

## Endometrial Integrin $\alpha V\beta_3$ and LIF mRNA Expression in Women with Recurrent Implantation Failure

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### Abstract

**Introduction:** To determine expression of integrin  $\alpha V\beta_3$  and leukemia inhibitory factor (LIF) in the endometrium from women with recurrent implantation failure (RIF) and to determine whether the differences in their expression levels in fresh and frozen cycles.

**Materials and Methods:** Thirty women with RIF and 15 healthy fertile women were recruited. The patients in the RIF were divided into two different groups. While in 15 cases RIF was diagnosed after the fresh cycle the remaining 15 cases RIF was diagnosed after the frozen cycle. RIF was defined as the failure to conceive after the transfer of three good-quality embryos in three or more fresh or frozen cycles. Endometrial biopsy samples were obtained from women with RIF and control women at the late follicular phase of the cycle. Expression of integrin  $\alpha V\beta_3$  and LIF were determined by RT-PCR.

**Results:** Expression of endometrial integrin  $\alpha V\beta_3$  and LIF were lower in the RIF group due to fresh cycle compared with the RIF group due to frozen cycle. But the differences failed to reach statistical significance. Expression of endometrial integrin  $\alpha V\beta_3$  in RIF women due to fresh cycle was lower than in the fertile controls. The difference between the RIF group due to fresh cycle and fertile controls was statistically significant in terms of integrin  $\alpha V\beta_3$  expression. Expression of endometrial LIF in RIF women due to fresh cycle was similar to LIF expression pattern of fertile controls. Likewise there was no significant difference in the expressions of LIF and integrin  $\alpha V\beta_3$  in RIF women due to frozen cycle compared with the fertile controls.

**Conclusion:** The expression of endometrial integrin  $\alpha V\beta_3$  decreases in RIF women due to fresh cycle. RIF is not associated with abnormal endometrial LIF expression in fresh or frozen cycles. In contrast to the expression of integrin  $\alpha V\beta_3$ , LIF appears to have no prognostic value in RIF etiology.

**Keywords:** Recurrent Implantation Failure; Endometrium; LIF; Integrin  $\alpha V\beta_3$ ; Frozen Cycle; Fresh Cycle

### Abbreviations

RIF: Recurrent Implantation Failure; mRNA: Messenger RNA; LIF: Leukemia Inhibitory Factor;  $\alpha V\beta_3$ : Alpha-v-Beta-3 Integrin; BMI: Body Mass Index; IVF: *In Vitro* Fertilization

### Introduction

The failure to achieve pregnancy after at least four good-quality embryo transfers over a minimum of three fresh or frozen *in vitro* fertilization (IVF) cycles is defined as recurrent implantation failure (RIF) [1]. Our understanding of the factors responsible for the failed

interaction between maternal and embryonic compartment leading to unsuccessful embryo implantation in RIF remains to be determined. In addition to homeobox genes, integrins and leukemia inhibitory factor (LIF) have been largely accepted as markers of endometrium receptivity [2,3]. Integrins are a family of cell adhesion molecules. The vitronectin receptor is an alpha-v-beta-3 integrin ( $\alpha V\beta_3$ ) that expresses during the window of implantation [4,5].  $\alpha V\beta_3$  expression down-regulates the estrogen and progesterone receptors. Endometrial integrin  $\alpha V\beta_3$  regulates angiogenesis and facilitates embryo attachment [5]. Leukemia inhibitory factor is a cytokine belongs to IL-6 family. It plays an important role in interaction between the embryo and endometrium during implantation period [6]. LIF regulates proliferation, differentiation, and cell survival in many biological environment including endometrium [7]. LIF binds with LIF receptor (LIFR) and activates the signal transducers and activators of transcription 3 (STAT3) signaling pathway [8]. Defective expression of LIF causes infertility in animals [9].

Expression pattern of LIF and integrin  $\alpha V\beta_3$  mRNA in women with RIF is variable [10,11]. Cyclic expression of LIF or  $\alpha V\beta_3$  may contribute to the establishment of a period of endometrial receptivity, whereby the endometrium becomes friendly to the implanting embryo [3,12,13]. The disturbed expression of LIF or  $\alpha V\beta_3$  may account for a significant number of cases of failed implantation in the female with implantation failure. Although  $\alpha V\beta_3$  and LIF are essential for embryo-endometrial attachment there is limited research regarding the expression levels of this two cytokines in RIF subjects. Moreover, LIF and integrins were studied individually in RIF patients, these two molecules were not studied together in RIF cases due to fresh or frozen cycles. We, therefore, planned this study to determine expression of integrin  $\alpha V\beta_3$  and leukemia inhibitory factor in the endometrium from women with recurrent implantation failure and to determine whether the differences in their expression levels in fresh and frozen cycles.

## Materials and Methods

### Study population

Thirty women with RIF who are 28 to 33 years old and failed to achieve a clinical pregnancy after 3 or more fresh or frozen embryo transfer cycles with at least one or two good-quality embryos transferred were included. They were registered at the Istanbul Bahcesehir University Medicalpark Hospital IVF Center, between January 2018 and December 2019. The patients in the RIF were divided into two different groups. While in 15 cases RIF was diagnosed after the fresh cycle the remaining 15 cases RIF was diagnosed after the frozen cycle. Fifteen healthy fertile women were recruited as control. In order to investigate possible endometrial etiology of previous failed IVF attempt and make a local endometrial injury both groups of RIF women underwent hysteroscopy. Hysteroscopy was scheduled during the late follicular phase of the menstrual cycle and endometrial samples were obtained. Likewise, endometrial tissue was also obtained with pipella endometrial canul from fertile control women during late follicular phase. The endometrial tissues were washed with a sterile saline solution to remove blood and transferred into RNA stabilization buffer and stored for future analysis. Expression of integrin  $\alpha V\beta_3$  and LIF were determined by RT-PCR. Presence of submucosal or intramural fibroids distorting endometrial cavity, endometrial polyps, uterine septum, antiphospholipid syndrome, diabetes or recurrent spontaneous abortion, uni- or bilateral hydrosalpinx, endometrioma, polycystic ovarian syndrome, endometrial hyperplasia, endometritis and uterine synechiae were excluded from the study. The study was approved by the local Research Committee of Göztepe Medicalpark Hospital IVF Center and all patients signed an informed consent before the inclusion.

### RT-PCR

Endometrial samples were transferred into RNA stabilization buffer (RNA Later; Qiagen) and then stored at  $-80^{\circ}\text{C}$  until used. Total RNA was extracted from decidua with the Rneasy Mini Kits (QIAGEN). RNA quantity and purity was measured spectrophotometrically with the use of the Maestronano. Complementary DNA (cDNA) was obtained with the use of the Quantitect Reverse Transcription Kit (Qiagen).  $\beta$ -Actin gene (ACTB) was used as housekeeping gene. Realtime PCR reaction was performed with the use of Quantitect Probe PCR Kit and

the RotorgeneQ (Qiagen) realtime PCR device. The primer sequences are the following: LIF forward: F 5'-GGA GGT CAC TTG GCA TTC AG-3'; R 5'-GG AAG AGA ACG AAG AAC CTA CC-3'; ITGB3 forward: F 5'-ACC ATC TCT TTA CCT CCT AAT TCC-3'; R 5'-CTG GCT CTA CAA TAG CAC TCT C-3'; ACTB forward: F 5'-GCA AGC AGG AGT ATG ACG AGT-3'; R 5'-CAA GAA AGG GTG TAA CGC AAC TAA-3'. Gene expression results are presented as Ct (cycle threshold),  $\Delta Ct$  and  $\Delta\Delta Ct$ . For calculation of average Ct values, each endometrial sample was studied three times. The relative gene expression was determined by means of the  $2^{-\Delta\Delta Ct}$  comparative method with the use of RT<sup>2</sup> Profiler PCR Array Data Analysis version 5.5 (SA Biosciences). All data were normalized according to mRNA of  $\beta$ -actin [3].

### Statistical analysis

The normality distribution of data was tested with the Kolmogorov-Smirnoff test. The continuous variables were analyzed by means of analysis of variance test with post hoc Tukey procedure and Mann-Whitney U test. The categoric data were analyzed by means of the Pearson chi-square test. Data are presented as the means  $\pm$  SD. A P value of  $< .05$  was considered statistically significant.

### Results

Age, BMI, history of smoking were not different between the RIF and control groups. RT-PCR was used to detect the expression of LIF and integrin  $\alpha V\beta_3$  genes in the endometrium. Average  $\Delta Ct$  value of integrin  $\alpha V\beta_3$  and LIF are 6.55 and 6.90 in women with RIF due to fresh cycle. Average  $\Delta Ct$  value of  $\alpha V\beta_3$  and LIF is 6.77 and 6.96 respectively in women with RIF due to frozen cycle. Expression of endometrial integrin  $\alpha V\beta_3$  and LIF were lower in the RIF group due to fresh cycle compared with the RIF group due to frozen cycle. But the differences failed to reach statistical significance. Average  $\Delta Ct$  value of integrin  $\alpha V\beta_3$  and LIF are 7.33 and 7.01 respectively in fertile controls. Expression of endometrial integrin  $\alpha V\beta_3$  in RIF women due to fresh cycle was lower than in the fertile controls. The difference between the RIF group due to fresh cycle and fertile controls was statistically significant in terms of integrin  $\alpha V\beta_3$  expression ( $\Delta Ct$  6.55 vs.  $\Delta Ct$  7.33,  $P < .029$ ). Expression of endometrial LIF in RIF women due to fresh cycle was similar to LIF expression pattern of fertile controls ( $\Delta Ct$  6.90 vs.  $\Delta Ct$  7.01,  $P > .012$ ). Expression of endometrial integrin  $\alpha V\beta_3$  ( $\Delta Ct$  6.77 vs  $\Delta Ct$  7.33,  $P > .05$ ) and LIF ( $\Delta Ct$  6.96 vs.  $\Delta Ct$  7.01,  $P > .05$ ) were lower in the RIF group due to frozen cycle compared with the fertile controls. But the differences failed to reach statistical significance.

### Discussion

In the present study, we showed that women with RIF following fresh cycle have decreased endometrial integrin  $\alpha V\beta_3$  mRNA expression and normal LIF expression, accompanied by failed implantation. Our results also indicated that LIF and integrin  $\alpha V\beta_3$  mRNA expression were similar in the RIF group due to frozen cycle as compared with fertile controls. These findings support a notion that RIF occurring after fresh cycle is associated with decreased integrin expression which is often related to failed implantation attempt. As a result, diminished expression of endometrial integrin  $\alpha V\beta_3$  may be one of possible causes of recurrent pregnancy losses or RIF [14].

RIF women with fresh cycle had significantly decreased integrin  $\alpha V\beta_3$  expression in endometrium, which reflect disturbed receptivity. Because integrins down-regulate estrogen and progesterone receptor expression the possible cause of decreased integrin expression in fresh cycles may be increased levels of estrogen and progesterone due to controlled ovarian stimulation. Previously, Celik, *et al.* demonstrated that in the functional deficiency of receptivity genes such as homeobox genes or integrins, endometrial receptivity diminishes, and embryo fail to implant [3]. Likewise, abnormal expression of integrin  $\alpha V\beta_3$  has been reported in infertile women with endometriosis [12]. Failed expression of integrin  $\alpha V\beta_3$  suggest that the eutopic endometrium of women with RIF may appear normal in hysteroscopy but in fact may be genetically abnormal during embryo transfer. In contrast to our finding, Coughlan, *et al.* [11] reported that RIF is not associated with failed endometrial integrin expression. They also noted that, the expression of integrin  $\alpha V\beta_3$  appears to have no prognostic value in subsequent IVF treatment.

In the present study, RIF women with fresh cycle had lower levels of LIF mRNA expression in endometrium compared with frozen cycle or fertile women. However, the difference failed to reach statistical significance. Likewise there was no significant difference in the exp-

ressions of LIF in RIF women due to frozen cycle compared with the fertile controls. Controversial results have been reported regarding endometrial LIF mRNA expression in women with reproductive disorders. Confirmation of our results comes from studies of LIF in endometrium of women with endometriosis. Recent study, Celik, *et al.* showed that endometrial LIF expression of women with endometrioma was similar with the fertile women [3]. They also noted that LIF mRNA expression was not changed significantly after endometrioma resection. Likewise, Mikolajczyk, *et al.* [15] demonstrated that endometrial LIF expression in infertile women with endometriosis and fertile controls were similar.

Conversely, significantly reduced endometrial LIF mRNA expression was noted in women with endometriosis [16]. Likewise, presence of hydrosalpinx causes reduced expression of LIF and integrin  $\alpha V\beta_3$  mRNA in the endometrium [17]. When reviewing the literature there is a few study investigating endometrial LIF expression in women with RIF. Concordantly, Huang, *et al.* [10] showed that LIF expression was significantly reduced by 50% in the endometrium of the RIF patients compared with the fertile controls. The discrepancy between the two studies may be related to endometrial biopsy time. We received endometrial samples in the late follicular phase while they received endometrial biopsy in the early follicular phase.

### Conclusion

Our study indicated that integrin  $\alpha V\beta_3$  mRNA expression was significantly lower, while LIF mRNA expression was similar in the RIF group due to fresh cycle as compared with fertile controls. The LIF and integrin  $\alpha V\beta_3$  expressions were similar between frozen and fresh cycles. The results of our study and other results when taken together we can suggest that diminished or unchanged LIF expression may be seen in some benign gynecological diseases but LIF expression pattern of patients with RIF is normal. We can also say that expression of endometrial LIF mRNA is not common future of RIF women following fresh or frozen cycles. Decreased integrin  $\alpha V\beta_3$  expression may be one of the basic mechanisms underlying RIF.

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