Familial aggregation of abnormal methylation of parental alleles at the \textit{IGF2/H19} and \textit{IGF2R} differentially methylated regions

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Loss of imprinting (LOI) has been observed in many types of human tumors and may be a predisposing event in some colon cancers. LOI is strongly associated with alteration of normal DNA methylation patterns in differentially methylated regions (DMRs) of affected loci but it is not known whether LOI is caused by stochastic, environmental or genetic factors. We have developed a simple, quantitative assay for measurement of allelic methylation ratios based on methylation-sensitive restriction endonuclease digestion of genomic DNA and ‘hot-stop’ PCR. We examined allelic methylation ratios at DMRs within the \textit{IGF2/H19} and \textit{IGF2R}-loci in a panel of 48 three-generation families. We observed familial clustering of individuals with abnormal methylation ratios at the \textit{IGF2/H19} DMR, as well as stability of this trait over a period of nearly two decades, consistent with the possibility that constitutional LOI at this locus is due largely to genetic factors. At the \textit{IGF2R} DMR, we observed more variability in the allelic methylation ratios over time but also observed familial clustering of abnormal methylation ratios. Overall, our observations at \textit{IGF2R} suggest that shared genetic factors are responsible for a major fraction of inter-individual variability in parental origin-dependent epigenetic modifications. However, temporal changes also occur in isolated cases, as well as within multiple individuals in the same family, indicating that environmental factors may also play a role.

INTRODUCTION

Genomic imprinting is a differential epigenetic modification of chromosomes that may result in the parent-of-origin specific transcription of some genes. DNA methylation (reviewed in 1), as well as various histone modifications (methylation, acetylation, phosphorylation and ubiquitination) (reviewed in 2,3), are likely to be important components of the somatically heritable imprint.

The great majority of transcriptionally imprinted genes show allelic differences in DNA methylation at so-called ‘differentially methylated regions’ (DMRs). ‘Loss of imprinting’ (LOI), or relaxation of imprinting (4,5), in which a gene transcribed normally from only one parent’s allele is transcribed from both alleles, has been observed in both pediatric and adult cancers (reviewed in 6), as well as normal tissues (7). There is a strong association between LOI and altered DNA methylation patterns at DMRs.

A number of studies suggest that there is inter-individual variability in allele-specific expression of some imprinted genes. For example, the \textit{IGF2R} gene, although differentially methylated on maternal and paternal alleles, is transcribed from both alleles in the vast majority of the population (8). However, a subset of individuals express predominantly or only the maternal allele (9). The \textit{WT1} gene has been reported to be transcribed from both alleles in the majority of placenta but placenta from some individuals express only the maternal allele (10). Further evidence for inter-individual variability in
imprinting comes from the observation of significant biallelic expression of the insulin-like growth factor II gene (IGF2) in about 10% of normal individuals (11). LOI at IGF2 also occurs in tumor tissue of 31% (12) to 44% (7) of sporadic colon cancer cases. Surprisingly, LOI is not thought to be a tumor-specific event in these cases, but is likely to be constitutional because both alleles of IGF2 are expressed in the normal colonic mucosa of 90% of these patients and also in the lymphocytes of one-third of these same individuals (7).

In the present study, we have attempted to determine whether stochastic, environmental or genetic factors are responsible for the potentially large population of ‘normal’ individuals (11) who have altered genome imprints. We have developed an assay based on methylation-sensitive restriction endonuclease digestion of genomic DNA and ‘hot-stop’ PCR. We used this assay to examine inter-individual variability in methylation at CpG sites within the IGF2/H19 DMR and the IGF2R DMR among a population of 680 individuals from 48 three-generation families. We also examined intra-individual variability in this phenotype by comparing peripheral blood DNA samples taken nearly two decades apart from a subpopulation of the same individuals as a way of detecting the influence of environmental or stochastic factors on parental origin-dependent epigenetic modifications over this time period.

RESULTS

Familial aggregation of individuals with abnormal methylation ratio at the IGF2/H19-DMR

Because we wished to screen a population of moderate size (680 individuals) for LOI using archival material, we developed an assay based on the correlation between parent of origin-specific transcription and methylation of CpG sites within DMRs (Fig. 1). Briefly, CpG sites within DMRs on the paternal allele at the IGF2/H19 locus are normally methylated, while those on the maternal allele are normally unmethylated (13). There are several DMRs at this locus but the most consistent observations indicating a role in the control of transcription of the IGF2 and H19 genes involve a CpG island located in a 5 kb region centromeric to the H19 gene (14). This is also the region that contains seven different binding sites for the CTCF protein (15) and the methylation status of the sixth binding site was found to be most consistently associated with the transcriptional status of both IGF2 and H19 (16). We used a single nucleotide polymorphism (a C/T polymorphism within a CfoI site, Fig. 1A) and pedigree analysis to identify maternal and paternal alleles of heterozygous individuals and a methylation-sensitive restriction endonuclease (MluI and, in selected cases, MaelI) to determine the methylation status of specific CpG sites within the DMR. If all paternal alleles are methylated and all maternal alleles are unmethylated at these sites in a sample of genomic DNA, then all maternal alleles should be cleaved by MluI while all paternal alleles will remain uncleaved. Amplification of the region by PCR using primers that flank the MluI site should amplify only paternal alleles (identified by post-PCR cleavage with CfoI). Amplification of maternal alleles indicates resistance to cleavage by MluI. This may occur as a result of methylation of the CpG site within the MluI recognition sequence (the principle upon which the assay is based), mutation of the MluI site or technical artifact. The latter two possibilities may be distinguished from the first by DNA sequencing, assay reproducibility and use of additional methylation-sensitive restriction endonucleases.

We identified 318 individuals (46.8%) who were heterozygous at the CfoI polymorphism (Fig. 1A) within the IGF2/H19 DMR and were able to determine unequivocally the parental origins of the alleles in 163 cases. Because previous studies have indicated that LOI is a quantitative trait (7), i.e. expression of the maternal IGF2 allele is variable and may not occur to the same extent in all individuals identified as having LOI, we calculated the ratio between the DNA methylation levels on maternal and paternal (M/P) alleles as an indicator of imprinting status (see materials and methods). A ratio of zero corresponds to exclusive methylation of the MluI site on the paternal allele, while a ratio of one signifies methylation of this site on an equal number of maternal and paternal alleles.

The distribution of individual M/P methylation ratios (Fig. 2A) shows that the great majority of individuals have a methylated paternal allele, with no detectable methylation of the maternal allele. (Because none of the 163 individuals for whom we could determine parental origin of alleles by pedigree analysis had a maternal allele that was more methylated than the paternal allele, we have assumed that the less methylated allele is maternal in the remainder of cases compiled in

Figure 1. (A) The IGF2/H19 DMR is located between the H19 gene and IGF2 and is normally methylated only on the paternal allele (13). A C/T polymorphism located at the sixth binding site for CTCF is recognized by CfoI. MaelI and MluI are methylation-sensitive restriction endonucleases. F3* is the primer that is 5’ end labeled for use in the ‘hot-stop’ PCR cycle. (B) The IGF2R DMR is located in the second intron and is normally methylated on the maternal allele. The C/T polymorphism is recognized by MspI. NotI and XhoI are methyl-sensitive restriction endonucleases. F4* is the primer that is 5’ end labeled in the ‘hot-stop’ PCR cycle.
Fig. 2A.) However, 21 individuals (6.6%) have M/P ratios greater than 0.1. We obtained similar ratios after digestion of genomic DNA with either MluI or MaeII, indicating that methylation of the maternal allele in these individuals is not restricted to the MluI site. We excluded the possibility that a subpopulation of lymphocytes from these individuals carried a mutation at the MluI or MaeII sites by DNA sequencing of the amplified maternal allele (Fig. 2C). An additional 28 individuals exhibit lower, but reproducible, levels of methylation on the maternal allele (M/P ratios between 0.05 and 0.1). While some of these individuals occur as isolated cases in some families, a significant proportion are found within families in which other members also have methylation of maternal alleles. Nine out of the 21 individuals (42.8%) with M/P ratios over 0.1 are found within two families (Fig. 3). In one of these families, six of 10 informative individuals have M/P ratios over 0.1 (for H0: six 'abnormal individuals' occur among 10 informative individuals by chance, given 6.6% population frequency, \( \chi^2 = 20.2, P < 0.001 \)). When a lower level of maternal allele methylation is considered (an M/P ratio over 0.05), the familial aggregation of individuals is even more pronounced, with 34 out of 49 (69.4%) individuals having at least one relative with this phenotype. Moreover, not every individual in these families is informative, and the true number of cases in which only a single family member is affected may be overestimated. We also note that 10 of the 15 individuals who have no relatives with methylation of the maternal allele are in the first generation of their pedigrees. In these cases, no sibling, and only a single offspring (either the mother or the father of the third generation), is available for analysis.

Familial aggregation of individuals with altered differential methylation at the IGF2R DMR

Although the human IGF2R gene is normally expressed from both alleles, differential methylation of maternal and paternal alleles is maintained in the human, as it is in the mouse (17), and some humans do appear to have transcriptional imprinting of IGF2R (9). We designed an assay (Fig. 1B) to examine relative levels of methylation at CpG sites on maternal and paternal alleles within the DMR of IGF2R (located in the second intron) using the same principles upon which the IGF2/H19 assay was based. We used a C/T polymorphism within anMspI site to distinguish heterozygous individuals, pedigree
analysis to determine maternal and paternal alleles and cleavage of genomic DNA by a methylation-sensitive restriction endonuclease followed by PCR using primers that flank the cleavage site to distinguish methylation of CpG sites on maternal and paternal alleles.

We observed 165 individuals (24.3%) who were informative for the MspI polymorphism found within the IGF2R DMR and we identified maternal and paternal alleles for 112 individuals by pedigree analysis. Although maternal and paternal alleles were distinguishable in almost all individuals on the basis of expected preferential methylation of the maternal allele, the distribution of paternal/maternal (P/M) methylation ratios at IGF2R DMR shows that, unlike the IGF2/H19 DMR, most individuals have a measurable level of methylation at the CpG within the XhoI cleavage site on the paternal allele (P/M > 0.1, Fig. 4). P/M ratios were similar for both XhoI and NotI restriction endonuclease digestion of genomic DNA (correlation coefficient for paired measurements  = 0.715), indicating, again, that the methylation of CpG sites within the paternal DMR is not restricted to a single site.

Because the distribution in Figure 4A is two-tailed (analogous to the distribution of P/M methylation ratios at IGF2/H19 DMR), we examined the distribution of individuals from each tail among the 48 families. Because the vast majority of individuals have some methylation of the paternal allele, this modification seems unlikely to inhibit transcription of the normally transcribed paternal allele. However, it is possible that individuals who have little or no methylation of the paternal allele are those individuals in which the paternal allele is predominately or exclusively transcribed, as reported by Xu et al. (9). We observed 19 individuals (11.5%) with P/M ratios under 0.1. Fourteen of these 19 individuals are distributed among five families, with five individuals being found in a single family (Fig. 5) for H0. Five ‘abnormal individuals’ occur among seven informative by chance, given 11.5% population frequency,  with Yates correction = 10.5,  < 0.01).

We also identified five individuals in which the CpG site being examined was methylated on approximately equal numbers of maternal and paternal alleles (P/M > 0.8). Two of these individuals are siblings within the same family (pedigree not shown).

Familial aggregation of individuals with abnormal methylation of IGF2/H19 and IGF2R DMRs; genetics or environment?

The familial clustering of individuals with abnormal allelic methylation ratios may be explained by the action of shared environmental factors or shared genetic factors. We have attempted to distinguish between these possibilities by genetic linkage analysis in those families in which multiple members are affected and by examining the temporal stability of the allelic methylation ratio over a period of nearly two decades.

Genetic linkage analysis of the abnormal methylation phenotype. The methylation of CpG sites within DMRs is one component of the overall chromatin structure of imprinted regions of chromosomes. Abnormal methylation of normally unmethylated CpG sites could result from some aspect of the structure of the particular chromosome in which it is found (an effect in cis) or result from the action of modifying genetic factors that do not reside in the vicinity of the DMR (an effect in trans). We may gain supporting evidence for the involvement of cis-acting factors by determining whether family members who share abnormal allelic methylation ratios also share the same chromosome haplotype in the vicinity of the DMR.

The assays used in this study identify abnormal methylation of the maternal IGF2/H19 DMR and abnormal methylation of the paternal IGF2R DMR. Therefore, we determined which maternal chromosome 11p15.5 was inherited by individuals in families in which more than one individual had an abnormal methylation ratio at the IGF2/H19 DMR and which paternal chromosome 6q26 was inherited by individuals in families in which more than one individual had an abnormal methylation ratio at the IGF2R DMR (see Materials and Methods).

We identified only one family (data not shown) in which all individuals with abnormal IGF2/H19 DMR methylation ratios shared the same maternal chromosome 11p15.5 haplotype. Cis-acting genetic factors may be involved in the abnormal methylation of maternal IGF2/H19 alleles in this family. However, some ‘normal’ individuals in this family inherited the same maternal chromosome 11p15.5 as did those individuals with abnormal methylation ratio so that there were no families...
We have not attempted to perform linkage analysis of candidate trans-acting genetic factors because the number of potential candidate genes is large and the likelihood of detecting spurious associations within any one of the families is high. However, the chromosome haplotype analyses we have performed indicate that if familial clustering of the abnormally methylated ratio trait is due to genetic factors, these factors must often act in trans.

Temporal stability of allelic methylation ratio. In the absence of a hypothesis on the specific identity of an environmental agent that might influence abnormal methylation of DMRs (and associated hypotheses on how and when such an agent might act), we sought to obtain evidence on whether any environmental factor might be involved in the generation of abnormal methylation ratios by comparing the allelic methylation ratios of the same individual at two different times. This approach is based on the premise that, if an environmental factor causes an alteration of the allelic methylation ratio, then one predicts a change in this phenotype between the time of establishment of the original, ‘normal’ ratio and the time at which the environmental factor exerts its effect.

To test the stability of the allelic methylation ratio over time, two sets of peripheral blood DNA samples taken from the same individuals (the first taken between 1982 and 1985 and the second in 2001) were analyzed. Both ‘old’ and ‘new’ samples were available for 133 individuals informative for the CfoI polymorphism at IGF2/H19 DMR and for 75 individuals informative for the MspI polymorphism at IGF2R DMR.

At the IGF2R DMR, only three individuals sampled at both times (individual nos 12, 100 and 125 in Fig. 6A) had M/P allelic methylation ratios of greater than 0.1 and these individuals had a ratio of greater than 0.1 at both sampling times. In fact, there was no case in which the methylation ratio at the IGF2R DMR changed substantially between the two sampling times (Fig. 6A). Because the level of methylation on the maternal allele at the IGF2R DMR locus was very close to zero for the majority of individuals in both ‘old’ and ‘new’ DNA samples, we did not attempt to assign statistical significance to small differences between individual paired results. For the data set, as a whole, a paired t-test indicates that there is no significant difference between the two time points (P = 0.1051).

At the IGF2R DMR, we observed eight cases in which a change in methylation ratio (only changes greater than three times the level of assay reproducibility [±0.067, see Materials and Methods] were considered as significant for this purpose) moved an individual from a normal to an abnormal category or vice versa. One individual (individual no. 43 in Fig. 6B) had a P/M methylation ratio of 0.2 in the first sample and this ratio was nearly one in the second sample. Two unrelated individuals (nos 32 and 38 in Fig. 6B) had P/M methylation ratios of less than 0.1 in the first sample and ratios of 0.26 and 0.37, respectively, in the second sample. Five individuals (individual nos 52, 54, 55, 57, and 61 in Fig. 6B) exhibited potentially significant changes in the opposite direction, i.e. attaining a
P/M methylation ratio of less than 0.1 in the second sample. Three of these individuals are from the same family (a mother and two offspring, pedigree not shown), suggesting a common environmental effect on this phenotype or a genetic predisposition to the observed change.

Overall, we observed very similar results for the two sets of measurements (correlation coefficient for paired measurements $r = 0.7821$) and the slope of the linear regression line (P/M methylation ratio at time 1 versus P/M methylation ratio at time 2) is $0.848 \pm 0.079$, near the expected value of 1.0 if no change in methylation ratio occurred in the interval between collection of the two samples. These data indicate that, although changes in allelic methylation ratio can be observed, this phenotype is temporally stable in the majority of the population.

**Bisulfite sequencing analysis of individuals with abnormal pattern of DNA methylation at IGF2/H19-DMR**

DNA sequence analysis of bisulfite treated DNA (18,19) has become the standard technique for determination of the methylation status of CpG sites in imprinted regions. The technique has the advantage that all CpG sites are amenable to analysis but has the disadvantage that it is labor-intensive and relatively expensive to use as a screening tool. In addition, the number of individual DNA molecules that must be sequenced to demonstrate a significant difference between individuals with a M/P allelic ratio of zero and individuals with a M/P allelic ratio of 0.1, for example, is much greater than the 10–20 molecules normally assayed in this procedure. These considerations led us to attempt this method only in those families in which we observed abnormal methylation ratios using the methylation-sensitive restriction endonuclease-based method.

As expected, we observed DNA molecules with an abnormal 'mixed' methylation pattern for many of these individuals (Fig. 7). However, we also analyzed a series of three single nucleotide polymorphisms present in the sequenced region of the individual shown in Fig. 7, as well as additional abnormal and informative individuals, in order to confirm the parental origin of the abnormal alleles. We found that the majority of these mixed methylation pattern molecules result from maternal/paternal heteroduplex products that are created during the PCR step of the assay and then repaired, randomly, by the bacterial mismatch repair system during the cloning procedure. We confirmed the potential for artifactual origin of molecules with a mixed methylation pattern by mixing the DNAs of individuals who are homozygous for different SNP haplotypes and detecting individual molecules with both mixed SNP haplotypes and mixed maternal/paternal methylation patterns. Because we were unable to establish conditions in which such heteroduplex molecules could be completely eliminated [including techniques designed to maintain favorable primer:template ratios (20) and techniques for eliminating heteroduplexes using enzymatic digestion with T7 endonuclease I (21); data not shown], we did not pursue this method of analysis further.

**DISCUSSION**

Characterization of variability in imprinting phenotype using allele-specific methylation assays

We have examined the relative level of methylation of CpG sites on maternal and paternal alleles within differentially methylated regions of the IGF2/H19 and IGF2R loci as a surrogate measure of imprinting. The distributions of these quantitative measures among informative individuals indicates...
that approximately 6.5% of the population has a significant fraction (>10%) of peripheral blood lymphocytes that have methylation of CpG sites on the maternal IGF2/H19 allele. We note that this estimate is similar to the fraction of the population designated as having LOI (10%) after measuring transcription of maternal and paternal IGF2 alleles (11).

The distribution of P/M allelic methylation at IGF2R is more variable than that at IGF2/H19. Approximately 11% of the population may be described as having abnormally low levels of methylation on the paternal allele (P/M allele ratios of <0.1) while a smaller fraction of individuals (about 3%) have P/M allelic methylation ratios greater than 0.8. Strong familial clustering of individuals from the tails of both distributions is observed (Figs 3 and 5), indicating that shared genetic factors or shared environmental factors are responsible for the majority of abnormal allelic methylation ratios (Table 1).

We attempted to obtain evidence that allelic methylation ratios may be affected by environmental factors by examining samples taken from the same individuals nearly 20 years apart. We did not detect significant changes at the IGF2/H19 DMR in any of the 133 individuals examined. Although we cannot eliminate the possibility that shared environmental factors may have acted before the first peripheral blood lymphocyte sample was taken, we suggest that familial clustering of abnormal methylation ratios at the IGF2/H19 DMR is most likely due to shared genetic factors. At the IGF2R DMR, significant changes in methylation ratio did occur in eight of 75 informative individuals. Three of these ‘changed’ individuals are from the same family, indicating that this acquired phenotype can also cluster in families and such familial clustering may be due to shared environmental factors or a genetic predisposition to temporal or environmentally induced changes.

We note that the restriction endonuclease/PCR-based assay used in our experiments provides a ratio of the number of maternal/paternal alleles at which a particular CpG site is methylated rather than the absolute number of DNA molecules on which a CpG site is methylated/unmethylated. For example, a sample in which neither the maternal nor the paternal IGF2/H19 DMR was methylated in 90% of cells but in which the maternal IGF2/H19 DMR was methylated in 10% of the remaining 10% of cells would give the same ratio as a sample in which the paternal DMR was methylated in all cells and the maternal DMR was methylated in 10% of all cells. These two possibilities may be distinguished by bisulfite sequencing because the vast majority of DNA molecules, be they paternal or maternal, will have the unmethylated pattern in the first case. We did not encounter such a situation (or the reverse) in any of the 20 ‘abnormal’ or four ‘normal’ samples that we examined by bisulfite sequencing. Nearly equal numbers of molecules with methylated and unmethylated patterns were found (disregarding molecules arising from repair of heteroduplexes). Corroborating evidence in favor of the second possibility was obtained by real-time PCR (see Materials and Methods) on several additional individuals, comparing methylation sensitive restriction endonuclease cleaved DNA with uncleaved DNA (data not shown).

Bisulfite sequencing assay versus methyl-sensitive restriction endonuclease assay

Bisulfite sequencing has become the standard method for single-molecule and single-base resolution analysis of DNA methylation (18,19). The main advantage of this method is the ability to examine the methylation of many CpG sites on a single DNA molecule. However, this method has some limitations (reviewed in 22,23), especially in situations in which inter-cellular or inter-individual variability itself is of interest. The most obvious disadvantage is the practical limitation on the number of chromosomes that can be analyzed. Sequencing of 20 DNA molecules from each individual, for example, yields an estimate of epigenetic variability based on a sample of only 10 cells.

An additional problem is the generation of heteroduplex artifacts during the amplification of mixed templates using universal primers. After bisulfite treatment a high diversity of template molecules will occur as the result of different genomic DNA methylation patterns between the parental chromosomes as well as differences in methylation of specific sites between cells. The presence of native single-nucleotide polymorphisms (SNPs) and any errors induced by the DNA polymerase will increase further the potential for heteroduplexes. When such a heteroduplex molecule is cloned, the host’s mismatch repair system can convert it into a single hybrid sequence (reviewed in 24). As the repair enzymes cannot identify a parent strand, either strand is chosen as a template for the synthesis of the ‘repaired’ strand. The repaired sequence will be a ‘mosaic’, i.e. composed of portions of the two parent heterologous
sequences. Several protocols to reduce the incidence of mosaic molecules have been described (20,21). Such protocols rely on maintaining a primer:template ratio that favors synthesis of new molecules over hybridization of ‘old’ strands, or post-synthesis enzymatic cleavage of heteroduplexes. However, it is difficult to eliminate completely the cloning of heteroduplex molecules and the utility of such data depends on the significance of these molecules to the interpretation of results. A mosaic pattern may be misinterpreted as abnormal hypermethylation or hypomethylation of some portion of a parental chromosome (Fig. 7). In our case, we could not tolerate the generation of heteroduplex artifacts because they occurred at levels greater than or equal to the fraction of endogenous molecules targeted for assay (those paternal IGF/H19 alleles and maternal IGF2R alleles that become methylated at a particular site, see Figs 2 and 4).

The main limitation of methyl-sensitive restriction endonuclease assays is the ability to analyze only one or a few CpG dinucleotides in a region. On the other hand, this method is likely to provide a better quantitative estimate of the ratio of methylation between the two parental alleles because a much larger number of chromosomes are analyzed. In a sample of 100 ng DNA, the methylation status of the selected CpG site is assayed on over 15,000 homologous pairs of a particular chromosome, compared with the larger number of CpG sites (but generally less than 50) that are analyzed on the five to 10 homologous chromosome pairs usually assessed by bisulfite-sequencing. The incorporation of the ‘hot-stop’ PCR technique circumvents the difficulty of analysis of heteroduplex molecules because only molecules synthesized during the last amplification cycle are radio-labeled (25).

LOI, parent of origin dependent methylation and cancer

The presence of LOI at IGF2 in both tumor cells and normal tissues raises the possibility that constitutional LOI represents a risk factor for cancer (7). Although use of the term ‘LOI’ implies a qualitative difference between individuals in whom there is parental-origin-dependent transcription of only one allele and individuals in whom there is transcription of both alleles, such differences are generally quantitative (7). Individuals characterized as having LOI exhibit a relaxation of allele-specific methylation with partial methylation on the normally unmethylated allele. This methylation ‘defect’ at IGF2/H19 is similar to what we found in the majority of individuals at the IGF2R DMR and one might argue that this circumstance may explain the normal biallelic expression of this gene. However, we also identified a number of individuals with nearly exclusive methylation of the maternal IGF2R allele. In these cases, one might hypothesize maternal allele-specific transcription, as described by Xu et al. (9). In this regard, the imprinting status of IGF2R is a potentially interesting variable in cancer susceptibility. A number of reports support its activity as a tumor suppressor gene in some types of tissues, including liver (26), breast (27) and kidney (28) and IGF2R is expressed from only the maternal allele in 50% of Wilms’ tumors (28), consistent with the hypothesis that the transcriptional imprinting of this gene is associated with tumorigenesis.

Assays of allele-specific methylation exploit the fact that differential DNA methylation of maternal and paternal alleles is one of the hallmarks of genome imprinting (reviewed in 29). Many imprinted loci show allele-specific DNA methylation, and in the absence of the maintenance methyltransferase, or in the presence of methyltransferase inhibitors, the imprinting of several genes is disrupted (30,31). A major advantage of allele-specific methylation assays is that the methylation phenotype of an individual can be scored in a DNA sample. A similar surrogate measure of the transcriptional status of the androgen receptor gene has been used extensively as an indicator of X-chromosome inactivation in the cells of human females (32,33). A disadvantage of surrogate measures of imprinting is the perception that methylation differences, in and of themselves, do not reflect ‘real’ imprinting unless they are also accompanied by parental allele-specific transcription (29). However, such differences must be at the root of the imprinting process, regardless of whether or not the process results in transcriptional differences between maternal and paternal alleles (34). The identification of inter-individual variability in the establishment or maintenance of epigenetic marks, and determining the causes of this variability, is an important step in determining the role of imprinting in evolution, development and cancer.

MATERIALS AND METHODS

Subjects

DNA samples obtained from unfractonated nucleated peripheral blood cells (not lymphoblast cell lines) from the Salt
Lake City collection of CEPH/Utah pedigrees between 1982 and 1984 (680 individuals from 48 three-generation families) were studied. For 25 of these families (288 individuals) a second set of DNA samples was obtained during 2001. All subjects gave informed consent under University of Utah I.R.B. approved protocol number 6090-96.

Epidermal growth factor receptor (IGF2R) gene polymorphism at intron 35 of IGF2R (recognized by MspI enzyme), and the C/T polymorphism in intron 2 of IGF2/H19 (recognized by CfoI) (16) and a C/T polymorphism (Fig. 1B). Parental origin of alleles of heterozygous individuals was determined by pedigree analysis.

A 100 ng sample of genomic DNA from informative individuals was digested overnight at 37°C with an excess of a methyl-sensitive restriction endonuclease: MluI or MaelI for the IGF2/H19-DMR and Xhol or NotI for the IGF2R-DMR respectively. In each experiment we used as control individuals who were homozygous for C alleles, homozygous for T alleles, as well as a mix of 50 ng genomic DNA from an individual homozygous for the C allele and 50 ng genomic DNA from an individual homozygous for the T allele. Digested DNA was then ethanol precipitated and amplified in a ‘hot-stop’ PCR assay using the following primers: F3, 5'-GAGATGGGAGGAGATAGAGG-3' and R3, 5'-GTCACTCAGTAAATGCTGG-3' for the IGF2/H19-DMR, and F4, 5'-GACAGACCCAGATTACGCTG-3' and R4, 5'-GGACATGCACCTTCCCCGTTG-3' for IGF2R-DMR. Briefly, ‘hot-stop’ PCR involves the addition, after 30 cycles, of a primer that has been radiolabeled at the 5’ position and allowing synthesis to take place for a final cycle (25). The labeled primer for the last cycle was one of the same primers used in the first 30 cycles. However, competition between labeled and unlabeled primer did not appear to be a significant problem because the concentration of the unlabeled primer was substantially reduced during the first 30 cycles and the labeled primer was added at the original concentration of the unlabeled primer (0.4 μM).

PCR products were digested with the enzyme used for identification of the parental origin of the alleles, the fragments were separated on 5% polyacrylamide gels and the intensity of the bands (alleles) was quantified using a Fuji BAS 2000 phosphorimager (33). In the case of controls, no uncleaved C alleles were detected in any C/C homozygous individuals (indicating that the CfoI and MspI enzymes cleaved the PCR products with >99% efficiency) and the mixture of C homozygote/T homozygote resulted in a 1:1 ratio of C/T intensity (data not shown).

All the assays were performed three times, and the average values were used. At the IGF2R-DMR, all samples were assayed using both Xhol and NotI, while at the IGF2/H19-DMR, MaelI was used only for those samples with abnormal patterns of methylation after MluI digestion and for a limited number of samples with a normal pattern of methylation.

Quantification of the methylation level using real-time PCR

Samples of 100 ng of genomic DNA were digested under the same conditions as mentioned above using MluI, Xhol or NotI restriction endonucleases. As controls, samples of 100 ng genomic DNA were digested using Dral which cleaves outside the region of interest. For both the IGF2/H19-DMR and IGF2R-DMR we selected representative samples with normal and abnormal methylation pattern in the previous method. After ethanol precipitation, PCR reactions were set up using 10 ng of DNA as template in the LightCycler DNA Master SY BR Green I kit (Roche), according to the manufacturer’s instructions. The efficiency of the methyl-sensitive restriction enzymes was quantified as the ratio of fluorescence between the PCR products from DNA samples digested with MluI, Xhol or NotI enzymes and PCR products from the same samples digested with Dral restriction enzyme. The results were similar using the two methods and suggest that abnormal ratios are the result of increased methylation of the normally unmethylated alleles (data not shown).

Bisulfite-sequencing analysis at IGF2/H19 DM R

A 2.5 μg aliquot of genomic DNA was denatured at 37°C for 20 min using freshly prepared 3 M sodium hydroxide (final concentration of 0.3 M). Denatured DNA was subsequently incubated at 55°C with monosulfite solution (2 M sodium metabisulfite and 1 mM hydroquinone) in the dark, overnight (19). After purification with the QIAquick PCR purification kit (Qiagen), the converted DNA was used as a template for a nested PCR. The first set of primers was F1, 5'-GTGTTTTGATTTA-TTTTAGGTGATTG-3' and R1, 5'-CACAACACCACAAATACTCTAACCCAAAAA-3' and the second was F2, 5'-GATTAAATAGGAGTTGTTGTTTATTT-3' and R2, 5'-CTAACACCTAAAAACTAAATTCAC-3'. The PCR products were separated in 1.5% agarose gels, purified using the QIAEX II gel extraction kit (Qiagen) and subsequently subcloned into a TA Cloning vector (Invitrogen) according to the manufacturer's instructions. The DNA from 15-20 individual clones was sequenced for each individual.

Haplotype analysis

In order to analyze the concordance between a shared haplotype and familial aggregation of individuals with abnormal methylation ratios, we determined the genotypes of these individuals for several SNPs adjacent to the IGF/H19 and IGF2R loci. For chromosome 11 we analyzed seven polymorphisms, in the following physical order: D11S2071 (0.29 M from the telomere), the HRAS1 minisatellite, the A/C polymorphism at exon 5 of the H19 gene (recognized by Rsal), the A/G polymorphism at exon 5 of H19 gene (recognized by Alul), the C/T polymorphism at IGF2/H19-DMR (recognized by CfoI), the A/G polymorphism at exon 9 of IGF2 gene (recognized by Apal enzyme), and the VNTR minisatellite at the 5’ flanking region of insulin gene (2.27 M from the telomere). For chromosome 6 we analyzed the C/T polymorphism in intron 2 of IGF2R (recognized by MspI) and the C/T polymorphism at intron 35 of IGF2R gene (recognized by
REFERENCES