JMG Online First, published on August 3, 2010 as 10.1136/jmg.2010.079343

Parthenogenetic chimaerism/mosaicism with a Silver-Russell syndrome-like phenotype

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Received 20 March 2010
Revised 6 May 2010
Accepted 6 May 2010

ABSTRACT

Introduction We report a 34-year-old Japanese female with a Silver-Russell syndrome (SRS)-like phenotype and a mosaic Turner syndrome karyotype (45,X/46,XX).

Methods/Results Molecular studies including methylation analysis of 17 differentially methylated regions (DMRs) on the autosomes and the *XIST*-DMR on the X chromosome and genome-wide microsatellite analysis for 96 autosomal loci and 30 X chromosomal loci revealed that the 46,XX cell lineage was accompanied by maternal uniparental isodisomy for all chromosomes (upid(AC)mat), whereas the 45,X cell lineage was associated with biparentally derived autosomes and a maternally derived X chromosome. The frequency of the 46,XX upid(AC)mat cells was calculated as 84% in leukocytes, 56% in salivary cells, and 18% in buccal epithelial cells.

Discussion The results imply that a parthenogenetic activation took place around the time of fertilisation of a sperm missing a sex chromosome, resulting in the generation of the upid(AC)mat 46,XX cell lineage by endoreplication of one blastomere containing a female pronucleus and the 45,X cell lineage by union of male and female pronuclei. It is likely that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of SRS phenotype, but not for the occurrence of other imprinting disorders or recessive Mendelian disorders.

Although a mammal with maternal uniparental disomy for all chromosomes (upd(AC)mat) is incompatible with life because of genomic imprinting,[1] a mammal with a upd(AC)mat cell lineage could be viable in the presence of a co-existing normal cell lineage. In the human, Strain *et al*[2] have reported 46,XX peripheral blood cells with maternal uniparental isodisomy for all chromosomes (upid(AC)mat) in a 1.2-year-old phenotypically male patient with aggressive behaviour, hemifacial hypoplasia and normal birth weight. Because of the 46,XX disorders of sex development, detailed molecular studies were performed, revealing the presence of a normal 46,XY cell lineage in a vast majority of skin fibroblasts and a upid(AC)mat 46,XX cell lineage in nearly all blood cells. In addition, although the data are insufficient to draw a definitive conclusion, Honke *et al*[3] have also identified 46,XX peripheral blood cells with possible upd(AC)mat in a phenotypically male patient through methylation analyses for plural differentially methylated regions (DMRs) in 11 patients with Silver–Russell syndrome (SRS)-like phenotype. This patient was found to have a normal 46,XY cell lineage and a triploid 69,XXY cell lineage in skin fibroblasts.

However, such patients with a upd(AC)mat cell lineage remain extremely rare, and there is no report describing a human with such a cell lineage in the absence of a normal cell lineage. Here we report a female patient with a upid(AC)mat 46,XX cell lineage and a non-upd 45,X cell lineage who was identified through genetic screenings of 105 patients with SRS-like phenotype.

MATERIALS AND METHODS

Case report

This Japanese female patient was conceived naturally and born at 40 weeks of gestation by a normal vaginal delivery. At birth, her length was 44.0 cm (−3.1 SD), her weight 2.1 kg (−2.9 SD) and her occipitofrontal head circumference (OFC) 30.5 cm (−2.3 SD). The parents and the younger brother were clinically normal (the father died from a traffic accident).

At 2 years of age, she was referred to us because of growth failure. Her height was 77.7 cm (−2.5 SD), her weight 8.45 kg (−2.6 SD) and her OFC 43.5 cm (−2.5 SD). Physical examination revealed several SRS-like somatic features such as triangular face, right hemihypoplasia and bilateral fifth finger clinodactyly. She also had developmental retardation, with a developmental quotient of 56. Endocrine studies for short stature were normal as were radiological studies. Cytogenetic analysis using lymphocytes indicated a low-grade mosaic Turner syndrome (TS) karyotype, 45,X[3]/46,XX[47]. Thus, a screening of TS phenotype[4] was performed, detecting horseshoe kidney but no body surface features or cardiovascular lesion. Chromosome analysis was repeated at 6 and 32 years of age using lymphocytes, revealing a 45,X[8]/46,XX[92] karyotype and a 45,X[12]/46,XX[88] karyotype, respectively. On the last examination at 34 years of age, her height was 125.0 cm (−6.2 SD), her weight 37.5 kg (−2.0 SD) and her OFC 51.2 cm (−2.8 SD). She was engaged in a simple work and was able to get on her daily life for herself.

Sample preparation

This study was approved by the Institutional Review Board Committees at National Center for Child health and Development. After obtaining written informed consent, genomic DNA was extracted from leukocytes of the patient, the mother and the brother and from salivary cells which comprise ~40% of buccal epithelial cells and ~60% of leukocytes[5] of the patient. Lymphocyte metaphase spreads and leukocyte RNA were also

obtained from the patient. Leukocytes of healthy adults and patients with imprinting disorders were utilised for controls.

Primers and probes

The primers utilised in this study are summarised in supplementary methods and supplementary tables 1–3.

DMR analyses

We first performed bio-combined bisulfite restriction analysis (COBRA)[6] and bisulfite sequencing of the *H19*-DMR (A) on chromosome 11p15.5 by the previously described methods[7] and methylation-sensitive PCR analysis of the *MEST*-DMR (A) on chromosome 7q32.2 by the previously described methods[8] with minor modifications (the methylated and unmethylated allele-specific primers were designed to yield PCR products of different sizes and the PCR products were visualised on the 2100 Bioanalyzer (Agilent, Santa Clara, California, USA)). This was because hypomethylation (epimutation) of the normally methylated *H19*-DMR of paternal origin and maternal uniparental disomy 7 are known to account for 35–65% and 5–10% of SRS patients, respectively.[9 10] In addition, fluorescence in situ hybridisation (FISH) analysis was performed with a ~84-kb RP5-998N25 probe containing the *H19*-DMR (BACPAC Resources Center, Oakland, California, USA). We also examined multiple other DMRs by bio-COBRA. The ratio of methylated clones (the methylation index) was calculated using peak heights of digested and undigested fragments on the 2100 Bioanalyzer using 2100 expert software.

Genome-wide microsatellite analysis

Microsatellite analysis was performed for 96 autosomal loci and 30 X chromosomal loci. The segment encompassing each locus was PCR-amplified, and the PCR product size was determined on the ABI PRISM 310 autosequencer using GeneScan software (Applied Biosystems, Foster City, California, USA).

PCR analysis for Y chromosomal loci

Standard PCR was performed for six Y chromosomal loci. The PCR products were electrophoresed using the 2100 Bioanalyzer.

Expression analysis

Quantitative real-time reverse transcriptase PCR analysis was performed for three paternally expressed genes (*IGF2*, *SNRPN* and *ZAC1*) and four maternally expressed genes (*H19*, *MEG3*, *PHLDA2* and *CDKN1C*) that are known to be variably (usually weakly) expressed in leukocytes (UniGene, http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene), using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). *TBP* and *GAPDH* were utilised as internal controls.

RESULTS

DMR analyses

In leukocytes, the bio-COBRA indicated severely hypomethylated *H19*-DMR, and bisulfite sequencing combined with rs2251375 SNP typing for 50 clones revealed maternal origin of 29 hypomethylated clones and non-maternal (paternal) origin of a single methylated clone in this patient (figure 1A). Thus, the marked hypomethylation of the *H19*-DMR was caused by predominance of maternally derived clones rather than hypomethylation of the *H19*-DMR of paternal origin. FISH analysis for 100 lymphocyte metaphase spreads excluded an apparent deletion of the paternally derived *H19*-DMR or duplication of the maternally derived *H19*-DMR (Supplementary figure 1). Methylation-sensitive PCR amplification for the *MEST*-DMR delineated a major peak for the methylated allele and a minor peak for the unmethylated allele (figure 1B). This also indicated the predominance of maternally derived clones and the co-existence of a minor portion of paternally derived clones. Furthermore, autosomal DMRs invariably exhibited markedly abnormal methylation patterns consistent with predominance of maternally inherited DMRs, whereas the methylation index of the *XIST*-DMR on the X chromosome remained within the female reference range (figure 1C). The abnormal methylation patterns were less obvious in salivary cells (thus, in buccal epithelial cells) than in leukocytes, except for the methylation index for the *XIST*-DMR that mildly exceeded the female reference range (figure 1A–C).

Microsatellite analysis

Major peaks consistent with maternal uniparental isodisomy and minor peaks of non-maternal (paternal) origin were identified for at least one locus on each autosome, with the minor peaks of non-maternal origin being more obvious in salivary cells than in leukocytes (figure 1D and supplementary table 4). Furthermore, the frequency of the upid(AC)mat cells was calculated as 84% in leukocytes, 56% in salivary cells and 18% in epithelial buccal cells, using the area under curves for the maternally and the non-maternally inherited peaks (supplementary note). Such minor peaks of non-maternal origin were not detected for all the 30 X chromosomal loci examined.

PCR analysis for Y chromosomal loci

PCR amplification failed to detect any trace of Y chromosome-specific bands in leukocytes and salivary cells (Supplementary figure 2).

Expression analysis

Expression analysis using control leukocytes indicated that, of the seven examined genes, *SNRPN* expression alone was strong enough to allow for a precise assessment (Supplementary figure 3). *SNRPN* expression was extremely low in this patient (figure 1E).

DISCUSSION

These results imply that this patient had a upid(AC)mat 46,XX cell lineage and a non-upd 45,X cell lineage. Indeed, methylation patterns of the *XIST*-DMR is explained by assuming that the two X chromosomes in the upid(AC)mat cells undergo random X-inactivation and that 45,X cells with the methylated *XIST*-DMR on a single active X chromosome[11] are relatively prevalent in buccal epithelial cells. Furthermore, lack of non-maternally derived minor peaks for microsatellite loci on the X chromosome is explained by assuming that the two X chromosomes in the upid(AC)mat cells and the single X chromosome in the 45,X cells are derived from a common X chromosome of maternal origin, with no paternally derived sex chromosome. It is likely, therefore, that a parthenogenetic activation took place around the time of fertilisation of a sperm missing a sex chromosome, resulting in the generation of the 46,XX cell lineage with upid(AC)mat by endoreplication (the replication of DNA without the subsequent completion of mitosis) of one blastomere containing a female pronucleus and the 45,X cell lineage with biparentally derived autosomes and a maternally derived X chromosome by union of male and female pronuclei (figure 2), although it is also possible that a paternally derived sex chromosome was present in the sperm but was lost from the normal

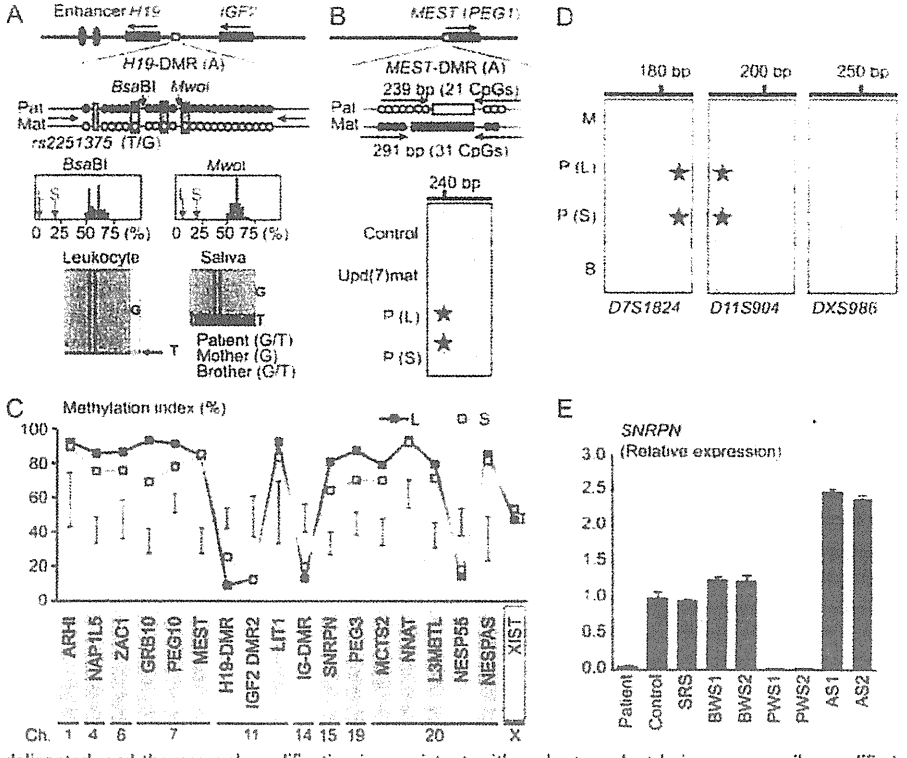

Figure 1 Representative molecular results. Pat, paternally derived allele; Mat, maternally derived allele; P, patient; M, mother; B, brother; L, leukocytes; and S, salivary cells. Filled and open circles in A and B represent methylated and unmethylated cytosine residues at the CpG dinucleotides, respectively. A. Methylation patterns of the *H19*-DMR (A) harbouring 23 CpG dinucleotides and the T/G SNP (*rs2251375*) (a grey box). The PCR products are digested with *Bsa*BI when the cytosine at the sixth CpG dinucleotide (highlighted in yellow) is methylated and with *Mwo*I when the two cytosines at the ninth and the 11th CpG dinucleotides (highlighted in orange) are methylated. For the bio-COBRA data, the black histograms represent the distribution of methylation indices (%) in 50 control participants, and L and S denote the methylation indices for leukocytes and salivary cells of this patient, respectively. For the bisulfite sequencing data, each line indicates a single clone. B. Methylated and unmethylated allele-specific PCR analysis for the *MEST*-DMR (A). In a control participant, the PCR products for methylated and unmethylated alleles are delineated, and the unequal amplification is consistent with a short product being more easily amplified than a long product. In a previously reported patient with upd(7)mat,[8] the methylated allele only is amplified. In this patient, major peaks for the methylated allele and minor peaks for the unmethylated allele (red asterisks) are detected. C. Methylation patterns for the 18 DMRs examined. The DMRs highlighted in blue and pink are methylated after paternal and maternal transmissions, respectively. The black vertical bars indicate the reference data (maximum–minimum) in 20 normal control participants, using leukocyte genomic DNA (for the *XIST*-DMR, 16 female data are shown). D. Representative microsatellite analysis. Minor peaks (red asterisks) have been identified for *D7S1824* and *D11S904* but not for *DXS986* of the patient. Since the peaks for *D7S1824* and *D11S904* are absent in the mother and clearly present in the brother, they are assessed to be of paternal origin. E. Relative expression level (mean ± SD) of *SNRPN* on chromosome 15. The data have been normalised against *TBP*. SRS, an SRS patient with an epimutation (hypomethylation) of the *H19*-DMR; BWS1, a BWS patient with an epimutation (hypermethylation) of the *H19*-DMR; BWS2, a BWS patient with upd(11)pat; PWS1, a PWS patient with upd(15)mat; PWS2, a PWS patient with an epimutation (hypermethylation) of the *SNRPN*-DMR; AS1, an Angelman syndrome (AS) patient with upd(15)pat; and AS2, an AS patient with an epimutation (hypomethylation) of the *SNRPN*-DMR.

cell lineage at the very early developmental stage. Hence, in a strict sense, this patient is neither a chimera resulting from the fusion of two different zygotes nor a mosaic caused by a mitotic error of a single zygote. In this regard, a triploid cell stage is assumed in the generation of a upid(AC)mat cell lineage, and such triploid cells may have been detected in skin fibroblasts of the patient reported by Horike *et al*.[7]

The upid(AC)mat cells accounted for the majority of leukocytes even in adulthood of this patient, despite global negative selective pressure.[12 15] This phenomenon, though intriguing, would not be unexpected in human studies because leukocytes are usually utilised for genetic analyses. Rather, if the upid(AC)mat cells were barely present in leukocytes, they would not have been detected. It is likely, therefore, that upid(AC)mat cells have occupied a relatively large portion of the definitive haematopoietic tissues primarily as a stochastic event. Furthermore, parthenogenetic chimera mouse studies have revealed that parthenogenetic cells are found at a relatively high frequency in some tissues/organs including blood and are barely identified in other tissues/organs such as skeletal muscle and liver.[15] Such a possible tissue-specific selection in favour of the preservation of parthenogenetic cells in the definitive haematopoietic tissues may also be relevant to the predominance of the upid(AC)mat cells in leukocytes. In addition, a reduced growth potential of 45,X cells[16] may also have contributed to the skewed ratio of the two cell lineages.

Clinical features of this patient would be determined by several factors. They include: (1) the ratio of two cell lineages in various tissues/organs, (2) the number of imprinted regions or DMRs relevant to the development of specific imprinting disorders (eg, plural regions/DMRs on chromosomes 7 and 11 for SRS[9 10] and a single region/DMR on chromosome 15 for Prader–Willi syndrome (PWS)),[17] (3) the degree of clinical effects of dysregulated imprinted regions/DMRs (an (epi)dominant effect has been

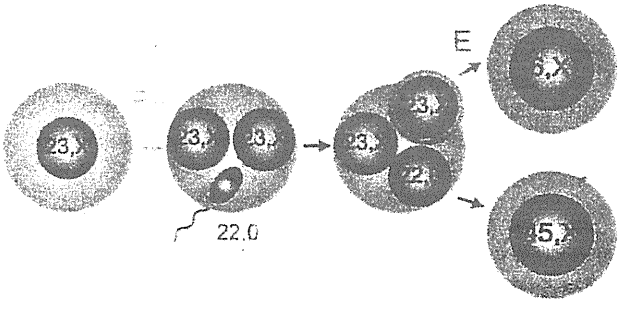

Figure 2 Schematic representation of the generation of the upid(AC)mat 46,XX cell lineage and the non-upd 45,X cell lineage. Polar bodies are not shown. PA, parthenogenetic activation; and E, endoreplication of one blastomere containing a female pronucleus.

assumed for the 11p15.5 imprinted regions including the *IGF2–H19* domain on the basis of SRS or Beckwith–Wiedemann syndrome (BWS) phenotype in patients with multilocus hypomethylation[16] and BWS-like phenotype in patients with a upid (AC)pat cell lineage,[17] a mirror image of a upid(AC)mat cell lineage), (4) expression levels of imprinted genes in upid(AC)mat cells (although *SNRPN* expression of this patient was consistent with upid(AC)mat cells being predominant in leukocytes, complicated expression patterns have been identified for several imprinted genes in androgenetic and parthenogenetic fetal mice, probably because of perturbed *cis*- and *trans*-acting regulatory mechanisms)[18] and (5) unmasking of possible maternally inherited recessive mutation(s) in upid(AC)mat cells.[19] Collectively, it appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of SRS phenotype and horseshoe kidney characteristic of TS[4] but remained below the threshold level for the occurrence of other imprinting disorders or recessive Mendelian disorders.

In summary, we identified a upid(AC)mat 46,XX cell lineage in a woman with an SRS-like phenotype and a 45,X cell lineage accompanied by autosomal haploid sets of biparental origin. This report will facilitate further identification of patients with a upid(AC)mat cell lineage and better clarification of the clinical phenotypes in such patients.

Acknowledgements We thank the patient and her family members for their participation in this study. We also thank Dr. Toshiro Nagai for providing us with blood samples of patients with Prader–Willi syndrome.

Funding This work was supported by grants from the Ministry of Health, Labor, and Welfare and from the Ministry of Education, Science, Sports and Culture.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Institutional Review Board Committees at National Center for Child health and Development.

Contributors Drs Kazuki Yamazawa (first author) and Kazuhiko Nakabayashi (second author) contributed equally to this work.

Provenance and peer review Not commissioned; externally peer reviewed.

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Obesity Research & Clinical Practice (2010) 4, e333–e337

ELSEVIER

SHORT REPORT

High cardiovascular risk factors among obese children in an urban area of Japan☆

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Received 6 July 2010; received in revised form 12 August 2010; accepted 12 August 2010

KEYWORDS
Obesity;
Childhood;
Cardiovascular risk factor;
Metabolic risk factor

Summary The association between degree of obesity and cardiovascular and related metabolic risk factors were examined in 355 Japanese obese school children from 11 to 12 years old. The parameters evaluated were blood pressure, serum lipids, fasting blood glucose, and serum ALT and AST. ALT, AST and triglycerides were more commonly evaluated in obese boys than in obese girls, while HDL-cholesterol was more commonly lowered in obese girls. Hypercholesterolemia was 2-fold, and abnormal liver functions were 3-fold more common in severely obese than in moderate obese children. Thus, cardiovascular and related metabolic risk factors are present in obesity in school-aged children, particularly in boys.

☆ Grant support: Supported by grants from the Showa Women's University Research Fund.
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1871-403X/$ – see front matter
doi:10.1016/j.orcp.2010.08.004

Introduction

There is growing concern over the worldwide increase in childhood obesity [1]. In Japan, the prevalence of obesity in children of primary school age increased by almost 1.7-fold between 1977 and 2005 [2]. In particular, the increase in severe obesity has attracting attention in view of the risk of future health problems. In recent years, the relationships between childhood obesity and cardiovascular and related metabolic risk factors have been discussed widely in Japan, and evaluation of accumulated epidemiological data suggests that further clinical studies are required. It has been established that childhood obesity is at an increased risk of adulthood obesity [3,4], with resultant high morbidity and mortality [5,6]. It is likely that this risk will be elevated if cardiovascular and metabolic risk factors are already present in childhood.

Several studies have shown an association of childhood obesity with cardiovascular and related metabolic risk factors [7–11]. However, the relationships of the degree of obesity, sex and age with cardiovascular and metabolic risk factors have yet to be clarified. In the present study, the subjects were limited to children aged 11–12 years old who were examined in the Setagaya Lifestyle-related Diseases Prevention and Screening Project, which is targeted at school children of this age. For stratification of obesity, in the previous studies has commonly used categories of moderate or severe. However, since we considered this may be too rough to provide an estimate for determining the associations of the degree of obesity with cardiovascular and related metabolic risk factors. We therefore chose to stratify subjects into 3 obesity categories in the present study.

Methods

Overview

The 'Setagaya Lifestyle-related Diseases Prevention and Screening Project' was started in 1978. The project is supported by the Setagaya Medical Association and Board of Education, and has the goal of promoting early treatment of obese children attending public schools in Setagaya ward, which is a relatively populous part of Tokyo. There were 20,953 public primary schoolchildren in Setagaya ward in 2000, and 21,052 in 2002. The prevalence of childhood obesity, (defined as body weight exceeding the standard weight by 30%), was essentially stable from 2000 to 2002 (4.9% and 4.7%, respectively), but the proportion of severely obese children in the ward increased from 18.0% to 19.2% from 2000 to 2002.

Participants

The subjects in the present study were recruited by the Setagaya Lifestyle-related Diseases Prevention and Screening Project in 2000 and 2002. Medical check-ups (including blood tests) were performed as part of the project, which was targeted mainly at 5th and 6th grade schoolchildren with obesity. Our data were obtained from 355 volunteers (235 boys and 120 girls) out of 917 obese children aged 11–12 years old. None of the subjects were receiving medical treatment for diseases related to obesity. The study protocol was approved by the Setagaya Medical Association and Board of Education. Informed consent was obtained from the children's parents.

Measurements

Weight and height were measured with a digital scale by a school nurse at each school, in accordance with methods recommended by the Japanese government. The degree of obesity was estimated using the obesity criteria of Murata's monogram [12]. These criteria rely on a weight-for-height by sex-and-age chart for Japanese children derived from a nationwide school health survey, and constitute conventional criteria for Japanese children. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and circulating metabolic parameters including fasting blood glucose (FBG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were also examined. Cut-off points for abnormal blood pressure and serum factors were defined according to the criteria of the Setagaya Medical Association: >149 mm Hg for systolic blood pressure (SBP), >89 mm Hg for diastolic blood pressure (DBP), >109 mg/dL for FBG, >200 mg/dL for TC, <35 mg/dL (boys) or <41 mg/dL (girls) for HDL-C, >170 mg/dL for triglycerides, >36 IU/L for AST, and >35 IU/L for ALT.

Data analysis

We categorized the subjects into 3 obesity groups based on the percentage relative body weight: 30–39% (moderate I), 40–49% (moderate II), and ≥50% (severe). A χ^2 test was used for comparison of proportions. Logistic regression models were used to estimate odds ratios (ORs) with corresponding 95% confidential intervals (CIs) for each risk fac-

Table 1 Prevalence of cardiovascular and metabolic risk factors and degree of obesity among participants in the Setagaya Lifestyle-related Disease Prevention Project (2000–2002).

	All	Boys	Girls	p^a	All			p^b	Boys			p^b	Girls			p^b
	$n=355$	$n=235$	$n=120$		Degree of obesity				Degree of obesity				Degree of obesity			
					Moderate I $n=223$	Moderate II $n=75$	Severe $n=57$		Moderate I $n=136$	Moderate II $n=57$	Severe $n=42$		Moderate I $n=87$	Moderate II $n=18$	Severe $n=15$	
Blood pressure	0.3	0.4	0.0		0.5	0.0	0.0		0.7	0.0	0.0		0.0	0.0	0.0	
FBG	5.7	5.6	5.9		6.4	5.3	3.6		6.7	5.3	2.4		5.8	5.6	7.1	
TC	17.5	17.4	17.5		14.8	18.7	26.3		14.0	15.8	31.0	*	16.1	27.8	13.3	
HDL-C	3.9	1.3	9.2	**	4.5	2.7	3.5		1.5	1.8	0.0		9.2	5.6	13.3	
TG	23.1	26.8	15.8	*	22.9	32.0	12.3	*	27.2	33.3	16.7		16.1	27.8	0.0	
AST	6.8	8.9	2.5	*	4.9	8.0	12.3		6.6	10.5	14.3		2.3	0.0	6.7	
ALT	12.4	16.6	4.2	***	8.1	14.7	26.3	*	12.5	19.3	26.2		1.1	0.0	26.7	**
Liver abnormality[c]	13.2	17.4	5.0	***	9.0	14.7	28.1	*	13.2	21.1	26.2		2.3	0.0	26.7	**
≥2 complications[d]	14.6	17.4	9.2	*	10.8	20.0	22.8	*	13.2	19.3	28.6		6.9	16.7	13.3	

This table shows numbers in percentage.

[a] p values in sex (boys vs. girls).

[b] p values in degree of obesity.

[c] Estimated by AST and/or ALT.

[d] Counted from blood pressure 6 serum biochemistry items.

* $p<0.05$.

** $p<0.01$.

*** $p<0.001$.

Table 2 Association of cardiovascular and metabolic risk factors and degree of obesity among participants in the Setagaya Lifestyle-related Disease Prevention Project (2000–2002).

Degree of obesity	Adjusted OR (95%CI)			
	FBG	TC	HDL-C	TG
Moderate I	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Moderate II	0.75 (0.24–2.37)	1.20 (0.59–2.44)	0.70 (0.15–3.35)	1.40 (0.78–2.52)
Severe	0.47 (0.10–2.12)	2.16 (1.08–4.30)*	1.28 (0.33–4.98)	0.40 (0.17–0.94)*
Degree of obesity	Adjusted OR (95%CI)			
	AST	ALT	Liver abnormality[a]	≥2 complications[b]
Moderate I	1.00 (ref)	1.00 (ref)	1.00 (ref)	1.00 (ref)
Moderate II	1.13 (0.37–3.42)	1.50 (0.65–3.47)	1.40 (0.61–3.24)	1.59 (0.77–3.23)
Severe	2.59 (0.97–6.90)	3.51 (1.61–7.63)**	3.62 (1.69–7.75)**	1.97 (0.93–4.18)

Odds ratio was adjusted by sex and age.
Blood pressure is not analyzed because of small number of cases.
[a] Estimated by AST and/or ALT.
[b] Counted from blood pressure 6 serum biochemistry items.
* $p<0.05$.
** $p<0.01$.

tor relative to each level of obesity, controlling for sex and age. All statistical analyses were performed using SPSS 14.0J for Windows. p value of <0.05 was considered to indicate a significant difference.

Results

Almost all the subjects (99.7%) had normal blood pressure. Approximately half (50.1%) had one abnormal serum parameter and 14.9% had at least 2 abnormalities among high blood pressure, hyperglycemia, hypercholesterolemia, low HDL-C, hypertriglyceridemia, and indicators of liver abnormality (elevated ALT and AST). The associations of the prevalence of cardiovascular and metabolic risk factors with sex are shown in Table 1. Elevated ALT and AST (indicating abnormal liver function), and elevated TG were significantly more common in boys than in girls, whereas lower HDL-C was significantly more frequent in girls. A significantly higher prevalence of at least 2 risk factors was observed in obese boys compared to obese girls.

Associations of each risk factor with the relative body weight are shown in Table 2. For FBG, OR tended to decrease with an increase of obesity, but the difference did not reach statistical significance. The risk of hypertriglyceridemia decreased in severely obese subjects (OR = 0.40). Severe obesity was also associated with a higher risk of hypercholesterolemia (OR = 2.16), and liver dysfunction (OR = 3.62). The OR for a risk of more than 2 abnormalities tended to gradually increase with an increase in obesity, but the differences among the categories of obesity were not significant.

Discussion

Our results showed a higher prevalence of elevated ALT and AST in obese boys compared to in obese girls, which suggest the presence of non-alcoholic fatty liver disease (NAFLD) in obese boys [13,14]. An increased prevalence of abnormal TG was also observed in obese boys. These results are consistent with the previous reports, showing high rates of cardiovascular and related metabolic disorders in childhood obesity [7–11].

We classified the subjects into 3 categories based on the percentage relative body weight. Moderate obesity (30–50%) was stratified into moderate I (30–39%) and moderate II (40–49%), and the third category was defined as severe (≥50%). To our knowledge, the characteristics of subjects with 40–49% obesity have not been examined previously. Our results suggest that liver-related risk factors (elevated ALT and AST) are more common in severely obese children, particularly in boys. Clustering of coronary risk factors including obesity, hyperglycemia, dyslipidemia and hypertension is defined as a metabolic syndrome in childhood [15,16], and this is a high risk condition for coronary heart disease in adulthood. To our surprise, more than 2 risk factors were already associated with in severely obese children particularly in boys. The finding of multiple associations of coronary risk factors with obesity in the present study is consistent with this syndrome.

In the present study, we found that severely obese children were more prone to have coronary and related metabolic risk factors, with this being

particularly apparent in boys. This may be due to multiple phenomena [17], including early adiposity rebound, in which BMI (body fat) rises during infancy, then declines to a nadir at 5–6 years of age, and then begins to accelerate again [18,19]; and nutritional transition, in which increased availability of food and reduced physical activity lead to increased obesity [19].

The interpretation of the results of the present study has several limitations. First, the participants were volunteers who accounted for 38.7% of the targeted obese children. Therefore, this may have produced a bias in our observation of the cardiovascular risk factors among obese children in Setagaya ward. Second, the number of metabolic parameters evaluated was limited due to financial constraints. In the Setagaya Lifestyle-related Diseases Prevention and Screening Project, several metabolic parameters have been added to the medical check-up (abdominal circumference, body fat, HbA1c) with revision of the cut-off points, and we have also extended the target population of children since 2006. This study is ongoing and we expect to improve the reliability of the findings.

In conclusion, coronary and metabolic risk factors are already evident in school-aged obese children particularly and in boys. Early detection and intervention is necessary for obese children to prevent the progression of metabolic disorders, which result in increased morbidity and mortality associated with obesity in adulthood.

Conflict of interest

None

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Hypertension Research (2011), 1–6

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ORIGINAL ARTICLE

Prediction of pregnancy-induced hypertension by a shift of blood pressure class according to the JSH 2009 guidelines

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Elevated blood pressure (BP) at early or mid pregnancy is a known risk factor for pregnancy-induced hypertension (PIH). However, the association between BP changes during the first half of pregnancy and subsequent PIH development is unknown. We used changes in maternal BP between 16 and 20 weeks of gestation to evaluate the risk of PIH. A total of 976 pregnant women with BP estimations recorded before 16 weeks and at 20 weeks of gestation participated in this study. BPs were classified by the Japanese Society of Hypertension 2009 Hypertension Treatment Guidelines (JSH 2009). There was a significant trend for future PIH in women whose JSH 2009 BP class increased between 16 and 20 weeks of gestation, and the risk of PIH was highest among women whose BP was Class IV Hypertension (systolic BP ⩾ 140 mm Hg and/or diastolic BP ⩾ 90 mm Hg). The risk of PIH increased in women whose BPs shifted from Classes I Optimal (systolic BP < 120 mm Hg and diastolic BP < 80 mm Hg) and II Normal (systolic BP 120–129 mm Hg and/or diastolic BP 80–84 mm Hg) before 16 weeks to Class III High-Normal (systolic BP 130–139 mm Hg and/or diastolic BP 85–89 mm Hg) at 20 weeks of gestation. These shifts in BP class were significantly correlated with the risk of PIH after adjustments for variables (*P*-value for trend < 0.05). Within JSH 2009 Classes I, II and III, a shift in BP from a low to a high class between 16 and 20 weeks of gestation predicts the subsequent development of PIH.
Hypertension Research advance online publication, 28 July 2011; doi:10.1038/hr.2011.107

Keywords: blood pressure; prediction; pregnancy-induced hypertension; risk factor

INTRODUCTION

Pregnancy-induced hypertension (PIH) refers to high blood pressure (BP) during pregnancy. PIH affects 3–10% of all pregnancies[1–3] and is associated with high levels of maternal, fetal, and neonatal morbidity and mortality.[1,4,5] Furthermore, the long-term prognosis of women with a history of PIH includes increased risks of cerebrovascular disease, ischemic heart disease and renal disease.[6–12] These data indicate that the early identification, and subsequent monitoring and management of PIH are critical for maternal and fetal well-being.

In normotensive women, BP in early pregnancy decreases up to 20 weeks of gestation, and gradually increases to normal or higher than pre-pregnancy levels before delivery.[13,14] A diagnosis of PIH includes a BP > 140/90 mm Hg in the late second or third trimester. Previous studies described successful screening for PIH development following a single estimation of maternal BP. However, the false positive rate and sensitivity of these studies varied widely, from 7 to 52% and 8 to 93%,[15–19] respectively, indicating that this method is not sufficient for effective PIH prediction. In contrast, systematic monitoring of changes in BP during the early to mid stages of pregnancy may predict the development of PIH more exactly. Systematic sampling with 48-h ambulatory BP monitoring indicated that PIH was associated with a stable BP in the first half of gestation and a greater increase to delivery than in healthy pregnancies.[20] In addition, the development of PIH in women with low education levels was related to the absence of a significant fall in diastolic BP at mid pregnancy compared with healthy pregnancies in women with higher education.[21] These data indicate that information describing the changes in BP during early to mid pregnancy may be more predictive of subsequent PIH development than data recorded at a single measurement.

In the present study, we examined whether BP changes from early to mid gestational age are capable of predicting the development of PIH. BP was classified according to the JSH 2009 for easy clinical use.[22]

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Received 17 March 2011; revised 1 May 2011; accepted 9 May 2011

METHODS

Study population and design

Our investigations are part of the Tokyo-Children's Health, Illness and Development Study. This is a unicenter, longitudinal, prospective observational birth cohort study conducted at the National Center for Child Health and Development (Tokyo, Japan). The aims of this cohort study are: (1) to investigate the influence of maternal weight gain and nutrition during pregnancy on the birth weight, growth and development of infants, (2) to identify the influence of maternal environment on the development of childhood allergies, (3) to study the influence of the prenatal and perinatal environment (fetus) on the psychological and psychiatric development of the infant, (4) to study the influence of parental attitude and knowledge about child rearing on the parent–child relationship and the development of children and (5) to determine the feasibility of using an electronic medical record system to conduct a birth cohort study. The participants in the Tokyo-Children's Health, Illness and Development Study were recruited at their first antenatal visit, before 16 weeks of gestation, from October 2003 to December 2005. Institutional review boards at the National Center for Child Health and Development approved our investigations.

We used the cohort data to analyze the relationship between BP changes during the first half of pregnancy and the onset of PIH. Our inclusion criteria accepted only participants with BP estimations recorded before 16 weeks and at 20 weeks of gestation (18–22 weeks of gestation), and who delivered at our institution after 22 weeks. Subjects with multiple gestations, pre-existing hypertension and pre-existing proteinuria were excluded. A total of 1019 women were initially included, from which 43 cases were excluded because of mismatched selection criteria and loss of information; therefore, data from 976 women were used in our analyses (Figure 1).

Measurement and classification of BP

After 5 min rest, BP was measured in the sitting position with the right arm held at heart level, using an automated sphygmomanometer (Omron BP-203RVIII oscillometer; Nippon Colin, Tokyo, Japan). BP monitoring was performed at two time points: before 16 weeks and between 18 and 22 weeks of gestation. If BP was measured on several occasions before 16 weeks, the average systolic and diastolic values were evaluated.

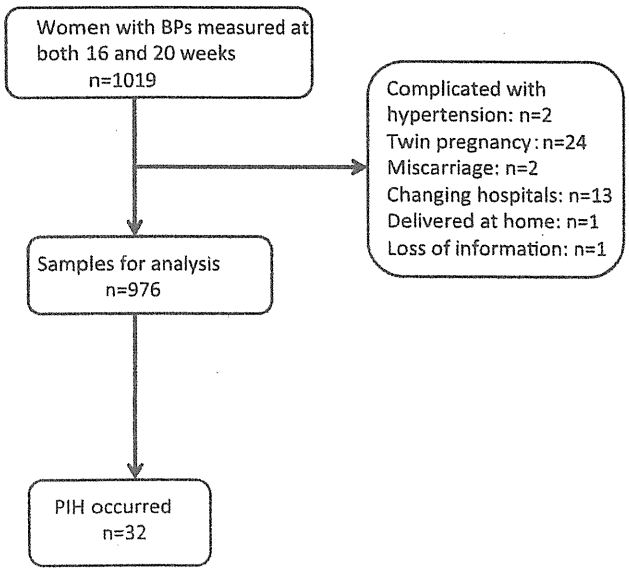

Figure 1 Flow diagram showing sample selection for our analyses. A total of 1019 pregnant women with BP estimations recorded before 16 weeks and at 20 weeks of gestation (18–22 weeks of gestation) were initially included in the study; 976 subjects were enrolled because of mismatched selection criteria and loss of information. Pregnancy-induced hypertension occurred in 32 women. BP, blood pressure; PIH, pregnancy-induced hypertension.

BPs were stratified into four groups based on Japanese Society of Hypertension 2009 Hypertension Treatment Guidelines (JSH 2009):[22] Class I (Optimal), systolic BP < 120 mm Hg and diastolic BP < 80 mm Hg; Class II (Normal), systolic BP 120–129 mm Hg and/or diastolic BP 80–84 mm Hg; Class III (High-Normal), systolic BP 130–139 mm Hg and/or diastolic BP 85–89 mm Hg; Class IV (Hypertension), systolic BP ≥ 140 or diastolic BP ≥ 90 mm Hg.

Definition of PIH

PIH was defined according to Guideline 2009 for care and treatment of hypertension in pregnancy by the Japan Society of the Study of Hypertension in Pregnancy[23] as: 'hypertension with or without proteinuria occurring after 20 weeks of gestation but resolving by twelve weeks postpartum.'. We excluded superimposed PIH, defined as: 'pre-existing hypertension with new onset of proteinuria after 20 weeks of gestation, or pre-existing proteinuria with new onset of hypertension.'

Other baseline data

Information describing sociodemographic, medical and behavioral data, past medical history, previous pregnancy complications, family history of hypertension or diabetes mellitus, smoking, education, family income and delivery were collected from the database of the cohort study.

Statistical analysis

Student's *t*-test and Mann–Whitney *U*-test were performed for analysis between two continuous variables, and χ^2-test or Fisher's exact test was used for discrete variables. The influence of BP class on the development of PIH was assessed by multiple logistic regression analysis. The probability of PIH occurrence was determined from the shifts in BP classes between 16 and 20 weeks of gestation and evaluated with odds ratios and *P*-values. All analyses were performed with the SPSS software (version 18 for Windows; SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Baseline patient characteristics are shown in Table 1. In 976 participants, 32 of the index pregnancies (3.3%) were eventually complicated with PIH. There were no significant differences in the gestational ages of participants at the times of BP monitoring. Maternal age, maternal pre-existing diabetes mellitus and previous history of PIH were significantly different between the PIH and non-PIH groups. Other variables (pre-pregnancy body mass index, rate of nulliparity, maternal pre-existing renal disease, previous pregnancy history of fetal growth restriction and placental abruption, rate of smoking, educational levels, distribution of family income, family history of diabetes mellitus, hypertension, ischemic heart disease, cerebrovascular stroke, chronic renal disease) were similar.

Pregnancy and delivery outcomes

Pregnancy and delivery outcomes in the non-PIH and PIH groups are shown in Table 2. The gestational age of delivery was significantly lower and the rate of preterm delivery was higher in the PIH group compared with non-PIH subjects. The rate of normal vaginal delivery was significantly lower and the rate of instrumental delivery was higher in the PIH group. The frequency of cesarean section (both planned and emergency) was similar between both groups. Placental weight and neonatal birth weight were significantly lower in the PIH group, and the number of neonates with an Apgar score of ≦7 at 5 min was significantly higher.

Analysis for PIH risk based on BP classification

Table 3 shows the crude and adjusted odds ratios, and confidence intervals of PIH occurrence according to class of BP (based on JSH

Table 1 Baseline characteristics of the PIH and non-PIH groups

Characteristics		*All (n=976)*	*PIH group (n=32)*	*non-PIH group (n=944)*	*P-value*
Maternal age (years)	mean (s.d.)	33.6 (4.1)	35.3 (4.5)	33.5 (4.1)	0.028
Maternal height (cm)	mean (s.d.)	159.4 (5.1)	158.7 (5.5)	159.4 (5.1)	NS
Maternal pre-pregnancy body weight (kg)	mean (s.d.)	51.3 (6.5)	52.0 (8.0)	51.3 (6.5)	NS
Pre-pregnancy BMI ($kg\,m^{-2}$)	mean (s.d.)	20.2 (2.4)	20.6 (2.4)	20.2 (2.4)	NS
Parity					
0	*n* (%)	489 (50.1)	19 (59.4)	470 (49.8)	NS
≧1	*n* (%)	487 (49.9)	13 (40.6)	474 (50.2)	
Mean gestational age before 16 weeks blood pressure	mean (s.d.)	14.3 (1.0)	14.3 (1.0)	14.2 (1.1)	NS
Mean gestational age at 20 weeks blood pressure	mean (s.d.)	20.0 (1.2)	20.0 (1.2)	20.1 (1.2)	NS
Maternal pre-pregnancy complications					
Diabetes mellitus	*n* (%)	6 (0.6)	2 (6.3)	4 (0.4)	0.014
Renal disease	*n* (%)	2 (0.2)	1 (3.1)	1 (0.1)	NS
Previous pregnancy complications					
PIH	*n* (%)	12 (1.2)	4 (12.5)	8 (0.8)	<0.001
Fetal growth restriction	*n* (%)	5 (0.5)	1 (3.1)	4 (0.4)	NS
Placental abruption	*n* (%)	2 (0.2)	1 (3.1)	1 (0.1)	NS
Smoking					
Never or former	*n* (%)	944 (96.9)	31 (96.9)	913 (96.9)	NS
Current	*n* (%)	30 (3.1)	1 (3.1)	29 (3.1)	
Education (high school or less)	*n* (%)	94 (10.1)	4 (12.9)	90 (10.0)	NS
Income (per year)					
<4 million yen	*n* (%)	55 (6.2)	3 (10.0)	52 (6.0)	NS
<6 million yen	*n* (%)	202 (22.6)	6 (20.0)	196 (22.7)	
<8 million yen	*n* (%)	198 (22.2)	9 (30.0)	189 (21.9)	
<10 million yen	*n* (%)	192 (21.5)	4 (13.3)	188 (21.8)	
over 10 million yen	*n* (%)	246 (27.5)	8 (26.7)	238 (27.6)	
Family History					
Diabetes mellitus	*n* (%)	73 (7.5)	0 (0.0)	73 (7.7)	NS
Hypertension	*n* (%)	72 (7.4)	4 (12.5)	68 (7.2)	NS
Ischemic heart disease	*n* (%)	38 (3.9)	1 (3.1)	37 (3.9)	NS
Cerebrovascular stroke	*n* (%)	18 (1.8)	0 (0.0)	18 (1.9)	NS
Chronic renal disease	*n* (%)	8 (0.8)	0 (0.0)	8 (0.8)	NS

Abbreviations: BMI, body mass index; NS, not significant; PIH, pregnancy-induced hypertension; s.d., standard deviation.

2009) before 16 weeks of gestation. Although the risk of PIH was significantly higher in Class III and IV subjects without adjustments for any variables, these risks became insignificant after variables were accounted for. However, the trend of PIH occurrence was statistically significant, regardless of any adjustments.

Table 4 demonstrates the crude and adjusted odds ratios of PIH occurrence based on BPs at 20 weeks of gestation. The risk of PIH was significantly greater in all Class II, III and IV subjects with or without adjustments for variables.

BP class shift from 16 weeks to 20 weeks gestation and the risk of PIH occurrence

Table 5 shows the shifts in BP classes between 16 and 20 weeks of gestation, and their associations with PIH occurrence. Odds ratios and 95% confidence intervals were calculated based on each BP class before 16 weeks of gestation. Women with Class IV (Hypertension) BPs before 16 or at 20 weeks of gestation were excluded, because they were already considered high risk for PIH as indicated in Tables 3 and 4.

The subjects with BPs that did not shift class between 16 and 20 weeks of gestation were referred to as baseline. The risk of PIH occurrence was significantly higher in subjects whose BP shifted from Class I at 16 weeks to Class III at 20 weeks of gestation. The risk of PIH occurrence was not statistically significant in subjects whose BP shifted from Class I to II at 16 and 20 weeks of gestation, respectively; however, the trend for PIH risk was significant (*P*-value for trend <0.05) and remained significant even after adjustments for all variables. When comparing two groups, one in which the BP class elevated to Class II or III at 20 weeks gestation and the other in which the BP class did not change, the sensitivity, false positive rate and positive predictive value of BP class elevation between 16 and 20 weeks of gestation were 33.3, 10.8 and 7.4%, respectively. The risk of PIH occurrence was significantly higher in subjects whose BP shifted from Class II at 16 weeks to Class III at 20 weeks of gestation, although this

Table 2 Pregnancy and delivery outcomes of the PIH and non-PIH groups

		All (n=976)	*PIH group (n=32)*	*non-PIH group (n=944)*	*P-value*
Gestational age (weeks)	mean (s.d.)	39.1 (1.8)	37.8 (1.9)	39.1 (1.7)	<0.001
<37	*n* (%)	59 (6.0)	11 (34.4)	48 (5.1)	<0.001
≧37	*n* (%)	917 (94.0)	21 (65.6)	896 (94.9)	
Stillbirth	*n* (%)	2 (0.2)	1 (3.1)	1 (0.1)	NS
Delivery mode					
Normal vaginal delivery	*n* (%)	614 (62.9)	11 (34.4)	603 (63.9)	<0.001
Instrumental delivery	*n* (%)	152 (15.6)	11 (34.4)	141 (14.9)	0.01
Total Cesarean section	*n* (%)	208 (21.3)	10 (31.3)	198 (21.0)	NS
Planned cesarean section	*n* (%)	124 (12.7)	6 (18.8)	118 (12.5)	NS
Emergency cesarean section	*n* (%)	84 (8.6)	4 (12.5)	80 (8.5)	NS
Placental weight (g)	mean (s.d.)	558.4 (104.6)	513.0 (100.9)	560.0 (104.4)	0.022
Birth weight (g)	mean (s.d.)	3003.6 (421.8)	2618.7 (538.7)	3016.6 (411.3)	<0.001
Head circumference (cm)	mean (s.d.)	33.1 (1.4)	32.4 (1.7)	33.2 (1.4)	0.007
Chest circumference (cm)	mean (s.d.)	31.5 (1.7)	30.0 (2.0)	31.6 (1.7)	<0.001
Apgar score at 5 min of ≦7	*n* (%)	18 (1.8)	3 (9.4)	15 (1.6)	0.02

Abbreviations: NS, not significant; PIH, pregnancy-induced hypertension; s.d., standard deviation.

Table 3 Unadjusted and multivariable adjusted ORs (95% CIs) of PIH occurrence classified by blood pressure before 16 weeks of gestation

	<16 weeks blood pressure OR (95% CI)				
Classification	*I*	*II*	*III*	*IV*	
n	*713*	*189*	*59*	*15*	
PIH	*18*	*7*	*5*	*2*	*P for trend*
Unadjusted	1	1.49 (0.61–3.61)	3.58 (1.28–10.00)	5.94 (1.25–28.28)	0.003
Age adjusted	1	1.51 (0.62–3.68)	3.25 (1.15–9.19)	5.15 (1.07–24.90)	0.006
Age+BMI adjusted	1	1.52 (0.62–3.71)	3.26 (1.12–9.54)	5.17 (1.03–25.94)	0.008
age+BMI+parity adjusted	1	1.51 (0.62–3.71)	3.13 (1.06–9.21)	5.59 (1.09–28.60)	0.009
Age+BMI+pre-existing DM adjusted	1	1.55 (0.63–3.83)	3.25 (1.08–9.77)	5.81 (1.16–29.16)	0.007
Age+BMI+pre-existing renal disease adjusted	1	1.62 (0.66–3.99)	3.49 (1.19–10.28)	5.56 (1.10–27.99)	0.005
Age+BMI+family history of HTN adjusted	1	1.51 (0.61–3.69)	3.22 (1.10–9.49)	5.53 (1.10–27.89)	0.008
Age +BMI+previous history of PIH adjusted	1	1.38 (0.55–3.45)	2.80 (0.92–8.47)	3.86 (0.70–21.36)	0.032
Fully adjusted (all above)	1	1.50 (0.59–3.82)	2.71 (0.85–8.63)	5.41 (0.90–32.50)	0.025

Abbreviations: BMI, body mass index; CI, confidence interval; DM, diabetes mellitus; HTN, hypertension; OR, odds ratio; PIH, pregnancy-induced hypertension.

Table 4 Unadjusted and multivariable adjusted ORs (95% CIs) of PIH occurrence classified by blood pressure at 20 weeks of gestation

	20 weeks blood pressure OR (95% CI)				
Classification	*I*	*II*	*III*	*IV*	
n	*782*	*143*	*44*	*7*	
PIH	*14*	*7*	*9*	*2*	*P for trend*
Unadjusted	1	2.82 (1.12–7.12)	14.11 (5.72–34.81)	21.94 (3.92–122.90)	<0.001
Age adjusted	1	2.78 (1.10–7.05)	13.57 (5.45–33.78)	19.10 (3.32–109.76)	<0.001
Age+BMI adjusted	1	2.89 (1.14–7.36)	14.84 (5.76–38.37)	21.02 (3.57–123.61)	<0.001
Age+BMI+parity adjusted	1	2.84 (1.11–7.24)	14.66 (5.68–37.84)	19.34 (3.23–115.71)	<0.001
Age+BMI+pre-existing DM adjusted	1	2.94 (1.15–7.50)	13.94 (5.29–36.73)	22.76 (3.85–134.50)	<0.001
Age +BMI+pre-existing renal disease adjusted	1	2.95 (1.16–7.54)	13.94 (5.31–36.62)	21.63 (3.66–127.85)	<0.001
Age+BMI+family history of HTN adjusted	1	2.88 (1.13–7.34)	14.40 (5.53–37.46)	21.58 (3.66–127.38)	<0.001
Age +BMI+previous history of PIH	1	2.98 (1.15–7.76)	13.96 (5.22–37.31)	23.50 (3.95–140.02)	<0.001
Fully adjusted (all above)	1	3.01 (1.14–7.98)	11.72 (4.13–33.26)	24.14 (3.81–152.99)	<0.001

Abbreviations: BMI, body mass index; CI, confidence interval; DM, diabetes mellitus; HTN, hypertension; OR, odds ratio; PIH, pregnancy-induced hypertension.

Table 5 Blood pressure class shift from 16 weeks to 20 weeks of gestation and the risk of PIH occurrence

	20 weeks (no. of PIH(+)/PIH(−))			
16 weeks	*I*	*II*	*III*	*P for trend*
I	12/619	3/65	3/10	<0.001
II	2/120	2/52	3/9	0.001
III	0/26	2/15	2/13	0.081
I	*12/619*	*3/65*	*3/10*	*P<0.001*
BP Class I before 16 weeks gestation (OR (95% CI))				
Unadjusted	1	2.38 (0.65–8.65)	15.47 (3.77–63.45)	0.001
Age adjusted	1	2.40 (0.66–8.74)	16.44 (3.96–68.34)	0.001
Age+BMI adjusted	1	2.40 (0.66–8.79)	16.47 (3.85–70.56)	0.001
Age+BMI+parity adjusted	1	2.43 (0.66–8.97)	14.78 (3.38–64.73)	0.002
Age+BMI+pre-existing DM adjusted	1	2.26 (0.604–8.44)	17.06 (3.97–73.33)	0.001
Age+BMI+pre-existing renal disease adjusted	1	2.47 (0.67–9.08)	12.98 (2.70–62.32)	0.009
Age+BMI+family history of HTN adjusted	1	2.40 (0.65–8.80)	16.63 (3.84–71.99)	0.001
Age +BMI+previous history of PIH adjusted	1	2.60 (0.70–9.63)	17.81 (4.11–77.08)	0.001
Fully adjusted (all above)	1	2.35 (0.58–9.47)	13.01 (2.64–64.16)	0.010
II	*2/120*	*2/52*	*4/9*	*P<0.001*
BP Class II before 16 weeks of gestation (OR (95% CI))				
Unadjusted	0.43 (0.06–3.16)	1	8.67 (1.27–159.35)	0.002
Age adjusted	0.45 (0.06–3.28)	1	7.40 (1.04–52.35)	0.003
Age+BMI adjusted	0.43 (0.06–3.18)	1	7.73 (1.08–55.54)	0.002
Age+BMI+parity adjusted	0.38 (0.05–2.82)	1	8.13 (1.06–62.26)	0.002
Age+BMI+pre-existing DM adjusted	0.4 (0.05–3.00)	1	4.78 (0.54–42.00)	0.008
Age+BMI+pre-existing renal disease adjusted	NA	NA	NA	NA
Age+BMI+family history of HTN adjusted	0.42 (0.06–3.15)	1	13.65 (1.64–113.53)	0.001
Age +BMI+previous history of PIH adjusted	0.27 (0.03–2.55)	1	8.21 (1.12–60.07)	0.001
Fully adjusted (all above)	0.22 (0.02–2.25)	1	8.44 (0.81–87.72)	0.008
III	*0/26*	*2/15*	*2/13*	*P=0.081*
BP Class III before 16 weeks of gestation (OR (95% CI))				
Unadjusted	NA	0.87 (0.11–7.05)	1	0.114
Age adjusted	NA	0.64 (0.06–6.28)	1	0.066
Age+BMI adjusted	NA	0.71 (0.07–7.62)	1	0.079
Age+BMI+parity adjusted	NA	0.72 (0.07–7.78)	1	0.080
Age+BMI+pre-existing DM adjusted	NA	0.43 (0.04–5.33)	1	0.053
Age+BMI+pre-existing renal disease adjusted	NA	NA	NA	NA
Age+BMI+family history of HTN adjusted	NA	0.44 (0.03–6.41)	1	0.086
Age +BMI+previous history of PIH adjusted	NA	0.74 (0.07–8.08)	1	0.083
Fully adjusted (all above)	NA	NA	NA	NA

Abbreviations: BMI, body mass index; BP, blood pressure; CI, confidence interval; DM, diabetes mellitus; HTN, hypertension; NA, not available; OR, odds ratio; PIH, pregnancy-induced hypertension.

risk became insignificant after adjustment for pre-existing diabetes mellitus. The risk of PIH occurrence was not statistically significant in subjects whose BP shifted from Class II at 16 weeks to Class I at 20 weeks of gestation. However, the trend for PIH risk was significant ($P<0.05$) and remained significant even after adjustments for all variables. When comparing two groups, one in which the BP class elevated to Class III at 20 weeks gestation and the other in which the BP class did not change or decreased to Class I, the sensitivity, false positive rate and positive predictive value of BP class elevation between 16 and 20 weeks of gestation were 50.0, 5.0 and 30.8%, respectively. Subjects whose BP shifted from Class III at 16 weeks to Classes I and II at 20 weeks of gestation were not associated with a significant risk reduction of PIH (Table 5).

DISCUSSION

In this study, we classified BP in pregnant women according to JSH 2009. Our results suggested that the risk of PIH could be predicted from BP class shifts between 16 and 20 weeks of gestation. The elevation of BP over the course of pregnancy was associated with a significant risk for future development of PIH even among women with Class I (Optimal) and II (Normal) BPs before 16 weeks of gestation, who are normally recognized as low risk for PIH.

In a previous study, Hermida *et al.*[20] compared the time course of BP changes during pregnancy in normotensive and PIH women. The normotensive women had a steady decrease in BP toward 20 weeks; this was absent in patients with gestational hypertension and pre-eclampsia. Silva *et al.*[21] investigated the effect of maternal education levels on BP alterations in pregnancy. They found the absence of a mid-pregnancy fall in diastolic BP in low educational groups with a high occurrence of PIH. However, both studies failed to analyze the direct association between changes in BP during pregnancy and the risk of PIH. In this study, we clearly demonstrated a significant association between an increased BP during the first half of pregnancy and the risk of PIH.

Our results are in accordance with those previously reported, which identified normal and high-normal BPs at early and mid pregnancy as predictors of subsequent PIH development.[5,23] We used the JSH 2009 classification to show a significant trend for risk of future PIH in women whose BP class increased both before 16 (Table 3) and 20 weeks (Table 4) of gestation, even after adjustment for all variables. Thus, JSH 2009 may be a novel, more accurate system for PIH prediction based on a single estimation of maternal BP during pregnancy, particularly if the measurement is taken at early-mid pregnancy, and especially if performed at 20 weeks.

In a previous report, Duckitt *et al.*[16] conducted a meta-analysis to evaluate patient characteristics recorded at antenatal booking as risk factors of PIH. These included age, body mass index, nulliparity, previous history of PIH and diabetes mellitus, multiple gestations, family history of PIH and antiphospholipid syndrome. In our study population, age, pre-existing diabetes mellitus and previous history of PIH were also identified as significant risk factors for PIH.

There were some limitations to our study, including the retrospective design allowing the possibility of some bias and the small size of the study population.

In conclusion, the JSH 2009 classification may be used early in pregnancy to identify women at risk of PIH even in those with optimal or normal BPs. A shift in BP class at 20 weeks of gestation is predictive of subsequent PIH development. JSH 2009 has potential widespread clinical application.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We wish to thank the women who participated in the T-CHILD study, and Drs H Saito, S Aizawa and R Horikawa, the principal members of the T-CHILD study. We also appreciate with Ms S Saito, Ms C Matsuzawa for management of this cohort data. This study was supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour, and Welfare of Japan (No 09158522). This study was also supported by the Grant of National Center for Child Health and Development (20A-1).

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PRENATAL DIAGNOSIS
Prenat Diagn (2011)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI:** 10.1002/pd.2675

Gene expression in chorionic villous samples at 11 weeks of gestation in women who develop pre-eclampsia later in pregnancy: implications for screening

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Objectives To determine the gene expression profile in chorionic villous samples (CVSs) of women destined to develop pre-eclampsia (PE).

Method Case-control study encompassing five women destined to develop PE [cases matched for gestational age with 30 controls]. We quantified mRNA expression on tissue samples from CVS of normal and PE patients. We then assessed mRNA expressions of cathepsin (CTSD), angiopoietin 2 (ANGPT2), interleukin 8, chemokine (C-X-C motif) ligand 10, neurokinin B (NKB), matrix metallopeptidase 9, major histocompatibility complex, class I, C (HLA-C)and human leukocyte antigen-G (HLA-G). Data were analyzed by nonparametric rank analysis.

Results For all the mRNA species considered in this study, except CTSD and ANGPT2, all the mean observed ranks in the PE group were significantly altered compared with the rank expectation among controls. mRNA for NKB and HLA-C were the markers with the highest degree of aberration in PE, compared with those in controls.

Conclusion Our study has directly showed that gene expressions relating to trophoblastic cell invasion or utero-placental hemodynamic adaptation are altered in the first trimester trophoblasts that go on to develop PE later. These results posit the use of residual CVS as a possible screening method for PE. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS: mRNA; screening for pre-eclampsia; real-time PCR; chorionic villous samples

INTRODUCTION

Pre-eclampsia (PE) is a serious complication of pregnancy affecting the mortality and the morbidity of both mothers and infants, with a prevalence of approximately 2–7% (De Groot *et al.*, 1999). Although the primary mechanism of PE is still unknown, it is characterized by impaired placental function, abnormal trophoblast invasion, deficient physiologic maternal spiral artery modification, increased apoptosis of trophoblastic cells and placental ischemia. Failure of normal invasion by trophoblast cells leads to inappropriate development of the maternal spiral artery (Walker *et al.*, 2000) which can interfere with normal villous development and reduce placental perfusion. PE is unique to human pregnancy and its clinical features are well recognized, characteristically manifesting in the second to third trimester. However, the underlying pathologic changes, related to early abnormal trophoblast invasion into the maternal vascular tissue associated with increased apoptotic and necrotic indices, occur much earlier. The earlier the gestational age (GA) at diagnosis, the higher the risk of maternal death (MacKay *et al.*, 2001). In fact, the increased risk of maternal death is fourfold if PE is diagnosed before gestational week 32 as compared with the risk after that GA. Given the morbidity associated with PE and the long preclinical phase before it manifests clinically, several studies have attempted to identify early-pregnancy proteins that might be predictive of PE and intrauterine growth restriction, offering the possibility of reducing maternal and fetal risks by administration of prophylactic low-dose aspirin started early during the gestation in high-risk pregnancies. PE can be classified as early- and late-onset PE, defined by the development of symptoms before or after 32 weeks of pregnancy, respectively. The early-onset form is more severe, frequently leading to the delivery of growth-retarded premature babies or poor outcome for the mother.

This study belongs to a series of already published studies tending to demonstrate that direct alterations in gene expression among trophoblasts will later develop into PE. We prospectively collected tissue samples of villous trophoblasts at the time of fetal karyotype analysis through chorionic villous sampling (CVS). mRNA expressions of factors that are reported to play

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Received: 8 August 2010
Revised: 30 October 2010
Accepted: 1 November 2010

roles in the development of PE were assessed and compared with the clinical outcomes.

METHODS

Thirty-five pregnant women in care at the Division of Prenatal Medicine at the University of Bologna, Bologna, Italy were enrolled in the present prospective case-control study. All the women were older than 35 years at delivery and bearing a single male fetus. Pregnancies with major fetal defects (like congenital heart diseases and aneuploidies) were excluded. CVS was performed for assessment of fetal karyotype. Residual CVS from five women with a diagnosis of PE (performed at the third trimester of pregnancy) was matched with 30 controls (1 : 6 ratio) for GA and fetal gender. GA was calculated by ultrasound measurements at 11 weeks of gestation. All women gave informed consent to participate in the study, which was approved by the local Institutional Review Board.

PE was defined as gestational hypertension (systolic pressure >140 mmHg or diastolic blood pressure >90 mmHg on ≥2 occasions after gestational week 20, with proteinuria (>0.3 g/day). Severe PE was defined by the presence of ≥1 of the following: (1) severe gestational hypertension (systolic pressure >160 mmHg or diastolic blood pressure >110 mmHg on ≥2 occasions after gestational week 20 or (2) severe proteinuria (≥5 g protein in a 24-h urine specimen). (ACOG practice bulletin, 2002)

Tissues and RNA preparation

Villous samples were centrifuged at 1500 rpm for 5 min at 4 °C, resuspended in 1 mL of phosphate buffered solution (PBS) and then centrifuged at 1500 rpm for 5 min at 4 °C. Each villous sample was mixed with 0.8 mL of Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and lysated by repetitive pipetting. For RNA extraction, 0.16 mL of chloroform was added to the sample; after vigorous shaking, the sample was incubated at 4 °C for 15 min and centrifuged at 12 000 rpm for 15 min at 4 °C; following centrifugation, the aqueous phase was transferred to a fresh microtube. Precipitation of total RNA from the aqueous phase was obtained by mixing with 0.4 mL of isopropyl alcohol, incubation at 4 °C for 15 min and centrifugation at 12 000 rpm for 15 min at 4 °C. The RNA pellet was washed once in 0.8 mL of 75% ethanol and briefly air-dried. Finally, the total RNA was dissolved in 20 μL of RNase-free water and stored at −80 °C.

Real-time quantitative reverse transcription-PCR

RNA samples were transferred to Japan at under −20 °C. Molecular analysis was performed in the Department of Obstetrics and Gynecology at Showa University School of Medicine, Tokyo. RNA was reverse-transcripted by an Omniscript RT kit (Qiagen, Hilden, Germany). cDNA products were amplified by real-time quantitative PCR according to the manufacturer's instructions (QuantiTect Probe PCR kit; Qiagen) using a 2-μL aliquot of cDNA and the kit components in a reaction volume of 20 μL. TaqMan PCR analyses for cathepsin D (CTSD), angiopoietin 2 (ANGPT2), interleukin 8 (IL8), chemokine (C-X-C motif) ligand 10 (IP-10), neurokinin B (NKB), matrix metallopeptidase 9 (MMP9), major histocompatibility complex, class I, C (HLA-C) and human leukocyte antigen G (HLA-G) mRNAs; (Applied Biosystems, Foster City, CA, USA). As an initial step, we verified that each PCR assay was specific to mRNA and not to genomic DNA. Amplification data were collected and analyzed with an ABI Prism 7900T Sequence Detector (Applied Biosystems). Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. The thermal profile used was as follows: 15 min of denaturation at 95 °C, followed by 15 s of annealing at 94 °C and 1 min of extension at 60 °C. Quantification of gene expression was performed by investigators blinded to the outcome of pregnancy. Amounts of mRNA samples were expressed in terms of copies per millilitre. To quantify these mRNA concentrations, we prepared plasmid DNA for calibration curves as previously described (Purwosunu *et al.*, 2007). As initial volumes of tissues could not be measured, the level of each gene expression was expressed as a ratio to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Statistical analysis

Data were matched for GA in a 1 : 6 case-control study design. Median mRNA concentrations of each available marker CTSD, ANGPT2, IL8, IP-10, NKB, MMP9, HLA-C and HLA-G in cases and controls were calculated. Rank analysis and nonparametric analysis (Mann–Whitney U test or Fisher exact test) were used to detect differences between cases and controls. Differences were considered significant for a p-value <0.05.

RESULTS

There were two cases of severe PE that were treated according to the guideline for 'expectant management' (Sibai *et al.*, 2007). Tables 1 and 2 report the clinical information available for the data set. The ranks of the mean mRNA levels from each PE sample were found to be significantly higher or lower than the expected rank for six controls. Table 3 reports the whole data set for PE cases and the medians [intraquartile range (IQR)] for controls and the mean observed ranks for PE cases and 30 controls generated by a 1 : 6 match showing a statistically different distribution between cases and controls. CTSD, ANGPT2, MMP9, HLA-C and HLA-G showed lower values than controls. IP-10, IL8 and NKB by contrast showed higher values. NKB and HLA-C were the markers with the highest difference, followed by MMP9.

Table 1—Demographic and clinical characteristics of patients. Data are expressed as medians (minimum–maximum) or percentages

Variable	PE $n = 5$	Controls $n = 30$	p Value[a]
Gestational age (days) at time of CVS	82 (81–84)	82 (81–84)	ns
Maternal age	36 (35–38)	36 (35–38)	ns
Percentage of primiparae	36	38	ns
Week at delivery	38 (35–41)	39 (36–41)	ns
Neonatal weight (g)	2850 (2000–3510)	3100 (2560–4180)	ns

[a] Mann–Whitney U test or Fisher exact test.
CVS, chorionic villous samples; PE, pre-eclampsia.

Table 2—Clinical data available for pre-eclampsia (PE) cases

Case ID	Week of PE insurgence	PE degree	Second-trimester Doppler of uterine arteries (mRI[a])	Type of delivery
SB	29	Severe	0.68, no incisura	Cesarean section
IR	31	Severe	0.50, monolateral incisura	Cesarean section
DM	33	Mild	0.38, no incisura	Cesarean section
AF	32	Mild	0.44, no incisura	Vaginal
MC	36	Mild	0.46, no incisura	Vaginal

[a] mRI = medium resistance index.

Table 3—Median value (×1000) for mRNA species in pre-eclampsia (PE) cases and controls (numbers in parentheses are the nonmedian raw values and the intraquartile range, respectively)

mRNA species	PE $n = 5$	Controls $n = 30$	Mean rank in PE	Mean rank in controls	p Value*	Expression in PE cases versus controls
CTSD	142 (104,136,1 871 947)	275 (149–483)	2.00	4.00	0.170	↓
ANGPT2	1621 (1134,1514,1945,2395)	4480 (1188–6943)	3.00	4.00	0.369	↓
IL8	2389 (2058,2123,2496,2566)	21322013–2259)	7.00	3.50	0.016	↑
IP-10	632 (309,418,731,989)	307 (84–420)	6.00	3.50	0.016	↑
NKB	6618 (2742,4369,8245,14 816)	2431 (1103–5426)	7.00	3.50	<0.001	↑
MMP9	1566 (1430,1451,2006,7742)	8720 (5810–10474)	1.00	4.50	0.007	↓
HLA-C	43 (11,19,44,60)	120 (70–257)	1.00	4.50	<0.001	↓
HLA-G	1634 (1173,1308,1698,1198)	2476 (1677–3047)	2.00	4.50	0.016	↓

Unit is copies per mL. The gene expression is normalized for GAPDH.
CTSD, cathepsin; ANGPT2, angiopoietin; IL8, interleukin; IP-10, ligand 10; NKB, neurokinin; MMP9, matrix metallopeptidase 9; HLA-C, major histocompatibility complex, class I, C; HLA-G, major histocompatibility complex, class I, G.
* Rank sum test.

NKB and HLA-C proved to be the markers with the lowest level of aberration in PE. CTSD and ANGPT2 did not reach the level of statistical significance.

DISCUSSION

This study, as an extension of the earlier study, prospectively examined CVSs from women at gestational week 11. We assessed eight kinds of mRNA expressions and compared them with clinical outcomes.

All of them had been previously evaluated in molecular, biochemical and immunohistochemistry studies and were associated with PE. They belong to different categories including trophoblast invasion, villous angiogenesis, remodelling of extracellular matrix, maternal immunity and hemodynamic adaptation. As is widely known, all of these are biological functions associated with PE development.

Matrix metalloproteinases (MMPs) play a crucial role in restructuring the extracellular matrix by activating the secretion of gelatinases, collegenases and proteolytic enzymes. The balance between MMPs and tissue inhibitors of matrix metalloproteinase (TIMPs); is likely to play an important role in remodeling uterine arteries in pregnancy and maintaining vasodilatation in later pregnancy (Kelly *et al.*, 2003). The maternal plasma concentration of MMP9 is about 15 times higher than in nonpregnant women. MMP9 has been associated with a prediction of PE but with discordant results. For example, trophoblasts and placentas from pre-eclamptic pregnancies were recently found to express lower levels of MMP9 (Kolben *et al.*, 1996; Campbell *et al.*, 2004; Montagnana *et al.*, 2009), but some investigators have found higher expression levels of MMP9 in placental sections from PE tissue (Wang *et al.*, 2009). Again, Poon *et al.* (2009), in disagreement with our results, demonstrate that the maternal serum concentration of MMP9 at 11 ± to 13+ weeks of gestation is increased in pregnancies that subsequently develop PE, suggesting an association with an underlying inflammatory process.

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DOI: 10.1002/pd

Natural Killer (NK) cells express an array of receptors, some of which are known to bind HLA-C molecules expressed by extravillous trophoblastic cells. Several recent studies have suggested that NK cell and trophoblast interaction are required to attract trophoblasts to the decidua and to promote placental vascular remodeling and differentiation (Hanna *et al.*, 2006; Le Bouteiller and Tabiasco, 2006). In PE, it has been found that some killer cell immunoglobulin-like receptor/HLA-C combinations appear unfavorable to trophoblastic cell invasion (Hiby *et al.*, 2004), suggesting a role for this gene in the pathogenesis of PE. Thus, in normal pregnancies NK cell activation through interaction with HLA-C in extravillous trophoblasts may promote placental development and maternal decidual spiral artery modifications. Insufficient NK cell activation would halt this process prematurely, resulting in poor decidual artery remodeling and increasing the risk of PE (Hiby *et al.*, 2004; Parham *et al.*, 2004). It is therefore also possible that a lower HLA-C expression, as shown by our results, could reduce and make less efficient such an interaction.

HLA-G is a nonclassic class I HLA molecule that is expressed in extravillous trophoblast cells. The molecular properties of HLA-G have recently been reviewed (Apps *et al.*, 2008). A number of studies have reported significantly lower concentrations of soluble HLA-G in maternal blood in cases of PE than in control pregnancies when measured at the end of the third trimester, (Yie *et al.*, 2004; Hackmon *et al.*, 2007), in the second trimester and in the first trimester (Yie *et al.*, 2005). Decreased HLA-G RNA (Hara *et al.*, 1996; Lim *et al.*, 1997; O'Brien *et al.*, 2000) and protein (Hara *et al.*, 1996; Lim *et al.*, 1997; Goldman-Wohl *et al.*, 2000a,b) as found in placental tissue of patients affected by PE suggests that lower levels of HLA-G do not protect the invading trophoblast from decidual NK cells. Thus, trophoblast invasion is defective, leaving a high degree of resistance in the uterine spiral arteries. Goldman-Wohl *et al.* (2000b) demonstrated HLA-G expression in anchoring extravillous trophoblasts with an increasing gradient of expression in more invasive cells. The authors suggested that HLA-G expression correlates with increased invasiveness and that HLA-G may be a necessary precondition for invasion. This indicates that, in PE, clusters of trophoblasts that do not express HLA-G may be unable to invade into maternal spiral arteries. Our results are in accord with these previous observations.

IP-10 is a chemokine of the CXC family (Neville *et al.*, 1997). Its principal biological activity is regulation and control of the basal homeostatic and inflammatory leukocyte movement (Rosenkilde *et al.*, 2004). It has potent angiogenic properties (Belperio *et al.*, 2000; Bernardini *et al.*, 2003; Rosenkilde *et al.*, 2004; Strieter *et al.*, 2005) and promotes adhesion, migration and invasion of trophoblast cells. PE is associated with a higher maternal serum concentration of IP-10 than normal pregnancy (Gotsch *et al.*, 2007). This result is consistent with the view that PE may reflect an anti-angiogenic state as well as an enhanced systemic inflammatory response.

IL8, a member of the CXC family of chemokines known as CXCL-8, is one of the main chemo-attractants for neutrophils and can activate the neutrophils (Goldsby *et al.*, 2003). It is also involved in angiogenesis (Baggiolini *et al.*, 1989). IL-8 and IP-10, produced by decidual NK cells, have been shown to attract trophoblasts expressing specific chemokine receptors. A block in trophoblast migration has been demonstrated by the addition of anti-IL-8 and anti-IP-10 monoclonal antibodies in culture (Hanna *et al.*, 2006). Scott Kauma *et al.* (2002) found that circulating plasma levels of IL8 were elevated in pre-eclamptic than in normal pregnant women (Sharma *et al.*, 2007). Although activated vascular endothelial cells are probably responsible, in part, for increased circulating IL-8 levels in pre-eclamptic women, other potential sources for these chemokines during pregnancy are the placenta, maternal decidua and circulating leukocytes (Denison *et al.*, 1997). Hence, higher mRNA expression in CVS, as shown in our results, is consistent with previous studies.

NKB belongs to a family of neuropeptides called the tachykinins. Traditionally, these peptides have been classified as neurotransmitters, being found in discrete neurons and immune cells (Page, 2004, 2005). Recently, this dogma was challenged when the placenta, a tissue devoid of nerves, was found to be a source of *TAC3* gene expression (Page *et al.*, 2000, 2001).

A role for the tachykinins in the placenta has remained as yet undefined. Nevertheless, recent and consistent evidence suggest that they may play a role in uteroplacental hemodynamic adaptation by inducing uterine and placental vasodilatation, thereby increasing placental blood flow (Page *et al.*, 2000, 2001; Brownbill *et al.*, 2003; D'Anna *et al.*, 2004; Laliberte *et al.*, 2004). Elevated circulating levels of the tachykinin NKB have been observed in women with PE during the third trimester of pregnancy and may also explain the sequelae of PE, including hypertension.

Currently, Doppler ultrasound analysis of the uteroplacental circulation combined with clinical history and, sometimes, with biochemical markers like pregnancy-associated plasma protein-A (PAPP-A), forms the most common screening tool in routine prenatal settings to detect women at increased risk for PE. Molecular screening tests (alone or combined with Doppler analysis) seem to have a sufficient predictive power and could be extensively used although the costs are still very high. For these reasons, it seemed promising to search for the best mRNAs with the highest predictive power, in order to reduce costs and optimize the screening results. This study presents eight new mRNAs that could be evaluated in further studies for a possible use in PE screening. However, screening PE by CVS analysis affords a slightly different point of view representing an extra opportunity for early screening. In fact, given that women who undergo CVS for fetal karyotype analysis are at higher risk for PE because of advanced maternal age, the mRNA dosage may be a possible way of detecting high-risk patients. Again, the CVS itself is considered, even if controversial, a possible extra risk for PE occurrence (Adusumalli *et al.*, 2007).

In conclusion, in this report we evaluated a new list of genes possibly involved in PE development and screening. They belong to categories widely associated with

PE onset including trophoblast invasion, villous angiogenesis, remodelling of extracellular matrix, maternal immunity and hemodynamic adaptation. All of them potentially open up new opportunities for screening the disease long before clinical onset; however, because of the small sample size only those genes that showed a higher degree of aberration have a higher chance to predict PE in prospective studies.

ACKNOWLEDGEMENTS

This study was supported in part by a PRIN 2007 (to A. Farina and N. Rizzo), Università di Bologna RFO to AF and CZ and a Corigliano City award (DM); in part also by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Nos. 20 591 930 and 20 591 308) and Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare, Japan.

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