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RESEARCH

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Investigation of *HER2* I655V and *PHB* 3′UTR C > T polymorphisms in azoospermic infertile males

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Abstract

Background Male infertility is a complex, multifactorial pathological condition with a highly heterogeneous phenotypic variation, from complete absence of spermatozoa in the testicles (azoospermia) to marked changes in sperm quality. The *Erb-B2* receptor tyrosine kinase 2 gene (*ERBB2*, also often called *HER2*) was hypothesized to be involved in spermatogenesis and Leydig cell steroidogenesis in males. Prohibitin (*PHB*) has been shown to regulate sperm motility in infertile men by changing mitochondrial membrane potential and increasing reactive oxygen species levels. In this study, we aimed to investigate the *HER2* I655V and *PHB* 3′UTR C > T polymorphisms in azoospermic infertile males.

Methods One hundred and thirty-three infertile males with idiopathic azoospermia and 100 healthy male controls were included in this study. Patient and control DNAs, which were extracted from peripheral blood, were analyzed using polymerase chain reaction and restriction fragment length polymorphism technics. For statistical evaluation, Chi-square and variance analyses were carried out using IBM SPSS (version 20.0) and Openepi (version 3.01) software programs.

Results We did not observe any differences between azoospermic infertile males and healthy male controls in terms of allele and genotype distributions of both the *HER2* I655V and *PHB* 3′UTR C > T polymorphisms ($p > 0.05$). Moreover, composite genotype analyses did not show any differences between two groups ($p > 0.05$).

Conclusions No association was found between *HER2* I655V and *PHB* 3′UTR C > T polymorphisms and azoospermic male infertility. The study can be expanded further by increasing the number of samples and studying in various populations.

Keywords Azoospermia, *Erb-B2* receptor tyrosine kinase 2, Prohibitin, Polymorphism

Background

Infertility, which is defined as the inability to have children at the end of one year despite regular and unprotected sexual intercourse, can cause loss of self-confidence and social withdrawal in couples [1, 2]. About 7% of men have male infertility, which is a complex, multifactorial pathological condition with highly heterogeneous phenotypic variations, from complete absence of spermatozoa in the testicles (azoospermia) to marked changes in sperm quality[3, 4]. The pathogenesis of male infertility has been linked to genital anomalies, varicocele, testicular pathologies, cancer, chemotherapy, urogenital infections, endocrine disorders and genetic anomalies[5]. Semen analysis results of male patients who applied to clinics due to infertility can vary from

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normozoospermia to azoospermia. Azoospermia can be detected in 1% of male cases and 10–15% of infertile male patients [6]. Undescended testis, orchitis, trauma, radiotherapy, chemotherapy, hypogonadism, congenital absence of vas deferens and Y chromosome microdeletion are well-known factors causing azoospermia. However, apart from these factors, the etiology of azoospermia cannot be completely determined even in the majority of the cases with non-obstructive azoospermia.

The *Erb-B2* receptor tyrosine kinase 2 gene (*ERBB2*, often referred to as *HER2*) is a 185-kDa transmembrane tyrosine kinase receptor belonging to the epidermal growth factor receptors (EGFRs) family. This gene is located on the long arm of chromosome 17 (17q12). HER1 (EGFR), HER3 (ERBB3) and HER4 (ERBB4) are other members of the EGFR family. All these tyrosine kinase receptors are single-subunit transmembrane glycoproteins and consisted of three domains: the extracellular ligand-binding domain, the transmembrane domain and the intracellular tyrosine kinase catalytic domain [7]. The levels of HER2 mRNA in the testicles have been linked to spermatogenic activity. It has been shown that active HER2 functions in spermatogenesis and Leydig cell steroidogenesis in males in conjunction with other erb type-1 tyrosine kinase receptors via the epidermal growth factor-growth factor (EGF-GF) signaling pathway [8]. The presence of a single nucleotide polymorphism (SNP) (change of isoleucine to valine) at codon 655 in the transmembrane coding region of the *HER2* gene has been reported in different cancer types [9, 10]. Codon 655 alteration from ATC to GTC results in enhanced HER2 dimerization and autophosphorylation as well as tyrosine kinase activity, which is known to trigger cell transformation [11]. This characteristic means that *HER2* I655V polymorphism may have an impact on sperm cell development.

Prohibitin (PHB), a highly conserved protein of the mitochondrial inner membrane, was directly linked to the process of spermatogenesis and the regulation of sperm quality. It is also a potential substrate for ubiquitination modifications [12]. PHB has been shown to regulate sperm motility in infertile men by changing mitochondrial membrane potential (MMP) and increasing reactive oxygen species (ROS) levels [13]. The 3′ untranslated region (UTR) of PHB has a positive effect on cell proliferation, and a C to T change in this region creates a variant with no antiproliferative effect [14, 15]. Hence, *PHB* 3′UTR C > T polymorphism may have a proliferative effect on sperm cells.

Previous studies have suggested a relationship between *HER2/PHB* genes and spermatogenesis/sperm motility. *HER2* I655V was found to induce the transformation of cells, and *PHB* 3′UTR C > T polymorphisms was shown to have proliferative effects on cells. So, we aimed to investigate the possible relationship between azoospermic male infertility and *HER2* I655V and *PHB* 3′UTR C > T polymorphisms in a Turkish population, in this study.

Materials and methods

Subjects

This study was included 133 infertile males who were examined and diagnosed with idiopathic azoospermia in Urology Clinic of Tokat Gaziosmanpasa University Research Hospital between 2009 and 2019. These 133 azoospermic infertile males were composed of individuals whose blood samples were taken and DNAs were obtained routinely for Y chromosome microdeletion analysis, and no mutations were detected in the Laboratory of Medical Biology Department, and who had signed a consent form during the routine examination. Hormone profile (FSH, LH, testosterone), physical examination, a detailed medical history, semen analysis, routine hematological and biochemical analysis were used to evaluate the patients. The study excluded individuals with chromosomal anomalies, vas deferens agenesis or obstructive azoospermia, and testicular trauma, testicular torsion or a history of undescended testis. The fertile group consisted of 100 male individuals who freely decided to participate in the study and completed the consent form. They had at least two children, no known hereditary or chronic disorders, and had applied to urology outpatient clinic for any reason other than infertility. The Tokat Gaziosmanpasa University Faculty of Medicine Clinical Research Ethics Committee determined that our investigation met ethical requirements (registration no.: 19-KAEK-179).

Genotyping

Blood samples were collected in ethylene diamine tetraacetic acid (EDTA)-coated tubes from azoospermic infertile males and fertile male controls. Genomic DNA was extracted from whole venous blood samples using a commercial DNA isolation kit (Invitrogen Life Technologies, Carlsbad, CA) and kept at 20 °C until use. PCR and RFLP assays were applied to determine the genotypes of *HER2* I655V (rs1136201) and *PHB* 3′UTR (rs6917) polymorphisms. The forward (F) 5′-CCA GCC CTC TGA CGT CCA T-3′ and reverse (R) 5′-TCC GTT TCC TGC AGC AGT CTC CGC A-3′ primers were used to detect *HER2* I655V polymorphism. The PCR amplification was performed in a total volume of 25 μL containing 200 ng of genomic DNA, 1 × PCR buffer ($MgCl_2$ supplied), 0.2 mM of each deoxyribonucleotide triphosphates, 5 pmol of each primer and 1U of Taq DNA polymerase (A.B.T). 148-bp PCR products were obtained after 5 min at 94 °C;

subsequent to 35 cycles of 94 °C for 30 s, 62 °C for 45 s, 72 °C for 30 s; and finally 7 min at 72 °C. For RFLP analyses, 10U *Alw26I* (*BsmAI*) (New England Biolabs) restriction endonuclease was used, and after digestion, 116 and 32 bp for the Val (GTC) allele and a single 148-bp fragment for the Ile (ATC) allele were observed (Fig. 1).

The F: 5′-AAG GTG GCT TTC TGG TGA-3′ and R: 5′-GGA AGG TCT GGG TGT CAT TT-3′ primers were used to detect *PHB* 3′UTR C>T polymorphism. The PCR amplification was performed in a total volume of 25 µL containing 200 ng of genomic DNA, 1×PCR buffer ($MgCl_2$ supplied), 0.2 mM of each deoxyribonucleotide triphosphates, 10 pmol of each primer and 1U of Taq DNA polymerase (A.B.T). 1237-bp PCR products were obtained after 5 min at 94 °C; subsequent to 35 cycles of 94 °C for 45 s, 60 °C for 30 s and 72 °C for 90 s; and finally 10 min at 72 °C. For RFLP analyses, 2U *Afl*III (New England Biolabs) restriction endonuclease was used, and after digestion, fragments of 671, 442 and 124 bp for CC genotype, fragments 671, 566, 442 and 124 bp for CT genotype and fragments 671 and 566 bp for TT genotype were observed (Fig. 2).

Statistical analysis

Statistical Program for the Social Sciences (IBM SPSS Statistics, version 20) and OpenEpi Info software package version 3.01 (www.openepi.com) were used for the statistical analysis. Chi-square (χ^2) test was used to compare allele, genotype and composite genotype frequencies between the control and patient groups. One-way analysis of variance (ANOVA) method was used to compare the clinical and demographic characteristics of the patients with the genotypes of each polymorphism. Hardy–Weinberg equilibrium (HWE) was assessed for each polymorphism by χ^2 test to evaluate the deviation of the study groups. Risk factors were determined by 95% confidence intervals (CI) and odds ratio (OR). Two-tailed *p* values were used, and values with p less than 0.05 were considered significant.

Results

In this study, 133 azoospermic infertile male patients and 100 healthy male controls were examined to investigate the relationship between *HER2* I655V (A>G) and *PHB* 3′UTR C>T polymorphisms and azoospermic male infertility. Median age for patients and healthy controls was 41.5 years (ranging 20–60 years) and 32.3 years (ranging 17–54 years), respectively. The *HER2* I655V (A>G) polymorphism was examined in 133 patients, of whom 76.6% had homozygous AA (Ile/Ile) genotypes and 23.3% had heterozygous AG (Ile/Val) genotypes. GG homozygous genotype was not observed in the patient

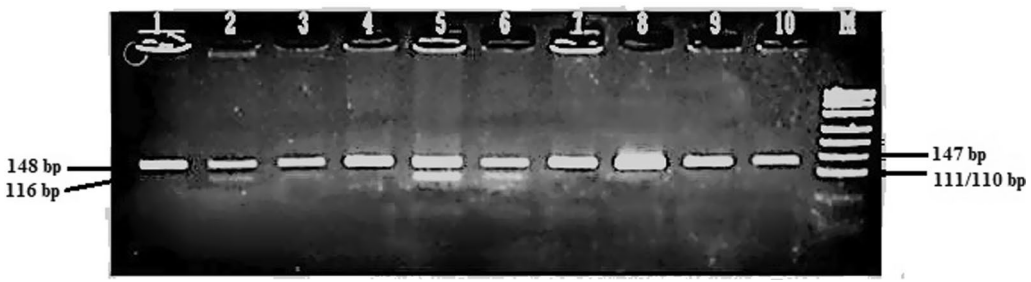

Fig. 1 The polymorphic alleles of I655V polymorphism of *HER2* gene, which is defined by the *BsmAI* restriction endonuclease. Lines 1, 3, 4, 7, 8, 9 and 10: homozygous AA genotypes; lines 2, 5 and 6: heterozygous AG genotypes. M: pUC 19 DNA/MspI (HpaII) marker. The DNA marker contains the following 11 visible fragments (in base pairs): 501/489, 404, 331, 242, 190, 147, 111/110, 67, 34

Fig. 2 The polymorphic alleles of 3′UTR C>T polymorphism of *PHB* gene, which is defined by the *AflIII* restriction endonuclease. Lines 3, 4, 5, 6, 7 and 8: homozygous CC genotypes; lines 2, 9 and 10: heterozygous CT genotypes; line 1: homozygous TT genotype. M: pUC mix marker. The DNA marker contains the following 12 visible fragments (in base pairs): 1118, 881, 692, 501/489, 404, 331, 242, 190, 147, 111/110.

group. One hundred controls were tested: 77.0% had AA homozygous genotypes, 22.0% had AG heterozygous genotypes and 1.0% had GG (Val/Val) homozygous genotype. The *PHB* 3′UTR C > T polymorphism was examined in 133 patients of whom 67.6% had CC homozygous genotypes, 27.1% had CT heterozygous genotypes and 5.3% had TT homozygous genotypes; 100 controls were tested: 65.0% had CC homozygous genotypes, 32.0% had CT heterozygous genotypes and 3.0% had TT homozygous genotypes. No statistically significant relationships between azoospermic male infertility and HER2 I655V (A > G) polymorphism or between azoospermic male infertility and PHB 3′UTR C > T polymorphism were found when the genotype and allele frequencies of the control and patient groups were compared ($p > 0.05$). When the *HER2* gene genotypes were compared as AA versus AG + GG between patients and controls, no statistically significant differences were observed ($p = 0.956$). When the *PHB* gene genotypes were compared as CC versus CT + TT, no statistically significant differences were observed ($p = 0.669$), either. Genotype distributions in azoospermic infertile males and healthy male controls were compatible with HWE for both the polymorphisms ($p > 0.05$) (Table 1).

No statistically significant relationship was observed between the age and clinical characteristics of the patients (FSH, LH, prolactin, estradiol, total testosterone) and *HER2* I655V (A > G) and *PHB* 3'UTR C > T

Table 1 Genotype and allele frequencies of *HER2* and *PHB* gene polymorphisms in infertile and fertile men

Gene (polymorphism)	Infertile men $n = 133$ (%)	Fertile men ($n = 100$)	*p*	OR (95% CI)
HER2 I655V (A > G)	HWE $p = 0.128$	HWE $p = 0.676$		
Genotypes				
AA	102 (76.6)	77 (77.0)	0.503	
AG	31 (23.3)	22 (22.0)		
GG	0	1 (1.0)		
AA: AG + GG	102:31	77:22	0.956	1.02 (0.55–1.9)
Allel				
A	235 (88.3)	176 (88.0)	0.909	0.97 (0.55–1.72)
G	31 (11.6)	24 (12.0)		
PHB 3′UTR C > T	HWE $p = 0.191$	HWE $p = 0.691$		
Genotypes				
CC	90 (67.6)	65 (65.0)	0.544	
CT	36 (27.1)	32 (32.0)		
TT	7 (5.3)	3 (3.0)		
CC: CT + TT	90: 43	65: 35	0.669	0.89 (0.51–1.54)
Allel				
C	216 (81.2)	162 (81.0)	0.956	0.99 (0.62–1.58)
T	50 (18.7)	38 (19.0)		

Data were analyzed by χ2 test. HWE, Hardy–Weinberg equilibrium; *HER2,* Human epidermal growth factor receptor 2; *PHB*, Prohibitin

Table 2 Clinical characteristics of infertile men stratified according to *HER2* and *PHB* gene polymorphisms

Characteristics	Total $n = 133$	*HER2* I655V (A > G)			*PHB* 3′UTR C > T		
		AA $n = 102$	AG + GG $n = 31$	*p* value	CC $n = 90$	CT + TT $n = 43$	*p* value
Mean age (years)	32.35 ± 6.76	32.27 ± 7.06	32.58 ± 5.76	0.826	32.64 ± 6.84	31.72 ± 6.62	0.463
FSH (IU/L)	15.48 ± 17.59	16.51 ± 19.24	12.11 ± 10.08	0.267	16.18 ± 18.66	14.02 ± 15.25	0.547
LH (IU/L)	8.53 ± 7.72	8.85 ± 8.56	7.47 ± 3.79	0.429	8.34 ± 8.21	8.91 ± 6.66	0.720
Prolactin (ng/ml)	11.24 ± 6.14	11.43 ± 6.24	10.68 ± 5.97	0.629	11.45 ± 6.22	10.86 ± 6.09	0.679
Estradiol (pg/mL)	23.90 ± 13.29	23.69 ± 14.88	24.48 ± 7.72	0.841	23.54 ± 13.66	24.88 ± 12.57	0.732
Total Testosterone (ng/mL)	3.64 ± 2.26	3.52 ± 2.45	4.0 ± 1.5	0.336	3.68 ± 2.47	3.56 ± 1.80	0.803

Data were analyzed by ANOVA. Mean ± standard deviation values were given for all characteristics. FSH, Follicle-stimulating hormone; *HER2,* Human epidermal growth factor receptor 2; *PHB,* Prohibitin

polymorphisms ($p > 0.05$) (Table 2). Routine hematologic and biochemical analysis of the patients was within normal limits.

In composite genotype analysis, composite genotype frequencies of *HER2* I655V (A > G)/*PHB* 3'UTR C > T gene polymorphisms were compared between azoospermic infertile men and healthy controls. As a result of this analysis, no statistically significant differences were observed between the patient and control groups ($p > 0.05$) (Table 3).

Discussion

The EGFR family member HER2 possess intrinsic tyrosine kinase activity. In addition to their critical roles in the signal transduction pathway, members of this family regulate a variety of cellular processes, including differentiation and proliferation [16]. EGF promotes proliferation of both epidermal and mesothelial cells. It has a stimulating effect on human sperm capacity by mediating biological processes such as apoptosis and differentiation. Male infertility was correlated with decreased EGFR expression, and gap junction development in spermatogenic cells, that is essential for intercellular communication [17]. It has been reported that the genetic polymorphism of *HER2* I655V (rs1136201) can alter receptor structure and activation, which can alter signal transduction and thus cell cycle regulation [18]. Because of this, *HER2* I655V polymorphism may have an impact on sperm cell development.

PHB encodes a 30-kDa protein that regulates both E2F transcription factors and p53 protein and is connected with proliferative and apoptotic pathways. Its deficiency in germ cells was associated with MMP and increased ROS production. It has been suggested that a change in MMP and a consequent decrease in PHB expression may be associated with poor sperm quality in infertile males [19]. In a previous study, it was observed that sperm with poor motility or low concentrations in infertile men were negatively correlated with mitochondrial ROS levels, but positively correlated with MMP. It has also been found that these cells have a low PHB expression levels. These findings suggested that PHB expression levels are critical for germ cell mitochondrial integrity and can be used as an indicator of human sperm quality [20]. In a recent study, it was also proposed that PHB is essential for preventing the energy loss brought on by poor mitochondrial function in granulosa cells of infertile endometriosis patients [21]. The 3′UTR of *PHB* gene encodes a trans-acting regulatory RNA molecule [22]. Due to their control over the expression of genes and proteins, 3′UTR polymorphisms have been linked to a variety of phenotypic effects [23]. The cytosine to thymine transition at position 1630 in the 3'UTR of *PON1* gene results in a variant that encourages carcinogenesis by reducing cell motility and losing antiproliferative function [24].In the present study, we were unable to find any data to support the claim that the *HER2* I655V and *PHB* 3'UTR C > T polymorphisms increase azoospermic male infertility risk, either overall or in subgroups based on age or clinical characteristics of the patients. SNPs and copy number variations (CNVs) have recently been identified as significant factors that affect male fertility [25, 26]. Although it is very plausible that genetic alterations affect male fertility, each polymorphism might only be a minor part of all occurrences of male infertility. To address the crucial questions about the association between SNPs, CNVs and male infertility, it will probably be necessary to create datasets containing genome-wide data from numerous institutions. Deepening our understanding of these topics might help us use assisted reproductive technology more successfully.

Table 3 Composite genotype analysis of two-way SNP–SNP interactions of *HER2* I655V (A > G)/*PHB* 3'UTR C > T polymorphisms in patient and control groups

Genotypes	Patient ($n = 133$)		Control ($n = 100$)		*p*
I655V (A > G)/3'UTR C > T	***n***	**%**	***n***	**%**	
CC/AA	67	50.3	52	52	0.806
CT/AA	30	22.5	21	21	0.776
CC/AG	23	17.2	14	14	0.496
TT/AA	5	3.7	2	2	0.711
CT/AG	6	4.5	9	9	0.167
TT/AG	2	1.5	1	1	0.790

Conclusions

In this preliminary study, no significant correlation was found between *HER2* I655V and *PHB* 3'UTR C > T polymorphisms and azoospermic male infertility. Since this study is the first and only study examining the relationship between azoospermic male infertility and *HER2* and *PHB* gene polymorphisms, and the distribution of polymorphisms differs among populations of different ethnic origins, the study should be expanded by increasing the number of samples and evaluating in different populations.

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Author contributions
The correspondence author carried out the experimental steps involving PCR and RFLP techniques, calculated the statistical analysis data and participated in designing the study and drafting the article. All authors have read and approved the manuscript.

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Availability of data and materials
Data sharing is not appropriate for ethical reasons.

Declarations

Ethics approval and consent to participate
The present study was approved by the ethically applicable at the Tokat Gaziosmanpasa University Faculty of Medicine Clinical Research Ethics Committee (Registration no: 19-KAEK-179). Informed consent was obtained from all participants. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication
We obtained written informed consent with signature from all the included participants or their legal guardians for publication.

Competing interests
The authors report no declarations of interest.

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