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# Expression of Immunosuppression-Related Genes in Fetal Chorionic Villi Derived from Recurrent Spontaneous Abortion Patients

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## 습관성 유산 환자의 융모막 조직에서의 면역억제유전자 발현 양상

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**연구목적:** 본 연구에서는 정상 환자와 습관성 유산 질환 환자에서 유래된 융모막 조직 내에서의 면역억제유전자들의 발현 양상에 대해 알아보고자 하였다.

**연구재료 및 방법:** 임신 6주와 8주의 습관성 유산 질환 환자와 정상 환자로부터 융모막 조직을 채취하였다 (Normal N=6; RSA N=6). 면역조직화학적 분석을 통해서 조직을 관찰하고 세포가 살아있음을 확인한 후에, 역전사 중합효소연쇄반응을 통해서 면역억제유전자인 *placenta protein 14* (*PP14*), *indoleamine 2,3-dioxygenase* (*IDO*) 그리고 *mucin1* (*MUC1*) 유전자의 발현 정도를 비교하였다. *GAPDH* 발현에 기준한 면역억제유전자 발현을 정량 분석하여 Student's *t*-test를 시행하였고, $p<0.05$를 유의성이 있는 것으로 판정하였다.

**결 과:** 습관성 유산 질환 환자의 경우, 임신 6주와 8주의 융모막 조직에서의 면역억제유전자 (*PP14, IDO, MUC1*) 발현이 현저하게 낮은 양상을 보이고 있었다. 습관성 유산과 면역억제유전자의 발현이 통계학적으로 유의성 있는 연관성을 가지고 있다는 것이 확인되었다.

**결 론:** 면역억제유전자 (*PP14, IDO, MUC1*)의 발현이 습관성 유산 질환 환자에서 특이적으로 낮게 나타나는 것으로 보아 습관성 유산 질환의 진단과 치료 연구 방안에 이 유전자들의 발현이 이용될 수 있을 것으로 사료된다.

**Key Words:** Chorionic villi, In situ hybridization, Pregnancy, Recurrent spontaneous abortion

During pregnancy, chorionic villi enlarge and blood vessels grow into them, forming highly vascularized structures, completely surrounding the chorion. These villi are important interface for diffusion of nutrients, oxygen and wastes between mother and embryo. In these interface tissues, mother and fetus could consider to be a foreign each other, since a half of the fetal genome is

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derived from the farther. Recent investigations have been focused on immunological feto-maternal reaction that may cause recurrent spontaneous abortion (RSA).[1] RSA or habitual abortion, which is defined as the loss of three or more consecutive pregnancies before the 20th week of gestation, occurs in approximately 2~5% of pregnant women.[2] Why pregnancy loss containing RSA takes place at the molecular level remains unclear. It has been previously demonstrated that RSA can be caused by multiple problems associated with chromosomal abnormality, endocrine dysfunction, autoimmune disorders, and other unknown factors.[3~5]

One of immunosuppression-related molecules is glycodelin. It has been called by various names; placental protein 14 (PP14), chorionic α2-microglobulin (CAG-2), progesterone-associated endometrial protein (PEP) and pregnancy-associated α2-microglobulin (α2-PEG).[6] *PP14* has been isolated from human placenta and is expressed in other normal tissues including the epithelium of the fallopian tube, ovarian surface epithelium, uterine cervix, breast tissue, sweat glands, and bone marrow aspirates.[7,8] One of biological functions for PP14 is to inhibit early events in the T-cell receptor signaling pathway.[6,9] This indicates that PP14 has important roles as a possible immunosuppressive molecule capable of successful pregnancy and growth of the fetus.[10,11] Mucin1 (MUC1) is a cell-surface and secretory molecule of endometrial epithelium and shows the highest expression in the mid-luteal phase.[12] Mucins including MUC1 have various functions including protection against bacterial infections,[13,14] protection of proteins and cells from proteolysis, inhibition of cell attachment, promotion of cell attachment, and inhibition of immune cell function.[15] Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catabolizes tryptophan, which induces the death of T-lymphocytes.[16] In murine pregnancy, IDO expression may suppress T-cell-mediated induction of C3 deposition at the maternal-fetal interface that increases the risk of allogeneic fetal loss.[17] Taken all together, immunological regulation of these gene products is required for keeping normal pregnancy.

In this study, we compared the expression of three genes (*PP14*, *MUC1* and *IDO*) involved in immunosuppression in chorionic villi from RSA and normal patients to define the molecular regulation of immunological processes at the feto-maternal interface during pregnancy.

## MATERIALS AND METHODS

### 1. Tissue specimens

A total number of 12 fetal chorionic villi samples, 6 from RSA patients and 6 from patients with electively terminated pregnancy, were obtained from 12 pregnant women between 6 and 8 weeks of gestation (three samples for each gestation period). The chorionic villi from RSA patients were obtained from patients who had at least three times of unexplained miscarriages and from patients with no history of abortion, ectopic pregnancy, pre-term delivery, or stillbirth as controls. The average ages of RSA patients and fertile controls were 34±6 and 32±4 years, respectively. All specimens were obtained within 30 minute of collection, frozen, and stored at -80℃ until use. Informed written consent was obtained from all patients.

### 2. Immunocytochemical staining for fetal chorionic villi

Fetal chorionic villi from normal controls and RSA patients were immunocytochemically stained using antibodies for proliferating cell nuclear antigen (PCNA) to detect the proliferative activity, and hematoxylin and eosin (HE) to observe mitotic figures. Chorionic villi samples from normal and RSA patients were washed three times, 5 min

**Table 1.** Primers used for RT-PCR analysis. Primers of immunosuppression-related genes (*PP14*, *IDO*, and *MUC1*) were made on the basis of published sequences in GenBank

| Immunosuppression-related genes | Accession numbers | Nucleotide sequences | Size of PCR products |
|---|---|---|---|
| Placental protein 14 | XM_005360 | 5' AAGTTGGCAGGGACCTGGCACTC 3' | 422 bp |
| | | 5' ACGGCACGGCTCTTCCATCTGTT 3' | |
| Indoleamine 2,3-dioxygenase | NM_002164 | 5' GCGCTGTTGGAAATAGCTTC 3' | 119 bp |
| | | 5' CAGGACGTCAAAGCACTGAA 3' | |
| Mucin 1 | NM_002456 | 5' AGACGTCAGCGTGAGTGATG 3' | 171 bp |
| | | 5' CAGCTGCCCGTAGTTCTTTC 3' | |
| GAPDH | BC009081 | 5' ACCACAGTCCATGCCATCAC 3' | 452 bp |
| | | 5' TCCACCACCCTGTTGCTGTA 3' | |

each, in a 50 μl drop of PBS. This was followed by fixation at room temperature for 6 hrs in neutral buffered formalin. Then samples were washed and postfixed in 70% ethanol for 10 min. Villi samples were incubated with a 1 : 100 dilution of monoclonal anti-PCNA antibody (NCL-PCNA, Novocastra Lab., Newcastle upon Tyne, UK) for 18 hrs. They were washed three times for 5 min each time in Tris-buffer (pH 7.2). For the visualization of PCNA-labeled nuclei, villi were incubated for 10 min in the presence of biotinylated rabbit anti-mouse immunoglobulins at a dilution of 1 : 250. Also villi were incubated for 20 min in the presence streptavidin labeled horseradish peroxidase, and washed three times for 5 min in Tris-buffer. For the visualization of all nuclei, diaminobenzidine (DAB, Sigma Chemical Co., MO, USA) and Mayer's hematoxylin added and thereafter washed three times for 5 min. The washed villi were placed onto a coverslip and immediately mounted in xylene.

**3. Total RNA preparation and cDNA synthesis**

Total RNA was obtained from stored chorionic villi at -80℃, using TRIzol reagent (Gibco BRL). cDNA was synthesized from total RNA according to the protocol of SuperScript Preamplification system (Gibco BRL). After incubation of 3 μg total RNA with 0.5 μg oligo $(dT)_{12\text{-}18}$ primer at 70℃ for 10 min, the reaction was carried out in 5X first strand buffer, 10 mM DTT, and 0.5 mM dNTP containing final volume of 20 μl mixture. Mixed contents of the tube were incubated at 42℃ for 2 min. And then SUPERSCRIPT™II (Gibco BRL) was added and incubated for 50 min at 42℃.

**4. Primers for reverse transcription-polymerase chain reaction (RT-PCR)**

Primers of immunosuppression-related genes (*PP14*, *IDO*, and *MUC1*) are made on the basis of published sequences in GenBank. (Accession numbers: XM_005360, NM_002164 and NM_002456, respectively) The sequences for primers and their expected size of PCR products are listed in Table 1. The specific primers for *GAPDH* for a control are designed based on GeneBank accession number BC009081.

**5. Semi-quantitative RT-PCR analysis**

The relative expression level of genes isolated from RSA and normal chorionic villi samples was determined according to the expression level of *GAPDH*, a housekeeping gene. PCR condition is following; after boiling at 94℃ for 4 min, am-

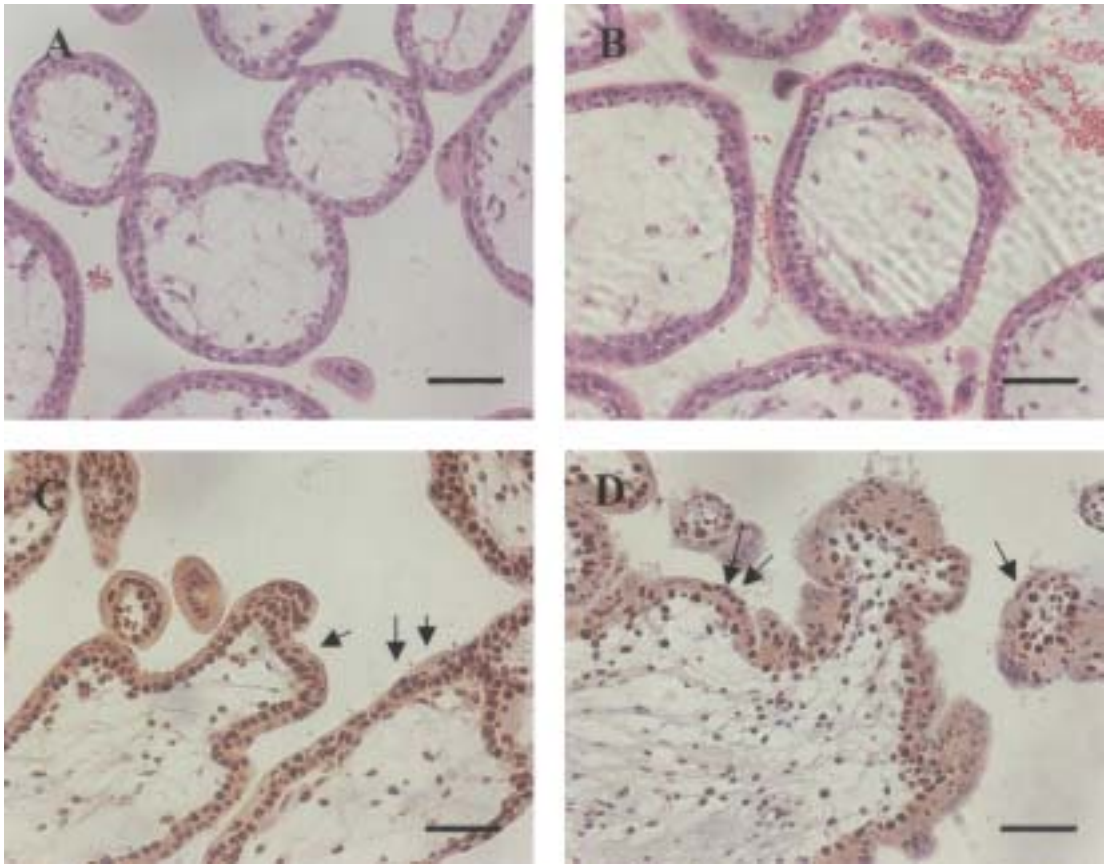


**Figure 1.** Immunocytochemical staining for fetal chorionic villi from normal and RSA patients. Fetal chorionic villi were immunocytochemically stained with either an antibody for PCNA to detect the proliferating activity or HE to observe mitotic figures. Magnification X100. Bar=100 μm.

plification was carried out for 30 cycles at 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 2 min, the mixture was then cycled at 72℃for 10 min to complete the elongation step and was finally stored at 4℃. The density of each band was measured using a Gel-Doc image analyzer (Vilver Loumat, France). The band intensities of each gene were normalized with those of corresponding *GAPDH* used as a control.

### 6. Statistic analysis

All statistical analyses of the data, obtained from semi-quantitative RT-PCR for 3 different immunosuppression-related genes using 6 pairs of chorionic villi samples (Normal N=6; RSA N=6), were performed using Student's *t*-test when normal and RSA groups were compared. Numerical data for the expression level of each gene are presented as mean±SD, and a p value less than 0.05 was considered statistically significant.

## RESULTS

The presence of live cells in two groups of chorionic villi derived from normal and RSA patients was confirmed by immunocytochemical analysis. Fetal chorionic villi from RSA patients and normal controls were immunocytochemically stained using hematoxylin and eosin (H/E) to observe mitotic figures and an antibody for prolife-

rating cell nuclear antigen (PCNA) to detect the proliferative activity. The analysis with hematoxylin and eosin (H/E) staining revealed that most of cells constituting chorionic villi in both groups are alive (Figures 1A and 1B). In addition, immunocytochemical analysis with anti-PCNA antibody (Figures 1C and 1D) showed that both groups of chorionic villi contain proliferative cells.

In order to investigate whether they are expressed abnormally in chorionic villi of RSA patients, we performed RT-PCR with primers generated for immunosuppression-related genes (*PP14, IDO* and *MUC1*). Semi-quantitative RT-PCR analysis showed that these genes were less expressed in chorionic villi from the RSA group than in those from the therapeutic abortion group (Figures 2 and 3). It is noticeable that three genes showed different levels of expression between chorionic villi from normal controls and chorionic villi from RSA patients at both 6- and 8-weeks in gestation. To confirm the similar amount of RNA used for semi-quantitative RT-PCR analysis, a housekeeping gene *GAPDH* was used as a control (Figures 2 and 3). For semi-quantitative analyses for RT-PCR products, we first equalized band densities for *GAPDH* expression and then the expression level of each gene was compared between RSA and normal control patients that were gestational age matched (6 weeks and 8 weeks, respectively). The ratios were determined by dividing the band density of each gene by that of *GAPDH*. The intensity of the EtBr staining was analyzed by densitometry as described in MATERIALS AND METHODS, and plotted as a bar graph (Figure 3).

Statistical analysis revealed that the expression level of immunosuppression-related genes (*PP14, IDO* and *MUC1*) in fetal chorionic villi from normal pregnant women was significantly higher than that in fetal chorionic villi from pregnant women

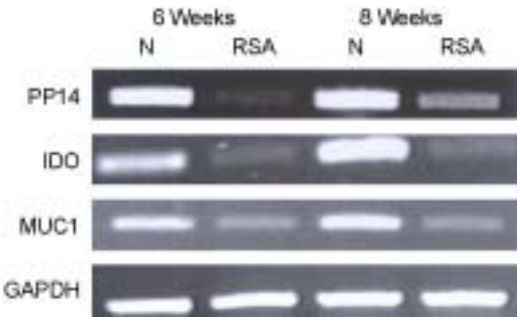


**Figure 2.** Semi-quantitative RT-PCR analysis in chorionic villi for immunosuppression-related genes. Immunosuppression-related genes tested in this study were PP14, IDO and MUC1. Samples were obtained from two groups, normal patients and RSA patients, at the gestation stages of 6 weeks and 8 weeks. (N; normal patients, RSA; RSA patients).

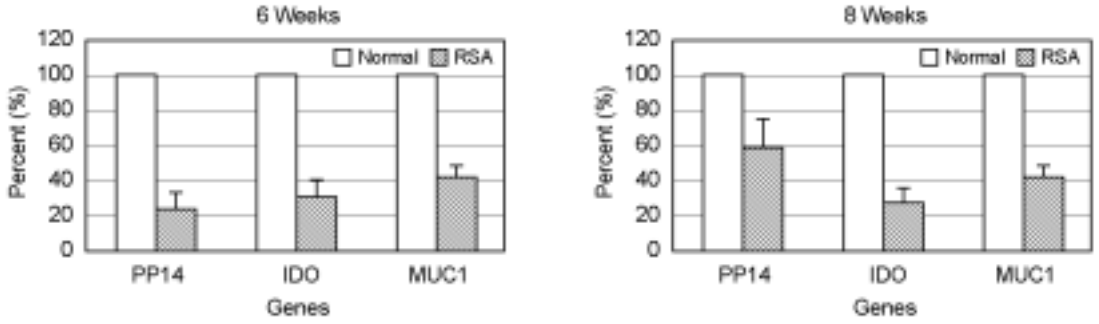


**Figure 3.** Semi-quantitative RT-PCR analysis in chorionic villi for immunosuppression-related genes. Immunosuppression-related genes tested in this study were PP14, IDO and MUC1. The percent ratios were calculated for the expression of each gene in chorionic villi tissues from normal patients (open bar) and RSA patients (solid bar), respectively. The percent ratios were determined by dividing the band density of each gene by that of *GAPDH* followed by the multiplication of 100. All statistical analysis of the data was performed using Student's *t*-test ($p<0.05$).

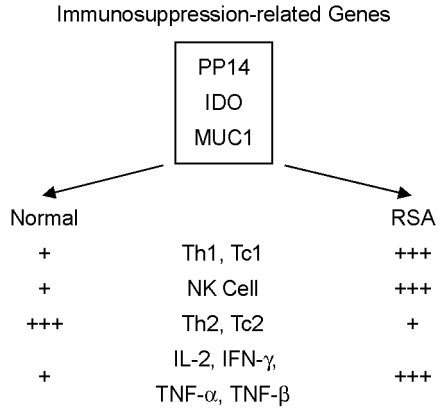


**Figure 4.** Hypothesis for *in vivo* function of immunosuppression-related genes (*PP14*, *IDO* and *MUC1*). In general, they down-regulate immune reactions by inhibiting the proliferation of T-lymphocytes. The degree of immunosuppression activity in pregnant women is determined by immune cells and related cytokines.

with the RSA ($p<0.05$). The higher expression of three different immunosuppression-related genes occurred in six independent experiments (Normal N=6; RSA N=6). Taken all together, PP14, IDO, and MUC1 may have important roles as a possible immunosuppressive molecule capable of normal pregnancy and growth of fetus.

## DISCUSSION

The conceptus expresses both paternal and maternal antigens. Therefore, it is possible that the maternal immune system recognizes paternal gene products generated by the fetus as immunologically foreign, resulting in rejection.[12,18,19] However, the frequency of this rejection is rare due to the fact that uterine decidua secrete soluble proteins capable of inhibiting cell-mediated immune responses, protecting the conceptus from maternal immune rejection during pregnancy.[20,21] Approximately 25% of RSA patients has been shown the elevation of immune response to trophoblast and increased proliferation of inflammatory cells.[22] This leads to the possibility that aberrant regulation of immunosuppression-related gene products may cause RSA or habitual abortion.

In this study, we investigated the possibility of aberrant expression level of immunosuppression-related genes (*PP14*, *IDO* and *MUC1*) in fetal chorionic villi of RSA patients. Molecular analysis showed that immunosuppression-related genes are expressed at a low level in chorionic villi from RSA patients than those from normal patients. This may result in high levels of immune reactions, following by RSA or habitual abortion. It has been demonstrated that PP14, IDO and MUC1 play a role in inhibiting the proliferation of T-lymphocytes.[16,23,24] In addition, down-regulation of the cellular immune response is dependent upon the suppression of T-helper (Th) 1 and T-cytotoxic (Tc) 1, which produce cytokines including IL-2, IFN-γ, and TNFβ.[25] In the case of RSA patients, NK cells and cytolytic activity are increased due to the inability of inhibition for T-lymphocyte proliferation and of down-regulation for the cellular immune response.[20,26] Therefore, the degree of immunosuppression activity in pregnant women is determined by immune cells and related cytokines (Figure 4). This indicates that the appropriate regulation of immunosuppression-related gene expression is required for the early pregnancy state. Confirmation at the protein level, which was impossible with limited amounts of chorionic villi samples in our study, will help to verify their roles for normal pregnancy. This leads to the possibility that the identification of prognostic markers for RSA using immunosuppression-related gene products such as PP14, IDO and MUC1 will be helpful to provide some insights into the prognosis of pregnancies with a high risk of RSA and management of those pregnancies.

In addition to these genes, it is expected that there should be more genes involved in the process of establishing and maintaining pregnancy. Previous investigations have demonstrated that

RSA is a complicated problem associated with endocrine dysfunction, autoimmune disorders, chromosomal abnormalities, advanced maternal and paternal age, infectious processes, environmental toxins, and congenital or structural uterine anomalies,[3,5,26~32] indicating that a number of gene products may modulate these biological processes. Taken all together, the diagnosis of early pregnancy disorders including RSA depends on an accurate assessment of potential pathophysiological mechanisms and this will identify pregnancies with a high risk of miscarriage and enable to manage those pregnancies effectively.

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