Original Article

Leukaemia Inhibitory Factor (*LIF*) Gene Mutations in Women Diagnosed with Unexplained Infertility and Endometriosis Have a Negative Impact on the IVF Outcome A Pilot Study

(leukaemia inhibitory factor (*LIF*) / mutation / infertility / *in vitro* fertilization)

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Abstract. The frequency of functionally relevant mutations of the leukaemia inhibitory factor (*LIF*) gene in infertile women is significantly enhanced in comparison with fertile controls. The objective of this retrospective cohort study was to evaluate the impact of *LIF* gene mutations on the outcome of the treatment in women with various causes of infertility. Fifteen infertile women with the G to A transition at position 3400 leading to the valine to methionine exchange at codon 64 were analysed. Group A was made up of women with diagnoses that are frequently accompanied by changes in humoral as well as cell-mediated immunity – idiopathic infertility and endometriosis (N = 7). Group B consisted of patients with polycystic ovary syndrome (PCOS), andrological factor, tubal factor and hyperprolactinaemia (N = 8). The control group comprised 136 infertile women with no *LIF* gene mutation diagnosed with idiopathic infertility and endometriosis (N = 37) (group C) and patients with PCOS, tubal and andrological factor (N = 99) (group D). Seven of the mutation-positive patients were successfully treated by *in vitro* fertilization (IVF), but nobody in this group was diagnosed with idiopathic infertility and only one with endometriosis, which means that there is a statistically significant difference in the pregnancy rates between groups A and B (P = 0.01, Fisher's 2 by 2 exact test) but no statistically significant difference when comparing patients with the *LIF* gene mutation (group A+B) to no *LIF* gene mutation (group C+D). The results suggest that in mutation-positive women the idiopathic infertility and endometriosis have a negative impact on the outcome of IVF treatment.

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Abbrevations: IVF – *in vitro* fertilization, LIF – leukaemia inhibitory factor, LIFR – LIF receptor, NK cells – natural killer cells, PCOS – polycystic ovary syndrome, PR – pregnancy rate, TGGE – temperature gradient gel electrophoresis.

Introduction

Female conditions of impaired fertility represent a heterogeneous group of disorders. The diagnoses are attributed to various anatomic, hormonal and immunological disturbances and the genetic background of impaired fertility is largely unknown.

The leukaemia inhibitory factor (LIF) (OMIM* 159540) is a pluripotent cytokine that plays a role in the control of embryo implantation. So far, the mechanisms of LIF action have not been fully understood. In the en-

dometrium of healthy women, LIF and LIF mRNA are expressed throughout the menstrual cycle with a striking increase in the midsecretory phase, coinciding with a supposed window of implantation. LIF acts on cells by binding to the LIF receptor (LIFR) and gp130, which belongs to the IL-6 receptor family. Human blastocysts express mRNAs for LIFR and gp130, participating actively in establishing contact with the endometrium. In the endometrium, LIFR and gp130 are expressed in the epithelium throughout the cycle, with a strong increase in the midsecretory phase (for review see Aghajanova, 2004; Králíčková et al., 2005).

A decrease in production of LIF in the uterine microenvironment was generally found in women in states of impaired fertility. No correlation between LIF in serum and uterine flushing was demonstrated, rendering LIF measurements in serum useless for the diagnosis of impaired infertility. On the other hand, LIF measurement in uterine flushing seems to be a useful but laborious diagnostic tool in the prediction of unsuccessful implantation (Mikołajczyk et al., 2003).

The frequency of functionally relevant mutations of the *LIF* gene in infertile women is significantly enhanced in comparison with fertile controls (Giess et al., 1999; Steck et al., 2004; Králíčková et al., 2006), but these alterations have not yet been characterized. The reason is that in the first two reports (Giess et al., 1999; Steck et al., 2004) there were only one or two women with the same potentially functional mutation identified. The only larger population with identical mutation, the G to A transition at position 3400 of the *LIF* gene, was studied in 2007 with the aim to characterize the clinical impact of this mutation. The study suggested that women with this *LIF* gene mutation have elevated levels of antiphospholipid antibodies in the serum (Králíčková et al., 2007). The authors hypothesized that this is due to the LIF impact on NK cell population, which is functionally connected to the presence of antiphospholipid antibodies in the serum (Roussev et al., 1996; Kaider et al., 1999; Sher et al., 2000). Our current population of 15 infertile women with identical mutation is the largest presented in the literature so far.

The involvement of changes in humoral as well as cell-mediated immunity in endometriosis and idiopathic infertility has been put forward by numerous investigators (Giudice et al., 2002; Mahutte and Arici, 2002; Antsiferova et al., 2005). However, the exact role of immunity disturbances in the pathophysiology of these diseases still remains controversial.

Endometriosis is a chronic inflammatory disease of multifactorial aetiology characterized by the implantation and growth of endometrial glands and stroma outside the uterine cavity. Reports on the role of LIF in the infertility of endometriosis patients are rather conflicting. Peritoneal fluid from women with endometriosis has a detrimental effect on embryo implantation, perhaps by adversely affecting LIF expression as well as uterine receptivity (Illera et al., 2000). LIF staining intensity in the glandular epithelium is significantly reduced in endometriosis patients compared to controls (Dimitriadis et al., 2006). On the other hand, in a recent study in which the uterine flushings and endometrial samples were collected 7–9 days after ovulation (at the time of a supposed implantation window) from both infertile patients with endometriosis and fertile, endometriosis-free controls, LIF was assessed and no statistically significant differences were found. The authors concluded that there is no receptivity defect with regard to LIF secretion by eutopic endometrium in infertile women with endometriosis (Mikołajczyk et al., 2006).

The NK and T cells are deregulated in endometriosis patients, too – inhibition of natural killer (NK) and cytotoxic T-cell function has been proposed as a mechanism of the pathogenesis of endometriosis (Maeda et al., 2002; Antsiferova et al., 2005; Matsuoka et al., 2005; Zhang et al., 2006). According to current knowledge, immune cell inhibition in endometriosis is mediated by factors other than HLA-G (the only MHC antigen expressed on cytotrophoblast cells of placenta) and cytokines in general have been suggested.

Idiopathic (unexplained) infertility patients suffer from various immunologic disturbances. They represent a heterogeneous group with many different findings – elevated NK-cell activity (Matsubayashi et al., 2001), antiphospholipid antibodies in the serum (Nouza et al., 1992; Ulčová-Gallová et al., 1998), and embryotoxic cytokines to name just a few. For example, sera and cervical mucus of idiopathic infertile women demonstrate significantly higher levels of interferon γ and tumour necrosis factor α compared to fertile controls (Naz et al., 1995).

The alterations in NK cell numbers and function are exclusive for endometriosis and idiopathic infertility, and the involvement of changes in immunity in other frequent infertility diagnoses (PCOS, tubal factor) only appears in a few studies and is never suggested as a key factor in the pathogenesis of the disease.

The objective of this retrospective cohort study was to evaluate the impact of the above-mentioned *LIF* gene mutations on the outcome of treatment in women with various causes of infertility.

Material and Methods

Patients

Fifteen women (ages ranging from 24 to 40 years with median age 32 years) with potentially functional *LIF* gene mutation, the G to A transition at position 3400 leading to valine to methionine exchange at codon 64 (V64M), in the AB loop region of the LIF protein (study group) and 136 infertile women without any *LIF* gene mutation (control group) were included in this retrospective cohort study. The group of mutation-positive women was divided into two parts: group A was made up of women with diagnoses that are frequently accompanied by various changes in humoral as well as cell-mediated immunity – idiopathic infertility (N = 4) and endometriosis (N = 3).

Group B comprised patients with PCOS (N = 3), andrological factor (N = 3), tubal factor (N = 1) and hyperprolactinaemia (N = 1). The control group consisted of 136 infertile women with no *LIF* gene mutation (ages ranging from 22 to 41 years with median age 31 years). It was made up of 10 patients diagnosed with endometriosis, 27 patients diagnosed with idiopathic infertility (group C), 41 patients diagnosed with male factor, 28 with tubal factor and 30 with PCOS (group D).

The endometriosis patients covered all stages of the disease. Patients classified as idiopathically infertile were documented as having patent tubes using laparoscopy, were free of pelvic adhesions and endometriosis, and were shown to have a normal uterine cavity using hysteroscopy or hysterosalpingography. They had normal ovulation and there was no evidence of male factor, antisperm or zona pellucida antibodies.

With regard to both follicular response and days of gonadotropin stimulation, the groups were similar. This study was approved by the Charles University Ethics Committee and informed consent was obtained from all individuals.

DNA extraction, polymerase chain reaction (PCR) and mutation status of the LIF gene

Peripheral blood leukocytes were used for DNA isolation in all cases. DNA was isolated using the DNeasy Tissue Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol. The coding regions and the exon-intronic junctions were analysed using temperature gradient gel electrophoresis (TGGE). Exon 3 was divided into three parts and the *LIF* gene was screened and divided into five partly overlapping fragments. PCR was performed using five sets of primers (Table 1). Primers were modified using the Poland java script (www.biophys.uni-duesseldorf.de/POLAND/poland.html) with the help of GC-clamp addition to create a thermostable domain suitable for TGGE. The reaction conditions were as follows: 12.5 µl of HotStart Taq PCR Master Mix (QIAgen), 10 pmol of each primer, 100 ng of DNA and distilled water up to 25 µl. The amplification programme consisted of denaturation at 95 °C for 15 min and then 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. PCR was completed by a final extension at 72 °C for 7 min. The amplification programme was the same for all analysed exons except exon 1, where the annealing temperature was 50 °C. The length and quality of the PCR products were checked in standard agarose gels.

Screening of mutations was performed using heteroduplex analysis on TGGE (Biometra, Goettingen, Germany) on 8% denaturing acrylamide gel (AA : bis-AA [37.5 : 1], 6 mol/l urea, 1x MOPS, 2% glycerol). The TGGE analysis was performed in two steps – firstly, for each exon, electrophoresis conditions for parallel gels had to be optimized using perpendicular gels. Then the parallel gels for patients' samples were run. The running time was always 1 h 30 min at a temperature gradient shown in Table 1. DNA bands were detected using the silver staining method already used by us previously, again according to a standard protocol (Králíčková et al., 2006).

The relevant DNA samples of all the women found to be positive in TGGE analysis were amplified and sequenced using automated sequencing with the aid of a Big Dye Terminator Sequencing Kit (PE/Applied Biosystems, Foster City, CA). The samples were run in an automated sequencer ABI Prism 310 Avant (PE/Applied Biosystems) at a constant voltage of 11.3 kV for 20 min. All PCR and sequencing experiments were repeated at least twice in TGGE-positive patients.

Statistical methods

The results were statistically assessed using the Fisher's 2 by 2 exact test; $P < 0.05$ was considered statistically significant.

Table 1. Table of primers and temperature gradients for parallel TGGE gels. GC clamps in italics

Exon	Names of the primers	Sequence 5 ,→ 3‘	Temperature gradients
E1	E1F-GC	*CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG* CTATGATGCACCTCAAACAA*	55°C–63°C
	E1R	GGGGCGGGTGTATTTA*	
E2	E2F	GCCACCCTTTCCTGCCTTTCTAC**	53°C–65°C
	E2R-GC	*CGGGCGGGGGCGGCGGGCCGGGCGCGGGGCGCGGCGGGCG* TCCCTGCCATCTCCTGTCAGTATC**	
E3.1	E3.1F-GC	*CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG* ACAATTCCAGATGCTTACAGGG **	58°C–66°C
	E3.1R-GC	*GCGGG* GCCAAGGTACACGACTATGC**	
E3.2	E3.2F	CCCAACAACCTGGACAAGCTATG**	60°C–65°C
	E3.2R-GC	*CGCCCGCCGCGCCCCGCGCCCGCCCCGCCGCCCCCGCCCC* CCGTAGGTCACGTCCACATG**	
E3.3	E3.3F-GC	*CCCGC* CCTCCTTAGCAACGTGCTGT*	58°C–62°C
	E3.3R-GC	*CGGGCGGGGGCGGCGGGCCGGGCGCGGGGCGCGGCGGGCG* ACATCTGGACCCAACTCCTG*	

* our original primers; ** primers inspired by Giess et al. (1999)

Table 2. Overview of all mutation-positive infertile women

Groups	Age	Diagnosis	Type of infertility	Outcome of the IVF treatment
Group A	30	Unexplained infert.	Primary	No pregnancy
	39	Unexplained infert.	Primary	No pregnancy
	40	Unexplained infert.	Secondary	No pregnancy
	34	Unexplained infert.	Primary	No pregnancy
	39	Endometriosis	Primary	No pregnancy
	33	Endometriosis	Primary	No pregnancy
	33	Endometriosis	Secondary	Pregnancy
Group B	27	PCOS	Primary	No pregnancy
	28	PCOS	Primary	Pregnancy
	28	PCOS	Secondary	Pregnancy
	31	Androlog. factor	Primary	Pregnancy
	31	Androlog. factor	Primary	Pregnancy
	24	Androlog. factor	Primary	Pregnancy
	31	Tubal factor	Primary	No pregnancy
	33	Hyperprolactinaemia	Secondary	Pregnancy

Results

In all studied groups (N = 151) there were fifteen positive samples identified by TGGE. All of them were in exon 3.2. By subsequent sequencing all these samples showed an alteration in the DNA sequence that was identical to the previously described potentially functional *LIF* gene point mutation, the G to A transition at position 3400.

Seven of the mutation-positive patients were successfully treated by the first cycle of IVF (Table 2), which means that the pregnancy rate was 47 %. If we then compare mutation-positive women (group A+B) to women with no *LIF* gene mutation (group C+D) regardless of their groupings according to their infertility diagnoses, there is no statistically significant difference in pregnancy rates.

Of the successfully treated group of seven mutation-positive women, not one had been diagnosed with idiopathic infertility and there was only one with endometriosis, which means that there was a statistically significant difference in the pregnancy rates between groups A and B (P = 0.01, Fisher's 2 by 2 exact test).

Comparing the two groups of women with endometriosis and idiopathic infertility, it was found that the mutation-positive group had a lower pregnancy rate (14 %) than the control group C (pregnancy rate 49 %), but due to the low number of patients in the groups it was not statistically significant (P = 0.11, Fisher's 2 by 2 exact test). The pregnancy rates of the groups with different diagnoses in the control population of our cohort were not significantly different statistically (Table 3).

Discussion

The frequency of the *LIF* gene mutations in the population of infertile women is significantly elevated in comparison with fertile controls. Nevertheless, the *LIF* gene mutations may be compensated and the treatment can succeed. The compensatory mechanisms for *LIF* defects have not been elucidated yet. The role of the second *LIF* gene allele and the hormonal stimulation were hypothesized (Steck et al., 2004), as well as the role of other regulatory molecules, most of them of cytokine nature (Králíčková et al., 2006).

Our results suggest that success of the infertility treatment in *LIF* gene mutation-positive women is influenced by the cause of infertility. Idiopathic infertility and endometriosis have a negative impact on the outcome of IVF. We suppose that this is due to the difference in NK cell counts and function in these two groups and the interaction of NK cells with variants of the LIF molecule.

We are aware of the fact that the statistical significance is not strong and that the compared groups are themselves heterogeneous, which makes our results even more disputable. Nevertheless, the group of 15 mutation-positive women is the largest published so far and the results can contribute to characterization of the influence of LIF and its gene mutations on infertility and its treatment.

Table 3. Overview of control population (LIF gene-negative infertile women)

Groups	Diagnoses	Number of patients	Pregnant after IVF	PR of the individual diagnoses	PR of the group
Group C	Unexplained infert.	27	14	51 %	49 %
mean age 31 ± 4 years	Endometriosis	10	4	40 %	
Group D	PCOS	30	15	50 %	
mean age 30 ± 6 years	Androlog. factor	41	23	56 %	52 %
	Tubal factor	28	14	50 %	
		136	70		51 %

The high pregnancy rate in group B can be influenced by lower average age of patients as well as by the indications to IVF/ICSI. Some authors (Arici et al., 1996; Omland et al., 2005) report that andrological factor, PCOS or hyperprolactinaemia are usually associated with relatively higher pregnancy rates after therapy than other indications.

The question of whether post-treatment pregnancy rates are influenced by the cause of infertility remains controversial. Some authors report a lower pregnancy rate in endometriosis patients (Arici et al., 1996; Omland et al., 2005; Ahinko-Hakamaa et al., 2007); some others report no differences between the various diagnoses (Burke et al., 2000; Calhaz-Jorge et al., 2004).

Human endometrium possesses a unique immunological environment enabling implantation of the semiallogeneic or allogeneic embryo. Large populations of macrophages and uterine-specific NK cells infiltrate the implantation site, believed to be important modulators of trophoblast invasion and decidualization (Johnson et al., 1999; Croy et al., 2003). However, our knowledge regarding selective recruitment of leukocyte subtypes is not complete.

LIF is produced by endometrial NK cells that interact with the invading trophoblast and although its effects on trophoblast are not yet clear, LIF appears to mediate interactions between maternal decidual leukocytes and the invading trophoblast. One of the actions of LIF on the endometrium is the regulation of the uterine leukocyte population. The *LIF* knockout mice have double the percentage of NK cells compared to wild-type mice at the time of the implantation window, indicating that LIF restricts the migration of NK cells to the uterus (Schofield and Kimber, 2005).

We are aware of the fact that this is just a pilot study and a larger number of *LIF* gene mutation-positive infertile patients is needed for further investigations.

A complete understanding of the complex regulatory mechanism may provide new therapeutic targets in female reproductive tract physiology.

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