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METHODS

Epigenetic Detection of Human Chromosome 14 Uniparental Disomy

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The recent demonstration of genomic imprinting of *DLK1* and *MEG3* on human chromosome 14q32 indicates that these genes might contribute to the discordant phenotypes associated with uniparental disomy (UPD) of chromosome 14. Regulation of imprinted expression of *DLK1* and *MEG3* involves a differentially methylated region (DMR) that encompasses the *MEG3* promoter. We exploited the normal differential methylation of the *DLK1/MEG3* region to develop a rapid diagnostic PCR assay based upon an individual's epigenetic profile. We used methylation-specific multiplex PCR in a retrospective analysis to amplify divergent lengths of the methylated and unmethylated *MEG3* DMR in a single reaction and accurately identified normal, maternal UPD14, and paternal UPD14 in bisulfite converted DNA samples. This approach, which is based solely on differential epigenetic profiles, may be generally applicable for rapidly and economically screening for other imprinting defects associated with uniparental disomy, determining loss of heterozygosity of imprinted tumor suppressor genes, and identifying gene-specific hypermethylation events associated with neoplastic progression. Hum Mutat 22:92–97, 2003. © 2003 Wiley-Liss, Inc.

KEY WORDS: uniparental disomy; monochromosomal hybrid; methylation; methylation-specific PCR; DLK1; MEG3; GTL2; imprinted gene; bisulfite sequencing

DATABASES:
DLK1 – OMIM 176290; GDB: 9958854, GenBank: NM_003836;
MEG3 – OMIM 605636; GDB: 11500849; GenBank: AB032607, AF052114, AK057522, AK055725, AF090934, AF119863, BC036882

INTRODUCTION

Uniparental disomy (UPD) of chromosome 14 causes multiple congenital anomalies and has been most commonly identified in individuals having two maternally derived copies [upd(14)mat] [Sutton and Shaffer, 2000; Kurosawa et al., 2002]. These individuals have certain phenotypic characteristics, including intrauterine growth retardation, short stature, scoliosis, hypotonia, obesity, distinctive facial appearance, mental delay, developmental delay, and precocious puberty. Paternal UPD14 [upd(14)pat] is less common and affected persons have a more severe phenotype as compared to upd(14)mat, including marked developmental delay, growth retardation, hirsute forehead, short palpebral fissures/blepharophimosis, small/abnormal ears, protruding/long philtrum, depressed nasal bridge, short neck, small thorax, abdominal muscle hypoplasia, joint contractures, and mental retardation [Sutton and Shaffer, 2000; Kamnasaran, 2001; Eggermann et al., 2002].

The phenotypes associated with maternal and paternal UPD14 have implied the presence of genes subject to genomic imprinting on chromosome 14. Such genes are characterized by expression from a single parental allele, unlike the vast majority of genes throughout the genome that are biallelically expressed. The monoallelic expression of imprinted genes suggests that gene copy number is an important factor in regulating gene function. Deleterious phenotypes linked to maternal and paternal UPD14 may therefore result from either the complete loss of expression or overexpression of imprinted genes on this chromosome. Consistent with this idea, mice having UPD for distal chromosome 12, syntenic to human 14q32, exhibit reciprocal phenotypes. Paternal duplication of this region leads to promotion of growth

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and late embryonal or neonatal lethality while maternal duplication is characterized by growth retardation and late embryonal lethality (see *MEG3*; MIM# 605636; also see Imprinting Maps of the Mouse, Mammalian Genetic Unit, Harwell, UK www.mgu.har.mrc.ac.uk/imprinting/imprin-viewmaps.html).

We have recently established the presence of two imprinted genes on human chromosome 14 [Wylie et al., 2000]. *DLK1* (delta, drosophila, homolog-like 1; MIM# 176290) and *MEG3* (maternally expressed gene 3; also referred to as *GTL2*, for gene trap locus 2; MIM# 605636) are paternally and maternally expressed genes, respectively. They are located approximately 90 kb apart on human 14q32, a region frequently affected in reported human UPD14 cases [Sutton and Shaffer, 2000; Coveler et al., 2002]. DLK1 encodes a 45–60 Kd protein member of the delta-notch family of proteins [Laborda et al., 1993; Smas and Sul, 1993] involved in cellular signaling and differentiation [Laborda, 2000], and may function in the mitogen activated protein kinase (MAPK) pathway [Ruiz-Hidalgo et al., 2002]. Evidence of an essential role for DLK1 in the etiology of upd(14)mat syndrome comes from a report of *Dlk1* null mice that recapitulate phenotypes associated with upd(14)mat and indicate that mouse Dlk1 is involved in early embryonic development, postnatal growth, and fat deposition [Moon et al., 2002]. The function of *MEG3* is currently not understood. *MEG3* is postulated to produce a non-coding RNA transcript due to lack of an extended open reading frame within the multiple alternatively spliced mRNAs [Schuster-Gossler et al., 1998].

The identification of paternally and maternally expressed genes on chromosome 14 along with the clinical description of UPD14 and the identification of cytogenetic subgroups with upd risk association [Papenhausen et al., 1999; Berend et al., 2000; Robinson, 2000; McGowan et al., 2002] prompted us to develop a direct diagnostic tool for detection of UPD14 based strictly on differences in the methylation profile of individuals with UPD14 vs. those with biparental chromosome 14 inheritance.

MATERIALS AND METHODS

Bisulfite DNA Modification

Sodium bisulfite modification of DNA was performed based on the method described by Grunau et al. [2001]. Briefly, 1 µg of genomic DNA was denatured with 3M NaOH for 20 min at 42°C followed by deamination in saturated sodium bisulfite/10mM hydroquinone (Sigma; St. Louis, MO) solution, pH 5.0 for 4 hr at 55°C. The DNA was desalted using the Wizard DNA Clean-up System (Promega; Madison, WI), then desulfonated in 3M NaOH (20 min at 37°C) and ethanol precipitated. The samples were resuspended in 25 µl Tris-Cl, pH 8.0 and stored at 4°C.

Methylation-Specific PCR

Bisulfite-treated genomic DNA was subjected to an optimized methylation-specific PCR protocol in 25 µl reactions using ~5 ng template (~50 ng of non-bisulfite treated DNA where applicable), 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM each M primer (MF: GTT AGT AAT CGG GTT TGT CGG C; and MR: AAT CAT AAC TCC GAA CAC CCG CG) and/or 0.8 µM each U primer (UF: GAG GAT GGT TAG TTA TTG GGG T; and UR: CCA CCA TAA CCA ACA CCC TAT AAT CAC A) [Kubota et al., 1997; Zeschnigk et al., 1997a]. The use of desalted primers (without further purification) gave unexpected results such that a weak band corresponding to the opposite parental region was sometimes present in addition to the correct band. In contrast, primers purified by polyacrylamide gel electrophoresis (Sigma-Genosys; The Woodlands, TX) accurately and reproducibly amplified the anticipated regions and were used for all experiments shown herein. Touchdown PCR was used as follows: 94°C for 3 min followed by five cycles of 94°C for 30 sec, 70°C for 30 sec, 72°C for 30 sec; five cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec; final 5 min extension at 72°C. The products were separated on 3% high resolution agarose gels and visualized by ethidium bromide staining.

Bisulfite Sequencing

Bisulfite-treated genomic DNA was amplified by PCR (3 min at 94°C, then 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec with a 5 min extension at 72°C) with primers specific for the bisulfite converted *MEG3* DMR (SQF1: GAT TTT TTT TAT ATA TTG TGT TTG and SQR: CTC ATT TCT CTA AAA ATA ATT AAC C) in 25 µl reactions with 5–25 ng template, 3 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 µM each primer. The 235-bp PCR products were resolved on an agarose gel, purified using Sigma GenElute spin columns (Sigma; St. Louis, MO), and cycle sequenced (Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit; Amersham Biosciences, Piscataway, NJ) using a nested primer (SQF2: GTG TTT GAA TTT ATT TTG TTT GG): 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min for a total of 35 cycles.

RESULTS

In our initial analysis of the epigenetic profile of the *DLK1/MEG3* region we demonstrated that the region encompassing the *MEG3* promoter is differentially methylated, although we had not determined the parental origin of the methylated chromosome [Wylie et al., 2000]. Studies in mice show that like *H19* [Kerjean et al., 2000], the paternal chromosome is methylated at the *Meg3* DMR [Takada et al., 2000; Paulsen et al., 2001]. The extent of the DMR of human *MEG3* was recently shown to span approximately 4 kb of genomic sequence that encompasses the putative promoter region and includes two consensus CTCF (CCCTC binding factor) binding sites that also exhibit differential methylation [Wylie et al., 2000] (Fig. 1). To determine the parental origin of methylation at this DMR as well as the feasibility of analyzing the methylation status of this region with a PCR-based method, we utilized DNA from individuals previously demonstrated to have UPD for chromosome 14 and DNA from normal human fetal liver tissue. DNA samples treated with sodium bisulfite [Grunau et al., 2001] were analyzed by methylation-specific multiplexed (MSM) PCR. Two independent primer sets were designed to amplify the methylated and unmethylated DMR such that the amplicons are distinguished by size on a non-denaturing agarose gel. As shown in Figure 2, the primers specific for methylated DNA (M) produced a 160-bp band in the normal and upd(14)pat samples (lanes 1 and 9), while the primers designed to amplify

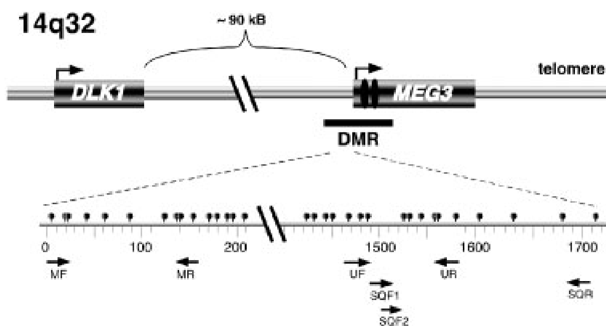

FIGURE 1. **Schematic representation of the *DLK1/MEG3* locus at human chromosome 14q32. The paternally expressed *DLK1* and maternally expressed *MEG3* are separated by approximately 90 kb. The promoter of *MEG3* is situated within an ~4-kb differentially methylated region (DMR) containing two consensus CTCF binding sites (ovals). The region analyzed by MSM-PCR spans ~1,700 bp. The positions of individual CpGs are denoted by lollipops, and the primers used for MSM-PCR and bisulfite sequencing are represented by arrows. MF and MR, forward and reverse primers specific to bisulfite converted methylated DNA; UF and UR, forward and reverse primers specific to bisulfite converted unmethylated DNA; SQF1 and SQR, primers used to generate PCR amplicons from bisulfite converted DNA for sequencing with primer SQF2. Position "0" corresponds to nt 64,450 of BAC AL117190 and the complement of nt 440,243 of contig NT_030824.**

unmethylated DNA (U) produced a 120-bp band in the normal and upd(14)mat samples (lanes 2 and 6). When the primers were multiplexed in the same reactions (M + U), they again amplified only their respective targets (lanes 3, 7, and 11). The specificity of the primers for bisulfite-treated DNA is demonstrated by the lack of PCR products for untreated DNA (lanes 4, 8, and 12). These results indicate that the M and U primer sets specifically amplify the methylated and unmethylated sequences, respectively, and furthermore that the maternal chromosome is unmethylated while the paternal chromosome is methylated within this *MEG3* DMR.

To carry out a more extensive test of the multiplexed PCR reaction, we analyzed additional DNA samples from patients previously diagnosed with UPD14 [Pentao et al.,

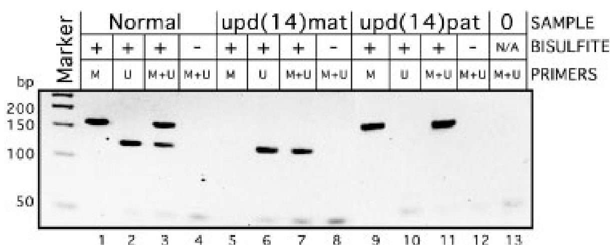

FIGURE 2. **Methylation-specific PCR primers specifically amplify the methylated and unmethylated copies of the *MEG3* DMR. Bisulfite-treated (+) or untreated (–) genomic DNA was subjected to methylation-specific PCR using the M or U primer pairs separately or multiplexed to generate 160-bp and/or 120-bp bands only from bisulfite-modified methylated (lanes 1, 3, 9, and 11) and unmethylated (lanes 2, 3, 6, and 7) template DNAs, respectively. These bands were absent with untreated genomic DNA template (lanes 4, 8, and 12). Samples: normal, liver DNA from a 122d human conceptus; upd(14)mat and upd(14)pat, DNA from individuals with maternal and paternal uniparental disomy of chromosome 14, respectively. Lane 13: negative control.**

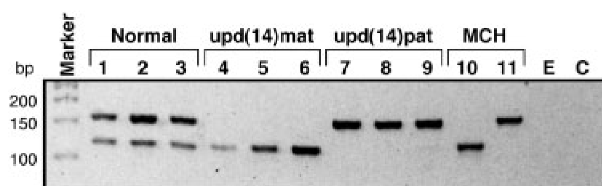

FIGURE 3. **MSM-PCR analysis of normal and UPD14 samples. PCR amplification of bisulfite-treated DNA from three individuals with biparental chromosome 14 inheritance (lanes 1–3), three upd(14)mat (lanes 4–6) and three upd(14)pat (lanes 7–9) was performed with the U and M primers in multiplexed reactions. Lanes 10 and 11: DNA from monochromosomal hybrid (MCH) cell lines carrying human maternal or paternal chromosome 14, respectively. The 160-bp and 120-bp amplicons are derived from methylated and unmethylated template DNA, respectively. Lane E, empty; lane C, negative control.**

1992; Papenhausen et al., 1995; Walter et al., 1996; Cotter et al., 1997; Berend et al., 2000; Towner et al., 2001a, b; Coveler et al., 2002; McGowan et al., 2002]. The chromosome 14 status of the samples was initially kept concealed to insure an unbiased assignment by the MSM-PCR technique. Normal controls included parental DNA samples from two of the UPD patients in addition to normal human fetal liver DNA. DNA from three normal, three upd(14)mat, and three upd(14)pat individuals was treated with sodium bisulfite and analyzed using MSM-PCR. The predicted UPD status of each sample based on the MSM-PCR result was confirmed when the identity of the samples was revealed. Figure 3 shows representative data obtained for these DNA samples, with amplification of both the 160- and 120-bp bands for samples with biparental chromosome 14 inheritance (lanes 1–3), the 120-bp band for upd(14)mat samples (lanes 4–6), and the 160-bp band for upd(14)pat samples (lanes 7–9). To further corroborate these data, DNA from monochromosomal somatic cell hybrid cell lines were assessed for methylation status of the *MEG3* DMR. The two monochromosomal hybrid cell lines were generated from lymphocyte DNA derived from the same individual such that the maternal and paternal chromosome 14 were separated into different rodent–human hybrids [Coveler et al., 2002]. These two samples also yielded the anticipated PCR products (lanes 10 and 11), based on the known parental identity from previous marker analysis (data not shown). Together, these results indicate that the multiplexed PCR reactions accurately identified the parental origins of chromosome 14 in the UPD samples based on the epigenetic profile of the *MEG3* DMR.

To confirm unambiguously that the identity of the UPD samples was assigned correctly by the MSM-PCR assay, we performed bisulfite nucleotide sequencing. The primers used for PCR amplification and sequencing of the DMR were designed to anneal to sequence devoid of CpG dinucleotides. As shown in Figure 4, the methylation profile of each sample was consistent with and corroborated the results obtained by MSM-PCR. Bisulfite sequencing of the monochromosomal hybrids showed that the paternally derived chromosome 14 was methylated completely while the maternally derived

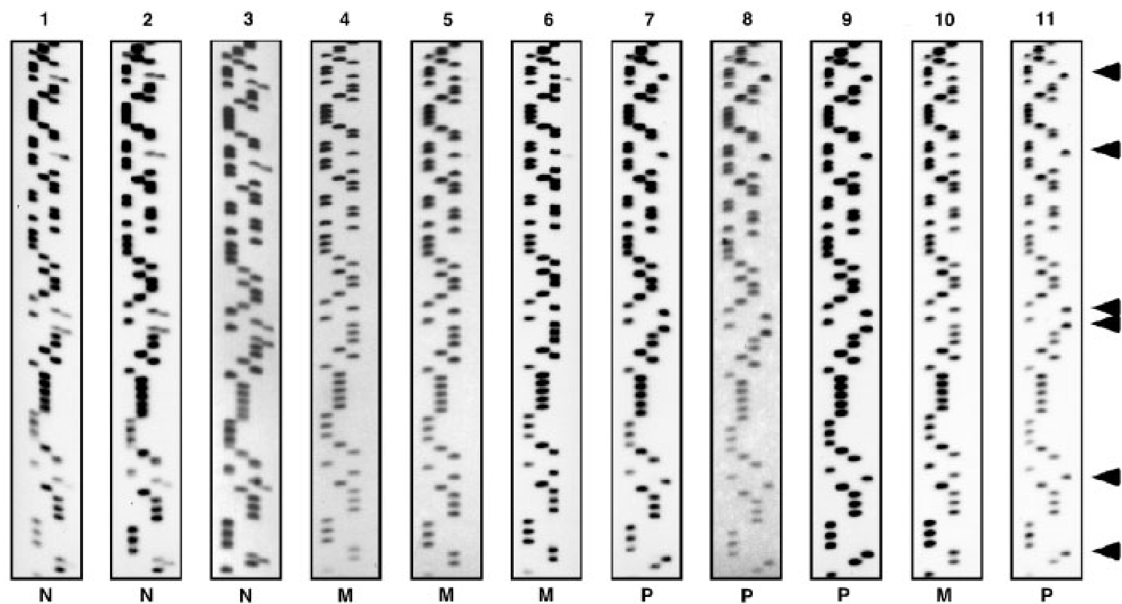

FIGURE 4. **Bisulfite sequencing of the *MEG3* DMR. Bisulfite-treated genomic DNA was amplified by PCR using primers SQF1 and SQR followed by nucleotide sequencing using primer SQF2. Lane order: G A T C. Cytosines in the context of 5′-CpG-3′ in the original unconverted sequence are designated by arrowheads at the right of panel 11. Normal individuals (N; panels 1–3) display an approximate 50:50 distribution of unmethylated to methylated cytosines. Individuals with upd(14)mat and the somatic cell hybrid carrying the maternal chromosome 14 (M; panels 4–6 and 10) lack methylated cytosines while individuals with upd(14)pat and the somatic cell hybrid carrying the paternal chromosome 14 (P; panels 7–9 and 11) have methylated cytosines. The panel numbers correspond to the same sample DNAs analyzed in Figure 3.**

chromosome 14 was unmethylated, indicating that the fusion event did not affect methylation status of the human chromosome 14 retained in the hybrid cell line. The three normal samples exhibited an approximate 50:50 distribution of methylated to unmethylated CpGs, the upd(14)mat samples were unmethylated, and the upd(14)pat samples were methylated at these positions. The reproducible detection of faint cytosine bands in one maternal UPD sample (panel 6, Fig. 4) is indicative of the presence of a low level (10–25% as quantitated by Phospho Imager analysis; Molecular Dynamics Storm 860, Amersham Biosciences, Piscataway, NJ) of site-specific methylation in this sample for two of six CpG sites analyzed. The conversion of unmethylated cytosines to uracils (resulting in thymines in the PCR products) by the sodium bisulfite appeared to be complete (Fig. 4 and data not shown). However, we cannot rule out the possibility that certain CpG dinucleotides are refractory to bisulfite conversion. Previous analyses did not detect mosaicism for UPD in this region [Pentao et al., 1992]. In addition, mixing experiments showed that the MSM-PCR assay accurately detects each parental chromosome when present at a 10-fold lower concentration relative to the other parental copy (data not shown), also supporting that mosaicism is an unlikely explanation in this case. The presence of specific sites of CpG methylation in a background of unmethylated CpGs is not unexpected. Previous reports in which individual cloned PCR products generated from DMRs of bisulfite-treated DNA have shown low level variability in the parental methylation status [Zeschnigk et al., 1997b; Vu et al., 2000; Li et al., 2002]. Importantly, the pattern of methylation observed in this sample (panel 6, Fig. 4) did not impede the accurate diagnostic and reproducible amplification by MSM-PCR (lane 6, Fig. 3).

DISCUSSION

With the recent identification of the imprinted genes *DLK1* and *MEG3* on human chromosome 14 and their association with a differentially methylated region [Wylie et al., 2000], an unresolved issue remained regarding the identity of the methylated parental chromosome. By analysis of documented cases of UPD14, we have shown here that it is the paternal chromosome that is methylated in this region, consistent with the demonstration of paternal methylation for the mouse *Dlk1/Meg3* DMR [Takada et al., 2000; Paulsen et al., 2001]. Paternal methylation is an unusual characteristic in terms of imprint regulation [Reik and Walter, 2001] and is only thus far known to be shared in humans with the *IGF2/H19* imprinted region on chromosome 11 [Kerjean et al., 2000].

Using this newly established methylation profile of the *DLK1/MEG3* imprinted domain, we have developed an assay for detection of UPD14 that eliminates the requirement for parental DNA and informative microsatellite marker analysis, and reduces the time requirement and expense of traditional diagnostic procedures. Indeed, the need for analysis of DNA only from the proband for UPD determination could lead to cost savings of two-thirds as compared to performing studies which include parental DNA samples. Further, the procedure can be completed within one day. It has

recently been suggested that UPD14 patients may be going unrecognized in part because many are karyotypically normal [Kurosawa et al., 2002]. The ability to rapidly screen for UPD14 using MSM-PCR will facilitate diagnosis of these individuals. Already, this assay has been successfully used to exclude UPD14 as a major etiologic component in 200 individuals that exhibit Prader-Willi like symptoms yet test negative for chromosome 15 abnormalities [Dietz et al., 2003].

One potential limitation of this technique is that individuals with mosaicism for UPD14 could theoretically be misclassified as having a normally methylated *MEG3* DMR depending on the extent of mosaicism. Although this was not seen in the assays we performed, prudent use of further testing will be required for these individuals if they exhibit phenotypic characteristics consistent with UPD14.

The utility of this type of assay is not limited to the detection of uniparental disomies. It can also be exploited to assess the methylation status of any genomic region that exhibits differences in methylation status due to imprinting defects, loss of heterozygosity for imprinted tumor suppressor genes, or hypermethylation in cancer, with care taken to reduce potential contamination from adjacent normal cells.

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