

Expression and cellular distribution of insulin-like growth factor 1 receptor during window of implantation in infertile women with intramural fibroids

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ABSTRACT

Background: The “window of implantation” (WOI) is a transient but well defined period during which the hostile endometrial lining is transformed to a surface receptive to accept the embryo. Recently, data are beginning to accumulate suggesting negative influence of non-cavity distorting intramural uterine fibroids (NCD-IMF) on endometrial receptivity that may have implications for implantation failure. However, molecular mechanisms underlying infertility associated with NCD-IMF remain unclear. The aim of present study was to examine the expression and cellular distribution of insulin-like growth factor 1 receptor (IGF1R) during WOI in infertile women with NCD-IMF and fertile controls. While, reports are available that support role of IGF1R in mediating adhesive interaction with the implanting blastocyst, the effect of NCD-IMF on IGF1R expression during the WOI is not defined.

Methods: Quantitative real-time polymerase chain reaction and immunohistochemistry were used to evaluate messenger RNA (mRNA) and protein expression of IGF1R in midsecretory endometrial biopsies obtained from infertile women with NCD-IMF (n=20) and healthy fertile controls (n=10).

Results: As compared to fertile controls, significantly higher IGF1R: i) mRNA levels (1.59 fold up regulation; p=0.044) and ii) immunoscore in the luminal epithelium (8.94±3.13 versus 6.31±1.49; p=0.009) were observed in infertile women with NCD-IMF.

Conclusions: Over expression of IGF1R in infertile women with NCD-IMF, during the window of receptivity, may result in altered ability of uterine epithelial cells for blastocyst adhesion and subsequent implantation, which might lead to poor reproductive outcome in these women.

Keywords: IGF1R, Immunohistochemistry, Intramural fibroids, RT-PCR, Window of implantation

INTRODUCTION

Uterine fibroids, also known as leiomyomas, are most common benign monoclonal tumors of the uterine smooth muscle that are reported to occur in 50-60% of the female population.^{1,2} Besides being associated with

conditions such as menorrhagia, pelvic pain, etc., they also have unfavorable impact on fertility. Surprisingly, one in ten women undergoing fertility treatment were observed to have fibroids.³ Submucosal or intramural fibroids that distort endometrial cavity have been linked with lower clinical pregnancy rates and increased

miscarriage rates after ART (Assisted Reproduction Technology) treatment.⁴ Lower pregnancy rates after ART have also been observed in women with NCD-IMF.⁵ Reports are available suggesting negative influence of NCD-IMF on endometrial function that may have implications for implantation failure.⁶⁻⁹ However, the molecular mechanisms underlying infertility associated with NCD-IMF remain unclear.

It is well established that for successful pregnancy initiation, a precise synchrony must occur between development of a fertilized egg to an implantation-competent blastocyst and succession of events that render the uterus receptive for embryo implantation. This hormone dependent timing event referred to as “window of implantation” involves structural and functional changes of the endometrium that allow apposition and attachment of the embryo to the epithelial cells and adequate invasion into the stroma.¹⁰

Experimental evidence is available that supports the opinion that several hormone-induced growth factors influence pre-implantation embryo development and implantation. Pertinently, a functional role for the insulin-like growth factor 1 (IGF1) in coordinating blastocyst implantation ability with endometrial receptivity has been described. The biological functions of IGF1 are mediated mainly by IGF1R, a tyrosine kinase receptor.¹¹

IGF1R was found to be located abundantly on the uterine epithelium in rat during the preimplantation phase, and was suggested to contribute to adhesive interaction with the implanting blastocyst.¹² Decrease in IGF1R expression in epithelial cells has been reported to result in destabilized embryo attachment in vitro.^{13,14}

Although role of IGF1R in endometrial receptivity has been shown in several experimental and in vitro studies, not much is known on the effect of NCD-IMF on IGF1R expression during the window of implantation in infertile women. In the present study, we investigated midsecretory endometrial expression of IGF1R in infertile women with NCD-IMF in comparison to healthy fertile women without fibroids serving as study controls.

METHODS

The study included healthy fertile women (n=10) with normal pelvic cavities, without uterine fibroids, no history of infertility or habitual abortion, undergoing tubal ligation or reversal of tubal sterilization at the Department of Obstetrics and Gynecology, King George's Medical University, Lucknow, India; and infertile women with NCD-IMF (n=20). All women were enrolled in the study after obtaining written informed consent. The use of human tissue for research was approved by the ethics committee of King George's Medical University, Lucknow, India. The study period was from January, 2017 to July, 2018.

Women in the fertile control group were aged between 26 and 38 years (mean±SEM: 32.7±1.23 years) with mean parity of 2.0 (range 1-3). Women in the infertile group were aged between 26 and 37 years (mean±SEM: 30.85±0.80 years) with a mean duration of infertility of 6.95±0.78 years. Uterine fibroids were diagnosed by transvaginal ultrasonography, magnetic resonance imaging or hysteroscopy. Each infertile woman had a single intramural fibroid >3cm in diameter (mean±SEM: 4.88±0.34cm; range 3.25-7.6cm). The fibroid was located in the anterior wall in 7 women and in the posterior wall in 13 women. The uterine cavity in infertile women was evaluated for mechanical distortion either by hysteroscopy, hysterosalpingography or sonosalpingography.

Inclusion criteria

- Regular menstrual cycles in all participants (between 27 and 32 days)
- Normal FSH (follicle stimulating hormone), LH (luteinizing hormone) E2 (17beta estradiol), TSH (thyroid stimulating hormone), prolactin and mid luteal P (progesterone) levels
- Presence of intramural uterine fibroid as the only detectable cause of infertility in the infertile women
- Normal hysterosalpingography in infertile women
- No history of contraceptive use or hormonal medication within six months before enrollment in the study.

Exclusion criteria

- Women with history of endometriosis, adenomyosis, polycystic ovarian disease, hydrosalpinx
- Women with uterine polyps, uterine septae, presence of submucous or subserosal fibroids
- Previous surgeries including myomectomy, myolysis, metroplasty etc.
- History of habitual abortions
- Mechanical distortion of the uterine cavity due to the presence of intramural fibroids
- History of uterine artery embolization
- Infertile patients with male factor infertility.

The participants underwent transvaginal ultrasonographic monitoring of ovulation with the help of 3.5 to 8.5 MHz vaginal transducer (Shimadzu SBU 350; Shimadzu, Japan).⁸ The midsecretory phase was calculated +6 to +8 days post ovulation and was confirmed by endometrial histologic dating and serum P levels (>10 ng/ml).

Endometrial samples and histological dating

Endometrial biopsies were obtained from the uterine fundus of each women during the midluteal phase (days 20 to 24 of menstrual cycle) with the help of an endometrial suction curette (Gynetics, Belgium) and divided into two portions. The first portion was processed

for histologic dating and IHC and the second portion was snap frozen and stored at -80°C until RNA isolation for RT-PCR. Histological dating of each endometrial sample was performed by the same pathologist (MMG) following the Noyes criteria.¹⁵ Biopsies dated as midsecretory were only included in the study.

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the frozen tissue samples using TRIzol reagent (Invitrogen, Carlsbad, California) following manufacturer's instructions. RNA was purified using DNase1 (Amplification grade; Invitrogen) and quantified on a Picodrop spectrophotometer (Picodrop, United Kingdom) at 260/280nm wavelength. For cDNA (complementary DNA) synthesis, 250ng of total RNA was subjected to reverse transcription using random hexamer primers with high-capacity cDNA Reverse Transcription kit (Applied Biosystems, California) following the standard manufacturer's protocol.

Quantitative polymerase chain reaction was performed on step one real-time PCR system (Applied Biosystems, CA, USA) using SYBR Green fluorescent dye. The reaction mixture consisted of cDNA, 2X POWER SYBR green master mix (Applied Biosystem), and $10\mu\text{mol/L}$ of the forward and reverse primers. The house keeping gene viz. β -actin was used as normalization control. The sequences of forward and reverse primers used in the study are i) β -actin: Forward $5'$ -GTGGGGCGCCCCAGGCACCA- $3'$; Reverse $5'$ -CTCCTTAATGTCACGCACGATTTTC- $3'$; ii) IGF1R: Forward $5'$ -CGCAACGACTATCAGCAGCT- $3'$; Reverse $5'$ -AGATGAGCAGGATGTGGAGGT- $3'$. All PCR primers were synthesized by MWG Operon, India and were cross checked by primer express software 3.0 (Applied Biosystems) and blast sequence analysis (NCBI, Bethesda, Maryland). Thermal cycling conditions used were: 10 minutes at 95°C , followed by 40 cycles of 15 second at 95°C and 1 minute at 60°C . A negative control without template was run to evaluate the overall specificity of the reaction. Each measurement was performed in triplicate. Melt curve analysis was carried out at the end of each PCR to confirm the specificity of PCR product. Relative gene expression was determined by the $2^{-\Delta\Delta\text{CT}}$ method and expressed as fold change from fertile control set at a value of 1.0.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on formalin fixed paraffin-embedded (FFPE), $4\mu\text{m}$ thick endometrial sections mounted on APS (3-aminopropyl triethoxysilane) coated glass slides. Slides were incubated at 60°C for 1h and the deparaffinized and rehydrated. Antigen retrieval was performed at 98°C for 15 min in a microwave oven (EZ antigen retriever system, Biogenex, USA) in Tris-EDTA buffer (pH 9.0) for antibody against β subunit of IGF1R (sc 713; Santa Cruz Biotechnology, Santa Cruz, CA). The antibody was used at a dilution of

1:50, and slides were incubated overnight at 4°C in a humidified chamber. Detection was performed using DAKO Envision FLEX detection system (DAKO, Denmark). Visualization of immunoreactivity was achieved using chromogenic substrate 3,3'-diaminobenzidine tetra hydrochloride followed by hematoxylin counterstain. Slides were then dehydrated with a series of graded ethanol washes, mounted with DPX mountant (Rankem, India) and cover slipped for bright field microscopy using NIS-elements (Nikon) software for image acquisition. Positive control was selected according to the manufacturer's recommendations. Endometrium processed without primary antibody served as the negative control.

Scoring of IHC staining

Scoring was conducted independently by two observers (AM and MMG) who were blinded to the clinical diagnosis of the patients. Immunostaining was evaluated in the luminal epithelium (LE), glandular epithelium (GE) and stroma of each endometrial specimen. Scoring was semi-quantitative, assessing the intensity and proportion of cells stained. The staining intensity was scored on a scale of 0: no staining, 1: light staining, 2: low intermediate, 3: high intermediate and 4: darkest brown stain. The proportion of staining was scored on a scale of 1: 0-25% of cells stain positive; 2: 26-50% of cells stain positive; 3: 51-75% of cells stain positive; 4: 76-100% cells stain positive. Immunointensity and immunopositivity scores were multiplied giving results that ranged from 0 to 16.

Statistical analysis

The RT-PCR results were evaluated by the Student's t-test. IHC data was analyzed by one-way ANOVA (unstacked) to compare means between two groups. Minitab 13.20 statistical software (Minitab Inc., State College, PA) was used for data analysis. A p-value <0.05 was considered as statistically significant.

RESULTS

To evaluate IGF1R expression at the mRNA and protein levels, we performed RT-PCR and IHC respectively in midsecretory endometrial biopsies from infertile women with NCD-IMF and fertile controls.

RT-PCR analysis revealed that the mRNA expression levels of the IGF1R were significantly higher (1.59 fold up regulation; $p=0.044$) in infertile women as compared to fertile controls (Figure 1). The results of IHC demonstrated cytoplasmic and membranous expression of IGF1R which was localized predominantly in luminal and glandular epithelium, with very weak expression in the stroma (Figures 2A-2B). Endometria from infertile women as compared to fertile controls showed higher immunoscore for IGF1R in the LE (8.94 ± 3.13 versus 6.31 ± 1.49 ; $p=0.009$) and GE (8.31 ± 2.76 versus

6.46±2.69; p=0.073), however, the increase reached statistical significance only in the LE.

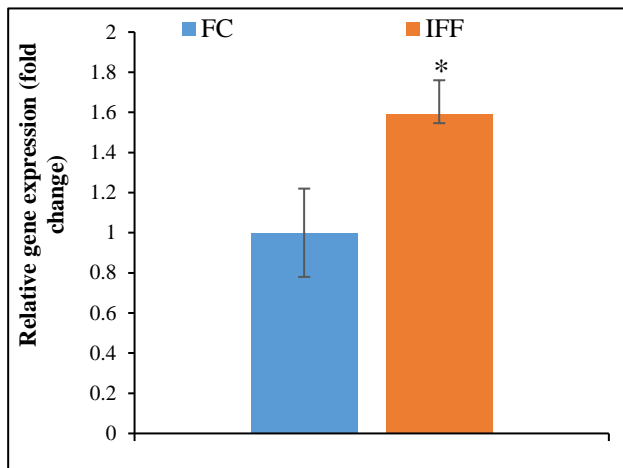


Figure 1: Analysis of relative expression levels of IGF1R gene by RT-PCR in fertile controls (FC) and infertile women with NCD-IMF (IFF). Data was analyzed by the 2- $\Delta\Delta$ CT method, normalized to β -actin and expressed as fold change from the fertile control set at a value of 1.0. Asterisk indicates statistically significant level at p<0.05, calculated by the Student's t-test.

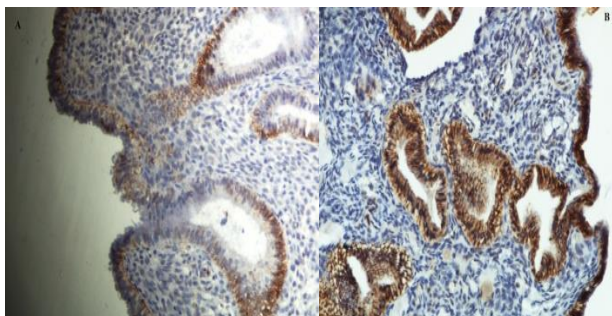


Figure 2: Representative microphotographs of immunohistochemical staining of IGF1R (β subunit) in midsecretory endometrium obtained from (A) fertile woman and (B) infertile woman with NCD-IMF. The luminal and glandular epithelial cells in the infertile group showed higher expression of cytoplasmic and membranous staining as compared to controls. The microphotographs are shown at an original magnification x 200.

DISCUSSION

The “window of implantation” is a transient but well defined period that requires a subtle collaboration of several cellular, biochemical and molecular events that transform the hostile endometrial lining to a surface receptive to accept the embryo. Interestingly, any aberration in the cellular or molecular interactions during the period of receptivity can result in infertility, implantation failure or early pregnancy loss.

To understand the role of IGF1R in fibroid associated infertility, we undertook a study to analyze IGF1R expression at the transcription level and temporal changes in its distribution in midsecretory endometrium from infertile women with NCD-IMF compared to that from women with proven fertility. We observed significant up regulation of IGF1R at the mRNA and protein level in infertile women as compared to controls.

Although immunostaining for IGF1R was observed both in the epithelial and stromal compartments of endometria, it was predominantly localized to the luminal and glandular epithelia during the period of receptivity in both groups. The observation is similar to that reported previously and is important due to the fact that endometrial IGF1R participates in mediating an adhesive interaction with the blastocyst during the receptivity period.^{16,17}

Our IHC findings are in agreement with the RT-PCR results suggesting regulation of IGF1R expression at the transcriptional level. An increase in IGF1R immunoexpression was evident in the LE and GE of endometria from infertile women as compared to fertile controls, however, the difference in intensity of IGF1R staining in GE between the two groups was less marked and failed to reach statistical significance.

Adherence of blastocyst to the uterus epitomizes the most crucial step of the implantation process. Over expression of IGF1R in the LE in the infertile group as compared fertile controls, observed in the present study, indicates its aberrant activity and may contribute to altered endometrial receptivity for blastocyst adhesion and implantation in these women. Although no reports are available on expression of IGF1R in infertile women with fibroids, Wu and Zhou observed that decreased IGF1R mRNA expression in relation with lower progesterone level, as compared to normal controls, may be one of the major causes of unexplained infertility.¹³ Kang et al, in their study with Ishikawa cells using a ligand-coated bead assay demonstrated that microRNA-145 suppresses embryo-epithelial juxtacrine communication at implantation by modulating maternal IGF1R, thereby confirming role of IGF1R in implantation.¹⁷ Ghazal et al, found that in women with endometriosis, alterations in H19 long noncoding RNA/microRNA Let-7/ IGF1R-mediated regulation of endometrial stromal cell proliferation may affect their endometrial preparation and receptivity to pregnancy and might represent a potential mechanism for infertility in these women.¹⁴

CONCLUSION

To conclude, results of the present study suggest that overexpression of IGF1R in infertile women with NCD-IMF, during the window of receptivity, may result in altered ability of uterine epithelial cells for blastocyst adhesion and subsequent implantation, which might lead to poor reproductive outcome in these women. Further

research in a larger patient population is required to confirm the findings and get a better insight into exploring the possibility of targeted IGF1R specific therapy, as a nonsurgical alternative for treatment of infertility in these women.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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