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Expression of the imprinted *IGF2* and *H19* genes in the endometrium of cases with unexplained infertility

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ABSTRACT

Objective: As genomic imprinting plays a critical role in the development of the placenta, the aim of this study was to detect whether the expression levels of the imprinted genes *IGF2* and *H19* in the endometrium differ between infertile and fertile women.

Study design: : Total RNA was extracted from 30 (15 unexplained infertile and 15 fertile) women's endometrial tissue. cDNA was synthesized from total RNAs of each sample. *IGF2* and *H19* mRNA expression levels were measured quantitatively using the Real Time PCR method. In order to determine the allelic expression of *IGF2* and *H19*, genomic DNA was extracted from endometrial tissues.

Results: When compared with the control group, increased mRNA expression of *IGF2* was detected (1.5-fold change, $P = 0.015$) in the unexplained infertility group. In contrast, *H19* expression was lower in the infertility group as compared to the control group (4-fold change, $P < 0.0001$). Restriction analysis of cDNA-derived PCR product showed that all patients and controls indicated monoallelic expression of *IGF2* and *H19*.

Conclusion: Our results showed that altered expression of these imprinted genes might affect implantation and that their timely and appropriate activation is important for proper functioning. To understand the molecular epigenetic basis of implantation and placental development, genomic imprinted genes should be further investigated.

1. Introduction

Implantation requires the participation of numerous molecules, some of which are regulated by imprinted genes [1]. Any alteration from normal genomic imprinting would result in abnormal development [2,3]. Approximately 90 genes have been described as being imprinted, although it is unlikely that all imprinted genes have been identified [4]. One of the hallmarks of imprinted genes is that many are found in clusters throughout the genome [5]. The best-characterized cluster that follows a strict insulator model for imprinted expression is the cluster containing *H19* and insulin-like growth factor 2 (*IGF2*) [6,7]. *H19* is an imprinted gene in which the maternal allele is expressed, but not translated to a protein, and which functions as an RNA molecule [8]. It is closely related to the oppositely imprinted paternally expressed *IGF2* [9]. While *IGF2* is well known as a growth factor essential for proliferation and differentiation in various tissues during embryonic development, the role of *H19* in the endometrial cycle is still far from being understood [10,11]. *H19* is highly expressed in several tissues during embryonic development; however, *H19* expression in these tissues is absent during adult life but reappears in these tissues when a malignant process occurs [12]. Among rare tissues in which *H19* expression persists during postnatal life are endometrium and ovary [10]. Its expression in these tissues fluctuates during menstrual cycle [10].

H19 and *IGF2* are two of the most studied imprinted genes. Several studies proved their important role during implantation at the side of the embryo [13–16]. However, comparison of the expression of imprinted genes *H19* and *IGF2* in infertile and fertile women has not been studied to date at the side of the human endometrium. Inspired by their high endometrial expression levels during the phase of the menstrual cycle overlapping with the "implantation window", we conducted this first study in the literature to investigate the roles of the imprinted genes *H19* and *IGF2* in the endometrium during implantation.

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2. Materials and methods

2.1. Criteria for selecting patients and tissue sampling

Baseline study of infertile couples includes demonstration of ovulation, hysterosalpingography and pelvic ultrasonography to prove tubal patency and a semen analysis to exclude male factor. This diagnosis points to a factor resulting in infertility that cannot be demonstrated by a basic work-up. Factors interfering with implantation are major concerns at this point. Patients were selected from couples with one year's infertility and normal baseline diagnostic work-up. All members of this group had at least three unsuccessful ovulation induction and intrauterine inseminations. Gazi University Faculty of Medicine, Ethics Committee approved the study. Informed consents of all participants were taken.

Pipelle biopsies of the endometrium were collected at Gazi University, Department of Obstetrics and Gynecology from 15 patients and 15 controls. Seven of these 15 patients had prior diagnostic laparoscopies which revealed no abnormalities. Two of these 15 patients had prior hysteroscopy. In one case, hysteroscopic polypectomy was performed. The other hysteroscopy revealed a normal cavity. The control group consisted of women who had given birth to at least one baby and who did not have any evidence of failed implantation in their obstetric history, such as spontaneous abortion, blighted ovum, missed abortion and ectopic pregnancy.

All endometrial biopsies were obtained during the luteal phase, between cycle days 20 and 26 [6,10]. Cycle days 20–26 include both the implantation window and maximum *H19* and *IGF2* expressions.

All endometrial tissue samples were washed in sterile serum physiologic (SF) before being frozen in liquid nitrogen and stored at −80 °C. To be sure that all biopsies were performed during late secretory phase covering the implantation window, serum progesterone levels were determined on the sampling day and some endometrial tissues were sent to the pathology department for histological dating according to the Noyes criteria [17]. For both infertile (21.20 + 2.27) and fertile (21.47 + 0.74) groups, histological dating according to Noyes criteria was consistent with the increased progesterone levels (infertile group 11.26 + 6.32; fertile group 9.69 + 3.47) (Table 1). In both groups, thyroid function tests and thrombophilia parameters (Factor V Leiden R506Q (rs6025), methylene tetrahydrofolate reductase (MTHFR) C677T (rs1801133) and A1298C (rs1801131), prothrombin G20210A (rs1799963) mutations) were obtained from medical records, as their abnormality might interfere with appropriate implantation [18]. Clinical and biochemical characteristics of women participating in this study are listed in Table 1.

2.2. RNA extraction and reverse transcription

Total RNA from endometrial tissues (approximately 50–100 mg) was extracted by Trizol reagent (peqGOLD TriFast™, peqlab, Erlangen, Germany). To avoid DNA contamination, we made modifications to manufacturer's instructions. Tissues were lyzed in 1 ml peqGOLD TriFast™ reagent following homogenization using pestle. After complete lysis of homogenate, 200 μl chloroform was added. The mixture was vigorously shaken with vortex for 15 s and then incubated for 5 min in room temperature to allow the phases to separate. After centrifugation for 15 min at 12,000 *g* and 4 °C, upper phase containing RNA was carefully transferred to a new tube. RNA was precipitated with the addition of 500 ml isopropanol and incubated at −20 °C for 1–1.5 h. After centrifugation for 10 min at 12,000 *g* and 4 °C, supernatant was removed. Then RNA-containing pellet was treated with approximately 1–5 U RNase-free DNase (DNaseI, Roche Diagnostics, GmbH, Mannheim, Germany) per μg RNA and incubated at 37 °C for 30 min before being washed with 75% ethanol to prevent DNA contamination. After centrifugation for 15 min at 12,000 *g* and 4 °C, ethanol was removed and then pellet was dissolved in 10–30 μl DEPC treated water after which it was air-dried. Isolated RNA was kept at −80 °C. The total RNA concentration and purity (only samples with an A260/A280 ratio from 1.7 to 2) were considered valid for Reverse Transcriptase. PCR of each sample was measured using the Nanodrop spectrophotometer (NanoDrop ND-1000, Nanodrop Technologies, Montchanin, DE, USA) at 260 nm and equal amounts of RNA were used for reverse transcription. cDNA was synthesized from 1 μg of total RNA with random hexamer primers using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Reactions were performed in the PCRSprint thermocycler (Hybaid, Heidelberg, Germany).

2.3. Quantitative Real Time PCR (QRT-PCR) analysis

IGF2 and *H19* mRNA expression levels were measured using Real Time PCR method with LightCycler® (Roche Diagnostics, GmbH, Mannheim, Germany). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as housekeeping gene in order to normalize *IGF2* and *H19* expression levels. Probes and intron spanning primers

Table 1
Clinical and biochemical characteristics of women participating in the study.

	Infertile ($n = 15$)	Fertile ($n = 15$)	P values
Age (years)	30.60 ± 5.44	31.67 ± 3.95	.738
Sampling day	21.80 ± 1.74	21.53 ± 0.83	.596
Histological dating according to Noyes criteria	21.20 ± 2.27	21.47 ± 0.74	.141
Progesterone (ng/ml)	11.26 ± 6.32	9.69 ± 3.47	.281
TSH (mIU/l)	1.86 ± 0.86	1.87 ± 0.65	.709
Free T3 (pg/ml)	2.60 ± 0.51	2.66 ± 0.53	.803
Free T4 (ng/dl)	1.05 ± 0.15	1.06 ± 0.29	.289
ATIII (%)	98.60 ± 7.26	100.86 ± 10.35	.723
Protein C (%)	84.73 ± 24.94	91.06 ± 31.37	.708
Protein S (%)	91.26 ± 30.66	86.53 ± 32.07	.693
APRC (%)	0.68 ± 0.17	0.64 ± 0.21	.588
Mean duration of infertility (months, range)	73.2 + 53.2 (18–216)	–	
Ovulation induction and intrauterine insemination			
rFSH	9	–	
Clomiphene citrate	2	–	
rFSH + clomiphene citrate	4	–	
Times of ovulation induction and intrauterine insemination (range)	3–8	–	
Mean gravida (range)	–	2.8 (1–8)	
Mean parity (range)	–	2.3 (1–4)	

APRC: Activated Protein C Resistance; ATIII: Antithrombin 3; rFSH: Recombinant follicle stimulating hormone.