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Epidermal growth factor +61A/G (rs4444903) promoter polymorphism and serum levels are linked to idiopathic male infertility

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Infertility is defined as a failure to achieve pregnancy despite one year of unprotected intercourse at regular intervals. In nearly 50% of infertility cases, the male has suboptimal sperm parameters [1]. Growth factors such as epidermal growth factor (EGF) play an important role in normal spermatogenesis and is primarily produced by Leydig cells in testis [2]. The expression of EGF and its receptor (EGFR) in the male germ cells plays as important paracrine and/or autocrine regulators of spermatogenesis [3]. The elimination of serum EGF suppresses spermatogenesis and decrease sperm content and motility in adult mice [4]. Decreased EGFR expression is linked to male infertility and EGF regulates gap junction formation in the spermatogenic cells necessary for intercellular communication [5]. EGF promotes the proliferation of both epidermal and mesothelial cells and mediates other biological processes such as programmed cell death, and differentiation and it has stimulatory effect on human sperm capacitation [6]. EGF plays a key role in spermatogonial proliferation in an endocrine manner through receptors including ErbB1, ErbB2, and ErbB4 expressed on Sertoli cells [7]. In foetal human testes, *HER2* is expressed in proliferating Sertoli and Leydig cells. Testicular her2 mRNA levels are closely related with spermatogenic activity. EGF/HER2 signalling is likely to participate in spermatogenesis and Leydig cell steroidogenesis via mediating EGF-GF signalling in conjunction with other members of the erb type-1 tyrosine kinase receptors [8]. We hypothesised links between *EGF* 61A>G (rs4444903) genetic variation and its serum levels in idiopathic male infertility.

We tested our hypothesis on 120 men with idiopathic infertility attending the Mehr infertility Institutes, Rasht, Iran. Patients with azoospermia factor (AZF) microdeletions, karyotype abnormalities, varicocele, Klinefelter's syndrome, cystic fibrosis, seminal infections, diabetes, and chronic diseases were excluded. These men were compared to 140 fertile men who had at least one child without assisted reproductive technologies. Written informed consent was obtained from all cases and control subjects. The study has been performed in compliance with the 1964 Helsinki declaration and local research ethics committee approval was obtained.

A two ml blood sample was taken and transferred in EDTA-containing tubes for DNA extraction and genotyping, a further two ml into anti-coagulant free tubes. Serum was separated by centrifugation at 2,000 rpm for 10 min at 4 °C and stored at −70 °C. Total genomic DNA was extracted from peripheral blood samples by the GPP Solution Kit (Gen Pajoohan Pouya,Tehran, Iran) according manufacturer's instructions and used as a template to evaluate the EGF A61G polymorphism by polymerase chain restriction fragment length polymorphism (PCR-RFLP) analysis. The primer sequences were: EGF-F: 5'TCCTCTTTGGCAGTCATCCC3' and EGF-R: 5' CATTTCCTGCGAGAGTACCTT3'. Primers were designed according to the published nucleotide sequence in ENSEMBL database and using oligo primer analysis software (version 7.54, Molecular Biology Insights Inc., Cascade, CO, USA). PCR amplification was performed in 20 µl reaction volumes containing 5 µl DNA, PCR master mix (Pishgam, Tehran, Iran), 3 µl deionized water, and 1 µl of forward and reverse primers under the following PCR cycling program: the initial denaturation at 94 °C for 5 min followed by 35 repetitive cycles, denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 45 s and final extension step of 72 °C for 5 min. The resulting 708 bp DNA fragment was digested with *CspCI* restriction enzyme (New England Biolabs Inc, London, UK) generating two fragments of 196 and 477 bp. DNA fragments were separated on 2% agarose gel. Genotyping was performed and interpreted independently by two investigators. Serum EGF concentration was measured by ELISA according to manufacturers' instruction kits (Abcam, Cambridge, UK). Statistical analyses were conducted using MedCalc statistical software (Version 17.9.7, Mariakerke, Belgium).

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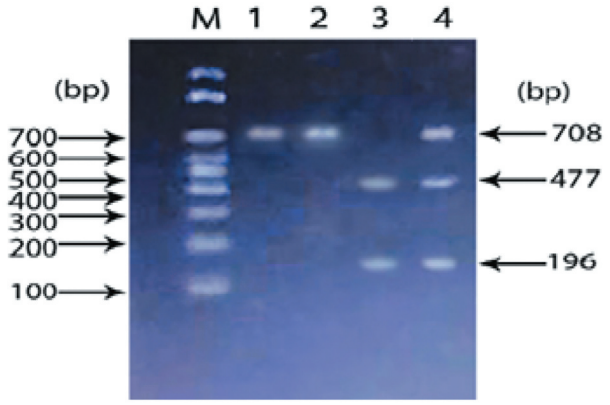

Figure 1. PCR-RFLP analysis of the A61G *EGF* polymorphism. Lanes1 and 2: uncut PCR product of 708 bp for A/A genotype, Lanes 3: two fragments of 196 and 477bp for GG genotype; Lanes 4: three fragments of 196, 477and 708bp for A/G genotype. M: molecular size marker.

The 120 idiopathic infertile cases were aged mean [SD] 36.4 [4] years, the 140 fertile men 38.6 [4] years (t test, p = 0.19) and were genotyped by PCR-RFLP (Figure 1). Genotype frequencies of the rs4444903 were in Hardy Weinberg equilibrium in both groups (P > 0.05). The frequencies of *EGF* AA, AG and GG genotypes are shown in Table 1. The cases were more likely to have the AG or GG genotypes, and the G allele. The mean [SD] EGF serum levels in cases and controls were 464 ± 66 pg/ml and 314 ± 45 pg/ml, respectively (t test p < 0.001).

We report a strong link between the AG and GG genotypes of *EGF* SNP (rs4444903) and male infertility, which adds to other literature on this gene in reproductive disease. In Chinese women, no association of *EGF* 61 G/A and *EGFR* +2073 A/T SNPs with endometriosis was reported, while the *EGFR* +2073 A/T SNP was linked to a higher risk to endometriosis [9]. Others showed links between SNPs rs11568943 and rs11569017 in *EGF* to the risk of abnormal prostate volume, EGF being significantly increased in-vitro spermatogonial cell cluster formation, and plasma EGF concentration to be associated with epididymal sperm count [10–12]. Decreased expression of the *EGFR* in azoospermic men has also been reported [5]. EGF can increase oocyte maturation rate in vitro, and significantly improve the oocyte quality, and several gene polymorphisms such as glial cell derived neurotrophic factor are associated with the risk of male infertility [13,14]. Different SNPs in the promoter region of *EGF* can influence the level of circulating EGF, and the *EGF* 61 G/A SNP increases serum EGF are significantly in several cancers [15].

Table 1. Genotype and allele frequencies of EGF +61A/G (rs4444903) gene polymorphism in cases and controls.

Genetic models	Cases n (%)	Controls n (%)	OR (95% CI)	P-value
Codominant				
AA	89 (74.2)	129 (92.1)	1.00	
AG	28 (23.3)	10 (7.2)	4.05 (1.87-8.77)	0.0004
GG	3 (2.5)	1 (0.7)	4.34 (0.44-42.48)	0.20
Dominant				
AA	89 (74.2)	129 (92.1)	1.00	
AG+GG	31 (25.8)	11 (7.9)	4.08 (1.95-8.55)	0.0002
Recessive				
AA+AG	117 (97.5)	139 (99.3)	1.00	
GG	3 (2.5)	1 (0.7)	3.56 (0.36-34.72)	0.27
Overdominant				
AA+GG	92 (76.7)	130 (92.85)	1.00	
AG	28(23.3)	10(7.14)	3.95 (1.83-8.54)	0.0005
Alleles				
A	206 (86)	268 (96)	1.00	
G	34 (14)	12 (4)	3.68 (1.86-7.29)	0.0002

We acknowledge certain limitations. Firstly, the size of sample was relatively small and the result should be interpreted with caution. Secondly as only one SNP in promoter region of *EGF* was studied, we cannot exclude the possibility that other SNPs could have a role in idiopathic male infertility as several factors may act individually and together to influence the risk of idiopathic male infertility. Accordingly, additional studies are required.

In summary, this work represents an advance in biomedical science because it indicates a link between the *EGF* +61A/G (rs4444903) SNP and its serum concentration with idiopathic male infertility.

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Disclosure statement

The authors report no conflict of interest.

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