Microarray analysis of differentially expressed genes in preeclamptic and normal placental tissues

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Summary

Purpose of investigation: To detect the candidate genes for preeclampsia (PE). *Materials and Methods:* The gene expression profiles in preeclamptic and normal placental tissues were analyzed using cDNA microarray approach and the altered expression of important genes were further confirmed by real-time RT-PCR (reverse transcription polymerase chain reaction) technique. Total RNA was extracted from placental tissues of three cases with severe PE and from three cases with normal pregnancy. After scanning, differentially expressed genes were detected by software. *Results:* In two experiments (the fluorescent labels were exchanged), a total of 111 differentially expressed genes were detected. In placental tissue of preeclamptic pregnancy, 68 differentially expressed genes were up-regulated, and 44 differentially expressed genes were down-regulated. Of these genes, 16 highly differentially expressed genes were confirmed by real-time fluorescent quantitative RT-PCR, and the result showed that the ratio of gene expression differences was comparable to that detected by cDNA microarray. *Conclusion:* The results of bioinformatic analysis showed that encoding products of differentially expressed genes were correlated to infiltration of placenta trophoblastic cells, immunomodulatory factors, pregnancy-associated plasma protein, signal transduction pathway, and cell adhesion. Further studies on the biological function and regulating mechanism of these genes will provide new clues for better understanding of etiology and pathogenesis of PE.

Key words: Microarray analysis; Gene expression profiles; Preeclampsia; Placenta.

Introduction

Preeclampsia/eclampsia (PE) is one of common and severe maternal complications of pregnancy and also the main cause of diseases and death in pregnant women and perinatal infants, with the incidence rate being 7-10% [1]. Since its pathophysiological changes are correlated to multiple systems and organs, and its etiology and pathogenesis have not been fully understood, PE is attracting much interest in maternity studies. Placenta is a unique critical organ that is essential for normal pregnancy maintenance and is responsible for supplying all nutrients for fetal growth and development. Abnormal pathophysiological changes of placenta will cause various maternal and fetal diseases including PE. It has been widely accepted that the clinical symptoms of PE such as hypertension and proteinuria are induced by pathological changes occurring during the development of placenta, leading to damage of vascular endothelial cells.

PE has a family genetic predisposition, which is mainly characterized by maternal inheritance. Chesley *et al.* [1-3] have found that the incidence rate of PE in daughters of women with PE (26%) was far beyond that (8%) in their brother's and sister's daughters. Pedigree analysis has indicated that PE is closely correlated to genetic factors [4-7]. Reincidence rate of PE in women with multiple pregnancies, who had changed their sexual partners, would be increased obviously, especially when the mother of their new sexual partners had a history of PE [8].

On the basis of predisposing genes of PE, the following chromosome segments have been found to be correlated to PE: chromosome 6, chromosome 17 (includes gene of angiotensin-converting enzyme), chromosome 21 (includes gene of superoxide dismutase), and chromosome 3 (includes gene of angiotensin II type 1 receptor) [9]. Presently PE is considered to be one of polygenic inheritance diseases associated with multiple predisposing factors [9]. To date, only a small number of candidate pathogenic genes of PE has been identified, including blood pressure regulatory gene, thrombosis gene, vascular endothelial injury gene, lipid metabolism gene, immune related genes, and mitochondria related genes [10-18].

Most previous studies on pathogenic genes of PE have been focused on maternal body, and the main purpose was to detect maternal gene abnormalities. These studies neglected one of the important characteristics of PE that it is one of diseases related to products of conception produced during special periods of pregnancy. The incidence, development, and turnover of PE are closely related to the placenta. Patients with preeclampsia recover from clinical symptoms of the disorder only after the delivery of the fetus and placenta. Since placenta is one of fetal appendixes and is obviously different from maternal tissues, genetic research of PE should not ignore the placenta. There

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is an emerging consensus that the placenta plays an essential role in the etiology of PE. Expression profiling using microarray has proved to be a powerful tool for studying complex disorders such as cancer, diabetes, hypertension, and PE. To date, there have been several reports that have addressed the gene expression profiles of the placentas from preeclamptic women. These studies have shown that genes such as the obesity-related genes, cytokine-receptor genes, host-pathogen interaction genes, lipid metabolism genes, carbohydrate metabolism genes, and -related genes are essential for or strongly associated with the development of PE [19-22].

The scarcity of early biomarkers for PE has hindered our ability to take effectively preventive and therapeutic measures to manage this dangerous disease in a timely manner. Thus, it is of clinical importance to compare the expression profiles between this disease and normally pregnancy and thereby increase our understanding of the etiologies of PE and aid in the future development of new therapies. In this study, placental tissue of preeclamptic pregnancy was analyzed by cDNA microarray in order to detect differentially expressed genes. The identified up- or down-regulated genes were further confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR).

Materials and Methods

Human subjects

Placental tissues were collected from three cases with severe PE and from three women with normal pregnancy, which underwent cesarean section delivery at the Affiliated Obstetrical and Gynecological Hospital of Fudan University (Shanghai, China) in August, 2005, and were well matched according to age, parity, and gestation. The including criteria for normal control group were: a) women with singleton pregnancy; b) women with 28-40 weeks of gestation; c) pregnant women without any medico-surgical complications such as diabetes, primary hypertension, nephritis, heart diseases, anaemia, hepatitis, intrahepatic cholestasis during gestational period, sexually transmitted diseases, and other internal or surgical diseases of pregnant women; and d) pregnant women without any complications of pregnancy and labor, including placenta previa, placental abruption, fetal distress, hydramnios or oligohydramnios, intrauterine growth retardation, large for date infant, fetal anemia, and congenital diseases. Women were confirmed with preeclamptic pregnancy according criteria of pregnancy-induced hypertension syndrome by the American College of Obstetricians and Gynecologists [1], and patients who met the PE criteria were included. Informed consent was obtained from each patient. The study was approved by the Ethics Committee for Human Studies at Fudan University.

Placental tissue biopsy collection

All of the placental biopsies both from preeclamptic and normal pregnancy were obtained after cesarean sections. To avoid the effect of labor on the expression profile, only placental samples that were obtained from the women who did not undergo labor were included in the study. After placenta expulsion, the maternal deciduas and amnionic membranes were removed and about one cm^3 of placental tissue was cut from the center of placental maternal surface between basal and chorionic plates. After being rinsed with saline, they were transferred into diethylpyrocarbonate (DEPC)-treated Eppendorf tubes, and frozen with liquid nitrogen and stored at -80°C until analysis.

Extraction and purification of RNA from placental tissues

Total RNA was extracted from 400 mg placental tissue using trizol and then precipitated with isopropyl alcohol and purified with RNeasy mini-kit. Both quality and quantity of extracted total RNA samples were examined by loading five μg of each sample on a denaturating agarose gel and staining with ethidium bromide. The RNA was quantified and evaluated for purity by UV spectrophotometry. To further evaluate the quality of the RNA, all specimens were tested by expression analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using RT-PCR. To test for possible contamination by maternal blood cells, the expression level of leukocyte-specific gene/leukocyte common antigen (LCA) was also examined using conventional semi-quantitative RT-PCR. The PCR products were electrophoresed in 2% agarose gel containing ethidium bromide, which were then visualized with ultraviolet.

cDNA microarray analysis

BioStarH140s cDNA microarray used in this study contained 14,112 human clones, mainly consisting of the following genes: 1) proto-oncogenes and anti-oncogenes; 2) cell signal transduction protein genes; 3) cyclin genes; 4) outer stress response protein genes; 5) cell regulatory protein genes; 6) apoptosis related protein genes; 7) DNA synthesis, repair, and recombination protein genes; 8) DNA binding, transcription, and transcription factor genes; 9) cell receptor genes; 10) cell surface antigen and attachment protein genes; 11) ion channel and transport protein genes; 12) metabolism genes; and 13) house-keeping genes.

To perform fluorescent labeling, the cDNA was reversely transcribed from ten μg total RNA primed by T-Oligo(dT)15, and then was purified with a PCR purification kit. An *in vitro* transcription was performed to produce biotin labeled cRNA from the cDNA. To normalize for fluorescent labeling, mRNA isolated from preeclamptic and normal placental tissues was firstly labeled with Cy3-dUTP and Cy5-dUTP, respectively; and then exchanged the labels with mRNA isolated from preeclamptic and normal placental tissues being labeled with Cy5-dUTP and Cy3-dUTP, respectively.

A total of three placentas from women with preeclampsia and three from normal subjects were used as test samples in the hybridizations. Glass slides containing the above labeled cDNA was transferred into 35 μl hybridization solution (3% sodium chloride sodium citric acid (SSC), 0.2% sodium dodecyl sulfate (SDS), 5% Denhart's, and 25% formamide solution) and incubated at 42°C overnight. The glass slides were rinsed with 2% SSC solution containing 0.2% SDS at 42°C for five minutes, and then rinsed with 0.2% SSC solution at room temperature for five minutes and dried while shaking.

The dried glass slides were scanned with a cDNA microarray dual channel laser scanner. The value and ratio of fluorescence intensities of Cy3 and Cy5 of each spot in the cDNA microarray were analyzed with GenePix Pro 4.0 and normalized by Lowess method. The average signal intensities were corrected for median background intensity and transferred with GenBank descriptors to a Microsoft Excel data spreadsheet. Those spots showing more than a twofold difference between the Cy5 and Cy3 signals, i.e., the ratio of Cy5 to Cy3 fluorescence intensity was no less than 2.0 or no more than 0.5, were considered as dif-

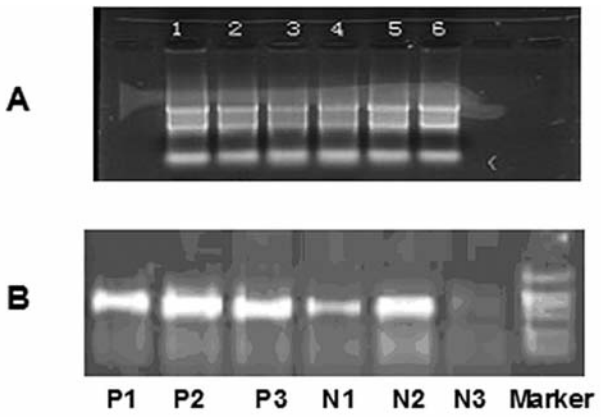

Figure 1. — RNA electrophoretogram. (A) Electrophoretogram of 18s PCR products; and (B) Electrophoretogram of RNA isolated from placenta samples. P1-3 indicates RNA electrophoretogram of placental tissues of preeclamptic pregnancy. N1-3 indicates RNA electrophoretogram of placental tissues of normal pregnancy.

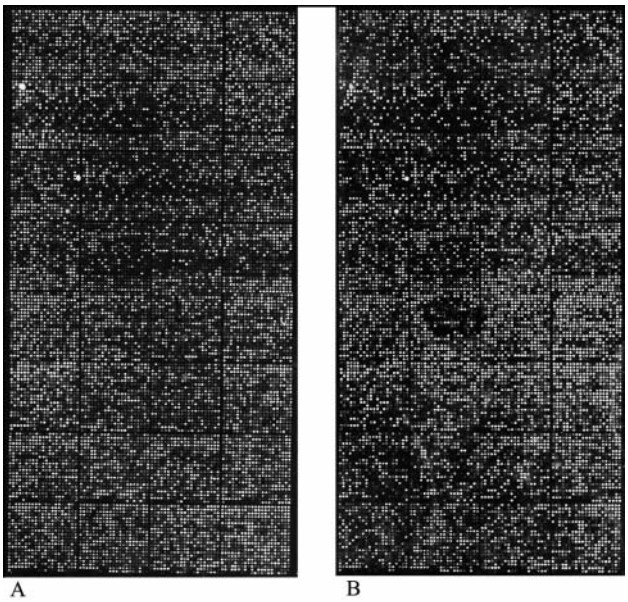

Figure 2. — Hybridization maps of cDNA isolated from preeclamptic and normal placental tissues. (A) positively labeled microarray; and (B) negatively labeled microarray.

ferentially expressed genes. In order to ensure the reliability of samples, two experiments with fluorescent labels being exchanged were performed.

Real-time fluorescent quantitative RT-PCR

Sixteen highly differentially expressed genes identified by cDNA microarray were confirmed by real-time fluorescent quantitative RT-PCR, using 18S house-keeping gene as calibration gene. Primer sequence and product sizes of theses 16 genes were listed in. All of the primers were provided by the Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. The total RNA isolated from preeclamptic and normal placental tissues was digested with RNA free DNase I at 37°C for 30 minutes. Then phenol/chloroform, 1/10 × 3 mol/l sodium acetate, and 2.5 × cold dehydrated alcohol were added in turn to the mixed solution to precipitate RNA, which was washed with 70% ethanol, and then was dried and re-dissolved in DEPC water solution. The first cDNA was reversely transcribed from 2.5 g total RNA using reverse transcriptase and then amplified by a PCR method. The PCR product was electrophoresed on 1% agarose gel. The 18S band was observed, which indicated that the total RNA was qualified for reverse transcription (Figure 1).

Amplification was performed on a real time-PCR machine. The analyses were carried out using a Light Cycler fast start DNA master SYBR green I kit. Data analysis and processing were made by using Rotor-Gene 5.0 and Excel 7.0 to obtain Ct values for each gene in each sample. In an effort to compare the differences at transcriptional level, Ct values of each target gene were normalized to the Ct value of a house-keeping gene using the following equation: $\Delta Ct = [Ct\ (target\ gene)] - [Ct\ (house\text{-}keeping\ gene)]$. In a typical reaction, the PCR product was produced exponentially, i.e., in a 2^n way (where n is the number of cycles). The concentration of the original template is $2\text{-}\Delta^{Ct}$.

Gene nomenclature

The human genes investigated in this study were named in accordance with the guidelines provided by Human Genome Organization (HUGO) Nomenclature Committee (http://www.gene.ucl.ac.uk).

Results

Hybridization maps and scatter plot of gene expression profiles

Figure 2A shows the overlaping between hybridization map of Cy3 labeled cDNA prepared from preeclamptic placental tissues and that of Cy5 labeled cDNA prepared from normal placental tissues. If the Cy3 signal of a spot was more intensive than its Cy5 signal and the spot was green, the spot represented a gene with a down-regulation tendency; in contrast, if the Cy5 signal of a spot was more intensive than its Cy3 signal and the spot was red, the spot represented a gene with an up-regulation tendency. Figure 2B shows the overlaping between hybridization map of Cy3 labeled cDNA prepared from normal placental tissues and that of Cy5 labeled cDNA prepared from preeclamptic placental tissues.

Scatter plot of microarray hybridization can directly reflect the differences of gene expression profiles of two different samples. The X-axis refers to the intensity of Cy3 signal, while the Y-axis refers to the intensity of Cy5 signal. Each data point represents the hybridization signal of one gene. If the data point is red and the ratio of its X value over its Y value is between 0.5 to 2.0, it represents a gene without expression differences; in contrast, if the data point is yellow and the ratio of its X value to its Y value is no more than 0.5 or no less than 2.0, it represents a gene with marked expression difference (Figure 3).

Profiles of differentially expressed genes

The cDNA microarray used in this study consists of most of the following genes: proto-oncogenes, anti-oncogenes, ion

channel and transport protein genes, cyclin genes, cytoskeleton and movement protein genes, apoptosis genes, DNA synthesis and repair genes, transcription factor genes, cell receptor genes, immune related genes, cell signal transduction genes, metabolism genes, and development genes. Microarray hybridization results showed that the exchange of fluorescent labels for cDNA prepared from preeclamptic and normal placental tissues could effectively eliminate false positive rate caused by nonspecific adsorption characters of fluorescent labels. In two experiments with fluorescent labels being exchanged, 382 and 394 differentially expressed genes were detected, respectively, with the number of genes detected in both of the experiments being 111, which accounts for 29.1% and 28.2% of total detected genes, respectively. Of these 111 genes, 68 were up-regulated with the ratio being no less than 2.0 and 44 were down-regulated with the ratio be no more than 0.5 in preeclamptic placental tissue.

Of 111 differentially expressed genes detected in this study, many of them are: 1) genes relating to infiltration of placenta trophoblastic cells such as latent transforming growth factor binding protein 2 (LTBP-2) gene (NM_000428), and insulin-like growth factor binding protein 1 (IGFBP1) gene (NM_000596); 2) immune related genes such as interferon (a, b and W) receptor 1 (IFNAR1) gene (NM_000629), and mannose receptor C1 (MRC1) gene (NM_002438); 3) pregnancy associated plasma protein (PAPP) and pregnancy specific glycoprotein (PSG) genes including PAPPE/PAPPA2 (NM_021936), PSG1 (NM_006905), and PSG4/PSG9 (NM_002780); the ratio of expression differences of PPAP-A detected by cDNA microarray technique was 12.406; 4) genes relating to cell signaling pathway such as expression of tissue factor pathway inhibitor-2 (TFPI-2) gene (NM_006528) and serine (or cysteine) proteinase inhibitor 1 (SERPINT1) gene (NM_001235); TFPI-2 gene was up-regulated 12.6 times; and 5) other genes such as keratin 15/type I cytoskeletal 15 (KRT15) gene (NM_002275), fragile X mental retardation (FMR1) gene (NM_002024), and cytochrome P450 (CYP19A1/aromatase) gene (NM_031226).

On the basis of previous reports and the results of our previous experiments, 16 key candidate genes were screened from numerous differential expressed genes of preeclamptic placental tissues according to the following criteria: 1) expression differences can be seen during positive and negative detecting process; 2) gene expression differences and protein differences were similar and significant; 3) genes relating to placental development, angiogenesis, and immune response; 4) bioinformatics information indicated that the protein of the candidate genes can interact with proteins relating to pathogenesis of hypertension; 5) the expression products of the candidate genes are transcription factors or the important regulators for other genes. Among the above criteria, the first one is essential, and the other ones are auxiliary and supportive (Table 1).

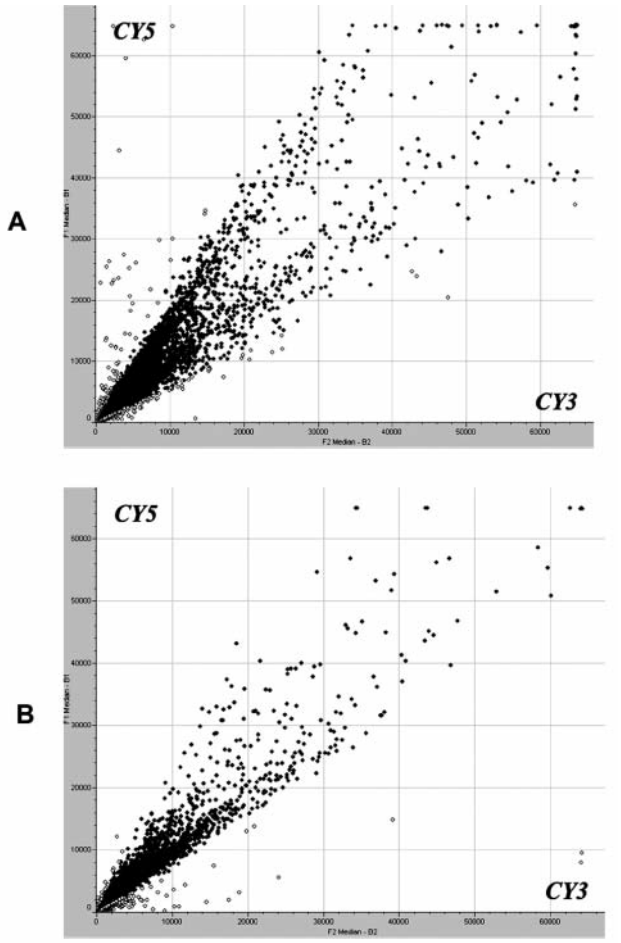

Figure 3. — Scatter plot of hybridization signal intensity of cDNA prepared from preeclamptic and normal placental tissues. (A) Scatter plot of positively labeled cDNA; and (B) Scatter plot of negatively labeled cDNA.

Gene expression profiles as determined by real-time quantitative RT-PCR

The differences (determined as differences of Ct value) of gene expression profiles between preeclamptic and normal placental tissues were analyzed using a Rotor Gene RG-3000 Real Time PCR machine. They showed that among the 16 highly differentially expressed genes, three were down-regulated, and 13 were up-regulated. The results from real-time RT-PCR were well correlated to those from microarray analysis, i.e., the two techniques showed not only the same change direction of gene expression, but also comparable magnitudes in altered gene expression for most genes screened (Figure 4). Among differentially expressed genes detected in this study, LTBP-2 gene was down-regulated in preeclamptic placental tissue (the ratio of expression differences was 0.324). The expression differences were confirmed by real-time RT-PCR and tissue microarray immunohistochemical analysis.

Discussion

Analysis of differentially expressed genes between preeclamptic and normal placental tissues and the significance

To date, the etiology and pathogenesis of PE is unclear. PE is considered a polygenic trait disease with impaired immune function, infiltration capacity of placenta trophoblastic cells ,and placental blood supply. The activated trophoblastic cells and other effector cells will release cytotoxic factors and vasoactive substances, which will induce the damage of vascular endothelial cells and arteriolar spasm [23]. It can be concluded that the etiology and pathogenesis of PE are correlated to changes of a multitude of genes and proteins, rather than abnormalities of a single gene or protein.

The technique of cDNA microarray was developed in the mid-1990s and has been widely used for gene analysis and detection [24]. Presently it was mainly used for high throughput analysis of gene expression and gene polymorphism. It can be used to compare gene expression profiles in certain tissue cells under different physiopathological states, which will benefit for the analysis of changing characters and law of certain gene group under different physiopathological states, and understanding of intergenic relationships, the biological functions of genes and their encoding proteins, especially understanding of pathogenesis of certain disease at gene level, which will provide new treatment method for the disease. BioStarH140s cDNA microarray used in this study contains 14,112 human clones. Differentially expressed genes between preeclamptic and normal placental tissues detected using the microarray include most of genes related major cell functions of placental tissue. The analysis of these differentially expressed genes selected from hundreds of genes, and studies on changes of cell proliferation, immunity, apoptosis, signal transduction, transportation and metabolism, and gene synthesis, repair, and regulation, will provide new clues and routes for elucidation of pathogenesis and pathophysiological changes of PE. In this study, some of differentially expressed genes detected by cDNA microarray were confirmed by real-time fluorescent quantitative RT-PCR.

Table 1. — *Primer sequence, product sizes, coding proteins, and gene ontology of 16 genes confirmed by real-time fluorescent quantitative RT-PCR.*

Gene name	Genbank No. & mRNA No.	Primer (5'→3')	Position	Product length (bp)
CYP19A1	NM_031226	5'-CTTGGTGTGGAATTATGAGG-3'	358	
		5'-GAGCGTGTTAGAGGTGTC-3'	856	499
ELTD1	NM_022159/BC025721	5"-TGGGTGTCATCTACAACAAGGG	116	
		5"- TTCGGTGCTAAGCCAACATACT	142	143
FMR1	NM_002024/BC067272	5"-TCCCTACAGCCTATTGGACACG	692	
		5"- AGAGCCATCAGTCCGATTACC	742	253
IFNAR1	NM_000629/AK123813	5'-CAGATGATGGTCGTCCTC-3'	145	
		5'-CCAAAGCCCACATAACAC-3'	622	478
IGFBP1	NM_000596/CR595377	5'-GTCTGGCTGGTACTGCTC-3'	318	
		5'-TTATCTCCGTGCTCTCTGG-3'	681	364
KRT15	NM_002275/AK122864	5"-TGGGGTGAAAGGCGAGTAA	669	
		5"- ACAGGGCACAGGGAAAGAGT	177	302
LTBP2	NM000428	5"-CCTCACGGAGAAAATCAAGAAG	504	
		5"- AGAGCCATCAGTCCGATTACC	582	118
MRC1	NM002438/BU198859	5"-TATGCCTACCATGCCCTCG	139	
		5"- GACCAGATTCCCTCCAAAGC	193	154
PAPPA	NM_002581/AK094182	5'-GATGGGTCGTGGGCATTC-3'	836	
		5'-TATCTCCCGCTGAGTCCTG-3'	1,185	350
PAPPE/PAPPA2	NM_021936/AF342989	5'-TTCAGGTAGCGAGGAGTGTG-3'	702	
		5'-ATGGGCAGACGAGAAAGAGG-3'	1,105	404
Predicted protein PP1665	BC033391	5'-TAGCAGAGCCAGGATTCG-3'	66	
		5'-GAGCGTGTTAGAGGTGTC-3'	449	384
PSG1	NM_006905/M23575	5'-AAGCCGAGCCAACCAAAG-3'	347	
		5'-GACCATTCATCCACCACAGG-3'	784	438
PSG4/PSG9	NM_002780/BC063127	5'-AGTGGTGGATGAATGGTC-3'	642	
		5'-CGGTAATAGGTGAATGAAGG-3'	1140	499
PSG6/PSG10	NM_002782/BC020652	5'-TCACTGTCACCTTATACTC-3'	509	
		5'-AACACATCCTTCTTCTCC-3'	869	361
SERPINH1	NM_001235/BC012609	5'-CAGCAACTCAGAGAATAACC-3'	38	
		5'-GAGCGGAAGGATGAATGG-3'	405	368
TFPI2	NM_006528/AK129833	5'-AGGAGCCAACAGGAAATAAC-3'	152	
		5'-CAGCCAGTATAGGTGAAAGC-3'	647	496

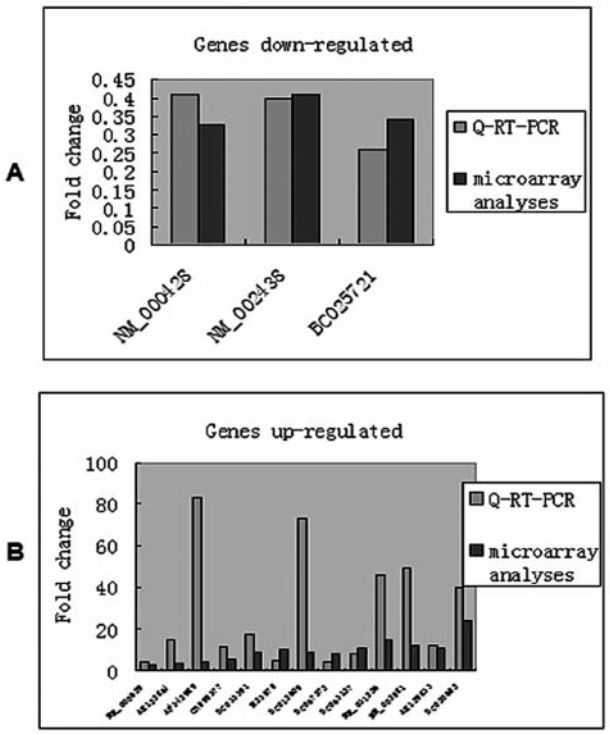

Figure 4. — Expression differences of down-regulated (A) and up-regulated (B) genes detected by real-time RT-PCR and cDNA microarray.

Table 2 shows that the ratios of gene expression differences between preeclamptic and normal placental tissues detected by the two techniques are comparable, indicating that the result of cDNA microarray analysis was accurate and reliable.

In this study, the expression profiles of differentially expressed genes known in previous studies, and also many unknown differentially expressed genes in preeclamptic placental tissue were detected. Many genes are found to be up- or down-regulated in the placental tissue in PE. LTBP-2 gene and insulin-like growth factor binding protein gene were down-regulated in preeclamptic placental tissues, probably resulting in the decrease of the adhesion of placenta trophoblastic cells to extracellular matrix, and the decrease of invasion and migration of placenta trophoblastic cells. In this study, it was found that mannose receptor gene was down-regulated, and interferon receptor gene was up-regulated. This may induce immunologic regulation disorder, and the increase of activities of lymphocytes and inflammatory cytokines, causing the damage of blood vascular endothelium. The expression of TFPI-2 gene, and TFPI-2 has significant inhibitory effects on four important matrix hydrolases including fibrinolysin, trypsin, and matrix metalloproteinases (MMP-2 and MMP-9) [25]. When these matrix hydrolases are inhibited, the invasion of placenta trophoblastic cells into helicine arteries would be inhibited. Pregnancy related protein genes and cytochrome P450 19A1 (CYP19A1/aromatase) gene may affect the pathophysiological process of PE by regulating immunity and mitochondria metabolism, respectively.

Expression of latent transforming growth factor b binding protein 2 (LTBP-2) gene in preeclamptic placental tissue and the significance

LTBP2 was identified in 1994 from human platelet and was mapped to chromosome 14q24. Having a size of 112,148 bp, the gene encodes a protein with 1,812 amino acids. Structural analysis showed that the gene has several repeat sequences such as epidermal growth factor (EGF)-like repeats and a repeat containing eight cysteine residues. Analysis of *in situ* hybridization showed that the gene is highly expressed in the lung, placenta, heart, liver, and muscle tissue [26]. To date, four isoforms of the gene were found, including LTBP-1,2,3 and 4, which were assigned to different chromosomes [27,28]. Previous studies have indicated that LTBP genes may play important roles in the following ways [29]: 1) regulating intracellular biosynthesis of TGF-β precursor; 2) promoting the binding of TGF-β precursor to specific connective tissues, and thus being named as "matrix receptor"; 3) inactivating TGF-β precursor and releasing mature TGF-β; and 4) maintaining the basal activity of TGF-β precursor on the cell surface. However, LTBP2 gene was not coordinately expressed with TGF-β gene [30], instead, it had no direct relationship with TGF gene during embryonic implantation. LTBP-2 may be one of components of important extracellular matrixes, or has regulatory effects on cell signal transduction [31-33]. In addition, LTBP-2 can promote migration of Bowes cells and adhesion of melanoma cells [34].

LTBP genes may play a critical role in the regulation of TGF-β. TGF-β is one of polypeptide growth factors and is composed of three isoforms in mammals, i.e., TGF-β1, TGF-β2, and TGF-β3. TGF-β is very important for the development of many tissues, since it is involved in regulation of cell proliferation, differentiation, and metabolism. TGF-β in placental tissue is involved in infiltration and differentiation of placenta trophoblastic cells, immunoloregulation, embryonic development, and the development of placental blood vessels [35]. It has been reported that TGF-β1, TGF-β receptor 1, and TGF-β receptor 2 genes are expressed in trophoblastic cells of placental villi and decidual cells, and the expression level in preeclamptic placental tissue is higher than that in normal placental tissue [36,37]. However, a very low expression level of TGF-β1 and TGF-β2 in uterine and placental tissue are also reported [38]. Thus, the role of TGF-β in the pathogenesis of PE is unclear.

The expression of LTBP-2 gene is down-regulated in preeclamptic placental tissue, and the gene may affect the pathological process and development of PE in various ways. The incidence of PE is closely correlated to shallow invasion of placenta trophoblastic cells into deciduomata

Table 2. — *Differences (ΔCt) of gene expression profiles between preeclamptic and normal placental tissues.*

GENBANK No.	Gene/Gene product (Protein)	PE-Ct	N-Ct	-ΔΔCt	$2^{-\Delta\Delta Ct}$	Microarray analyses
NM_000428	LTBP2/Latent transforming growth factor β binding protein 2	32.83	31.34	-1.29	0.409	0.324
NM_002438	MRC1/Mannose receptor C type 1	31.22	29.70	-1.32	0.401	0.41
BC025721	ELTD1/EGF, latrophilin and seven transmembrane domain containing 1	23.88	21.73	-1.95	0.259	0.342
NM_000629	IFNAR1/Interferon (α, β and Ω) receptor 1	18.65	20.41	1.96	3.89	2.689
AK122864	KRT15/Keratin 15, type I cytoskeletal 15	21.42	25.11	3.89	14.83	3.363
AF342989	PAPPE/Pregnancy-associated plasma protein E1	19.89	26.06	6.38	83.29	4.019
CR595377	IGFBP1/Insulin-like growth factor binding protein 1	28.63	31.81	3.38	11.43	5.335
BC033391	Hypothetical protein PP1665	25.34	29.26	4.12	17.39	8.57
M23575	PSG1/Human pregnancy-specific β-1 glycoprotein	28.96	31.01	2.25	4.76	9.624
BC012609	SERPINH1/Serine (or cysteine) proteinase inhibitor	24.30	30.29	6.19	73.01	8.619
BC067272	FMR1/Fragile X mental retardation protein	20.74	22.47	1.93	3.81	8.416
BC063127	PSG4/Pregnancy specific β-1-glycoprotein 4	24.14	26.90	2.96	7.78	10.982
NM_031226	CYP19A1/Cytochrome P450 19A1	24.23	29.55	5.52	45.89	14.824
NM_002581	PAPPA/Pregnancy-associated plasma protein A	30.28	35.70	5.62	49.18	12.158
AK129833	TFPI2/Tissue factor pathway inhibitor 2 precursor	22.83	26.22	3.59	12.04	10.814
BC020652	PSG6/Pregnancy specific b-1-glycoprotein 6	26.74	31.85	5.31	39.67	23.98
NR_003286	18s ribosomal RNA	16.82	16.62			

tissue. Perhaps inhibition on invasion ability of placenta trophoblastic cells is one of major mechanisms of LTBP-2 gene. The relationship between LTBP2 and invasion ability of placenta trophoblastic cells is unclear. On one hand, as one of components of extracellular matrixes, LTBP2 has regulatory effects on invasion process of placenta trophoblastic cells, decrease the differentiation of invasive placenta trophoblastic cells needed by helicine arteries reconstruction process, and thus decrease the number of reconstructed helicine arteries, resulting in the decrease of blood flow in intervillous lacuna, the increase of ischemic damages in deciduomata and placental tissue, and finally the incidence of PE. On the other hand, with the decrease of expression level of LTBP2 gene, the level of active TGF-β1 is increased. Possible mechanisms underling inhibitory effects of TGF-β1 on invasion process of placenta trophoblastic cells include: a) induction of the production of tissue inhibitor of metalloproteinase-1 (TIMP-1), which has the function of blocking the infiltration of placenta trophoblastic cells; b) down-regulation of the production of urokinase-type plasminogen activators (u-PA) in placenta trophoblastic cells; c) induction of the overexpression of integrin, resulting in more tight adhesion between placenta trophoblastic cells and extracellular matrix, and then inhibition of the invasion of placenta trophoblastic cells; and d) altered proliferation and differentiation of peripheral placenta trophoblastic cells, promoted production of syncytiotrophoblasts, and inhibited infiltration of placenta trophoblastic cells [39-41], resulting in incomplete reconstruction of helicine arteries, placental hypoxia and ischemia, activation and damage of vascular endothelial cell, and finally the incidence of PE. The abnormal down-regulation of LTBP2 gene in preeclamptic placental tissue reflects dysfunction of placenta trophoblastic cells and abnormal placentation. However, the function of LTBP2 gene and its effects on pathophysiological process of PE should be further studied.

Expression of IGFBP1 gene in preeclamptic placental tissue and the significance

IGFBPs are regulatory proteins which can bind to insulin-like growth factors (IGFs). They can regulate the binding ability of IGFs to their receptors, and the intensity of downstream signal transduction of IGFR, and thus regulate the growth and proliferation of target cells. To date, ten IGEBPs selectively expressed in different tissue cells have been found, and their genes have been assigned to chromosome 2, 4, 6, 7, 8, 12, and 17, respectively. IGFBPs are carrier proteins of IGFs, and have the function of prolonging the half-life of IGFs. However after the IGF-IGFBPs complex is transported to places nearby target cells, IGFBPs will again become the main obstacle of the binding between IGFs and IGFR, since IGFBPs can competitively inhibit the binding between IGFs and IGFR. Therefore, IGFBPs may inhibit the duplication, growth, and proliferation of DNA in this way [42].

IGFBP-1, which is the first member of IGF binding protein family and has been mapped to chromosome 7p12-p14, composed of 234 amino acids, and has a molecular weight of 25.3~31 kD. IGFBP-1 is one of important regulators of IGF-1. In addition, it has important effects on the incidence and development of many diseases, especially such as endocrine diseases such (e.g. diabetes, hyperthyreosis, and obesity), polycystic ovarian syndrome, and sexual development disorders [43]. The exact physiological function and regulating mechanism of IGFBP-1 gene is unclear. The

expression of IGFBP-1 gene can be regulated by insulin, adrenocorticotropin, cAMP, cytokines, and estrogen [44-48]. As an important multifunctional protein with significant biological meanings, IGFBP-1 can: a) regulate the transportation and metabolism of IGF-1 by binding to IGF-1; influence the transportation of IGF across vascular endothelial cell; b) prolong the half life of IGF-1, and regulate the clearance rate of IGF; c) determine tissue specificity of IGF by regulating the distribution of IGF in specific tissues and cells; d) regulate the interaction between IGF and its receptor, and thus regulate the biological activity of IGF-1; and e)inhibit metabolism, promote cell proliferation, and induce the increase of blood sugar through IGF-independent pathways.

IGFBP-1 is one of the proteins in decidua that have the highest expression level, indicating that it has regulatory effects on part of maternal-fetal interaction process. Abnormal expression of IGFBP-1 gene will induce a series of pregnancy-associated diseases. Giudice *et al*. [47] showed that the concentration of IGFBP-1 in serum of women with PE was five times of that of normal control group, and the concentration was correlated to the severity of PE. Angiospasm and the decrease of blood flow can be observed in preeclamptic placental tissue results in insufficient nutrient supply, which will cause hypoglycemia and hypoinsulinemia. Large scale biosynthesis and secretion of IGFBP-1 in decidua and liver can be induced by hypoinsulinemia. Other studies showed that unbalance between stimulating factors (e.g., adrenocorticotropin, progesterone, and cAMP) and inhibitory factors (e.g., insulin, IGF, and IL-1β) relating to the release of IGFBP-1 will increase the level of IGFBP-1. The increase of progesterone and antagonists of IL-1 receptor in pre-eclamptic placental tissue may increase the level of IGFBP-1. IGFBP-1 can bind to placenta trophoblastic cells at RGD-independent integrin binding site (α5β1), resulting in inhibition on the invasion ability of placenta trophoblastic cells. Paul *et al.* showed that the invasion of placenta trophoblastic cells into decidua in IGFBP-1 transgenic pregnant mice was obviously shallower than that in wild-type pregnant mice, indicating that the overexpression of IGFBP-1 may inhibit invasion process of placenta trophoblastic cells. IGFBP-1 can also inhibit the invasion of placenta trophoblastic cells into decidua by inhibiting the expression of MMP29 gene and inducing the expression of TIMP-1 gene. IGF-I cannot only promote the proliferation, differentiation, and migration of placenta trophoblastic cells, but also the protease secretion and blastocyst implantation. IGFBP-1 can bind to IGF-I, inhibit the biological activity of IGF-I, resulting in invasion disorder of placenta trophoblastic cells, placenta shallow implantation, and finally the incidence of PE [49].

Shang *et al.* [50] analyzed IGFBP-1 in serum and placental tissue of preeclamptic and normal pregnancy by enzyme linked immunosorbent assay (ELISA) and immunohistochemical analysis, showed that IGFBP-1 level in serum and the positive rate of IGFBP-1 were higher in preeclamptic placental tissue than in normal placental tissue, and the level of IGFBP-1 in serum and placental tissue was positively correlated to the incidence and development of PE. In this study, the results of cDNA microarray analysis and real-time RT-PCR showed that the expression level of IGFBP-1 was obviously higher in pre-eclamptic placental tissue than in normal placental tissue, indicating that IGFBP-1 gene may be involved in the incidence and development PE during gene expression process and metabolism process, and IGFBP-1 level in serum can objectively reflect placental functions and may be used in the clinic.

IGFBP-1 can regulate the activity of IGF-1 via self phosphorylation. After dephosphorylation, the affinity of IGFBP-1 to IGF-1 is decreased, and then the activity of IGF-1 is increased. On the contrary, phosphorylated IGFBP-1 will inhibit the activity of IGF-1. Therefore, further studies on expression regulation, post-translational modification of IGFBP-1 gene, and its action mechanism in the pathogenesis of PE may benefit for better understanding of the change of ischemia and metabolism in preeclamptic placenta trophoblastic cells, and open up a broad prospect for studies on etiology, prevention, diagnosis, and treatment of PE.

Expression of pregnancy specific proteins in preeclamptic placental tissue and the significance

Pregnancy specific glycoproteins (PSGs) include pregnancy-associated plasma protein A (PAPP-A), PAPP-B, and PAPP-C. PAPP-A is synthesized by syncytiotrophoblast and decidual cells and then is secreted in blood circulation [51]. Cultures of placenta trophoblastic cells and decidual cells cannot synthesize PAPP-A by themselves unless pregnancy serum is added into, indicating that pregnancy serum may have a special inducer that can induce the production of PAPP-A in placenta trophoblastic cells and decidual cells [52]. PAPP-A gene was mapped to chromosome 9q33.1. It has a molecular weight of 750~ 820 kD, and is stable at pH 4-10, but will be fully damaged at pH<2 or pH >10. It has regulatory effects on activity of cell factors. Lawrences *et al.* [53] showed that PAPP-A is one of proteases relating to IGFBP-4. Intact IGFBP-4 can inhibit the activity of IGFs, but degraded IGFBP-4 loses the inhibitory effect, indicating that IGFBP-4 can be used as one of positive regulators of active IGFs strain. PAPP-A is one of special proteins of pregnancy serum that can be detected in four to six weeks after last menstrual period. PAPP-A level in pregnancy serum is increased more obviously than human chorionic gonadotropic (hCG) along with the increase of pregnant weeks, and will reach the peak at term (at that time, the PAPP-A level in serum is ten times of that in amniotic fluid). After childbirth, PAPP-A level is decreased gradually, with the half life being three to four days. Six weeks after childbirth, PAPP-A in serum can no longer be detected. The change rule of PAPP-A in amniotic fluid is sim-

ilar to that in maternal blood. PAPP-A cannot be detected in cord blood and the body of fetus. Since it does not secrete in kidney, PAPP-A cannot be detected in pregnancy urine throughout the whole duration of pregnancy.

PAPP-A level in pregnancy serum may have relationships with placental functions and maturity. Therefore, detection of PAPP-A level in pregnancy serum can be directly used to monitor placental maturity, and indirectly reflect fetal growth and development. PAPP-A level in pregnancy serum presents a high value in twin pregnancy [54], and presents a low value in the condition of spontaneous abortion, ectopic pregnancy, intrauterine growth retardation, fetal death, fetal anomaly, and pregnancy associated with diabetes because the synthesis of PAPP-A is inhibited by placenta insufficiency [54-56]. Hence, detection of PAPP-A level in maternal serum can be used as an auxiliary index to determine placental functions and maturity. It was demonstrated that PAPP-A level in pregnancy serum could be detected as early as when any overt symptoms could be observed and other proteins in pregnancy serum were still not affected by the increase of PAPP-A level, and the increase amplitude of PAPP-A level was positively correlated to illness degree [57,58]. Therefore, some researchers proposed that detection of PAPP-A level in maternal serum during early pregnancy can be used to reflect placental functions and predict and monitor the pathogenesis of PE.

In this study, the expression of PAPP-A gene was obviously up-regulated in preeclamptic placental tissue. As one of endocrine organs, placenta can produce PAPP-A, which will then enter into maternal and fetal blood circulation, and have various effects on metabolic and immune system. As a protease inhibitor, PAPP-A can benefit for maintenance of placental barrier through its activation effect on complements, and its inhibitory effect on proteolysis of maternal phagocytes [59]. Placental ischemia and hypoxia, and placenta shallow implantation induced by the incidence of PE, and response of placenta trophoblastic cells to placental hypoxia will increase the expression level of PAPP-A, which will prevent fetus away from immune rejection through its immunosuppressive action. Simultaneously increased PAPP-A in maternal serum may reflect the degree of placental function decrease and the severity of hypoxia. Therefore, the high expression level of PAPP-A gene in preeclamptic placental tissue and the increase of PAPP-A level in maternal serum are secondary changes of PE, rather than the etiology of PE. However, the molecular biological functions of PAPP-A, the regulatory effects of PAPP-A on placenta trophoblastic cells, and the action mechanism of PAPP-A in pathogenesis of PE should be further studied.

PSG is a large group of carcinoembryonic antigens (CEA). It is composed of 11 isoforms (PSG1-8 and PSG11-13), and has been mapped to q13.2 of chromosome 19 together with seven cellular adhesion molecules relating to CEA. PSG is synthesized in syncytiotrophoblast and decidual cells and then is secreted in blood circulation. It can be detected in seven days after embryonic implantation. It will reach peak at between 34 to 38 pregnant weeks, and even reach 200 mg/l at term [60]. It can also be detected in maternal blood, amniotic fluid, cord blood, and milk of normal pregnant women. Biological functions of PSG are still unclear. Low expression level of PSG may have relationships with pathological pregnancy, including fetal distress, fetal growth restriction, and spontaneous abortion [61-63]. It was demonstrated that the binding of PSG to monocyte receptors may have inhibitory effects on cytotoxic action of macrophages and nitric oxide secretion of macrophages, antagonistic effect on lipopolysaccharide-induced macrophage activation, and inhibitory effects on proliferation of T-cells [64]. Since it can induce the production of anti-inflammatory cytokines, interleukin-10 (IL-10), IL-6, and PGE_2 in monocytes [65], and promote the transformation of Th cells to Th2 cells, PSG is considered to have inhibitory effect on immune reactions, which is very important for inhibition of maternal immune rejection and the improvement of fetal survival [66]. The differences of PSG expression play a decisive role for the maintenance of normal pregnancy and the protection of fetal growth and development [67,68].

In this study, the expression of PSG3, PSG3, and PSG6 genes were obviously up-regulated in preeclamptic placental tissue, indicating that neutrophilic granulocytes and monocytes in placenta-site of decidua were activated during late pregnancy, the incidence of PE, or in the condition of placental hypoxia, which would cause up-regulation of PSG in placental trophoblastic cells and the increase of PSG excretion, probably resulting in inhibition of maternal immune rejection, prolongation of pregnancy time, and the improvement of fetal survival. However, the regulating mechanism and the structure and functions of PSGs should be further studied.

Conclusions

In conclusion, differentially expressed genes in preeclamptic placental tissue were analyzed by cDNA microarray technique, and many genes are likely related to the pathophysiology of PE. The gene expression profile of preeclamptic placental tissue is obviously different from that of normal placental tissue, indicating that the pathophysiology of PE is very complicated and is involved in a wide range of multiple changes relating to cell metabolism, cell cycle, gene regulation, and cell signal transduction. The result of this study showed that during late period of PE high level of gene expression in placental trophoblastic cells, which are the immunologic barrier between pregnant women and fetus, is the response to maternal immune reaction, which will keep fetus away from maternal immune rejection and improve the survival of fetus. Further studies on the biological function and regulating mechanism of these genes will provide new clues for better understanding of etiology and pathogenesis of PE.

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