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Comparison of gene expression at the feto–maternal interface between normal and recurrent pregnancy loss patients


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Abstract. Normal pregnancy requires a series of immunological, metabolic, vascular and endocrine regulating processes. However, the specific genes and proteins involved in these processes are not well defined. Aberration of these processes may lead to problems in pregnancy. One of these problems may be recurrent pregnancy loss (RPL). Little information is available on the level of expression of genes that may play a role in normal pregnancy. Therefore, this study determined whether different levels of gene expression at the feto–maternal interface could be associated with factors for RPL. The expression patterns of genes isolated from subtractive hybridization analysis performed with chorionic villi from normal and abnormal pregnancies were investigated. Eight genes classified into groups, including immunosuppression-related, embryo attachment-related and angiogenesis-related, were isolated.

Introduction

The maintenance of human pregnancy involves immunological, metabolic, vascular and endocrine regulatory processes that are genetically controlled. Abnormal regulation of these processes may be involved in recurrent pregnancy loss (RPL), which is defined as the loss of three or more consecutive pregnancies before the twentieth week of gestation, and which affects 2–5% of couples trying to conceive (Coulam et al. 1997). Why pregnancy loss occurs remains largely ill defined other than for chromosomal anomalies. Epidemiological surveys indicate that the aetiology for 24–60% of all cases of RPL remains unresolved (Wouters et al. 1993).

Previous investigations have focused on parental chromosome abnormalities (Costa et al. 1993; Lanasa et al. 2001), congenital malformations of the uterus, endocrine abnormalities (Costa et al. 1993; Bussen et al. 1999; Rai et al. 2000), immunological disorders (Lim et al. 1996; Ebelen et al. 2000; Lee et al. 2001), and haemostatic and metabolic abnormalities (Coumans et al. 1999). Since the conceptus contains paternal gene products and its immune differentiating antigen, it is possible that the maternal immune system recognizes these gene products as immunologically foreign, resulting in rejection (Matsui et al. 1989; Hey et al. 1995; Dalton et al. 1998). However, the incidence of this is most likely rare owing to the fact that uterine decidua secrete soluble proteins capable of inhibiting cell-mediated immune responses, potentially protecting the conceptus from maternal immune rejection during pregnancy (Bolton et al. 1987; Kamarainen et al. 1996).

Abnormal expression of several endometrial proteins, including PP14 (also known as pregnancy-associated endometrial α-2 globulin or glycofetin), CA125, and MUC1 (mucin 1), has been described for RPL (Dalton et al. 1995; Hey et al. 1995; Kamarainen et al. 1996). Since they are secreted by the endometrial glandular epithelium, the concentration of these proteins can be detected by endometrial flushing. PP14 is secreted by the ovary and the endometrium under the influence of progesterone and may serve as an immunosuppressor potentially by protecting the feto–maternal tissues (Dalton et al. 1995; Kamarainen et al. 1996). The concentration of PP14 has been reported to be significantly lower in endometrial and uterine flushings from RPL patients than from normal fertile controls (Dalton et al. 1995, 1998). CA125 is also produced by both the ovary and the endometrium (Zeimet et al. 1993; Dalton et al. 1998), and it has reportedly been found less in the endometrial flushings from RPL patients than from normal fertile controls (Dalton et al. 1998). In addition to PP14 and CA125, MUC1 has also been reported to be lower in uterine flushings from women suffering RPL than in normal fertile controls (Hey et al. 1995). Therefore, it is expected that there are genes expressed to an aberrant extent in RPL patients. In this study, we determined gene expression levels in normal pregnancy and in pregnancy resulting in or from RPL, using molecular cloning and the expression pattern of genes in chorionic villi.
Materials and methods

Tissue specimens and karyotyping

Informed written consent was obtained from all patients. A total of twelve chorionic villi samples (six from RPL and six from electively terminated pregnancies) were obtained from 12 pregnant women between 6 and 8 weeks of gestation (three samples for each gestation period). The chorionic villi were obtained from patients who had had at least three unexplained miscarriages (RPL), and from patients with no history of abortion, ectopic pregnancy, pre-term delivery or stillbirth (controls). The average age of the RPL patients and fertile controls were 34 ± 6 and 32 ± 4 years respectively. Chorionic villi, confirmed as such by immunocytochemical analysis with proliferating cell nuclear antigen and haematoxylin and eosin staining followed by histological examination, were used for karyotyping and cDNA subtractive analysis. Therapeutic and RPL samples were morphologically and histologically indistinguishable. Karyotyping, showing the entire 46XX genotype, was performed on all samples using the standard G-banding technique (Luciani et al. 1975). All specimens were processed within 30 min of collection, frozen, and stored at –80°C until use.

Total RNA preparation and reverse transcription

Total RNA was obtained from stored chorionic villi at –80°C, using TRIzol reagent (Gibco BRL, Rockville, MD, USA). cDNA was synthesized from reverse transcribed cDNA by the PCR-select cDNA subtraction Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) and cDNA synthesis primer (5′-TTTTGTCAGAACGTCTTGGN3′). Double strand (ds) cDNA was synthesized from reverse transcribed cDNA by the PCR-select cDNA Subtraction Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) and each ds cDNA was digested with Rsal (New England BioLabs, Beverly, MA, USA) to obtain shorter, blunt-ended molecules. These were divided into a tester for normal control and a driver for RPL samples. The tester cDNA was subdivided into two portions and then a different adaptor (adapter 1: 5′-CTAATACGACTCACTATAGGGCTCAAGGCGGCCGAGGT-3′ with a hanging fragment of 3′-GGCCCGCTCCA-5′; adapter 2R: 5′-CTAATACGACTCACTATAGGGCCAGGTGGTCGCGGCCGAGGT-3′ with a hanging fragment of 3′-GCCGCTCCA-5′) was ligated to each tester portion. Two consecutive hybridizations were carried out for subtraction. In the first hybridization, excess driver cDNA was added to each adaptor-ligated tester cDNA in order to heat-denature and anneal, generating a hybrid between tester and driver cDNA. Two primary cDNA hybridization samples were combined, rendering the remaining single-stranded, adaptor-ligated testers hybridizable. Finally, polymerase chain reaction (PCR) amplification was performed at 94°C for 10 s, 68°C for 30 s, and 72°C for 1.5 min for a total of 12 cycles using nested primers (primer 1: 5′-TCGAGCCGCGCCGCGCCGAGGT-3′; primer 2: 5′-AGCGTGGTCCGGGCAGGAGGT-3′) to obtain different gene sequences, which were further enriched.

Cloning

After subtraction, the PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and then transformed by heat shock at 42°C for 1 min in 50 µL of Escherichia coli DH5α competent cells. Colonies were cultured overnight at 37°C on Luria-Bertani (LB; DIFCO, Sparks, MD, USA) agar plates containing ampicillin (Sigma, St Louis, MO, USA), X-Gal (Gibco BRL), and isopropyl-β-thiogalactopyranoside (Gibco BRL).

Selecting the different sequences

White colonies screened were isolated and cultured in 4 mL of ampicillin-containing LB medium overnight. Plasmid DNA was purified with the QIAprep Spin Miniprep kit (Qiagen Inc.) from an insert-containing colony and the insert of each colony was identified through EcoRI (New England BioLabs, Beverly, MA, USA) digestion (Fig. 1). Selected clones were sequenced, and their sequences were identified with a BLAST analysis in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

Semi-quantitative reverse transcriptase–polymerase chain reaction and statistical analyses

The relative expression level of genes isolated from RPL and normal chorionic villi samples was determined according to the expression level of the housekeeping G3PDH gene. Primers are made on the basis of gene sequences identified with a BLAST search. The PCR conditions were as follows: after boiling at 94°C for 4 min, amplification was performed for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; the mixture was then cycled at 72°C for 10 min to complete
the elongation step and was finally stored at 4°C. The sequence of PCR products was confirmed by automated sequencing using ABI Prism (Applied Biosystems, Foster City, CA, USA). The density of each band was measured using a Gel-Doc image analyser (Bio-Rad, Hercules, CA, USA). The band intensities were normalized with those of the corresponding G3PDH. Statistical analysis of the data for each gene was performed using Student’s t-test when two groups were compared. Data were presented as mean ± SD and a value of $P<0.005$ was considered to indicate a statistically significant difference.

**Results**

**Subtractive hybridization analysis**

To compare the differences between genes expressed in chorionic villi from RPL patients and those from elective terminations of apparently normal pregnancies, we performed subtractive hybridization analysis. Tester cDNA generated by reverse transcription using RNA derived from chorionic villi of therapeutic abortion patients, was ligated with adaptors and then hybridized with a sufficient amount of driver cDNA generated by reverse transcription using RNA derived from RPL patients that were matched for gestational age, resulting in the subtraction of similar DNA fragments. The DNA fragments left after subtraction were considered to be genes that were expressed more or only in the tester. After cloning these DNA fragments into a T vector, we isolated a total of 348 clones. Of these, 166 potentially different clones were sequenced (Table 1). Figure 1 shows the EcoRI digestion of different clones, indicating that there were different sizes of insert. Each individual band represents a partial sequence of different genes isolated by subtractive hybridization. Sequence analysis of those inserts indicated that they encoded a partial sequence of 33 known genes and two unknown genes (Table 1). After identifying genes by sequence analysis, we performed reverse transcriptase–polymerase chain reaction (RT-PCR) with primers generated for these genes (Table 2).

**Semi-quantitative reverse transcriptase–polymerase chain reaction**

We identified eight genes that were expressed at different expression levels. They are PP14, MUC1, MMP-2 (matrix metalloproteinase-2), fibronectin, hCG (human gonadotrophin), HBG2 (haemoglobin-γ globulin), and two as yet unidentified genes. Semi-quantitative RT-PCR analysis indicated that these genes were more abundantly expressed in chorionic villi from the therapeutic abortion group than in those from the RPL group (Fig. 2). Interestingly, the newly found unknown genes showed a different level of expression between the normal control group and the RPL group. To confirm the similar amount of RNA used for semi-quantitative RT-PCR analysis, a housekeeping gene, G3PDH, was used as the control (Fig. 2). First, band densities for G3PDH expression analysed by RT-PCR were equalized and then the expression level of each gene was compared between RPL and normal control patients that were matched for gestational age. The ratios were determined by dividing the band density of each gene by that of G3PDH. The intensity of the

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**Table 1. The number of selective clones from subtractive analysis**

<table>
<thead>
<tr>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total clones</td>
</tr>
<tr>
<td>Sequenced clones</td>
</tr>
<tr>
<td>Positive clones (unknown and known gene)</td>
</tr>
<tr>
<td>Negative clones (vector and genomic DNA)</td>
</tr>
</tbody>
</table>

**Table 2. Primers used for reverse transcriptase–polymerase chain reaction analysis to confirm the positive clones**

<table>
<thead>
<tr>
<th>Sequence identification</th>
<th>Accession No.</th>
<th>Size of nucleotide sequence</th>
<th>Size of PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP14 (placental protein 14)</td>
<td>XM_005360</td>
<td>5′ AAGTTGGCACGGACTGGCACTC 3′</td>
<td>443 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ ACGGCCAGGGCTTCCATGGCTT 3′</td>
<td></td>
</tr>
<tr>
<td>MUC1 (mucin 1)</td>
<td>NM_002456</td>
<td>5′ AGACGTAGCGTGAGTGGATG 3′</td>
<td>171 bp</td>
</tr>
<tr>
<td>MMP-2 (matrix metalloproteinase-2)</td>
<td>NM_004530</td>
<td>5′ CAAGCCACTGACGCCCTT 3′</td>
<td>233 bp</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>X02761</td>
<td>5′ CCTGAAGCCTGGTGGATG 3′</td>
<td>274 bp</td>
</tr>
<tr>
<td>HCG (chorionic gonadotrophin)</td>
<td>M13504</td>
<td>5′ GCACAAAGGATGGAGTGGT 3′</td>
<td>119 bp</td>
</tr>
<tr>
<td>HBG2 (haemoglobin-γ globulin)</td>
<td>XM_006556</td>
<td>5′ ATGGGTCATTTCACAGAGG 3′</td>
<td>443 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ GTCTAAGGTGATG 3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ ACCACAGTCATATGCAGAGA 3′</td>
<td></td>
</tr>
<tr>
<td>Unknown 1</td>
<td>–</td>
<td>5′ GGCCAGGTATTACGGAGA 3′</td>
<td>102 bp</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>–</td>
<td>5′ CTTAAACCCAGGGATG 3′</td>
<td>376 bp</td>
</tr>
<tr>
<td>G3PDH (glyceraldehyde-3-phosphate dehydrogenase)</td>
<td>BC009081</td>
<td>5′ ACCACAGTCATATGCAGAGA 3′</td>
<td>452 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′ ACCACAGTCATATGCAGAGA 3′</td>
<td></td>
</tr>
</tbody>
</table>
EtBr staining was analysed by densitometry and plotted as a bar graph (Fig. 3). Statistical analysis revealed that the expression level of the normal group was significantly higher than that for the RPL group \((n = 6; P < 0.005)\).

**Classification of recurrent pregnancy loss-related genes**

The eight genes found to be more abundantly expressed in chorionic villi from normal controls than from RPL patients were classified into four groups based on their known functions. They are immunosuppression-related (PP14), embryo attachment-related (MUC1), angiogenesis-related (MMP-2 and fibronectin), and other genes (hCG, HBG2 and two unidentified genes). Our results suggest that the lower transcriptional level of immunosuppression-related genes, including PP14, and embryo attachment-related genes, including MUC1, in chorionic villi of RPL is related to the lower protein expression level of these genes in the endometrial flushings from RPL patients.

**Discussion**

Biological processes of implantation and the maintenance of pregnancy are mediated, in part, by regulated gene expression. Aberrant gene expression for these processes may cause a number of problems including RPL. To characterize the molecular mechanisms regulating these processes during early human development, we performed subtractive hybridization analysis using cDNAs from human chorionic villi of normal and RPL patients. Subtractive hybridization analysis is a powerful tool used to compare two groups of mRNA and we obtained clones of genes that are expressed more in one group but not in the other. In this study, we identified eight genes that are expressed at significantly lower levels in the chorionic villi from RPL patients than from electively terminated pregnancies. The identified genes are PP14 (immunosuppression-related), MUC1 (embryo attachment-related), MMP-2 and fibronectin (angiogenesis-related), hCG, HBG2 and two unidentified genes.

We expect that a number of genes are expressed at different levels in normal and RPL patients as several proteins, including PP14, hCG and MUC1, have been reported to present at reduced levels in flushings of the uterus and the endometrium from RPL patients. We found eight genes by cDNA subtractive hybridization analysis and confirmed that they are differentially expressed by semi-quantitative RT-PCR analysis. These observations support the notion that many genes are expressed at different levels between normal and RPL patients. Our results showed that the levels of PP14, hCG and MUC1 transcripts are reduced in RPL patients. This is in agreement with previous reports of reduced protein levels of these genes in RPL patients.

![Fig. 2](image1.png)

**Fig. 2.** Quantitative analysis of mRNA expression level in chorionic villi by RT-PCR. Primers were made from sequences of eight genes (PP14, MUC1, MMP-2, fibronectin, hCG, HBG2 and two unknown genes). Samples obtained from two groups, RSA and therapeutic abortion, at the gestation stages of six and eight weeks were analysed. TA, therapeutic abortion; RSA, recurrent spontaneous abortion.

![Fig. 3](image2.png)

**Fig. 3.** Semi-quantitative expression analysis of eight genes (PP14, MUC1, MMP-2, fibronectin, hCG, HBG2 and two unknown genes) expressed in chorionic villi tissues from patients with recurrent pregnancy loss (open bars) and patients with therapeutic abortion (solid bars). Biopsy was performed at the gestation stages between six and eight weeks. The ratios were determined by dividing the band density of each gene by that of G3PDH. *\(P < 0.005\) represents a statistically significant difference.
patients (Dalton et al. 1995, 1998; Hey et al. 1995; France et al. 1996), indicating that the function of PP14, hCG and MUC1 genes is affected at the level of transcription in these patients.

It is worth noting that some of the gene products we identified regulate the function of T lymphocytes in immune responses (Fig. 4). PP14 is an immunosuppressive molecule and inhibits the proliferation of T lymphocytes. Therefore, PP14 may potentially protect the conceptus from the maternal immune system (Kamarainen et al. 1996). Fibronectin stimulates the production of MMP-2 and MMP-9 from T lymphocytes and blocks the cytotoxic effect of Th1 cytokines (Kamarainen et al. 1996; Esparza et al. 1999; Pijnenborg et al. 2000). These observations indicate that regulation of the maternal immune system is important for the establishment and the maintenance of the pregnancy.

Usually, a normal concentration of hCG protein is maintained until the ninth week of pregnancy, but the level of hCG protein is markedly reduced in RPL patients (France et al. 1996). Therefore, hCG may be a useful marker for the prognosis of early miscarriage. It is not known whether the protein levels of MMP-2, fibronectin, HBG2 or two, as yet unidentified, genes are affected in RPL patients, and it is not yet clear whether they are related to RPL. Further research on the mechanisms of transcriptional and post-transcriptional regulation of the genes identified in this study will help us understand better the molecular mechanisms for maintaining normal pregnancy.

Interestingly, we identified two unknown genes and found that they are located on chromosomes 9 and 21, based on the human genome sequence database. The cloning of these genes is underway. Characterization of the molecular mechanisms for these gene products is required to understand their biological functions in maintaining normal pregnancy.

The PCR-based subtractive hybridization analysis has been used for investigating a variety of subjects in living organisms (Diatchenko et al. 1996). We performed subtractive hybridization analysis and RT-PCR analysis of cDNAs from the chorionic villi of normal and RPL patients, and identified eight genes, including the two unidentified genes, whose expression is significantly reduced in RPL patients. Four of the genes have been reported to regulate embryo attachment, angiogenesis or immunosuppression. Characterizing their functions will help us understand the process of establishing and maintaining pregnancy. In addition, more

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**Fig. 4.** A model for functional interactions among some of the genes isolated. PP14, an immunosuppressive-related gene, is known to inhibit the proliferation of T lymphocytes in immune reactions. Fibronectin stimulates the production of MMP-2 and MMP-9 from T lymphocytes and functions to block the cytotoxic effect of Th1 cytokines (TNF-α).
detailed studies of their expression in normal and RPL patients are needed to evaluate their clinical relevance. Further identification of genes aberrantly expressed in RPL patients will help the prognosis of the pregnancy, identifying pregnancies with a high risk of miscarriage and enabling management of those pregnancies.

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References


