

Expression of extracellular matrix metalloproteinase inducer (EMMPRIN) in the endometrium of patients with repeated implantation failure after *in vitro* fertilization

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Abstract. – **AIM:** To compare the immuno-histochemical expression of extracellular matrix metalloproteinase inducer (EMMPRIN) in repeated implantation failure (RIF) patients with normal fertile controls.

PATIENTS AND METHODS: The study group consisted of primary infertile patients with RIF and normal fertile controls between January 2011 and February 2013. Endometrial samples received at the luteal phase were exposed to immunohistochemical staining for EMMPRIN antibodies. EMMPRIN expression of endometrial glandular epithelial cells, stromal cells and vascular endothelial cells were evaluated. The main outcome measure was defined as immunohistochemical score with regard to the severity and extent of staining.

RESULTS: The study group consisted of 26 primary infertile patients, whereas the control group consisted of 40 normal fertile controls. The fertile group was found to have stronger expression of EMMPRIN than the study group when endometrial glandular epithelial cells, stromal cells and vascular endothelial cells were evaluated with regards to the severity of staining ($p < 0.001$), the extent of staining ($p < 0.001$) and total staining score ($p < 0.001$).

CONCLUSIONS: This is the first study showing low expression of EMMPRIN in the endometrial cells of the patients with RIF compared with fertile healthy controls. We suggest that reduced EMMPRIN expression in the human endometrium may lead to poor endometrial receptivity.

Key Words:

Endometrium, *In vitro* fertilization, Repeated implantation failure, Endometrial receptivity, EMMPRIN.

tempts¹. A functioning and receptive endometrium is crucial for embryo implantation. During the menstrual cycle, the endometrium undergoes both morphologic and biologic changes, during which it becomes prepared for interaction with the embryo, leading to successful implantation. Once all biological changes are adequate, the embryo can attach, invade the endometrium, and finally implant².

During the estrous cycle and the establishment of pregnancy, endometrial cells undergo rapid growth and differentiation, extracellular matrix (ECM) break down and remodeling³. Around implantation, a number of molecules are expressed at the embryo-maternal interface including interferon-tau, cytokines, growth factors, hormones, and matrix metalloproteinases (MMPs)⁴. These changes in the endometrium are partly modulated by the expression of the MMP system, a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-1, and extracellular matrix metalloproteinase inducer (EMMPRIN) in coordination with ovarian steroids⁵⁻⁷. Despite many advances in assisted reproductive technologies (ART), contributed to this limited time span, low implantation rate is still the most important factor negatively affecting success rates. No definite clinically applicable receptivity marker has been discovered yet. Current approaches of the endometrium are focusing on factors emerging in the implantation window or retrieved from animal or *in-vitro* studies^{8,9}.

To our knowledge, no data exists regarding the role of EMMPRIN expression in the endometrium of the patients with implantation failure after repeated IVF attempts in humans. The aim of this present study was to compare the immunohistochemical expression of EMMPRIN in RIF patients with normal fertile controls.

Introduction

Repeated implantation failure (RIF) is determined when transferred embryos fail to implant after several *in vitro* fertilization (IVF) treatment at-

Patients and Methods

Study Design

Ethics Committee approval was obtained from the local Institutional Review Board and confirmed written consent forms were obtained from all the participants. The study group consisted of primary infertile patients with RIF after IVF and normal fertile controls admitted to the Department of Obstetrics and Gynecology between January 2011 and February 2013.

The repeated implantation failure was defined as failure to conceive after two or more IVF attempts in which at least one good quality embryo was transferred. All patients in the study group had clarified fallopian tubes opened with hysterosalpingography or laparoscopy. Patients diagnosed with endometriosis were excluded. The control group consisted of fertile women who had at least one uncomplicated pregnancy and no history of abortion.

Patients whose implantation rates were adversely affected by antiphospholipid syndrome, male infertility, parental chromosomal abnormalities, intracavitary fibroids, endometrial polyps, intrauterine synechiae, and factors such as uterine anomalies were excluded from the study group. Women with irregular menstruation, such as women with polycystic ovary syndrome, and with apparent endometrial pathology were also excluded.

Endometrial samples were examined according to histologic endometrial dating by the Noyes et al criteria¹⁰. The patients in the study group were evaluated for the duration of their infertility and the number of unsuccessful IVF attempts. Hysteroscopic examination was performed in all patients under local or general anesthesia. After hysteroscopic examination, endometrial biopsy samples were obtained by silastic suction curettage in the luteal phase. Endometrial samples were examined by a single pathologist for endometrial histological suitability and pathological evaluation. Endometrial samples were stored in paraffin blocks. Hysteroscopic and pathologic examination revealing polyps, inflammation, hyperplasia (such as leiomyoma), or endometrial pathology excluded patients from the study as these would affect the expression of EMMPRIN.

Immunohistochemistry

Five-micrometer thick sections from paraffin blocks were selected. Slides were stored in a 62°C oven for 60 minutes. For the deparaffinization process, paraffin blocks were soaked for 4 to

5 minutes in xylene, and for 4 to 5 minutes in a 96% alcohol solution. For the purpose of antigen retrieval, a citrate solution of pH 6 was added, and then heat shocked at 125°C in a high-pressure Biocare's Decloaking Chamber. The mixture was allowed to stand for 20 minutes of protein block (Ultra V Blok, Freemont, CA, USA, ScyTek, Logan, VT, USA). As the primary antibody, CD147 (Abcam Inc., Cambridge, MA, USA) was incubated for 120 minutes, then linked with a biotinylated antibody (ScyTek, Logan, VT, USA) and Streptavidin/HRP solution (ScyTek, Logan, VT, USA) for 20 minutes. It was then allowed to stand for 10 minutes in instilled AEC (3-amino-9-ethylcarbazole). Single solution and washed with distilled water. The slides were then counterstained with Mayer's hematoxylin (Bio-Optica, Milan, Italy) for one minute, dehydrated and mounted with an aqueous mounting medium (ScyTek Laboratories, Logan, UT, USA).

Scoring Analysis of Immunoreactivity

EMMPRIN slides stained with hematoxylin and eosin (H&E) were evaluated with Nikon Eclipse 80i light microscope (Nikon, Melville, NY, USA) by an expert pathologist. At least five fields for each tissue were randomly selected and calculated based on average score at 200x magnification. The results were scored according to endometrial glandular, stromal and vascular endothelial cells, depending on the severity and extent of staining. The severity of staining was assigned semiquantitatively on a 4-point scale from 0 to 3 (0 = no staining, 1 = weak, 2 = moderate, 3 = strong). Then, each severity score was added by their extent score from 1 to 3 (1 = less than one third of the area stained, 2 = more than one third but less than two thirds stained, 3 = more than two thirds stained). The maximum score of immunoreactivity was six for each compartment.

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS) software (version 18.0 for Windows, Chicago, IL, USA). Parametric tests were applied to data of normal distribution and non-parametric tests were applied to data of questionably normal distribution. Independent-samples *t*-test and Mann-Whiney U-test were used to compare independent groups. All differences associated with a chance probability of 0.05 or less were considered statistically significant. Continuous variables are presented as mean±SD.

Table I. Mean clinicopathologic characteristics of study and control group.

	Study group (n = 26) (mean ± SD)	Control group (n = 40) (mean ± SD)	p values
Age	31.8 ± 3.0	33.0 ± 2.9	0.207
Histologic dating to Noyes et al criteria ¹⁰	22.2 ± 0.7	22.0 ± 0.8	0.284
Duration of infertility	4.0 ± 1.4		
Previous failed cycles	2.6 ± 0.6		

SD = standard deviation.

Results

The study group consisted of 26 primary infertile patients with RIF after IVF, whereas the control group consisted of 40 normal fertile controls. The mean age of the study group was 31.8±3.0 (range 26 to 38) years; while the mean age of the control group was 33.0±2.9 (range 29 to 39) years. Histologic dating to Noyes et al criteria¹⁰ was 22.2±0.7 for the study group, and 22.0±0.8 for the control group. Between patients in the study and control groups, there was no significant difference in age and the histological data based on Noyes et al classification ($p = 0.207$ and $p = 0.284$, respectively). In the study group, the average duration of infertility was 4.0±1.4 years and the average recurrent IVF failure was 2.6±0.6 attempts (Table I).

As for the expression of EMMPRIN in endometrial glandular, stromal and vascular endothelial cells, the mean severity score of staining was 1.3±0.5 for the study group, and 2.2±0.7 for the control group ($p < 0.001$); while the mean extent score of staining was 1.7±0.5 for the study group, and 2.7±0.5 for the control group ($p < 0.001$). The EMMPRIN stained slides from the study and control groups were examined with regard to total staining score, the endometrial glandular epithelial cells, stromal cells, and vascular endothelial cells were found to have a lower expression of EMMPRIN in the study group compared with control group ($p < 0.001$) (Table II) (Figure 1). Considering the strength of individual

cases of EMMPRIN expression in epithelial, stromal and vascular endothelial cells was found to be correlated with the cases on an individual basis; epithelial, stromal, and endothelial cells were found to be correlated with the strength of the expression of EMMPRIN.

Discussion

This is the first study evaluating EMMPRIN expression in endometrial samples of the patients with RIF after IVF. In the present study, EMMPRIN expression was found to be lower in the endometrial glandular, stromal and vascular endothelial cells of the patients with RIF after IVF compared with fertile healthy controls.

Maternal causes (anatomical factors, decreased endometrial receptivity, thrombophilia, immunological factors) and embryonic causes (genetic factors, inadequate development of the embryo, male factor) are included among the causes of RIF after IVF¹¹. Implantation is an intricate process through which the blastocyst attaches itself to the uterine endometrium leading to the formation of the placenta, which will provide an interface between the growing fetus and the maternal circulation. Many conditions are required for successful implantation to take place: a receptive endometrium, a normal and functional embryo at the blastocyst developmental stage, and a synchronized dialogue between maternal and embryonic tissues¹².

Table II. Mean EMMPRIN expression of the study and control group.

	Study group (n = 26) (mean ± SD)	Control group (n = 40) (mean ± SD)	p values
EMMPRIN expression Severity Score	1.3 ± 0.5	2.2 ± 0.7	< 0.001
EMMPRIN expression Extent Score	1.7 ± 0.5	2.7 ± 0.5	< 0.001
EMMPRIN expression Total Score	3.0 ± 0.8	4.9 ± 1.0	< 0.001

SD = standard deviation.

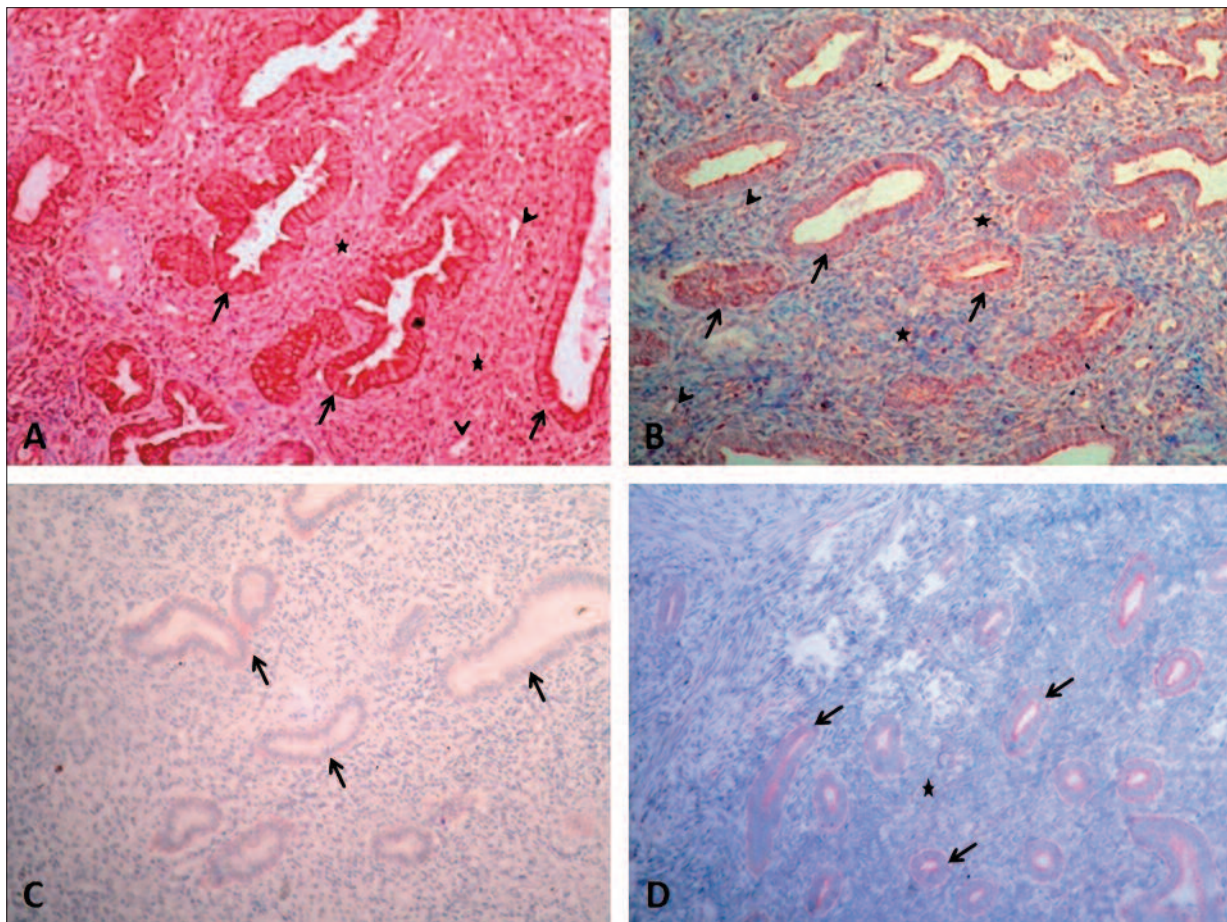


Figure 1. Photomicrographs showing EMMPRIN expressions in endometrium [all scored as severity: 3, extent: 3 and total score: 6 (immunoperoxidase, 200x)]. **A**, Stronger EMMPRIN expression in endometrial gland epithelium (arrow), vascular endothelial (arrowhead) and stromal cells (stars) in control group scored as severity: 3, extent: 3 and total score: 6. **B**, Stronger EMMPRIN expression in endometrial gland epithelium (arrow), vascular endothelial (arrowhead) and stromal cells (stars) in control group scored as severity: 2, extent: 3 and total score: 5. **C**, Lower EMMPRIN expression in endometrial gland epithelium (arrow), vascular endothelial and stromal cells in IVF group scored as severity: 1, extent: 1 and total score: 2. **D**, Lower EMMPRIN expression in endometrial gland epithelium (arrow), vascular endothelial and stromal cells (stars) in IVF group scored as severity: 1, extent: 2 and total score: 3.

The MMPs comprise a family of zinc-dependent endopeptidases that mediate the proteolytic remodeling of the extracellular matrix. Members of the MMP family are distinguished by substrate specificity, regulatory mechanisms, mode of action, and localization¹³. Several MMPs are expressed in the endometrium, and their expression patterns change throughout the menstrual cycle. Most MMPs are up-regulated when estrogen levels are rising and are down-regulated once progesterone levels increase. The activity of MMP activity, including up- or down-regulation of expression by inducers/inhibitory factors, the proteolytic activation of catalytic activity, and direct inhibition through the activators and tissue in-

hibitors of MMPs (TIMPs)¹⁴. Most studies of MMPs and TIMPs have emphasized the key role of MMPs in the breakdown of ECM that ultimately leads to rupture of the fetal membranes and detachment of the placenta from maternal uterus at human parturition. A marked increase in expression of several MMPs in placenta and fetal membranes or amniotic fluid occurs just after the onset and during parturition in association with a significant decrease in the expression of TIMPs^{15,16}.

Within the last decade, a major inducer of MMPs has been identified as EMMPRIN. It was expected that EMMPRIN would play a fundamental role in various physiological and patho-

logical processes because of its broad distribution and effects on MMP production¹⁷. Because EMMPRIN is expressed by the human placenta and fetal membranes and the levels of glycosylated EMMPRIN increase selectively in association with labor. Previous studies have shown that placental syncytiotrophoblasts, chorion trophoblasts, and amnion epithelium are also major sites of MMP expression, consistent with the distribution of EMMPRIN in these tissues¹⁸. Thus, changes in EMMPRIN expression may indirectly influence MMP action to enhance tissue degradation, leading to further rupture of the fetal membranes and detachment of placenta and fetal membrane from uterus.

A recent study by Noguchi et al¹⁹ reported that EMMPRIN protein is expressed in the human endometrium throughout the menstrual cycle. As for the problem of infertility and recurrent cases of unsuccessful IVF, there is not enough information about the expression of EMMPRIN and the mechanism of EMMPRIN action during implantation. In this study, EMMPRIN expression in patients RIF was lower than the control group, which is thought to be important for implantation in the luteal phase.

EMMPRIN is known to be expressed in glandular epithelia and stroma of human eutopic and ectopic endometria and in epithelial and stromal cells of human endometrial tissues^{20,21}. In the present study, the mid luteal phase endometrium in patients with failed IVF was evaluated and EMMPRIN expression was found to be decreased in endometrial glandular epithelial cells, stromal cells, and vascular endothelial cells which may play role in the fertilization process.

Conclusions

The reduced EMMPRIN expression in the human endometrium may lead to poor endometrial receptivity. Our results support a role for the immune system in patients with RIF. However, more research needs to be completed in order to understand the signaling pathways involved in regulation of MMPs in endometrium. These findings can be used to develop new therapeutic agents in infertile patients.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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