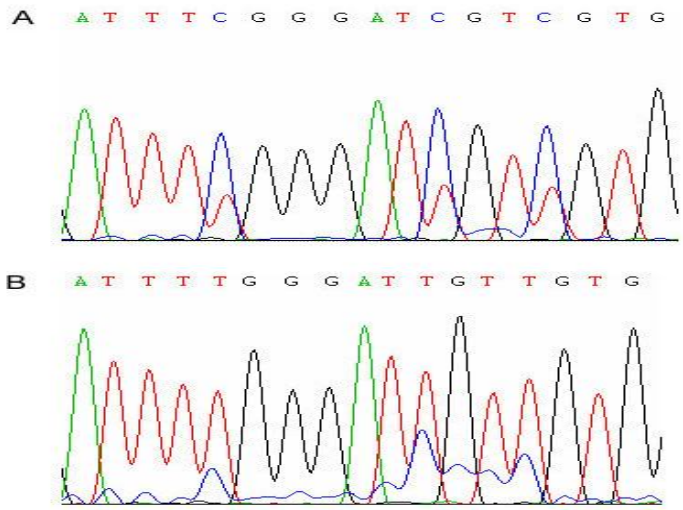


Figure 2-17 Loss of methylation at *PEG1* DMR in a patient with Beckwith-Wiedemann syndrome

Bisulphite genomic sequencing trace at the *PEG1* DMR. (A) Normal control. DNA sequencing shows both a cytosine and a thymine peak at CpG sites 11, 12 and 13, representing methylated and unmethlated alleles respectively, with taller cytosine peaks. (B) Patient with LOM at *PEG1*. DNA sequencing shows reversal of the expected peaks with taller thymine peaks at CpG sites 11, 12 and 13, indicating a loss of methylation. [(Lim *et al.*, 2009a) by permission of Oxford University Press]

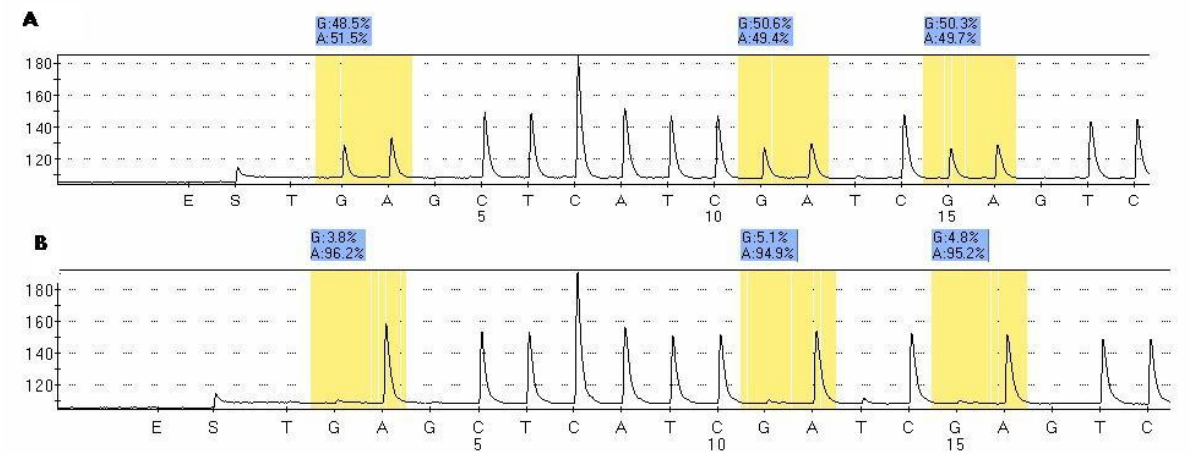


Figure 2-18 Loss of methylation at *SNRPN* DMR in a patient with BWS

Reverse strand pyrosequencing trace. Percentage of methylated cytosines is represented as the percentage of guanine (G) and the percentage of unmethylated cytosines which normally will be represented by thymine is represented by alanine (A) on the reverse strand. (**A**) Normal control, (**B**) patient with LOM at *SNRPN* [(Lim *et al.*, 2009a) by permission of Oxford University Press]

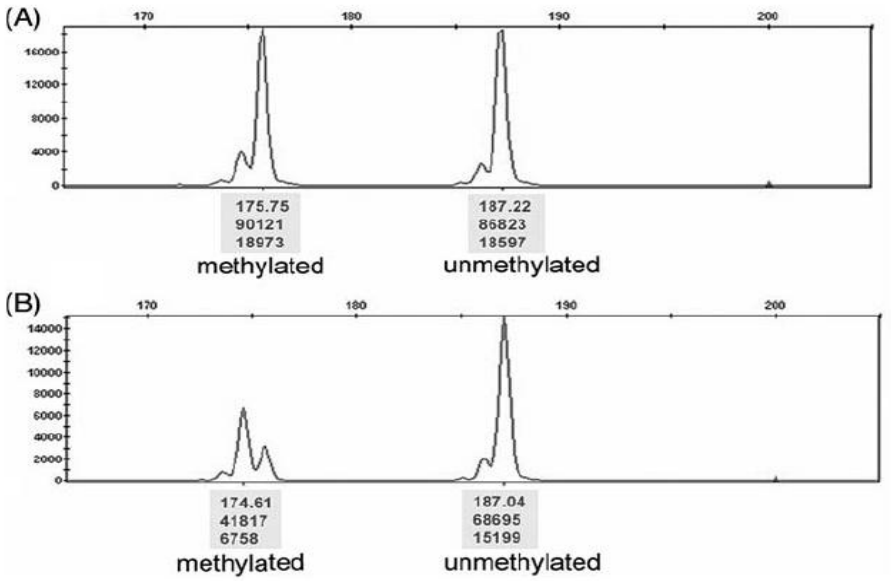


Figure 2-19 Loss of methylation at *PLAGL1* DMR in a patient with BWS

Electropherogram of amplification products of MS-PCR. The X axis represents the calculated product size (in bp and also represented as the top number in the box). The Y axis represents the peak height (bottom number in the box). The methylated to unmethylated ratio was calculated as the area under the curve (middle number in the box) of methylated versus unmethylated amplified products. (**A**) Normal Control (ratio 1.04) (**B**) Patient with LOM at *PLAGL1* DMR (ratio 0.61) [(Lim *et al.*, 2009a) by permission of Oxford University Press]

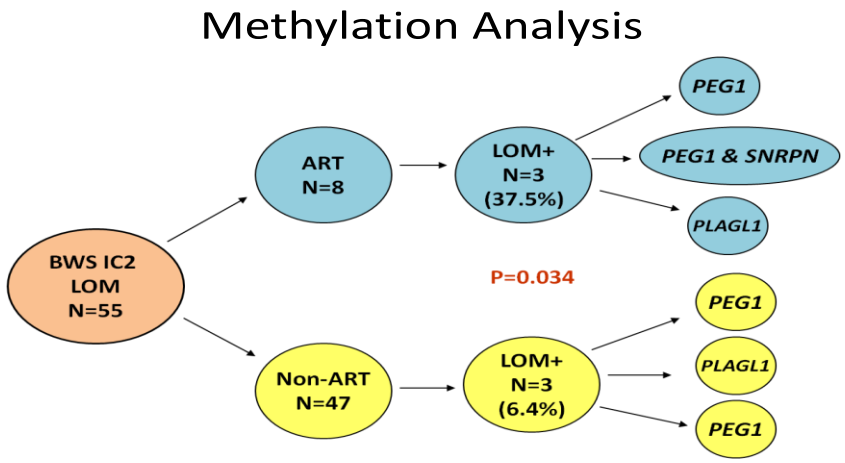


Figure 2-20 Diagrammatic representation of methylation analysis results in BWS IC2 epimutation cases

The frequency of loss of methylation at additional DMRs was significantly higher in the assisted reproductive technologies group. ART: Assisted reproductive technologies. LOM+ : loss of methylation at additional DMRs.

2.3.4.2 Analysis of paternally methylated DMR

The methylation status at the *DLK1* DMR at 14q32 were analysed in the 6 cases found to have multiple maternal DMR hypomethylation above. None of these 6 cases had methylation abnormalities at this DMR.

2.3.4.3 Analysis of clinical features of additional LOM cases

No major phenotypic differences were evident between the children with LOM at additional loci and those without this epigenotype. Table 7 summarises the clinical and molecular characteristics of the 6 cases with additional LOM.

In two of the three naturally conceived BWS cases with LOM at additional loci, there were no reports of fertility problems in the parents or the use of ovarian stimulation. Unfortunately, there was no information on the use of ovarian stimulation in the 3rd couple apart from the child was naturally conceived.

Table 7. Clinical and molecular characteristics of Imprinting Centre 2 epimutation Beckwith-Wiedemann syndrome patients with additional loss of methylation at other imprinted loci

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Sex	M	F	M	F	M	M
ART	IVF	ICSI	ICSI	No	No	No
Pregnancy	Singleton	Singleton	Twin	Singleton	Singleton	Singleton
Macrosomia	No	NR	No	No	Yes	Yes
Exomphalos	Yes	No	No	Yes	Yes	No
Umbilical Hernia	No	Yes	Yes	No	No	Yes
Macroglossia	No	Yes	Yes	Yes	Yes	Yes
Hemihypertrophy	Yes	No	No	No	No	Yes
Embryonal Tumour	No	No	No	No	No	No
Ear creases	Yes	No	Yes	NR	Yes	Yes
Neonatal Hypoglycaemia	No	Yes	Yes	Yes	Yes	Yes
Facial Naevus Flammeus	Yes	Yes	Yes	No	Yes	Yes
Intellectual Disability	No	No	No	No	No	No
6q24 (*PLAGL1*) methylation	Normal	Normal	LOM	Normal	Normal	LOM
MI N= (0.65-1.78)	1.25	1.23	0.55	1.22	0.93	0.61
7q32 (*PEG1*) methylation	LOM	LOM	Normal	LOM	LOM	Normal
15q13 (*SNRPN*) methylation	Normal	LOM	Normal	Normal	Normal	Normal
MI N= (0.55-1.1)	0.84	0.05	0.98	0.82	0.77	0.89
14q32 (*DLK1*) methylation	Normal	Normal	Normal	Normal	Normal	Normal
MI N= (0.5-1.4)	0.91	0.82	0.83	0.85	0.76	0.88
11p15.5 (KvDMR1) methylation	LOM	LOM	LOM	LOM	LOM	LOM
MI	0.04	0	0	0.12	0	0.02

M, male; F, female; IVF, in vitro fertilisation; ICSI, intra-cytoplasmic sperm injection; LOM, loss of methylation; MI, methylation index; NR, not recorded; N, normal range [(Lim *et al.*, 2009a) by permission of Oxford University Press]

2.4 DISCUSSION

Our BWS research group in Birmingham previously reported that six out of 149 UK children with BWS had a history of conception using ART (4% of BWS cases versus 1.2% of the general population, P=0.009)(Maher *et al.*, 2003). Groups in USA and France also reported similar findings in their BWS cohorts (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003). Subsequent studies has shown a 9 to 10 fold increased risk of BWS following ART compared to natural conceptions (Halliday *et al.*, 2004; Hiura *et al.*, 2012). This project identified IC2 epimutations in 24 of 25 post ART BWS patients. Previously IC2 epimutations was described in 11 of 12 ART conceived BWS patients by 2 groups (DeBaun *et al.*, 2003; Gicquel *et al.*, 2003). Therefore, IC2 epimutations appear to occur more commonly in ART conceived BWS cases then expected when compared to the 50% expected in unselected BWS cases (Cooper *et al.*, 2005). This provides further evidence for a causal link between ART and IC2 epimutations. However, the cause for this association remains unclear.

The process of ART with IVF requires oocyte collection and *in vitro* culture before the embryos are implanted in the womb. ICSI requires an additional step of direct injection of the sperm into the ovum. Many parts of the ART process could potentially affect the developing epigenome. These include artificial hyperstimulation of ovulation, the culture conditions of the embryos, effect of embryo cryopreservation and timing of embryo transfer. The pattern of mosaicism of the imprinting defects suggests they occurred post-fertilisation by some mechanism that impairs the maintenance of imprints. The underlying mechanism allowing failure to protect imprinted loci against demethylation is unclear but suggests that it may fail in ART causing tissue-specific loss of imprints (Chiba *et al.*, 2013).

ART is associated with an increased relative risk of imprinting disorders although the absolute risk is small. Experiments with animals suggest that in vitro embryo culture may be associated with epigenetic alterations (Dean *et al.*, 1998; Khosla *et al.*, 2001; Reik *et al.*, 1993). In particular, the Large Offspring syndrome in cattle and sheep undergoing ART has similar phenotypic similarities with BWS and have been associated with loss of maternal allele methylation at the *IGF2R* DMR (Young *et al.*, 2001) and more recently IC2 epimutations (Chen *et al.*, 2015). However, it has been argued that either the initial infertility or even the use of ovarian stimulation may predispose to epigenetic errors (Ludwig *et al.*, 2005).

The underlying cause of infertility in the couple should also be considered as a potential cause of the methylation disturbance. Couples requesting ART have reduced fertility, increased reproductive loss rate and are usually of advanced parental age. These can all be associated with various congenital and developmental anomalies. There are reports that some infertile men with oligozoospermia have pre-existing imprinting errors in their sperm (Kobayashi *et al.*, 2007; Marques *et al.*, 2004). Therefore there are many confounding factors that make it difficult to be certain of the role of ART from the underlying fertility problems. Unfortunately in my study, insufficient data and difficulty obtaining further information on the rates of miscarriage or years trying to conceive in both cohorts made it difficult to study this hypothesis.

Some insights into the association into infertility as a cause of perturbation in genomic imprinting come from studies into familial biparental complete hydatidiform molar

pregnancies where biallelic mutations in a maternal effect gene *NLRP7* in the female predisposes to their conceptions being at high risk of reproductive wastage and biparental hydatidiform moles (Murdoch *et al.*, 2006). Together with my colleague Dr Esther Meyer, we reported that in a consanguineous family with a recurrence of BWS in a pair of siblings due to IC2 epimutation, homozygous mutations in the maternal effect gene *NLRP2* in the mother was found to be the underlying cause (Meyer *et al.*, 2009). MLID was present in one of the affected BWS siblings and the mother also had a hydatidiform molar pregnancy. More recently, mutations in the *NLRP5* gene in females have been described as another maternal effect gene that can cause MLID and infertility and reproductive wastage in their conceptions (Docherty *et al.*, 2015). The MLID cases reported by Docherty include clinical phenotypes of BWS (IC2 epimutation), SRS (H19 hypomethylation) as well as clinically non-specific –MLID. The NLRP5 protein is known to be a component of the subcortical maternal complex (SCMC) of proteins which also includes KHDC3L, TLE6 and OOEP (Zhu *et al.*, 2015). The SCMC proteins have been shown to be important for progression of development beyond the first zygotic cell divisions. Interestingly *KHDC3L* (previously known as *C6orf221*), is also a maternal effect gene which causes females with biallelic mutations to develop high rates of pregnancy loss and biparental complete hydatidiform moles (Parry *et al.*, 2011). Therefore these observations in *NLRP5* and *KHDC3L* suggest a role of the SCMC in postzygotic maintenance of DNA methylation at imprinted genes. It will be interesting to see if NLRP2 and/or NLRP7 turn out to be either further components of the SCMC or influences function of the SCMC. NLRP2 and NLRP7 have similar NACHT nucleotide binding domain and

Leucine-rich repeat (LRR) domains as NLRP5 and other proteins in the NLRP family (Meyer *et al.*, 2009).

These maternal effect genes *NLRP2*, *NLRP5* and *NLRP7* are examples of *trans*-acting genes that appear to regulate genomic imprinting in their offspring. This is different to the *trans*-acting gene *ZFP57* in which mutations predisposes to TNDM with MLID in the affected probands themselves (Mackay *et al.*, 2008). The emerging role of the SCMC in human developing embryos may also provide insights into the role of ART in imprinting disorders looking in particular at the various processes of ART and their effects on the SCMC increasing the risk of certain imprinting disorders or MLID.

Genotype-phenotype correlations with regards to Wilms tumour and exomphalos in BWS have been described previously by a number of groups (Bliek *et al.*, 2001; Cooper *et al.*, 2005; DeBaun *et al.*, 2002; Engel *et al.*, 2000; Gaston *et al.*, 2001; Ibrahim *et al.*, 2014; Lam *et al.*, 1999; Maas *et al.*, 2016; Mussa *et al.*, 2015; Sparago *et al.*, 2007; Weksberg *et al.*, 2001). BWS patients due to UPD or IC1 epimutation (H19 DMR gain of methylation) are at significantly increased risk of Wilms tumour. To date, Wilms tumour has very rarely been reported in BWS patients with IC2 epimutations or with mutations in *CDKN1C*. By contrast, exomphalos occurs more commonly in IC2 epimutation and *CDKN1C* mutation cases but is rarely seen in UPD or IC1 epimutation cases.

Chang and colleagues previously reported no phenotypic differences between post-ART and naturally conceived BWS cases (Chang *et al.*, 2005). However, they did not compare like with like in terms of looking at the molecular causes in the naturally conceived group. As

described above, because the predominant molecular subtype involved in ART conceived BWS cases is an IC2 epimutation, in this project I compared this group with a control cohort of naturally conceived BWS cases with IC2 epimutations. Therefore a more direct comparison can be made to draw conclusions from. My data suggest that the ART conceived cases had a significantly lower risk of exomphalos compared to naturally conceived cases. In addition, there is a significantly increased risk of non-Wilms tumour neoplasia risk in the ART conceived group. The increased neoplasia risk, although statistically significant, should be considered a preliminary finding as it is based on only two cases. Caution should be exercised before recommending any surveillance in the ART cohort for these as the numbers are small. In addition, the effectiveness of surveillance for such tumours has to be considered.

No Wilms tumour has been identified in both naturally conceived and ART-conceived BWS cases in this study. This provides further evidence that Wilms tumour surveillance is not indicated in BWS IC2 epimutation cases.

It is interesting to note that a childhood tumour was present in two of the 19 post-ART conceived BWS children reported by Chang and colleagues (Chang *et al.*, 2005). Rossignol and colleagues reported 2 of 11 BWS children with LOM at multiple loci developed an embryonal tumour (a rhabdomysarcoma and a hepatoblastoma) but neither of these 2 cases were conceived using ART (Rossignol *et al.*, 2006). Due to lack of DNA, I was unable to test the 2 ART conceived BWS cases who develop non-Wilms tumours in my project, to determine if they had LOM at multiple loci. However, it is hoped that our finding will prompt

other groups to examine their data to better define the relationship between ART and MLID in BWS children with non-Wilms tumour neoplasia.

With regards to the lower incidence of exomphalos in the ART group, although it might be suggested that less severe KvDMR1 hypomethylation in the ART conceived cases might lead to a milder phenotype, comparison of KvDMR1 methylation indices from DNA extracted from peripheral leucocytes in the two groups did not show any statistically significant difference. However, methylation patterns may differ in different tissues. Nevertheless, this explanation would not account for the possible higher risk of neoplasia in the ART conceived cases in this study.

The finding that BWS children with IC2 epimutations might also display loss of methylation at multiple imprinted loci was reported initially by Rossignol and colleagues, Bliek and colleagues and subsequently by Azzi and colleagues (Azzi *et al.*, 2009; Bliek *et al.*, 2009; Rossignol *et al.*, 2006). In this study, I found significantly higher frequencies of loss of methylation at non-11p15.5 DMRS in the ART conceived cases compared to naturally conceived cases. This is in contrast with the Rossignol study and Azzi study which found similar rates in both groups. The Bliek study did report among the 16 BWS IC2 epimutations patients they detected with MLID, one child born following IVF. However, the overall number of BWS cases which were conceived with ART tested in their cohort was not reported to make comparisons.

Table 8. Studies reporting Multi-Locus Imprinting Disturbance (MLID) in BWS

	Rossignol et al 2006	Bliek et al 2009	Azzi et al 2009	Tee et al 2013	Poole et al 2013	Court et al 2013	Maeda et al 2014	Lim et al 2009 (this thesis)
No of BWS cases	40 IC2	81 IC2	68 IC2	187 IC2	21 IC2	42 IC2	44 IC2	55 IC2
No of imprinted DMRs tested	4	9	7	3	12	27	29	3
Number of MLID cases	10/40 (25%)	17/81 (21%)	16/68 (24%)	33/187 (18%)	8/21 (38%)	14/42 (33%)	15/44 (34%)	6/55 (11%)
ART with MLID	3/11 (27%)	1 ART case (total unknown)	4/12 (25%)	7/14 (50%)	0/2 (0%)	Not specified	2/5 (40%)	3/8 (37.5%)
Non-ART with MLID	7/24 (29%)	16 non-ART (total not clear)	12/56 (21%)	26/173 (15%)	8/12 (42%)	Not specified	13/39 (33.3%)	3/47 (6.4%)

Subsequently, more studies have been published with increasing number of imprinted DMRs tested in BWS patients (Court *et al.*, 2013; Maeda *et al.*, 2014; Poole *et al.*, 2013; Tee *et al.*, 2013). Comparison of the results of this thesis with the other published studies looking at incidence of MLID in BWS including ART and number of imprinted DMRs tested are summarised in Table 8. There continues to be variability in the incidence of MLID comparing ART and naturally conceived BWS IC2 cases. The reasons for this variability may be due to ascertainment bias and size of cohort studied, number of imprinted DMRs studied and the technique used to detect the methylation changes with certain techniques having better sensitivity at detecting mosaic methylation changes.

The hypothesis remains consistent that differences in the phenotype between ART conceived and naturally conceived BWS patients might be caused by epigenetic differences at these additional imprinted DMRs. Further analysis of a larger number of DMRs in more extensive cohorts of patients should provide further information on the relative frequency of hypomethylation at different loci in ART conceived and naturally conceived BWS patients.

The additional DMR loss of methylation found in this study has been at DMRs which are normally methylated in the maternal allele. Due to limited amount of DNA available, the methylation status at the *DLK*-IG DMR on 14q32 was only tested in the 6 patients found to have additional loci LOM and there was no abnormal methylation levels detected. This DMR is normally methylated on the paternal allele. Our finding agrees with the finding in the Bliek study which also did not find any additional LOM affecting this DMR in their BWS cohort. In addition, a study of another imprinting disorder, transient neonatal diabetes mellitus

(TNDM) due to abnormal imprinting at *PLAGL1* revealed that loss of methylation at additional loci also occurred in this group of patients but also restricted to normally maternally methylated DMRs (Mackay *et al.*, 2008). They studied the *DLK*-IG DMR and did not find any abnormalities as well. Our results provide further evidence that the maternal allele methylated DMRs are more susceptible to epigenetic alterations. Subsequent to completion of this BWS project, it has now been reported that both loss and gain of methylation in both paternal and maternal allele methylated DMRs have been identified in BWS patients with MLID. *ZBFDF2*, *NESP* and *ZNF597/NAA60* are paternally methylated DMRs that have been reported to gain methylation (Court *et al.*, 2013; Maeda *et al.*, 2014). These gains of methylation have been reported to be due to a concomitant loss of methylation of the *GPR1-AS*, *GNAS* and *ZNF597* DMRs (all maternally methylated) nearby which have been shown to regulate methylation of these somatic DMRs in a hierarchical fashion (Court *et al.*, 2014; Kobayashi *et al.*, 2013; Liu *et al.*, 2005). To date the most frequent DMRs affected in BWS patients with MLID are the *PLAGL1*, *GRB10*, *PEG1/MEST*, *GNAS*, *IGF2R* and *ZNF331* (Court *et al*., 2013; Docherty *et al*., 2014; Tee *et al*., 2013).

Analysing the phenotypes of naturally conceived cases with and without MLID did not identify any differences. However, the numbers were small. It will be interesting to compare the phenotypes of BWS cases with MLID with other imprinting disorders including a subgroup of TNDM patients with MLID in whom overlapping phenotypes have been described (Mackay *et al.*, 2006b).

Recently, Poole and colleagues found that mild to moderate developmental delay/intellectual disability was found to occur in 8/8 of BWS patients with MLID compared to 3/11 non-MLID BWS patients (Poole *et al.*, 2013). This reached statistical significance. This is in contrast to my study where the frequency of intellectual disability/developmental delay (4%) was similar in both the ART and naturally conceived BWS IC2 epimutations groups. There were no occurrences of intellectual disability in the MLID patients in my study. Previously developmental delay and intellectual disability was found to be mainly found in BWS cases due to duplications involving the paternal copy of 11p15.5 (Slavotinek *et al.*, 1997). Therefore more studies are required to clarify the risk of developmental delay/intellectual disability in BWS and in MLID.

The finding that MLID was more frequently seen in ART conceived BWS cases could have implications for other ART conceived children. This raises the possibility of developmental defects, abnormal growth or childhood cancers in ART conceived children might be due to variable combinations of epigenetic alterations at imprinted DMRs. Williams carried out a large UK population-based cohort study over 17 years (1992-2008) looking at overall risk of cancer in ART conceived children under the age of 15 years and found no increase in the overall risk compared to the expected risk (Williams *et al.*, 2013). Interestingly, they did however find an increased risk of two rare specific cancers rhabdomyosarcoma and hepatoblastoma, both of which were identified in our cohort of ART conceived BWS cases. In the children that were identified by the Williams study with both these tumours, none were reported to have an imprinting disorder. Despite the increased risk identified for these two tumours, the absolute excess risks remain small. Though this might be due to chance,

the finding of these two tumours in our ART conceived BWS patient cohort raise the possibility of an epigenetic cause (an imprinting disorder or MLID) being a risk factor.

In summary, this study provides further evidence that locus heterogeneity at 11p15.5 affects phenotype in BWS. Disruption of IC1 or overexpression of *IGF2* remains the main cause of Wilms tumour in BWS and Wilms tumour rarely occurs in BWS cases due to IC2 epimutations/deletions or mutations in *CDKN1C*. *Trans* acting and environmental factors can influence the variability in phenotype in BWS as evidenced in the difference in the clinical phenotype in ART conceived cases of BWS when compared to the naturally conceived BWS cases with the same underlying molecular defect.

CHAPTER 3. THE CLINICAL AND MOLECULAR GENETIC INVESTIGATION OF BIRT-HOGG-DUBE SYNDROME

DECLARATION:

My work setting up the *FLCN* locus specific mutation database (LSDB) as part of the BHD project in collaboration with the European BHD Consortium and curation of variants into the database have been published in the journal Human Mutation (Lim *et al.*, 2010a). In this thesis analysis of variants in the database was performed following a cut off date of 11/08/2011.

3.1 INTRODUCTION

3.1.1 BIRT-HOGG-DUBE SYNDROME

Birt-Hogg-Dube syndrome (BHD) was first described in 1975 by Drs. Arthur Birt, Georgina Hogg and James Dube in Canada. They described a large family segregating cutaneous manifestations of fibrofolliculomas, acrochordons and skin tags with an autosomal dominant inheritance pattern (Birt *et al.*, 1977). As more "BHD families" were reported, an association was then made with the cutaneous manifestations of BHD with the occurrence of spontaneous pneumothorax due to the presence of lung cysts (Binet *et al.*, 1986; Toro *et al.*, 1999; Zbar *et al.*, 2002). Subsequently, an increased risk of renal cancer in BHD was established following an increasing number of reports of renal tumours in patients with BHD syndrome (Pavlovich *et al.*, 2005; Roth *et al.*, 1993; Toro *et al.*, 1999; Zbar *et al.*, 2002).

3.1.1.1 Skin manifestations

The principal skin manifestations of BHD syndrome are fibrofolliculomas. These are benign tumours of the hair follicles. They appear as white or flesh coloured papules and increase in number and size with age. Studies on a cohort of BHD patients in North America report that the fibrofolliculomas usually occur after the age of 20 years. The typical areas that develop fibrofolliculomas include the face, neck, chest and upper back. They can vary in number and size between affected members in a particular family and therefore are highly variable. They typically do not cause any symptoms. The numbers can range from very few in mild cases to many hundreds in severe cases (Menko *et al.*, 2009; Schmidt *et al.*, 2005; Toro *et al.*, 2008).

The initial description of the cutaneous features of BHD syndrome in addition to fibrofolliculomas also includes trichodiscomas and acrochordons (Birt *et al.*, 1977). Trichodiscomas were initially defined as benign tumours of the hair discs and was not clinical discernible from fibrofolliculomas by clinical examination alone (Pinkus *et al.*, 1974). However, they are now thought to represent the same morphological spectrum of fibrofolliculomas upon closer histological examination and better sectioning techniques (Schulz *et al.*, 1999; Vincent *et al.*, 2003). Acrochordons or skin-tags seen in BHD can also occur in the general population. As they are often easier to identify just by direct clinical examination due to their pedunculated appearance, they are rarely biopsied for histological analysis in BHD. However, a few papers that studied the histological appearance of some of these lesions showed that they are actually pedunculated fibrofolliculomas that can give the same macroscopic appearance of an acrochordon (Balus *et al.*, 1983; De la Torre *et al.*, 1999). Therefore, it is likely that fibrofolliculomas, trichodiscomas and some of the

acrochordons in BHD may represent the same cutaneous lesion (De la Torre *et al.*, 1999; Vincent *et al.*, 2003).

3.1.1.2 Lung manifestations

Lung cysts which predisposes to spontaneous pneumothorax was found to occur in around 80% of BHD patients. The cysts are often bilateral and typically occur in the basal regions of the lungs (Toro *et al.*, 2007; Toro *et al.*, 2008). This is in contrast to sporadic primary pneumothorax where cysts occur typically in the apical regions of the lungs (Sahn *et al.*, 2000). These cysts are usually detectable by CT scanning of the thorax which is more sensitive than plain chest radiographs (Grant *et al.*, 2009). Respiratory function is usually unaffected despite the presence of multiple lung cysts (Toro *et al.*, 2007).

Spontaneous pneumothorax may present symptomatically as chest pain or breathlessness. The risk of pneumothorax in BHD patients is increased by 50 fold compared to the general population and is attributed to the presence of the lung cysts (Zbar *et al.*, 2002). Despite the high number of BHD patients who have lung cysts, the prevalence of pneumothorax has been reported as 24% with a median age of first occurrence of 38 years (Toro *et al.*, 2007). The youngest reported pneumothorax in BHD occurred in a 7 year old child (Bessis *et al.*, 2006). BHD patients may develop a single pneumothorax but recurrent episodes are common (Toro *et al.*, 2007). Recurrent exposure to changes in atmospheric pressures such as flying and deep sea diving is the major risk factor for spontaneous pneumothorax.

3.1.1.3 Renal manifestations

The risk of renal cancer is the most threatening manifestation of BHD. The histological types that occur in BHD renal tumours are chromophobe tumours, oncocytomas, clear cell, although the most characteristic is a mixed hybrid of chromophobe and oncocytomas. Papillary renal cancer and other mixed histology have also been reported in BHD (Fahmy *et al.*, 2007; Janitzky *et al.*, 2008; Kluijt *et al.*, 2009; Pavlovich *et al.*, 2005; Pavlovich *et al.*, 2002).

The estimated lifetime risk of renal cancer in BHD was estimated at 27% with a mean age of diagnosis of 50.4 years (range 31-74 years) in a series of BHD patients reported by Pavlovich and colleagues (Pavlovich *et al.*, 2005; Pavlovich *et al.*, 2002). The youngest age of onset of renal cancer in a BHD patient has been reported at the age of 20 years (Khoo *et al.*, 2002). The difficulty with assessing the true risk of renal cancer in BHD arises from the clinical variability of the phenotype in BHD, and the low number of patients identified with the condition. There have not been many large studies in BHD to further define the risk. In addition, there may be ascertainment bias in the recruitment of patients where more severely affected patients are identified and subsequently recruited for studies.

The renal tumours that occur in BHD show similarities in features with other renal tumour predisposing genetic conditions such as von Hippel-Lindau disease (VHL). Bilateral and multifocal tumours can be seen as well as a younger age of onset of renal tumours compared to sporadic renal cancers. However, relatively few patients with metastatic renal cancer have been described in the literature (Pavlovich *et al.*, 2005).

In addition to renal cancer, cysts in the kidneys can also be found in BHD patients when undergoing renal imaging surveillance (Kluijt *et al.*, 2009; Toro *et al.*, 1999). However, it is difficult to be certain if the incidence of renal cysts in BHD is different from renal cysts in the general population, as the latter is currently unknown.

3.1.1.4 Other tumours

Apart from renal cell carcinoma, other tumours that have been reported in individuals with BHD include colorectal cancer and polyps (Khoo *et al.*, 2002; Toro *et al.*, 2008), malignant melanoma (Houweling *et al.*, 2011; Toro *et al.*, 1999; Toro *et al.*, 2008), parotid gland tumours (Schmidt *et al.*, 2005; Toro *et al.*, 2008), basal cell carcinoma (Leter *et al.*, 2008; Toro *et al.*, 1999; Toro *et al.*, 2008), breast cancer (Leter *et al.*, 2008; Toro *et al.*, 2008), prostate cancer (Houweling *et al.*, 2011; Toro *et al.*, 1999; Toro *et al.*, 2008), bladder cancer, sarcoma, pituitary adenoma, phaeochromocytoma, astrocytoma, gastric carcinoma (Houweling *et al.*, 2011), cutaneous leiomyosarcoma, uterine cancer, dermatofibrosarcoma protuberans, Thyroid cancer, Hodgkin disease, squamous cell carcinoma of cervix (Toro *et al.*, 2008) and skin squamous cell carcinoma (Leter *et al.*, 2008).

3.1.2 MOLECULAR GENETICS OF BHD SYNDROME

3.1.2.1 Identification of the gene responsible for BHD

The chromosomal locus for the gene responsible for BHD was located at chromosome 17p11.2 by linkage analysis on BHD families (Khoo *et al.*, 2001; Schmidt *et al.*, 2001).

Subsequently, truncating mutations in a novel gene at that locus, *FLCN* coding for a unknown protein called Folliculin, was identified in BHD patients (Nickerson *et al.*, 2002).

3.1.2.2 The Folliculin (*FLCN*) gene

The *FLCN* gene has 14 exons with a transcriptional start site in exon 4. *FLCN* encodes a 579 amino acid protein folliculin which is expressed in most tissues including the skin, lung, kidney and in fetal lung, kidney liver and brain tissue (Nickerson *et al.*, 2002). The function of folliculin is yet to be fully understood. Folliculin and its interacting proteins Folliculin interacting protein 1 (FNIP1) and Follculin interacting protein 2 (FNIP2), are thought to be involved in the energy and nutrient sensing pathways via the AMPK (5'AMP-activated protein kinase) and mTOR (mammalian target of rapamycin) signalling pathways (Baba *et al.*, 2006; Hasumi *et al.*, 2008; Takagi *et al.*, 2008).

There is a mutation hotspot in a hypermutable polycytosine (C8) track in exon 11 of *FLCN* where around 50% of BHD patients have a germline duplication (formerly insertion) or deletion of a cytosine in the tract (Schmidt *et al.*, 2005). Detection of a mutation by gene sequencing is estimated to be in the region of 88% of BHD families which fulfil a clinical diagnosis of BHD (Toro *et al.*, 2008). Recently, there have been several reports of large intragenic deletions and duplication detected in BHD patients by techniques such as Multiple Ligation-Dependent Probe Amplification assay (MLPA) which can detect copy number changes within a gene that is not detectable by normal gene sequencing (Benhammou *et al.*, 2011; Kunogi *et al.*, 2010). Using techniques such as MLPA will increase the diagnostic yield of *FLCN* mutation testing by a further 5% in BHD. However, there

remains around 5% of clinically diagnosed BHD patients in which current sequencing and copy number detection techniques will not identify a causative mutation (Benhammou *et al.*, 2011).

3.1.2.3 Diagnostic criteria for BHD

Prior to the identification of *FLCN* as the causative gene in BHD, the diagnosis of BHD was made clinically by the presence of at least 5-10 cutaneous fibrofolliculomas, of which at least one must be histologically confirmed on biopsy (Khoo *et al.*, 2002; Toro *et al.*, 1999). Recently, the European Birt-Hogg-Dube consortium has suggested major and minor diagnostic criteria for the diagnosis of BHD syndrome in the light of DNA testing for BHD (Menko *et al.*, 2009). BHD patients should fulfil one major or two minor criteria below:

Major Criteria:

- At least 5 fibrofolliculomas or trichodiscomas, at least one histologically confirmed, of adult onset
- Pathogenic *FLCN* germline mutation

Minor Criteria:

- Multiple lung cysts: bilateral basally located lung cysts with no other apparent cause with or without spontaneous primary pneumothorax
- Renal cancer: early onset (<50 years) or multifocal or bilateral renal cancer, or renal cancer of mixed chromophobe and oncocytic histology
- A first degree relative with BHD

These diagnostic criteria take into account *FLCN* mutation analysis, penetrance and clinical variability.

3.1.2.4 Folliculin is a tumour suppressor gene

The functions of folliculin are largely unknown. Studying the renal tumours that occur in BHD patients by DNA sequencing to detect somatic mutations detected loss of heterozygosity of *FLCN* as well as acquired mutations in the wild type allele (Vocke *et al.*, 2005). This is in keeping with a tumour suppressor gene and follows Knudson's two hit hypothesis. A first "hit" in a tumour suppressor gene is not sufficient for the development of a tumour. However the acquisition of a second "hit" in the remaining wild type allele due to loss of heterozygosity or mutation will result in the loss expression of that tumour suppressor gene and can drive tumour proliferation. In patients with cancer predisposition syndromes due to a germline mutation in a tumour suppressor gene (e.g. *RB1* in retinoblastoma), a mutation is already present in one allele of the tumour suppressor gene in every cell of the body. Therefore the acquired second "hit" occurs at a younger age compared to sporadic cancers, and can also result in bilateral and multifocal tumours (Knudson, 1971). Several tumour predisposing genetic syndromes including BHD show these hallmarks with younger age onset tumours, bilateral and multifocal tumours.

In addition loss of *FLCN* mRNA expression was also detected in renal tumours from BHD patients (Warren *et al.*, 2004). However, studies on the fibrofolliculomas of the skin showed no loss of mRNA expression (Warren *et al.*, 2004). In addition, loss of heterozygosity was not

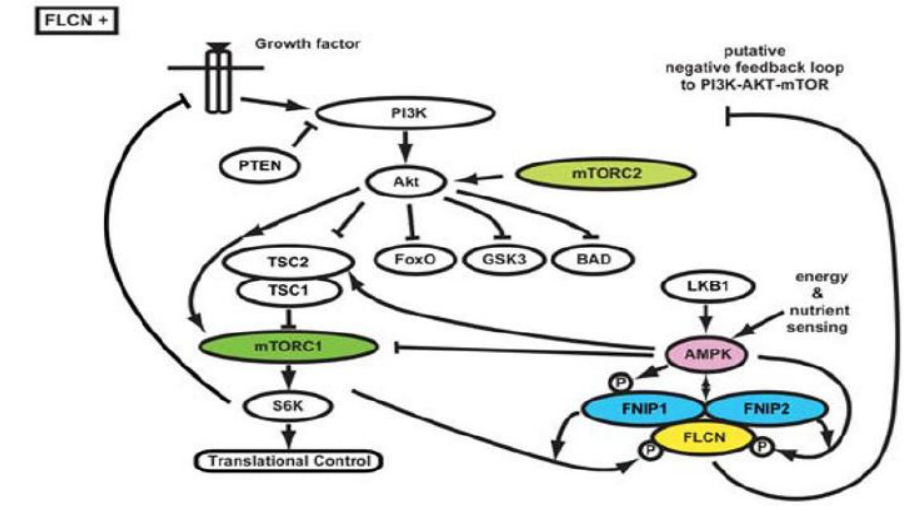
detected in fibrofolliculomas, therefore suggesting that the mechanism of tumourigenesis in the skin is different from the kidneys (van Steensel *et al.*, 2007).

3.1.2.5 The mTOR signalling pathway and Folliculin

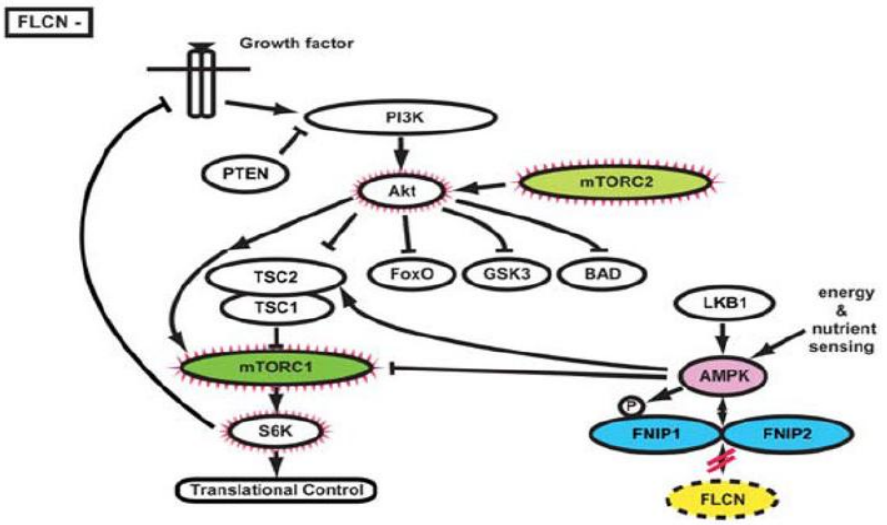
The energy-sensing mammalian target of Rapamycin (mTOR) signalling pathway plays an important role in cell growth regulation and receives input from multiple signalling pathways such as nutrients and growth factors resulting in protein synthesis. Dysregulation of the mTOR pathway has been implicated in a number of hamartoma tumour predisposition syndromes such as Tuberous Sclerosis (due to mutations in *TSC1*/*TSC2*)(Inoki *et al.*, 2005), PTEN hamartoma tumour syndrome (due to mutations in *PTEN*)(Eng, 2003) and Peutz-Jegher syndrome (due to mutations in *LKB1*)(Shaw *et al.*, 2004). These disorders are all caused by mutations in tumour suppressor genes which normally negatively regulates the mTOR signalling pathway .

Recent studies have shown that folliculin interacts with proteins that affect the mTOR pathway. Folliculin was shown to form a complex with its interacting proteins FNIP1 and FNIP2 that binds to AMPK via its gamma subunit. AMPK is involved in energy and nutrient sensing and is one of the negative regulators of the mTOR signalling pathway. The C-terminus of folliculin is involved with binding to FNIP and FNIP2 (Hasumi *et al.*, 2008).

The precise role of folliculin in the mTOR pathway remains to be fully elucidated. Folliculin appear to have several functions and it will be important to clarify these functions with respects to renal cancer development. These will help researchers into developing effective targeted therapies for renal cancer.



A



B

Figure 3-1 The FLCN pathway.

A. The FLCN/FNIP1/FNIP2 complex binds AMPK and FLCN is phosphorylated by a rapamycin-sensitive kinase (mTORC1). B. When FLCN is deficient, AKT, mTORC1 and mTORC2 are activated. Adapted by permission from Macmillan Publishers Ltd: Nature reviews. Urology (Linehan *et al.*, 2010), copyright 2010

3.1.2.6 Naturally occurring animal models

Two naturally occurring animal models of BHD exist.

3.1.2.6.1 Hereditary multifocal cystadenocarcinoma and nodular dermatofibrosis

An exon 7 missense substitution H255R (which is also reported in human) in the canine BHD gene is seen in German Shepherd dogs who develop a condition called hereditary multifocal renal cystadenocarcinoma and nodular dematofibrosis (Lingaas *et al.*, 2003).

These dogs develop bilateral, multifocal tumours in the kidneys, cutaneous nodules consisting of dense collagen fibres and uterine leiomyomas. The kidney tumours that develop in these dogs show evidence of a second hit in the canine *FLCN* gene with loss of heterozygosity of the second wild type allele in most of the kidney tumours and also in around 30%, of early cystic renal lesions (Bonsdorff *et al.*, 2008; Bonsdorff *et al.*, 2009). Loss of heterozygosity was not found in the cutaneous tumours in these dogs, therefore showing similar results as humans.

3.1.2.6.2 The Nihon Rat

The Nihon rat has a germline single nucleotide insertion leading to a frameshift mutation in the rat *FLCN* orthologue (Okimoto *et al.*, 2004). The heterozygotes develop renal cancer from preneoplastic lesions appearing from 3 weeks to adenocarcinoma by 6 months of age. The histological features of the renal tumours resemble human chromophobe renal cancer (Kouchi *et al.*, 2006). Loss of heterozygosity was demonstrated in the kidney tumours. Introduction of wild type *Flcn* (*Bhd*) suppressed the renal carcinogenesis (Togashi *et al.*, 2006). Homozygote rats are embryonically lethal.

3.1.2.7 Experimental animal models

Homozygous BHD knockout mouse have been shown by Hasumi and colleagues to be embryonically lethal (Hasumi *et al.*, 2009). In their knockout mouse model, heterozygotes develop renal tumours including chromophobe, hybrid oncocytomas and clear cell histology with concomitant loss of heterozygosity of wildtype *Flcn*. The kidney tumours that develop from the heterozygotes showed evidence of mTOR pathway activation (both mTORC1 & mTORC2).

Chen and colleagues generated conditional knockdown mice in which *Flcn* was only inactivated in the kidneys (Chen *et al.*, 2008). These mice developed renal hyperplasia, multiple cysts and died with renal dysfunction by 3 weeks of age. Treatment with the mTOR inhibitor Rapamycin prolonged survival of these mice and inhibited the development of renal cysts. However, the cysts recurred and they died within 10 days of stopping treatment.

Working on a xenograft model, Hong and colleagues introduced UOK257 cancer cell line into immunodeficient nude mice (Hong *et al.*, 2010). UOK257 cell line is a renal cell carcinoma cell line from a BHD patient. These cells are *FLCN* null with the exon 11 common mutation c.1285dupC and deletion of the second wild type allele. Following the xenografting of the cell line, the mice developed tumours. Upon reintroduction of wild type *FLCN* into UOK257, these suppressed the growth of the tumours, thus providing further evidence for the role of folliculin as a tumour suppressor.

3.1.3 FAMILIAL PRIMARY SPONTANEOUS PNEUMOTHORAX AND FLCN MUTATIONS

FLCN mutations have also been identified in patients presenting with familial primary spontaneous pneumothorax (PSP) (MIM#173600) (Frohlich *et al.*, 2008; Graham *et al.*, 2005; Gunji *et al.*, 2007; Ishii *et al.*, 2009; Painter *et al.*, 2005; Ren *et al.*, 2008). These patients have lung manifestations of BHD but without any cutaneous manifestations or renal tumours. However, since the diagnostic criteria proposed by the European BHD consortium, these patients with *FLCN* mutations would be diagnosed as having BHD. For the purpose of genotype-phenotype correlation analysis, I will analyse PSP patients and *FLCN* mutations as having PSP to aid analysis.

3.1.4 FAMILIAL NON-SYNDROMIC CLEAR CELL RENAL CELL CARCINOMA AND FLCN MUTATIONS

Woodward et al (2008) detected germline *FLCN* mutations were detected in 4.3% of probands with features of inherited non-syndromic clear cell renal cell carcinoma (FcRCC) (Woodward *et al.*, 2008). These patients are reported (at the time of ascertainment) to not have any other manifestations of BHD. In view of their findings, they recommended adding *FLCN* testing to the panel of investigations for potential cases of inherited RCC together with *VHL* gene mutation analysis and constitutional cytogenetics.

However, as for PSP, the diagnostic criteria for BHD proposed by the European BHD consortium will also result in *FLCN* mutation positive inherited RCC cases being diagnosed with BHD syndrome.

3.1.5 SUMMARY

I have provided an overview on BHD and the current understanding about the manifestations of the condition, the gene responsible for BHD, the pathways that are thought to be involved in tumourigenesis in BHD, along with experimental model results. The association of BHD with the three main manifestations affecting the skin, lung and kidney are well established. However, other tumours have been described in BHD such as colorectal cancers and malignant melanomas, and the risks of these are still yet to be clarified. Further studies on large cohorts of BHD patients and possible genotype-phenotype correlations are needed to help understand further the many aspects of the condition and identify causes of phenotypic variability.

3.1.6 AIMS OF PROJECT

The aims of the BHD project are to study a large cohort of BHD families in the UK to collect and analyse phenotypic and natural history data and study the genetic factors that may affect clinical variability in BHD.

1. To define the natural history and lifetime risk of the various manifestations in BHD

To date there are less than 150 families with BHD reported in the medical literature. Due to phenotypic variability, it is important to try and further define the natural history of BHD with regards to the lifetime risks of the various manifestations. This will help advice patients and define a surveillance programme for the tumours that develop in BHD patients.

2. To collect detailed phenotypic information on a large cohort of BHD patients

Detailed phenotypic examination of patients will help identify previously unrecognised or rarer manifestations in BHD. Analysis of phenotype may also provide novel insights into the aetiology and possible pathways that may be affected in BHD, and thus help inform experimental studies.

3. To collate published and unpublished novel *FLCN* sequence variants and establish a mutation database/locus specific-database for *FLCN*

The establishment of an online database for *FLCN* variants/mutations will provide a useful resource for clinicians/researchers/scientists working on BHD. Analysis of the database with regards to the variants will help in genotype-phenotype analysis.

4. To study the factors that affect phenotypic variability in BHD

Genotype-phenotype studies can be done in a large cohort of BHD patients to help identify those most at risk of kidney cancers or the other tumours that may develop in BHD. In addition, analysis can be performed to look for other factors such as genetic/environmental modifiers which may affect phenotype.

3.2 METHODS

3.2.1 PATIENT RECRUITMENT AND ASCERTAINMENT

The West Midlands Regional Genetics Laboratory is the only laboratory in the UK offering a diagnostic service for *FLCN* gene testing by sequencing and MLPA. Since the service was set up in 2007, a list of *FLCN* mutation positive patients was recorded. Patient information leaflets and consent forms were sent to the patient's referring clinician to disseminate to their patients.

3.2.2 CONSENT AND ETHICS APPROVAL

Informed consent was obtained from all participants. All clinical research adhered to principles outlined by the Declaration of Helsinki. The BHD study is a sub-study of the Molecular Pathology of Human Genetic Disease study which has received ethical approval by the South Birmingham Research Ethics Committee (REC) (REC reference 5175) prior to my involvement in the project. However upon completion of my project, I have been involved in revising the information leaflet and consent form to an updated version that was submitted for an amendment in 2011 which received a favourable ethical opinion of the amendment by the South Birmingham REC. These are included in Appendix F.

3.2.3 PATIENT ASSESSMENT

I carried out clinical examination on patients who consented to take part in the study at clinics and from home visits to characterise the phenotype of each affected individual (clinical data collection proforma in Appendix B). Portable dermoscopy was used to look for characteristic dermoscopic findings of fibrofolliculomas on affected patients to confirm clinical appearance of fibrofolliculomas in individuals who have not required histological

diagnosis (Jarrett *et al,* 2009). Further clinical information was also obtained from medical records. Confirmation of cancer diagnosis was obtained from cancer registries.

3.2.4 CALCULATION OF LIFETIME RISKS

The lifetime risk (up to the age of 70) for the various manifestations in BHD were calculated using statistic software GraphPad PRISM and MedCalc by generating Kaplan-Meier survival curves.

3.2.5 STATISTICAL SIGNIFICANCE IN GENOTYPE-PHENOTYPE CORRELATION ANALYSIS

Statistical significance when comparing Kaplan-Meier survival statistics between different genotypes were taken at the 5% level.

3.2.6 EVIDENCE OF GENETIC MODIFIERS:

A similar approach was taken to that used to identify genetic modifier effects in neurofibromatosis type 1 and von Hippel-Lindau disease (Easton *et al.*, 1993; Webster *et al.*, 1998) such that the similarity of disease was compared in close and more distant relatives and between families in kindreds with identical *FLCN* mutations. To quantify disease similarity we assigned values for age at onset of fibrofolliculomas and then compared the age of onset of fibrofolliculomas index in *FLCN* mutation positive patients with variable degrees of relatedness. Correlation between age of onset and degree of relationship between the two individuals was analysed by generating sequential paired data and correlation analysis was performed by the Spearman correlation co-efficient analysis. The degree of relationship was classified as first degree relationship (Parent-child or sibling relationships), second degree relationships, third or more distant relationships. The age of

onset was classified into groups of 10 years. For mutation carriers who have not manifested cutaneous signs, the age at examination/censure was used.

3.2.7 Software/Platform for the Locus Specific Database for Folliculin

I used the Leiden Open Variation Database (LOVD) software as a repository for all published sequence variants in *FLCN* in the medical literature as well as unpublished novel sequence variants submitted by collaborating diagnostic laboratories in Europe. The database is available publically at www.lovd.nl/flcn and is hosted by the University of Leiden who maintain the technical aspects of webhosting and maintenance of servers.

3.2.8 Ascertainment and curation of sequence variants for the FLCN LSDB

A literature search was performed using PubMed with the keywords "Birt-Hogg-Dube", "BHD", "Folliculin", "FLCN", "Primary Spontaneous Pneumothorax", "Hereditary Kidney Cancer" and "Familial Kidney Cancer". All reported *FLCN* sequence variants and their reported pathogenicity was listed and uploaded using the LOVD software. I also checked the references section of each paper to further widen the literature to ensure maximal data capture.

Through collaboration with the European BHD consortium, an email was sent to research and diagnostic laboratories in member countries to publicise the setting up and ongoing plan of the LSDB. Subsequently, a list of novel variants was added to the online database.

Mutation/Variant names are given according to the Human Genome Variation Society (HGVS) nomenclature. The numbering of the cDNA sequence of *FLCN* (+1 = A of the initiation codon ATG) was obtained from the National Centre for Biotechnology Information

(NCBI) database (accession number NM_144997.5) (Appendix C). Previously reported mutations are renumbered based on the HGVS nomenclature and numbering system before curating onto the database. However, the original nomenclature used in the report is also included in the corresponding entry in the database to allow cross-reference.

For each variation, information is provided at the molecular level. This includes the DNA change, exon, protein change (predicted), type of mutation, the reported and concluded pathogenicity of the variant, source of DNA, and technique used, brief clinical information (reported disease phenotype, patient unique identifier and hyperlink to published reference or submitter contact information for unpublished variants) and also the submitter ID.

Clinical context and molecular findings were used to guide assignment of pathogenicity. All putative novel mutations were identified in affected individuals, segregated with disease status and were not detected/reported in control individuals. The putative mutations were graded according to type of mutation. Frameshift and nonsense mutations were considered pathogenic; splice site variants were considered pathogenic if predicted to disrupt the consensus donor or acceptor splice sites; missense variants were classified as “probably pathogenic” unless proven by experimental evidence or detected in multiple families.

Contributors can easily submit their variants to the online database. Upon registering on the homepage with a username and password, instant access is granted to submit data. Data is provided in two parts: 1) Variant details (details of the variants at the molecular level) and 2) Patient details (details of anonymous clinical information) linked to the variant. Upon submission of data, an email is generated to the curator (me) informing of the data

submission. An email is also sent to the submitters to confirm submission of the data. I can then log in to the database and check the details of the data submitted and verify the nomenclature and conclude the pathogenicity before curating onto the database and making the entry publically available.

3.3 RESULTS

3.3.1 PATIENT RECRUITMENT AND ASSESSMENT

A total of 118 *FLCN* mutation positive individuals from 39 families in the UK were recruited and assessed (Table 9). The male to female ratio is 1:1.1 (56 males, 62 females). The mean age of the cohort is 50.7 years (range 22-85).

3.3.2 ASCERTAINMENT OF PROBANDS FROM EACH FAMILY

The most common manifestation that led to the diagnosis of BHD in the 39 probands was cutaneous fibrofolliculomas (Table 9) (Figure 3-2). 31/39 (80%) probands were diagnosed with BHD following investigation for their skin manifestations. Interestingly, in those 31 probands, 10 had a previous medical history of recurrent spontaneous pneumothorax with hospital admissions. 2/39 (5%) probands were diagnosed with BHD following presentation with recurrent pneumothorax and 7/39 (15%) probands were diagnosed following diagnosis of renal cancer. There was 1 proband (BHD2) who was only diagnosed with BHD following presentation with renal cancer despite earlier manifestations of fibrofolliculomas and recurrent pneumothoraces.

Table 9. Features of the 39 recruited families with 118 *FLCN* mutation positive individuals

No	Family ID	Mutation	No of *FLCN* +ve individuals [Total: (M/F)]	No of mutation carriers with FF	No of mutation carriers with PTX	No of mutation carriers with Renal Ca	No of mutation carriers with Colorectal cancer	Manifestation leading to diagnosis in proband	Earlier manifestation in proband (if applicable)	Other tumours	
1	BHD1	c.1285dupC	20 (8/12)	16	3	7	3	Renal Ca		Malignant melanoma	Calcified tumour heart ventricle
2	BHD2	c.1389C>G	1 (1/0)	1	1	1	0	Renal Ca	FF, PTX	-	
3	BHD3	c.-227-?_c.1432+?dup	5 (3/2)	5	0	1	1	FF		-	
4	BHD4	c.1285dupC	8 (5/3)	8	4	1	0	FF	PTX	Eccrine spiroma of scalp	
5	BHD5	c.890_893delAAAG	1 (0/1)	1	0	0	0	FF		Thyroid folicullar adenoma	
6	BHD6	c.890_893delAAAG	2 (1/1)	2	1	1	0	Renal Ca		-	
7	BHD7	c.1285dupC	7 (5/2)	7	1	0	0	FF		-	
8	BHD8	c.1318_1334dup17	5 (1/4)	5	1	1	0	PTX	FF	-	
9	BHD9	c.1285dupC	1 (0/1)	1	1	0	0	FF	PTX	-	
10	BHD10	c.1657T>C	1 (1/0)	1	1	0	0	FF		-	
11	BHD12	c.1597_1598del	2 (0/2)	1	1	1	0	FF		-	
12	BHD13	c.-227-?_c.1432+?dup	2 (2/0)	2	0	0	0	FF		-	
13	BHD14	c.1285dupC	3 (1/2)	3	1	0	0	FF		-	
14	BHD15	c.1076delC	2 (0/2)	2	1	0	0	PTX		-	
15	BHD16	c.1318_1334dup17	1(0/1)	1	0	0	0	FF		-	

16	BHD17	c.890_893delAAAG	4 (1/3)	4	1	1	0	FF		Breast Cancer
17	BHD20	c.1285dupC	2 (1/1)	2	1	0	0	FF	PTX	-
18	BHD24	c.836_839delCCGA	1 (0/1)	1	0	0	0	FF		-
19	BHD25	c.1285dupC	2 (0/2)	2	1	0	0	FF	PTX	Adrenal tumour
20	BHD27	c.1318_1334dup17	4 (1/3)	3	0	3	0	PTX		Lung cancer
21	BHD29	c.1285dupC	3 (1/2)	3	0	1	0	FF		Malignant melanoma
22	BHD31	c.1285dupC	4 (3/1)	4	1	0	1	FF	PTX	-
23	BHD35	c.1429C>T	1 (1/0)	1	0	0	0	FF		Multiple Basal cell carcinomas
24	BHD36	c.469_471delTTC	1 (1/0)	1	0	0	0	FF		-
25	BHD40	c.890_893delAAAG	2 (1/1)	1	2	0	0	FF	PTX	-
26	BHD50	c.1285dupC	1 (0/1)	1	0	0	0	FF		-
27	BHD51	c.510C>A	1 (1/0)	1	0	0	0	FF		-
28	BHD52	c.1318_1334dup17	4 (2/2)	1	0	2	0	Renal Ca		-
29	BHD53	c.1285dupC	2 (2/0)	2	0	0	0	FF		-
30	BHD54	c.240delC	1 (0/1)	1	0	0	0	FF		Angiomyolipoma
31	BHD57	c.1389C>G	1 (1/0)	1	0	1	0	FF		-
32	BHD59	c.1367_1398del32	1 (0/1)	1	0	0	0	FF		Angiomyolipoma
33	BHD60	c.689dupT	8 (5/3)	7	2	0	0	FF	PTX	-
34	BHD61	c.1318_1334dup17	3 (0/3)	3	2	2	0	FF	PTX	-
35	BHD62*	c.715C>T	1 (1/0)	1	0	1	1	Renal Ca		Oesophageal Cancer
36	BHD64	c.1285dupC	2 (2/0)	1	0	0	0	FF		-
37	BHD65	c.1285dupC	2 (0/2)	1	1	0	0	FF	PTX	Thyroid Follicular Cancer / Adrenal tumour
38	BHD70	c.1285delC	5 (4/1)	5	2	0	0	FF	PTX	-
39	BHD71	c.1A>G	1 (0/1)	1	0	0	0	FF		-

(FF=Fibrofolicullomas, PTX = Pneumothorax, Renal Ca = Renal cancer) *=patient subsequently shown to also harbour a germline *TP53* variant

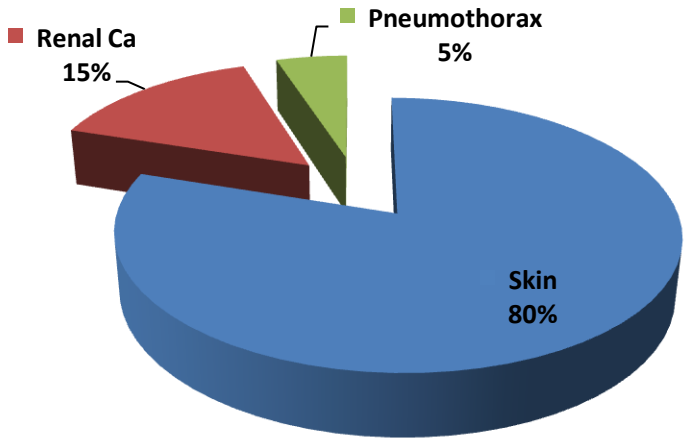


Figure 3-2 Manifestation that led to the diagnosis of BHD in the probands of each family

Majority of probands were diagnosed following seeking medical attention of their skin manifestations.

3.3.3 INITIAL MANIFESTATIONS OF BHD SYNDROME

At the time of study, 8 subjects were unaffected (mean age 26.9 years) and 110 had developed one or more manifestations of BHD syndrome. Fibrofolliculomas were the most frequent initial manifestation (87.3%) followed by pneumothorax (7.3%), renal cancer (3.6%) and colorectal cancer (1.8%).

At the time of study, 64 patients had just a single manifestation of BHD syndrome (mean age of first manifestation = 34.3 years): most commonly fibrofolliculomas (n=61) followed by renal carcinoma (n=2) and pneumothorax (n=1). 37 patients had developed two manifestations of BHD syndrome (mean age at diagnosis of second manifestation = 45.1 years): most commonly fibrofolliculomas and pneumothorax (n=20, mean age at second diagnosis = 36.95 years) and fibrofolliculomas and renal cancer (n=14, mean age at second

diagnosis = 54.3 years) and 3 patients had developed a combination of fibrofolliculomas and colorectal cancer (mean age at second diagnosis = 57 years). 9 patients (mean age at third diagnosis = 47.9 years) had developed three features of BHD syndrome (FF+PTX+RCC = 6; PTX+RCC+CRC= 1; FF+RCC+CRC =1; FF+PTX+CRC = 1). Age related curves for developing one or more manifestations of BHD syndrome were constructed (Figure 3-3).

There was no clear pattern of patients with multiple manifestations being clustered in particular families.

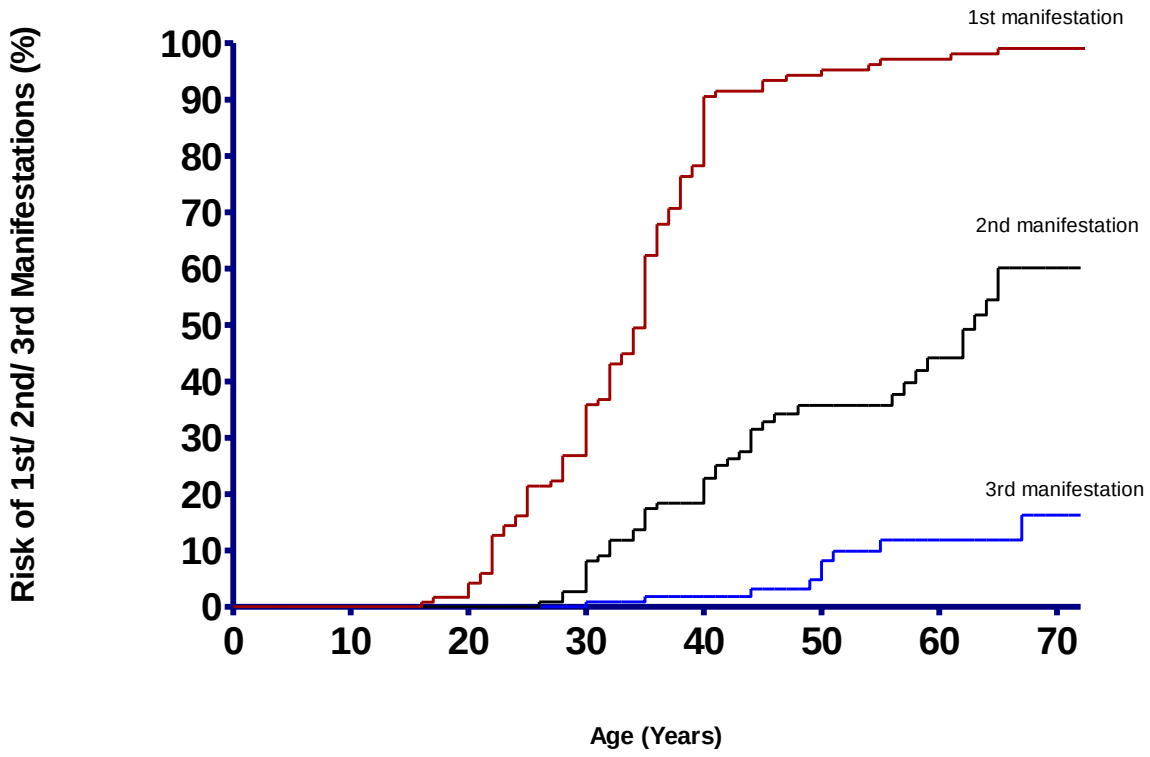


Figure 3-3 Kaplan-Meier curves for probability of 1st, 2nd and 3rd manifestations of BHD syndrome in *FLCN* mutation positive individuals

3.3.4 SKIN MANIFESTATIONS

The mean age of onset of fibrofolliculomas in the cohort was 34.1 years with the youngest age of onset of 20 years and the oldest age of onset of 77 years. Figure 3-4 shows the Kaplan-Meier curve for the age related risk of developing fibrofolliculomas from our cohort. Fibrofolliculomas were highly penetrant but may not be present in some individuals with *FLCN* mutations. At the age of 40 years, around 88% of *FLCN* mutation carriers would have manifested fibrofolliculomas. However, 12% of mutation carriers will not have manifested the cutaneous manifestations. The lifetime risk (age 70 years) of developing fibrofolliculomas is 97%.

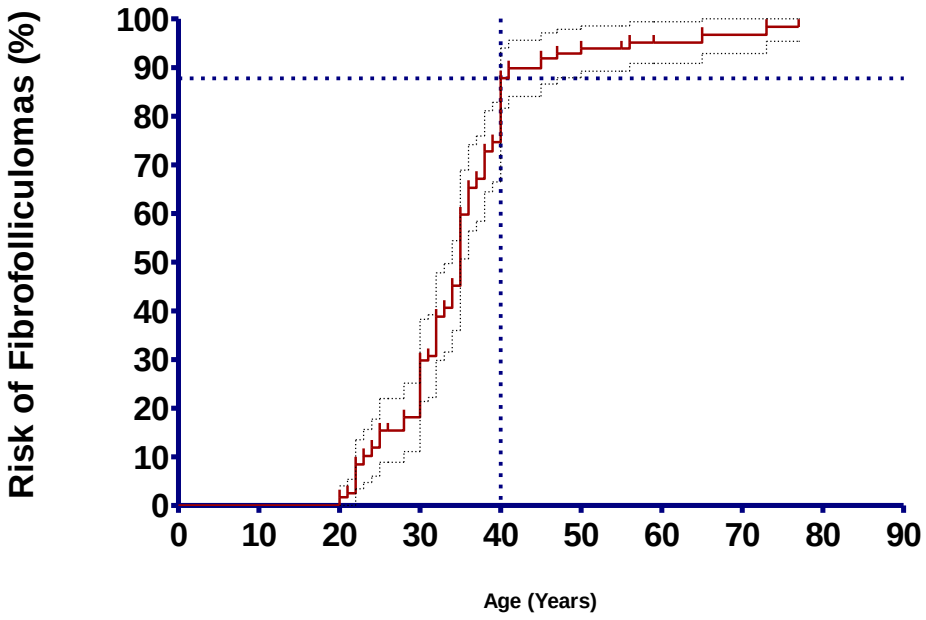


Figure 3-4 Kaplan-Meier curve (with 95% confidence intervals) for age related risk of developing cutaneous manifestations of BHD in patients with *FLCN* mutations

The fibrofolliculomas that develop increase in size and number with increasing age. The fibrofolliculomas show phenotypic variability where some patients are only mildly affected (Figure 3-5) where as other patients are more severely affected (Figure 3-6). They show both inter-familial and intra-familial variability.

The fibrofolliculomas rarely cause any symptoms but may sometime interfere in shaving in males, especially if they are numerous around the face and neck region. They may sometime coalesce and give an appearance of a larger lump or tumour, but on closer inspection, distinct fibrofolliculomas are usually clearly visible (Figure 3-7). Although they do not cause any symptoms *per se*, over 75% of patients report that they are burdened by the cosmetic appearance of their skin.

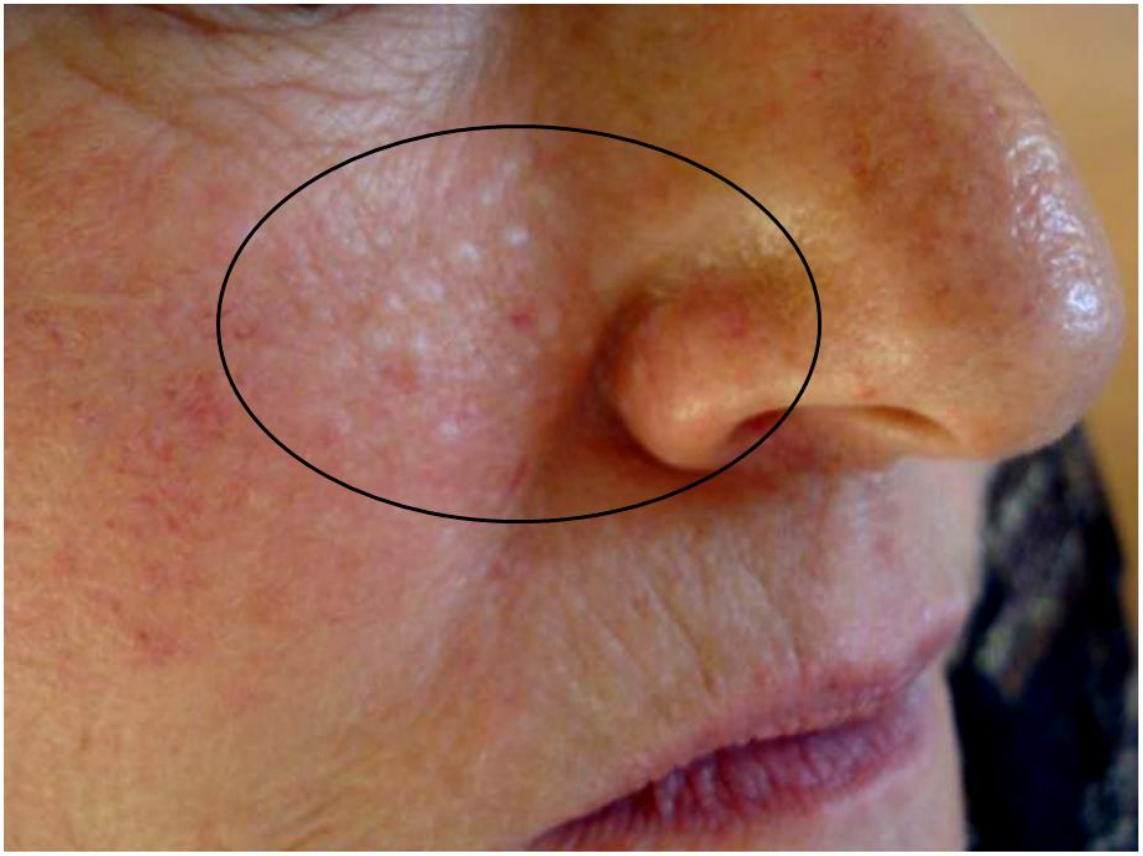


Figure 3-5 Photograph of the skin of a mildly affected patient with fibrofolliculomas

20 papules on the left cheek and nose (area circled) histologically confirmed as fibrofolliculomas seen on this 50 year old patient with the exon 11 common mutation c.1285dupC (p.His429ProfsX27). The cutaneous lesions were only restricted to the area shown and were not found anywhere else on the body.

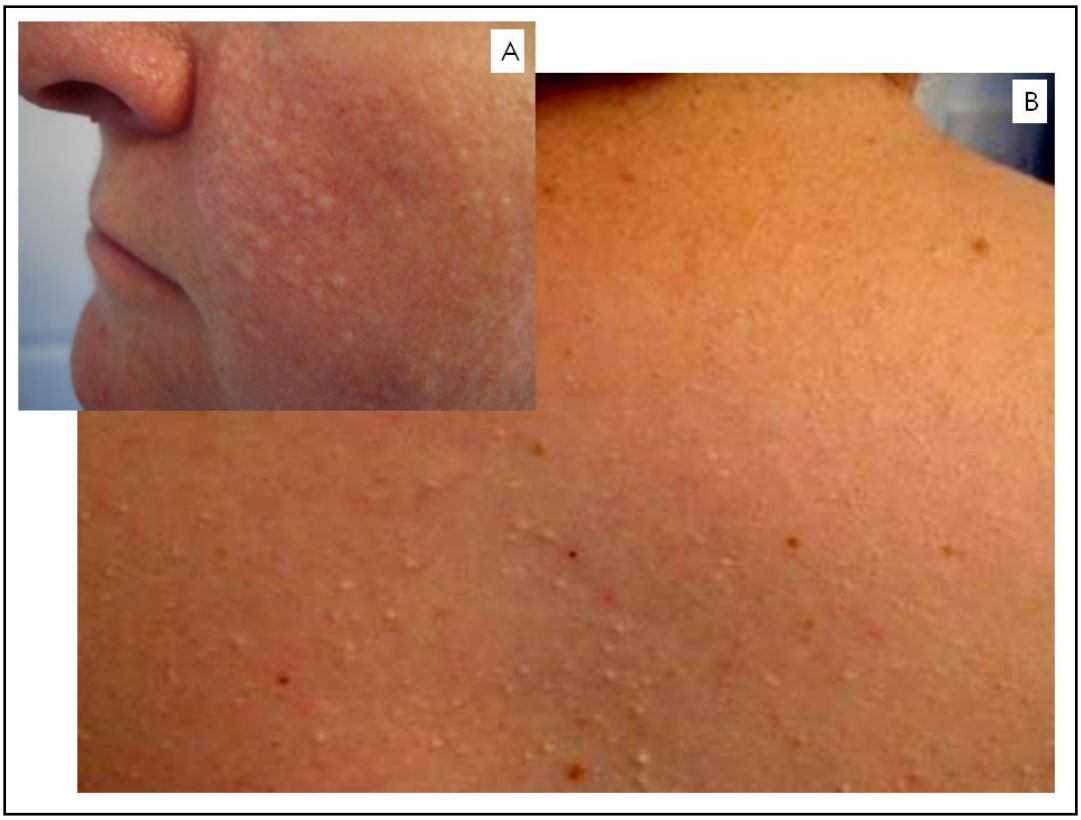


Figure 3-6 Photograph of the skin of a severely affected patient with fibrofolliculomas

This patient has hundreds of papules histologically confirmed as fibrofolliculomas over the face, chest, neck and back, Note the patient is the same age and has the same exon 11 common mutation as the patient in Figure 3-5 above.

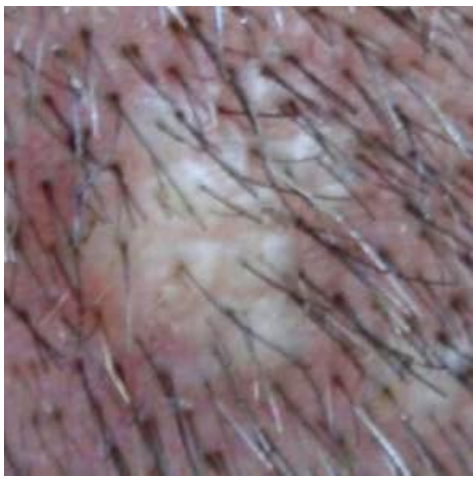


Figure 3-7 Photograph of a collection of fibrofolliculomas appearing as a larger tumour

On closer inspection there are around 8 fibrofolliculomas adjacent to one another coalescing to give an appearance of a larger lump.

3.3.5 Evidence of Genetic Modifiers: Age of onset of Fibrofolliculomas

Table 10 summarises the results of the pair wise analysis of age of onset of fibrofolliculomas and the different degree of relatedness. First degree related individuals are more closely correlated in terms of their age of onset of fibrofolliculomas with siblings more closely correlated compared to parent-child. The correlation coefficient drops by 50% in second degree relationships. In third degree or more distant relationships, the correlation does not reach statistical significance. These results provide evidence for the presence of genetic modifiers influencing the age of onset of fibrofolliculomas in *FLCN* mutation positive individuals.

Table 10. Pairwise analysis of correlation between degree of relatedness and age of onset of fibrofolliculomas

Pair group	Spearman Correlation coefficient	P value
All First degree relatives	0.63	<0.000001
- Siblings	0.68	<0.000001
- Parent-child	0.53	0.0003
Second degree relatives	0.34	0.021
Third degree and more distant relatives	0.23	0.110

3.3.6 LUNG MANIFESTATIONS

3.3.6.1 Pneumothorax

29/118 (24%) patients (17 males, 15 females) had a history of pneumothorax. In 20 (69%) (9 males, 11 females) of them the pneumothorax was recurrent. The median age of first pneumothorax in the cohort is 32 years (range 16-63). The lifetime risk of developing a pneumothorax (up to age 70 years) is 33% (Figure 3-8).

Typical symptoms reported by BHD patients who have had a pneumothorax are identical to sporadic pneumothoraces with chest pain and breathlessness. All of the pneumothoraces that have occurred were spontaneous and not a secondary result of trauma.

3.3.6.2 Lung cysts

32 *FLCN* mutation positive individuals had a CT scan of the thorax. Lung cysts were detected in 22 (68%) of these patients. In all 22 cases, cysts were detected bilaterally and were present in the lung bases. 12 (55%) patients with lung cysts had a history of pneumothorax. Of the 29 patients in the overall BHD cohort with a history of pneumothorax, only 12 had CT scans of their thorax and all had detectable lung cysts. There were 10 patients (45%) who had detectable cysts on CT scan but not developed a pneumothorax (median age 46 years, range 35-69). Of the 20 patients that had recurrent pneumothoraces, only 9 (41%) had a CT scan of the thorax looking for lung cysts.

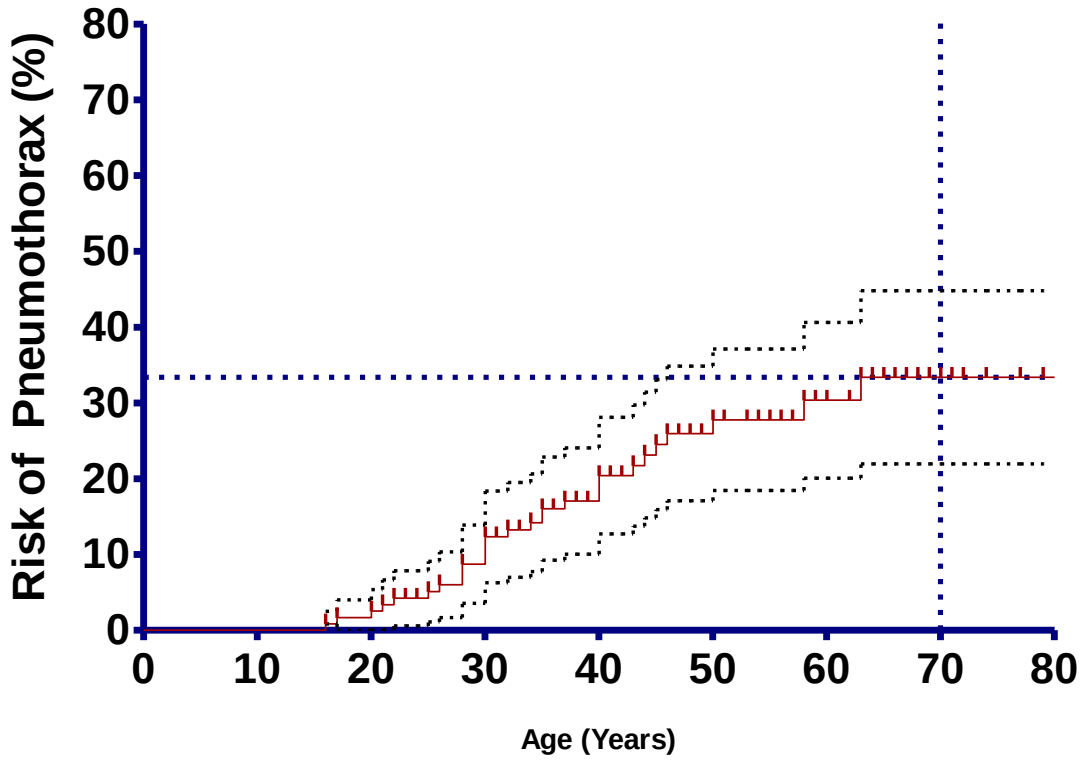


Figure 3-8 Kaplan-Meier curve (with 95% confidence intervals) for age related risk of developing pneumothorax in patients with *FLCN* mutations

3.3.7 RENAL CANCER

24 patients (16 females and 8 males) from 14 families were diagnosed with a renal tumour. Table 11 summarises the patients with renal tumours. The male to female ratio is 1:2. The median age of diagnosis of a renal tumour was 52 years (range 25-85). 11/24 (46%) tumours were diagnosed before the age of 45 years. 13/24 (54%) tumours were detected following routine surveillance renal imaging following a molecular diagnosis of BHD. The lifetime risk of developing a renal tumour in this cohort is 36.8% (95% CI 20-50%) (Figure 3-9).

Table 11. *FLCN* mutation +ve individuals with renal tumours

No	ID	Sex	Age (y)	Unilateral/ Bilateral/ Multifocal (U/B/M)	Histology	Presentation	*FLCN* mutation	Exon
1	BHD1-6	M	62	B	-	Symptoms	c.1285dupC	11
2	BHD1-4	F	67	B	Clear cell & oncocytoma	Surveillance	c.1285dupC	11
3	BHD1-10	F	79	B M	N/A	Surveillance	c.1285dupC	11
4	BHD1-11	F	59	U	Clear cell	Surveillance	c.1285dupC	11
5	BHD1-12	F	62	B	Clear cell	Symptoms	c.1285dupC	11
6	BHD1-13	F	57	U	-	Symptoms	c.1285dupC	11
7	BHD1-14	F	35	U	Clear cell	Surveillance	c.1285dupC	11
8	BHD2-1	M	44	B M	Clear cell	Symptoms	c.1389C>G	12
9	BHD3-1	F	36	U	Sarcomatoid	Symptoms	c.-227-?_c.1432+?dup	2-11
10	BHD4-1	M	31	U	N/A	Surveillance	c.1285dupC	11
11	BHD6-1	F	25	U	Clearcell	Symptoms	c.890_893delAAAG	9
12	BHD8-3	F	44	U	N/A	Surveillance	c.1318_1334dup17	12
13	BHD12-2	F	60	U	Hybrid oncocytoma/ chromophobe	Surveillance	c.1597_1598del	14
14	BHD17-4	F	44	U	Clear cell	Symptoms	c.890_893delAAAG	9
15	BHD27-2	F	28	U	Clear cell	Symptoms	c.1318_1334dup17	12
16	BHD27-3	F	65	B	N/A	Surveillance	c.1318_1334dup17	12
17	BHD27-4	M	42	U	-	Symptoms	c.1318_1334dup17	12
18	BHD29-2	F	84	U	N/A	Surveillance	c.1285dupC	11
19	BHD52-2	M	54	B M	Chromophobe	Symptoms	c.1318_1334dup17	12
20	BHD52-4	M	55	B M	Chromophobe	Surveillance	c.1318_1334dup17	12
22	BHD57-1	M	41	B	N/A	Surveillance	c.1389C>G	12
21	BHD61-2	F	65	U	Oncocytoma	Symptoms	c.1318_1334dup17	12
23	BHD61-3	F	55	U	N/A	Surveillance	c.1318_1334dup17	12
24	BHD62-1*	M	32	U	Chromophobe	Surveillance	c.715C>T	7

*patient subsequently found to also harbour a germline *TP53* variant

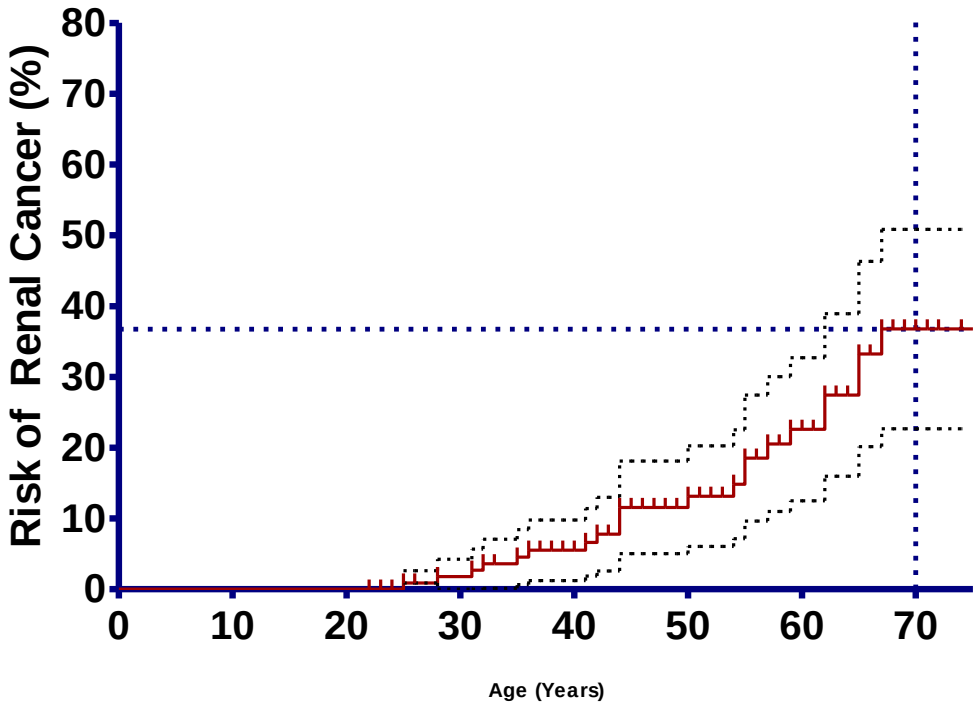


Figure 3-9 Kaplan-Meier curve (with 95% confidence intervals) for age related risk of developing renal cancer in patients with *FLCN* mutations

15 patients had unilateral tumours, 5 with bilateral tumours and 4 with bilateral multifocal tumours. Tumour histopathology was available for 14 patients. There were 7 clear cell, 3 chromophobe, 1 oncocytoma, 1 sarcomatoid and 1 hybrid oncocytoma/chromophobe. In addition one patient with bilateral tumours was found to have a histology of a clear cell tumour in one kidney and an oncocytoma in the contralateral kidney. There were no incidence of metastatic disease.

3.3.8 *COLORECTAL NEOPLASIA*

Colonoscopies were carried out in 21/118 (17.8%) patients (mean age 52.5years range 41-72) either as a result of a family history of colorectal cancer (n=10), symptoms (7) or surveillance following a diagnosis of BHD (4). A total of 6 patients from 4 different families were diagnosed with a colorectal cancer. A total of 3 patients from 3 families developed colorectal polyps. Table 12 summarises the features of these patients. The median age of diagnosis for colorectal cancer was 55 years (range 27-64). The male to female ratio was 5:1. The lifetime risk of developing colorectal cancer (up to age 70 years) is 13% (Figure 3-10).

Table 12. Features of *FLCN* mutation +ve individuals that developed colorectal neoplasia

No	Family ID	Mutation	Sex	Type	Age of diagnosis (years)	Location
1	BHD1-1	c.1285dupC	Male	CRC	51	Colon
2	BHD1-4	c.1285dupC	Female	CRC	61	Colon
3	BHD1-5	c.1285dupC	Male	CRC	48	Colon
4	BHD1-2	c.1285dupC	Male	Single Polyp - hyperplastic	56	Rectum
5	BHD3-5	c.-227-?_c.1432+?dup	Male	CRC	64	Caecum
6	BHD8-1	c.1318_1334dup17	Female	Single Polyp- tubular adenoma	69	Sigmoid
7	BHD14-2	c.1285dupC	Female	Multiple polyps - metaplastic	41	Colon
8	BHD31-4	c.1285dupC	Male	CRC	59	Colon
9	BHD62-1*	c.715C>T	Male	CRC	27	Rectum

*patient subsequently found to also harbour a germline *TP53* variant, CRC=colorectal cancer

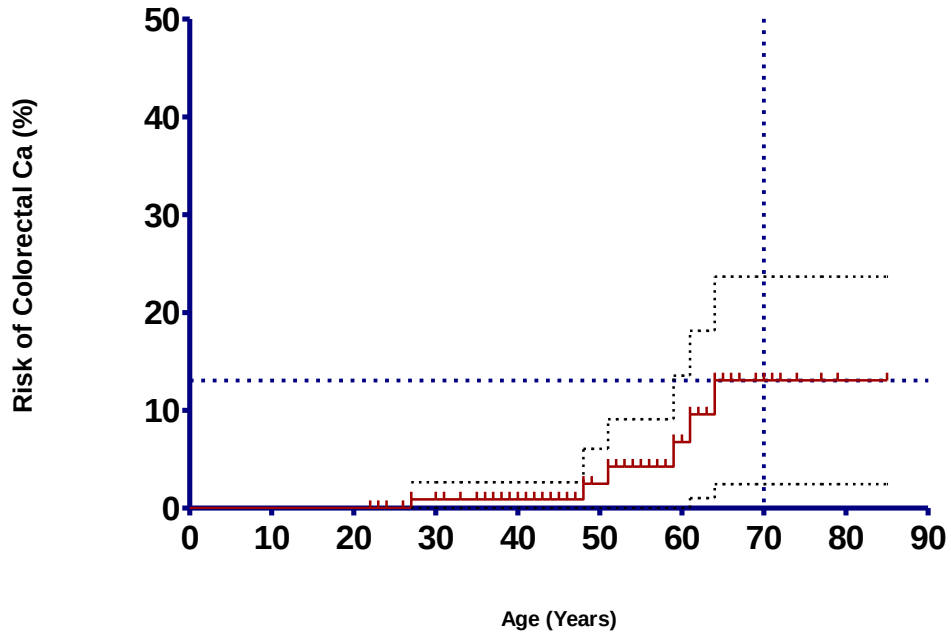


Figure 3-10 Kaplan-Meier curve (with 95% confidence intervals) for age related risk of developing a colorectal cancer

3.3.9 OTHER TUMOURS

9 different tumours in addition to colorectal cancer, not recognised as a cardinal tumour type in BHD have been identified in this cohort (Table 9). 3 patients from 2 families developed a malignant melanoma. One female patient from BHD1 was diagnosed at age 77 years (see multiple primary tumours below), and her nephew was diagnosed with 2 melanomas at ages 27 and 37 years. A female patient from BHD29 was diagnosed at age 55 years.

An adrenal cortical tumour was also diagnosed in 2 unrelated patients. Angiomyolipomas were also diagnosed in 2 unrelated patients. There were also single occurrences of breast cancer (age 45 years – see multiple primary tumours below), and a calcified tumour of the

heart ventricle. One patient had a thyroid follicular adenoma and another unrelated patient was diagnosed with a thyroid follicular cancer. One patient also had multiple basal cell carcinomas. He did not have any clinical evidence of Gorlin syndrome (naevoid basal cell carcinoma syndrome). One patient was diagnosed with an oesophageal adenocarcinoma at the age of 32 years. He was also diagnosed with a colorectal cancer at age 27 years and with a chromophobe renal tumour at age 32 years. He was subsequently found to have a germline variant in the *TP53* gene (see case report below).

3.3.10 Multiple Primary Tumours

4 patients in the cohort developed multiple primary tumours (excluding fibrofolliculomas). These patients are summarised in Table 13.

3.3.10.1 Case report:

Patient BHD62-1 had a medical history of ulcerative colitis and required a pan-proctocolectomy at the age of 27 years where an incidental rectal adenocarcinoma was detected during pathological examination of the resected specimen. He then went on to be diagnosed with a gastro-oesophageal junction adenocarcinoma on endoscopic evaluation at age 32 following symptoms of dysphagia. Upon this discovery, an incidental 6cm renal tumour was detected on staging imaging which required a nephrectomy and was histologically confirmed as a chromophobe tumour. *FLCN* testing identified a pathogenic missense variant c.715C>T (p.Arg239Cyst). Skin examination confirmed the presence of 10 facial fibrofolliculomas.

In collaboration with my colleague Dr James Whitworth, who is studying genetic factors in patients with multiple primary tumours, genetic testing using next-generation sequencing of 94 inherited cancer genes on the TruSight cancer panel identified, in addition to the *FLCN* missense variant, a likely pathogenic *TP53* missense variant (c.526C>T, p. Cys176Arg).

Table 13. *FLCN* mutation +ve individuals with multiple primary tumours

No	ID	Sex	Tumour 1 (Age of diagnosis)	Tumour2 (Age of diagnosis)	Tumour 3 (Age of diagnosis)	Mutation
1	BHD1-4	F	Colorectal Cancer (61)	RCC: Clear cell & oncocytoma (67)	Malignant Melanoma (77)	c.1285dupC
2	BHD17-4	F	RCC: clear cell (44)	Breast Cancer (45)	-	c.890_893delAAG
3	BHD27-4	M	RCC (42)	Lung cancer (46)	-	c.1318_1334dup17
4	BHD62-1*	M	Rectal adenocarcinoma (27)	Oesophageal adenocarcinoma (32)	RCC:chromophobe (32)	c.715C>T

*patient subsequently found to also harbour a germline *TP53* variant, RCC=renal cell carcinoma

3.3.11 GENOTYPE-PHENOTYPE CORRELATION

No clear differences were detected between the ages at onset of fibrofolliculomas according to the germline *FLCN* mutation (c.1285dupC vs c.1318_1334dup17, c.1285dupC vs c.890_893delAAAG). However, the exon 12 frameshift mutation c.1318_1334dup17 (p. L449QfsX255) was associated with a statistically significant higher lifetime risk (74% at age 70 years) of developing renal cancer than in BHD patients with the exon 11 common mutation c.1285dupC (p.H429Pfsx27) (29% at age 70 years) (p=0.0034) (Figure 3-11).

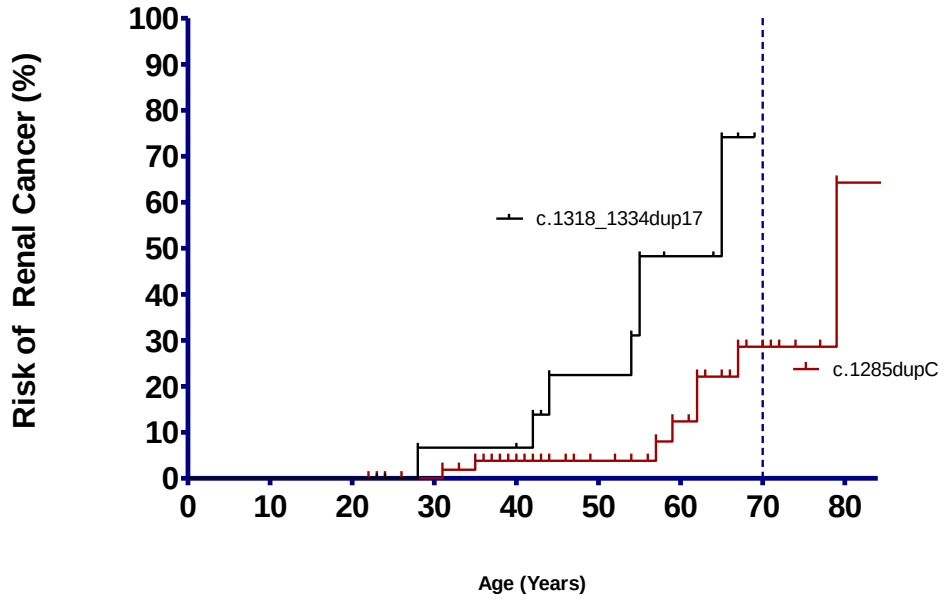
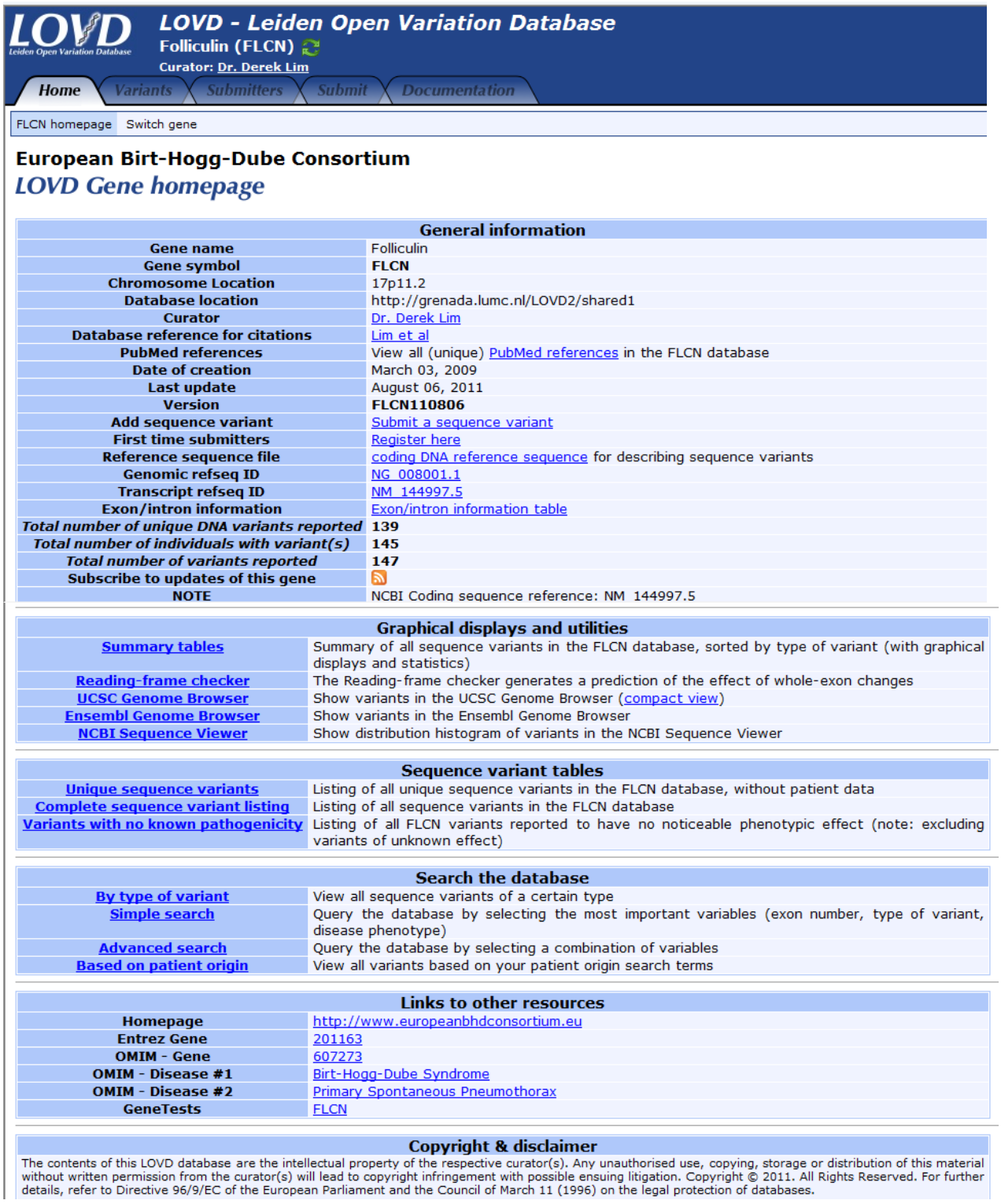


Figure 3-11 Kaplan-Meier curves for risk of renal cancer in *FLCN* mutation patients with c.1318_1334dup 17 vs c.1285dupC mutation

3.3.12 FLCN MUTATION DATABASE

At a cut off date of 06/08/2011 for the purpose of this chapter, there were 140 unique germline *FLCN* variants curated onto the *FLCN* mutation database available at www.lovd.nl/flcn (Figure 3-12). These included published variants (Benhammou *et al.*, 2011; Bessis *et al.*, 2006; Cho *et al.*, 2008; Frohlich *et al.*, 2008; Fuertes *et al.*, 2009; Gad *et al.*, 2007; Graham *et al.*, 2005; Gunji *et al.*, 2007; Imada *et al.*, 2009; Ishii *et al.*, 2009; Kawasaki *et al.*, 2005; Khoo *et al.*, 2002; Kim *et al.*, 2008; Kluger *et al.*, 2009; Kluijt *et al.*, 2009; Koga *et al.*, 2009; Kunogi *et al.*, 2010; Lamberti *et al.*, 2005; Leter *et al.*, 2008; Maffe *et al.*, 2011; Meyer *et al.*, 2009; Murakami *et al.*, 2007; Nahorski *et al.*, 2011; Nickerson *et al.*, 2002; Painter *et al.*, 2005; Palmirotta *et al.*, 2008; Ren *et al.*, 2008; Schmidt *et al.*, 2005; Sempau *et al.*, 2010; So, 2009; Tomassetti *et al.*; Toro *et al.*, 2008; van Steensel *et al.*, 2007; Warwick *et al.*, 2010; Woodward *et al.*, 2008) and novel variants that are unpublished. The database is publically available with useful search functions, database statistics, and hyperlinks to genome browsers and other resources including PubMed references.



LOVD - Leiden Open Variation Database

Folliculin (FLCN)

Curator: Dr. Derek Lim

Home | Variants | Submitters | Submit | Documentation

FLCN homepage | Switch gene

European Birt-Hogg-Dube Consortium

LOVD Gene homepage

General information	
Gene name	Folliculin
Gene symbol	**FLCN**
Chromosome Location	17p11.2
Database location	http://grenada.lumc.nl/LOVD2/shared1
Curator	Dr. Derek Lim
Database reference for citations	Lim et al
PubMed references	View all (unique) PubMed references in the FLCN database
Date of creation	March 03, 2009
Last update	August 06, 2011
Version	**FLCN110806**
Add sequence variant	Submit a sequence variant
First time submitters	Register here
Reference sequence file	coding DNA reference sequence for describing sequence variants
Genomic refseq ID	NG_008001.1
Transcript refseq ID	NM_144997.5
Exon/intron information	Exon/intron information table
Total number of unique DNA variants reported	**139**
Total number of individuals with variant(s)	**145**
Total number of variants reported	**147**
Subscribe to updates of this gene	
NOTE	NCBI Coding sequence reference: NM_144997.5

Graphical displays and utilities	
Summary tables	Summary of all sequence variants in the FLCN database, sorted by type of variant (with graphical displays and statistics)
Reading-frame checker	The Reading-frame checker generates a prediction of the effect of whole-exon changes
UCSC Genome Browser	Show variants in the UCSC Genome Browser (compact view)
Ensembl Genome Browser	Show variants in the Ensembl Genome Browser
NCBI Sequence Viewer	Show distribution histogram of variants in the NCBI Sequence Viewer

Sequence variant tables	
Unique sequence variants	Listing of all unique sequence variants in the FLCN database, without patient data
Complete sequence variant listing	Listing of all sequence variants in the FLCN database
Variants with no known pathogenicity	Listing of all FLCN variants reported to have no noticeable phenotypic effect (note: excluding variants of unknown effect)

Search the database	
By type of variant	View all sequence variants of a certain type
Simple search	Query the database by selecting the most important variables (exon number, type of variant, disease phenotype)
Advanced search	Query the database by selecting a combination of variables
Based on patient origin	View all variants based on your patient origin search terms

Links to other resources	
Homepage	http://www.europeanbhdconsortium.eu
Entrez Gene	201163
OMIM - Gene	607273
OMIM - Disease #1	Birt-Hogg-Dube Syndrome
OMIM - Disease #2	Primary Spontaneous Pneumothorax
GeneTests	FLCN

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Figure 3-12 Screenshot of www.lovd.nl/flcn homepage.

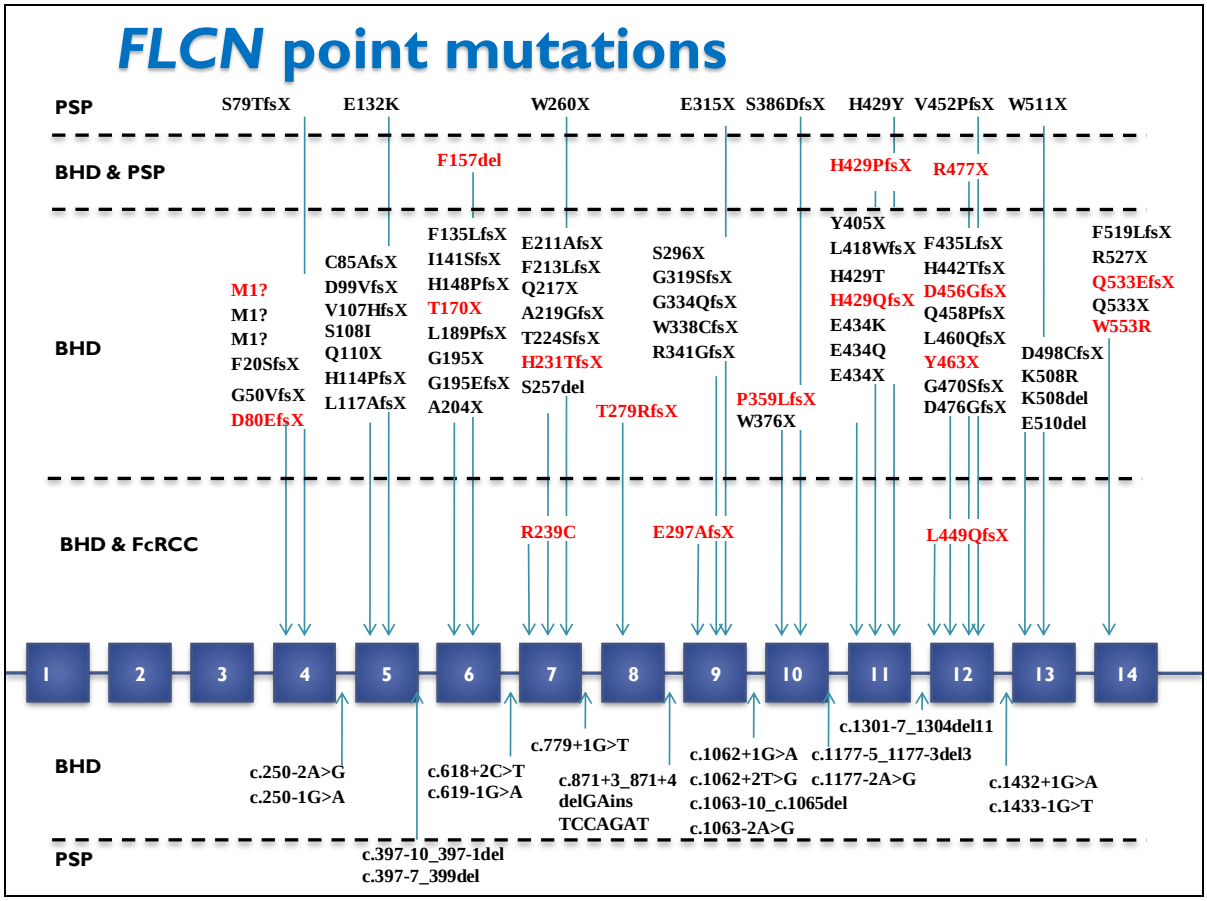

Figure 3-13 *FLCN* point mutations in the database (www.lovd.nl/flcn)

Distribution map of mutations in the *FLCN* gene and their associated phenotype. The blue boxes represent the exons. Mutation below the genogram represent intronic mutations affecting splice sites and displayed as the cDNA sequence change. Mutations above the exons represent mutations in the coding exons and are displayed as the protein change. The mutations in red represent the mutations that have been detected in the UK cohort of BHD patients in this thesis. BHD: Birt-Hogg-Dube; PSP: Primary Spontaneous Pneumothorax; FcRCC: Familial/inherited non-syndromic renal cell carcinoma. Genogram not drawn to scale.

Table 14. Point mutations listed on the *FLCN* mutation database

Exon	Mutation	Protein Change (Predicted)	Type	Concluded Pathogenicity	Reported Phenotype
4	c.1A>G	p.M1?	Missense	Probably pathogenic	BHD
4	c.3delG	p.M1?	Missense	Probably pathogenic	BHD
4	c.3G>A	p.M1?	Missense	Probably pathogenic	BHD
4	c.59delT	p.F20SfsX	Frameshift	Pathogenic	BHD
4	c.147delA	p.G50VfsX	Frameshift	Pathogenic	BHD
4	c.235_238delTCGG	p.S79TfsX	Frameshift	Pathogenic	PSP
4	c.240delC	p.D80EfsX	Frameshift	Pathogenic	BHD
4i	c.250-2A>G		Splicesite	Pathogenic	BHD
4i	c.250-1G>A		Splicesite	Pathogenic	BHD
5	c.252delC	p.C85AfsX	Frameshift	Pathogenic	BHD
5	c.296delA	p.D99VfsX	Frameshift	Pathogenic	BHD
5	c.319_320delGTinsCAC	p.V107HfsX	Frameshift	Pathogenic	BHD
5	c.323G>T	p.S108I	Missense	Probably pathogenic	BHD
5	c.328C>T	p.Q110X	Nonsense	Pathogenic	BHD
5	c.340dupC	p.H114PfsX	Frameshift	Pathogenic	BHD
5	c.347dupA	p.L117AfsX	Frameshift	Pathogenic	BHD
5	c.394G>A	p.E132K	Missense	Probably pathogenic	PSP
5i	c.397-10_397-1del		Splicesite	Pathogenic	PSP
5i	c.397-7_399del		Splicesite	Pathogenic	PSP
6	c.404delC	p.F135LfsX	Frameshift	Pathogenic	BHD
6	c.420delC	p.I141SfsX	Frameshift	Pathogenic	BHD
6	c.443_459del	p.H148PfsX	Frameshift	Pathogenic	BHD
6	c.469_471delTTC	p.F157del	Inframe deletion	Probably pathogenic	BHD,PSP
6	c.510C>A	p.T170X	Nonsense	Pathogenic	BHD
6	c.566_577delTGCTGGGGAAGGinsCC	p.L189PfsX	Frameshift	Pathogenic	BHD
6	c.583G>T	p.G195X	Nonsense	Pathogenic	BHD
6	c.584delG	p.G195EfsX	Frameshift	Pathogenic	BHD
6	c.610_611delGCinsTA	p.A204X	Nonsense	Pathogenic	BHD
6i	c.618+2C>T		Splicesite	Pathogenic	BHD
6i	c.619-1G>A		Splicesite	Pathogenic	BHD
7	c.632_633delAGinsC	p.E211AfsX	Frameshift	Pathogenic	BHD
7	c.637delT	p.F213LfsX	Frameshift	Pathogenic	BHD
7	c.649C>T	p.Q217X	Nonsense	Pathogenic	BHD
7	c.655dupG	p.A219GfsX	Frameshift	Pathogenic	BHD
7	c.671_672delCA	p.T224SfsX	Frameshift	Pathogenic	BHD
7	c.689dupT	p.H231TfsX	Frameshift	Pathogenic	BHD
7	c.715C>T	p.R239C	Missense	Pathogenic	BHD,FcRCC
7	c.769_771delTCC	p.S257del	Inframe deletion	Probably pathogenic	BHD
7	c.779G>A	p.W260X	Nonsense	Pathogenic	PSP
7i	c.779+1G>T		Splicesite	Pathogenic	BHD
8	c.836_839delCCGA	p.T279RfsX	Frameshift	Pathogenic	BHD
8i	c.871+3_871+4delGAinsTCCAGAT		Splicesite	Pathogenic	BHD
9	c.887C>A	p.S296X	Nonsense	Pathogenic	BHD

9	c.890_893delAAAG	p.E297AfsX	Frameshift	Pathogenic	BHD,FcRCC
9	c.943G>T	p.E315X	Nonsense	Pathogenic	PSP
9	c.923_950dup	p.G319SfsX	Frameshift	Pathogenic	BHD
9	c.997_998dupTC	p.G334QfsX	Frameshift	Pathogenic	BHD
9	c.1013delG	p.W338CfsX	Frameshift	Pathogenic	BHD
9	c.1021delC	p.R341GfsX	Frameshift	Pathogenic	BHD
9i	c.1062+1G>A		Splicesite	Pathogenic	BHD
9i	c.1062+2T>G		Splicesite	Pathogenic	BHD
9i	c.1063-10_c.1065del		Splicesite	Pathogenic	BHD
9i	c.1063-2A>G		Splicesite	Pathogenic	BHD
10	c.1076delC	p.P359LfsX	Frameshift	Pathogenic	BHD
10	c.1127G>A	p.W376X	Nonsense	Pathogenic	BHD
10	c.1156_1175del	p.S386DfsX	Frameshift	Pathogenic	PSP
10i	c.1177-5_1177-3del3		Splicesite	Pathogenic	BHD
10i	c.1177-2A>G		Splicesite	Pathogenic	BHD
11	c.1215C>G	p.Y405X	Nonsense	Pathogenic	BHD
11	c.1252delC	p.L418WfsX	Frameshift	Pathogenic	BHD
11	c.1285dupC	p.H429PfsX	Frameshift	Pathogenic	BHD,PSP
11	c.1285delC	p.H429TfsX	Frameshift	Pathogenic	BHD
11	c.1286dupA	p.H429QfsX	Frameshift	Pathogenic	BHD
11	c.1285C>T	p.H429Y	Missense	Probably pathogenic	PSP
11	c.1300G>A	p.E434K	Splicesite	Pathogenic	BHD
11	c.1300G>C	p.E434Q	Splicesite	Pathogenic	BHD
11	c.1300G>T	p.E434X	Nonsense	Pathogenic	BHD
11i	c.1301-7_1304del11		Splicesite	Pathogenic	BHD
12	c.1305delT	p.F435LfsX	Frameshift	Pathogenic	BHD
12	c.1323delCinsGA	p.H442TfsX	Frameshift	Pathogenic	BHD
12	c.1318_1334dup17	p.L449QfsX	Frameshift	Pathogenic	BHD,FcRCC
12	c.1340_1346dupCCACCCT	p.V452PfsX	Frameshift	Pathogenic	PSP
12	c.1367_1398del32	p.D456GfsX	Frameshift	Pathogenic	BHD
12	c.1372dupC	p.Q458PfsX	Frameshift	Pathogenic	BHD
12	c.1379_1380delTC	p.L460QfsX	Frameshift	Pathogenic	BHD
12	c.1389C>G	p.Y463X	Nonsense	Pathogenic	BHD
12	c.1408_1418delGGGAGCCCTGT	p.G470SfsX	Frameshift	Pathogenic	BHD
12	c.1426dupG	p.D476GfsX	Frameshift	Pathogenic	BHD
12	c.1429C>T	p.R477X	Nonsense	Pathogenic	BHD,PSP
12i	c.1432+1G>A		Splicesite	Pathogenic	BHD
12i	c.1433-1G>T		Splicesite	Pathogenic	BHD
13	c.1487_1490dupCTGT	p.D498CfsX	Frameshift	Pathogenic	BHD
13	c.1523A>G	p.K508R	Missense	VUS	BHD
13	c.1522_1524del	p.K508del	Inframe deletion	Pathogenic	BHD
13	c.1528_1530delGAG	p.E510del	Inframe deletion	Pathogenic	BHD
13	c.1533G>A	p.W511X	Nonsense	Pathogenic	PSP
14	c.1557delT	p.F519LfsX	Frameshift	Pathogenic	BHD
14	c.1579C>T	p.R527X	Nonsense	Pathogenic	BHD
14	c.1597_1598del	p.Q533EfsX	Frameshift	Pathogenic	BHD
14	c.1597C>T	p.Q533X	Nonsense	Pathogenic	BHD

14	c.1657T>C	p.W553R	Missense	Probably pathogenic	BHD

Mutations in yellow denote the point mutations detected in the UK BHD cohort in this thesis. (i = intron, BHD = Birt-Hogg-Dube syndrome, PSP = Primary Spontaneous Pneumothorax, FcRCC = Familial/inherited non-syndromic clear cell renal cell carcinoma)

3.3.13 FLCN VARIANTS/MUTATIONS

41/139 of the variants uploaded on to the database are classified as single nucleotide polymorphisms. In the remaining 98 putative mutations, 88 were concluded as pathogenic, 9 probably pathogenic, and 1 variant of unknown significance. Figure 3-13 and Figure 3-14 displays the putative mutations in relation to the *FLCN* gene. Table 14 summarises the point mutations.

Mutations are found in all coding exons (4-14) and a mutation hotspot exists in the polyC(8) tract in exon 11 with the c.1285dupC (previously reported as c.1733insC, c.1740dupC and C.1277insC) being the commonest mutation.

Leaving out SNPs and large intragenic copy number changes, at the nucleotide level, the majority of putative mutations are deletions (36/81, 44.4%) and single base substitutions 36/81, 44.4%). In addition, duplications (11/81, 16%), and deletion/insertions (6/81, 7.4%) make up the rest of the mutations.

The majority of mutations in *FLCN* are predicted (in the absence of nonsense-mediated mRNA decay) to lead to protein truncation and loss of function. The most frequent mutational consequences are frameshifts (43/88, 48.9%), which are predicted to result in a

premature stop codon. In addition there are 19 splicesite (19/88, 21.6%), 16 nonsense (16/88, 18.2%), 9 missense (9/88, 10.2%), 4 in-frame deletions (4/88, 4.5%) and 7 large intragenic copy number changes (7/88, 7.9%).

A total of 13 mutations have been reported in PSP. 3 of these mutations, the exon 11 common mutation c.1285dupC (p.His429ProfsX27), an exon 12 nonsense mutation c.1429C>T (p.Arg477X) and an exon 6 in-frame deletion c.469_471delTTC (p.Phe157del), are reported in both BHD and PSP.

In FcRCC families, 3 mutations, 2 frameshift mutations in exon 9 (c.890_893del, p.Glu297AlafsX25) and exon 12 (c.1318_1334dup17, p Leu449GlnfsX25) and a missense substitution in exon 7 (c.715C>T, p.Arg23Cyst) were detected. These 3 mutations have subsequently been found in patients presenting with classic BHD syndrome phenotype.

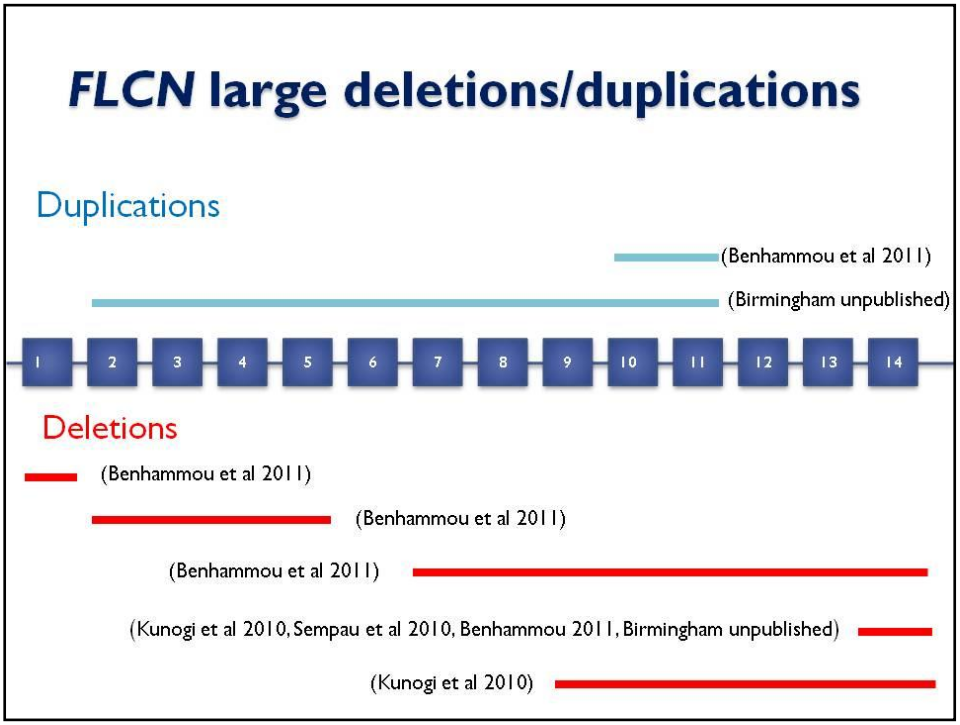

Figure 3-14 Large intragenic deletions and duplications in *FLCN* in the database

3.3.14 FLCN INTRAGENIC DELETIONS/DUPLICATIONS

7 large intragenic copy number changes have been identified in *FLCN* (Figure 3-14 above). 5 large intragenic deletions in *FLCN* have been reported in BHD. One of the deletions, the exon 14 deletion has been reported by 3 studies and in an unpublished UK family (Benhammou *et al.*, 2011; Kunogi *et al.*, 2010; Sempau *et al.*, 2010). Isolated exon 1 deletions have been reported by Benhammou and colleagues in 4 unrelated families (Benhammou *et al.*, 2011).

2 large intragenic duplications in *FLCN* have been identified to date. A duplication involving exons 10 and 11 was reported by Benhammou and colleagues in a North American BHD family. In the UK cohort, 2 unrelated families with BHD (BHD3 & BHD13) have been found to have a large duplication spanning exons 2 to 11. This duplication was found to be in tandem by analysis with long-range PCR by the diagnostic laboratory at the West Midlands Regional Genetics Laboratory.

3.3.15 GENOTYPE-PHENOTYPE CORRELATIONS FROM THE MUTATION DATABASE

Figure 3-13 above displays the point mutations that have been discovered in the *FLCN* gene and their corresponding disease of BHD, PSP or FcRCC. Point mutations have been identified in all the coding exons. There appear to be no strong correlation with a particular mutation in a particular exon with the development of just PSP or FcRCC nor clustering of mutations in particular exons for PSP or FcRCC.

3.4 DISCUSSION

BHD syndrome is a rare disorder and information on the frequency, natural history and nature of the clinical features is limited. This represents the first large cohort of BHD syndrome patients reported from the UK and there are only two other large series of BHD patients (from the USA and the Netherlands). Schmidt and Toro reported a total of 196 patients from 102 families from the National Cancer Institute (NCI) from the USA (Schmidt *et al.*, 2005; Toro *et al.*, 2008). Houweling described 115 patients from 35 families in the Netherlands (Houweling *et al.*, 2011). In contrast to these two studies we only included patients with a detectable *FLCN* mutation. In general the clinical features apparent in the UK cohort are similar to those in the other two large series.

80% of probands from the 39 families in the UK BHD cohort were diagnosed following investigations for skin manifestations. In addition, 87.3% of the symptomatic *FLCN* mutation positive individuals presented with fibrofolliculomas as their initial manifestation of BHD. The finding that some of these skin papules can be very mild leads to the impression that BHD is likely to be both under-diagnosed and under-recognised. Some of the mildly affected patients may not present to a doctor for treatment or diagnosis as they usually do not cause any symptoms. Therefore it is likely that there are BHD patients in the population who have not sought medical attention especially if there is no personal or family history of recurrent pneumothorax or kidney cancers. In addition, despite the presence of fibrofolliculomas, the underlying diagnosis of BHD may be delayed or overlooked in patients presenting with

pneumothorax or kidney cancer. This is shown in our cohort from the 10 probands who had required hospital admissions for recurrent pneumothoraces who were not diagnosed with BHD despite the presence of the skin papules at the time of the episodes of pneumothoraces. Therefore an earlier diagnosis of BHD could have been made in these families if the skin features were recognised. This suggests that there is still under recognition of BHD among non-dermatology clinicians.

There is wide variability in the severity of the fibrofolliculomas that develop in BHD patients. It shows both inter and intra familial variability. The fibrofolliculomas are the most penetrant of all the manifestations of BHD. 88% of patients will have manifested these skin papules by the time they reach the age of 40 years. Conversely, 12% of patients will not have manifested any of these skin papules by the age of 40 years. This has important considerations for clinicians who are assessing patients with a possible diagnosis of BHD. In order to avoid morbidity and mortality associated with late diagnosis of RCC, it is paramount that clinicians who are assessing patients with possible features of BHD have access to molecular genetic testing and that once a molecular diagnosis is made, all at risk relatives are offered predictive (presymptomatic) testing and regular clinical follow up and surveillance for RCC.

In the context of genetic counselling for pre-symptomatic testing for a known familial *FLCN* mutation, clinical geneticists offering testing to an apparently unaffected at risk family member should take into consideration the age of the patient and the likelihood of the age-related penetrance and variability of the skin features during the counselling and

assessment. In particular, caution should be taken in offering reassurance in the absence of skin manifestations during the pre-symptomatic testing counselling.

Looking at the initial manifestations in our cohort, fibrofolliculomas is the most common first manifestation followed by pneumothorax. 54% have only a single manifestation of BHD, with fibrofolliculomas the most likely. 31% of patients developed two manifestations, with the combination of fibrofolliculomas and pneumothorax the most common (54%) followed by fibrofolliculomas and renal cancer (38%). The patients with fibrofolliculomas and pneumothorax tend to develop their second manifestation at an earlier mean age (36.95 years) compared to patients who develop fibrofolliculomas and renal cancer (54.3years). Only 7.6% of patients developed 3 manifestations of BHD with a mean age of 47.9 years. There were no clear patterns of patients with multiple manifestations being clustered in particular families. The Kaplan-Meier curve generated (Figure 3-3) provides information on the age related probability of developing a first, second and third manifestation of BHD. This will be useful in the context of counselling of patients in the clinical genetics clinic.

The lifetime risk of pneumothorax in our cohort is 33% and the prevalence of lung cysts (among those that had CT scans) was 68%. In keeping with previous reported cohorts, the presence of lung cysts is the major determinant factor for pneumothorax (Toro *et al.*, 2007). Unfortunately, CT scanning of the thorax is not routinely offered to all all newly diagnosed patients but, if it were, it would improve knowledge of the natural history of the lung manifestations of BHD and inform individualised risk estimates of pneumothorax. CT scans of the thorax were only performed in 32 patients. There is also a lack of recognition of a

possible diagnosis of BHD in patients presenting with recurrent spontaneous pneumothorax. 20 patients had recurrent pneumothoraces in the cohort, but only less than half (41%) were referred for a CT scan of the thorax. Although we are not able to make a direct conclusion from this, this observation may suggest that there may be families presenting with recurrent pneumothoraces without a family history of BHD who may not be investigated by CT scans for the aetiology of their pneumothoraces and therefore not detecting the classical lung cysts that involve the lung bases. This could therefore miss a possible clinical diagnosis of BHD syndrome. As shown by the proband analysis earlier, there is also under recognition of the BHD skin lumps by clinicians managing patients with recurrent pneumothoraces.

In view of the high prevalence of lung cysts in BHD patients in this cohort, looking for the presence of typical BHD lung cysts by CT scans can help establish a diagnosis in the small percentage of suspected or possible BHD patients with normal *FLCN* testing.

The lifetime risk of renal cancer in this cohort is quite high at 36.8% compared to the Dutch cohort (16%) recently reported. One possibility for this difference may be due to ascertainment bias. However, analysis of the proband of each family in our cohort showed that the majority of BHD probands in our cohort were diagnosed following presentation of skin manifestations. The youngest age of diagnosis of a renal tumour in our cohort is at age 25 years. Therefore annual screening in BHD patients from the age of 20 years by renal imaging should be offered as recommended by the European BHD Consortium (Menko *et al.*, 2009). In our cohort renal surveillance has detected a number of asymptomatic renal tumours which provides further cause for the usefulness of surveillance. In keeping with

other renal cancer predisposition syndromes, renal tumours in our cohort of BHD patients include bilateral (37%) and multifocal tumours (17%). The management of renal tumours in BHD therefore is similar to von Hippel-Lindau disease. Nephron sparing surgery is appropriate once a solid tumour reaches 3cm in size.

Looking at the histology of the tumours, in our cohort clear cell histology was most common followed by chromophobe tumours. In contrast to the literature whereby a mixed/hybrid oncocytoma-chromophobe histology was reported to be the most common tumour type in BHD, this histology was only found in one patient in our cohort. A possible explanation for the discrepancy may be the element of bias in that 4 out of the 7 clear cell tumours in our cohort involved patients from the same large family (BHD1). Another explanation for the discrepancy may be that there may be histological appearances that are similar between clear cell RCC and chromophobe RCC both with granular/floccular eosinophilic cytoplasm (Houweling *et al.*, 2011). In the Dutch study this led to a classification of tumours being intermediate between clear cell and chromophobe carcinoma (CC/CPH). We did not go back to re-classify the tumour histology in the UK cohort. The other possibility for the discrepancy is that more than half (54%) of the patients with renal tumours were detected on surveillance scans and were asymptomatic. As the management of these tumours is to monitor the size until partial nephrectomy is required at 3cm, the histology of these tumours were not available. Looking at the tumour types that can occur in BHD, slow growing benign tumours such as oncocytomas can be present in patients for many years with slow growth and therefore never requiring surgical resection and this may cause an element of bias with regards to histology. Thus faster growing tumour types (such as clear

cell RCC) are more likely to be resected leading to available histology. In addition to the one case of a sarcomatoid transformation of RCC in the Dutch cohort, one patient in our UK cohort also developed a sarcomatoid renal carcinoma. We were unable to establish from the histology report what the possible original histology of the RCC was.

The European BHD consortium proposed diagnostic criteria for the diagnosis of BHD syndrome based on the presence of Major and/or Minor criteria (Menko *et al.*, 2009). Renal cancer of early onset (<50 years) or multifocal or bilateral renal cancer, or renal cancer of mixed chromophobe and oncocytic histology make up one of the minor criteria. Looking at the histological types of RCC that have been detected in our cohort, only one was found to have the mixed chromophobe and oncocytic histology. Therefore the diagnostic criteria may have to be revised as the Dutch cohort also found only 1 tumour of this mixed chromophobe-oncocytic histology.

The association between possible colorectal neoplasia and BHD was postulated following the recognition that Hornstein's report in 1976 of colonic polyps and generalised dermal perifollicular fibromas were in fact patients with what is now known as BHD syndrome (Hornstein, 1976). To date it is still debated whether BHD is associated with an increased risk of colorectal cancer. Zbar and colleagues did not identify any increased risk in their cohort from the NCI (Zbar *et al.*, 2002). However, Khoo reported a large French family harbouring an exon 11 c.1285delC mutation where 6 out of 8 mutation positive family members developed colonic polyps (Khoo *et al.*, 2002). They postulated that colorectal

neoplasia risk might apply to certain subgroups with possible environmental factors or genetic modifiers playing a role.

In collaboration with my colleague Dr Michael Nahorski and the group from the Netherlands (Dr Fred Menko, VUMC Amsterdam), we looked at a total of 149 patients with a clinical diagnosis of BHD from 51 families (32 with confirmed germline *FLCN* pathogenic mutations) from the UK (including 19 reported in this cohort/thesis: BHD1, BHD2, BHD3, BHD5, BHD6, BHD7, HD8, BHD9, BHD10, BHD12, BHD13, BHD14, BHD15, BHD16, BHD17, BHD20, BHD24, BHD27 & BHD31) and the Netherlands and found a lifetime risk of colorectal cancer of 11% up to age 70 years (Nahorski *et al.*, 2010). In the UK, the lifetime risk for developing colorectal cancer is 7.3% in men and 5.5% in women. (CRUK,http://www.cancerresearchuk.org/health-professional/cancer-statistics/risk/lifetime-risk#heading-One, Accessed May 2016). In my study (this thesis), which concentrates on families only with proven germline *FLCN* mutations, we identified colorectal neoplasia in 9 patients from 6 families who developed colorectal neoplasia of which there were 6 patients with colorectal cancer and 3 patients with colonic polyps. The lifetime risk in our cohort of colorectal cancer up to the age of 70 years is 13%. The median age for the diagnosis of colorectal cancer in this cohort was 55 years. Of note the patient from BHD62-1 suffered from ulcerative colitis and as part of his treatment for this he had a panproctocolectomy at age 27 which showed an incidental rectal adenocarcinoma. He subsequently developed further cancers and was also subsequently found to also have a germline variant in the *TP53* gene (see later).

In this cohort, 3 out of the 6 families that developed colorectal neoplasia (cancer or polyps) carry the c.1285dupC common mutation. This mutation occurs in a hypermutable mononucleotide tract (also called C_8 mononucletiode tract or poly C tract) in exon 11. In collaboration with Nahorski et al, we also looked at possible genotype-phenotype correlations for colorectal neoplasia in BHD. Looking at a combined total of 15 families from the UK (including BHD1, BHD9, BHD14, BHD20, BHD26 and BHD31) and the Netherlands with two recurrent mutations in *FLCN* showed a statistically significant increased lifetime risk of colorectal neoplasm in the c.1285dupC mutation carriers compared to the c.610_611delGinsTA mutation (Nahorski *et al.*, 2010). Therefore the risk of colorectal neoplasia in *FLCN* mutation positive individuals may be dependent on the genotype and suggest allelic heterogeneity. However other genetic modifiers and or environmental factors may be involved as only 3 out of 13 families (23%) with the c.1285dupC mutation reported individuals who developed colorectal neoplasia in this study/thesis. Another limitation of generating conclusions from this study is that only a small percentage (21/118, 17.8%) of *FLCN* mutation positive carriers in this cohort had a colonoscopy. 53 patients above the age of 45 years have not had a colonoscopy.

Interestingly, the family reported by Khoo with colorectal neoplasia harbour a mutation in the C_8 monucleotide tract (c.1285delC) (Khoo *et al.*, 2002). Both the c.1285dupC and c.1285delC frameshift mutations would result in truncation of the FLCN protein (p.His429ProfsX27 –lacking 126 aminoacids and p.His429THrfsX39 –lacking 114 respectively), in the absence of nonsense mediated decay. This may result in a dominant-negative effect in colorectal cells compared to the nonsense mutation c.610_611delGinsTA

(p.Ala204X – lacking 377 amino acids). Although both the mutations in the C_8monucleotide tract and the c.610_611delGinsTA mutation would result in loss of the C-terminus which contains the FNIP binding site, there may be other functions of FLCN (which is not fully understood currently) that may be altered differently by these different mutant proteins.

It is known that these monucleotide repeat tracts are hypermutable and susceptible to instability. Defects in mismatch repair genes (e.g. in Lynch syndrome) can result in increased microsatellite instability and contribute to tumourigenesis by predisposing to somatic mutations in colorectal cancer tumour suppressor genes that contain short repeat coding sequences (Lynch *et al.*, 2003; Markowitz *et al.*, 1995). Although Kahnoski and colleagues (Kahnoski *et al.*, 2003) did not detect any *FLCN* C_8 monunucleotide tract mutation in 8 microsatellite instable sporadic colorectal cancers, Nahorski found somatic *FLCN* C_8 monucleotide tract mutations in 23% of sporadic colorectal cancers which are microsatellite unstable (Nahorski *et al.*, 2010). This is in keeping with Shin and colleagues who reported a detection rate of 16% in 32 sporadic microsatellite instable colorectal cancers (Shin *et al.*, 2003).

In view of the finding by Woodward that 5% of patients with familial non-syndromic renal cell carcinoma have a germline *FLCN* mutation (Woodward *et al.*, 2008), Nahorski also looked at the rate of detection of *FLCN* germline mutations in 50 unrelated individuals with familial non-syndromic colorectal cancer cases (with no evidence of familial adenomatous polyposis or germline mismatch repair genes). No germline *FLCN* mutations were detected. Therefore it is unlikely that patients with BHD would present with familial non-syndromic

colorectal cancer. In addition, as the age of diagnosis of colorectal cancer in this UK BHD cohort is 55 years, it is less likely that BHD patients who develop colorectal cancer would fall under the category of familial colorectal cancer as the criteria for this diagnosis is biased towards a younger age of diagnosis (e.g. the Amsterdam criteria in Lynch syndrome). As a large proportion of older patients with *FLCN* mutations would be expected to have developed fibrofolliculomas, I suggest that in the absence of medical history or family history of BHD (skin fibrofolliculomas, history of pneumothorax or renal cancer) *FLCN* mutation testing is not indicated in this group of familial colorectal cancer patients.

Like many other genodermatoses there is wide variability between patients in the severity of BHD syndrome. Analysis of renal tumours from patients with BHD syndrome demonstrates loss of the wild type allele in tumour tissue consistent with "recessive tumour suppressor gene" model of tumourigenesis (Vocke *et al.*, 2005) and so stochastic factors will influence whether a patient develops RCC or not. In other inherited disorders with overlapping phenotypes such as VHL disease, both genotype-phenotype correlations associated with allelic heterogeneity and modifier effects may influence the risk of individual tumour types (Ong *et al.*, 2007; Webster *et al.*, 1998). No clear genotype-phenotype correlations have been described in BHD syndrome. Previously Schmidt and colleagues reported the c.1062+2T>G (previously called IVS9+2) splice site mutation conferred a possible higher risk of renal cancer (Schmidt *et al.*, 2005). However in a follow up study, this observation was not confirmed (Toro *et al.*, 2008). Benhammou and colleagues reported a possible genotype-phenotype correlation in BHD where families with an intragenic deletion of exon 1 (possible promoter region) detected on MLPA may have a

lower risk for the development of renal cancer (Benhammou *et al.*, 2011). However, the numbers were too small to make clear conclusions. None of the families in the UK cohort here and the Dutch cohort (Houweling *et al.*, 2011) were found to have an exon 1 deletion.

We found that the Leu449PhefsX9 mutation, identified in 4 families in our UK cohort, was associated with a high risk of RCC (8 mutation positive individuals from these 4 families developed renal cancer with a lifetime risk of renal cancer of 68%) compared to other *FLCN* mutation positive individuals. This candidate novel genotype-phenotype correlation will require confirmation in prospective analysis and in other cohorts of patients. Furthermore it would be of interest to know whether this mutation had differential effects on folliculin protein function compared to mutations with lower risks of RCC. However the function of the folliculin protein is not well defined. Both the Leu449PhefsX9 and the common His429ProfsX27 mutation would be expected to compromise binding of folliculin to FNIP1/2 and though folliculin has been reported to have a number of cellular functions including regulation of mTOR (Baba *et al.*, 2006; Hartman *et al.*, 2009), TGFbeta (Hong *et al.*, 2010), Rho (Nahorski *et al.*, 2012), HIF (Preston *et al.*, 2011) and other signalling pathways, it is not clear which functions are most relevant to folliculin renal tumour suppressor activity.

Previously Webster demonstrated that genetic modifier effects can influence age at diagnosis in VHL disease (Webster *et al.*, 1998). Here we found the first evidence of such effects in BHD syndrome with significantly better correlation of age at onset of fibrofolliculomas in close relatives than in more distantly related pairs. The strongest correlation was between first degree relatives (siblings and parent-child).

Patient BHD62-1 is one of four patients in the cohort who presented with more than one primary tumour type (rectal adenocarcinoma, oesophageal adenocarcinoma and chromophobe RCC). *FLCN* mutation testing identified a missense variant (c.715C>T, p. Arg239Cys) which has been shown to affect protein stability and therefore classified as a pathogenic mutation (Nahorski *et al.*, 2011). (To further confirm pathogenicity, this missense mutation has also been reported in a female patient who was previously thought to have familial non-syndromic clear cell RCC with a clear cell RCC at age 46 and a contralateral multicentric clear cell RCC at age 55 years (Woodward *et al.*, 2008). She would now be classified as having BHD in view of the diagnostic criteria proposed by the European BHD Consortium). The finding of a second likely pathogenic variant in a cancer predisposition gene, a *TP53* missense variant (c.526C>T, p. Cys176Arg) could explain the multiple tumours and early age of onset of tumours in this patient. This patient has now been written up as part of a case series with Dr James Whitworth (Whitworth *et al.*, 2015). Germline mutations in *TP53* are associated with the cancer predisposition syndrome Li Fraumeni syndrome (LFS). Individuals with LFS have a high lifetime risk of developing cancer with an estimated risk of 50% by age 30 years and 90% by age 60 years (Lustbader *et al.*, 1992). The main cancers associated with LFS are soft tissue sarcomas, osteosarcomas, breast cancer, adrenocortical carcinomas, brain tumours and leukaemias. Among other tumours reported as also occurring more frequently in LFS are gastrointestinal cancers (including oesophageal cancers) and genitourinary cancers (including renal cell carcinoma) and affected individuals have a high risk of developing multiple primary cancers and cancers at a young age (Gonzalez *et al.*, 2009; Lustbader *et al.*, 1992). Although the patient's

oesophageal cancer, rectal cancer (additional risk factor of ulcerative colitis) and RCC would be compatible with LFS, the chromophobe histology of the RCC would be more in keeping with the RCC seen in BHD. The *TP53* missense variant found in this patient has been described before as a somatic mutation in multiple tumours including colorectal adenocarcinoma. *In silico* prediction tools also predict a damaging or function altering effect on the protein (see (Whitworth *et al.*, 2015) and references within). Whitworth also identified a further two BHD *FLCN* mutation positive patients (not in this cohort) with multiple primary tumours harbouring a second cancer predisposition gene mutation (*FLCN* & *NF1* and *FLCN* & *MSH2*). We proposed the name Multiple Inherited Neoplasia Alleles Syndrome (MINAS) to describe patients with 2 or more inherited cancer predisposition alleles causing multiple primary tumours. In the case series, a neurofibromatosis type 1 patient with a *NF1* mutation was also found to have a *BRCA2* mutation and a patient with *MLH1* mutation also was found to have a homozygous *XPA* splice mutation. Therefore another explanation for phenotypic variation in some patients with BHD syndrome could be the presence a second cancer predisposition gene allele.

Environmental modifying factors can also be responsible for the phenotypic variability seen in BHD. Previous studies have shown that smoking increases the risk of cancers including kidney cancer as well as an increased risk of pneumothorax (Bense et al 1987, Cheng et al 2009). Although in this study I collected data on smoking status, the data returned was not complete in sufficient numbers to calculate pack years to allow us to make direct analysis of correlation between cigarette smoking and risk of fibrofolliculomas, lung cysts, pneumothorax or kidney tumour. Data collection on occupational sun exposure only

identified a small number of patients (n=3) with a high risk occupation (working long hours outdoors and/or significant time working in the middle-east). Therefore I was not able to study this with regards to either age of onset of fibrofolliculomas or severity of fibrofolliculomas in terms of number, distribution and growth. It would be interesting to compare this with a cohort of BHD patients in another country such as Australia with an increased sun exposure compared to the UK.

At the start of this project, I established and curated variants into the *FLCN* Locus Specific Database (available at www.lovd.nl/flcn) and analysed the contents of the database. Mutations in the *FLCN* gene have been reported spanning all coding exons (Lim *et al.*, 2010a; Wei *et al.*, 2009). Recently, large intragenic deletions and duplications have also been reported by using copy-number detection techniques such as MLPA (Benhammou *et al.*, 2011; Kunogi *et al.*, 2010; Sempau *et al.*, 2010). Therefore, mutation negative patients with clinical features of BHD who were investigated a number of years ago only by sequencing of *FLCN* should have updated molecular testing by MLPA. This would be expected to increase the mutation detection rate by a further 5%. From my results although there appear to be no genotype-phenotype correlations evident with regards to the location of the mutation with a corresponding phenotype of BHD, PSP or FcRCC, however, this may reflect the criteria for diagnosis of these conditions and the effect of variability of the clinical phenotype. For example, from the results of our study, the skin manifestations can be quite mild or may not have manifested yet in a *FLCN* mutation carrier depending on the age whereby the patient has been assessed. In addition, using the new diagnostic criteria proposed by the European BHD consortium, individuals who have been diagnosed as having

PSP or FcRCC who have a germline *FLCN* mutation would now be classified as having BHD, despite the absence of the other features of BHD.

In summary this BHD clinical and molecular study contributes a further 118 *FLCN* mutation positive BHD patients from 39 families to the medical literature and represent the largest cohort of BHD patients in the UK. It presents a total of 5985 patient years of natural history data with age related penetrance of the various manifestations of BHD. The study provides further evidence of allelic heterogeneity and modifying genetic factors as a potential cause of variability of the phenotype in BHD.

CHAPTER 4. OVERALL CONCLUSIONS

4.1 CLINICAL AND EPIGENETIC INVESTIGATIONS OF BWS

I studied a cohort of ART conceived BWS patients and found that the predominant (96%) molecular defect causing BWS in this group are IC2 epimutations (loss of methylation at KvDMR1). This is in contrast to naturally conceived sporadic BWS cases where 50% of cases have IC2 epimutations. Comparing the phenotypes of ART conceived and naturally conceived BWS patients with IC2 epimutations, I found phenotypic differences: a reduced incidence of exomphalos, increased facial naevus flammeus and an increased risk of non-Wilms tumour neoplasia in the ART conceived cases. There were no incidences of Wilms tumour in both cohorts. I studied the possible cause of this variation by looking for evidence of additional loss of methylation (epimutations) at other imprinted loci that are normally methylated in the maternal allele at 6q24 (*PLAGL1* DMR), 7q32 (*PEG1* DMR) and 15q13 (*SNRPN* DMR) and found additional loss of methylation cases in both cohorts. The frequency of additional loss of methylation were significantly higher in the ART conceived BWS cohort (37.5% vs 6.4% p=0.034). In the subgroup of BWS patients with additional loss of methylation at multiple imprinted loci I looked for evidence of loss or gain of methylation at the *DLK1* DMR at 14q32 and found none. The observations in this study suggest that the process of ART act as an environmental modifier that increases the risk of epimutations at imprinted DMRs. The variation in phenotype between the ART conceived and naturally conceived BWS cases could be caused by these methylation abnormalities acting as epigenetic modifiers of the phenotype.

4.2 NATURAL HISTORY OF BIRT-HOGG-DUBE SYNDROME

Studying the *FLCN* Mutation positive BHD patients in this UK cohort the following observations are made. The three main organ systems that are affected in BHD are the skin, lungs and kidneys. The most penetrant manifestation is the skin fibrofolliculomas with a lifetime risk of 97% and a mean age of onset of 34.1 years. The second most common manifestation are the lung cysts with a prevalence of 68%. The lifetime risk of developing a pneumothorax is 33%. The lifetime risk of renal cell carcinoma in this cohort is 36.8% with a median age of diagnosis of 52 years (range 25-85). The renal tumours that occur in BHD show features of other kidney cancer predisposition syndromes including the presence of bilateral and multifocal tumours. Therefore BHD patients will require annual surveillance for renal tumour by MRI scans. The lifetime risk of colon cancer is 13% with a median age of diagnosis of 55 years (range 27-64) and may be mutation-dependant. There is inter- and intra-familial variation in the various features of BHD that could be explained by allelic heterogeneity and modifying genetic factors. I also identified a novel genotype-phenotype correlation with the Leu449Phen frameshift mutation that increases the risk of renal cell carcinoma in BHD.

4.3 CAUSES OF PHENOTYPIC VARIABILITY IN BECKWITH-WIEDEMANN SYNDROME AND BIRT-HOGG-DUBE SYNDROME

Variability in the phenotype of genetic disorders can occur as a result of multiple causes including allelic heterogeneity, locus heterogeneity, genetic and epigenetic modifiers, environmental modifiers and chance. In my MD project, I studied two genetic kidney tumour predisposition syndromes Beckwith-Wiedemann syndrome (BWS) and Birt-Hogg-Dube syndrome (BHD) to study factors that affect variability in these two conditions.

4.3.1 BECKWITH WIEDEMAN SYNDROME

4.3.1.1 Locus Heterogeneity

In BWS, I have shown that in agreement with previous studies, locus heterogeneity at 11p15.5 plays an important role in the variability of the phenotype in BWS. In the BWS IC2 epimutation cohort (both ART and naturally conceived) studied here, there was no occurrence of Wilms tumour. This is in keeping with previous findings and studies suggesting that the increased Wilms tumour risk is associated with an overexpression of *IGF2* due to upd(11)pat and IC1 epimutation (*H19* DMR hypermethylation).

Locus heterogeneity has also been demonstrated in other human imprinting syndromes. In Silver-Russell syndrome (SRS), phenotypic differences have been described between SRS cases with *H19* DMR hypomethylation and upd(7)mat cases (Wakeling *et al.*, 2010). Learning difficulties and speech delay were more frequent in the upd(7)mat group but congenital anomalies and fifth finger clinodactyly occurred more frequently in the *H19* DMR hypomethylation group.

4.3.1.2 Epigenetic Modifiers

I also demonstrated that *trans*- acting epigenetic modifiers (loss of methylation at various imprinted loci) can affect the phenotype in BWS. This study shows that there is variation in the phenotype of BWS cases conceived with ART compared to naturally conceived BWS cases with IC2 epimutations. There was a reduced incidence of exomphalos, an increased incidence of facial naevus flemmeus and more importantly a potentially increased incidence of non-Wilms tumour embryonal tumours in the ART conceived BWS cases. This difference in phenotype could be potentially explained by the presence of further epigenetic alterations by the finding of additional loss of methylation at other imprinted loci. This variation in phenotype due to additional loss of methylation at other imprinted loci has also been seen in another human imprinting syndrome Transient Neonatal Diabetes (TNDM) (Mackay *et al.*, 2006a).

4.3.1.3 Environmental modifiers

The use of assisted reproductive technologies (ART) is a possible environmental factor that can affect phenotype by increasing the risk of loss of methylation at multiple imprinted loci. In BWS cases where sporadic naturally conceived cases have been shown to be due to IC2 epimutation (KvDMR1 hypomethylation) in 50% of cases, in ART conceived BWS cases, almost all cases are due to this epimutation. The incidence of multi imprinted loci loss of methylation was also significantly higher in BWS cases conceived by ART. As discussed above, this could explain the difference in phenotype between the two groups of BWS patients.

4.3.2 BIRT-HOGG-DUBE SYNDROME

4.3.2.1 Allelic Heterogeneity

In BHD syndrome, I have showed that there is both intra and inter-familial variation in expression of the clinical phenotype as well as penetrance of the various features (fibrofolliculomas, lung cysts, pneumothorax, renal cancer and colorectal cancer). I also found that different mutations can apparently influence the risk of specific tumours that occur in BHD syndrome. A particular frameshift mutation c.1318_1334dup17 (p.Leu449PhenfsX27) conferred a higher risk of renal cancer in the UK cohort BHD patients compared to the exon 11 common mutation. In addition, the incidence of colorectal cancer appears to be higher in patients with the presence of the exon 11 common mutation and appears to segregate in some families.

In another genetic kidney predisposition syndrome VHL disease, similar allelic heterogeneity has been described with the variable risk of certain tumour types. Phaechromocytoma is reported to be associated with an increased risk in the presence of certain missense mutations in the *VHL* gene and a decreased risk in the presence of truncating mutations or large deletions (Chen *et al.*, 1995; Crossey *et al.*, 1994; Maher *et al.*, 1996).

4.3.2.2 Genetic Modifiers

In the BHD study I have shown evidence of the likely presence of genetic modifiers influencing the age of onset of the skin fibrofolliculomas in *FLCN* mutation positive BHD patients. By comparing the age of onset of fibrofolliculomas between different degrees of relationships between the *FLCN* mutation positive patients, I have shown a statistically

significant closer correlation of the age of onset of fibrofolliculomas in first degree relatives (siblings and parent-child) compared to more distantly related relatives.

Similarly in VHL disease, pair wise analysis looking at the number of ocular angiomas that occurred identified evidence for genetic modifiers with first degree relative (siblings and parent-child) showing statistically significant correlation (Webster *et al.*, 1998).

The finding of a second genetic cancer predisposition allele (a *TP53* missense variant) in one of the *FLCN* mutation positive BHD patients in this cohort with multiple primary tumours together with other reported *FLCN* mutation positive BHD patients with MINAS (multiple inherited neoplasia allele syndrome) developing multiple primary tumours reveals a “trans-acting” genetic modifier. Other examples of these in the literature include children with more than one autosomal recessive condition due to consanguinity in the family (Marin *et al.*) and co-occurrence of rare genetic diseases in non-consanguineous families (Adams *et al.*, 2014), both of which can causes overlapping or complex phenotypes.

CHAPTER 5. FUTURE DIRECTIONS

5.1 BECKWITH-WIEDEMANN SYNDROME

5.1.1 DISCOVERY OF NEW EPIGENETIC LOCI MODIFIERS

Further analysis of a larger number of DMRs in more extensive cohorts of BWS patients should provide further information on the relative frequencies of abnormal methylation at different imprinted loci in ART conceived and naturally conceived BWS patients. Whole genome methylation array and whole genome bisulphite sequencing can be considered to determine extent of methylation disturbance on a larger scale.

5.1.2 IDENTIFICATION OF GENETIC BASIS TO EXPLAIN RISK OF BWS IN ART

The use of next generation sequencing techniques such as whole exome or genome sequencing of trios of parents and affected child in ART conceived BWS compared to controls may help elucidate the genetic basis of why only some children who are conceived with ART develop BWS.

5.1.3 IDENTIFICATION OF TRANS-ACTING GENES THAT AFFECT IMPRINTING

The occurrence of MLID is not restricted to ART conceived BWS cases but is also seen, albeit less frequently, in naturally conceived BWS cases. Currently there are 4 known *trans*-acting genes that can be associated with MLID (*NLRP2*, *NRLP5*, *NRLP7* and *ZFP57*). Whole exome or genome sequencing of cohorts of BWS patients with multiple imprinted DMR hypomethylation (affected child and unaffected parent trio) can be undertaken to identify known or novel *trans*-acting genes that affect imprinting in the proband. Sequencing the grandparents in addition can also help clarify significance of variants identified, clarify phase

and parent of origin effects, and help interpret variants in mothers of affected probands in potential maternal effect genes affecting imprinting in *trans*.

5.2 BIRT-HOGG-DUBE SYNDROME

5.2.1 LARGE PROSPECTIVE COHORT STUDIES

The observations in this MD project provide a basis for large collaborative studies to further delineate the natural history as well as investigate the molecular basis of our findings in a large cohort of BHD patients. As BHD remains a rare and under-recognised condition, such a study would have to be collaborative and multinational to achieve such an objective. International consensus on management of the cancer risk and surveillance can be guided by the results of such studies.

5.2.2 MULTIPLE PRIMARY TUMOUR STUDY

Another avenue for investigation in BHD is to undertake exome or whole genome sequencing of constitutional and tumour DNA in affected patients with multiple primary tumours (and tumours that do not fit with a single genetic cause) to detect cohorts that may have multiple inherited neoplasia allele syndrome (MINAS).

5.2.3 IDENTIFICATION OF MOLECULAR CAUSE OF BHD IN FLCN MUTATION NEGATIVE CASES

In a cohort of families with a clinical diagnosis of BHD who test negative for *FLCN* mutations and copy number changes, further studies by whole exome or genome sequencing have the potential to identify the molecular cause of their disease. This could be a novel gene or a mutation not currently detectable using current techniques of sequencing and copy number detection.

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APPENDIX

Appendix A BECKWITH WIEDEMANN SYNDROME CLINICAL INFORMATION QUESTIONNAIRE

Questionnaire Beckwith- Wiedemann Syndrome

Name:

Date of Birth:

Clinical history:

Regarding pregnancy:

History of IVF or ICSI	☐ yes ☐no ☐ unknown
Duration of pregnancy	.....weeks
Pre-eclampsia	☐ yes ☐no ☐ unknown
polyhydramnios	☐ yes ☐no ☐ unknown

Regarding perinatal period (< 1 month):

birth weight	.....gram
length	.....cm
Placenta larger than usual	☐ yes ☐no ☐ unknown
Feeding difficulties	☐ yes ☐no ☐ unknown
Apnoea	☐ yes ☐no ☐ unknown

Regarding postnatal period (>1month)

Feeding difficulties	☐ yes ☐no ☐ unknown
Apnoea	☐ yes ☐no ☐ unknown
Cyanosis	☐ yes ☐no ☐ unknown
Seizures	☐ yes ☐no ☐ unknown
Visual defect	☐ yes ☐no ☐ unknown
Hearing loss	☐ yes ☐no ☐ unknown
Mental retardation	☐ yes ☐no ☐ unknown

Family:

Are there any more relatives with the	
Beckwith-Wiedemann Syndrome	☐ yes ☐no ☐ unknown
if yes, who?	.....

Laboratory findings:

Neonatal hypoglycaemia	☐ yes ☐no ☐ unknown
Neonatal polycythaemia	☐ yes ☐no ☐ unknown

CLINICAL FEATURES:

In general:

Height	.....cm (age.....years)
Weight	.....kg (age.....years)
Head circumference	.....cm (age.....years)
hemihypertrophy	☐ yes ☐no ☐ unknown
If yes which parts of body?	.....
Advanced bone age	☐ yes ☐no ☐ unknown

Head/Neck

Macroglassia	☐ yes	☐no	☐ unknown
Macrostomia	☐ yes	☐no	☐ unknown
Prognathism	☐ yes	☐no	☐ unknown
Facial naevus flammeus	☐ yes	☐no	☐ unknown
Maxillary hypoplasia	☐ yes	☐no	☐ unknown
Prominent occiput	☐ yes	☐no	☐ unknown
Cleft palate	☐ yes	☐no	☐ unknown
Other dysmorphias	☐ yes	☐no	☐ unknown

if yes, which ? .....

Earlobe anomalies:

Earlobe creases	☐ yes	☐no	☐ unknown
Helical ear pits	☐ yes	☐no	☐ unknown

Thorax:

Congenital cardiac malformations	☐ yes	☐no	☐ unknown

if yes, which .....

Abdomen:

Exomphalos	☐ yes	☐no	☐ unknown
Umbilical hernia	☐ yes	☐no	☐ unknown
Malrotation of the gut	☐ yes	☐no	☐ unknown
Diastasis recti	☐ yes	☐no	☐ unknown
Inguinal hernia	☐ yes	☐no	☐ unknown
Cryptorchidism	☐ yes	☐no	☐ unknown
Hypospadia	☐ yes	☐no	☐ unknown
Clitoromegaly	☐ yes	☐no	☐ unknown

Organomegaly:

Nephromegaly	☐ yes	☐no	☐ unknown
Splenomegaly	☐ yes	☐no	☐ unknown
Hepatomegaly	☐ yes	☐no	☐ unknown

Tumours:

Wilms' tumour	☐ yes	☐no	☐ unknown
Adrenal cortical carcinoma	☐ yes	☐no	☐ unknown
Rhabdomyosarcoma	☐ yes	☐no	☐ unknown
Hepatoblastoma	☐ yes	☐no	☐ unknown
Neuroblastoma	☐ yes	☐no	☐ unknown

Other features .....

Thank you very much for your help.

Filled in by.....
Phone.....*Date*.....

Professor E. R. Maher
Division of Medical Genetics
University of Birmingham
Birmingham Women's Hospital
Edgbaston
BIRMINGHAM B15 2BR

Appendix B BIRT-HOGG-DUBE CLINICAL INFORMATION PROFORMA

THE UNIVERSITY OF BIRMINGHAM

BIRT-HOGG-DUBE PROFORMA

Name: ***FLCN* mutation:** ☐Y ☐N

DOB: **Sex:** ☐M ☐F

Date of assessment: **Ethnicity:**

Patient ID: **Contact number:**

Skin manifestations:

Fibrofolliculomas?	☐Y	☐N	
Histological diagnosis?	☐Y	☐N	Biopsy done at:
Facial FFs	☐Y	☐N	Number:
Truncal FFs	☐Y	☐N	Number:

Age FFs diagnosed: Age patient noticed FFs:

Other skin lesions/tumours and age of diagnosis:

Other skin conditions (eg. Eczema):

Lung manifestations:

Pneumothorax?	☐Y	☐N	Number of Pneumothoraces:

Age of 1st pneumothorax:

Asymptomatic lung cysts?	☐Y	☐N	Age diagnosed:

Date of last chest scan: Date of normal chest scan:

Type of chest scan: Where scans performed:

Frequency of scans:

Kidney manifestations:

Renal Tumour?	☐Y	☐N	Bilateral	☐Y	☐N

Age renal tumour diagnosed: Pathology:

Age last normal MRI/CT/USS kidney: Tumour detected by screening or symptoms:

Type of Scan: Screening Symptoms

Where scans performed: List symptoms:

Name: Patient ID:

Colon manifestations:

Colorectal Polyp? ☐Y ☐N Age of diagnosis of polyps

Location of polyps:

Polyps identified by screening or symptoms:

Screening: Type of scan

Symptoms: List symptoms:

Colorectal cancer ☐Y ☐N Age of diagnosis of cancer:

Age last normal colonoscopy:

Identified by screening or symptoms:

Screening: Type of scan

Symptoms: List symptoms:

Other manifestations:

Malignancies: Age malignancy diagnosed

1.

2.

Other cystic lesions (kidney/liver/pancreas):

1.

Other diagnosis: Age of other diagnosis

1.

2.

3.

Lifestyle:

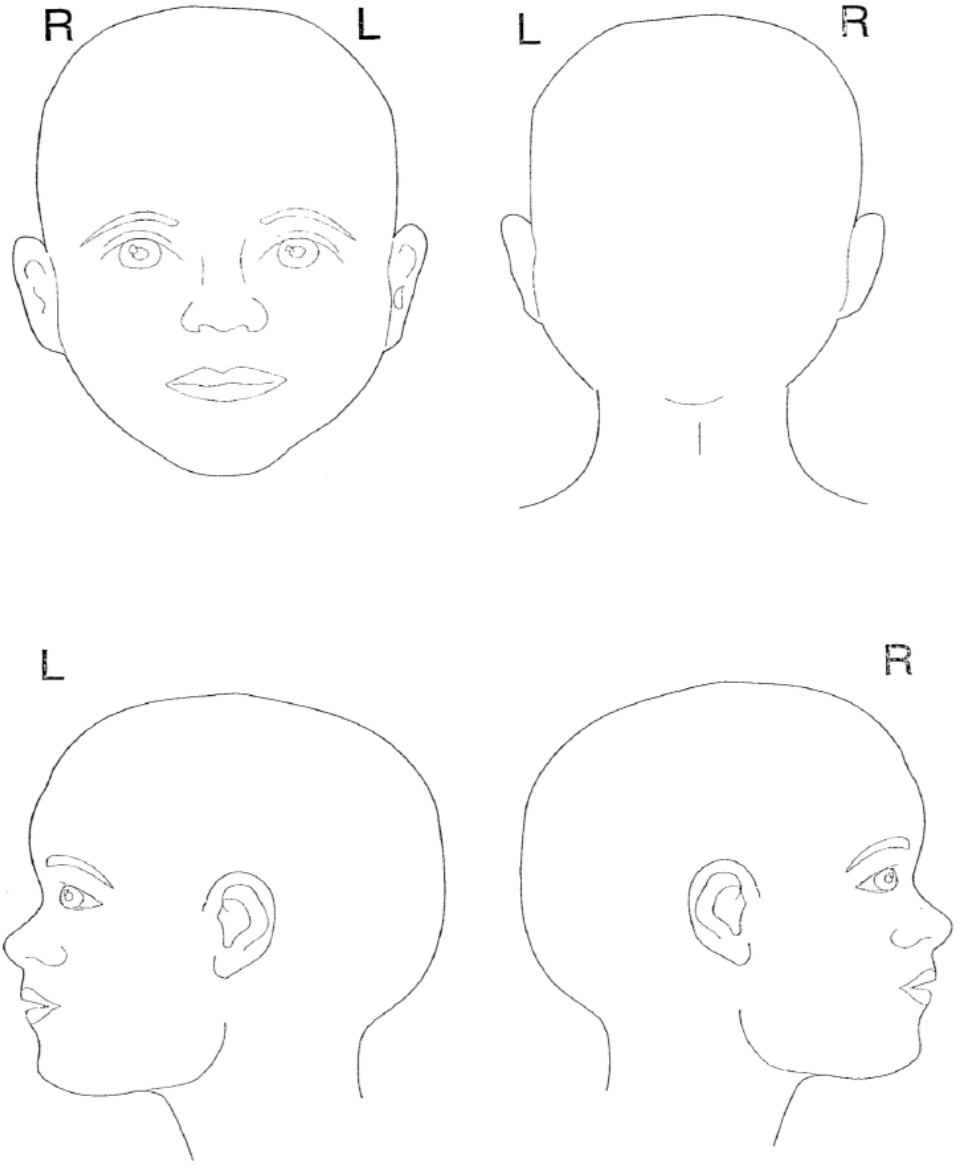
Smoker ☐Y ☐N Duration: Cigarettes per day:

Average alcohol consumption per week: units

Occupation:

Name: Patient ID:

Skin:

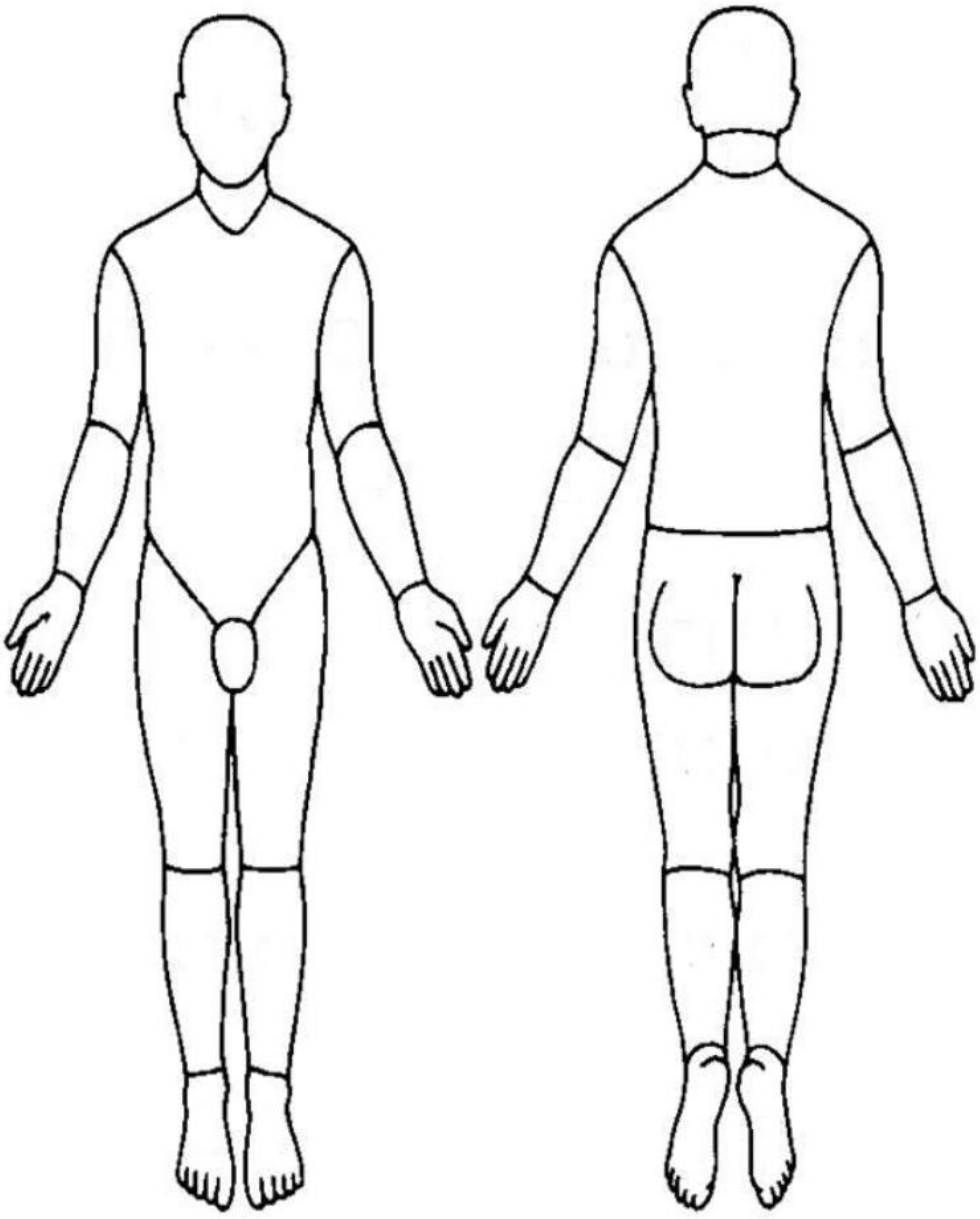


Head circumference (cm):

Name: Patient ID:

THE UNIVERSITY OF BIRMINGHAM

BIRT-HOGG-DUBE PROFORMA



Name: Patient ID:

Notes:

Appendix C FOLLICULIN (FLCN) CODING DNA REFERENCE SEQUENCE

(used for mutation description) (last modified June 18, 2009)

The sequence was taken from NM_144997.5 *Please note that introns are available by clicking on the exon numbers above the sequence.*

```
(upstream sequence)
                                                .         .              g.5024
                                          gagtcacgcgcctgggtgtcggcg       c.-481

.         .         .         .         .         .                      g.5084
gggctgcgggaccgcgagtgagtgtggtcgctcctggttctgccagctcccctgagagcc             c.-421

.         .         .         .         .         .                      g.5144
tgaacccgggcttgagagcctcgccaccccgggtgacatccctgccgtgggcttgggggc             c.-361

.         .         .         .         .         .                      g.5204
tctgggtgtgattccgccggtccgggtcccgcagcgaccacctacccagcgcagtcaggg             c.-301

.         .         .         .         .         .                      g.5264
gcgggggctgggacccagagcgggaccccggctgccgagtccaggtgtcccgcgggcctcg             c.-241

.         .    | 02   .         .         .         .                   g.9334
atttggggagcag | aaaacgccaggtcttcaagggtgtctgccaccaccatgcctgaccca         c.-181

.         .         .         .         .         .                      g.9394
tttggcagcagcctcgtgtgtggtggtctggtgtggacggtggaagcgtgattctgctga           c.-121

.       | 03 .         .         .         .         .                   g.10359
gtgtcag | tgtgaccactcgtgctcagccgtatctcagcaggaggacaggtgccggagcag         c.-61
```

```
.         .         .         .      | 04 .         .            g.14051
ctcgtgcagctaagcagccaactgcagaaacgtcag | gcctgttgcagtctccaaggcacc    c.-1

          .         .         .         .         .         .      g.14111
ATGAATGCCATCGTGGCTCTCTGCCACTTCTGCGAGCTCCACGGCCCCCGCACTCTCTTC      c.60
M  N  A  I  V  A  L  C  H  F  C  E  L  H  G  P  R  T  L  F       p.20

          .         .         .         .         .         .      g.14171
TGCACGGAGGTGCTGCACGCCCCACTTCCTCAAGGGGATGGGAATGAGGACAGTCCTGGC      c.120
C  T  E  V  L  H  A  P  L  P  Q  G  D  G  N  E  D  S  P  G       p.40

          .         .         .         .         .         .      g.14231
CAGGGTGAGCAGGCGGAAGAAGAGGAAGGTGGCATTCAGATGAACAGTCGGATGCGTGCG      c.180
Q  G  E  Q  A  E  E  E  E  G  G  I  Q  M  N  S  R  M  R  A       p.60

          .         .         .         .         .         .      g.14291
CACAGCCCCGCAGAGGGGGCCAGCGTCGAGTCCAGCAGCCCGGGGCCCAAAAAGTCGGAC      c.240
H  S  P  A  E  G  A  S  V  E  S  S  S  P  G  P  K  K  S  D       p.80

          | 05      .         .         .         .         .      g.15917
ATGTGCGAG | GGCTGCCGGTCACTTGCTGCAGGGCACCCGGGATATATCAGCCATGATAAA    c.300
M  C  E   | G  C  R  S  L  A  A  G  H  P  G  Y  I  S  H  D  K       p.100

          .         .         .         .         .         .      g.15977
GAGACCTCCATTAAAATACGTCAGCCACCAGCACCCCAGCCACCCCCAGCTCTTCAGCATT      c.360
E  T  S  I  K  Y  V  S  H  Q  H  P  S  H  P  Q  L  F  S  I       p.120

          .         .         .      | 06 .         .         .   g.18069
GTCCGCCAGGCCTGTGTCCGGAGCCTGAGCTGTGAG | GTCTGCCCTGGCCGTGAAGGCCCC    c.420
V  R  Q  A  C  V  R  S  L  S  C  E   | V  C  P  G  R  E  G  P      p.140
```

```
        .         .         .         .         .         .       g.18129
ATCTTCTTCGGAGATGAGCAGCACGGCTTTGTGTTCAGCCACACCTTCTTCATCAAGGAC       c.480
I  F  F  G  D  E  Q  H  G  F  V  F  S  H  T  F  F  I  K  D        p.160

        .         .         .         .         .         .       g.18189
AGCCTGGCCAGGGGCTTCCAGCGCTGGTACAGCATCATCACCATCATGATGGACCGGATC       c.540
S  L  A  R  G  F  Q  R  W  Y  S  I  I  T  I  M  M  D  R  I        p.180

        .         .         .         .         .         .       g.18249
TACCTCATCAACTCCTGGCCCTTCCTGCTGGGGAAGGTCCGGGGAATCATCGATGAGCTC       c.600
Y  L  I  N  S  W  P  F  L  L  G  K  V  R  G  I  I  D  E  L        p.200

        .       | 07        .         .         .         .       g.19569
CAGGGCAAGGCGCTCAAG | GTGTTTGAGGCAGAGCAGTTTGGATGCCCACAGCGTGCTCAG       c.660
Q  G  K  A  L  K   | V  F  E  A  E  Q  F  G  C  P  Q  R  A  Q        p.220

        .         .         .         .         .         .       g.19629
AGGATGAACACAGCCTTCACGCCATTCCTACACCAGAGGAACGGCAACGCCGCCCGCTCG       c.720
R  M  N  T  A  F  T  P  F  L  H  Q  R  N  G  N  A  A  R  S        p.240

        .         .         .         .         .       | 08
g.20561
CTGACATCGCTGACAAGTGATGACAACCTGTGGGCGTGCCTGCACACCTCCTTTGCCTG | G    c.780
L  T  S  L  T  S  D  D  N  L  W  A  C  L  H  T  S  F  A  W  |     p.260

        .         .         .         .         .         .       g.20621
CTCCTGAAGGCGTGTGGCAGCCGGCTGACCGAGAAGCTCCTGGAAGGTGCTCCGACCGAG       c.840
L  L  K  A  C  G  S  R  L  T  E  K  L  L  E  G  A  P  T  E        p.280

        .         .         . | 09        .         .         .       g.23008
```

```
GATACCTTGGTCCAGATGGAGAAGCTCGCTG | ATTTAGAAGAGGAATCAGAAAGCTGGGAC    c.900
D  T  L  V  Q  M  E  K  L  A  D |  L  E  E  E  S  E  S  W  D      p.300

         .        .        .        .        .        .             g.23068
AACTCTGAGGCTGAAGAGGAGGAGAAAGCCCCTGTGTTGCCAGAGAGTACAGAAGGGCGG      c.960
N  S  E  A  E  E  E  E  K  A  P  V  L  P  E  S  T  E  G  R       p.320

         .        .        .        .        .        .             g.23128
GAGCTGACCCAGGGCCCGGCAGAGTCCTCCTCTCTCTCAGGCTGTGGGAGCTGGCAGCCC      c.1020
E  L  T  Q  G  P  A  E  S  S  S  L  S  G  C  G  S  W  Q  P       p.340

         .        .        .        .  | 10     .        .        g.25024
CGGAAGCTGCCAGTCTTCAAGTCCCTCCGGCACATGAGGCAG | GTCCTGGGTGCCCCTTCT    c.1080
R  K  L  P  V  F  K  S  L  R  H  M  R  Q  | V  L  G  A  P  S      p.360

         .        .        .        .        .        .             g.25084
TTCCGCATGCTGGCCTGGCACGTTCTCATGGGGAACCAGGTGATCTGGAAAAGCAGAGAC      c.1140
F  R  M  L  A  W  H  V  L  M  G  N  Q  V  I  W  K  S  R  D       p.380

         .        .        .     | 11 .        .        .        g.25709
GTGGACCTCGTCCAGTCAGCTTTTGAAGTACTTCGG | ACCATGCTTCCCGTGGGCTGCGTC    c.1200
V  D  L  V  Q  S  A  F  E  V  L  R  | T  M  L  P  V  G  C  V      p.400

         .        .        .        .        .        .             g.25769
CGCATCATCCCATACAGCAGCCAGTACGAGGAGGCCTATCGGTGCAACTTCCTGGGGCTC      c.1260
R  I  I  P  Y  S  S  Q  Y  E  E  A  Y  R  C  N  F  L  G  L       p.420

         .        .        .        . | 12     .        .        g.26892
AGCCCGCACGTGCAGATCCCCCCCCACGTGCTCTCCTCAG | AGTTTGCTGTCATCGTGGAG    c.1320
S  P  H  V  Q  I  P  P  H  V  L  S  S  E |  F  A  V  I  V  E      p.440
```

```
        .         .         .         .         .         .       g.26952
GTCCACGCAGCCGCACGTTCCACCCTCCACCCTGTGGGGTGTGAGGATGACCAGTCTCTC       c.1380
V  H  A  A  A  R  S  T  L  H  P  V  G  C  E  D  D  Q  S  L         p.460

        .         .         .         .         .   | 13    .     g.27106
AGCAAGTACGAGTTTGTGGTGACCAGTGGGAGCCCTGTAGCTGCAGACCGAG | TGGGCCCC     c.1440
S  K  Y  E  F  V  V  T  S  G  S  P  V  A  A  D  R  V |  G  P       p.480

        .         .         .         .         .         .       g.27166
ACCATCCTGAATAAGATTGAAGCGGCTCTGACCAACCAGAACCTGTCTGTGGATGTGGTG       c.1500
T  I  L  N  K  I  E  A  A  L  T  N  Q  N  L  S  V  D  V  V         p.500

        .         .         .       | 14        .         .       g.28354
GACCAGTGCCTCGTCTGCCTCAAGGAGGAGTGGATGAA | CAAAGTGAAGGTGCTTTTTAAG     c.1560
D  Q  C  L  V  C  L  K  E  E  W  M  N | K  V  K  V  L  F  K       p.520

        .         .         .         .         .         .       g.28414
TTCACCAAGGTGGACAGTCGACCCAAAGAGGACACACAGAAGCTGCTGAGCATCCTGGGT       c.1620
F  T  K  V  D  S  R  P  K  E  D  T  Q  K  L  L  S  I  L  G         p.540

        .         .         .         .         .         .       g.28474
GCGTCCGAGGAGGACAATGTCAAGCTGCTGAAGTTCTGGATGACTGGCCTGAGCAAGACC       c.1680
A  S  E  E  D  N  V  K  L  L  K  F  W  M  T  G  L  S  K  T         p.560

        .         .         .         .         .         .       g.28534
TACAAGTCACACCTCATGTCCACGGTCCGCAGCCCCACAGCCTCGGAGTCTCGGAACTGA       c.1740
Y  K  S  H  L  M  S  T  V  R  S  P  T  A  S  E  S  R  N  X         p.579

        .         .         .         .         .         .       g.28594
```

cccgtcacacacacctgcc**taa**agacagggatggctgtccacaggatcctccagccccgt c.*60

. . . . . g.28654

gagagggactgtccct**tga**gtttctcaactgctggaaggagctgtgtcccagcaaggaag c.*120

. . . . . g.28714

ggaaaccatcagggctgggctcggccctgtcaggtttggggcctgtgtgcttcccagact c.*180

. . . . . g.28774

ctccctccagccgttggaatcgctgaagatggcaatgaaaggcggagggatgatgggctc c.*240

. . . . . g.28834

tctctgtgttcaaactccttggagagacgac**tag**gaggacagcttgcctcccaggcccct c.*300

. . . . . g.28894

tgtggacttagactcaaaacccgcaggagaaacaggtccgactcagtatgcagtcgcaat c.*360

. . . . . g.28954

aacatgtctgctcccgaggttaacattcaagcgtttctactttgaaattcagcaagagtt c.*420

. . . . . g.29014

tctgggccttatgtttgagggtacctttgctgcagttgtgaatattcagtacattgcca c.*480

. . . . . g.29074

gctcttggtcactgag**tga**ttgagt**tag**ggctccgcaagagactttggggag**tga**agtgg c.*540

. . . . . g.29134

atctcttcctcatcttttggtcctc**tga**aatgtgtgttctgaagccatggggctcgtctt c.*600

. . . . . g.29194

ctggggtgttcccctgcaggtgctggtgaaggtaacctggggcttaatgatggagtccct c.*660

. . . . . g.29254

gatcatttttgcacaagacaggttgctgaggggtcggcaagcatctgacttgcccaatcc c.*720

. . . . . g.29314

cctggatatggtgagccccgccatgcttttattctgtatcgcttttgtctttattgctgc c.*780

. . . . . g.29374

tttcaacatttacgtttggttacagttaactattttcggagtgtgg**tga**ttgaagacaat c.*840

. . . . . g.29434

ttcatcatcccactgtacttttttttttgagagggagtttcactcttgttgcccaggctg c.*900

. . . . . g.29494

gagtgcaatggcacgatcttggctcactgcaacctctgcctcctgggttcaagcaattct c.*960

. . . . . g.29554

cctgcctcagcctccagag**tag**ctggaactacaggtgcccgccactatgcccagc**taa**tt c.*1020

. . . . . g.29614

tttgtatttttagtagagacggggtttcaccgtgttggccgggctggtctcaaactcct c.*1080

. . . . . g.29674

gacctcaggtgatccacccacctcagcctcccaaagtgctgggattacaagcgtgagcca c.*1140

. . . . . g.29734

ctgtgcctggccctttttttttttttttttttttttttaaagagatggcatcttgctat c.*1200

. . . . . g.29794

gtcgtccaggctggtcttgaactcc**tga**gttcaagcagtcctcctgcttcaacatacagc c.*1260

. . . . . g.29854

tacaggtacccccactatacattttaataaggattcatggctcagagggattttctga c.*1320

. . . . . g.29914

tggttttgctgatttgtttctagttttttgtgtttatatttaaca**tga**agaccaagttt c.*1380

. . . . . g.29974

atataac**tag**gtatctgta**taa**tgcaacaacattggaacacaa**taa**agatgtatttttgt c.*1440

g.29977

aaa c.*1443

(downstream sequence)

Legend:

Nucleotide numbering (following the rules of the HGVS for a 'Coding DNA Reference Sequence') is indicated at the right of the sequence, counting the A of the ATG translation initiating Methionine as 1. Every 10th nucleotide is indicated by a "." above the sequence. The Folliculin protein sequence is shown below the coding DNA sequence, with numbering indicated at the right starting with 1 for the translation initiating Methionine. Every 10th amino acid is shown in bold. The position of introns is indicated by a vertical line, splitting the two exons. The start of the first exon (transcription initiation site) is indicated by a '\', the end of the last exon (poly-A addition site) by a '/'. The exon number is indicated above the first nucleotide(s) of the exon. To aid the description of frame shift mutations, all **stop codons in the +1 frame are shown in bold** while all stop codons in the +2 frame are underlined.

Appendix D PUBLICATIONS ARISING FROM THIS THESIS

Lim, D, Bowdin, SC, Tee, L, Kirby, GA, Blair, E, Fryer, A, Lam, W, Oley, C, Cole, T, Brueton, LA, Reik, W, Macdonald, F, Maher, ER (2009a) Clinical and molecular genetic features of Beckwith-Wiedemann syndrome associated with assisted reproductive technologies. *Hum Reprod* **24**(3)**:** 741-747.

Lim, DH, Maher, ER (2009b) Human imprinting syndromes. *Epigenomics* **1**(2)**:** 347-369.

Lim, DH, Rehal, PK, Nahorski, MS, Macdonald, F, Claessens, T, Van Geel, M, Gijezen, L, Gille, JJ, Giraud, S, Richard, S, van Steensel, M, Menko, FH, Maher, ER (2010a) A new locus-specific database (LSDB) for mutations in the folliculin (FLCN) gene. *Hum Mutat* **31**(1)**:** E1043-1051.

Lim, DHK, Maher, ER (2010b) DNA methylation: a form of epigenetic control of gene expression. *The Obstetrician & Gynaecologist* **12**(1)**:** 37-42

Appendix E PRESENTATIONS ARISING FROM THIS THESIS

Platform presentations:

Clinical investigation of the UK cohort of FLCN mutation positive Birt-Hogg-Dube syndrome patients:

1. The 91st Annual Meeting of the British Association of Dermatologist (BAD): London July 2011 (Plenary session)
2. American Society of Human Genetics (ASHG) conference: Washington DC November 2010 (Concurrent session)
3. British Human Genetics Conference: Warwick September 2010 (Concurrent session)

Investigating the Folliculin (FLCN) mutation positive Birt-Hogg-Dube syndrome patients in the UK: Clinical and Molecular Genetic analysis:

1. 3rd International Birt-Hogg-Dube syndrome Symposium: Maastricht May 2011

Germline mutation in NLRP2 (NALP2) in a familial imprinting disorder (Beckwith-Wiedeman Syndrome):

1. European Society of Human Genetics: Human Genetics Conference: Vienna May 2009 (Concurrent session)
2. Epigenome Network of Excellence (NOE) meeting: Edinburgh June 2009

Clinical and molecular genetic features of Beckwith-Wiedemann Syndrome associated with Assisted Reproductive Technologies:

1. UK Clinical Genetics Society (CGS) Spring meeting: Liverpool May 2008

Seminars:

Clinical and molecular genetic investigation of Birt-Hogg-Dube syndrome:

1. West Midlands Regional Genetics Service – Genetics Directorate lunchtime Seminar September 2010
2. Department of Genetic Medicine Manchester – Genetics seminar October 2012

3. West Midlands Dermatogenetics meeting - Seminar November 2009

Clinical and molecular genetic features of Beckwith-Wiedemann Syndrome associated with Assisted Reproductive Technologies:

1. West Midlands Regional Genetics Service – Genetics Directorate lunchtime Seminar September 2009

Poster presentations:

The Folliculin (FLCN) mutation database: an online resource for FLCN sequence variants involved in Birt-Hogg-Dube syndrome

1. The 5th National Cancer Research Institute (NCRI) Cancer Conference: – Birmingham October 2009
2. UK Cancer Genetics Group (CGG) Spring Meeting – Glasgow May 2009 – **BEST POSTER PRIZE**

Clinical and molecular genetic features of Beckwith-Wiedemann Syndrome associated with Assisted Reproductive Technologies:

1. University of Birmingham College of Medical and Dental Sciences Research & Enterprise Gala April 2010

Appendix F PATIENT INFORMATION SHEETS AND CONSENT FORMS CREATED

Birmingham Women's NHS
NHS Foundation Trust

PRINCIPAL INVESTIGATOR:
PROFESSOR EAMONN MAHER

UNIVERSITY OF BIRMINGHAM
Medicine within the:
College of Medicine and Dental Sciences
Medical and Molecular Genetics
The Medical School
Edgbaston
Birmingham B15 2TT
United Kingdom
Study Coordinator
Sarah Hadfield Tel: (0121) 627 2723

INVITATION TO TAKE PART IN A PROJECT
TO INCREASE RESEARCH INTO THE CLINICAL AND GENETIC FEATURES OF:
BECKWITH WIEDEMANN SYNDROME

You are invited to take part in a research project. Before you decide whether or not you wish to take part it is important for you to understand why the project is being done and what it will involve if you take part. Please read the following information carefully. Discuss it with your friends and relatives if you wish. Ask us if there is anything you don't understand or if you would like more information. You will be given as much time as you want to make a decision.

WHAT ARE WE AIMING TO DO?

We would like to study the gene(s)/genetic factors involved in Beckwith Wiedemann Syndrome, and may be the cause the condition in you and/or your family.

WHY ARE WE ASKING FOR YOUR HELP?

We are seeking your help as we need to investigate the genes/genetic factors of many individuals and/or families with exactly the same condition. The cells in your blood carry a complete set of your genetic material (genes), which we can study. We would like to store some of the genetic material in the laboratory because it may take some years and many experiments to understand the genetic factors that cause the condition in you and/or your family.

WHAT WILL YOU HAVE TO DO?

We will ask you and other members of your family to consider giving us a small blood sample **(e.g. 10mls/2 teaspoons)** taken through a needle from a vein in your arm. Usually this only causes brief discomfort and occasionally a small bruise. The sample will be used to obtain the genetic material from the white cells. Sometimes we may use alternative samples to obtain genetic material (e.g. saliva, mouth swab, tissue samples from biopsies, surgery or stored pathology specimens). We will ask some questions about

your medical and family history. We may want to ask for your permission to look at your medical records. We will ask for your permission to put information about your family onto a computer database to store the information. We will remove all personal details, such as names and addresses, so your family cannot be recognized from it.

WILL THIS PROJECT BENEFIT YOU OR YOUR FAMILY?

We cannot guarantee to discover anything that will directly benefit you or your family. However, we hope to find the gene(s)/genetic factors causing the condition in you or your family. If this happens then genetic tests may be available for your relatives to find out if they are at risk of developing the condition. In this case we will inform you (unless you indicate you would not wish to be contacted). We hope that in the long term future, this research could lead to improved treatments for the condition.

DO YOU HAVE TO TAKE PART?

Your taking part in this project is voluntary. If you would prefer not to take part you do not have to give a reason. You may also withdraw from the project at any time. This will not affect your or your family's medical care. If this research leads to the development of a new treatment or medical test, you will NOT benefit financially from this. If in the future you lose the ability to understand the research or die, you may still want us to carry on the research (unless your family states otherwise).

WHO WILL KNOW ABOUT YOU TAKING PART?

The information collected about you during the course of the research project will be kept strictly confidential and you will not be identifiable from it. Any results arising from this research work will be kept strictly confidential. If any research results are published in medical articles as a result of this project all personal details will be removed so that your family cannot be recognized from it. With your permission your G.P. will be told that you have agreed to be involved in the project.

WHAT OTHER INFORMATION MAY BE PRODUCED BY THE RESEARCH?

You will NOT be told about any genetic alterations which are identified as a by-product of this research that are not relevant to you or your family's illness. In some cases the genetic information produced by studying your genetic material may be placed in an electronic archive with no connection to your name or other personal identifier. This archive will only be accessible to appropriate doctors and researchers who have been approved by a committee set up to ensure the results are only used to advance scientific and medical understanding. Although there is a theoretical possibility that you could be identified by the deposited information (e.g. if you are entered into another independent genetic study), this is extremely unlikely.

Principal Investigator ER Maher, Professor of Medical Genetics and Honorary Consultant in Clinical Genetics

WHO DO YOU CONTACT WITH ANY CONCERNS?

If you have any questions or concerns about this project, please contact Prof. Eamonn Maher **or the Patient Advise & Liaison Service (PALS) on 0121 627 2747**. Alternatively you can write to the following contact addresses:

Prof. Eamonn Maher
Medical and Molecular Genetics
(University of Birmingham)
Norton Court
Birmingham Women's Hospital
Edgbaston
Birmingham
B15 2TG

Principal Investigator ER Maher, Professor of Medical Genetics and Honorary Consultant in Clinical Genetics
Telephone 0121 627 2741 Fax 0121 627 2618 Email E.R.Maher@bham.ac.uk

Birmingham Women's NHS
NHS Foundation Trust

UNIVERSITY OF BIRMINGHAM

PRINCIPAL INVESTIGATOR:
PROFESSOR EAMONN MAHER

Eamonn Maher
Professor of Medical Genetics
BSc MBChB MRCP FRCP MD MA FMed Sci
Department of Medical and Molecular Genetics
(University of Birmingham)
Norton Court
Birmingham Women's Hospital
Edgbaston Birmingham, B15 2TG
Tel: (0121) 627 2630 Fax: (0121) 627 2618
Study Coordinator
Sarah Hadfield Tel: (0121) 627 2723

Family no:
Patient Identification Number:

CONSENT FORM

Title of Project: Molecular Pathology of Human Genetic Disease

A PROJECT TO STUDY THE CLINICAL AND GENETIC FEATURES OF:
BECKWITH WIEDEMANN SYNDROME

Name of Researcher: Prof. Eamonn Maher

Please initial boxes

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

2. (a) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

   (b) I give you permission to continue to use the samples and information collected as part of this research in the event of my losing capacity or dying unless my next of kin requests otherwise. ☐

3. I understand that relevant sections of any of my/ my child's medical notes and data collected during the study, may be reviewed by individuals from the project team, from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. (a) I agree to allow medical information about my family to be entered on a confidential computer database. ☐

   (b) In addition, in some cases the genetic information produced by studying my/ my child's DNA may be placed in an electronic archive with no connection to my/ my child's name or other personal identifier. I understand that this archive will only be accessible to appropriate doctors and researchers who have been approved by a committee set up to ensure the results are only used to advance scientific and medical understanding. Although there is a theoretical possibility that I/ my child could be identified by the deposited information (e.g. if I/ my child is entered into another independent genetic study), this is extremely unlikely. ☐

   (c) If further medical information is requested by members of the project team, I agree to be contacted again for this purpose. ☐

5. (a) I agree to provide a sample, or for..... (my child) to provide a sample, which will be stored and may be used for genetic research studies appropriate to my/ my family's condition. I understand that any results arising from this research work will be kept strictly confidential. ☐

(b) I understand that the techniques used are NOT suitable for diagnostic testing for known genetic defects, and that I will NOT be told about any genetic alterations which are identified as a by-product of this research that are not relevant to my/ my family's illness. This will not affect my/ my child's access to clinically approved genetic advice and testing through other doctors caring for me in any way. ☐

(c) If a genetic test becomes available as a result of medical research on my/ my family's sample(s) I would like to have the opportunity to discuss the implications of these findings with appropriate medical experts. ☐

6. I understand that I/ my child will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

_____	_____	_____
Name of Participant	Date	Signature (if signing on behalf of Participant state your name & relationship to them)
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

(1 for participant; 1 for researcher; 1 for hospital notes)

UNIVERSITY OF BIRMINGHAM

Birmingham Women's NHS
NHS Foundation Trust
PRINCIPAL INVESTIGATOR:
PROFESSOR EAMONN MAHER

School of Clinical and Experimental Medicine within the:
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United Kingdom
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E mail g.a.kirby@bham.ac.uk

INVITATION TO TAKE PART IN A PROJECT
TO INCREASE RESEARCH INTO THE CLINICAL AND GENETIC FEATURES OF:
BIRT HOGG DUBE

You are invited to take part in a research project. Before you decide whether or not you wish to take part it is important for you to understand why the project is being done and what it will involve if you take part. Please read the following information carefully. Discuss it with your friends and relatives if you wish. Ask us if there is anything you don't understand or if you would like more information. You will be given as much time as you want to make a decision.

WHAT ARE WE AIMING TO DO?

We would like to study the gene(s)/genetic factors involved in Birt Hogg Dube, and may be the cause the condition in you and/or your family.

WHY ARE WE ASKING FOR YOUR HELP?

We are seeking your help as we need to investigate the genes/genetic factors of many individuals and/or families with exactly the same condition. The cells in your blood carry a complete set of your genetic material (genes), which we can study. We would like to store some of the genetic material in the laboratory because it may take some years and many experiments to understand the genetic factors that cause the condition in you and/or your family.

WHAT WILL YOU HAVE TO DO?

We will ask you and other members of your family to consider giving us a small blood sample **(e.g. 10mls/2 teaspoons)** taken through a needle from a vein in your arm. Usually this only causes brief discomfort and occasionally a small bruise. The sample will be used to obtain the genetic material from the white cells. Sometimes we may use alternative samples to obtain genetic material (e.g. saliva, mouth swab, tissue samples

from biopsies, surgery or stored pathology specimens). We will ask some questions about your medical and family history. We may want to ask for your permission to look at your medical records. We will ask for your permission to put information about your family onto a computer database to store the information. We will remove all personal details, such as names and addresses, so your family cannot be recognized from it.

WILL THIS PROJECT BENEFIT YOU OR YOUR FAMILY?

We cannot guarantee to discover anything that will directly benefit you or your family. However, we hope to find the gene(s)/genetic factors causing the condition in you or your family. If this happens then genetic tests may be available for your relatives to find out if they are at risk of developing the condition. In this case we will inform you (unless you indicate you would not wish to be contacted). We hope that in the long term future, this research could lead to improved treatments for the condition.

DO YOU HAVE TO TAKE PART?

Your taking part in this project is voluntary. If you would prefer not to take part you do not have to give a reason. You may also withdraw from the project at any time. This will not affect your or your family's medical care. If this research leads to the development of a new treatment or medical test, you will NOT benefit financially from this. If in the future you lose the ability to understand the research or die, you may still want us to carry on the research (unless your family states otherwise).

WHO WILL KNOW ABOUT YOU TAKING PART?

The information collected about you during the course of the research project will be kept strictly confidential and you will not be identifiable from it. Any results arising from this research work will be kept strictly confidential. If any research results are published in medical articles as a result of this project all personal details will be removed so that your family cannot be recognized from it. With your permission your G.P. will be told that you have agreed to be involved in the project.

WHAT OTHER INFORMATION MAY BE PRODUCED BY THE RESEARCH?

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Principal Investigator ER Maher, Professor of Medical Genetics and Honorary Consultant in Clinical Genetics
Telephone 0121 627 2741 Fax 0121 627 2618 Email E.R.Maher@bham.ac.uk

WHO DO YOU CONTACT WITH ANY CONCERNS?

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Birmingham Women's NHS
NHS Foundation Trust

UNIVERSITY OF BIRMINGHAM

PRINCIPAL INVESTIGATOR:
PROFESSOR EAMONN MAHER

Eamonn Maher
Professor of Medical Genetics
BSc MBChB MRCP FRCP MD MA FMed Sci
Department of Medical and Molecular Genetics
(University of Birmingham)
Norton Court,
Birmingham Women's Hospital,
Edgbaston, Birmingham, B15 2TG
Tel: (0121) 627 2741 Fax: (0121) 627 2618
Research Nurse
Gail Kirby Tel: (0121) 623 6859

Family no:
Patient Identification Number:

CONSENT FORM

Title of Project: Molecular Pathology of Human Genetic Disease

A PROJECT TO STUDY THE CLINICAL AND GENETIC FEATURES OF:
BIRT HOGG DUBE SYNDROME

Name of Researcher: Prof. Eamonn Maher

Please initial boxes

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

2. (a) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

   (b) I give you permission to continue to use the samples and information collected as part of this research in the event of my losing capacity or dying unless my next of kin requests otherwise. ☐

3. I understand that relevant sections of any of my/ my child's medical notes and data collected during the study, may be reviewed by individuals from the project team, from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. (a) I agree to allow medical information about my family to be entered on a confidential computer database. ☐

   (b) In addition, in some cases the genetic information produced by studying my/ my child's DNA may be placed in an electronic archive with no connection to my/ my child's name or other personal identifier. I understand that this archive will only be accessible to appropriate doctors and researchers who have been approved by a committee set up to ensure the results are only used to advance scientific and medical understanding. Although there is a theoretical possibility that I/ my child could be identified by the deposited information (e.g. if I/ my child is entered into another independent genetic study), this is extremely unlikely. ☐

   (c) If further medical information is requested by members of the project team, I agree to be contacted again for this purpose. ☐

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(b) I understand that the techniques used are NOT suitable for diagnostic testing for known genetic defects, and that I will NOT be told about any genetic alterations which are identified as a by-product of this research that are not relevant to my/ my family's illness. This will not affect my/ my child's access to clinically approved genetic advice and testing through other doctors caring for me in any way. ☐

(c) If a genetic test becomes available as a result of medical research on my/ my family's sample(s) I would like to have the opportunity to discuss the implications of these findings with appropriate medical experts. ☐

6. I understand that I/ my child will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

_____	_____	_____
Name of Participant	Date	Signature (if signing on behalf of Participant state your name & relationship to them)
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

(1 for participant; 1 for researcher; 1 for hospital notes)

Appendix G PATIENT INFORMATION LEAFLETS REVIEWED FOR WWW.BHDSYNDROME.ORG

Birt-Hogg-Dube syndrome: Clinical Introduction

www.bhdsyndrome.org/wp-content/uploads/2012/05/pamphlet-clinician-info-2015.pdf

Birt-Hogg-Dube syndrome: Diagnosis Information

www.bhdsyndrome.org/wp-content/uploads/2013/11/BHD-pamphlet-diagnosis-2015.pdf

Birt-Hogg-Dube syndrome: Skin Symptoms and Treatment Options

www.bhdsyndrome.org/wp-content/uploads/2013/11/BHD-pamphlet-skin-2015.pdf

Birt-Hogg-Dube syndrome: Kidney Symptoms

www.bhdsyndrome.org/wp-content/uploads/2013/11/BHD-pamphlet-kidney-symp-2015.pdf

Birt-Hogg-Dube syndrome: Kidney Treatment Options

www.bhdsyndrome.org/wp-content/uploads/2013/11/BHD-pamphlet-kidney-treatment-2015.pdf

Birt-Hogg-Dube syndrome: Lung Symptoms and Treatment Options

www.bhdsyndrome.org/wp-content/uploads/2013/11/BHD-pamphlet-lung-v61.pdf