The parent-of-origin effect of 10q22 in pre-eclamptic females coincides with two regions clustered for genes with down-regulated expression in androgenetic placentas

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By affected sib-pair linkage analysis of 24 families with pre-eclampsia, we confirm a susceptibility locus on chromosome 10q22.1 in Dutch females: a multipoint non-parametric linkage score of 3.6 near marker D10S1432 was obtained. Haplotype analysis showed a parent-of-origin effect: maximal allele sharing in the affected sibs was found for maternally derived alleles in all families, but not for the paternally derived alleles. As matrilineal inheritance suggests the presence of maternally expressed imprinted genes, while imprinting operates predominantly in (extra)embryonic tissues, all genes (n = 132) known on 10q22 between GATA121A08 and D10S580 were screened for seven sequence-related features associated with imprinting and subsequently tested for expression in first trimester placentas. Placental expression of genes selected in this way (n = 55) was compared with expression in androgenetic placentas of identical gestational age. Two regions on 10q22 were identified with developmentally co-expressed genes with non-random chromosomal distribution. Interestingly, these two clusters, near CTNNA3 and KCNMA1 and each containing five genes with down-regulated expression in androgenetic placentas, coincided with developmentally co-repressed genes with non-random chromosomal distribution. Interestingly, these two clusters, near CTNNA3 and KCNMA1 and each containing five genes with down-regulated expression in androgenetic placentas, coincided with two regions clustered for genes (Lachmeijer et al., 2001; Kanayama et al., 2002). In this respect, pregnant mice heterozygous for the maternally expressed imprinted Cdkn1c gene show all features of pre-eclampsia, both in the mother and in the placenta (Kanayama et al., 2002). Although the pregnant Cdkn1c−/− mice carry conceptuses both with and without Cdkn1c expression, only conceptuses without Cdkn1c expression develop a placental phenotype with lack of trophoblast invasion followed by maternal symptoms. In the placenta, lack of Cdkn1c expression is dependent on the parent-of-origin of the mutant allele: only alleles inherited from the mother become phenotypically dominant and functionally mutant as a consequence of imprinting. Alleles derived from the father, even when mutated, have no effect by being transcriptionally silent (Kanayama et al., 2002). The combined genetic and experimental data not only confirm the essential and primary role of trophoblast invasion in the pathogenesis of pre-eclampsia, but also point towards the contribution of epigenetic features, i.e. imprinting (Feinberg et al., 1991; Georgiades et al., 2001; Kanayama et al., 2002). In this respect, pregnant mice heterozygous for the maternally expressed imprinted Cdkn1c gene show all features of pre-eclampsia, both in the mother and in the placenta (Kanayama et al., 2002). Although the pregnant Cdkn1c−/− mice carry conceptuses both with and without Cdkn1c expression, only conceptuses without Cdkn1c expression develop a placental phenotype with lack of trophoblast invasion followed by maternal symptoms. In the placenta, lack of Cdkn1c expression is dependent on the parent-of-origin of the mutant allele: only alleles inherited from the mother become phenotypically dominant and functionally mutant as a consequence of imprinting. Alleles derived from the father, even when mutated, have no effect by being transcriptionally silent (Kanayama et al., 2002).
Although the human CDKN1C gene is clearly no candidate gene for pre-eclampsia as no linkage has been demonstrated for 11p15 and pre-eclampsia (Table I), the mode of linkage seen in the mouse Cdkn1c model has never been tested systematically in the context of genetic and other studies of pre-eclampsia.

Therefore, non-Mendelian inheritance of placentally expressed genes subject to imprinting should be considered in the linkage and aetiology respectively of gestational hypertension and related syndromes associated with trophoblast dysfunction (Tables I and II). Consequently, linkage and other analyses performed for this purpose should be corrected likewise. In this study, this was done for chromosome 10q, which had been previously suggested to be linked to pre-eclampsia in The Netherlands (Lachmeijer et al., 2001).

### Materials and methods

#### Linkage analysis of pre-eclampsia families

Twenty-five nuclear multiplex pre-eclampsia families with two affected sibs (sisters) having either pre-eclampsia (n = 49), or eclampsia (n = 1) in each family (Lachmeijer et al., 2001) were analysed by non-parametric allele-sharing analysis following detailed fragment analysis with additional markers on chromosome 10. Markers were selected close to D10S1432 (Lachmeijer et al., 2001). Families included both parents of the pre-eclamptic sisters. In the mothers, previous pregnancies were either normal (n = 5), pre-eclamptic (n = 5) or accompanied by pregnancy-induced hypertension (PIH) (n = 15). One mother had a previous pregnancy complicated by the HELLP syndrome. Paternal DNA was available in 18 families (72%). Maternal DNA was available in 23 families (92%). The pedigree structures of all families are shown in supplementary files 1–6.

#### Diagnostic criteria for gestational hypertension with or without proteinuria

- **Pre-eclampsia**: de novo hypertension (diastolic blood pressure $\geq 90$ mmHg with increment $\geq 20$ mmHg from first trimester diastolic blood pressure) and proteinuria $\geq 300$ mg/24 h or at least twice $\geq 1 \text{+}$ on semiquantitative analysis; eclampsia: seizures in hypertensive pregnancy with or without proteinuria; HELLP syndrome: lactate dehydrogenase $\geq 600$ IU/l, aspartate aminotransferase and alanine aminotransferase $\geq 70$ IU/l and $\leq 100$ platelets $\times 10^7$/l; and PIH: de novo hypertension in pregnancy without proteinuria.

The marker alleles in individual samples were analysed by PCR of the DNA fragment lengths (Dib et al., 1996). The PCR product lengths were determined by capillary electrophoresis using the ABI Prism 310 Genetic Analyzer (Gudbjartsson et al., 1999). The PCR product lengths of the polymorphic markers D10S537 and D10S580 with oligonucleotide primers that were labelled with the fluorophores 6-FAM or TET. PCR was performed as described previously (Leegwater et al., 1999). The PCR product lengths were determined by capillary electrophoresis using the ABI Prism 310 Genetic Analyzer (Perkin Elmer). DNA from Centre d'Etude du Polymorphisme Humain (CEPH) individual 1331-01 were considered the standard for polymorphic markers D10S537 and D10S580.

#### Table I. Genome-wide linkage studies of gestational hypertension

<table>
<thead>
<tr>
<th>Study and country</th>
<th>Genome-wide scan</th>
<th>Markers</th>
<th>n</th>
<th>Clinical groups</th>
<th>Chromosome</th>
<th>Logarithm of the odds (LOD)</th>
<th>NPL</th>
<th>cM</th>
<th>Marker</th>
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<tbody>
<tr>
<td>Harrison et al. (1997, Australia)</td>
<td>Low density</td>
<td>90</td>
<td>15</td>
<td>General (incl. PIH)</td>
<td>4q34</td>
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<td>General (incl. PIH)</td>
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<td>144.7</td>
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<tr>
<td>Arraignmentson et al. (1999, Iceland)</td>
<td>Medium density</td>
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<td>124</td>
<td>General (incl. PIH)</td>
<td>11q23-24</td>
<td>2p12**</td>
<td>121.3</td>
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<td>Lachmeijer et al. (2001, The Netherlands)</td>
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<td>293</td>
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<td>Strict (E/PE)</td>
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<td>93.9</td>
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<tr>
<td>Laivuori et al. (2003, Finland)</td>
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<td>163</td>
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<td>9p11</td>
<td>2.20</td>
<td>49.9</td>
<td>D9S187</td>
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</tbody>
</table>

**a** Chromosomal position of marker D2S286 linked with pre-eclampsia in Iceland has been reassigned according to NCBI Human Genome Resources.

NPL = non-parametric linkage score; PIH = pregnancy-induced hypertension; E = eclampsia; PE = pre-eclampsia.

#### Table II. Genetic causes of defective endovascular trophoblast invasion in relation to pre-eclampsia

<table>
<thead>
<tr>
<th>Clinical entity</th>
<th>Invasion</th>
<th>Genetics/cause</th>
<th>Loci</th>
<th>Pre-eclampsia</th>
<th>Reference</th>
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<td>A. Human: clinical</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td>PAPS</td>
<td>Reduced</td>
<td>Fetal trisomy (7, 9, 20, 22), maternal in origin</td>
<td>Fetal</td>
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<td>Sebire et al. (2002)</td>
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<td>IUGR</td>
<td>Reduced</td>
<td></td>
<td></td>
<td></td>
<td>Sheppard and Bonnar (1976)</td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>Reduced</td>
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<td></td>
<td></td>
<td>Meekins et al. (1994)</td>
</tr>
<tr>
<td>Miscarriage</td>
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<td>Michel et al. (1990)</td>
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<tr>
<td>CHM</td>
<td>Reduced</td>
<td>Androgensis</td>
<td>Fetal</td>
<td>Yes/no</td>
<td>Sebire et al. (2001)</td>
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<td>Trisomy</td>
<td>Reduced</td>
<td>Fetal trisomy 13q</td>
<td>Fetal</td>
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<td>Feinberg et al. (1991)</td>
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<tr>
<td>Trisomy</td>
<td>Unknown</td>
<td>Placenta confined trisomy 16</td>
<td>Fetal</td>
<td>Yes/no</td>
<td>Brandenburg et al. (1996)</td>
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<td>PHM</td>
<td>Unknown</td>
<td>69 XXY, paternal in origin</td>
<td>Fetal</td>
<td>Yes</td>
<td>Schinzel et al. (1975)</td>
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<tr>
<td>B. Mouse: experimental</td>
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<tr>
<td>pUPD12</td>
<td>Reduced</td>
<td>Paternal uniparental disomy 12</td>
<td>Fetal</td>
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<td>p57kip2</td>
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<td>IGFBP-1</td>
<td>Reduced</td>
<td>Decidual IGFBP-1 † †</td>
<td>Maternal</td>
<td>Unknown</td>
<td>Crossley et al. (2002)</td>
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<td>BPH/5</td>
<td>Unknown</td>
<td>Brother–sister matings of BPH/2</td>
<td>Maternal</td>
<td>Yes</td>
<td>Davison et al. (2001)</td>
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</tbody>
</table>

PAPS = primary antiphospholipid syndrome; IUGR = intrauterine growth restriction; CHM = complete hydatidiform mole; PHM = partial hydatidiform mole; pUPD12 = paternal uniparental disomy 12; IGFBP-1 = insulin growth factor binding protein 1.

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Table I. Genome-wide linkage studies of gestational hypertension

- **Study and country**: Harrison et al. (1997, Australia); Moses et al. (2000, Australia/NZ); Arraignmentson et al. (1999, Iceland); Lachmeijer et al. (2001, The Netherlands); Laivuori et al. (2003, Finland).
- **Genome-wide scan**: Low density, Medium density.
- **Markers**: 90, 400, 440, 293, 435.
- **n**: 15, 34, 124, 72, 38, 453.
- **Clinical groups**: General (incl. PIH), Strict (E/PE), Strict (E/PE), General (incl. PIH), Strict (E/PE), Strict/general, Strict/general, Strict/general, Strict/general.
- **Chromosome**: 4q34, 2q23, 11q23-24, 2p12, 2q12, 2p12, 2q12, 2p25, 9p13, 4q32, 9p13.
- **Logarithm of the odds (LOD)**: 2.9, 2.58, 2.02, 4.77, 3.23, 2.38, 2.41, 2.51, 2.22, 2.96, 2.20.
- **NPL**: 178.7, 144.7, 121.3, 93.9, 32.4, 21.70, 38.90, 163, 49.9.
- **cM**: D4S450-S610, D2S112-S1511, D11S925-S4151, D2S211-S1394, D2S685, D2S168, D9S169, D4S413, D9S187.

Table II. Genetic causes of defective endovascular trophoblast invasion in relation to pre-eclampsia

- **Clinical entity**: A. Human: clinical; B. Mouse: experimental.
- **Invasion**: Reduced, Reduced, Reduced, Reduced, Unknown.
- **Genetics/cause**: Fetal trisomy (7, 9, 20, 22), maternal in origin, 2p12, 2p25, 9p13, Androgensis, Fetal trisomy 13q, Placenta confined trisomy 16, 69 XXY, paternal in origin, Paternal trisomy 12, p57kip2-neg placenta, Decidual IGFBP-1 † †, Brother–sister matings of BPH/2.
- **Loci**: Fetal, Maternal, Fetal, Fetal.
- **Pre-eclampsia**: No, Yes, Yes/no, Yes/no, Yes.
the non-parametric method. The scoring function used was $S_{all}$. Allele frequencies for the markers were calculated from the available family material.

The order and positions (in cM) (Kong et al., 2002) of the markers were: GATA12A108 (88.41), D10S538 (89.16), D10S1432 (93.97), D10S850 (95.52), D10S3227 (96.86) and D10S2470 (110.74).

Contig comparison

The gene sequences ($n = 132$) present and expected (NCBI genome scan) on contig NT_008583 between ZNF365 and KCNMA1 were compared to five other contigs to identify related genes. This criterion assumes the existence of the situation observed for Vanishing White Matter (VWM); two similar genes (EIF2B5 and EIF2B2) on different chromosomes (3q and 14q respectively) turned out to be responsible for the same disease (VWM), yet segregating in different populations as a consequence of different founders (Leegwater et al., 2001). The selection criterion in our study assumes the same: regions with linkage to pre-eclampsia (2p12, 2p25, 9p13) could contain similar genes functioning in the same pathway yet segregating with different populations (Finland, Iceland, The Netherlands).

Contig NT_022182 contains the D2S286 marker on 2p12 with linkage in the Icelandic population (Arngrimsson et al., 1999). Contig NT_005334 contains marker D2S168 on 2p25 with linkage in Finland (Laivuo et al., 2003). Contigs NT_023974 and NT_037734 are adjacent contigs spanning the region near marker D9S169 with linkage in Finland (Laivuo et al., 2003). Contig NW_000027 is of murine origin, contains marker D10Mit20 (Wright et al., 1999; Davison et al., 2001) and corresponds to a region on 10B5.

Blot searches were performed for all 132 genes to identify: (i) the presence of Unigene clusters associated with sense–antisense orientation (Shendure and Church, 2002), (ii) the existence of related family members on other autosomes subject to genomic imprinting with maternal expression (www.mgu.har.mrc.ac.uk), (iii) the absence of LINE (type 1 and 2) and SINE (Alu/Mir) repeats within the 5 kb upstream or downstream of the initiation and stop codons respectively (Grealy, 2002; Ke et al., 2002), (iv) the presence of CpG islands within the 1 kb region overlapping or following the last exon (Strichman-Almashanu et al., 2002) and having homology to the differentially methylated region (DMR) (accession no. hCG1659616) on 9p13, (v) the presence of homologous sequences within the CITE databases (http://fantom2.gsc.riken.go.jp/imprinting), (vi) the existence of complementary transcripts including antisense in the Fantom database (Kiyosawa et al., 2003; Nikaido et al., 2003; Numata et al., 2003) or other files (Yang et al., 2003). The DMR on 9p13 was selected given its location in the region linked with pre-eclampsia in Finland (Laivuo et al., 2003). Alignment with the 9p13 DMR was done with repeat-masked sequences only (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker). The 10B5 mouse contig was used to confirm their presence on human chromosome 10q22, to identify additional orthologous genes and to demarcate the genomic boundaries of synteny between human 10q22 and mouse 10B5.

Expression analysis in normal and androgenetic placental tissues

First trimester placental tissues were obtained from pregnancy terminations with ethical approval and informed consent. RNA from normal first trimester placental tissues ($n = 4$) (gestational weeks 8–12) was extracted using RNazol B, followed by RQ1 DNase treatment, and following precipitation stored as 5 µl aliquots at −80°C. To maximize RNA yield by RNAzol extraction, the use of multiple (50–100) cryostat sections (20 mmol/l each) was preferred over mechanical homogenization. RNA aliquots were thawed only once. Besides determination of purity by OD260/280 measurement, RNA integrity was checked by gel electrophoresis in formaldehyde gels. RNA from androgenetic placental tissues ($n = 4$) (gestational weeks 8–12) was obtained from two complete hydatidiform moles of dispermic origin and two complete hydatidiform moles of monospermic origin as confirmed by microsatellite analysis (Shahib et al., 2001). Tissue fragments of molar pregnancy (4) (gestational weeks 8–12) was extracted using RNazol B, followed by inactivation of RT enzyme at 95°C; annealing: 1 min at annealing temperatures optimal for each gene as calculated by the Oligo program; extension for 2 min at 72°C and a final extension for 10 min at 72°C. The characteristics of the primer sequences used and corresponding annealing temperatures used are described in the supplementary file 7. To permit reliable comparison between normal and androgenetic placentas by semiquantitative RT–PCR, samples were pre-screened for absolute PBGD expression levels using real-time quantitative RT–PCR as described in detail elsewhere (Westerman et al., 2002). In this way, the amounts of input RNA used subsequently for large-scale semiquantitative RT–PCR were corrected accordingly with an RNA input for each sample corresponding to 50 000 copies of PBGD mRNA molecules. In addition, the semiquantitative approach was validated using the CDKN1C gene as model system representative of an imprinted maternal expressed gene with down-regulated expression in androgenetic placentas (Castrillon et al., 2001; Fisher et al., 2002). For this, real-time Q-RT–PCR (Westerman et al., 2002) was done for both Porphobilinogen deaminase (PBGD) and CDKN1C in normal and androgenetic placentas. PBGD-normalized expression levels of CDKN1C corresponded to 22 000 CDKN1C copies per 50 ng total RNA in normal placentas and to either 6000–7000 copies or <2000 copies per 50 ng total RNA in androgenetic placentas. By semiquantitative RT–PCR, these copy numbers were found to correspond to PCR bands presenting as strongly positive, weakly positive and negative bands respectively (supplementary file 8). Controls consisting of RT–PCR reactions lacking the RT enzyme were negative.

Results

**Chromosome 10q22 contains a susceptibility locus for pre-eclampsia in Dutch families**

Twenty-four pre-eclampsia families with two affected sibs in each family were evaluated by linkage analysis of six markers including:

<table>
<thead>
<tr>
<th>HUMAN</th>
<th>MOUSE</th>
<th>HUMAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>10q22.1</td>
<td>BPH/2</td>
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</tr>
<tr>
<td>Netherlands</td>
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<td>Iceland</td>
</tr>
<tr>
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<td>TACR1</td>
</tr>
<tr>
<td>P4HA1</td>
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</table>

**Figure 1.** Similarity of chromosomal regions linked with pre-eclampsia. Chromosome 10q22.1 near D10S1432 (A) shows significant linkage with pre-eclampsia in Dutch families (NPL 3.6). This region demarcated by the EGR2 and P4HA1 genes is syntenic to a region on mouse chromosome 10 (Marker D10mit20) (B) with genome-wide linkage (lod 4.9) with high blood pressure in BPH/2 mouse. These mice develop pre-eclampsia in the BPH/5 backcrosses (Wright et al., 1999; Davison et al., 2001) Homologous genes (C) are located on human chromosome 2p12 near marker D2S286 linked with pre-eclampsia in Icelandic families (lod 4.77) (Arngrimsson et al., 1999).
D10S1432 spanning 24 cM on 10q22. Mothers of the pre-eclamptic sisters in each family had experienced either pregnancy-induced hypertension (n = 14) (group A), pre-eclampsia (n = 5) (group B) or no pregnancy complications (n = 5) (group C). Both sisters in all families had pre-eclampsia. In these families, chromosome 10q22.1 near D10S1432 reached genome-wide significance with a multipoint non-parametric linkage score (NPL) of 3.6, confirming that this region contains a susceptibility locus for pre-eclampsia in Dutch patients (Lachmeijer et al., 2001).

The region on 10q22 is homologous to the regions on mouse chromosome 10B5 and human 2p12 linked with pre-eclampsia

Interestingly, the human 10q22 region (Figure 1a) is homologous to the region on mouse chromosome 10B5 (Figure 1b) in BPH/2 mice that showed significant linkage in a genome scan for blood pressure loci (Wright et al., 1999). An LOD score of 4.9 was observed near marker D10Mit20. Subsequent breeding by brother–sister matings of the BPH/2 strain led to the induction of new-onset hypertension during pregnancy with late-gestational proteinuria accompanied by progressive glomerulosclerosis in the BPH/5 strain (Wright et al., 1999; Davison et al., 2001). Not only are human 10q22.1 (near marker D10S1432) and mouse 10B5 (near marker D10Mit20) syntenic over a large region, human 10q22.1 is homologous to 2p12 (near marker D2S286) as well (Figure 1c). This indicates that the pre-eclampsia locus on chromosome 10q22 in Dutch patients is not only related to the locus on 10B5 in the BPH/2 mouse, but also to the locus on 2p12 in the Icelandic population (Arngrimsson et al., 1999). The homology between these chromosomal regions as well as to 9p13 was subsequently used as an additional selection criterion for the selection of candidate genes on 10q22 (see below). This criterion is similar to the situation observed for Vanishing White Matter (VWM): two similar genes (EIF2B5 and EIF2B2) on different chromosomes (3q and 14q, respectively) are responsible for the same disease (VWM), yet segregate in different populations as a consequence of different founders (Leegwater et al., 2001).

Figure 2. Sharing of maternal haplotypes in affected sisters with pre-eclampsia. (a) In sibpairs with pre-eclampsia, the maternal haplotypes flanking marker D10S1432 on 10q22 are identical in all families (n = 24) indicating a parent-of-origin effect. This is not seen for the paternal haplotypes. Families are grouped according to the obstetric histories of the mothers who experienced either pregnancy-induced hypertension (A) (grey circle) (n = 14), pre-eclampsia (B) (black circle) (n = 5) or had normal pregnancies (open circle) (n = 5). Only one representative pedigree is shown for each group. The pedigree structures of all families can be seen in the supplementary files 1–6. (b) Sharing of maternally inherited alleles in pre-eclamptic sisters is maximal at markers D10S1432 and GATA121A08. Alleles shared are coloured yellow. Non-identical alleles are boxed.

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Parent-of-origin effect of the chromosomal 10q22 region in pre-eclamptic patients

Analysis of the parental origin of the haplotypes shared between the affected sibs in our pre-eclampsia families showed a parent-of-origin effect: the alleles shared between the two affected sisters were always maternal in origin (Figure 2a). Sharing was maximal near marker D10S1432 (Figure 2b). The pedigree structures of all families are shown in the supplementary files 1–6. A second region with uninterrupted maternal sharing was found near GATA121A08. In fact, when 18 out of 24 pre-eclampsia families were selected, LOD and NPL scores increased to 4.99 and 4.09 respectively, accompanied by a shift towards marker GATA121A08. The criterion for selection of these 18 families was based on the presence of gestational hypertension (with or without proteinuria) or intrauterine growth retardation in at least one of the sisters during the respective pregnancies of their mothers. The inclusion of type II intrauterine growth retardation assumes a common aetiology, i.e. both syndromes are related to trophoblast invasion (Table II).

Chromosome 10q22 contains two regions enriched for genes with down-regulated expression in molar pregnancies

Parent-of-origin effects with matrilineal transmission involving neither Mendelian inheritance involving either (i) genomic imprinting, (ii) the involvement of an autosomal locus with effect on mitochondrial DNA transcription or function, (iii) aberrant expansions of DNA repeats (triplet repeats) during maternal meiosis or early embryogenesis or (iv) genes expressed from the maternal haploid genome during early development (fertilized oocyte and cleavage stage embryo).

Given the proven association of imprinting with pre-eclampsia in heterozygous Cdkn1c mice (Kanayama et al., 2002), the fact that imprinting predominantly operates in developing tissues (i.e. embryo and placenta) and the reported clinical associations between pre-eclampsia and parent-of-origin effects (Schinzel et al., 1975; Sebire et al., 2001; Billieux et al., 2004; Vatish et al., 2004), we explored the presence of maternally expressed imprinted genes on 10q22 that function in the early placenta. For this, a novel combinatorial approach was used with complementary use of structural and functional genomics. Structural genomics was done by bioinformatical screening for seven sequence- and expression-related features associated with imprinted genes that have become available recently. Functional genomics was based on expression analysis of molar pregnancies: placentaically expressed genes subject to genomic imprinting with preferential expression of the maternal allele become down-regulated in complete hydatidiform moles (Jinno et al., 1995; Castrillon et al., 2001; Fukunaga, 2002; Fisher et al., 2002).

In the structural genomic approach the genes present or expected (n = 132) on chromosome 10q22 region (between ZNF365 and KCNMA1) (contig NT_008583) were scored for positivity for each of 10 different selection criteria (Figure 3). Three criteria were based on the presence of related genes on 2p12 (Arngrimsson et al., 1999), 2p25 (Laivuori et al., 2003) or 9p13 (Laivuori et al., 2003) (Figure 3, columns B–D). Seven criteria were based on homology or identity with sequence- or transcript-related features (Figure 3, columns E–K) known to be associated with maternally expressed imprinted genes (Greally, 2002; Ke et al., 2002; Shendure and Church, 2002; Strichman-Almashanu et al., 2002; Kiyosawa et al., 2003; Nikaido et al., 2003; Numata et al., 2003; Yang et al., 2003). Examples are the exclusion of short interspersed transposable elements (Greally, 2002) or the presence of complementary sense–antisense transcripts (Shendure and Church, 2002). A total of 55 genes (Figure 3, column A) reached a score of ≥1 (Figure 3, column L). These genes were subsequently tested by RT–PCR for reduced or absent expression in androgenetic placentas in comparison with normal placentas of identical gestational age. Three differential expression patterns were seen with respect to changes in transcript levels in androgenetic placenta compared to normal placentas of identical gestational age. The first pattern, equal expression, was seen for 26 genes out of 50 genes screened (Figure 3, columns M, N). The second pattern, reduced levels in the majority of molar pregnancy samples compared to normal placentas, was found for nine genes (Figure 4: JDP1, SIRT1, MAWBP, SUPV3L1, MYST4, C10orf35, LOC142893, C10orf11 and LOC142955). This pattern was independent of the di- or monospermic origin of the molar pregnancy samples but identical to the pattern observed for CDKN1C (Figure 4). CDKN1C was used as positive control representative of an imprinted maternally expressed gene with down-regulation in molar placenta samples (Castrillon et al., 2001) permitting validation of the semiquantitative approach used. The third pattern, absent expression in all molar pregnancy samples, was found for three genes: CTNNA3, DFKZP564G092 and KCNMA1 (Figure 4).

Interestingly, out of the total of 15 genes with reduced or absent expression in molar pregnancy samples, 10 of these genes were found to be clustered in two separate regions each containing five genes (Figure 3, columns M, N). Moreover, both regions coincided with the regions with maximal allele sharing between affected pre-eclamptic sisters (Figures 2b and 3). Region 1 contains five differentially expressed genes (CTNNA3, JDP1, SIRT1, DFKZP564G092, MAWBP) and is located near marker GATA121A08. This region is homologous to mouse chromosome 10B5 containing marker D10Mit20. Region 2 also contains five genes (MYST4, LOC142893, C10orf11, LOC142955, KCNMA1) and is located near marker D10S1432. This region is homologous to mouse chromosome 14A2.

Discussion

Here we present evidence that chromosome 10q22.1 contains a susceptibility locus for pre-eclampsia in Dutch patients. This region shows a parent-of-origin effect with matrilineal inheritance by the affected sisters of the maternal alleles in all families. Maximal sharing is seen near markers D10S1432 and GATA121A08. The very same chromosomal regions with maximal sharing of maternal alleles were found to be enriched for genes with down-regulated expression in placentas of androgenetic origin. Our linkage and expression data are compatible with the concept that pre-eclampsia involves placentaically expressed genes subject to imprinting with preferential expression of the maternal allele, and involves related genes of the same gene family yet located on different chromosomes (2p12, 2p25, 9p13 and 10q22) (Arngrimsson et al., 1999; Laivuori et al., 2003; this study). These genes are involved in trophoblast function (Alexander et al., 2001; Brosens et al., 2002) and code for proteins that become phenotypically dominant and functionally mutant by preferential expression of variations inherited from the mother. This mode of inheritance is similar to the situation seen in pregnant pre-eclamptic mice heterozygous for the imprinted Cdkn1c gene (Kanayama et al., 2002). In this view, the genomic variations involved can occur frequently in the normal population, presenting as polymorphic variations. In the normal population, these variations have no effect. However, in pre-eclampsia these variations become phenotypically dominant, functionally mutant and disease inducing by the combination of tissue- (placenta)
Figure 3. Candidate imprinted genes on chromosome 10q22.1. Pre-eclampsia (PE) candidate genes known or predicted ($n = 132$) on chromosome 10q22 (contig NT_008583) between KIAA0844 and KCNMA1 were selected (column A) for expression analysis by RT–PCR in normal and androgenetic placentas if they met at least one of the selection criteria, based either on homology (columns B–D) or on imprinting features (columns E–K). Homology was scored for the presence of homologous genes on chromosomes with genome-wide linkage to pre-eclampsia in other populations: 2p12 (column B), 2p25 (column C), or 9p13 (column D). Imprinting features were scored positive when homologous for expressed genes (RNA) with proven or suspected imprinting (column E), or homologous to non-coding sequence repeats recently identified as being shared by and conserved between imprinted genes, such as the differentially methylated region (DMR) on 9p13 (column F), lack of LINE (type 1 and 2) repeats in the upstream 5 kb upstream region (column G), or the lack of SINE (Alu/Mir) repeats in the downstream 5 kb region (column H). Final selection criteria consisted of the presence of related human (lane I) or mouse (lane J) sequences identified in the Functional Annotation of Mouse (Fantom) database including antisense RNA (lane K) (http://genome.gsc.riken.go.jp). In this way, out of 132 genes known or predicted on chromosome 10q22 (contig NT_008583) between KIAA0844 and KCNMA1, 55 genes reached a score of $\geq 1$ (lane L) and were subsequently screened for down-regulated expression in androgenetic placentas (lane N) compared to expression in normal placentas (lane M) of identical gestational age (weeks 8–12). Fifteen genes (marked with yellow in lanes L and M) were identified with down-regulated expression in androgenetic placentas indicative of genes subject to imprinting in the early placenta with preferential expression of the maternal allele. Ten of these genes were found to be clustered (indicated by small boxes) in two separated clusters (near CTNNA3 and KCNMA1) with each cluster containing five co-repressed genes. The location of the genes in these clusters as well as the locations of SUPV3L1 and C10orf35 coincided with the regions (indicated by large boxes) with maximal linkage to GATA121A08 and D10S1432 respectively. Scores for LINE (type 1 and 2) and SINE (Alu/Mir) repeats (columns G and H) are presented as number of repeats for each subtype. The three character designations in columns I, J and K correspond to the last three characters of the corresponding sequences in the Fantom imprinting (http://fantom2.gsc.riken.go.jp/imprinting) and antisense (http://genome.gsc.riken.go.jp/m/antisense) databases. Sequences used are: 1300116P1 2, 4933408A16, 6430563K19, AF214646, B230311F01, A530096011, 4921531D01, 6330415E02, F730003L23, 2310005G07, A130082D14, B230337M17, B130019B13, A230052B14, A730077G03, 4921518C22, 9430097D22, 5430425E15, NT = not tested; Impr = imprinting; DMR = differentially methylated region; Hum = human, AS = antisense; Andro = androgenic.
and allele-specific (maternal allele only) activation in the early placenta due to genomic imprinting. It should be noted that although the genotypic effect is one of preferential transcriptional activation, the phenotypic effect at the protein level is that of loss of function. In the Cdkn1c model, this defect is complete by wholesale loss of expression of the maternal allele in the placenta. In the human situation, this protein defect is more likely to be partial and variable. However, in both the human situation and in the mouse model, the result is loss of function, i.e. being reduced or absent respectively. In addition, in the mouse model, no genetic variation is present (isogenic mouse). In humans, this is certainly not the case. In this respect, the existence in humans of variable penetrance of the imprinting mechanism itself (polymorphic imprinting) cannot be excluded. Whether this is related to the phenomenon seen in our pre-eclampsia families where the affected sibs always had pre-eclampsia yet the mothers could have PIH or pre-eclampsia (Figure 2a) requires further exploration. This epigenetic model of pre-eclampsia is schematically represented in Figure 5. The fact that in our families, pedigrees were also observed where both sisters had pre-eclampsia while the mother had uncomplicated pregnancies (Figure 2a, supplementary files 1–6) is compatible with this concept as well. In these families, the genomic variation involved can be expected to originate from the grandfather (generation I in Figure 5) with the imprint being reset and maternally marked during maternal germline transmission in their daughters (generation II in Figure 5). This resetting reverses the genomic blueprint of the alleles involved with respect to parental origin. Subsequent transmission to the next generation (indicated by generation III in Figure 5 and by 0 and 1 in pedigree C in Figure 2a) leads to pre-eclampsia when expressed from the maternal allele in the placenta of the children carried by these females. Moreover, in this concept loci on the chromosomes linked with pre-eclampsia in other populations (such as 2p12, 2p25 and 9p13) can be expected to be subject to imprinting and should also be tested for imprinting. For at least one locus (2p12) this is likely although it might involve imprinting paternally expressed genes rather than imprinted maternally expressed genes. A parent-of-origin effect with linkage for paternal identity-by-descent sharing has been observed for 2p12 (Francks et al., 2003). Maximal linkage was found for marker D2S417 located at 2p11.2–2p12, the very same region linked with pre-eclampsia in Icelandic patients (Arngrimsson et al., 1999).

Sequence analysis to identify polymorphic DNA variations followed by allele-specific RT–PCR in heterozygous informative samples (Oudejans et al., 2001) will be needed to confirm that one or more genes identified in our study are subject to imprinting and harbour nucleotide variations that segregate with the pre-eclamptic phenotype. In this respect, our approach with the identification of a feasible number of candidate genes in a chromosomal region linked with pre-eclampsia in Dutch patients will greatly facilitate this analysis. This is supported by our recent observation that at least one locus in this region, i.e. CTNNA3, is subject to imprinting. The maternal allele of CTNNA3 is expressed preferentially in the placenta, although imprinting is restricted to the villous trophoblast (M.van Dijk et al., unpublished observations).

Of the candidate genes located within the two clusters on 10q22 with maximal allele sharing, certain genes are involved in pathways known to be disturbed in pre-eclampsia. CTNNA3 codes for the alpha-T-catenin protein involved in cell adhesion (Janssens et al., 2001). Abnormal expression of adhesion molecules by invasive cytotrophoblasts is one of the earliest hallmarks of pre-eclampsia (Zhou et al., 1993). JDP1 codes for a member of the HSP40/DNAJ protein family (Lee et al., 2000). Abnormal placental levels of heat shock proteins have been described in relation to oxidative stress, which is a prominent feature of the placenta in pre-eclampsia (Hung et al., 2001; Vaughan and Walsh, 2002). Other genes are unrelated to pathways known to be involved in pre-eclampsia, yet cannot be
excluded as candidate genes for the following reasons. SIRT1 deacetylates histone H3/H4 (Senawong et al., 2003), while MYST4 is a novel histone acetyltransferase with multiple functional domains (Champagne et al., 1999). Histone modifications, in particular of histones H3 and H4, form essential modulating components of the epigenetic marking system of transcriptionally active or silent chromatin states (Jenuwein and Allis, 2001). SUPV3L1 codes for a helicase containing a DEAD-box domain (Minczuk et al., 2002). Within the 10q22 region with linkage, SUPV3L1 is flanked by two additional genes with DEAD-box helicase activity: DDX50 and DDX21. DEAD-box and related RNA helicases have recently been recognized as maternal effect genes required for early embryo development that function potentially by RNA silencing (Golden et al., 2002). In this scenario, besides a parent-of-origin effect through imprinting, expression of these genes from the haploid maternal genome in the earliest stages of development (fertilized oocyte and early cleavage stage pre-embryo) is also compatible with our linkage data. Early maternal expression of DEAD-box helicases with a subsequent silencing effect on RNA or DNA sequences from the diploid fetal genome mark the epigenotype of the offspring and lead to later effects in (extra)embryonic development (Pickard et al., 2001). DKFZP564G092 and KCNMA1 code for proteins with ubiquitin protein ligase activity and calcium-sensitive potassium transport activity respectively (Alioua et al., 2002). Homologues of these genes are known to be imprinted. The proposed function of the KCNMA1 protein, i.e. control of vasoconstriction (Alioua et al., 2002), is interesting in this context. If KCNMA1 expression includes extravillous trophoblast cells situated within and around the maternal spiral arteries, the KCNMA1 protein could control maternal vasoconstriction locally at the level of the placental bed. The functions of the proteins encoded by MAWBP, C10orf35, LOC142893, C10orf11 and LOC142955 are unknown. Although the recent burst of data from the Fantom database (Kiyosawa et al., 2003; Nikaido et al., 2003; Numata et al., 2003) will enhance the continuing search for novel imprinted genes, it must be stressed that many transcripts are likely to exist with differential expression between andro- and parthenogenetic embryos not because they are imprinted, but because it is difficult or impossible to match these uniparental embryos developmentally. In this respect, we feel that our approach, which combines bioinformatics, genetic linkage and functional analysis, is a powerful addition or alternative for this purpose. Guided by shared sequence characteristics and complemented by data from the imprinting databases, the bioinformatics data can be genetically supported by linkage analysis and experimentally

Figure 5. Epigenetic model of pre-eclampsia. Pre-eclampsia model based on epigenetic transmission of a genetic heterozygous variation that becomes phenotypically dominant and functionally mutant in the placenta by preferential expression of maternally inherited alleles. In families (left pedigree structure indicated with grandmaternal origin), where the mother as well as the affected sib pairs in the second and third generations have pre-eclamptic pregnancies (dark circles), the mutant allele (A, marked in red) is derived from the grandmother. In families (right pedigree structure indicated with grandpaternal origin), where the mothers in the second generation had normal pregnancies (open circles), while the affected daughters in the third generation have pre-eclampsia (dark circles), the mutant allele (G, marked in red) is derived from the grandfather. In the latter families, due to resetting of the imprint during maternal germline transmission, the grandpaternal allele (G) becomes maternally marked, but remains silent (no pre-eclampsia) in daughters of the second generation, and maternally marked, but active (pre-eclampsia) in daughters of the third generation. In this model, the epigenetic defect occurs within the placenta (indicated in yellow). plac = placenta; mut = mutation.
verified by expression analysis of uniparental tissues as shown in this study. Although our approach was targeted towards identification of maternally expressed imprinted genes only, the same strategy can be applied towards the identification of paternally expressed imprinted genes and applied to other organs.

Finally, human chromosome 10 contains two sets of developmentally co-repressed genes with non-random chromosomal distribution that share similarities with a recent observation in Drosophila cells (Greil et al., 2003). The induction and maintenance of patterns of gene expression during development involves a hierarchical higher-order phenomenon with the recruitment of heterochromatin protein complexes with histone methyltransferase properties that associate with specific sets of developmentally co-repressed genes (Greil et al., 2003). This stable epigenetically form of gene silencing classically known as position effect variegation (PEV) involves euchromatin as well. The human SUV39H1 protein is one of the essential heterochromatin proteins involved (Greil et al., 2003) and is located on the X chromosome, which is particularly important for mammalian extraembryonic development (Hemberger, 2002).

Acknowledgements
Special thanks to Drs Pieters, Van De Berk, Janssens, de Leeuw and Tjou, the gynecologists of 21 hospitals and all families for their enthusiastic participation. This work is supported by grants NWO 95-10-612, HRDC 28-2593 and KWF VU-99-1993 (B.A.W.).

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Submitted on April 16, 2004; resubmitted on May 25, 2004; accepted on May 31, 2004