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**A familial disorder of altered DNA-methylation**

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**ABSTRACT**

**Background**

In a subset of imprinting disorders caused by epimutations, multiple imprinted loci are affected. Familial occurrence of multi-locus imprinting disorders is rare.

**Purpose/Objective**

We have investigated the clinical and molecular features of a familial DNA-methylation disorder.

**Methods**

Tissues of affected individuals and blood samples of family members were investigated by conventional and molecular karyotyping. Sanger sequencing and RT-PCR of imprinting associated genes (*NLRP2, NLRP7, ZFP57, KHDC3L*, *DNMT1o*), exome sequencing, and locus-specific, array-based, and genome-wide technologies to determine DNA-methylation were performed.

**Results**

In three offspring of a healthy couple we observed prenatal onset of severe growth retardation and dysmorphism associated with altered DNA-methylation at paternally and maternally imprinted loci. Array-based analyses in various tissues of the offspring identified the DNA-methylation of 2.1% of the genes in the genome to be recurrently altered. Despite significant enrichment of imprinted genes (OR: 9.49), altered DNA-methylation predominately (90.2%) affected genes not known to be imprinted. Sequencing of genes known to cause comparable conditions and exome sequencing in affected individuals and their ancestors did not unambiguously point to a causative gene.

**Conclusion**

The family presented herein suggests the existence of a familial disorder of DNA-methylation affecting imprinted but also not imprinted gene loci potentially caused by a maternal effect mutation in a hitherto not identified gene.

**INTRODUCTION**

Genomic imprinting leads to parent-of-origin specific DNA-methylation and gene expression(1). Imprinting defects in humans contribute to several recognizable syndromes like Beckwith-Wiedemann (BWS), Silver-Russell (SRS), Prader-Willi (PWS) or Angelman (AS) syndrome. These imprinting disorders show DNA-methylation changes at the disease specific imprinted locus, however, more recent studies suggest that a subset of individuals with imprinting disorders shows changes of DNA-methylation at multiple imprinted loci. These multi-locus imprinting disorders seem to be particularly prevalent in individuals with BWS, SRS, and Transient Neonatal Diabetes Mellitus (TNDM), the latter has been associated with a “maternal hypomethylation syndrome” (2-4).

Familial occurrence of true imprinting defects, i.e. changes of DNA-methylation at imprinted loci without causative mutations at the imprinted locus itself in *cis* is rare. An autosomal recessive trait has been described in a subset of the “maternal hypomethylation syndromes” in which affected individuals carry biallelic mutations in the *ZFP57* gene(5). An alternative mechanism leading to familial occurrence of imprinting defects is the presence of “maternal effect mutations” in genes important for establishing or maintaining genomic imprints in early development. Indeed, two evolutionary closely related genes located head-to-head in chromosomal region 19q13, *NLRP2* and *NLRP7*, have been associated with such “maternal effect mutations”. A homozygous mutation in *NLRP2* has been described in a woman giving birth to siblings with BWS due to an imprinting defect in 11p15 and partial loss of methylation in *PEG1* in one child(6). In women with biallelic mutations of *NLRP7,* pregnancies completely fail to develop properly but instead result in hydatidiform moles, which also show alterations at multiple imprinted loci (for an overview see(7)). Recently, in women with familial biparental hydatidiform moles (FBHM) without *NLRP7* mutations changes in *KHDC3L* were observed(8-10).

Here we describe a family, in which two fetuses and one child of a couple showed altered DNA-methylation patterns not only of maternally and paternally imprinted genes but also of various other genes suggesting a more widespread disorder of DNA-methylation.

**PATIENTS AND METHODS**

**Case reports**

We report on two fetuses (III-1, III-3) and one child (III-2) of a healthy non-consanguineous Turkish couple (II-3, II-4). Family histories were unremarkable on both sides. In the first pregnancy (III-1) omphalocele and shortened femora were noticed at 21 weeks of gestation. At 33 weeks hypoplastic thorax and clover leaf skull were noted and the pregnancy was terminated. On pathological examination marked lung hypoplasia was confirmed and abnormal lung lobulation, gall bladder agenesis, hydronephrosis, and further abnormalities were noted. As X-rays showed no signs of skeletal dysplasia or craniosynostosis the tentative diagnosis of SRS was suggested. At that time only maternal UPD7 was known as cause for SRS but microsatellite analyses on cultured amniocytes ruled this out. The second pregnancy (III-2) was complicated by early diagnosis of molar changes in the placenta, asymmetrical fetal growth restriction, omphalocele, and massively elevated β-human chorionic gonadotropin (β-HCG). The child was born at 32 weeks. After birth coarse facial features, facial hemangioma, omphalocele, and asymmetric growth restriction were suggestive both of SRS and BWS which prompted methylation studies. Postnatally body proportions harmonised. The child is developmentally delayed. In the 3rd pregnancy (III-3) early asymmetric growth retardation, elevated β-HCG, and molar changes of the placenta were noted. The pregnancy ended by spontaneous fetal demise one week after chorionic villi sampling at 12 weeks. For further details see Figure 1A, online supplementary results, supplementary Table S1, and supplementary Figure S1.

Materials

Various specimens of the affected individuals (III-1, III-2, III-3) and peripheral blood samples of further family members were investigated (see online supplementary Table S2). Detailed information on analysed materials and used methods (incl. PCR conditions and primer sequences) are provided in the online supplementary appendix.

**Cytogenetic analyses**

Karyotyping, FISH, and molecular karyotyping was performed according to standard techniques and manufacturers´ instructions.

**Molecular studies**

Mutation analysis of *NLRP2* (NM\_001174081)*, NLRP7* (NM\_001127255)*,* *ZFP57* (NM\_001109809)*,* *KHDC3L* (NM\_001017361), and of the oocyte specific variant of *DNMT1* (NM\_001130823) was performed by Sanger sequencing. Segregation of *NLRP7/NLRP2* alleles was verified by microsatellite analysis on chromosome 19.

Exome enrichment using the NimbleGen Human SeqCap EZ v3.0 Kit followed by sequencing on an Illumina HiSeq 2000 system and data analysis was carried out on DNA from two affected children (III-1 and III-2), the parents (II-3 and II-4), and the maternal grandparents (I-3, I-4) as detailed in the supplementary appendix.

**DNA-methylation analysis**

Locus-specific DNA-methylation analysis was performed using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), methylation-specific PCR (MSP), sequence-based quantitative methylation analysis (SeQMA) and bisulfite pyrosequencing (BS-PS). Global DNA-methylation was analysed using LUminometric Methylation Assay (LUMA). For array-based DNA-methylation quantification of 27,578 CpG sites the HumanMethylation27 DNA Analysis BeadChip (Illumina) was applied. Raw hybridisation signals were interpreted using GenomeStudio software (GSE47879)(11). A detailed description of the bioinformatic analysis of array based methylation data is provided in the online supplementary appendix.

RESULTS AND DISCUSSION

In order to rule out a chromosomal aberration as cause of the phenotype, we performed conventional chromosome analyses in both parents and all three offspring, molecular karyotyping on both parents as well as individuals III-1 and III-2, and FISH analyses of chromosomes X, Y, 13, 18, and 21 on amniotic fluid samples of III-1 and III-2. All these analyses lacked evidence for any kind of chromosomal aberration as cause of the phenotype in the offspring III-1, III-2, and III-3(see online supplementary Table S3).

As several phenotypic characteristics of the affected individuals resembled features of imprinting disorders, DNA-methylation analyses of known imprinted loci was initiated. Results of locus-specific methylation analyses are shown in Table 1. In all three offspring, DNA-methylation changes at multiple imprinted loci were identified. Remarkably, these affected both maternal and paternal imprints. The only hypermethylation was observed for the *NESP* somatic DMR but this is most likely caused by hypomethylation of the *GNAS* DMR. Remarkably, DNA-methylation at the imprinted loci was not fully lost and the extent of DNA-methylation changes varied between the different individuals as well as between the tissues from the same individual (Table 1). These observations strongly suggested a DNA-methylation defect to underlie the phenotype in the offspring. Moreover, the extent and variation of DNA-methylation levels at imprinted loci suggested mosaicism for the DNA-methylation changes which could explain the phenotypic variability of the three offspring.

The DNA-methylation analyses excluded a maternal hypomethylation syndrome but instead proved that the underlying disorder affects bi-parental imprints(4,5). Such familial occurrence of a multilocus imprinting disorder affecting both paternal and maternal imprints has yet been rarely described(12,13).

We next wondered whether the DNA-methylation changes extended over differentially methylated regions of imprinted loci. Thus, in order to rule out a global disturbance of the DNA-methylation we performed LUMA in two of the three affected offspring and the parents. By this approach, no overall aberrations of DNA-methylation were detected (see online supplementary Figure S2).

As LUMA is biased towards the determination of DNA-methylation levels in repeat regions, we additionally aimed at investigating single-copy locus DNA-methylation. To this end, we performed array-based DNA-methylation quantification of 27,578 CpG sites in the human genome. DNA-methylation levels in different accessible tissues from the affected individuals were compared to matched tissue samples from healthy controls (for details see online supplementary appendix). In line with previous studies demonstrating the validity of the array(14), there was a good agreement of the array-based results and the above shown results of locus specific analyses of imprinted loci. In order to get an initial estimation of the extent of the DNA methylation changes in the offspring, we performed a supervised comparison of all samples of the three affected individuals compared to all control samples independent of the tissue origin. By this global approach, we identified 95 CpG loci corresponding to 87 genes to be differentially methylated (t-test, FDR<0.01; online supplementary Figure S3 und Table S4). In line with the molecular analyses described above, these included CpGs from several imprinted genes, like *GNAS, H19, KCNQ1, MEG3, NNAT* and *PLAGL1*. Formal testing showed known or previously suggested imprinted genes to be strongly enriched among the affected genes (OR=13.90, RR=12.12, p<0.001). Moreover, GATHER maploc analysis(15) revealed an enrichment for loci mapping to chromosome band 11p15 (p<0.01, Bayes factor > 14.3) containing the imprinted gene cluster associated with BWS and SRS.

Obviously, DNA-methylation patterns can be tissue specific(16). Thus, in a second approach to estimate the extent of the DNA methylation changes we aimed at identifying CpG loci differentially methylated between corresponding tissue of affected and control individuals. The sum of tissues showing aberrant DNA-methylation between affected and control individuals was calculated for each CpG locus. In total, 287 genes accounting for 2.1% of the genes analysed by the array were affected recurrently, i.e. in at least two comparisons (online supplementary Figure S4 and Table S5). These 287 genes were again significantly enriched for known or previously suggested imprinted genes (OR=9.49, RR=8.17, p<0.0001). Moreover, 18 of these 287 genes overlapped with the 87 genes identified by the first approach, which included imprinted genes like *GNAS, KCNQ1, L3MBTL, MEG3, NAP1L5, NNAT, PLAGL1,* and *ZNF597*. These 18 genes also included the *RB1* gene, which we could recently prove to be imprinted based on the findings in this family(17).

Both approaches concordantly showed clearly strong and significant enrichment of imprinted loci among the aberrantly methylated loci in the affected individuals of the family. However, the vast majority of the aberrantly methylated genes (75/87, 86.2% and 259/287, 90.2%, respectively) have yet not been described to be imprinted. In line with this, we could recently prove by targeted deep bisulfite sequencing that the aberrant methylation at least of some of the aberrantly methylated genes in III-2 is independent of the parental allele(11). Moreover, the aberrant methylation was not restricted to hypomethylation as we observed both increased and decreased methylation in the affected individuals as compared to the controls (see online supplementary Figure S3)

As the above described findings indicated that the DNA-methylation disorder in the family presented is not restricted to imprinted loci and moreover might have features different from previously described multi-locus methylation disorders we performed *in silico* analysis of the aberrantly methylated genes. The aberrantly methylated genes derived from both approaches were enriched for genes with low CpG content promoters (OR=2.59, RR=2.57, p<0.001 and OR=1.89, RR=1.86, p<0.0001, respectively), while they were significantly depleted for high CpG content promoters (OR=0.40, RR=0.40, p<0.001 and OR=0.43, RR=0.44, p<0.0001) and CpG-islands (OR=0.29, RR=0.29, p<0.001 and OR=0.37, RR=0.38, p<0.0001) (Figure 1B). Furthermore, the aberrantly methylated genes were significantly enriched for target genes of several transcription factors including SMAD3 (p< 0.01; Bayes factor > 8.0, online supplementary table S6). Remarkably, SMAD3 has recently been shown to co-localize with CTCF to the *H19* imprinting control region and has been proposed to play a role in chromatin cross-talk organized by the *H19* ICR(18).

Next, we aimed at investigating the mechanism which might lead to the DNA-methylation defect in the family. Besides *NLRP2, NLRP7*, *ZFP57* and *KHDC3L* which have yet been predominately linked to changes of maternal marks at imprinted DMRs with high CpG content we considered *DNMT1o* as potential candidates. We failed to identify potentially pathogenic coding mutations in any of these genesin the family. Nevertheless, a heterozygous missense variant c.2156C>T (p.A719V) in *NLRP7* was identified in the mother (II-4) of the affected individuals (Figure 1C, online supplementary Figure S5 and Table S7)(19). *NLRP7* mutations in the sense of so called “maternal effect mutations” have been previously associated with recurrent hydatidiform moles, which show a disturbance of imprinting at multiple loci. Indeed, the very same variant p.A719V has been detected in heterozygous state by Messaed and colleagues in a woman with four spontaneous abortions, one of which led to a gestational trophoblastic disease(20). Nevertheless, this variant is also listed in the 1000 Genome (<http://www.1000genomes.org>) and in the dbSNP database (build 138, rs104895526) with very low frequencies and always in a heterozygous state. This is in line with a recent observation which gave a minor allele frequency of 0,017/1 for this change (21). Moreover, in the family presented herein the maternal grandmother also carried the mutated allele. Together, considerable evidence suggests that this maternal change alone is not sufficient to cause the phenotype in the offspring, or that a stochastic process is involved. In this context it is remarkable, that in the array-based methylation analysis we noticed the mother (II-4) of the affected offspring to show a strong hypomethylation (normalised methylation value: -1.2) at one CpG (cg16106497) in the region containing the 5’ ends of both genes *NLRP2* and *NLRP7* as compared to controls (mean: 2.0, range: -0.2 to 3.9, see online supplementary Figure S6). Though it is tempting to speculate that this variant reduces *NLRP7* expression so that the mother is functionally compound heterozygous at this locus a maternal dysfunction of *NLRP7* could not ultimately be proven (see online materials).

To explore whether changes in genes other than those known to be related to imprinting disorders might cause the phenotype we performed exome sequencing of two affected children (III-1 and III-2), the parents (II-3 and II-4), and the maternal grandparents (I-3, I-4). Since the inheritance pattern could be most likely explained by a mutation in a maternal effect gene we searched for i) *de* *novo* mutations in the mother by comparing exome data of the mother and her parents; ii) homozygous and compound heterozygous mutations in the mother and iii) paternally inherited mutations in the mother not present in the maternal grandmother and the cohort of healthy individuals of our in-house data base. The query for *de novo*, compound heterozygous and homozygous mutations yielded no hit. In the third approach 110 mutations including 83 missense mutations were discovered (see supplementary Table S8). The unexpected high number of mutations/variations is likely due to the ethnic differences between the family and our in-house healthy controls. The variant in *NLRP7* was confirmed, but as it is listed in dbSNP it has not been included in Supplementary Table S8. We also analysed the exome data in regard to a paternal contribution, although we think it unlikely that this inheritance model would be compatible with the observed effects. The query showed 35 variants present in the father and the two siblings investigated by exome sequencing (III-2 and III-1; see supplementary Table S9). These variants were not present in the mother or the maternal grandparents. Moreover, no homozygous or compound-heterozygous variants present in both investigated siblings could be identified. Thus, the exome sequencing approach in this single family did not unambiguously point to a causative gene.

The family presented herein along with the molecular studies suggests the existence of a hitherto unrecognised familial disorder of altered DNA-methylation which besides paternally and maternally imprinted loci also affects a considerable number of loci not known to be associated with parent-of-origin specific DNA-methylation. Whether the disorder is linked to allelic methylation or other mechanisms remains speculative at this stage. The same holds true for the pathogenic role of the *NLRP7* variant. Nevertheless, based on the findings presented herein future studies of individuals with multi-locus imprinting disorders should also include the analysis of DNA-methylation of loci not known to be imprinted.

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**Contributions** RS, AC, BH and KB designed the study; NS, EJ and LS performed histopathologic review and provided material; JR, AH, JB, DK, BK, SB, DM, IN, IV and KB performed the experiments; JR, OA, JIMS and DK performed data analysis; AC, CSvK and RS performed clinical characterization and provided material of the family members; IKT, BH was involved in data interpretation; AC, JR, OA and RS wrote the manuscript, all authors approved the manuscript.

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**Table 1:** DNA-methylation analysis of imprinted gene loci.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **name** | **methyl allele** | **phenotype** | **chr region** | **method** | **II-3** | **II-4** | **III-1** | **III-2** | **III-3** |
| **pb** | **pb** | **mu** | **pb** | **lym cl** | **fib cl** | **bs** | **CVS** | **mu** |
| ***ARHI/DIRAS3*** | M |  | 1p31.3 | MSP | ↔ | ↔ | ▼ | ▼ | ▼ | ▼ | ▼ | ↓ | na |
| BS-PS | ↔ (54) | ↑ (67) | ↓ (40) | ↓ (13) | na | na | na | na | ↔ (42) |
| ***PLAGL1*** | M | TNDM | 6q24.2 | MSP | ↔ | ↔ | ▼ | ▼ | ▼ | ↓ | na | ↓/↔ | na |
| BS-PS | ↔ (31) | ↔ (37) | ↓ (6) | ↓ (9) | ↓ (11) | na | na | na | ↓ (25) |
| ***GRB10*** | M | SRS | 7p12.2 | MSP | ↔ | ↔ | ▼ | ▼ | ▼ | ▼ | na | ↔ | na |
| BS-PS | ↔ (26) | ↔ (22) | ↓ (7) | ↓ (11) | ↓ (12) | na | na | na | ↔ (37) |
| ***MEST*** | M |  | 7q32.2 | MSP | ↔ | ↔ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓/↔ | na |
| BS-PS | ↔ (29) | n.d. | ↓ (6) | ↓ (18) | na | na | na | na | ↔ (38) |
| ***KCNQ1OT1*** | M | BWS | 11p15.5 | MLPA | ↔ (50) | ↔(50) | ↓ (5-10) | ↓ (10-15) | ↓ (10-15) | ↓(25) | ↓ (15-20) | ↓(25) | na |
| ***KCNQ1*** | BS-PS | ↔ (20) | ↔ (16) | ↓ (10) | ↓ (7) | ↓ (7) | na | na | na | ↓ (7) |
| ***IGF2*** | P | SRS | BS-PS | ↔ (52) | ↔ (45) | ↔ (45) | ↓ (25) | ↓ (19) | na | na | na | ↔ (43) |
| ***H19*** | MLPA | ↔(50) | ↔(50) | ↔(50) | ↓ (10) | ↓ (10-15) | ↓(25) | ↓ (20-25) | ↔(50) | na |
| ***H19\_*6CTCF** | BS-PS | ↔ (23) | ↔ (31) | ↔ (28) | ↓ (5) | ↓ (5) | na | na | na | ↔ (19) |
| ***H19\_*3CTCF** | BS-PS | ↔ (19) | ↔ (15) | ↔ (22) | ↓ (9) | na | na | na | na | na |
| ***RB1*** | M |  | 13q14.2 | MSP | ↔ | ↔ | ↓ | na | ↓ | na | na | na | na |
| ***MEG3*** | P | TS | 14q32 | BS-PS | ↔ (41) | ↔ (42) | ↓ (6) | ↓ (19) | na | na | na | na | ↓ (24) |
| ***MEG3*\_a** | MSP | ↔ | ↔ | ↓ | ↓ | ↓ | ↓ | na | na | na |
| ***MEG3*\_b** | MSP | ↔ | ↔ | ▼ | ↓ | ▼ | ↓ | ↓ | na | na |
| **IG-DMR *DLK1/GTL2*** | MSP/SeQMA | ↔ | ↔ | ↓ | ↓ | ↓ | ↓ | ↓ | ↔ | na |
| ***SNRPN*** | M | AS | 15q11.2 | MLPA | ↔(50) | ↔(50) | ↔(50) | ↓ (20-25) | ↓ (15) | ↓ (10-20) | ↓ (30) | ↔(50) | na |
| BS-PS | ↔ (38) | ↔ (39) | ↔ (35) | ↓(17) | ↓ (17) | na | na | na | ↔ (38) |
| ***NDN*** | MLPA | ↔(50) | ↔(50) | ↔(50) | ↓(20) | ↓ (15) | ↓(20) | ▼ | ↔(35\*) | na |
| BS-PS | ↔ (47) | ↔ (41) | na | ↓ (37) | ↔ (40) | na | na | na | ↓ (29) |
| ***PEG3*** | M |  | 19q13 | MSP | ↔ | ↑ | ▼ | ↓ | ↓ | ▼ | na | ↓ | na |
| BS-PS | ↔ (33) | ↔ (38) | ↓ (5) | ↓ (23) | ↓ (24) | na | na | na | ↔ (33) |
| ***GNAS*** | M | PHP1b | 20q13.2 | MSP | ↔ | ↔ | ↓ | ↓ | ↓ | ↓ | ↓ | na | na |
| ***NESPAS*** | MSP | ↔ | ↔ | ↓ | na | ▼ | ↓ | na | ↓ | na |
| ***NESP*** | P | MSP | ↔ | ↔ | ↑ | na | ↑ | ↑ | ↑ | ↑ | na |

methyl allele: methylated allele, P: paternal, M: maternal, TNDM: transient neonatal diabetes mellitus, SRS: Silver-Russell syndrome, BWS: Beckwith-Wiedemann syndrome, TS: Temple syndrome, AS: Angelman syndrome, PHP1b: Pseudohypoparathyroidism type 1b, chr region: chromosomal region of the imprinted loci, MSP: methylation-specific PCR, BS-PS: bisulfite-pyrosequencing, MLPA: multiplex ligation-dependent probe amplification, SeQMA sequence-based quantitative methylation analyses, pb: peripheral blood, mu: muscle, lym cl: lymphoblastoid cell line, fib cl: fibroblast cell line, bs: buccal swab, CVS: chorionic villi, ↔: normally methylated, ↑: hypermethylated, ↓: hypomethylated, ▼: unmethylated, ↓/↔: marginal hypomethylated, na: not analysed, \**NDN* is always hypomethylated in CVS.

DNA-methylation of 21 imprinted loci of 17 known imprinted regions was investigated by different methods in nine samples of the parents (II-3, II-4) and their three children (III-1, III-2, III-3). The results are summarised with respect to the analysed region and which known syndromes/phenotypes are affected if the genes are hypomethylated (column 3). All values are rounded up. The methylation value per locus is given in the parenthesis.

**FIGURE LEGEND**

*Figure 1: Pedigree of the family and results of NLRP7 mutation and DNA-methylation analyses*

(A) Pedigree of the family. Presence of the *NLRP7* variant (c.2156C>T), segregation of chromosome 19 determined by microsatellite (D19S927, D19S926 and D19S418) and SNP analysis of the *NLRP7/NLRP2* genes are shown.

(B) Enrichment analysis of the genes differentially methylated in affected individuals independent of the tissue. Genes (n=87) differentially methylated between patient samples and tissue-matched controls were significantly enriched for promoters with low CpG content (p<0.001, chi square test) as well as for imprinted genes (p<0.001). In contrast, differentially methylated genes were depleted for promoters with high CpG content (p<0.001) and CpG island (p<0.001). The enrichment was calculated comparing the proportion of the respective group of the array to the differentially methylated genes. Grey bars indicate the proportion presented on the array and black bars the proportion presented on the 87 differentially methylated genes. HCP: promoters of high CpG content, ICP: promoters of intermediate CpG content, LCP: promoters of low CpG content, \*\*\*: p<0.001 (chi square test), ns: not significant (p>0.05).

(C) *NLRP7* mutation analysis in the parents identifies a heterozygous c.2156C>T (p.A719V) variant in the mother (II-4) of the affected individuals. The father carries only the wildtype allele.