Aberrant genomic imprinting in chromosome 11p15-associated congenital growth disorders: consequences for DNA-diagnostics
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Chapter 10

Summary and Discussion
SUMMARY

Beckwith Wiedemann Syndrome (BWS) is congenital growth disorder, with a variable phenotype. Children born with this syndrome have an increased birth weight, an enlarged tongue and abdominal wall defects. Other features typical for BWS are earpits and creases, hemihypertrophy, organomegaly, naevus flammeus and neonatal hypoglycaemia.

In case of severe abdominal wall defects, surgery is needed soon after birth and neonatal hypoglycaemia can be treated effectively. Sometimes surgical removal of a part of the enlarged tongue is necessary for cosmetic reasons and for normal speech development. The overgrowth features disappear slowly after birth. In most cases no differences in height and weight are observed in BWS patients compared to normal controls around the start of puberty.

A major problem in the clinical management of BWS patients is the fact that these patients have an increased risk of around 10% for the development of childhood cancer. The most frequently observed tumour type is Wilms tumour, but also hepatoblastoma, neuroblastoma and other embryonal tumour have been found. BWS patients are advised to follow a stringent screenings protocol, with three/four monthly ultrasound of the abdomen for the early detection of these tumours. This puts a heavy burden on the patients and their parents. The identification of those patients with an increased tumour risk is one of the main objectives of diagnostic testing.

So far no clear correlation between clinical phenotype and tumour risk has been observed, apart from an increased incidence of hemihypertrophy in BWS patients who developed a tumour. With the revelation of the genetic causes of BWS additional factors may be detected in the delineation of the tumour risk of individual BWS patients.

Less then a decade ago diagnostic testing for the Beckwith Wiedemann Syndrome consisted of karyotyping and FISH for the detection of chromosomal translocations and duplications. The only DNA test available was the detection of uniparental disomy of chromosome 11p15 by the analysis of polymorphic CA repeat markers. This led to a laboratory diagnosis in 20% of the patients.

The rare translocations that were found were always of maternal origin and the breakpoints of the translocations were clustered in three regions on chromosome 11p15. The uniparental disomies were always of paternal origin and involved the tip of chromosome 11p15 as well.

This led to the identification of two clusters of imprinted genes located within the SRO of the pUPD patients. The first cluster is the Beckwith Wiedemann Cluster region 1 (BWSIC1), this cluster contains amongst others a non-coding RNA H19 and the embryonal growth factor 2 (IGF2). The two genes are reciprocally expressed and imprinted expression within this cluster is regulated via an in imprinting centre (IC). The IC contains a stretch of differentially methylated CpG islands, called DMR1.
(differentially methylated region 1). DMR1 is methylated on the paternal allele, when methylated H19 is repressed and IGF2 becomes active.

Adjacent to BWSIC2 a second cluster of imprinted genes (BWSIC2) resides. This cluster also contains a non-coding RNA (KCNQ1OT1) and a number of other genes, one of which is a cell-cycle inhibitor (CDKN1C). Co-regulation of imprinted expression of genes in this cluster is also mediated via an imprinting centre that contains a differentially methylated region, DMR2. DMR2 is methylated on the maternal allele, when methylated KCNQ1OT1 is repressed and other imprinted genes in the region are expressed.

In BWS patients epigenetic defects in both imprinting clusters have been detected. Gain of Methylation (GOM) of DMR1 is observed in some BWS patients, the normally unmethylated paternal allele becomes methylated and IGF2 is expressed from this normally silent allele. In BWS patients with GOM of DMR1 overexpression of this embryonal growth factor is the key determinant in the development of the overgrowth phenotype. Overexpression of *Igf2* in mice results in an overgrowth phenotype that shares many features with BWS.

In some patients Loss of Methylation (LOM) of DMR2 is observed. The normally methylated maternal allele becomes demethylated, and, among others, CDKN1C expression is silenced from the normally active chromosome. In BWS patients with LOM of DMR2 the absence of the cell cycle inhibitor CDKN1C is the key determinant in the development of the overgrowth phenotype. The disruption of *Cdkn1c* in mice results in a phenotype that shares some features with BWS.

In patients with paternal UPD both alleles have the paternal imprint i.e. DMR1 is methylated and DMR2 is unmethylated, resulting in overexpression of IGF2 and suppression of CDKN1C.

In BWSIC2 LOM of DMR2 can be complete but in BWSIC1 pUPD and GOM of DMR1 are always present in a mosaic form. This suggests that maternal contribution of BWSIC1 to the epigenotype is necessary for embryonal survival. A gene that has been cloned in our laboratory *ASCL2* (homolog of drosophila acheate-scute complex 2), might explain this phenomenon. It is paternally imprinted and expressed only in placenta, mice deficient of this gene die at 10 days post coitum because of placental failure. In complete paternal UPDs and patients with complete GOM of DMR1 this gene is not expressed, leading to early lethality.

With the discovery of methylation defects of the two DMRs within the 11p15 region, a new era started in DNA diagnostics of BWS. This thesis starts in chapter 2 with the optimization of a test for aberrant methylation in either of the two DMRs. The test is performed on DNA isolated from peripheral blood and based on digestion with methylation sensitive enzymes in combination with southern blot hybridisation. This optimization led to a sensitive and reproducible test able to detect methylation defects in about 80% of all BWS patients.

With the introduction of this test, the molecular study of a large cohort of Dutch BWS patients with a known phenotype became possible. It gave insight in the
distribution of BWS patients among the different genetic subgroups. In 55% of the BWS patients the normally methylated maternal allele of DMR2 showed loss of methylation (LOM) whereas in 7% of the case the normally unmethylated maternal allele of DMR1 became methylated (GOM). In 20% of cases uniparental disomy of paternal origin was found resulting in the presence of two methylated copies of DMR1 (GOM) and two unmethylated copies of DMR2 (LOM). No defect could be detected in either region in 18% of the patients.

In contrast to previous studies, LOM of DMR2 was found in a mosaic form in 29/40 of the patients with this epigenotype. Moreover, LOM of DMR2 could also be detected in patients with only a partial BWS phenotype but also in patients not diagnosed as BWS patients at all.

Since BWS patients have an increased risk for the development of specific childhood tumours a possible correlation of epigenotype and tumour risk would be valuable for the screening protocols applied to BWS patients. In this first study the majority of tumours were found in BWS patients with GOM of DMR1, whereas no tumours were found in patients without a methylation defect.

The number of patients who developed a tumour in that series was small. Therefore we extended (in chapter 3 of this thesis) our cohort of BWS patients with cancer. We reviewed data available in the literature to obtain a population large enough to obtain significance.

Indeed, in a total of 278 patients, clear correlations between epigenotype and tumour risk were found. Patients with GOM of DMR1 had a very high tumour risk; the tumours that they developed were always Wilms tumours. Patients with pUPD and patients without detectable defect showed an intermediate tumour risk, these patients developed Wilms tumours or other tumours. Patients with LOM of DMR2 showed the lowest tumour risk, and Wilms tumours were never observed.

Since this study was published screenings protocols for BWS patients were changed in many countries, excluding patients with LOM of DMR2 from a stringent protocol of three monthly abdominal screening for early detection of Wilms tumour.

Among cancer patients an increased number of isolated hemihypertrophy (IH) patients were observed. Hemihypertrophy is one of the common features of BWS, the genetic defects found in BWS patients are also observed among IH patients. Therefore, in chapter 4 we applied the same strategy to IH patients with a tumour in order to improve screenings protocols for these patients. Together with data from the literature, we obtained epigenetic data of in total 125 IH patients. The most striking observation was the fact that in large fraction of IH patients no epigenetic defect could be detected (75% compared to 18% among BWS patients). This limits the use of epigenetic screening in the prediction of tumour risk among IH patients.

In 2005 the spectrum of epigenetic defects in the DMRs on chromosome 11p15 was expanded: LOM of DMR1 was observed in patients with the Silver Russell syndrome. This syndrome has an opposite phenotype to the BWS phenotype; it is
characterized by pre- and postnatal growth retardation. In chapter 5 we analysed the methylation status of chromosome 11p15 in 9 patients with an SRS or SRS-like phenotype, and we were able to show complete LOM of DMR1 in 2/3 patients meeting the clinical criteria for SRS. However, we also observed mosaic LOM of DMR1 in a number of patients with an incomplete phenotype.

The technique used to confirm the BWS diagnosis in DNA diagnostics based on southern blot was very laborious and required large amount of DNA. Recently, a new technique became available for the detection of DNA mutations called high resolution melting analysis (HRMA). This technique is, as is described in chapter 6, successfully adapted for the detection of changes in differentially methylated regions. This technique is PCR based and therefore needs relatively little hands-on time and only a small amount of DNA. After intensive technical validation this method has been introduced as standard tool in the DNA diagnostics of BWS and SRS.

In 2006 demethylation of a number of maternally imprinted loci outside the chromosome 11p15 imprinted region has been described. In chapter 7 we conducted a multicentre study on HIL (hypomethylation at maternally imprinted loci) in BWS patients. A cohort of Dutch and Italian BWS patients has been screened for methylation defects in 10 imprinted loci outside the 11p15 imprinted region. HIL was present only in patients with LOM of DMR2, and included MEST, GNAS/NESPAS, PLAG1 and IGF2R. Patients showing HIL had atypical BWS features, although because of the diversity of methylation defects, no significant association between the genes outside 11p15 and the phenotype could be detected.

The DNA-diagnostic laboratory of the Dept. of Clinical Genetics of the Academic Medical Centre has collected a large number (~400) of clinically well defined BWS patients. Of these, 13 are discordant BWS twins. In chapter 8 we characterized these twins. Information on the clinical features of the twins was collected, together with additional data on placentation and amnionicity. Methylation analysis was performed on blood lymphocytes and buccal swabs of the twins. Apart from the two BWS imprinting clusters on chromosome 11p15, 7 imprinted loci on other chromosomes were analysed. This study revealed some new insights in the causes of twinning. Methylation defects involved the DMR2 region, and in a high percentage (5/10) MZ twin HIL throughout the genome was also observed. HIL is normally associated only with maternally imprinted genes, but in two cases HIL extended to paternally imprinted genes.

Similar methylation defects were found in blood lymphocytes of the affected and non-affected twins that share a placenta. This can not solely be explained by blood sharing via the placenta (vascular anastomosis) so we propose an additional mechanism to explain this phenomenon.

Methylation levels found in a discordant BWS triplet support the theory that methylation defects proceed and possibly trigger the twinning process.
In the 9th chapter of this thesis two unique three generation families have been studied.

In one family a translocation involving chromosome 4 and 11 is transmitted either paternally or maternally. When the translocation is inherited in an unbalanced form from the father, the epigenotype of the extra copy of 11p15 obtains the paternal imprint resulting in increased methylation levels of DMR1 and decreased methylation levels of DMR2. Phenotypically this results in the BWS syndrome. If the same translocation is inherited in an unbalanced form from the mother, this results in an opposite methylation defect (LOM of DMR1 and GOM of DMR2). The patient carrying this unbalanced translocation showed the opposite phenotype, growth retardation.

In the second family a small interstitial duplication on chromosome 11p15 including only H19 and a few surrounding genes (not the DMR2 region). Upon paternal transmissing of the duplication this resulted in an extra copy of DMR1 with a paternal imprint. In methylation testing an increased methylation level was detected. The patient carrying the paternally transmitted duplication showed a mild BWS phenotype. The father of this patient inherited the same duplication from his mother but has a normal phenotype. The presence of an extra copy of the DMR1 with the maternal imprint apparently has no influence on the phenotype.

In both families the parental mode of transmission influences the phenotype of the recipient child.
In this thesis a variety of genetic and epigenetic defects are described in BWS patients. Observations made about (epi)genotype and phenotype raise questions that are important for the general understanding of imprinting disorders. In this chapter we discuss the background of the (epi)genetics defects found in BWS and SRS patients. We postulate models that may cause these epigenetic changes and propose new directions for research. Secondly, we introduce possible causes for the BWS phenotype in patients with no detectable epigenetic defect on chromosome 11p15. Then we discuss the implications of the fact that epigenetic changes in the BWS imprinted regions are also found in patients with an incomplete phenotype. And finally the tumour risk of BWS patients is reviewed.

(Epi)genotype

A large number of different genetic and epigenetic defects have been described involving the two differentially methylated regions located in close vicinity on chromosome 11p15 (see fig 1).

**Figure 1.** Overview of the different genetic and epigenetic changes that have been described involving DMR1 and DMR2, located on chromosome 11p15.
Only in a small number of cases genetic defects, such as mutations (e.g. CDKN1C), small interstitial deletions (DMR1 and DMR2 deletions) or translocations involving chromosome 11p15, were found in BWS patients.

CDKN1C mutations are genetic defects directly resulting in disruption of the protein function of the affected gene and hence the BWS phenotype.

In all other cases the underlying defect leads to an epigenetic change. Improper methylation of regulatory sequences within a cluster of imprinted genes results in aberrant imprinting. The role of CTCF binding sites and non-coding RNAs in the regulation of imprinting within these clusters is currently investigated.

In 20% of the patients the cause of the epigenetic defect is clear: a mitotic recombination error (UPD) leads to a disturbance of the balance between maternal and paternal contribution of imprinted genes.

In rare cases, chromosomal rearrangements leading to unbalanced translocations and also small duplications have been found. In these patients the methylation pattern of the duplicated region is defined by its parental origin, leading to differences in phenotype upon maternal and paternal transmission. Also in these cases the disturbance in maternal and paternal contribution results in an epigenetic defect.

In a small number of patients deletions in one of the two DMRs are observed. This leads directly to a disturbance of the regulation of imprinted expression of genes in either cluster.

However, in the majority of the patients (65%) the chromosomal distribution is normal in all cells, with one paternal and one maternal copy. In these cases a true imprinting defect in one of the two clusters affects methylation patterns of either one of the DMRs. The exact nature of the underlying defect that causes this imprinting defect is unknown.

Imprinted methylation is established during embryogenesis. The parental imprint has to be reset in the gametes. In the oocyte methylation of the paternal imprinted genes has to be removed while paternal non-methylated genes have to be methylated. In sperm, the original maternal genes have to undergo a reciprocal process. This imprint has to be maintained in the gametes. In contrast to oocytes, sperm cells undergo many rounds of mitotic divisions before maturation. Prior to fertilization methylation errors may lead to defects in imprinted expression. After fertilization, the imprint has to be maintained in each round of cell division in the developing zygote.

In any of these processes errors can occur resulting in aberrant methylation patterns in the zygote (fig. 2).

Loss of imprinted methylation

In the majority (93%) of BWS patients with a methylation defect, loss of methylation of DMR2 is observed (figure 2A). In these patients the normally methylated maternal copy of DMR2 becomes demethylated.
A. Possible causes of LOM of DMR2 in BWS patients

B. Possible causes of LOM of DMR1 in SRS patients
In 75% of LOM DMR2 patients demethylation is restricted to DMR2. In 25% of the LOM DMR2 patients loss of methylation extends to regions outside DMR2. In these patients additional maternally methylated DMR’s on other chromosomes become demethylated (hypomethylation of multiple imprinted loci HIL). In these patients the imprinting defect is more generalized. Demethylation of the other loci is also observed in a mosaic form. It is unknown whether demethylation of all loci involved takes place in the same cells, or that the observed mosaicism reflects the different cell fractions in which one of the DMRs becomes demethylated (as postulated by MacKay, pers. communication). In the study described in chapter 7 we screened 11 DMRs outside chromosome 11 in 81 patients with LOM of DMR2. In 17 patients one or more DMRs lost methylation on the maternal allele. In mice there are about 100 imprinted genes presently known, these genes are grouped in about 18-20 clusters. It is possible that in the patients in whom HIL could not be detected in this study, one of the imprinted loci that was not included in the study is affected.

Loss of methylation of DMR1 is also observed. This results in the SRS phenotype (figure 2B). In SRS patients with LOM of DMR1, failure to maintain methylation is, in contrast to LOM of DMR2, restricted to the paternal allele of DMR1 resulting in two unmethylated copies of DMR1.
**Gain of imprinted methylation**

Gain of methylation is less common. 7% of BWS patients are characterized by a methylation defect of DMR1 (figure 2C). In these patients the normally unmethylated maternal allele of DMR1 becomes actively methylated.

Gain of methylation of DMR2 has not been observed so far. The predicted phenotypic result is growth retardation but among the SRS patients we have screened to date, no such defect has been found.

**Mechanisms involved in imprinting errors**

Most imprinting defects in BWS are present in a mosaic form, so only in a fraction of the cells, while the remaining cells have normal imprinting patterns. This implies that the methylation defect must have been caused postzygotically, by a failure to maintain imprinting patterns in the early zygote (stage C in figure 2). Errors in stage A and B would result in a generalized imprinting defect in the complete zygote.

We hypothesize two possible mechanisms leading to disturbances of imprinted expression, what we call ‘genetic’ and ‘stochastic’ models for imprinting errors.

**The genetic model**

The genetic model is based on the presence of inactivating mutations/deletions in one of the genes in the methylation machinery.

Candidate genes are the DNMT’s (DNA (cytosine-5)-MethylTransferases), a gene family involved in maintenance of imprinting patterns of the genome. After the formation of the zygote, the RNA machinery is suppressed during the first rounds of cell division. All processes in the early zygote are regulated by proteins that originate from the oocytes since sperm cells contain mainly DNA that is packaged tidy into the spermhead.

A candidate gene for loss of methylation is DNMT1o. DNMT1o is an oocyte specific isoform of DNMT1 which is synthesized and stored in the cytoplasm of the oocyte. It is translocated to the cell nucleus during early embryonic development. It is not involved in the establishment of the female methylation pattern (1) but functions only during one round of replication in the eight-cell embryo (2). Dnmt1o deficient mice show reduction of methylation in the embryo of the usually methylated alleles irrespective of the parental origin.

Other candidates are the DNMT3 genes (reviewed by (3). DNMT3A and DNMT3B are enzymes that establish methylation on unmethylated substrates (de novo methylation) whereas DNMT3L acts as a stimulator of de novo methylation activities. Disruption or Dnmt3A and Dnmt3L in mice results in both activation and inactivation of maternally imprinted genes. DNMT3A and DNMT3B are expressed during gametogenesis, embryonic development and in some adult tissues, DNMT3L
is restricted to prospermatogonia and growing oocytes, at the moment the imprint is being set in the gamete.

Another group of candidate genes might not directly be involved in DNA methylation. In patients with transient neonatal diabetes mellitus (TNDM) aberrant methylation of PLAG1 on chromosome 6q is observed. In a subpopulation of TNDM patients aberrant methylation of DMR2 is observed. Linkage studies in families with HIL led to ZFP57 on chromosome 6p. In more than 50% of the TNDM patients with HIL, mutations in ZFP57 have been detected (4). This ZincFinger gene codes for a transcription factor and is expressed in mouse oocytes and the early zygote. CTCF is a transcription factor that binds to CTCF binding sites in unmethylated DMRs. CTCF also contains ZincFinger motives, possibly competition for the same binding sites between both proteins may influence regulation of imprinting.

More details about the background of the ZFP57 mutations in TNDM patients are not available.

**The stochastic model**

The stochastic model is not based on mutations in specific genes; it involves just a failure to maintain imprinting at one stage of embryogenesis. The process of resetting the imprint is complex and involves many steps of removing and adding methylation groups to cytosines of the DNA strands. The result of the process is very precise, so even single errors can disturb imprinting. If a simple error occurs in an early stage of embryogenesis, this error is transmitted to the cells of subsequent cell divisions. The stage in which this error occurs, defines the number of aberrant imprinted cells in the zygote. There may be a critical period, if the error occurs very early in embryogenesis only a few normal cells are present leading to loss of the embryo. If the error occurs late in embryogenesis, the defect only affects a small number of cells and does not result in a clinical phenotype. Whether or not environmental factors are involved is unknown. The methylation defects found in children born with the use of ART are thought to be caused during the culturing period of the zygote. In those cases it is conceivable that external factors induce the imprinting defects.

These errors may also occur in other sequences in the genome, although at the time only methylations errors in a small number of imprinted regions are known to result in phenotypic changes. It might well be that (mosaic) defects of the imprinted regions on chromosome 11p15 allow the fetal growth. Errors in other imprinted regions may not result in a change in phenotype or may just be lethal and not result in a pregnancy.

To investigate whether the genetic model is involved, each gene described above should be screened for mutations. No extensive studies have been performed in large cohorts of BWS patients to find mutations in any of these genes. In chapter 7
of this chapter we screened two mothers of BWS patients with hypomethylation at imprinted loci and found no evidence for mutations. ZFP57 has not been screened in BWS patients. Since mutations in this gene are common in TNDM patients with HIL, the gene might also be involved in BWS patients with HIL. Previously we reported three BWS translocation breakpoint clusters on chromosome 11p15 (5). The central cluster, that is located 4 Mb proximal to DMR1 and DMR2, disrupts another ZincFinger containing transcription factor, ZnF215. No mutations in BWS patients have been described so far although a number of UV are present in the region that possibly might be associated with BWS (6).

We propose screening of BWS patients with HIL for mutations in both ZnF proteins.

The stochastic model is far more difficult to study. It concerns processes that take place in an very early stage of zygote development, a stage that is difficult to mimic in vitro in humans.

Although none of the models fully explains the involvement of specific DMRs, the stochastic model is able to explain why only specific imprinted loci are involved. A mutation in any of the genes of the methylation machinery would be expected to result in generalized methylation defects in all imprinted gene clusters. An indication why some DMRs are preferentially involved might come from the results of a recent study by Sazhenova et al (7). They studied the effects of demethylating agents in foetal fibroblasts cell cultures. BWSIC2 showed a relatively high susceptibility to demethylation agents compared to BWSIC1 and the SNURF-SNRPN cluster on chromosome 15. This might also reflect the fact that aberrant methylation of this cluster is also frequently found in children born after IVF.

Studies like these on foetal cell cultures may give more insights in the processes involved in the non-genetic model.

**Patients with a BWS phenotype without the epigenotype**

By routine diagnostic screening for methylation defects in both imprinting clusters on chromosome 11p15, the clinical BWS diagnosis can be confirmed in 80% of the patients. The remaining 20% of the patients show no detectable defect in these regions. Still, these patients fulfill the most stringent clinical criteria of BWS. It is unknown what causes the overgrowth phenotype in these patients.

A number of explanations might account for this phenomenon.

First, in routine diagnostics methylation testing is always performed on blood lymphocytes. Maybe we are looking in the wrong tissue. Grati et al (8) determined the percentage of UPD cells in different tissues of two BWS foetuses. The number of disomic cells correlated with the degree of overgrowth of the tissue affected. The highest degree of disomic cells was observed in the most enlarged tissues, although the defect could also be detected in blood lymphocytes. This might also be the case for DMR1 and DMR2 defects. In chapter 8 of this thesis we compared the methylation level in blood lymphocytes and tongue tissue of a BWS patient with
macroglossia and detected equal levels (fig 4). In 8 other cases (unpublished results) we were able to perform methylation testing on tongue biopsies and always detected similar methylation levels compared to blood lymphocytes of the same patient.

However, more extensive studies on different tissues are needed to determine whether methylation defects can be restricted to affected tissues only and therefore be missed by routine diagnostic screening.

Second, we only determine methylation levels at two specific DMRs in the region, there are at least two other DMRs present in the IFG2 coding region itself and one in CDKN1C. We recommend screening other DMRs in the 11p15 region.

Third, apart from defects in methylation of DMRs other mechanisms may disturb regulation of imprinted expression. In some cases of BWS biallelic IGF2 expression is accompanied by monoallelic H19 expression. These cases show normal methylation and expression of H19 from the maternal allele with biallelic IGF2 expression (9). Apart from cytosine methylation specific histone modification proteins are involved in silencing of gene expression. Mutations in CTCF binding sites may alter binding of transcription factors and result in loss of imprinted expression.

Looking directly at expression of the imprinted genes itself is expected to be a more direct tool in diagnostic testing. However, postnatal diagnostic testing for BWS based on protein expression is heavily hampered. One of the key proteins involved, IGF2, is only monoallelically expressed before birth when a foetal promoter P0 initiates transcription from the paternal allele. Other promoters are used after birth but they drive expression from both alleles. Therefore IGF2 cannot not used as a postnatal diagnostic marker. More insights in regulation of imprinted expression and disturbances of the processes involved may reveal additional genetic defects in BWS patients.

Fourth, the genetic defect may not involve chromosome 11p15 at all. Although all chromosomal defects observed in BWS patients involve this locus, one exception was observed. One patient with BWS and duplication 4q/deficiency 18p as the result of an unbalanced paternal translocation has been described (10). No imprinted genes in any of the two regions involved are known, this finding indicates that there may be other loci involved in BWS.

And finally, BWS patients without a methylation defect in the 11p15 region may have a phenotype that might be attributed to other overgrowth disorders. Other genes involved in prenatal growth may be affected in these patients.

One candidate gene is GPC3 on chromosome X. Mutations in this gene have been described in patients with an overlapping overgrowth syndrome, the Simpson-Golabi-Behmel syndrome (SGBS). Simpson-Golabi-Behmel syndrome shares the following features with BWS: macrosomia, visceromegaly, macroglossia, and renal cysts. We screened about 80 male patients for GPC3 mutations but found no mutations in any of the patients (unpublished results). In today’s diagnostic
practice, GPC3 is only screened when the phenotype directs towards a possible SGBS diagnosis.

Sotos syndrome is characterized by pre- and post-natal overgrowth and variable mental retardation. Deletions and point mutations of the NSD1 gene account for >60% of cases of Sotos syndrome. Two cases have been reported of NSD1 mutations in individuals with features of BWS and mental retardation (11). We screened 20 BWS patients with mental retardation for mutations in NSD1, in one patient a NSD1 mutation was detected (unpublished results).

GPC3 and IGF2 are part of a complex growth regulating pathway. Other genes in the same pathway may be affected. Screening for mutations in these genes in BWS patients without detectable methylation defects may reveal new genetic causes of BWS. An example is IGF1R on 15q25-q26. Although imprinted in mice, IGF1R is expressed biallelically in humans. Monoallelic expression from the maternal allele has been observed in normal kidney and blood lymphocytes of a BWS patient (12). The background of this change to monoallelic expression is not known, but screening of more patients might reveal more cases of altered expression of IGF2R.

In chapter 7 we screened 48 patients without a detectable imprinting defect for loss of methylation on loci outside the 11p15 region. Although HIL is only observed in BWS patients without LOM of DMR2, another gene involved in prenatal growth should be considered. In about 10% of the Silver-Russell syndrome patients maternal uniparental disomy of chromosome 7 is observed. This region contains the imprinted gene GRB10. No mutations in GRB10 have been detected in SRS patients, but in view of the imprinted status of the gene methylation defects are more likely then pure mutations at least in SRS patients. In BWS patients no chromosomal aberrations are known in this region. Screening for methylation defects in the DMR of this gene however might reveal epigenetic changes in BWS.

**PATIENTS WITH A BWS EPIGENOTYPE WITHOUT THE PHENOTYPE**

An ongoing discussion between clinicians and geneticists concerns the question what defines the Diagnosis BWS: fulfilling the clinical criteria for BWS or the presence of the genetic defects associated with the syndrome.

It is clear that not all patients with a methylation defect on chromosome 11p15 fulfill the clinical criteria for BWS, as shown in chapter 2 of this thesis. Even in patients with isolated hemihypertrophy (IH) methylation defects associated with BWS are found. Also among patients that do fulfill the BWS criteria and do show typical methylation defects, there is a large variation in clinical presentation. In an unpublished study in patients with LOM of DMR2, we investigated whether the degree of the methylation defect influences the severity of the phenotype.

We defined a scoring method to quantify the severity of the syndrome. The important features of BWS (overgrowth, macroglossia and abdominal wall defects) were given a score of 3, whereas all additional BWS related features were given a score of 1.
From these data we conclude that there is no clear correlation between the phenotype and the degree of demethylation of DMR2 in these patients. Even in patients with a high number of aberrant cells present, there are sometimes minimal effects on the phenotype. A extreme example is patient 22, this patient has a methylation index between 0.1 and 0.2 but the only features present are organomegaly and (maybe as a result) an abdominal wall defect.

In chapter 4 of this thesis we show that isolated hemihypertrophy patients, so patients with overgrowth of part of the body in absence of any other BWS related features, show in 25% of cases methylation defects on chromosome 11p15.

Both studies implicate that methylation analysis for laboratory confirmation can be offered to a broad range of phenotypic aberrations and should not be limited to those patients fulfilling the BWS criteria. Since these tests are mainly offered to BWS patients it remains unclear with which frequency imprinting defects on chromosome 11p15 are found in patients with partial phenotypes. To get more insight in this phenomenon, more patients showing single or few features related to BWS should be tested.

Data on tumour risk of non-BWS patients with a methylation defect are only available for isolated hemihypertrophy, although the cohort studied was too small to correlate tumour risk and epigenotype. Our study on the effect of the methylation defect on the severity of the syndrome was performed on patients with LOM of DMR2 only, the patient group with a relative low risk of developing cancer. Extended screening of partial phenotypes may therefore also generate data on tumour risks.

**TUMOUR RISK**

In chapter 3 we studied the correlation between tumour risk and epigenotype in a large series of 278 BWS patients, data obtained from our own laboratory combined with large studies in literature. From the results we concluded that the individual epigenetic profile of BWS patients could be used to delineate the patients tumour risk and the type of tumour it might develop.

A small group of BWS patients (5%), with gain of methylation of DMR1, have a very high risk of developing cancer. These patients develop uniquely Wilms’ tumours. Gain of methylation of H19 is usually observed in sporadic Wilms’ tumours. That results in decreased expression of H19 and concomitant loss of imprinting of IGF2 expression. Parallel changes are observed in the epigenotype and the tumour that these patients develop.

These patients should be screened intensively for the presence of renal abnormalities by ultrasound investigation, which is embedded in all BWS tumour screening protocols.

More then half of the BWS patients (55%) show loss of methylation of DMR2. These patients have a relative low tumour risk. These patients never developed Wilms’ tumours. These data are in favour of an adaptation of the stringent tumour
### Table 1 and Figure 3

Plot of the degree of demethylation versus the severity factor (SF) of 41 BWS patients.
screening protocol. Still, for a long time the advice was to maintain current protocols until more data were available (13) (www.genetests.org). In 2005 the Wilms’ tumour surveillance group in the UK refined their screening protocol, Patients with a tumour risk of less then 5% are excluded from renal ultrasonography every three to four months. Since Wilms’ tumours have never been described in BWS patients with LOM of DMR2 these patients are no longer included in the screening protocol.

Since this study on tumour risk in BWS patients was published in 2004, no new reports on patients with LOM of DMR2 that developed a Wilms’ tumour were published. Currently Dutch paediatricians and clinical geneticists are reconsidering the Dutch preventive protocol in adaptation of these genetic findings.

BWS showing loss of methylation at DMR2 do sporadically develop tumours, such as rhabdomyosarcoma and gonadoblastoma. It is likely that other genetic pathways in embryonal tumour development are involved. Imprinting defects of DMR2 have not been observed in Wilms’ tumour, but no data are available on the methylation status of DMR2 in these more rare tumour types. Although CDKN1C functions as a cell cycle inhibitor, loss of CDKN1C expression has not been observed in tumours. It is possible that the other imprinted genes (Phlda2, Slc22a18, Kcnq1, Cd81 and Ascl2 (14)) in BWSIC2 play a role in tumour development. Not many data are available on the role of these genes in tumour development, but at least in one of them, SLC22A18/BWR1A, a homozygous mutation has been reported in a rhabdomyosarcoma (15). The region also contains two tumour suppressor genes, TSSC4 and TSSC6, that are involved in transformation into malignancy. Since both genes escape imprinted expression their role in tumour development is unclear.

The analysis of these genes in tumour genesis may gain insights in the tumour risk of BWS patients with a defect in DMR2.

The remaining BWS patients, those with UPD (20%) and with no detectable genetic defect (20%) have an intermediate tumour risk, and develop both Wilms’ tumour and other childhood tumours. In UPD patients both imprinted clusters are affected, so tumour types associated with both clusters are found. The pathway involved in tumour genesis in patients without a methylation defect in DMR1 and DMR2 is unknown. However, it is conceivable that, like UPD, both clusters are involved, since the tumour types between UPD patients and patients without a defect are similar.

The screening protocol for the early detection of cancer should be maintained for these patients.

The current predictive tools are only able to correlate a specific epigenotype to a relative tumour risk, not to predict which patients actually develop a tumour. This holds for all cancer predisposition syndromes. For example carriers of mutations in BRCA1 and BRCA2 mutations have a 60-80% chance to develop breast cancer. Not all carriers do develop a tumour. In these adult cases this can be explained by the two-hit hypothesis of Knudson (16), involving a germ line mutation (the first ‘hit’) of a
tumour suppressor gene (TSG) and a second (somatic) hit on the second allele of the same TSG. The longer a carrier of a germline mutation lives, the higher the chances are that this second hit takes place in one of the cells in the tissue at risk. Certain environmental effects increase the chance for a second hit, and therefore have a carcinogenic effect.

This is in contrast to what is observed among BWS patients. The tumour risk of BWS patients decreases at the age of 5-8 years, and tumours are rarely observed after the age of 8 years. Maybe this can be explained by the fact that all tumours found in BWS patients originate from reminiscent embryonic cells present in the affected tissues. Moreover, the TSGs involved BWS patients are imprinted genes, loss of imprinted expression directly results in over-expression of growth factors still active in these embryonic cells. But then, all patients with disturbed imprinting would be expected to develop cancer. Clearly additional (epi)genetic events are needed.

Insight has to be gained into the background of tumour development in BWS patients so that tools for more precise prediction of tumour risk and tumour type can be improved.

**REFERENCE LIST**


