**Genome-wide methylation profiling of Beckwith Wiedemann syndrome patients without molecular confirmation after routine diagnostics**

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**Abstract**

Beckwith Wiedemann syndrome (BWS) is caused by disturbance of imprinted genes at chromosome 11p15. Molecular confirmation of this syndrome is possible in approximately 85% of cases; in 15% of cases, the underlying defect remains unclear. The goal of our research was to identify new epigenetic loci related to BWS. We studied a group of 25 patients with a clinical diagnosis of BWS but without molecular conformation after DNA diagnostics and performed a whole genome methylation analysis using the HumanMethylation450 Array (Illumina).

We found hypermethylation throughout the methylome in two BWS patients. Hypermethylated sites in these patients overlapped and included both non-imprinted and imprinted regions. This finding was not previously described in any BWS diagnosed patient.

Furthermore, in one BWS patient we detected aberrant methylation in four maternally methylated regions: *IGF1R, NHP2L1, L3MBTL*, and *ZDBF2* that overlapped with differentially methylated regions (DMRs) found in BWS patients with multi-locus imprinting disorders (MLID). This finding suggests that the BWS phenotype may result from MLID without detectable methylation defects in the primarily disease-associated loci (11p15). In one patient we detected small but significant aberrant methylation in disease associated loci at 11p near *H19* possibly confirming the diagnosis in this patient.

KEYWORDS: BWS, MLID, DNA-methylation, Imprinting Disorders

**Introduction**

Beckwith Wiedemann Syndrome (BWS) (OMIM 130650)is an overgrowth disorder with predisposition to embryonal tumor development. The clinical symptoms are macrosomia, macroglossia and abdominal wall defects, while minor features are ear pits, hypoglycemia, nephromegaly and hemihypertrophy. BWS is clinically heterogeneous and the major features may not manifest themselves immediately after birth but become visible in the first months of life [1].

Most cases (about 85%) of BWS result from various genetic and epigenetic aberrations in the imprinted region on chromosome 11p15.5. This region contains two imprinting centers, *H19* TSS DMR (Transcription Start Site Differentially Methylated Region) and *KCNQ1OT1* TSS DMR. *H19* TSS DMR regulates transcription of the genes *IGF2* and *H19,* whereas *KCNQ1OT1* TSS DMRregulates, amongst others, *KCNQ10T1* and *KCNQ1* [2]in atissue dependent manner. About 50% of cases are caused by a loss of methylation at *KCNQ1OT1* TSSDMR on the maternal chromosome and 20% by paternal uniparental disomy (UPD) of 11p15.5. Up to 5% have a gain of methylation on the maternal chromosome at the *H19* TSS DMR. Between 5% and 10% of cases are caused by a mutation in the maternal copy of *CDKN1C*. Finally, some cases are caused by chromosomal aberrations on chromosome 11, for example: microdeletions (<1%), translocations (<1%), inversions (<1%) or duplications (<1%) within the imprinted region of 11p15 (2). Furthermore, in a large number of imprinting disorders (IDs), hypomethylation occurs not only at a single locus but also at other imprinted loci. This phenomenon is called multi-locus imprinting disturbance (MLID) and this is also seen in about 30% of BWS with hypomethylation at *KCNQ1OT1* [3]. In about 15% of BWS no (epi)genetic cause can be found (Figure 1) [4].

The aim of the current study was to identify new BWS related epigenetic loci in a group of patients with unknown (epi)genetic cause. We searched for regions with altered methylation in known imprinted genes in each patient and regions with altered methylation shared between patients in the whole methylome.

BWS

SRS

**Methods**

DNA was isolated from whole blood samples using Gentra Chemicals (Qiagen).

*Patient population*

BWS patients

Twenty-five BWS patients (BWS1-BWS25) without any methylation defects in the imprinted region on chromosome 11p15.5, and without a mutation in *CDKN1C* as determined in the genome diagnostics department of the Academic Medical Center (the Netherlands). The imprinting defect at 11p15.5 was excluded using high resolution melting analysis (HRMA)[5] and/or methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA). For both methods the limit of detection was 10% of alteration of methylation in comparison to negative controls. Maternally inherited mutations in *CDKN1C* were excluded using Sanger sequencing. (See Additional information)

 All patients fulfilled the BWS criteria of DeBaun , displaying at least two of five the most common BWS features, including macrosomia, macroglossia, abdominal wall defects, ear creases/pits , and hypoglycemia[6, 7].

Positive controls (MLID-BWS)

We used five DNA samples from MLID-BWS patients with known hypomethylation at *KCNQ1OT1* TSS DMRand other imprinted loci as a positive control group to determine the robustness of this test. Hypomethylation in this patients was previously determined and described by J.Bliek et al in 2008 [8]and confirmed using MS-MLPA. The additional hypomethylated loci in these patients were as follows:

MLID-BWS1 - *MEST, GNAS\_AS1*;

MLID-BWS2 - *GNAS\_AS1;*

MLID-BWS3 - *GNAS\_AS1, DIRAS3*;

MLID-BWS4 - *MEST;* and

MLID-BWS5 - *GBR10A*, *GNAS\_AS1, SNRPN*.

Control group

Twenty-six DNA samples from healthy anonymous individuals were used as a normal control group.

*HumanMethylation450 array*

The HumanMethylation450 array (Illumina, San Diego, USA) was used to obtain genome-wide methylation levels. This array comprises 485,000 CpG sites throughout the genome and covers 99% of RefSeq genes and 96% of CpG islands. In order to reduce technical bias, patients and controls were randomly divided over the analysis. DNA was bisulfite converted using the EZ DNA methyklation kit of ZYMO® and subsequently submitted for analysis on the HumanMethylation450 array, outsourced to GenomeScan, Leiden, The Netherlands (ISO/IEC 17025 approved). The quality of raw methylation data was assessed using the MethylAid script in R (GenomeScan’s Guidelines for Successful Methylation Experiments Using the Illumina Infinium® HumanMethylation BeadChip). All samples met the quality criteria. To analyze our data we used a statistical method for single sample analysis described by Rezwan et al. [9] adapted to a MINFI package in R (based on the Crawford-Howell t-test). Prior to this analysis, all probes known to involve polymorphic sites (minor allele frequency - MAF>0.01), cross-hybridization probes and probes located on sex chromosomes were removed from the dataset. The work flow and QC details are depicted in the supplemental information. The 450k data was normalized with the function preprocessFunnorm of MINFI [10].

Differentially methylated positions (DMP) with assigned adjusted P-value for M-values smaller than 0.05 were considered as significant (adjusted according to the false discovery rate - FDR).

*Searching for DMRs at imprinted loci*

Next, we determined whether significant DMPs were located in the imprinted regions. To correctly identify imprinted regions in our data, we used chromosomal locations for imprinted DMRs published by the European Network for Human Congenital Imprinting Disorder. These regions will be referred to as COST regions. (Standardized nomenclature for imprinted loci/DMR; <http://www.imprinting-disorders.eu/?page_id=3154>). Note that, COST regions in imprinted loci are differentially methylated alleles. (GenomicRanges version 1.28; R-software)

We calculated the percentage of significant DMPs that were located in COST regions for each patient. Further, to avoid false positive results only COST regions with two or more significant DMPs were considered as potential aberrantly methylated regions.

*Searching for new DMRs shared between BWS patients*

To test whether significant DMPs occurred in two or more BWS patients, we carried out a comparative analysis. (i) First, we selected significant DMPs based on the adj.Pvalue\_M < 0.05 per patient taking into account the direction of aberration (hypo-; hypermethylated) (ii) Then we compared selected DMPs between patients and filtered out DMPs that occurred in more than one BWS patient. (iii) The last filter step was a selection of at least 2 significant DMPs within the region (based on the gene name and index number of CpG sites). MLID-BWS patients were included in this analysis.

The implementation of these filter steps allowed for identification of DMPs showing recurrent altered methylation in BWS patients rather than the biggest effect sizes and/or the smallest p-value.

**Results**

*HumanMethylation450 array*

In order to test the ability of the HumanMehylation450 array to detect alterations of methylation in BWS, we tested five MLID-BWS patients with known epimutations. Significant DMPs were seen in all known aberrantly methylated regions *(KCNQ1OT1, DIRAS3, GNAS-AS1, MEST, GRB10*, and *SNRPN*), indicating that this platform can detect methylation defects in BWS. Moreover, using this method, we identified hypomethylation in 18 other imprinted loci *(DIRAS3\_Ex2, IGF1R, GNAS-XL, WRB, FAM50B, FANCC, GNAS-A/B, NHP2L1, ERLIN2, MAGEL2, MCTS2P, PPIEL, PEG10, RB1, NDN, SNRPN\_5'DMR4, SNRPN\_variant4,* and *L3MBTL1*) and hypermethylation in two imprinted loci (*ZDBF2,* *and GNAS-NESP*) that were not known to be aberrantly methylated in these five MLID-BWS patients(Table 1).

We performed a single sample analysis for each patient in our BWS cohort. The number of significantly hypomethylated DMPs per patient ranged from 184 in (BWS3) to 967 (BWS24). The number of significantly hypermethylated DMPs ranged from 148 (BWS16) to 12931 (BWS24) (Table 2).

In two patients, BWS24 and BWS25 we found hypermethylation throughout the methylome. We identified almost 13 000 hypermethylated DMPs in BWS24 which is more than 3% of all informative probes included in the analysis. In BWS25 significantly hypermethylated DMPs accounted almost 2% of all informative probes. The percentage of hypermethylated DMPs in other patients did not exceed 0.44%.

BWS24 and BWS25 had also a slightly increased number of hypomethylated DMPs in comparison to others BWS patients but not different from MLID-BWS patients. Significantly hypomethylated DMPs in these two patients accounted for 0.23% in BWS24 and 0.15% in BWS25 of all informative probes included in the analysis. In MLID-BWS patients this percentage ranged from 0.11% to 0.21%.

The analysis of genomic distributions of significant DMPs of these two patients showed that that both of them are very similar (Figure 2) Hypermethylated significant DMPs were over-represented in 3’ untranslated region (3’UTR), body, OpenSea, and enhancers while hypomethylated significant DMPs were over-represented in Islands, DNase I Hypersensitivity Site (DHS), and Promoter\_Associated.

To exclude that these methylation patterns in these two patients are caused by cell type skewing, we estimated the distribution of cell types ( function: estimateCellCounts, R package: FlowSorted.Blood.450k). Both patients had comparable cell type profiles to the other patients in this group.

All significant DMPs per patients are listed in the additional table 1 and 2.

The estimation of the distribution of cell types and calculated P-values are listed in the additional table3 and 4.

*Searching for DMRs at imprinted loci*

We calculated the percentage of total significant DMPs located in COST regions for each patient and in further analysis we considered only COST regions with two or more significant DMPs as significantly aberrant (to avoid false positive results).

MLID-BWS patients showed the highest percentage of significant DMPs assigned to COST regions, ranging from 3.73% to 17.97%, as expected (Table 3). In BWS24 and BWS25 the percentage of total significant DMPs located in COST regions was slightly increased but lower than in MLID-BWS patients. In BWS24 this percentage was 0.55% and in BWS25 – 1.19%. In contrast to MLID-BWS, BWS24 and BWS25 displayed hypermethylation in COST regions but the number of significant DMPs in these regions were relatively small and DMPs were not consecutive. However, both of them displayed hypermethylation in *H19* TSS DMR(BWS24 – 8 DMPs, BWS25 – 26 DMPs) and BWS24 additionally near *IGF2\_*3'UTR (3 DMPs).

In BWS7 we detected three hypomethylated DMRs (near *NHP2L1, IGF1R*, *and L3MBTL)* and one hypermethylated DMR (near *ZDBF2*) with 2 or more significant DMPs that overlapped with COST regions found in MLID-BWS patients. (Figure 3, Additional table 5 ).

In BWS4, we detected alteration of methylation in the primary BWS associated locus on 11p. In *KCNQ1OT1*, 2 consecutive hypomethylated DMPs were annotated with delta difference -0.37 and -0.45 These two DMPs have stable methylation status in controls with beta mean adequately 0.56 and 0.51 and standard deviation (SD) 0.03 and 0.04. Hypomethylation in this locus was not detected with standard diagnostics (see supplemental information). Additional sequencing of this region (Sanger sequencing) showed the presence of SNP ([rs190535862](https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=190535862); minor allele frequency (MAF) : A=0.005; G>A; ) in one of two hypomethylated CpGs positions in this patient explaining the detected hypomethylation.

BWS22 displayed hypermethylation in *H19* TSS DMR, in three nonconsecutive probes. The absolute delta difference in only one of these three DMPs was larger than 10%, however all of them had significant adj.P-value\_M (all delta differences and adj.P-values assigned to each DMPs per patients are listed in the supplemental data 1). The methylation status of all three DMPs in controls was stable with SD 0.02, 0.01, and 0.02. Sanger sequencing did not show the presence of a SNP in this region. Moreover, we detected hypermethylation in this patient in two other imprinted loci assigned to *MEST* and *PEG13* (2 significant DMPs in each one).

The genomic locations of COST regions and the complete COST regions analysis are listed in the additional table 6, 7, and 8. .

*Searching for DMRs shared between BWS patients in the methylome*

We compared significant DMPs between patients including MLID-BWS patients.

In the hypermethylation comparative analysis we detected 6613 significant DMPs shared between a minimum of two BWS patients. The majority of them were shared between BWS24 and BWS25 in whom we detected hypermethylation throughout the methylome. We observed an increased number of hypermethylated DMPs annotated to the protocadherin gamma gene cluster located on chromosome 5. Thirty DMPs assigned to this cluster displayed hypermethylation in three MLID-BWS patients and BWS7. Further inspection of the 130 healthy individuals previously collected in our lab for diverse genome-wide methylation studies, did not show any abnormal methylation in this region suggesting that alteration of methylation of this locus is specific for these patients (Table 4). Moreover, unsupervised hierarchical clustering of this region showed that the DNA pattern of BWS7 and three MLID-BWS patients fell in the same cluster (Figure 4B).

We detected 1521 significantly hypomethylated DMPs shared between a minimum of two patients. More than 20% were annotated to imprinted loci (as reported above in the COST regions analysis) and shared between MLID-BWS patients. The majority of shared probes represented single DMPs.

The most often altered methylated region was annotated to *DUSP22.* Ten DMPs located in this region shared hypomethylation between seven patients: MLID-BWS1, BWS1, BWS2, BWS3, BWS11, BWS19, and BWS22. The visualization of 130 healthy individuals showed variability in this specific region in healthy individuals (Figure 4A). That strongly suggests that hypomethylation of DMPs located near *DUSP22* detected in the current study is not specific for BWS.

The complete comparative analysis is listed in the additional table 9.

**Discussion**

We analyzed the methylation status of DNA in the cohort of 25 BWS patients without any known (epi)genetic cause. We also included 5 MLID-BWS patients with known hypomethylated regions. Besides the known loci, we identified 18 other significantly hypomethylated and two hypermethylated imprinted regions. The percentage of significant DMPs in COST regions was higher in MLID-BWS than in other patients. That indicates that the disruption of methylation is enriched in imprinted genes in MLID rather than genome wide loss of methylation.

In two patients BWS24 and BWS25 we detected hypermethylation throughout the methylome. BWS24 displayed hypermethylation in 3% and BWS25 in almost 2% of all informative probes. This percentage is very high compared to other patients in whom hypermethylated DMPs did not exceed 0.44%. Both of them also displayed hypermethylation in imprinted loci, among others in *H19* TSS and additionally in the *IGF2*\_3'UTR in BWS24. However, the changes in methylation in these regions are subtle and do not clearly explain the development of BWS phenotype in these patients. The number of significant DMPs located in COST regions was lower than in MLID-BWS patients, indicating that aberrant methylation is not specific for imprinted regions, which suggests a different underlying mechanism than in MLID patients. It has been shown recently that mutations in chromatin modifying genes cause aberrant methylation patterns throughout the genome. Possibly the methylation profile in our patients is caused by a mutation in such gene [11].

In patient BWS7 we detected several hypomethylated imprinted loci that overlapped with MLID-BWS patients (*NHP2L1*, *IGF1R*, *L3MBT*L and *ZDBF2*). All detected regions were previously described in not only MLID-BWS patients but also other imprinting disorders that show MLID. For example, Docherty et al. (2014)described hypomethylation in the *NHP2LI* locus in Silver- Russell syndrome with MLID [12] and Rochtus et al. (2016) described hypomethylation in the *IGF1R* locus in patients with pseudohypoparathyroidism [13].

The most interesting of these four loci seems to be a locus situated near the *IGF1R*.  *IGF1R* is a receptor for *IGF1* and *IGF2*. *IGF2* is over-expressed in BWS cases with gain of methylation at *H19* TSS DMR. *IGF1R* is normally biallelic expressed. However, Howard et al. (1993), reported one BWS case with monoallelic expression of the maternal *IGF1R* in normal kidney, in Wilms tumor and lymphocytes. It is worth mentioning here that BWS patients with gain of methylation in *H19* TSS DMR have a risk of Wilms tumor development [14]. In addition, many publications demonstrate that *IGF1R* affects birth weight and postnatal growth. This applies to both the overgrowth and to the growth retardation. Okubo et al. (2003) reported two children with an abnormal growth related to *IGF1R*. The first child with a growth retardation and hypoglycemia had only one copy of *IGF1R* . The second child with overgrowth showed three copies of *IGF1R*. It suggests that loss of expression of *IGF1R* leads to growth retardation and over expression leads to overgrowth [15]. In BWS7, MLID-BWS3, MLID-BWS4 and MLID-BWS5 we detected a significant hypomethylation in the locus near *IGF1R*. The major feature of BWS is overgrowth and we can speculate that hypomethylation of the *IGF1R* locus may lead to the over-expression of this gene and cause the overgrowth in our patients. However, additional studies are necessary to ascertain this causal relationship.

In BWS4 we observed hypomethylation in two consecutive DMPs located in imprinted locus *KCNQ1OT1,* primarilyassociated with BWS. A SNP was present at the hybridization site of the probes of both CpGs. Knowing that the existence of a SNP in the examined CpG have an impact on methylation readouts [16] we believe that hypomethylation of the other DMP is a result of the cross-reacting of two probes. That led to the conclusion that this finding is probably not the cause of BWS in this patient and one should consider the presence of a SNP when only one or two nearby CpG show hypomethylation.

BWS22 displayed gain of methylation in three nonconsecutive DMPs in the imprinted *H19* locus and only one DMP had a delta difference larger than 10%. Although significant, it is not clear whether such small number of aberrant DMPs can be causative for BWS. It may be that, due to tissue mosaicism, other tissues in this patient are more affected and aberrant methylation covers a larger region. Alders et al. (2014) reported three BWS patients with epigenetic changes in tongue and buccal swab but with normal methylation status in blood [17]. The tissue mosaicism may also be a cause of BWS in other patients in our cohort.

We observed enriched hypermethylation in 30 DMPs annotated to the protocadherin gamma gene cluster located on chromosome 5 in three of five MLID-BWS patients and BWS7. The additional visualization of methylation patterns in healthy individuals showed that hypermethylation in this locus was specific for these three patients. Interestingly, BWS7 showed disturbed methylation in imprinted regions overlapping with MLID-BWS cases as well. This strengthens our hypothesis that BWS7 has MLID and led us to conclude that MLID patients display similar methylation patterns not only in imprinted but also in non-imprinted regions.

One region near the *DUSP22* gene showed profound hypomethylation in seven BWS patients, involving ten DMPs. A similar hypomethylation pattern was seen in 27 of 130 healthy individuals which means that hypomethylation in this locus is not specific for BWS. The differential methylation in this gene was so far reported in several methylation studies [18-20]. We observed aberrant methylation in this gene in other cohorts (data not published), as well. W. Steegenga et al. (2014) described changes in methylation in *DUSP22* in an age related study. They showed that changes in the methylation in this gene do not influence gene expression and aberrant methylation may be interindividual variation [21]. Our results are in agreement with this hypothesis.

In summary, in our cohort of 25 BWS patients without a molecular conformation we identified one patient with MLID, two patients with genome wide hypermethylation and one patient with only very subtle hypermethylation at the H19 locus. The results described here suggest that the HumanMethylation array is highly sensitive and can detect even small alteration of methylation, which is helpful in confirming the diagnosis.

**Conclusions**

BWS diagnosis cannot always be confirmed by routine diagnostic tests.

The results of our study indicate that the BWS phenotype may result from different epi(genetic) aberrations and these aberrations do not necessarily have to be in the primarily disease associated locus on 11p15. Additionally, we showed that the HumanMethylation450 array may be used to extend BWS diagnostics. This study points to unknown epi(genetic) mechanisms and paves the way for development of new diagnostics tests for BWS.

**Limitations**

Our study was limited to lymphocytes and we were not able to determine the methylation status in the imprinted region on chromosome 11p in other tissues and thereby exclude tissue mosaicism.

Moreover, the single case method does not include a correction for blood cell distribution which may influence the results.

**List of abbreviations**

BWS - Beckwith Wiedemann syndrome

CNV - Copy Number Variation

DHS - DNase I Hypersensitivity Site

DMP - Differentially Methylated Position

DMR - Differentially Methylated Region

FDR - False Discovery Rate

HRMA – High Resolution Melting Analysis

IDs - Imprinting Disorders

MAF – Minor Allele frequency

MLID - Multi-locus Imprinting Disturbance

MS-MLPA - Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

SNP – single nucleotide polymorphism

TSS – Transcription Start Site

QC – Quality Control

**Additional files:**

Additional table 1: Significant hypermethylated DMPs per patient.

Additional table 2: Significant hypomethylated DMPs per patient.

Additional table 3: The estimation of the cell types distribution.

Additional table 4: The calculation of P-values of the cell types distribution per patient.

Additional table 5: The summary of identified aberrant CpGs within COST regions .

Additional table 6: Genomic locations of imprinted loci – COST regions.

Additional table 7: Significant hypermethylated DMPs within the COST regions (per patient. BWS patients that did not display aberrant methylation in imprinted COST regions are not included in this table.

Additional table 8: Significant hypomethylated DMPs overlapped with COST regions. BWS patients that did not display aberrant methylation in imprinted COST regions are not included in this table.

Additional table 9: Results of the comparative analysis.

**Additional information:** Contains additional information about (1) Quality control, (2) pre- processing data and statistical methods, (3) estimation of the cell type distribution, (4) standard diagnostics, and (5) additional inspection of primary BWS associated loci at 11p15.5.

**Declarations**

**Ethics approval and consent to participate**

METC waived (anonymous study, further study in line with clinical question).

**Consent for publication**

 Not applicable

**Availability of data and material**

All HumanMethylation450 data are available on request.

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**Competing (financial) interest**

The authors declare that they have no competing (financial) interests.

**Authors’ contributions:**

MA, MM, JB designed the study; SM diagnosed and selected patients of the study; FR, DM designed the statistical method; IK, MA, PH, AV performed the statistical analysis; IK, KL, AM, AV performed the lab experiments; IK,MA, MM contributed to the manuscript writing and revision. All authors reviewed the final version of the manuscript.

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