Evaluation the Association of Different Genotypes Variant of Transforming Growth Factor Beta 1 (TGIFβ1) and Fork Head Box Protein 3 (FOXP3) with Unexplained Females Infertility

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Abstract
Background: infertility is failure to achieve a pregnancy in a 12-month period for patients under 35 years of age and failure to conceive in a 6-month period for the over 35 years.
Aim: The present study was conducted to investigate the association between multiple polymorphisms in TGF-β1 and FOXP3 by Investigate the role of polymorphisms of rs1800471 (G74C) of TGF-β1 gene, (rs2232368-G/A) of FOXP3 gene polymorphisms with the susceptibility and risk for unexplained female infertility and identify the power of Combined genotypes as a risk factor for infertility. Methods: A total of 50 female patients with unexplained infertility were included in the study who were admitted to hospital from the period November 2021 to the February 2022, and other groups consist of 50 apparently healthy individuals. A five ml of blood samples from each patients and control groups were collected by vein puncture using disposable syringes under aseptic technique, the five ml of each samples were transferred into with EDTA tube and immediately frozen at -20 ◦Cuntil further use to avoid repeated thawing and freezing of sample for detection of TGFB1 and FOXP3 gene polymorphism by(Tetra ARMS-PCR) technique.Results: The results of the association between TGFB1gene polymorphism and risk of infertility revealed that the heterozygous genotype GC was considered as a risk factor for infertility according to odds ratio that equal to 4.329 (95% confidence interval of 0.84 -22.06) and an etiologic fraction of 0.769 . On the other hand, the homozygous genotype CC was a risk factor for infertility with an odds ratio of 6.184 (95% confidence interval of 0.69 – 55.21) and an etiologic fraction of 0. 838.While the association between TGFB1 allele polymorphism and risk of infertility observed there is a significant association between C allele and risk of infertility with p value equal to (P = 0.002). Therefore, C allele was a risk factor for infertility, on the other hand the association between FOXP3 gene polymorphism and risk of infertility is showed the heterozygous genotype GA was a risk factor for infertility according to odd ratio of 2.504 (95% confidence interval of 0.97 -6.46) and an etiologic fraction of 0.607, also the homozygous genotype AA was a risk factor for infertility with significant p value (P = 0.017). While the association between FOXP3allele polymorphism and risk of infertility is showed there is a significant association between the A allele and risk of infertility with p value (P = 0.001). Conclusion: A significant higher risk of developing infertility was observed in subjects with genotypes containing 3 or more risk alleles than those whose genotypes containing 0-1 risk alleles.

Keywords: Idiopathic female infertility, TGFB1, FOXP3

1. Introduction

Infertility will be considered as one of the problems that threaten human societies, and lead to the dissolution of family ties, which prompted many researchers to search for its causes to find an effective treatment for some of its possible cases. Infertility is defined as a fruitless marriage, or the failure of pregnancy after one year of continuous marriage, uninterrupted and without the use of contraceptives, even if both spouses are clinically healthy (1). The WHO ranks infertility in the young population as the fifth highest serious global disability. According to the Maternal Health Task Force 2010, 50 million couples worldwide are infertile (2).

Unexplained infertility accounts for nearly 40% of female infertility and 8% to 28% of infertility in couples The reported incidence of unexplained infertility varies according to the age and selection criteria in the study population (3).Unexplained infertility (UI) is a diagnosis of exclusion made after standard infertility investigations involving tests of ovulation, tubal patency, and standard semen analysis have failed to reveal an underlying absolute cause as a barrier in causing natural conception(4) Unexplained infertility is a source of anxiety for couples desiring pregnancy (5). It does not mean there is no physical explanation for the infertility, but that is just, standard infertility tests have not

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identified any specific problems. A quarter of infertility range (25%) cannot be explained because of current tests are not perfect in finding all problems., the problem preventing pregnancy is not covered by the usual range of tests for assessing infertility or causes which are not yet understood by scientists (6). In some cases, simply getting support and guidance to continue to try to conceive naturally may be sufficient. This can be done while working to improve the health if necessary (7).

It is admitted today that unexplained infertility is often associated to immunologic factors, this condition may be a result of autoimmunity (in man and woman) or of isoimmunity (in woman), an autoimmune disease occurs when the immune system attacks self-molecules as a result of a breakdown of immunologic tolerance to autoreactive immune cells, (8). Autoimmune diseases present with a clear gender bias with a greater prevalence amongst women, occurring at a rate of 2 to 1. Many autoimmune disorders tend to affect women during periods of extensive stress, such as pregnancy, or during a great hormonal change. A far greater number of women are affected every year with autoimmune diseases (9) Studies have demonstrated that prominent changes are made to create a maternal immune tolerance to the fetus during pregnancy, whereas imbalance in their status is involved in the initiation of inflammatory process and pathology of infertility, especially UI (10).

Moreover, some infertile women (around 5%–10%) may have principal genetic anomalies such as chromosome abnormalities, single or multiple gene mutations, and/or polymorphisms (11). However, apart from clearly defined genetic diseases, there is evidence that female infertility, especially unexplained infertility, may also be associated with single nucleotide polymorphisms (SNPs) of specific genes, (12).

Transforming growth factor beta 1 (TGFβ1) is multifunctional cytokine and produced mainly by T regulatory (Treg) lymphocytes. This cytokine plays an important role in physiology of normal pregnancy. because the important role of TGFB1 in physiology of normal pregnancy therefore aberration or change in gene structure could be associated with idiopathic infertility (13). Also, FOXP3, is a member of the fork head transcription factor family cytokines. Unlike other members, it is mainly expressed in a subset of CD4+ T-cells that play a suppressive role in the immune system (14), because suppressive process is important to prevent decidual maternal immune systems fetal allograft therefore polymorphisms of the foxp3 gene may change foxp3 functionally or quantitatively, therefore leading to lack of functional CD4b CD25b Tregs that have important role in suppression process, resulting in autoimmune diseases (15).

2. Materials and Methods

The current study was carried out on (50) females patients suffering from unexplained infertility (either primary or secondary) with age range 18-44 years old from the period from November 2021 to the February 2022. Other groups consist of 50 apparently healthy fertile woman without any history of systemic disease were clinically considered as healthy also included in this study as a control group. Any infertile woman with known etiological factors, either hormonal or structural, Pelvic health problems, primarily with the fallopian tubes or uterus have not been included in this study. A five ml of blood samples from each patient and control groups were collected by vein puncture using disposable syringes under aseptic technique, the five ml of each sample were transferred into with EDTA tube and immediately frozen at -20◦Cuntil further use to avoid repeated thawing and freezing of sample for detection of TGFB1 and FOXP3 gene polymorphism by (ARMS-PCR) technique. This study was in agreement with ethics of infertility Center at Al-Imam Al Sadiq Teaching Hospital, also infertility center at Maternity and children hospital and verbal informed consent was obtained from all participants.

3. Results

The present study enrolled 50 female patients with infertility and 50 apparently healthy subjects. The demographic characteristics of patients and control subjects are shown in table (1). The mean age of patients was 31.52 ± 6.11 and that of control subjects was 29.24 ± 8.16 years and there was no significant difference between patients and control subjects in mean age (P = 0.117). The frequency distribution of patients and control subjects according to age was also shown in table (1). Again, there was no significant difference in the frequency distribution of patients and control subjects according to age (P = 0.241).

The above results have ensured statistical matching between patients group and control group regarding age which is a prerequisite for such case control study.The distribution of TGFB1 Polymorphism was detected by Tetra ARMS-PCR technique. At this locus there are three genotypes: GC, GG and CC. The (GG) wild type homozygote was showed in G allele only, the (CC) mutant type homozygote was showed in C allele only, whereas the (G/C) heterozygote were showed in both G and C allele. The presence of G or C allele were observed at 307 bp product size, as show in figure (2). The genotype distribution had no deviation from Hardy-Weinberg equilibrium. The association between TGFB1 allele polymorphism and risk of infertility is shown in table (2). C allele was more frequent in patients' group in comparison with control group, 17 versus 4, respectively and the difference was significant (P = 0.002). Therefore, C allele was a risk factor for infertility with an odds ratio of 4.96 (95% confidence interval of 1.59 -15.1) and an etiologic fraction of 0.796. The distribution of FOXP3 rs2232368 (G/A) polymorphism was detected by Tetra ARMS–PCR technique. At this locus there are three genotypes: GA, GG and AA. The (GG) wild

type homozygote was showed in G allele only, the (AA) mutant type homozygote was showed in an allele only, whereas the (G/A) heterozygote were showed in both G and A allele. The presence of G or A allele were observed at 149 bp product size, figure (3). The genotype distribution had no deviation from Hardy-Weinberg equilibrium. The association between *FOXP3*allele polymorphism and risk of infertility is shown in table (4). Allele A was more frequent in patients' group in comparison with control group,38 versus 18, respectively and the difference was significant (*P* = 0.001). Therefore, genotype A was a risk factor for infertility with an odds ratio of 2.79 (95% confidence interval of 1.45-5.35) and an etiologic fraction of 0.641.

4. Discussion

The present results show the mean age of patients are 31.52 ± 6.11 and 29.24 ± 8.16 for control group (P= 0.117), these results agree with results of Mouavi et al., (2014) (16), which showed the mean age of the patients at diagnosis time was 29.2 years old (at the range of 14 to 40 years). The present results lower than the results of Piscopo et al., (2020) (17), which showed the mean and standard deviation of age was 36.3±4.6 and their age ranged from 22 to 48 years.

The age range of patients at diagnosis was wide (between 18 years and 44 years). There is a fluctuating tendency of infections among early adulthood and middle-aged women. The present study indicates most patients with infertility were diagnosed at the age between 30-39 years old, 22 (44.0 %), followed by the age group between 20-29 years old, 20 (40.0 %), but these results suggested no significant difference in the frequency distribution of patients and control subjects according to age (P= 0.241), table (1). The present results indicate the prevalence of infertility increased with increasing age before premenopausal age. Age of the female is the main player in the infertility issue especially ovulatory cause and this simply because of decrease of ovarian reserve due to aging process and oxidative stress (Enas,2019) (18). After age of 37 years old the female has lower ovarian reserve and depressed health of their follicles pool. They often face distressing issue of decreasing fertility and need for IVF. Their gonadotropins response, cancellation rate, implantation and miscarriage will deteriorate. Chronological age is the most important factor in ovarian response to stimulation whether superovulation or controlled ovarian hyperstimulation in IVF but the rate of reproductive aging and ovarian sensitivity to gonadotropins defer between women. Biological age and chronological age are not always equivalent. Biological aging causes resistance to ovarian stimulation and poor ovarian response with low volume of developing oocytes (19). The present results inconsistence with Al Lily et al., (2020) (20), which showed factors causing infertility that occur in women include age with an age range of 20-29 years old (64.5%), higher than age of 30- 39 years old (20%) and age of 40-49 years old (11.8%).

Age affects the fertility of a woman where the fertile age range is 15-49 years old, but the peak of fertility is at the age of 20-30 years old. At this age, the woman is in the reproductive period that still experiences regular menstruation where the possibility of getting pregnant is higher than the increased age. Then, the ability of the ovaries to produce ova has decreased. The results of this study indicate that there are women in 31-40 years old experiencing infertility (21). According to Marettih., (2012) (22), an increase in age will decrease the number of ova in the ovary so that the ovary is unable to stimulate the estrogen and progesterone hormones which affect the menstrual cycle.

Age is a major factor that affects vitamin D levels. The elderly is usually more vulnerable to the defect of vitamin D due to the reduction of vitamin synthesis in the skin with age, in addition to a decrease in skin thickness and weakness. Intestinal absorption and lower liver and kidney hydroxylase (Atteritano et al., 2018) (23). Vitamin D concentration is negatively renal connected follicle-stimulating hormone levels in premenopausal women, and this indicates that Vitamin D may influence ovarian reserve, and thus may it have effects on fertility in women with age and has demonstrated that endometriosis is capable of synthesizing vitamin D which is transformed through endometrial receptors and modifying gene expression or regulating response (Lips et al., 2014) (24).

The Frequencies of gene and allele of TGFB1 (rs1800471 Polymorphism) were measured in 50 patients with unexplained female infertility and 50 healthy controls for this study, figure (2), in overall, statistically significant differences can be found in the distribution of the genotype frequencies of TGF-β1 (rs1800471) between the infertility patients and control subjects (P= 0.041). The current results consistence with Marhemati et al., (2020) (25), which indicated significant association of TGF-β1 (rs1800471) polymorphism and infertility patients' disease (p=0.007). The frequencies of the GG, GC, and CC genotypes were 38, 7, 5 and 47, 2, 1, among infertility patients and controls, respectively (P=0.041). The homozygous genotype CC was more frequent in patients' group in comparison with control group, 5 versus 1, respectively, and may be considered as significant risk factor with an OR of 6.184 which means that patients with homozygous CC genotype are approximately six time more liable to develop infertility disease in comparison with patients with other genotypes, but the heterozygous GC genotype was not a significant risk factor (OR= 4.329). The present results similar to the results of Marhemati et al., (2020) (25), which reveals that infertility patients with TGF-β1 polymorphism would have 3 times risk to develop infertility in general than those without polymorphism.

statistically analysis revealed that allele C was more frequent in patients' group than in healthy controls group, 17 versus 4, respectively, and the difference

was significant (p = 0.002). Similar to present finding Marhemati et al., (2020) (25), showed C allele conferred high risk for female infertility disease with OR= 2.44.

The present results confirm the association of TGF-β1 polymorphism and as a novel candidate gene for female infertility in Iraqi population. The current study hypothesize that gene are functionally involved in the cascade of events that triggers infertility formation.

Also, the Frequencies of gene and allele of foxp3 (rs2232368 Polymorphism) were measured in 50 female patients of infertility and 50 healthy controls for this study, figure (3), in overall, significant differences can be found in the distribution of the genotype and allele frequencies of FOXP3 (rs2232368) between the infertility patients and control subjects (P= 0.023). The frequencies of the GG, GA, and AA genotypes were 23, 16, 11 and 36, 10, 4, among infertility patients and controls, respectively (P= 0.023). The heterozygous genotype GA was more frequent in patients' group in comparison with control group, 16 versus 10, respectively and the difference was non-significant (P = 0.054) but may be considered as risk factor for infertility with an odds ratio of 2.504 (95% confidence interval of 0.97 -6.46) and an etiologic fraction of 0.607, as shown in table (4). Also, there was increased homozygous genotype AA of infertility in comparison with control group 11 versus 4, respectively and the difference was significant (P = 0.017). Therefore, genotype AA was a risk factor for infertility with an odds ratio of 4.304 (95% confidence interval of 1.22 -15.15) and an etiologic fraction of 0.767. While the wild GG genotype showed more frequent in control group in comparison with patients' group, 36 versus 23, respectively, and may be considered as protective factor, table (4). Our results consistence with Andre et al., (2011)26, which indicated AA genotype more frequency in patients 16 (22.5%) in compared to healthy controls, 18 (10.5%), but the difference was significant (p=0.017), also demonstrated an association between idiopathic infertility and the rs2232368 polymorphism.

statistically analysis revealed that allele A was more frequent in patients' group than in healthy controls group, 38 versus 18, respectively, and the difference was highly significant (P < 0.001). Therefore, genotype A was a risk factor for infertility with an odds ratio of 2.79 (95% confidence interval of 1.45 - 5.35) and an etiologic fraction of 0.641, as shown in table (5). This results agreement with Andre et al., (2011) (26) who studied 71 cases diagnosed with Idiopathic infertility, which found A allele frequencies higher than in patients than control groups, 54 (38.0) versus 95 (27.8) respectively. Finally, it was found that the FOXP3 (rs2232368) A allele was a hazardous factor in infertility.

5. Conclusions

In conclusion the present results confirm the *TGF-β1* as a novel candidate gene for female infertility in Iraqi population. The current study hypothesize that gene are functionally involved in the cascade of events that triggers infertility formation. Finally, it was found that the *FOXP3 (rs2232368)* A allele was a hazardous factor in infertility.

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subjects

Table (1): Demographic characteristics of female patients with infertility and control			
Characteristic	Patients n =50	Control n = 50	P
Age (years)			
Mean ±SD	31.52 ± 6.11	29.24 ± 8.16	0.117 † NS
Range	18.00 – 44.00 years	18.00– 43.00 years	
< 20, n (%)	2 (4.0 %)	7 (14.0 %)	0.241¥ NS
20-29, n (%)	20 (40.0 %)	22 (44.0 %)	
30-39, n (%)	22 (44.0 %)	15 (30.0 %)	
≥ 40, n (%)	6 (12.0 %)	6 (12.0%)	

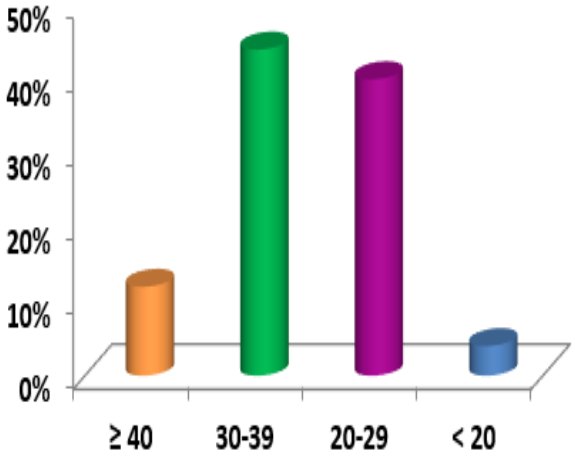

Figure (1): Distribution of female patients with infertility according to Age groups.

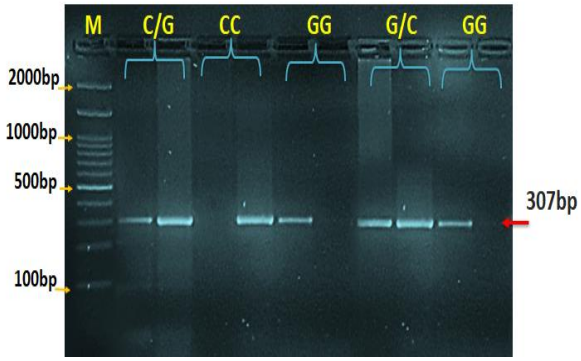

Figure (2): Agarose gel electrophoresis image that showed the ARMS-PCR product analysis of TGFβ1 rs1800471 (G/C) gene polymorphism in patients' samples. Where M: marker (2000-100bp). The (GG) wild type homozygote was showed in G allele only, the (CC) mutant type homozygote was showed in C allele only, whereas the (G/C) heterozygote were showed in both G and C allele. The presence of G or C allele were observed at 307bp product size.

Table (2): TGFB1 rs1800471 G/C allele frequency in female patients with infertility and control group.							
TGFB1 rs1800471	Patients n = 100	Control n = 100	P	OR	95%CI	EF	PF
G	83	96	0.002 ¥ S	0.203	0.06 -0.62	-	0.796
C	17	4		4.96	1.59-15.1	0.796	-

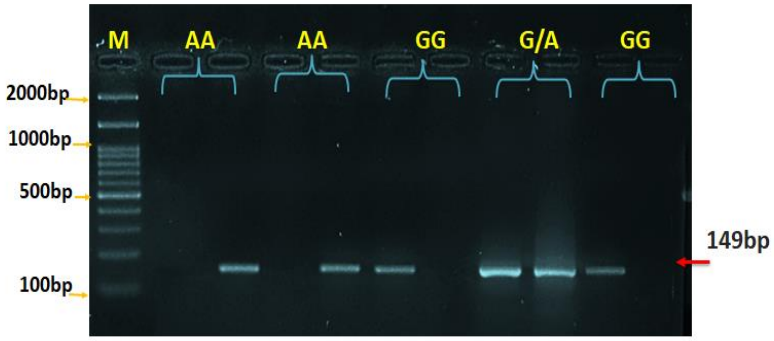

Figure (3): Agarose gel electrophoresis image that showed the ARMS-PCR product analysis of FOXP3 rs2232368 (G/A) gene polymorphism in patients' samples. Where M: marker (2000-100bp). The (GG) wild type homozygote was showed in G allele only, the (AA) mutant type homozygote was showed in an allele only, whereas the (G/A) heterozygote were showed in both G and A allele. The presence of G or A allele were observed at 149bp product size.

Table (3): FOXP3 rs2232368 (G/A) POLY allele frequency in female patients with infertility and control group.

FOXP3rs2232368 (G/A)	Patients n = 100	Control n = 100	P	OR	95%CI	EF	PF
G	62	82	0.001 ¥ S	0.358	0.18 -0.68	-	0.641
A	38	18		2.79	1.45-5.35	0.641	-