Aberrant genomic imprinting in chromosome 11p15-associated congenital growth disorders: consequences for DNA-diagnostics

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Chapter 1

Introduction
Beckwith Wiedemann Syndrome (BWS) is an overgrowth syndrome, characterized by a spectrum of growth related symptoms. Pre- and postnatal overgrowth, macroglossia and anterior abdominal wall defects, occur in most cases. In addition, ear pits and creases, nevus flammeus, neonatal hypoglycaemia, organomegaly, renal abnormalities and hemihypertrophy can be observed. Children with BWS have an increased susceptibility (7.5%) to childhood tumours compared to normal children (0,17%). The DNA-diagnostic laboratory of the Department of Clinical Genetics of the Academic Medical Centre in Amsterdam offers diagnostic testing for BWS patients for many years now. The diagnostic tools have changed over the years; from 1999 onwards the laboratory is the reference laboratory for BWS diagnostics in the Netherlands.

This introduction summarizes the current knowledge of the clinical and molecular aspects of the BWS syndrome and defines the research topics of the different chapters in this thesis.

**THE CLINICAL DIAGNOSIS OF BWS**

BWS was first described in 1963 by JB Beckwith and in 1964 by HR Wiedemann as a combination of prenatal overgrowth (=gigantism or macrosomia) in combination with an enlarged tongue (=macroglossia) and abdominal wall defects (omphalocele, umbilical hernia or diastasis recti). BWS was previously designated the EMG syndrome, after the distinctive features Exomphalos, Macroglossia and Gigantism.

An expanding spectrum of clinical features has been described in a number of papers. The features most frequently found in BWS patients are listed in table 1 and shown in figure 1.

Less common features associated with BWS are cleft palate, hemangioma, advanced bone age, enlarged labia, cliteromegaly, hypospadias and cryptorchidism. Sporadically there is a positive family history. Monozygotic twinning is increased among BWS patients, most twins being female. The sex-ratio for BWS patients is around 1 (106:100).

Although BWS is referred to as an overgrowth syndrome, at birth macrosomia is not present in all BWS patients. In some of the cases without prenatal overgrowth increased growth is observed in the first months of life. In all cases (early) adolescence growth curves approach the norm, but remain between P75 and P90 (the 50th percentile is the average height for any given age).

Patients with BWS have an increased risk for the development of embryonal tumours in childhood. The three largest studies show frequencies of 7.5% (29/388), 7.1% (13/183) and 14% (22/159), whereas the risk for these embryonal tumours is 0,17% in normal Dutch children. The most common tumour reported in BWS patients is Wilms tumour, a tumour of the kidney, also called nephroblastoma (43%). However, a wide variety of other tumours are seen, like neuroblastoma, rhabdomyosarcoma and adrenocortical carcinoma. Ninety % of Wilms tumours have been diagnosed at the age of 7 years, at the age of 10 years.
Table 1. Common clinical features and their prevalence.

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased birth weight</td>
<td>38.08</td>
</tr>
<tr>
<td>Postnatal gigantism</td>
<td>32.7</td>
</tr>
<tr>
<td>Macroglossia (enlarged tongue)</td>
<td>97.53</td>
</tr>
<tr>
<td>Omphalocele (exomphalos)</td>
<td>75.83</td>
</tr>
<tr>
<td>Umbilical hernia</td>
<td>49.3</td>
</tr>
<tr>
<td>Diastasis recti</td>
<td>33.33</td>
</tr>
<tr>
<td>Anterior linear ear lobe creases/posterior helical ear pits</td>
<td>66.23</td>
</tr>
<tr>
<td>Visceromegaly involving one or more intra-abdominal organs</td>
<td>97.63</td>
</tr>
<tr>
<td>involving liver, heart, spleen, kidneys, adrenal glands,</td>
<td></td>
</tr>
<tr>
<td>and pancreas</td>
<td></td>
</tr>
<tr>
<td>Hemihiyerplasia (asymmetric overgrowth of one or more</td>
<td>32.3</td>
</tr>
<tr>
<td>regions of the body)</td>
<td></td>
</tr>
<tr>
<td>Polyhydramnios</td>
<td>51.03</td>
</tr>
<tr>
<td>Neonatal hypoglycemia</td>
<td>60.9</td>
</tr>
<tr>
<td>Midfacial hypoplasia</td>
<td>81.33</td>
</tr>
<tr>
<td>Nevus flammeus</td>
<td>62.53</td>
</tr>
</tbody>
</table>

Features marked in bold are shown in figure 1.

the tumour risk approaches the baseline of the risk of cancer in the normal population.

Clinical features like hemihypertrophy and especially organomegaly may indicate a higher tumour risk. Wiedemann and Goldmann reported a tumour risk of more than 20% in BWS patients with hemihypertrophy. Hemihypertrophy and malignant tumours occur more frequently in patients with Uniparental Disomy (UPD) of chromosome 11p15. Hemihypertrophy can be found in association with other characteristics of BWS, but it can also occur as an isolated entity. A subgroup of individuals with apparently isolated hemihyperplasia may have BWS with minimal clinical findings. Children with isolated hemihyperplasia carry an increased tumour risk of 5.9%. Diagnosis is sometimes hampered by the heterogeneity of the clinical presentation. Children presenting with overgrowth in combination with an enlarged tongue, omphalocele or other umbilical abnormalities, are easily recognized at birth as BWS patients. But many patients however lack one or more of these distinctive features, where on the other hand overgrowth is present also in the normal population (>P97 indicates that 3/100 normal children fulfill one of the major criteria of BWS). Macroglossia is the most characteristic feature that leads paediatricians to the diagnosis BWS, with or in the absence of general prenatal overgrowth. Ninety-five % of BWS patients present an enlarged tongue. However, macroglossia is also present in other diseases like type 2 glycogen storage disease (MIM 232300) neurofibromatosis (MIM 162200), congenital hypothyroidism, and it can also be present in an isolated form.
Figure 1. Major characteristics of the Beckwith Wiedemann syndrome A Discordant monozygotic twinpair, BWS twin on the left. B. Macroglossia C. Ear pits D. ear creases E. abdominal mass defects (omphalocele), F. (Prenatal) Gigantism; Photographs were reproduced with informed consent of the parents of the patients.
Figure 2. Imprinting mechanisms in BWS. Me: methylated
Prenatal overgrowth, although typical for BWS, is also associated with other syndromes, referred to as the overgrowth syndromes (OGS)\(^{17}\); Simpson-Golabi-Behmel syndrome, Perlman syndrome, Sotos syndrome, Proteus syndrome, Bannayan-Riley-Ruvalcaba syndrome, macrocephaly-cutis marmorata syndrome, Marshall-Smith syndrome, Weaver syndrome and Klippel-Trenaunay syndrome belong to the OG syndromes.

Like BWS, all OGS have an increased tumour risk. Simpson-Golabi-Behmel syndrome (SGBS, MIM 312870) is an OGS that shares two of the three major features with BWS; prenatal overgrowth and macroglossia. Also seen are cleft palate, visceromegaly, earlobe creases, hernias and neonatal hypoglycemia. However, it can be distinguished from BWS when distinctive facial features i.e. cleft lip, skeletal abnormalities, including polydactyly or mental retardation are present. In 30% of individuals with SGBS deletions and mutations in the glypican-3 gene (GPC3, MIM 300037) are detected\(^{18}\).

Several attempts have been made to define criteria for clinical diagnosis to differentiate between BWS and other OG syndromes. In 1994 Elliot et al\(^{4}\) clinically characterized 76 BWS patients. Based on their findings features were classified as major (commonly present) or minor (less frequently present). They formulated criteria for diagnosis; a positive Diagnosis can be made if 3 major features or 2 major features plus 3 minor features are present. Major features are anterior abdominal wall defects, macroglossia and pre- and/or postnatal growth >90th centile; minor features are ear creases or pits, naevus flammeus, hypoglycaemia, nephromegaly and hemihypertrophy.

In 1998 DeBaun et al\(^{11}\) set new, less strict criteria to define the syndrome. His criteria for the disease are the presence of two or more of the most common features (macroglossia, macrosomia, abdominal wall defects, hypoglycaemia and creases and pits).

In 2001 Weksberg et al\(^{19}\) extended the list of major features with hemihyperplasia, embryonal tumours, adrenocortical cytomegaly, visceromegaly, renal abnormalities, cleft palate and a positive family history. If there are fewer than three of these common features the following minor features may support the diagnosis: polyhydramnios and pre-mature birth, enlarged placenta, cardiomegaly or structural cardiac anomalies, nevus flammeus or other hemangiomata, advanced bone age, midfacial hypoplasia and monozygotic twinning.

To be able to perform reliable genotype/phenotype correlation studies, agreement on criteria based on detailed characterization of patients is important. To obtain these data the DNA-diagnostic laboratory of Dept. of Clinical Genetics of the Academic Medical Centre has been sending questionnaires to all referring paediatricians. The questionnaire used for this purpose was designed by a clinical geneticist specialized in BWS (S. Maas). Patients were either classified as EE (fulfilling the criteria of Elliot\(^{4}\)), EB (fulfilling the criteria DeBaun\(^{11}\)) or neither of these two sets of criteria. All clinical and genetic data were collected in a BWS
database, now containing more than 400 BWS patients. Only those patients of whom clinical data are available have been included in the database. When patients were misdiagnosed and later appeared not to have BWS but another syndrome, they were excluded from the BWS database. Patients with an incomplete phenotype remain included in the database but are marked as not fulfilling the criteria for BWS.

**THE MOLECULAR GENETIC DIAGNOSIS OF BWS**

The majority of BWS patients have a normal karyotype, only a few cases with a chromosomal abnormality involving chromosome 11p15 have been reported. These cases include partial duplications of 11p15, often resulting from unbalanced translocations\(^20, 21\). The extra copy is always inherited from the father. Balanced translocations have also been described, all of maternal origin\(^22, 23\).

In 20% of BWS cases, uniparental disomy (UPD) of chromosome 11p15 can be detected\(^14, 24, 25\). These patients have two copies of the chromosome present, however both copies (or a part) of the chromosome originate from the father whereas no maternal copy is present.

These parent-of-origin effects in chromosomal abnormalities associated with BWS indicate that BWS might arise from altered expression of imprinted genes.

**GENOMIC IMPRINTING**

Some genes in the human genome escape Mendelian inheritance i.e. their expression depends on the parental origin of the chromosome on which the gene resides. This phenomenon is called genomic imprinting. The molecular basis of genomic imprinting is epigenetic. That is, heritable changes in gene expression are not the result of mutations in the DNA sequence itself, but from modifications such as DNA methylation and changes in chromatin structure. The process of genomic imprinting involves the establishment of parent-specific epigenetic modifications in the germ line that result in monoallelic expression of genes in a parent-of-origin dependent manner.

The most broadly accepted hypothesis for the existence of genomic imprinting is the parental conflict model first proposed by Moore and Haig \(^26, 27\). Imprinted genes are often involved in foetal growth\(^28\). In mammals nutrients for the offspring are provided exclusively by the mother through exchange via the placenta and then through lactation. The mother needs to conserve resources to ensure her own survival and the survival of future offspring. Therefore, maternally derived genes will reduce growth. In contrast, it is in the father’s genetic interest to have large, strong offspring; growth promoting genes are often paternally derived.

Imprinted genes are clustered in chromosomal domains and monoallelic expression of the genes in these domains is controlled by imprinting control regions (ICRs or ICs). ICRs are often methylated in a parent-of-origin-specific manner.

Most ICRs are less than a few kilobases in size and comprise sequences that are rich in CpG dinucleotides (CpG islands). The cytosine residues in these CpG islands
are either methylated on the paternal or the maternal chromosome. Regions in ICRs containing these CpG islands are called Differentially Methylated Regions (DMRs). Methylation of cytosine residues in DMRs is accompanied by changes in the chromatin structure; methylated DMRs show methylation of histone H3 lysine 9 and lysine 27 whereas unmethylated DMRs have histone modifications such as H3 and H4 acetylation and H3 lysine 4 methylation.

Targeted deletion of ICRs in mice suggest that they operate in cis to impose monoallelic expression in imprinted domains\textsuperscript{29, 30}. ICRs can induce gene silencing by two mechanisms: CCCTC-binding factor (CTCF) mediated insulation (figure 2C) and transcription of antisense RNAs (asRNA) (figure 2B).

An insulator is defined as a DNA sequence with binding sites for the CCCTC binding factor (CTCF). Binding of these proteins prevents enhancers from interacting with gene promoters if they are positioned between the enhancer and the target gene. The CTCF binding factors can bind to insulators only if they are unmethylated, on the unmethylated allele expression of the target gene is prevented\textsuperscript{30, 31}.

Most imprinted domains contain antisense RNAs (asRNA) that often overlap in anti-sense direction with protein coding genes. Expression of both transcripts show reciprocal imprinting implicating that asRNAs are involved in allele specific silencing of overlapping genes\textsuperscript{32}. Evidence for the involvement of asRNAs imprinted silencing of autosomal genes comes from the mouse \textit{Igf2r} locus, in which truncation of the \textit{Air} antisense transcript results in allele specific silencing of coding genes in the imprinted domain\textsuperscript{33}.

Propagation of imprinting pattern to the offspring implies resetting of the imprint in the germline. The process of gamete imprinting was reviewed in 2006 by Tresler\textsuperscript{34}. DNA methylation patterns on imprinted genes are, for the most part, erased in premordial germ cells (PGCs) and then re-acquired at gender-specific times during spermatogenesis and oogenesis. In the pre-implantation embryo genome wide demethylation takes place, with exception of the imprinted genes. Methylation of non-imprinted sequences is regained in the blastocyst stage and will persist through life. Histone modifications accompany methylation to inactivate genes\textsuperscript{35}.

Methylation of DNA is catalyzed by a family of DNA (cytosine-5)-MethylTransferases (DNMTs). DNMT\textsubscript{1}\textsuperscript{36} is a hemimethyltransferase, it is the most important gene involved in maintenance of DNA methylation patterns in the genome. During DNA replication the methylation patterns of the original strand are copied to the newly synthesized strand. The role of DNMT\textsubscript{2}\textsuperscript{37} remains to be elucidated. The DNMT\textsubscript{3} genes (DNMT\textsubscript{3a}\textsuperscript{38}, DNMT\textsubscript{3b}\textsuperscript{38} and DNMT\textsubscript{3L}\textsuperscript{39}) are involved in the acquisition of new methylation patterns or \textit{de novo} methylation. In male DNMT\textsubscript{1} is not expressed in spermocytes at the time imprinting patterns are initially acquired. DNMT\textsubscript{3a} and DNMT\textsubscript{3L} are predominantly involved in initial methylation of imprinted genes in the male germ cells. In the female germ line all three DNMT\textsubscript{3}s are expressed at the time when methylation imprints are acquired. In oocytes a
specific form of DNMT1 (DNMT1o⁴⁰) is expressed but knockouts in mice show that it is not involved in the establishment of the female methylation pattern. However, these DNMTo deficient mice die in late gestation, due to abnormal expression of imprinted genes implicating that DNMTo expression is essential for maintenance methylation during pre-implantation development⁴¹.

**GENOMIC IMPRINTING IN BWS**

Breakpoints of maternally transmitted chromosomal translocations found in BWS patients are clustered in three regions on chromosome 11p15, designated Beckwith-Wiedemann chromosomal regions (BWSCR)1, 2 and 3⁴². The most telomeric region, BWSCR1, is located within an imprinted domain on 11p15.5. This domain contains two distinct clusters of imprinted genes involved in the genetics of BWS, Beckwith Wiedemann Imprinting Cluster 1 and 2 (BWSIC1 and BWSIC2)⁴³-⁴⁵. The homologous region in the mouse is located on chromosome 7. Both clusters contain a number of paternally and maternally imprinted genes. Expression of these genes is controlled by imprinting centres that shows characteristics of a classical ICR (figure 2A).

**IMPRINTING CLUSTER 1**

BWSIC1 is the most distal cluster. Monoallelic expression in this cluster is controlled by IC1 which contains a DMR (DMR1), and the promoter of an untranslated RNA, *H19*. The region contains a number of imprinted genes, one of which is the Insulin-like growth factor 2 (*IGF2*) gene. *IGF2* expression is the key determinant in the role of BWSIC1 in BWS.

*IGF2* (MIM 147470) is expressed from the paternal allele and encodes an embryonic growth factor. The human *IGF2* is expressed in 4 isoforms, the promoters P2, P3 and P4 are foetal promoters and are subject to imprinting whereas P1 is used postnatal and drives expression from both alleles, mainly in liver⁴⁶. In mice postnatal expression of *Igf2* confined to the choroid plexus and the leptomeninges of the central nervous system⁴⁷. When the mouse *Igf2* gene is disrupted in heterozygous mice, the offspring show inhibited foetal growth if the disrupted allele is paternally inherited but develop normally after birth⁴⁸. Overexpression of *Igf2* results in most of the symptoms of Beckwith-Wiedemann syndrome, including prenatal overgrowth, polyhydramnios, fetal and neonatal lethality, disproportionate organ overgrowth including tongue enlargement, and skeletal abnormalities⁴⁹.

This cluster contains a non-coding RNA, the *H19* gene (MIM 103280). It is 2.7 kb long and includes 4 small introns. *H19* is abundantly expressed in both extra-embryonic and foetal tissues. The product of the *H19* gene is expressed exclusively from the maternal chromosome during mammalian development⁵⁰; the paternal allele is methylated. In knockout mice where the complete H19 coding sequence is removed but the promoter and surrounding transcription unit are left intact there was no effect on the imprinted expression of *IGF2*⁵¹. This suggests that the RNA itself has no function. However, a recent study suggests that H19 functions as a
primary microRNA precursor involved in the posttranscriptional downregulation of specific mRNAs during vertebrate development. The ICR in this cluster mediates opposite imprinted expression of H19 and IGF2 via CCCTC-binding factor (CTCF) mediated insulation. The CTCF protein binds to a DMR in the close vicinity of the H19 promoter (DMR1) which contains 7 CTCF binding sites. On the unmethylated maternal allele CTCF binds to target sites in the DMR preventing the enhancers downstream of H19 from accessing the IGF2 promoters. On the paternal allele, DNA methylation blocks CTCF binding. The absence of CTCF allows the enhancers to access IGF2 promoters resulting in IGF2 transcription. Deletion of H19 leads to loss of imprinting and thus biallelic expression of Igf2 in mice.

These findings support the hypothesis that gain of methylation (GOM), thus inactivation of the maternal copy of H19, results in over expression of IGF2 and the development of BWS. In 1997 Catchpoole et al showed that all UPD patients showed gain of methylation (GOM) at the promoter region of the H19 gene whereas GOM was also observed in 8% of non-UPD patients. The detection of aberrant methylation at this locus is since then included in the diagnostics of BWS.

**IMPRINTING CLUSTER 2**

BWSIC2 is located centromeric of cluster 1. Monoallelic expression of BWSIC2 is regulated by the IC2, which like IC1 contains a DMR (KvDMR/DMR2) and the promoter of a ncRNA, KCNQ1OT1 (LIT1). This cluster contains a number of imprinted genes, one of which is the cyclin dependent kinase inhibitor (CDKN1C) gene (Figure 2 cluster 2). Expression of CDKN1C is the key determinant in the role of BWSIC2 in BWS. CDKN1C (p57kip2, MIM 600856) is a kinase inhibitor and a negative regulator of cell proliferation. Mice lacking Cdkn1c expression show some of the features of BWS like exomphalos and adrenal cortex dysplasia, but lack typical features like overgrowth and macroglossia. These mice show altered differentiation and are not viable.

Mutations in the CDKN1C gene account for up to 40% of familial BWS cases and 5-10% of sporadic patients. Functional analysis of CDKN1C germ line mutations detected in two BWS patients showed the loss of cell cycle inhibition.

This cluster contains an antisense RNA; the KCNQ1OT1 gene. It is a very large RNA (>300 kb), expressed only from the paternal allele. Its promoter is located in exon 10 of KCNQ1. Hypomethylation of DMR2 in BWS is associated with silencing of CDKN1C.

The DMR2 contains both the promoter for the KCNQ1OT1 ncRNA and two CTCF binding sites.

KCNQ1OT1 mediates imprinted expression of the autosomal genes in the cluster by antisense silencing. In mice, deletion of Kcnq1ot1 results in biallelic expression of eight maternal-specific genes on distal chromosome 7 in all embryonic and extra-embryonic tissues, including Cdkn1c. Fetuses and adult mice that inherited this
deletion from their fathers were 20-25% smaller than their wild type littermates\textsuperscript{45}. However, truncation of the \textit{Kvlqt1ot1} transcript does not affect imprinted expression of \textit{Cdkn1c} in a subset of embryonic tissues despite loss of paternal specific methylation of \textit{Cdkn1c}. Growth deficiency in these mice is less severe than observed in mice with deletions of DMR2. This indicates that the DMR2 locus can silence \textit{Cdkn1c} via a mechanism independent of \textit{KCNQ1OT1} transcription, perhaps by CTCF-associated repression\textsuperscript{66}.

These findings support the hypothesis that LOM, thus activation of the maternal copy of \textit{KCNQ1OT1} results abnormal silencing of \textit{CDKN1C} and the development of BWS. In 1999 Lee et al\textsuperscript{43} first described aberrant methylation at the \textit{KCNQ1OT1} locus in 58% of non-UPD patients without aberrant methylation of \textit{H19}.

**MOSAICISM**

LOM of \textit{KCNQ1OT1} can be complete but pUPD and GOM of \textit{H19} are always present in a mosaic form. The percentage of cells having lost the maternal epigenotype varies between >0\% and <100\%.

This suggests that maternal contribution of BWSIC1 to the epigenotype is necessary for embryonal survival. The presence of a paternally imprinted gene in this region\textsuperscript{67}, the homolog of drosophila Achaete-scute complex 2 (\textit{ASCL2}, MIM 601886), might explain this phenomenon. \textit{ASCL2} is expressed only in the extravillous trophoblasts of the developing placenta, mice deficient of this gene die at 10 days post coitum because of placental failure\textsuperscript{68}. In complete paternal UPDs this gene is not expressed, leading to early lethality.

The term mosaicism in this context indicates that there is a mixed population of cells, some with a normal imprinting pattern (and an active copy of \textit{ASCL2}), and a subset with either two copies of the paternal chromosome or an imprinting defect of IC1 or IC2.

**OTHER ABNORMALITIES ASSOCIATED WITH METHYLATION DEFECTS ON CHROMOSOME 11P15**

BWS is an overgrowth syndrome caused by aberrant methylation of genes on chromosome 11p15. These defects are also found in a number of other growth related diseases. The first is isolated hemihypertrophy (IH, MIM 235000). Hemihypertrophy is often associated with various syndromes, one of which is BWS, but can also be present in an isolated form. Methylation defects in both imprinting clusters on chromosome 11p15 have been described in 30\% of IH patients\textsuperscript{69}.

A syndrome with clinical characteristics opposite to BWS is the Silver-Russell Syndrome (SRS or RSS, MIM 180860). The main features of SRS are severe intrauterine and postnatal growth retardation and a characteristic small, triangular face. The disease is associated with additional dysmorphic features including fifth finger clinodactyly and hemihypoplasia\textsuperscript{70, 71}. SRS is a heterogeneous disorder, therefore clinical criteria have been set. Patients are diagnosed with SRS when they
fulfill the following criteria: intrauterine growth retardation (IUGR: birth weight 2 SD or more below the mean), postnatal growth retardation (length or height 2 SD or more below the mean), normal head circumference, fifth-finger clinodactyly and limb-length asymmetry. Additional features that can aid in the diagnosis are short stature with normal upper- to lower-segment ratio, normal skeletal survey, and frequently delayed bone age, typical facial phenotype of broad prominent forehead with small triangular face, small narrow chin, and down-turned corners of the mouth, hypoglycaemia, brachydactyly, camptodactyly, café au lait spots and arm span less than height.

Like BWS, most cases of SRS are sporadic. Several SRS patients have structural aberrations affecting numerous chromosomes, but only chromosomes 7, 11 and 17 were consistently involved in individuals fulfilling strict diagnostic criteria of SRS. A few translocations all involving 17q25 have been described in SRS patients. Maternal uniparental disomy (UPD) of chromosome 7 has been implicated in 7 - 10% of SRS patients. Two imprinted genes are located in the smallest region of overlap (SRO): \textit{GRB10}, which maps to 7p11.2-p12 and \textit{MEST} (also known as \textit{PEG1}), which maps to 7q32. \textit{GRB10} is paternally imprinted and \textit{MEST} is maternally imprinted. Maternal overexpression of \textit{GRB10} can result from gene duplication or maternal uniparental disomy of chromosome 7.

In 2005 Gicquel et al detected methylation defects in the most distal imprinting cluster (BWSIC1) on chromosome 11p15 that is involved in BWS.

**DIAGNOSTIC TESTING FOR BWS**

In theory, overexpression of \textit{IGF2} would be the most relevant diagnostic tool for BWS, but unfortunately, this is not useful in practice. The postnatal level of circulating \textit{IGF2} protein does not reflect the tissue-specific over-expression of \textit{IGF2} that occurs prenatally and a source of \textit{IGF2} expressing tissue is required for direct measurement of expression levels.

DNA diagnostics is based on epigenetic and genetic defects that have been observed among BWS patients:

- Chromosomal abnormalities (translocations/partial duplications involving 11p15)
- Paternal uniparental disomy of 11p15
- LOM of \textit{KCNQ1OT1}
- GOM of \textit{H19}
- Mutations in \textit{CDKN1C}

The DNA-diagnostic laboratory of the Dept. of Clinical Genetics of the Academic Medical Centre offers genetic testing for all defects present in BWS patients. All diagnostic tests are performed on DNA isolated from blood lymphocytes. First, the methylation status of \textit{KCNQ1OT1} and \textit{H19} are determined. In most studies in this
thesis (except chapter 6-8), aberrant methylation is detected by a technique based on southern blot hybridisation (see figure 3).

DNA is first digested until completion with a methylation sensitive restriction enzyme, either NotI (KCNQ1OT1) or SmaI (H19). These enzymes only cut unmethylated DNA, leaving the methylated allele intact. The resulting DNA fragments are separated on an agarose gel and transferred to a immobilization membrane by southern blotting. Subsequently the membrane is hybridized with a radio active labelled probe, specific for the DMRs of KCNQ1OT1 or H19. Radioactive signals are detected with the use of a phosphorimager, which allows quantification of the signals. With each probe two bands are detected: a small digested non-methylated band and a larger non-digested methylated band. The relative intensity of the two bands represents the methylation profiles at each locus. In normal controls the two bands are present in equal intensities, one allele is methylated and one allele is unmethylated in all cells. A methylation defect of KCNQ1OT1 is observed when the smaller, digested unmethylated band of KCNQ1OT1 has a higher intensity, indicating that some cells have lost methylation of the maternal copy of the gene. For H19 the opposite stands true, the larger, undigested methylated band

Figure 3. Methylation analysis. Left panel: southern blot hybridisation, Right panel: schematic representation of experiment. A: probes LIT1 and NotC (control), B: probes SmaC (control) and H19. Meth: methylated, M.I. Methylation Index.
is more intense, indicating that there are more cells present in which the maternal copy of the gene has become methylated.

When both genes show aberrant methylation, this results either from paternal UPD or a duplication of chromosome 11p15.5. Both defects are no true imprinting defects, but the result of the presence of two copies of the paternal allele and hence two copies of the gene with a paternal imprint.

To confirm pUPD, a number of polymorphic CA-repeat markers on chromosome 11p15 are tested for allelic imbalance. For this test DNA from both parents must be available. Allelic ratios are determined by comparing the peak-areas of the paternal and maternal fragments.

Duplications are confirmed by FISH analyses or MLPA.

When no methylation defect is present in a BWS with a clear phenotype, and in familial cases, the complete coding region of the CDKN1C gene is analysed by direct sequencing.

**OUTLINE OF THE THESIS**

At the start of the studies described in this thesis, aberrant methylation patterns of H19 and KCNQ1OT1 were observed as a research finding in BWS patients.

At first our objective was to adapt the methods for the detection of methylation defects for a routine diagnostic setting. This indicated that the protocol needed to be sensitive, accurate and reproducible. There was a need for controls within the test for complete digestion with the methylation sensitive restriction enzymes. This study is described in chapter 2 of this thesis. We had previously collected a large number (>100) of clinically well characterized BWS patients. We performed methylation analysis, UPD screening and CDKN1C mutation analysis to be able to establish the prevalence of each genetic defect known to be associated with BWS.

In chapter 3 of this thesis, we focussed on the BWS patients with cancer. We investigated the possibility to distinguish between BWS patients with a low and a high tumour risk on the basis of genetic findings. For this study it was a necessity to include as many patients as possible. Therefore this study was performed in collaboration with a French research group of Dr. Christine Gicquel (Paris). Combined results on 114 BWS patients were obtained. We reviewed data from the literature to establish tumour risk and tumour types in a total of 287 BWS patients.

In chapter 4 of this thesis we performed the same analysis on isolated hemihypertrophy (IH) patients. Also in this study data were obtained from a large series (74) of well defined IH patients that were referred to our hospital. We further reviewed data from the literature to establish tumour risk and tumour types in a total of 114 IH patients.

In chapter 5 we analyzed methylation patterns in a syndrome that presents with symptoms opposite to the BWS syndrome. Patients with Silver-Russel syndrome (SRS) are small at birth and have a characteristic facial appearance. A small
fraction of SRS show loss of methylation of H19. We analysed methylation patterns of H19 in SRS patients and SRS patients with an incomplete phenotype.

In chapter 6 we developed a new technique that permits the detection of methylation changes in BWS patients. Until now, routine diagnostic testing is performed by southern blot, this method is sensitive but time consuming and needs a large amount of DNA. PCR based techniques require less DNA but do not differentiate between methylated and unmethylated alleles. Bisulfite treatment of DNA causes deamination of unmethylated cytosines to uridine, thereby enabling discrimination between unmethylated and methylated cytosine residues. The difference in the DNA sequence that arises from this treatment can be used to discriminate between the methylated and unmethylated alleles.

In 2006 Rossignol et al described LOM of multiple paternally imprinted genes outside the BWS regions in patients with LOM of KCNQ1OT180. In chapter 7, we screened a large series of BWS patients with an IC2 defect for loss of methylation in paternally imprinted genes. Patients were clinically characterized, to obtain possible correlations between the phenotype and (epi)genotype. This study was performed in collaboration with the Italian research group of Prof. Andrea Riccio (Naples) and the laboratory of Dr. Deborah MacKay (Salisbury).

Discordant MZ female twins have been described among BWS patients. In chapter 8, we characterize 13 BWS twins. We established zygosity and placentation of the twins and collected clinical data. In all twins we established the methylation status of genes on chromosome 11p15 and other paternally imprinted genes. The objective of this study is to gain further insight in the mechanisms underlying the aetiology of these remarkable twins.

In the last publication of this thesis, chapter 9, we report on a familial transmitted duplication and a familial transmitted translocation of the distal 11p15 region. Depending on the parental origin of the chromosomal abnormality, differences in phenotype occur. In this chapter we analyze the mechanism underlying this phenomenon.

In chapter 10 and 11 we summarize and our findings and discuss the implications for further research.

**REFERENCE LIST**


O’Keefe, D. *et al.* Coding mutations in p57KIP2 are present in some cases of Beckwith-Wiedemann syndrome but are rare or absent in Wilms tumors. *Am. J. Hum. Genet.* 61, 295-303 (1997).


