Non-random, individual-specific methylation profiles are present at the sixth CTCF binding site in the human H19/IGF2 imprinting control region

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ABSTRACT

Expression of imprinted genes is classically associated with differential methylation of specific CpG-rich DNA regions (DMRs). The H19/IGF2 locus is considered a paradigm for epigenetic regulation. In mice, as in humans, the essential H19 DMR—target of the CTCF insulator—is located between the two genes. Here, we performed a pyrosequencing-based quantitative analysis of its CpG methylation in normal human tissues. The quantitative analysis of the methylation level in the H19 DMR revealed three unexpected discrete, individual-specific methylation states. This epigenetic polymorphism was confined to the sixth CTCF binding site while a unique median-methylated profile was found at the third CTCF binding site as well as in the H19 promoter. Monoallelic expression of H19 and IGF2 was maintained independently of the methylation status at the sixth CTCF binding site and the IGF2 DMR2 displayed a median-methylated profile in all individuals and tissues analyzed. Interestingly, the methylation profile was genetically transmitted. Transgenerational inheritance of the H19 methylation profile was compatible with a simple model involving one gene with three alleles. The existence of three individual-specific epigenotypes in the H19 DMR in a non-pathological situation means it is important to reconsider the diagnostic value and functional importance of the sixth CTCF binding site.

INTRODUCTION

Imprinted genes are expressed from only one of the parental chromosomes (1,2). Generally they are located in clusters and epigenetically marked by DNA methylation, histone acetylation/deacetylation and histone methylation and often associated with antisense RNAs (3,4). In mice, extensive studies of the paradigmatic imprinted H19/Igf2 region revealed that the physical contacts between differentially methylated regions (DMRs), containing insulators, silencers and activators, lead to transcriptional regulation of both H19 and Igf2 genes (5,6). Methylation of the paternally derived allele at an imprinting control region (ICR) located 2 kb upstream of H19 (H19 DMR) is required to silence H19 and to activate Igf2 on the chromosome of paternal origin. Reciprocally, absence of methylation on the maternal allele in the H19 DMR leads to expression of the maternal H19 allele and the silencing of Igf2 throughout development (7). Mechanistically, the H19 DMR is the biological target for...
CTCF, a zinc finger CCCTC-binding factor, which can bind to four sites on the unmethylated maternal H19 DMR (8,9). The binding of CTCF creates a physical boundary on the maternal allele and inhibits interaction of downstream enhancers with Igf2 promoters (10). In addition, CTCF binding to H19 DMR might be necessary to prevent de novo methylation of the maternal allele (11).

Comparative analysis of a paternal-specific methylation imprint at the human H19 locus revealed the presence of several highly similar 400 bp repeats containing CpG islands, although no significant sequence homology to the corresponding region of the mouse H19 DMR could be identified (12). Much less is known about the functional regulation in humans compared to mice. The human H19 DMR encompasses seven different binding sites for CTCF. Only the sixth CTCF binding site has been reported to act as a key regulatory domain for switching between H19 and IGF2 expression, whereas the other sites appear to be hyper-methylated in a study by Takai et al. (13). Anomalies in methylation patterns of the H19/IGF2 locus have been consistently proposed as epigenetic markers of human disease (14–18).

However, the correlation between H19 DMR methylation and expression is not always strictly defined. In normal human brain, biallelic expression of IGF2 and/or H19 is found despite differential methylation and CTCF binding (19). Also an inter-individual variability in IGF2 imprinting with biallelic expression has been observed in lymphocytes of ~10% of normal individuals (20). In addition, a familial aggregation of ‘abnormal methylation’ in ~7% of analyzed samples was identified at the sixth CTCF binding site (21). These studies suggest overall that the regulation of methylation near imprinted genes might be leakier in human compared to mice.

Imprinted genes are probably the most important buffering factors for regulating the day-to-day trades between mother and foetus in placental mammals. Therefore understanding their regulation is of particular importance for understanding human placental physiopathology. Expression and gene invalidation data demonstrate the great importance of the H19/IGF2 locus in placental development and physiology (22–24). Both genes are expressed at very high levels and with high specificity in human placenta (25). In this tissue, the analysis of the H19 DMR methylation profile yielded contrasting results, with either an early monoallelic expression of H19 established as soon as the first trimester (8–12 weeks) (26) or a progressive acquisition of monoallelic expression between the first trimester and normal term (~39 weeks) (27). This discrepancy between the methylation of H19 DMR and expression of H19/IGF2, raises the general question of the correlation and anteriority between DNA methylation, histone modification, and the ultimate mark of imprinting, monoallelic expression.

Due to the high expression of both genes in placenta and its importance in placental development, we chose to analyze systematically methylation patterns at the sixth CTCF binding site in DNAs extracted from placentas of two developmental stages in a normal population. We further analyzed lymphocyte DNA as a second tissue to identify tissue-specific effects. DNA methylation patterns are mainly investigated by cloning and subsequent sequencing, a time consuming process restricting the number of analyzed molecules to the order of tens. Recently, the pyrosequencing technology was developed and validated for DNA methylation analysis of numerous individual samples providing excellent quantitative resolution (28–30).

In this study we demonstrate the existence of three individual-specific methylation profiles in human lymphocyte DNA as well as in placenta at the sixth CTCF binding site. This observation, although not correlated with a loss of monoallelic expression of H19 and IGF2 raises important questions about the functional importance of the sixth CTCF binding site in humans as well as its implication in regulation at this locus. Furthermore, we can show that the three levels of methylation could be explained by a genetic model involving a unique effecting locus. This represents the first example of transgenerational heritability of DNA methylation levels in a human non-pathological situation. Finally, these results further challenge the diagnostic value of methylation profiles in the H19/IGF2 imprinting control region as a marker for cancer or epigenetic diseases such as the Beckwith–Wiedemann or Silver–Russell syndrome.

**MATERIALS AND METHODS**

**Sample collection**

Sample collection (blood, placental villi and spermatozoa samples) was performed under a protocol approved by the institutional ethics committee. No individual identifiers or history, other than lack of known malformations were recorded. Informed consent was obtained from all individuals for the use of samples of placenta, blood and spermatozoa. Fifteen early term (between 8 and 14 weeks of amenorrhoea) and 40 full term placentas (37–40 weeks of amenorrhoea) were analyzed. In 29 cases, the placenta-maternal blood sample pair was collected. In 10 cases, the placenta-cord blood and maternal blood samples were collected. Blood DNA samples from 37 individuals from 6 two- to four- generation families and from a second independent cohort of 19 adult patients were collected and analyzed. DNAs of 30 individuals from 8 families extracted from lymphoblastoid cell lines of the CEPH/Utah collection were obtained from the DNA banking facility of the CNG (Evry, France). In addition, spermatozoa from 10 normozoospermic individuals were collected.

**Nucleic acid isolation**

Genomic DNA was isolated from adult lymphocytes, from dissected and washed placental villi and from spermatozoa by incubation with protease K (0.2 mg/ml) in Lysis Saline Buffer following standard methods. RNA extraction was performed from placental villi using Trizol reagent (Invitrogen Life Technology, Cergy, France).

**Methylation assays**

The bisulphite conversions of genomic DNA (1 µg) and pyrosequencing analysis were performed as previously described (29,30). Supplementary Table 1 lists the accession numbers and nucleotide positions of the analyzed regions, the PCR primers and annealing temperatures used, the size of the PCR products and the number of CpGs analyzed. Primers for PCR amplification, pyrosequencing and primer extension
reactions were purchased from Biotez (Buch, Germany). Briefly, 50 ng of bisulphite converted DNA was added as template into PCR buffer with 1 mM MgCl₂, 200 μM dNTP, 0.4 μM of each forward and reverse biotinylated primer and 2.0 U Platinum Taq polymerase (Invitrogen Life Technology). The PCR program consisted of a denaturing step of 4 min at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at the hybridization temperature (see Supplementary Table 1) and 20 s at 72°C, with a final extension of 5 min at 72°C. Purification of the PCR product with streptavidin Sepharose⁶⁸ HP beads (GE Healthcare, Uppsala, Sweden) and hybridization of the biotinylated PCR products and the sequencing primer were conducted following the PSQ96 sample preparation guide using a vacuum filtration sample transfer device (Pyrosequencing AB, Uppsala, Sweden). Sequencing was performed on a PSQ 96MA system with the PyroGold SQA reagent kit according to the manufacturer’s instructions (Pyrosequencing AB). All PCRs were checked for the absence of preferential amplification by mixing known ratios of whole-genome amplified and in vitro methylated (Sssl, Ozyme, St Quentin-en-Yvelines, France) DNA. Pyrosequencing primers and templates were controlled for self-extension or primer-dimer formation.

The verification of the methylation status of selected CpG positions was performed by MALDI mass spectrometry as previously described (29,31). For cloning and sequencing, the PCR products were subcloned into a TA cloning vector (pGemT vector, Promega, Charbonnieres, France) DNA. Pyrosequencing primers and templates were controlled for self-extension or primer-dimer formation.

Allele-specific expression
Allele-specific expression was carried out by genotyping of the Rsai polymorphism (rs3741219) in exon 5 of H19 and the Apal polymorphism (rs680) in exon 9 of IGF2, respectively. RT–PCR products were analyzed for samples heterozygous for the respective SNPs (n = 34 for H19 and n = 10 for IGF2, respectively). Primers are listed in Supplementary Table 2. After the cDNA synthesis, PCR products were either analyzed by standard Sanger sequencing on a 3730 ABI sequencer using the above described primer set (rs3741219, rs2839704 and rs2839703) for the H19 gene, or digested by Apal and electrophoresed on a 2% agarose gel to analyze the IGF2 polymorphism.

Quantitative RT–PCR
Real-time PCR was performed using a Lightcycler (Roche™ Diagnostics, Mannheim, Germany) and SYBR Green master mix (Invitrogen) to quantify gene expression. Primers (Supplementary Table 2) were designed to amplify a region encompassing exons 7, 8 and 9 of IGF2 and a region in exon 5 of H19. Relative expression of IGF2 and H19 is shown normalized to the expression of the succinate dehydrogenase A (SDHA).

Genotyping of polymorphisms
Genomic DNA was analyzed for two SNPs (rs10732516 and rs 2071094) and RT-PCR products were analyzed for three SNP (rs2839703, rs2839704 and rs3741219). Genotyping of polymorphisms were performed by pyrosequencing using

the SNP genotyping kit according to the manufacturer’s instructions (Pyrosequencing AB).

Statistical analysis
A Mann–Whitney rank test was used to determine whether the epigenotypes (hypo-, median- and hyper-methylated profiles) were associated with tissue type (placenta versus lymphocytes) and to test the distribution of the H19 and IGF2 expressions in early term and full-term placentas. A Monte Carlo based permutation test was used to evaluate the statistical significance of the distribution of the methylation profiles to exclude random effects. Student’s t-test was used to test the significance of gene expression differences between groups. A χ² test was used to determine whether the proportion of the different genotypes (CC, TT and CT, SNP rs10732516) was similar in the general population and in individuals analyzed in this study.

RESULTS
Methylation analysis of the sixth CTCF binding site in the H19 DMR
We analyzed the methylation levels at sixteen consecutive CpGs at the sixth CTCF binding site in the H19 DMR in DNA from lymphocytes (maternal blood cells, blood 1) as an adult control tissue, and in DNA from human placentas at two different stages of placental development (early gestation, 8–14 weeks of amenorrhoea, or at full term gestation, 37–40 weeks of amenorrhoea) by pyrosequencing. While an overall methylation level of 50% was expected, a high variability of methylation levels between individuals and consecutive CpGs was found leading to a surprisingly complex methylation pattern in both adult lymphocytes and placentas (Figure 1A–C). The fifth CpG in the DMR was completely unmethylated for some samples, which is explained by the existence of a C/T SNP (rs10732516). DNA samples harboring a T were therefore completely unmethylated, which explains the dip in the methylation level at this position for several of the DNA samples. This polymorphism did not affect the global methylation level of the DMR. After exclusion of the fifth CpG the methylation profiles in the H19 DMR could be grouped into three discrete methylation categories in maternal blood cell DNA (Figure 1A): a hyper-methylated profile (average methylation > 66%: 80.2 ± 8.2%), a median-methylated profile (33% < average methylation < 66%: 44.5 ± 5.1%) and a hypo-methylated profile (average methylation < 33%: 17.7 ± 7.5%). The median-methylated profile was encountered in 59% of the lymphocyte samples, while the hypo- and hyper-methylated represented 28% and 13%, respectively. A Monte-Carlo based permutation test (n = 5000) was used to test objectively for this discrete distribution into three categories (from 0 to 33%, from 33 to 66% and from 66 to 100% of methylation, respectively). Compared with the simulated dataset, probabilities of P < 0.008, P < 0.002 and P < 0.03 were obtained for the three categories, respectively. It can thus be concluded that the repartition was non-random and results from genuine biological differences. To exclude any sampling bias, we confirmed the distribution of the methylation status in a second independent cohort of nineteen adult individuals (Figure 1D, blood 2).
Further investigation revealed that these levels were not restricted to lymphocyte DNA. Similar profiles were also observed in placental tissue DNA (Figure 1B and C), albeit with a less clear division between the hypo- and median-methylated profiles, consistent with the classical observation of a placental hypo-methylation compared to other tissues. The frequencies of the three epigenotypes were not different between the two independent cohorts of lymphocyte DNAs (maternal or from anonymous adult individuals), early term or full term placentas (Figure 1D). Also inside each methylation group the inter-individual variations were not significantly different between placenta and lymphocyte samples.

The parental origin of the methylated strands was addressed by cloning and Sanger sequencing of PCR products from three placental samples corresponding to each one of the three different methylation profiles, consistent with the classical observation of a placental hypo-methylation compared to other tissues. The frequencies of the three epigenotypes were not different between the two independent cohorts of lymphocyte DNAs (maternal or from anonymous adult individuals), early term or full term placentas (Figure 1D). Also inside each methylation group the inter-individual variations were not significantly different between placenta and lymphocyte samples.

The parental origin of the methylated strands was addressed by cloning and Sanger sequencing of PCR products from three placental samples corresponding to each one of the three different methylation profiles and heterozygous for the single nucleotide polymorphism rs2071094 (Figure 2). The hyper-methylated profile corresponds mainly to methylated DNA molecules from both the maternal and paternal origin. Similarly, the hypo-methylated profile corresponded mainly to unmethylated molecules from both parental alleles. Consistent with the classical model, the median-methylated profile corresponded to the presence of methylated molecules of paternal origin.

Validation of the epigenetic profiles at the sixth CTCF binding site

Since our results were unexpected and somewhat contradictory to published observations of the homologous mouse DMR, we controlled and reproduced the results to exclude any technical artefacts in addition to the cloning and sequencing experiment described above.

(i) To rule out incomplete bisulphite conversion of the genomic DNA, the bisulphite treatments were standardized and verified. Systematically, 1 μg of genomic DNA was used for bisulphite conversion and 50 ng of converted DNA for PCR amplification. Conversion rates were confirmed by cloning and sequencing PCR products. This revealed that >99% of unmethylated cytosines were converted. Four independent replicates of the bisulphite treatment followed by PCR amplification were carried out for six placental DNA samples, displaying a very good reproducibility of the process (SD < 5.0%; Supplementary Figure 1A). The same methylation profiles were obtained using a second primer set encompassing the previous primer positions (n = 6, Supplementary Figure 1B).

(ii) Since the structure of the H19 DMR is very complex and consists of highly similar repeats (12), the specificity of the amplified PCR products was checked by systematically reanalyzing the amplified sequence at each base pair to demonstrate that the DMR encompassing the sixth CTCF binding site was exclusively amplified. Supplementary Figure 2 shows the alignment of the different consecutive binding sites proving that the sixth CTCF site is clearly distinguishable from the others after pyrosequencing. Any additional or missing base in the analyzed sequence—even partially—would induce
failure of the pyrosequencing procedure, which is based on the sequential input of the correct nucleotides into the elongating DNA strand.

(iii) Results were further confirmed by an independent quantitative method using a single base primer extension assay combined with MALDI mass spectrometric detection for several CpG inside the analyzed DMR (31).

The methylation level of the H19 DMR is individual-specific but does not depend on the tissue analyzed

We analyzed DNAs originating from the placenta and from blood of the foetus, taken at the time of birth for 10 individuals. The methylation profile was found to be identical in the two different tissues from the same individual (Figure 3). This showed that the epigenotype (hypo-, median- or hyper-methylated) is acquired early in development, probably before the separation of the syncytiotrophoblast from the inner cell mass. This early determination supports a strict determinism of the methylation status, which is maintained through the individual’s life.

Familial transmission of H19 DMR epigenotypes

The literature evoked the existence of ‘aberrant’ familial aggregation of methylation at the sixth CTCF binding site (21). Since different epigenotypes were often observed...
in the analysis of the mother/placenta pairs, we envisaged that these different epigenotypes could be due to familial segregation of ‘methylated’ alleles. Therefore we tested the possibility of genetic transmission of the three observed methylation levels. We analyzed the epigenetic profiles of 6 two- to four-generation families encompassing a total of 37 children (Figure 4, only the four most informative families are presented), as well as the epigenotypes of 29 mother/placenta pairs. When considering the transmission pattern from parents (both sexes) to children, 83 transmissions could be followed, which were 9 hypo-, 52 median- and 22 hyper-methylated. In a random situation, the epigenotype of the descendant would not be influenced by the epigenotype of the parent. Under this hypothesis, for a given child epigenotype, the probability of each parent epigenotype is 1/3. In fact, the actual proportions varied according to each epigenotype. These proportions were tested by a \( \chi^2 \) test and were found to be highly significant \( (P = 0.00014) \). This indicated that the transmission from parent to child was not random. In addition, we tested the epigenotypes of lymphocytes from 48 unrelated individuals (blood 1 and blood 2 cohorts), yielding 27, 59 and 14% of hypo-, median- and hyper-methylated epigenotypes, respectively. This sample was used as a reference population. These proportions were markedly different from that observed in our six families \( (P = 0.00006) \). These data showing different proportions in familial structures compared to a cohort of unrelated individuals strengthen the hypothesis of a genetic transmission of the epigenotypes. Without exception, our familial transmission patterns were compatible with a model of a single gene presenting three alleles and a strict model of dominance: median- > hypo- > hyper-methylated. The model was compatible with Hardy–Weinberg equilibrium.

In order to further validate the likelihood of our model we tried to follow methylation profiles in families from the CEPH-Utah collection analysing 30 different individuals from 8 families. However, all samples displayed a median-methylated profile with an average methylation of 38%. This homogeneity has probably been acquired during immortalization of the cells or cell culture.

To test for a correlation between the genetic structure of the locus and the methylation profile we compared genotype data from haplotype-tagging SNPs in the DMR upstream of \( H19 \), in \( H19 \) itself and in \( IGF2 \) (J. Tost, unpublished data) to the epigenetic profile, but no association was found. In DNA from purified spermatozoa, the analysis of the \( H19 \) DMR methylation profile by pyrosequencing showed complete hyper-methylation, as expected for spermatozoa \( (n = 10) \). This suggests that the individual-specific methylation status of \( H19 \) DMR observed in lymphocytes and in placentas is acquired after fertilization during the differentiation of somatic cell lineages.

**Spatial extension of the three methylation profiles**

*Methylation profiles are restricted to the sixth CTCF site.* To determine the extent of the variable epigenic profiles, we analyzed 10 CpGs encompassing the third CTCF site (Figure 5) as well as 9 CpGs in the direct proximal promoter region of \( H19 \). All samples displayed \( \sim 40\% \) methylation at these two loci.

*Methylation status of \( IGF2 \) DMR2 is independent of the epigenotype at the sixth CTCF site in the \( H19 \) DMR.* In mice, the \( H19 \) DMR is needed on the maternal allele to protect the \( Igf2 \) DMR1 and 2 from methylation \( (7) \). To assess a putative link of the methylation level at the sixth CTCF site of the \( H19 \) DMR with the methylation level in the \( IGF2 \) DMR2 we analyzed the methylation status of the \( IGF2 \) DMR2 by pyrosequencing. Two regions encompassing 17 CpGs (278 bp, \( IGF2 \) DMR2a) and 9 CpGs (195 bp, \( IGF2 \) DMR2b), separated by 265 bp and located in exons 8 and 9 of \( IGF2 \) (Figure 6) were quantitatively screened for individual-specific differential methylation. A highly specific and reproducible profile was observed for the DMR, irrespective of the sample used (placentas or maternal blood cells). Strong variability of methylation was observed between consecutive CpGs. The average proportion of methylated molecules in the first region \( (IGF2 \) DMR2a) was \( 62.9 \pm 7.3\% \) in lymphocytes (maternal blood cells, MBC), \( 47.5 \pm 4.9\% \) in early gestation placenta (ETP) and \( 41.1 \pm 5.4\% \) in term placenta.

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**Figure 4.** Familial segregation of individuals with various methylation profiles analyzing DNA samples from fresh blood.
The methylation of placenta samples was significantly lower than in adult lymphocytes ($P < 0.0001$). Similarly, the IGF2 DMR2b was hypo-methylated in placenta samples: $35.0 \pm 8.9\%$ in ETP, $34.0 \pm 3.6\%$ in FTP and $57.4 \pm 3.9\%$ in maternal blood cells, respectively (Figure 6). Again the difference between [FTP-ETP] and MBC methylation levels was highly significant ($P < 0.0001$). By contrast, the methylation status of both DMR2a and 2b were never significantly different between early gestation and full term placentas.

The comparative analysis of the H19 and IGF2 DMRs from the same samples revealed that their methylation levels were not correlated in humans.

Relationship between H19 DMR epigenotypes and expression of both H19 and IGF2 genes in human placenta

We then addressed the question whether the epigenetic polymorphism (three profiles) at the sixth CTCF binding site had an effect on the allele-specific expression of either H19 or IGF2 as well as on the quantity of the mRNA of both genes. As H19 and IGF2 are highly expressed in placenta but not in lymphocytes, 34 placental DNA, heterozygous for the SNP rs3741219 (RsaI cleavage site) in the last exon of H19, were selected (Supplementary Table 3). These samples were used to follow H19 allelic expression by RT–PCR followed by conventional Sanger sequencing. A single allele was exclusively expressed in 25 cDNA samples (83.3%) and predominantly expressed in five cDNA samples (16.7%). In this case, 80% of the cDNA molecules originated from one allele and 20% from the second. There was no correlation with the methylation profile of the corresponding genomic DNA samples (among the three samples where H19 was not exclusively expressed from the maternal allele, one was hyper-, one was hypo-, and one was median-methylated). Similarly, samples informative ($n = 10$) for the ApaI polymorphism in exon 9 of IGF2 demonstrated the allele-specific expression of the gene without exception. Therefore, the imprinting status of both genes is maintained whatever the methylation profile of the sixth CTCF binding site.

We measured the overall expression levels of IGF2 and H19 in early gestation and full-term placental villi by quantitative RT–PCR. A very strong positive correlation was found between the IGF2 and H19 mRNA levels in ETP ($R^2 = 0.87$) and a less strong in FTP ($R^2 = 0.36$, Figure 7). Very clearly, both genes are expressed at a much lower level at full-term compared to early gestation placentas (Mann–Whitney test: $P = 0.008$). Finally, the overall level of H19 and IGF2 expression was analyzed with regard to the methylation profile of the H19 DMR, but no significant differences could be found. In conclusion our results dissociate the methylation status of the sixth CTCF site of the H19 DMR from the allele-specific expression of both genes in the placenta.

**DISCUSSION**

This sixth CTCF site has previously been postulated as being the only one of the seven CTCF sites that is used for imprinting control (13). Therefore, the sixth CTCF binding site might be considered as the human functional homologue of the allele-specifically methylated region in the mouse H19/IGF2 ICR despite low sequence conservation between the two species. Our study shows that the sixth CTCF binding site is not consistently allele-specifically methylated in humans. However, the expected median-methylated pattern is found in another region of the DMR, as well as in the H19 promoter. Our observations suggest that the sixth CTCF binding site is at least not always involved in the allele-specific expression of H19 and IGF2 and that either
other CTCF binding sites or an overall allele-specific methylation state of the locus determines imprinted expression. Alternatively, species-specific mechanisms might exist in the onset and/or maintenance of imprinting in different mammals. In addition, we found that the methylation profile for a given individual is determined early in development (before the division of the inner cell mass lineage and the trophoderm lineage) and presumably genetically determined.

DNA methylation is generally addressed by cloning and DNA sequencing of PCR products from bisulphite treated DNA. This has become the standard technique for determining the methylation status of CpG imprinted regions. The major limitation of this technique is that it extrapolates methylation data from a reduced number of independently sequenced DNA molecules (generally 10–20) to a whole tissue. DNA methylation analysis by pyrosequencing has become more and more widespread as it combines important features such as ease-of-use, quantitative accuracy and high-throughput possibilities. For the presented study, the use of pyrosequencing also minimized the risk of technical artifacts. The sequences of the different CTCF sites are very similar, but do differ at some nucleotides. Any unspecific amplification of, e.g. several binding sites, would therefore be seen in the resulting pyrogram and would not pass quality control. Two recent studies demonstrated the presence of microdeletions fusing different CTCF binding sites in the \textit{H19 DMR}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Profiles of IGF2 DMR methylation obtained by pyrosequencing. The degree of methylation (\%) was analyzed for two differentially methylated regions, 17 CpGs in DMR2a and 9 CpGs in DMR2b. Analysis was performed on maternal blood cell DNA samples (A, Blood \( n = 17 \)), on placenta samples at early term of gestation (B, \( n = 6 \)) and at full term (C, \( n = 16 \)).}
\end{figure}
that lead to the hyper-methylation at the sixth CTCF binding site in Beckwith–Wiedemann syndrome patients (32,33). Although we can not exclude microdeletions in other parts of the DMR, we can be sure that the results are not due to a deletion or fusion of CTCF sites as this would, for the above described reasons, be visible in the obtained sequences. As the same methylation profile is found in different tissues from the same individual (blood and placenta), any artifact in the preparation of the DNA from the placenta and the lymphocytes can clearly be ruled out.

When analyzing the four functional CTCF sites in mice in ten different organs by pyrosequencing, we found a unique methylation profile in all organs as well as in the different analyzed mice (J. Tost, unpublished data). In humans, previous work showed that paternal specific methylation at H19 DMR is very well conserved not only in tissues that have parental specific expression of the two genes, but also in tissues such as the choroid plexus where expression is biallelic (34). Our results show that the opposite situation exists, i.e. tissues where the typical imprinted pattern of expression does not directly correlate with specific methylation levels. Indeed, while a major part of the human population exhibited the expected allele-specific methylation pattern, 34 to 65%, of the human population escapes the classically described expected allele-specific methylation pattern, 34% to 65%, while a major part of the human population exhibited the directly correlate with specific methylation levels. Indeed, our results show that the opposite situation exists, i.e. tissues such as the choroid plexus where expression is biallelic (34).

Aberrant methylation in the H19 DMR has previously been reported, mainly in analyses in the context of cancer. For example Cui et al. reported hyper-methylation at the first CTCF site in Wilms tumour with loss of imprinting, but also Wilms tumour samples with normal imprinting of IGF2 did not necessarily show a normal pattern of methylation at the H19 DMR. A recent study on head and neck carcinomas showed aberrant hyper-methylation at the sixth CTCF binding site in the control population (35). While Takai et al. (13) only found allele-specific methylation at the sixth CTCF binding site when analysing a few normal tissues, Sandovici et al. (21) reported on a familial aggregation of ‘aberrant’ methylation in a non-pathological situation. Our results support the findings reported by Sandovici et al. (21) which observed a sporadic or familial abnormal methylation status of H19 DMR as well as stability of this trait over a period of nearly two decades. However, due to the method based on methylation sensitive restriction endonucleases used in that study, they were only able to detect hyper- and median-methylated samples and therefore did not understand the full extent of the epigenetic polymorphism. By their method, a hypo-methylated profile is indistinguishable from the expected median-methylated methylation level as in both cases one allele is retrieved after digestion. Only a complete loss of methylation would result in a different result. This would also explain their higher percentage of median-methylated samples as they would have classed all but the hyper-methylated samples in this category. Their reported observations were also consistent with the possibility that a H19 DMR epigenotype is largely due to genetic factors.

Germline transmitted epimutations has been found in several plants and two metastable transposon-associated epimutations were described in mice (36). The maternally transmitted epigenetic state of the Agouti viable yellow allele determines coat color (37) and a parent-of-origin independent transmission of differential methylation at an internal retrotransposon of the AxinH19 allele leads to a presence or absence of the phenotype, a kinked tail (38). The only previously reported transgenerational inherited human epiallele concerns the mutL homolog 1 (MLH1) where methylated alleles confer an increased risk to develop cancer (39,40). We have shown that the observed epigenetic profiles at the sixth CTCF binding site are non-random and follow a pattern of inheritance that might be determined by a single genetic factor. Further work on familial genotyped DNAs from non-immortalized cell lines will be necessary to map the putative locus or gene responsible for the three methylation profiles and ascertain the proposed model of dominance.

Our findings suggest the absence of a direct connection between H19 monoallelic expression and H19 DMR allele-specific methylation at the sixth CTCF binding site. Allele-specific methylation at this locus might therefore play only a secondary role as an epigenetic mark in humans. Alternatively, it could be a primary mark early in development, which is then lost after the blastocyst stage. A contrasted situation was found in mice with respect to the imprinting centre located in the Kcnq1 domain on distal chromosome 7. In the embryo, imprinting is stably maintained only on genes that have promoter DNA methylation whereas in the placenta, the paternal repression along this domain involves the acquisition of histone H3 methylation without the involvement of promoter DNA methylation (41,42).

The correlation between the total expression levels of IGF2 and H19 suggests a co-regulation by a process acting simultaneously on the two homologous chromosomes and equilibrating the amount of transcript of both genes at specific stages of developing placenta. This could for example be achieved by a limiting amount of transcription factor. This hypothesis is strengthened by a systematic bioinformatic analysis of promoters from the H19/IGF2 locus demonstrating that the transcription factor binding site content of the P3 of IGF2 is highly similar to that of the P1 of H19 (D. Vaiman et al., unpublished data).

In conclusion, we could demonstrate the existence of three distinct non-random individual-specific methylation levels at the sixth CTCF site in the H19 DMR, which are probably set by a so far unidentified genetic factor. An immediate consequence of our study is the need for re-consideration of
methylations of at least the sixth CTCF site as diagnostic markers for cancer or epigenetic ‘diseases’ such as Silver–Russel and Beckwith–Wiedemann syndrome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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