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Gene expression and single nucleotide polymorphism (rs 1042838) of progesterone receptor gene in Iraqi females with recurrent spontaneous abortion(RSA).

[1]Shireen Hamid Farhan, [2]Ismail A. Abdul-Hassan, [3]Ruoida Abdul-Rahman

ABSTRACT--*The proposal target was determining the gene expression and single nucleotide polymorphism (**rs1042838** G>T) of progesterone receptor gene in blood sample of recurrent spontaneous abortion RSA Iraqi females. Fifty females (n=50) with RSA consulted Al-Elwiya teaching hospital from February to June 2019 and apparently healthy fertile control (n=50) were enrolled. Following the serum hormonal assay, DNA and RNA were extracted from leucocytes to detriment the SNP and gene expression were carried out by (RT-q PCR). The results showed significant disruption of hormones between the two groups and non -significant difference (p>0.05) in the mean of threshold cycle(ΔCt) of progesterone receptor gene between female with RSA and control. Assessing the $2^{-\Delta\Delta Ct}$ in female with RSA showed non -significant increased expression of folding in progesterone receptor m RNA compared with control. The genotypes and allele frequencies in the two groups showed significant decrease in the mutant (TT) genotype frequency in female with RSA. In conclusion, up-regulation of progesterone receptor gene expression was observed in female with RSA, the mutant genotype may play as protective SNP against RSA.*

Keywords-- *gene expression, polymorphism, progesterone receptor, recurrent spontaneous abortion*.

I. INTRODUCTION

Losing more than three or more repeated pregnancies before 20 weeks referred to as Recurrent spontaneous abortion, its multifactorial disorder including uterine anomaly, chromosomal abnormalities, endocrine and immune dysfunction, life style and maternal infections [1]. Progesterone hormone secreted from the granulosa-lutein cells that found in the corpus luteum and syncytiotrophoblasts of placenta during gestation also it secreted from the adrenal cortex in few amount less than 1 mg/day [2]. It influences the functions of that done by female reproductive systems include initiation of ovulation, assistance of implantation and preservation of gestation [3]. In pregnancy it essential for endometrial proliferation and maintain feto-placental functions and maturation as well as uterus - placental circulation and prevents myometrium contractility by decrease the uptake of extracellular calcium that is needed for contraction also progesterone inhibits lymphocyte cytotoxicity, natural killer cell degranulation and release of pro inflammatory cytokines [4].

The disorder in progesterone function increase the risk of recurrent spontaneous abortion, the action of progesterone is done by progesterone receptor (PGR) or/and by mechanisms suggest that modification of the

[1] *Department of Pathological Analysis, Applied Science, University of Fallujah.*

[2] *Institute of Genetic engineering and Biotechnology for postgraduate studies, University of Baghdad.*

[3] *Department of Pathological Analysis, Applied Science, University of Fallujah.*

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isoforms ratio change the expression level of PGR [5]. Progesterone receptor is a member of steroid nuclear receptor family of ligand dependent transcriptional factors [6]. The action of progesterone mediated by the PGR is genomic action, when progesterone hormone binds to its receptor a complex activation cascade occurs, this activation leads to conformational changes began with protein phosphorylation, dimer formation, detachment from special proteins called heat shock protein then nuclear transport complex binds to particular DNA sequence and act as transcriptional elements for target genes [7]. PROGINS, referred to as polymorphic variant in the progesterone receptor, they represent as a risk factor in some gynaecological diseases, [8] First description to PROGINS was as an Alu element in intron 7, recently added a silence SNP in exon 5 and a missense SNP in exon 4 (V660L, rs1042838) [9]. These variants were factors participate in several progesterone-dependent neoplasms, for example endometrial cancer, ovarian cancer and breast cancer [10]. The outcome of PROGINS variants were established by increasing transcription activity for mutated copies and decreasing the response to the hormone by influencing gene expression and mRNA constancy [11].

The goal of this proposal is to investigate the relation of RSA with the SNP in exon 4 and the expression of progesterone receptor.

II. SUBJECTS, MATERIAL AND METHOD

2.1 subjects

This study included fifty females (n=50) who had idiopathic RSA (mean age 35.06 ±0.9) and consulted Al Elwiya teaching hospital, Baghdad, Iraq between January 2019 and April 2019 and fifty normal fertile females(n=50) with at least 2 live labours and without history of abortion. Patients with anatomical, infection, endocrine. metabolic disorder and autoimmune diseases were excluded. For the study ethical approval was obtained from Al Elwiya teaching hospital.

1.2 Hormonal assay

Hormonal analysis for progesterone, testosterone and estradiol was performed by using Automated Immune Assay (AIA) by the VIDAS auto analyzer, (BioMérieux Company, France) in Hormonal Laboratory at El-Elwiya and Al-Yarmouk teaching hospitals.

2.3 DNA extraction and genotypes analysis

The DNA was extracted from blood by using kit (Quick-gDNA ™ Blood MiniPrep, Zymo research /USA), Nanodrop was used to estimate the purity and the concentration for DNA samples. Genotyping analysis was performed using Real Time PCR by predesigned TaqMan fluorescent oligonucleotide probes and primers ordered from integrated DNA technologies /USA for examined SNP rs1042838 in exon 4 of progesterone gene were stored lyophilized at -20°C. Taq man SNP genotyping assay using real time thermocycler according to the protocol recommended by the manufacturer as showed in table (1) and (2).

Table (1) the component reaction.

Component	20 µl (final volume)
2X TaqMan ®Master	10 µl

20X Assay Working	1µl
Nuclease –free	-
DNA sample volume	9 µl

Table (2) the thermocycler program.

Steps	Predesigned SNP		
	Temperature	**Duration**	**Cycles**
Enzyme activation step	95 ℃	10 minutes	hold
Denaturation step	95℃	15 seconds	40
Annealing extension step	60℃	1 minute	

2.4 RNA extraction and gene expression

The gene expression of progesterone receptor was set by the reverse transcription quantitative polymerase chain reaction (RTqPCR) method. Isolation of total RNA done by special reagent (TRIzolTM LS Reagent; Thermo Fisher Scientific; USA). The isolated RNA was reversely transcribed to cDNA using prime Script™ RT reagent Kit. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was carried out using the KAPA SYBER FAST qPCR Master Mix Kit (Universal, Germany) and cDNA as a template, forward and reverse oligonucleotide primers of progesterone receptor gene were designed and showed in table (3). The forward and reverse primers of the housekeeping gene GAPDH (reference gene: glyceraldehyde-3-phosphate dehydrogenase) were also given.

Table (3) the primers of gene expression.

Primer	Sequence	Tm (°C)	GC (%)
Forward of progesterone receptor gene	**TCACATCTGGTTCAATGCTCA**	**54.3**	**42.9**
Reverse of progesterone receptor gene	**TGAGAGCACTGGATGCTGTT**	**56.5**	**50**
Forward of GAPDH (reference gene	**AGGTCATCCCTGAGCTGAA**	**52.1**	**45**
Reverse of GAPDH (reference gene	**CTGCTTCACCACCTTCTTGAT**	**55.6**	**47.6**

The reaction mix was set to a final volume of 20 µl as recommended by the manufacturer: 10µl KAPA SYBR FASR_ qPCR Master Mix (2X), for 2-Step RT-qPCR, 0.4µl of each primer (0.2 µM), 3 µl cDNA, and nuclease-free water. The mix put in a real time thermocycler (Sacace Real-time PCR System, Italy) which was programmed for the optimized cycles. These include first initial denaturation step for 5 min at 95C°(one cycle), the second step was 40 cycles of denaturation each cycle 20 sec. at 95C°, annealing step take place in 20 sec. at 56 C°and extension

step for 20 sec. at 72 C°, and finally one cycle of melt curve for15 sec. at 90 C°. The expression was approaching as $2^{-\Delta\Delta Ct}$, which represents the relative fold change. The findings were expressed as a fold change in the expression grade of an objective gene that was normalized to endogenous control (reference gene) and relative to a calibrator.

2.5 Statistics:

The Statistical Analysis System- SAS (2012) program was used to determine the effect of difference factors in parameters of the study. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability). Estimate of Odd ratio and CI IN this study [12].

III. RESULT

3.1 The hormonal results

In the table (4) showed different distribution in the levels of hormones, progesterone and estradiol levels decrease significantly (P>0.001) in female with RSA than the control while testosterone level was same in two groups.

Table (4) hormonal results

Groups / Hormone s	Control (Means ±SD)	Female with RSA (Means ±SD)	P-value	LDS
Progesterone	20.75±0.69	10.58±0.36	0.0001	1.453**
Testosterone	2.438±0.22	2.445±0.16	NS	0.979
Estradiol	32.39±1.31	19.37±1.11	0.0001	3.442**

** means significant difference (p>0.001), NS= non-significant

3.2 The genotype results

The SNP of progesterone receptor gene a missense SNP in exon 4 (V660L, rs1042838) was present in three genotypes GG, GT, TT and two alleles (G and T) as showed in table (5)

As related with GG and GT genotype no significant difference in frequency percentage were noted between the control and female with RSA, whereas the frequency of AA genotype was significant lower in female with RSA than control (20% versus 28% respectively, X^2=4.39, OR=0.672, P<0.05).

The frequency of G allele was 0.74 in female with RSA and 0.68 in control, while the frequency of T allele was 0.26 in female RSA and 0.32 in control.

Table (5) genotype and allele frequencies of rs1042838 SNP in exon 4 of progesterone receptor gene

Group	Control		Female with RSA		χ^2	OR	CI
	No.	Percentage	No.	Percentage			
GG	32	64%	34	68%	1.04 NS	0.085	0.78-1.57
GT	4	8%	6	12%	1.04 NS	0.085	0.78-1.57

TT	14	28%	10	20%	4.39 *	0.672	0.86-1.63
Allele frequency							
G	0.68		0.74		--	--	--
T	0.32		0.26		--	--	--

***significant difference (p>0.05), x^2=chi squared, OR =odd ratio, CI=confidence interval.**

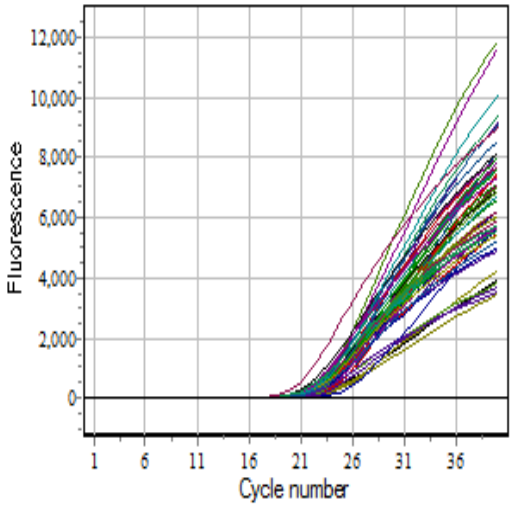

Figure (1) FAM (curves of G allele)

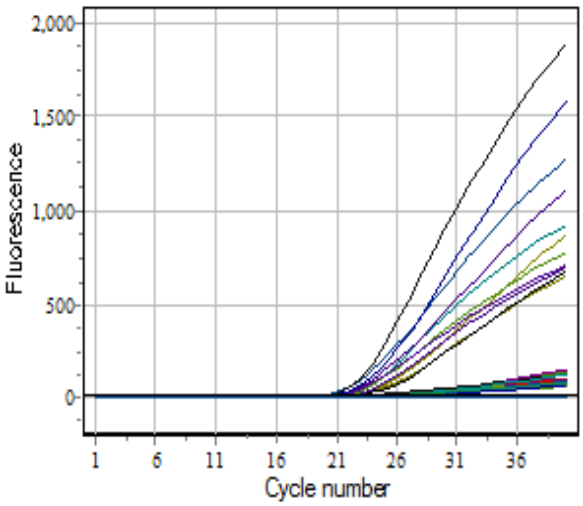

Figure (2) HEX (curves of B allele)

3.3 Gene expression result

The expression of gene was detected successfully by using new molecular technique which is Real time PCR (qRT-PCR) with used specific primer. Table (5) present the Ct and ΔCt of the reference gene. In table (6) the Ct and ΔCt of progesterone receptor showed a non- significant differences between control and female with RSA.

Table (5) the Ct and ΔCt values of reference gene.

Group	Ct means of GAPDH reference gene	2^{Δ}-ct	Fold of gene
Control	20.39	7.27	1
Female with RSA	20.53	6.6	0.9
LSD	0.530 **NS**	0.06 NS	0.01 NS
P-value	**0.119**	**0.1**	**0.9**

Table (6) the Ct and ΔCt values of progesterone receptor gene.

Group	Ct Means of progesterone receptor gene	Δ Ct	$2^{-\Delta ct}$

control	34.97± 0.15	14.58	4.08
Aborted female	34.78±0.21	14.25	5.13
LSD	0.511 NS	0.698 NS	1.061 NS
P -value	0.463	0.086	0.463

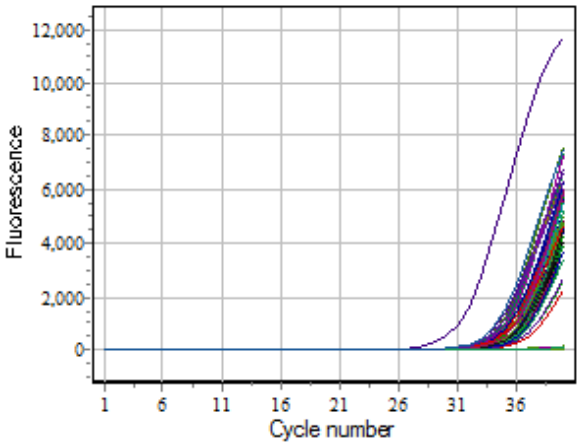

Figure (3) the Ct value of progesterone receptor gene

The table (7) show the fold of progesterone receptor gene expression depending on $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods, the folding of the gene is non-significantly up-regulated once and a quarter times.

Table (7) the fold of progesterone receptor gene expression depending on $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods

Parameters	**$2^{-\Delta Ct}$**		
	groups		
	Control	**Patients**	**P –value**
$2^{-\Delta Ct}$ Target	4.08	5.13	0.463NS
Experiment /control	4.08/4.08	5.13/4.08	
Fold of gene expression	1	1.25	0.52 NS
Parameters	**$2^{-\Delta\Delta Ct}$**		
	Groups		
	Control	**Patients**	
ΔCt Calibrator	12.9	12.9	1.00 NS
ΔΔCt	1.68	1.35	0.88 NS
$2^{-\Delta\Delta Ct}$	0.312	0.392	0.88 NS
Experiment/control	0.312/0.312	0.392/0.312	----------
Fold of gene expression	1	1.25	0.9 NS

IV. DISCUSSION

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Some researchers have established that progesterone supplement for RSA does not progress the implantation in some females, as mentioned before the problem may not just the availability of hormone but the abnormalities of PR gene [13]

Progesterone and estradiol hormones produce by ovaries in the earlier of pregnancy the placenta plays this role, these hormones are essential for endometrial proliferation, implantation and prevent myometrium contractility, the decrease in their levels in this study may be caused by the failure of ovaries and placenta to produce enough concentration of them [3].

The up-regulation of PR gene expression may occur to make up for the shortfall of the hormone in patients. This explanation depends on the studies that show that the expression of PR gene modifies during pregnancy in human endometrium [14]. This result disagrees with a study on Iranian female with RSA which find that the expression of PR gene was not affected [15].

The rs1042838 that examined in this study is one of three polymorphism form a PROGINS the SNP change G to T in exon 4 of PR gene this leads to the amino acid change Leu660Val in the hinge region of the receptor, this area binds the DNA-binding domain to ligand binding domain and it is highly variable [16]. This region is thought to be participate in dimerization of receptor and holds a nuclear localization signal [17] Also this region enhances the receptor –ligand interaction of the ligand binding domain [18].

The increase of mutant allele in control than female with RSA may play as protective genetic variation. There are four published articles investigated the relationship of PROGINS and RSA two of these articles linked the PROGINS with increased risk of RSA one of them in German population [19] and the other in Taiwanese Han women [20]. The two other studies in Australian and Indians populations assumed that there is on association between the RSA and polymorphism of the progesterone receptor gene [21][22].

This construct because the great difference in the frequencies of the allele and genotypes among ethnical population, the mutated T allele is rare in Asians and Africans, 4.1% in Taiwanese, 2% in Chinese Hans, 0% in Japanese but much more in Europeans 21%.

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