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# Expression of heparanase in the spontaneously aborted human chorionic villus

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**Summary**

*Objective:* Heparan sulfate proteoglycans (HSPG) are main components of remodeling the extracellular matrix (ECM). Heparanase (HPSE) can cleave the HSPG and alter the structural integrity of the ECM. The present research was to investigate the role of HPSE in recurrent spontaneous abortion (RSA). *Study Design:* Reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical methods were used to determine the expression of heparanase (HPSE) in the villus of the human normal pregnancy and RSA. *Results:* The expressions of HPSE mRNA and protein in the normal group were significantly greater in comparison with those in the spontaneous abortion group ($p < 0.05$). *Conclusion:* The HPSE participates in the procedure of normal pregnancy, and the lower expression of HPSE may lead to the spontaneous abortion.

*Key words:* Recurrent spontaneous abortion; HPSE; Reverse transcription-PCR; Immunohistochemical methods.

## Introduction

An estimated 1–3% of women have experienced three or more consecutive miscarriages prior to 20 weeks gestation, and this is defined as recurrent spontaneous abortion (RSA) [1]. RSA is a disease which usually occurs in gynaecology and obstetrics, and it is believed to be associated with the heredity, anatomy, endocrine secretion, immunity and infection, but the true etiology of this disease is still not known [2].

Implantation is a critical step to the survival of the embryo species. The processes of embryo implantation and placentation involve a complex coordination among multiple cell types of the embryo/fetus and mother [3]. Seventy-five percent of human blastocysts failed implant and therefore not clinically recognized as pregnancy [4]. Successful implantation that leads to the establishment of pregnancy, requires an orchestrated synchrony of complex interactions between an appropriately developed embryo and the hormonally primed receptive endometrium [4]. Remodeling the extracellular matrix (ECM) occurs during implantation and placental development in the uterus. Heparan sulfate proteoglycans (HSPG) are major components of the extracellular matrix, and provide natural support cell adhesion, migration, and differentiation [5]. Heparanase (HPSE) is a single enzyme to degrade HS. Cleavage of HS side chains is therefore expected not only to alter the structural integrity of the ECM, but also to release and modulate the activities of HS-bound biological mediators. HPSE promotes cell adhesion, migration, and neovascularization all of which potentially take place during the embryo implantation [6]. However, the expression and function of HPSE in recurrent spontaneous abortion have not been reported.

The aim of the present investigation was to study expression of HPSE in the spontaneously aborted human chorionic villus and normal group, and to evaluate the possible role of HPSE in recurrent spontaneous abortion and normal pregnancy, for potentially developing guidelines for the RSA clinical treatment.

## Materials and Methods

The study was approved by the institutional ethics committee on human research. After obtaining informed content, 28 cases of miscarriage of women with recurrent fetal losses were studied. All of cases with a diagnosis of recurrent miscarriage, defined as three or more verified consecutive miscarriages in the first or second trimester of pregnancy (five to 21 gestation weeks). Women who were known at risk for recurrent miscarriage, such as systemic lupus erythematosus, diabetes mellitus type 1, severe thrombophilia, and major chromosomal aberrations were not included in the study. The patients did not receive any therapy during these pregnancies. The patient's age ranged from 24 to 33 years. Thirty-five control cases were pregnancy terminations of women with normal obstetric history, and they chose artificial abortion to termination pregnancy. The age of control group ranged from 22 to 35 years. The control subjects (n=35) were matched for age at first planned pregnancy and were randomly chosen from the Datong Hospital among pregnant women. In the control group, none had a history of miscarriage and all had at least two spontaneous pregnancies, including the ongoing pregnancy. Beyond that, the same

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inclusion and exclusion criteria were applied for the control group as for the cases. Every pregnancy villus was confirmed by pathological diagnosis and chromosomal abnormalities analysis. The villi of abortion tissues were collected and all specimens were subdivided into two equal portions. One sample was frozen and stored at -80°C until for RNA extraction. The second sample was fixed in 10% formaldehyde solution for immunohistochemistry examination.

Total RNA was extracted from the tissue specimens using the Trizol reagent as described by the manufacturer. RNA concentration was quantified by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The cDNA preparation was obtained using oligo (dT) priming and reverse transcriptase enzyme as described as operating instruction. The reverse transcription polymerase chain reaction (RT-PCR) reaction was performed using PCR amplification kit on Mastercycler nexus gradient thermal cycler. The primer sequences used were as follows: HPSE forward primer: 5'- CACAAACACTGACAATCCAAGG -3'; HPSE reverse primer: 5'- CCATTGAGTTGGA CAGATTTGG -3'; β-actin forward primer: 5'- CAAAGACCTGTACGCCAACAC -3'; β-actin reverse primer: 5'-ATACTCCTGCTTGCTGATCC-3'. The expected lengths of HPSE and β-actin PCR products were 180 and 128 bp, respectively.

The amplification programme included an initial denaturation at 94°C for four minutes, followed by 40 repeated thermocycles of 94°C(30 seconds), 60°C (30 seconds), and 72°C (60 seconds). A portion of the amplified reaction mixture (ten μl), as well as a DNA molecular weight marker were electrophoresed on 2% agarose gels stained by ethidium bromide, and photographed using IQ300 gel imaging system. A negative control was performed using water instead of the extracted cDNA as template.

Expression of HPSE protein was evaluated by immunohistochemistry using the strepravidin-biotin method. Briefly, the specimens were fixed in 10% buffered formalin and processed routinely for paraffin embedding. Five-micro sections for immunohistochemistry were cut and placed on super plus slides. Slides were deparaffinized with xylene, rehydrated, and endogeous peroxidase activity was quenched for ten minutes using 3% hydrogen peroxide in PBS. Then the slides were subjected to antigen retrieval in 0.01 mol/lL citrate buffer (pH 6.0) for 30 minutes at 100°C. Cooled slides were rinsed with PBS and blocked with 10% normal goat serum for 15 minutes at room temperature. Followed by incubation for 24 hours at 4°C with anti-HPSE antibody (1:100), slides were washed using PBS and then incubated with Biotin labeling secondary antibodies for 15 minutes at room temperature. Then horseradish peroxidase labeling work liquid was added and incubated for 15 minutes at room temperature. Following additional washing, color was developed with the DAB, counterstained with hematoxylin, rehydrated in alcohol and xylene and mounted in resin. Expression of HPSE protein was observed in the cytoplasm of cells, and appeared as yellow or brown staining.

All statistical analyses were performed with SPSS 17.0. The expression of HPSE mRNA is shown as the percentage of positive number using the $\chi^2$ test. The data of HPSE protein was presented as *means* ± *SD* using and *t*-test. $P < 0.05$ was considered statistically significant.

## Results

In specimens from patients with spontaneous abortions, HPSE mRNA expression rate was much lower (16/28, 57.14%) than that in women with normal pregnancy (30/35, 85.71%). The low rate of expression with HPSE in spontaneous abortions suggests that HPSE is probably of etiological importance to spontaneous abortion in this region where the samples were collected (Figure 1, Table 1).

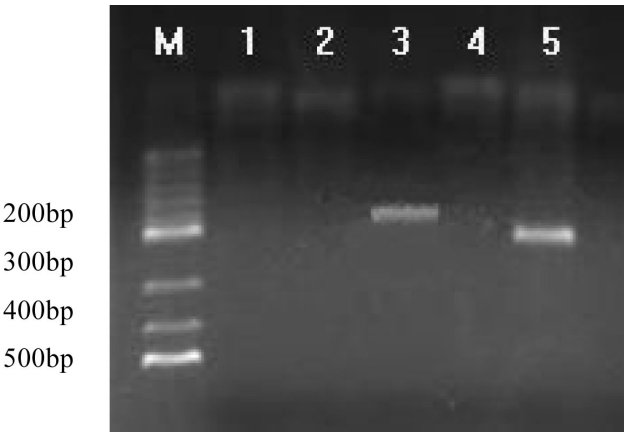


Figure 1. — Agarose gel electrophoresis of the HPSE RT-PCR production in various villus. M: Marker (500 bp). Lanes 1, 2, 4: The expression of HPSE mRNA (180 bp) in the spontaneous abortion villus. Lane 3: The expression of HPSE mRNA (180 bp) in the normal villus. Lane 5: The expression of β-actin mRNA (218 bp) in the normal villus.

Table 1. — *HPSE mRNA expression in normal pregnancy group and recurrent spontaneous abortion group.*

| Groups | Positive cases | Negative cases | $\chi^2$ *value* | *p value* |
|---|---|---|---|---|
| Normal pregnancy | 30 | 5 | 6.445 | 0.011 |
| Recurrent spontaneous abortion | 16 | 12 | | |

Table 2. — *HPSE protein expression in normal pregnancy group and recurrent spontaneous abortion group.*

| Groups | Means ± SE | *t* value | *p value* |
|---|---|---|---|
| Normal pregnancy (n=35) | 176.03 ± 40.11 | 4.3893 | <0.001 |
| Recurrent spontaneous abortion (n=28) | 136.66 ± 29.31 | | |

HPSE immunoreactivity was detected in the cytoplasm and appeared as yellow or brown staining (Figure 2). Among normal pregnancy specimens, the grey value is 176.03 ± 40.11, which is in sharp contrast to the much lower expression in patients with recurrent spontaneous abortion (136.66 ± 29.31). These observations suggest the HPSE expression was generally down-regulated in women with recurrent spontaneous abortion (Table 2).

## Discussion

Spontaneous abortion is a common complication of pregnancy; once pregnant women had spontaneous abortion for

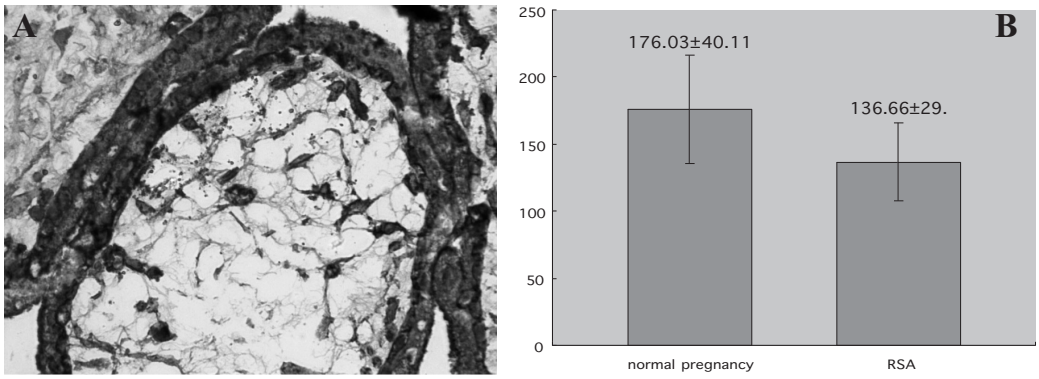


Figure 2. — A) The expression of HPSE protein immunohistochemical staining in normal pregnancy group (×400). B) HPSE protein staining intension in the normal pregnancy group and recurrent spontaneous abortion group. Using *t*-test there were significant differences found between two groups ($p < 0.05$).

the first time, the risk of repeated miscarriage is as high as 33.1% [7]. Despite significant progress in reproductive research, many fundamental questions about implantation remain to be resolved. Embryo implantation is a highly coordinated and complex process. To achieve successful implantation, proper embryo-maternal interactions must be maintained [4,5].

Embryo implantation in mammalian uterus is initiated by the formation of a direct cell to cell contact between the trophoblast and the uterine epithelium. Invasive trophoblasts share features of migration that are characteristic of cancer cells. Both cell types are invasive, but cancer cell invasive is not controlled, whereas trophoblast migration into the uterus is highly regulated, both temporally and spatially [4, 5]. Studies of the involvement of ECM macromolecules in cell attachment, growth, and differentiation have revealed a central role HSPG in embryogenesis, placentation, and trophoblast cell invasion. These HS-bind growth factors can be released by HPSE during tissue remodeling. Released growth factors can then interact with their receptors to elicit biological responses [6, 8].

Applying RT-PCR and immunohistochemistry, the present authors have demonstrated the expression levels of HPSE in the spontaneous abortion compared to normal pregnancy. The higher expression levels of HPSE mRNA and protein in normal pregnancies from the present study confirmed its fundamental role in the placental development and hemostatis. The reduction in the HPSE expression adversely affects the process of normal implantation. The lower expression of HPSE in the spontaneous abortion may inhibit trophoblastic cell invasion into the endometrium and decrease angiogenesis.

HPSE affects various biological processes through disintegration of the ECM and release of HS-bound growth factors, cytokines, and enzymes. Studies showed that the HPSE altered the integrity and functional state of tissues and provided a mechanism by which cells can respond rapidly to changes in the extracellular environment [9-11]. A number of functional assays indicate that HPSE participates in the early stages of embryo attachment through cleaving HS [12, 13]. Therefore the reduction in the expression of HPSE may influence embryo implantation, result in poor implantation, and consequently miscarriage appears. A better understanding of the mechanisms responsible for implantation and placentation at molecular level will increase the ability to treat disorders related to early RSA.

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