# Expression profile of trophoblast invasion-associated genes in the pre-eclamptic placenta

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**ABSTRACT**

The primary pathology of pre-eclampsia is thought be a defect in placentation due to failure of trophoblast invasion. Here, we aim to identify the expression profile of invasion-associated genes in the pre-eclamptic placenta. Messenger RNA (mRNA) expression levels of extracellular matrix molecule-related genes in five pre-eclamptic placentas and in five strictly matched normal placentas were assayed using complementary DNA (cDNA) microarrays representing over 220 human cytokine-associated or hormone-associated genes. Results demonstrated greater than two-fold higher expression of 18 extracellular matrix molecule genes, including cadherin, collagen, integrin and selectin, in the pre-eclamptic placenta. Extracellular matrix molecule degradation-related genes, including matrix metalloproteinase (MMP)-10, MMP-13, MMP-15, tissue inhibitor of metalloproteinase (TIMP)-2, TIMP-3, plasminogen and plasminogen activator, were also highly expressed in the pre-eclamptic placenta, compared to the normal placenta. Results suggest that the abnormal expression profiles of extracellular matrix molecules and degrading proteinases might be associated with the pathogenesis of pre-eclampsia.

KEY WORDS: Genes. Placenta. Pre-eclampsia. Matrix metalloproteinases.

## Introduction

Pre-eclampsia, the second most common cause of maternal mortality in the USA, complicates 6-8% of all pregnancies.[1] In developed nations, pre-eclampsia accounts for 10% of preterm births, ranks seventh in importance in perinatal death,[2] and is a major cause of maternal morbidity and mortality. Endothelial dysfunction appears to be central to the pathophysiology of pre-eclampsia[3] and has also been reported in the fetus,[4,5] but the mechanisms involved remain to be clarified.

The primary pathology of pre-eclampsia is thought to be a defect in placentation due to failure of the second wave of trophoblast invasion between eight and 18 weeks' gestation.[6] During early human pregnancy, cytotrophoblasts invade the uterine spiral arteries, replacing the endothelial layers of these vessels and subsequently destroying the medial elastic, muscular and neural tissue. In pre-eclampsia, however, invasion of the spiral arteries is limited to the proximal decidua, with 30-50% of the spiral arteries in the placental bed escaping endovascular trophoblast remodelling.[7,8] This failure of trophoblast invasion in pre-eclampsia results in a reduction in uteroplacental perfusion, with the placenta becoming increasingly ischaemic as gestation progresses.

Extracellular matrix molecules and degrading enzymes play an important role in regulating trophoblast invasion. For example, inappropriate integrin expression by the extravillous cytotrophoblast is associated with the shallow pattern of invasion and lack of arterial remodelling that occurs in pre-eclampsia.[8]

Thus, to investigate the mechanism of shallow trophoblast invasion in pre-eclampsia, this study aims to identify the expression of invasion-associated genes, such as integrins, in placentas from patients with pre-eclampsia, using DNA microarrays.

## Materials and methods

*Microarrays and reagents*

Commercially available microarrays were obtained from Takara Shuzo (Otsu, Shiga, Japan). One was arrayed with 221 DNA fragments of human genes, the other contained 243 immobilised human DNA fragments on a glass slide. The chips also included housekeeping (control) genes including β-actin, $\alpha_2$-tubulin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), hexokinase, transferrin receptor, ribosomal protein S5 and general transcription factor IIB. The controls were used to correct for signal intensity between the two fluorochromes used and to adjust the background in image processing.

The following reagents were used: Trizol (GibcoBRl, Life Technologies, Breda, The Netherlands); AMV Reverse Transcriptase XL (Life Sciences, USA); MicroSpin G-50 columns, 1 mmol/L FluoroLink Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, USA); Oligo dT primer, RNase inhibitor, 10× LowT dNTP mix (5 mmol/L dATP, 5 mmol/L dGTP, 5 mmol/L dCTP, 2 mmol/L dTTP), 5× Competitor I (Human Cot I DNA 7.5 μg/μL, Poly dA 4 μg/μL, yeast tRNA 5 μg/μL), glass cover slips, rubber cement and a humid hybridisation chamber (Takara Shuzo); and Oligotex mRNA kit (Qiagen, Valencia, CA, USA). Other reagents were all of analytical grade.

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*Tissue samples*
Placental tissue was collected from five normal pregnancies (>37 weeks gestational age) and five patients with pre-eclampsia. The latter patients were identified after 20 weeks' gestation with at least three new-onset symptoms including raised blood pressure (≥ 140/90 mmHg), proteinuria (≥ 2+) and oedema. Maternal consent was obtained according to Human Ethics Committee guidelines.

Patients in the two groups were strictly matched for age (24/25 years old), weight and body shape, and had no history of cardiovascular, renal or other hypertension-associated disease. Tissue blocks (1 cm cubes) were cut from the same place on the maternal face of each placenta. Chorionic villi were dissect from the blood vessels and connective tissue, stored in liquid nitrogen and used within a month.

*RNA isolation*
Total RNA was extracted from frozen villous tissues by a Trizol method described previously,[9] but was further purified by a second phenol/chloroform extraction. Pelleted RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water. The *A*260/*A*280 ratio of the RNA sample was over 1.8. To assess quality, isolated RNA (1 mg/lane) was electrophoresed for 3 h at 50 V on a 1% agarose/formalin gel and stained with ethidium bromide. It appeared as two bright and clear bands, representing 28S and 18S ribosomal RNA (rRNA), respectively; the ratio of intensities being approximately 2 to 1. Total RNA samples from each of the five normal placentas and each of the five pre-eclampsia placentas were pooled separately. Messenger RNA (mRNA) was purified from the total RNA sample following instructions included with the Oligotex mRNA kit.

*Probe labelling and purification*
Fluorescence-labeled probes used in microarray analysis were prepared using a reverse transcription method. Reaction mixture containing 300 pmol oligo dT primer, 2 μg polyA+ RNA and DEPC-treated water was prepared to a total volume of 15 μL, incubated at 70°C for 10 min and chilled on ice for 5 min. To the mixture was added 4 mL 10× AMV reaction buffer, 4 μL 10× LowT dNTP mix, 4 μL Cy3- or Cy5-dUTP (1 mmol/L) and 50 units of RNase inhibitor. Mixture volume was adjusted to 40 mL with DEPC-treated water. After adding 50 units of AMV Reverse Transcriptase XL, the mixture was incubated at 42°C for 1 h. Additional 50-unit amounts of AMV Reverse Transcriptase XL were added, and the mixture was incubated for a further 1 h at 42°C.

Reverse transcription was stopped by incubating the mixture at 70°C for 10 min. Unincorporated fluorescence-labelled nucleotides were removed through a G50 Microspin column, following the manufacturer's instructions. The Cy3- and Cy5-labelled complementary DNA (cDNA) probes were mixed and further purified using phenol/chloroform/isoamyl alcohol. Finally, precipitated mixed cDNA probes were dissolved in the appropriate amount of hybridisation buffer (6× SSC, 0.2% SDS, 5× Denhardt's solution, 0.1 mg/mL denatured salmon sperm DNA).

*Hybridisation*
Microarray slides were placed in a Coplin jar containing 5× SSC, 0.1% SDS and 1% bovine serum albumin, and incubated at 65°C for 2 h. The slides were then washed with MilliQ water and isopropanol, and then air-dried. The Cy3- and Cy5-labelled probe mixture was heated at 95°C for 3 min to denature it, then centrifuged at 12,000 ×*g* for 10 min at 25°C. The labelled probe was then applied to a prehybridised microarray slide and placed in a sealed hybridisation chamber to which 20 mL water was added. The sealed chamber was placed in a 65°C water bath and incubated for 12 h. The slide was removed from the chamber and placed in a staining dish containing low-stringency wash buffer consisting of 2× SSC and 0.2% SDS at 55°C. The coverslip was removed gently while the slide was in the solution and agitated for 5 min. The wash step was repeated and followed by immersion in 2× SSC and 0.2% SDS at 65°C for 5 min. Following a wash in 0.05× SSC at room temperature for 5 min, the slide was dried with compressed nitrogen gas and scanned immediately.

*Slide scanning*
An Affymetrix/GMS 418 scanner (Affymetrix, Santa Clara, CA, USA) was used to detect the fluorescence signal from Cy5 and Cy3 at 635 nm and 532 nm, respectively. Hybridised slides were scanned first in the Cy5 channel and then in the Cy3 channel.

*Data analysis*
Scanning data were analysed using the ImaGene 3.0 analysis programme. Background was calculated locally for each spot, and the background-subtracted hybridisation intensities for each spot were then calculated. Measured Cy5 and Cy3 values were normalised by reference to the housekeeping genes included on the slides. Mean intensities were used to calculate measured Cy3 to Cy5 ratio values to identify differentially expressed genes. A post-normalisation cut-off of a two-fold change in up- or down-regulation was applied to define differential expression.

## Results

*Gene expression profile of pre-eclamptic placentas compared to normal placentas*
Expression of 464 genes was detected in normal and pre-eclamptic placental tissue using the DNA microarrays. Scatter diagrams (Figure 1a and 1b) illustrate the relative expression of Cy5 and Cy3 (Cy5 on the Y axis, Cy3 on the X axis). The 45° line indicates equal expression of the genes in both normal and pre-eclamptic placental tissue.

*Expression profile of extracellular matrix molecule genes in pre-eclamptic placentas*
Expression of extracellular matrix molecule genes in normal and pre-eclamptic placentas, including cadherin, collagen, integrin and selectin, were analysed. Eighteen genes showed a more than two-fold up-regulation in the pre-eclamptic placental tissue, compared with the normal placental tissue. Four genes (GenBank accession numbers X63432, AF018081, AC002310 and AL161972) remained unchanged (Table 1).

*Expression of extracellular matrix degrading proteinase genes in pre-eclamptic placentas*
Among the genes detected by the DNA microarrays, several coded for matrix metalloproteinase (MMP) and tissue

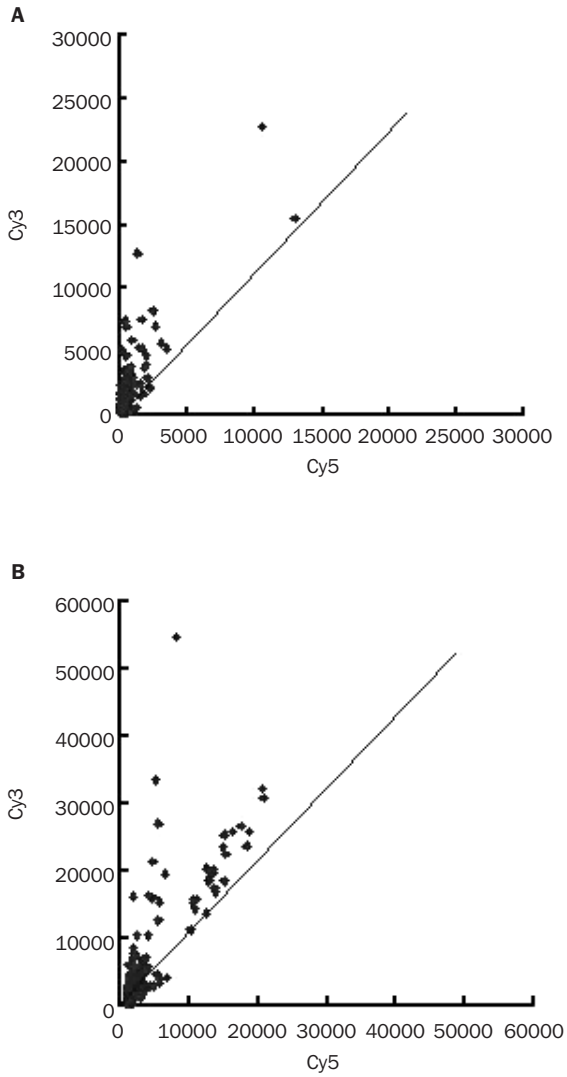


**Fig. 1.** Scatter plots of hybridising signals on gene chips: A) human cytokine, B) endocrine disruption study.

inhibitor of metalloproteinase (TIMP), which are associated with extracellular matrix degradation. Expression of MMP-10, MMP-13, MMP-15, plasminogen, plasminogen activator, TIMP-1, TIMP-2 and TIMP-3 were all higher in pre-eclamptic placental tissue than in normal placental tissue (Table 2).

## Discussion

In humans, trophoblast invasion into the decidualised endometrium and the inner third of the myometrium is of vital importance for both the anchoring of the placenta and connection with the maternal vascular system. This process is limited physically and restricted to the first 18 weeks of pregnancy.[10, 11] During this process, trophoblasts come into contact with the spiral arteries and replace the endothelial cells.

Pre-eclampsia is characterised by restricted endovascular invasion, in which physiological changes within the spiral arterioles are confined to the decidual portion of the arteries. Thus, some 30-50% of the spiral arteries in the placental bed show no evidence of endovascular trophoblast invasion.[12]

Using immunohistochemical methods, Zhou *et al*.[13] showed that cytotrophoblasts adopt an adhesion-receptor profile similar to that of endothelial cells, including decreased expression of E-cadherin and integrin α1vβ4 (a laminin receptor), and increased expression of vascular endothelial cadherin (VE-cadherin), platelet-endothelial cell adhesion molecule-1 (PECAM-1), vascular endothelial cell adhesion molecule-1 (VECAM-1) and integrin α1vβ1 (a fibronectin receptor). This might cause villous cytotrophoblasts to detach from their basement membrane and move into the fibronectin-rich matrix of the invasive cell columns.

In pre-eclampsia, however, the invading cytotrophoblasts express different adhesion molecules and integrins, thereby failing to adapt from an adhesion type that is characteristic of trophoblasts to one characteristic of endothelial cells.[14] Fisher *et al*.[14] showed that increased expression of integrin α1vβ1, which is a feature of invasive trophoblasts, is not found in trophoblasts isolated from pre-eclamptic patients and cultured *in vitro*.

The plasma contents of other extracellular matrix molecules are also changed in patients with pre-eclampsia. For example, elevated levels of cellular fibronectin and vascular cell adhesion molecule (VCAM) have been detected in the plasma of pre-eclamptic patients.[10] Plasma concentrations of L-selectin and P-selectin are also increased in patients with pre-eclampsia.[15,16] Additionally, there is evidence to show that expression of intercellular adhesion molecule-1 (ICAM-1) is also changed.[17]

Results from the present study, using DNA microarrays, showed that many extracellular matrix molecule genes, including cadherin, collagen, integrin, selectin and VCAM, are up-regulated in pre-eclamptic placental tissue. This indicates that the placenta might be an important source of the adhesion molecules that are increased in the plasma of patients with pre-eclampsia. Abnormal expression of some cadherin and integrin profiles, and possibly the collagen, selectin and VCAM profiles, are thought to be associated with the shallow trophoblast invasion that is a feature of pre-eclampsia. However, in the present study, not all integrin or adhesion molecule expression was changed in pre-eclamptic placental tissue, suggesting that some are not involved in the shallow trophoblast invasion or the pathogenesis of pre-eclampsia.

Extracellular matrix-degrading enzymes also play an important role in placentation and may participate in the development of pre-eclampsia. Trophoblasts facilitate invasion by secreting proteinase,[18] and serine protease, cathepsin and metalloproteinase have been implicated in the invasion process. MMPs, also called matrixins, are a family of human zinc-dependent endopeptidases capable of degrading all components of the extracellular matrix (ECM). Members of the MMP family can be classified, according to their substrate specificity and structure, into four subgroups.[19] Gelatinases (MMP-2 and MMP-9) digest collagen type IV (the major constituent of basement membranes) and denatured collagen (gelatin); collagenases (MMP-1, -8, -13) digest collagen types I, II, III, VII and X; and stromelysins (MMP-3, -7, -10, -11 and -12) digest collagen types IV, V, VII, as well as laminin, fibronectin, elastin, proteoglycans and gelatin. The substrate of the membrane-type metalloproteinases (MMP-14, -15, -16) is essentially proMMPs.

**Table 1.** Expression of extracellular matrix molecule genes in pre-eclamptic placentas compared to normal placentas

| GenBank Accession | UniGene Build 116 | Gene name | Cy3 | Cy5 | Cy3/Cy5 |
|---|---|---|---|---|---|
| Z13009 | 194657 | Cadherin 1, E-cadherin (epithelial) | 5450.89 | 1391.85 | 3.916296 |
| S42303 | 161 | Cadherin 2, N-cadherin (neuronal) | 1059.68 | 222.5 | 4.762613 |
| AF044209 | 75929 | Cadherin 11 (OB-cadherin, osteoblast) | 3693.52 | 414.16 | 8.918094 |
| X63432 | | Cadherin 11 (OB-cadherin, osteoblast) | 10592.51 | 9321.84 | 1.136311 |
| AF018081 | 178452 | Catenin (cadherin-associated protein), alpha 1 (102 kDa) | 263.74 | 258.86 | 1.01886 |
| X87838 | 171271 | Catenin (cadherin-associated protein), beta 1 (88 kDa) | 2020.96 | 305.79 | 6.608979 |
| M84349 | 119663 | CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344) | 11211.93 | 2391.99 | 4.68728 |
| J03464 | 179573 | Collagen, type I, alpha 2 | 19897.45 | 3860.86 | 5.153631 |
| X68742 | 116774 | Integrin, alpha 1 | 2172.41 | 134.38 | 16.16618 |
| M59911 | 265829 | Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor) | 1006.67 | 296.96 | 3.389918 |
| AF032108 | 74369 | Integrin, alpha 7 | 908.11 | 211.27 | 4.298353 |
| D25303 | 222 | Integrin, alpha 9 | 1413.55 | 54.97 | 25.71501 |
| AC002310 | 174103 | Integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide) | 548.00 | 361.16 | 1.517325 |
| M14648 | 118512 | Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51) | 6935.94 | 439.99 | 15.76388 |
| M24283 | 168383 | Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor | 1677.35 | 79.2 | 21.17867 |
| AL161972 | 83733 | Intercellular adhesion molecule 2 | 1948.37 | 1488.51 | 1.308939 |
| L34657 | 78146 | Platelet/endothelial cell adhesion molecule (CD31 antigen) | 21363.40 | 10604.15 | 2.014628 |
| AL021940 | 89546 | Selectin E (endothelial adhesion molecule 1) | 2613.97 | 169.4 | 15.43077 |
| M25280 | 82848 | Selectin L (lymphocyte adhesion molecule 1) | 2052.02 | 230.78 | 8.891681 |
| NM_003005 | 73800 | Selectin P (granule membrane protein 140 kDa, antigen CD62) | 441.99 | 130.01 | 3.399685 |
| M73255 | 109225 | Vascular cell adhesion molecule 1 | 2032.56 | 51.36 | 39.57475 |
| X51521 | 155191 | Villin 2 (ezrin) | 10386.46 | 3973.68 | 2.613815 |
| Z19554 | 2064 | Vimentin | 42796.54 | 6510.42 | 6.573544 |

**Table 2.** Expression of extracellular matrix degrading proteinase genes in pre-eclamptic placentas compared to normal placentas

| GenBank Accession | UniGene Build 116 | Gene name | Cy3 | Cy5 | Cy3/Cy5 |
|---|---|---|---|---|---|
| X07820 | 2258 | Matrix metalloproteinase 10 (stromelysin 2) | 983.48 | 62.21 | 15.80909 |
| X75308 | 2936 | Matrix metalloproteinase 13 (collagenase 3) | 283.32 | 8.06 | 35.15092 |
| Z48482 | 80343 | Matrix metalloproteinase 15 (membrane-inserted) | 1647.43 | 71.76 | 22.95753 |
| L07899 | 75576 | Plasminogen | 301.24 | 150.4 | 2.002909 |
| U09937 | 179657 | Plasminogen activator, urokinase receptor | 330.57 | 52.21 | 6.33155 |
| X03124 | 5831 | Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor) | 581.39 | 141.13 | 4.119568 |
| AL110197 | 6441 | Tissue inhibitor of metalloproteinase 2 | 5408.86 | 1609.5 | 3.360589 |
| U44385 | 267585 | Tissue inhibitor of metalloproteinase 2 | 3069.26 | 1555.81 | 1.972774 |
| AL023282 | 245188 | Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory) | 7003.26 | 2651.58 | 2.641169 |

Most MMPs are secreted as inactive proenzymes (proMMPs), which are activated in extracellular compartments. Several enzymes are capable of activating proMMPs, the most prominent being plasmin. The activity of MMPs in the extracellular space can be specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind specifically to the highly conserved zinc binding site of active MMPs at molar equivalence. The TIMP family consists of four structurally related members (TIMP-1, -2, -3 and -4). MMP-2 and MMP-9 are closely associated with the regulation of trophoblast invasion and are thus involved in the pathogenesis of pre-eclampsia. [20]

In the present study, TIMP-1 and TIMP-2 expression was up-regulated in pre-eclamptic placental tissue, which was consistent with the shallow pattern of trophoblast invasion in pre-eclampsia. However, in contrast, expression of metalloproteinases such as MMP-10, -13 and -15 was also higher in pre-eclamptic placental tissue than that in normal placental tissue, as was the expression of plasminogen and plasminogen activator. We suspect that this might be a stress response to the shallow trophoblast invasion that occurs in pre-eclamptic patients. □

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