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CTLA-4 Gene Polymorphism in Women with Idiopathic Recurrent Pregnancy Loss

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Abstract: Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is considered as a negative regulator of T cell activation and its role in maintaining immune tolerance is well established. The present case-control study aimed to investigate the CTLA-4 +49 A/G, -1661 A/G, -318 C/T and -1722 T/C single nucleotide polymorphisms (SNPs) and predisposition to recurrent pregnancy loss (RPL) in Gaza Strip - Palestine. The study was performed on 200 women with a history of two or more pregnancy losses (case group) and 200 control women with at least two live births and without any previous history of abortion. PCR-based restriction fragment length polymorphism (RFLP-PCR) method was used for genotyping *CTLA-4* polymorphisms. Study results revealed that there is no significant association between the allele/genotype frequencies of the four investigated *CTLA-4* SNPs and RPL. This trend remained true under dominant, co-dominant and recessive models. The A\G genotype of -1661 A\G polymorphism was higher in patients (45%) as compared to controls (39.5%) but without statistical significance. The minor allele frequencies (MAFs) of the *CTLA-4* gene polymorphisms in the patient/control group were as follows: +49A>G: 0.22/0.22, -318 C>T: 0.15/0.11, -1661 A>G: 0.30/0.26 and -1722 T>C: 0.08/0.08. The four investigated *CTLA-4* polymorphisms do not contribute to the risk of RPL in the study population. Testing other *CTLA-4* gene polymorphisms and the level of *CTLA-4* expression in RPL patients is recommended.

Keywords: Recurrent Pregnancy Loss, *CTLA-4*, Gene Polymorphism, PCR-RFLP

1. Introduction

Recurrent pregnancy loss, the occurrence of 2 or more consecutive miscarriages, is one of the most common pregnancy complications with a prevalence of 1-2% among pregnant women in reproductive life [1].

Despite years of effort to determine the factors involved in miscarriage, the cause remains unclear in around 50% of the cases hence, this reflects the heterogeneous nature of this malady [2].

Several mechanisms have been proposed to function actively in the protection of the semi-allogeneic fetus from maternal immune system. The presence of regulatory T cells (Tregs) and the expression of immunomodulatory molecules at the fetal maternal interface have been identified as crucial factors for fetomaternal tolerance [3].

Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) is constitutively expressed in Foxp3+ Tregs and induced in conventional T cells following activation [4]. CTLA-4 has a suppressive effect on T cell activation and might be involved in establishing immune tolerance by blocking CD28-dependent T cell activation through interactions with its ligand CD80/86 on antigen presenting cells in the decidua. The CTLA-4/B7 complex can compete with the CD28/B7 complex and convey an inhibitory influence to the T cell affecting T cell development and functions [5, 6]. CTLA-4 dysregulation, therefore, has the potential to affect fetal tolerance through altered activation of T cells to fetal antigens.

CTLA-4 gene is located on the chromosomal region 2q33 and contains more than 100 polymorphic sites. Distinct single nucleotide polymorphisms (SNPs) such as +49A/G (rs231775), -318C/T (rs5742909), -1661A/G (rs4553808) and -1722T/C (rs733618) have been associated with autoimmune diseases and preeclampsia [7].

CTLA-4 expression in placental fibroblasts and deciduas supports its role in the maintenance of pregnancy and fetomaternal tolerance [1]. Therefore, it is suggested that abnormal expression of *CTLA-4*, effected by genetic polymorphism(s), may be associated with RPL. Indeed, some *CTLA-4* single nucleotide polymorphisms (SNPs) have been implicated as potential risk factors for RPL in certain populations [8, 9, 10].

The present study was designed in order to investigate the association between four *CTLA-4* gene polymorphisms namely, +49A/G, -318C/T, -1661A/G and -1722T/C and RPL in a group of Palestinian women.

2. Materials and Methods

2.1. Study Subjects

The study group (n=200) included women aged 20-35 years who had experienced at least two unexplained spontaneous abortions before 20th week of gestation. The control group (n=200) consisted of women who had delivered at least one healthy child and had no previous history of pregnancy loss. Controls were matched with study subjects for all other possible characteristics. None of the individuals included in the study population used oral contraceptives, hormonal, or any serious medication affecting body vital functions. Individuals with known causes of RPL were excluded from the study group.

2.2. Genotyping

DNA extraction and polymorphism determination

About 2.0 ml of venous blood were drawn into sterile EDTA tubes under quality control and safety procedures. Genomic DNA was isolated from blood using Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer instructions.

The four SNPs were genotyped using PCR-RFLP technique. The specific PCR primers were designed using online Primer3 software (http://primer3.ut.ee/) based on the genomic sequence deposited in gene bank and the sequence of each SNP was retrieved from NCBI-SNP database (http://www.ncbi.nlm.nih.gov/snp/). Then restriction enzymes required for the PCR-RFLP identification of each SNP were selected from new England Biolabs database by using NEB cutter software (http://nc2.neb.com/NEB Cutter2/) (Table 1).

PCR primers and conditions, restriction enzyme digestion and results interpretation were done as indicated in (Tables 1 & 2).

Table 1. *Primers and restriction enzymes used for PCR-RFLP genotyping of the polymorphism*

SNP	Enzyme	Primers5'-3'	PCR product size
+49A/G rs231775	APeK1	F: TCCTGAAGACCTGAACACCG R: TGCCTTTGACTGCTGAAACA	222 bp A-allele: uncut G-allele : 79 bp + 143 bp
-318C/T rs5742909	Mn1I	F: GGCTCAGAAAGTTAGCAGCC R: ACAACCTCAAGCACTCAACTG	247 bp C-allele: 96+70+67+14 bp T-allele: 137 +96 + 14 bp
-1661A/G rs4553808	DRA1	F: CTAAGAGCATCCGCTTGCACCT R: TTGGTGTGATGCACAGAAGCCTTT	486 bp A-allele : 334 + 152 bp G-allele : uncut
-1722 T/C rs733618	APeK1	F: CTAAGAGCATCCGCTTGCACCT R: TTGGTGTGATGCACAGAAGCCTTT	486 bp T-allele:uncut C allele : 270 + 216 bp

Table 2. *The PCR reaction mix and conditions used for genotyping of CTLA-4 gene SNPs.*

SNP	PCR program	PCR mix and conditions
+49A/G rs231775	94°C, 5 min, 94°C, 30 sec; 56°C, 45 sec; 72°C, 45 sec; 35 cycle 72°C, 5min	Total volume of 20 µL, with 10 µL Taq PCR Master mix (Promega, USA), 2µL (10pmol) of primers, 4µL Nuclease-free water, 2µL (50ng) of genomic DNA
-318C/T rs5742909	94°C, 5 min 94°C, 30 sec; 57°C, 45 sec; 72°C, 45 sec; 35 cycle 72°C, 5min	
-1661A/G rs4553808	94°C, 5 min 94°C, 30 sec; 60°C, 45 sec; 72°C, 45 sec; 35 cycle 72°C, 5min	Total volume of 30 µL, with 15 µL Taq PCR Master mix (Promega, USA), 2µL(10pmol) of primers, 9µL Nuclease-free water, 2µL (50ng) of genomic DNA.
-1722 T/C rs733618		

2.3. Ethical Considerations

Informed consent was taken from all the subjects who participated in the study. The objective of the study was fully explained to all participants and their consent was taken.

2.4. Statistical Analyses

The genotype, allele frequency in RPL patients and the controls were analyzed by standard Chi-square test and odds ratio (OR) for risk of RPL at 95% confidence intervals (CI). Hardy-Weinberg equilibrium (HWE) was tested in the control group using a freely available software (http://www. oege. org/software/hwe-mr-calc.shtml). P-values of 0.05 or less were regarded as statistically significant.

3. Results

Table 3 illustrates genotypes and alleles frequencies, odds ratio, 95% confidence intervals and *P* values for the four tested *CTLA-4* polymorphisms among RPL patients and controls.

Statistical analyses of genotypic and allelic frequencies for the tested SNPs revealed no significant (all P values are > 0.05) difference between RPL patients and controls.

Moreover, statistical analyses of the four SNPs genotypes under recessive, dominant, and co-dominant models indicated no significant difference between the two study groups.

Hardy-Weinberg equilibrium for investigated SNPs

The distribution of the genotypes of -318C\T, -1661 A\G and -1722 T\C SNPs conformed with Hardy–Weinberg equilibrium as there was no significant difference between the expected and the observed genotypes. *CTLA-4* +49A\G genotypes, however, showed a significant deviation from Hardy-Weinberg equilibrium with a *P*-value =0.0001.

Table 3. *Genotype and allele frequencies of CTLA-4 gene polymorphisms in RPL patients and controls.*

SNP	Allele/ genotype	Patients n=200	Controls n=200	OR (95%CI)	P-Value
CTLA-4+49A/G	A\A	111 (55.5%)	112 (56%)	0.98 (0.66 to 1.45)	0.92
	A\G	89 (44.5%)	88 (44%)	1.02 (0.68 to 1.51)	0.92
	G\G	0	0	-	-
	A- allele	311 (77.75%)	312 (78%)	0.98 (0.70 to 1.37)	0.93
	G- allele	89 (22.25%)	88 (22%)		
CTLA-4-318C/T	C\C	145 (72.5%)	159 (79.5%)	0.68 (0.43 -1.08)	0.1
	C\T	51 (25.5%)	37 (18.5%)	1.04 (0.64 -1.7)	0.86
	T\T	4 (2%)	4 (2%)	1 (0.24 - 4.05)	1
	C- allele	341(85.25%)	355 (88.75%)	0.73 (0.48 to 1.10)	0.14
	T- allele	59 (14.75%)	45 (11.25%)		
CTLA-4-1661A/G	A\A	95 (47.5%)	108 (54%)	0.77 (0.52 to 1.14)	0.19
	A\G	90 (45%)	79 (39.5%)	1.25 (0.84 to 1.86)	0.26
	G\G	15 (7.5%)	13 (6.5%)	1.16 (0.54 to 2.52)	0.7
	A-allele	280 (70%)	295 (73.75%)	0.83 (0.61 to 1.13)	0.23
	G-allele	120 (30%)	105 (26.25%)		
CTLA-4-1722 T/C	T\T	170 (85%)	167 (83.5%)	1.11 (0.65 to 1.92)	0.68
	T\C	30 (15%)	33 (16.5%)	0.9 (0.52 to 1.53)	0.68
	C\C	0	0	-	-
	T-allele	370 (92.5%)	367 (91.75%)	1.10 (0.66 to 1.85)	0.73
	C-allele	30 (7.5%)	33 (8.25%)		

4. Discussion

Idiopathic RPL is a heterogeneous condition and its complexity might be due to the additive effect of several genes, and their interactions with each other and with environmental factors. From the immunology of pregnancy standpoint, major efforts are concerned with the role of sequence variants (e.g., SNPs) of immune tolerance related genes (e.g., *CTLA-4*) in the etiology of RPL.

There have been several studies that couple CTLA-4 deficiency to adverse pregnancy outcomes such as recurrent pregnancy loss (RPL), placental abrubtion, and pre-eclampsia (PE). Moreover, polymorphisms in the *CTLA-4* gene have been associated with dysregulated CTLA-4 production and function. For instance, heterozygosity of the *CTLA-4* A49G allele, has been reported as a predisposing factor to severe PE and placental abruption in some populations [8, 11]. Moreover, stretches of AT repeats in the 3'-untranslated region of the *CTLA-4* gene has been suggested to affect mRNA stability and fetal survival in humans [12].

The four polymorphisms targeted in this study affect CTLA-4 expression. The exon 1 +49A/G leads to less efficient glycosylation and reduced expression of membrane CTLA4 [13, 14, 16]. The -318 C/T, -1661 A/G and -1722 T/C polymorphisms lie in the promoter region and affect the level of transcription of *CTLA-4* [15, 16, 7]. Reduced *CTLA-4* expression and/or change in CTLA-4 activity result in uncontrolled T-cell regulation.

Results of the present work showed that no statistically

significant differences were evident between RPL cases and controls in terms of the allelic and genotypic distributions of *CTLA-4* "-318 C/T, -1661 A\G, -1722 T\C and +49A\G" polymorphisms.

Regarding lack of association of *CTLA*-4 "+49 A/G" with RPL, similar findings were reported by (Chaili, 2010; Pendeloski et al., 2011; Bonyadi et al., 2014; Naderi, 2014 and Hayashi et al., 2015) [17, 18, 6, 10, 19]. Whereas (Wang et al., 2005; Gupta et al., 2012; Messaudi et al., 2014 and Rasiti and Nasiri, 2016) [9, 5, 20, 1] showed that there is a significant association between *CTLA-4* +49 A\G polymorphism and RPL.

Interestingly, the homozygous "GG" genotype was not observed in the present study. The absence of this genotype in our population may be a consequence of selection against this genotype. This also explains the observed deviation of this SNP genotypes from Hardy-Weinberg equilibrium.

Likewise, the *CTLA-4* "-318 C/T" allele/genotype distribution did not show significant difference between the two study groups. Studies from different populations showed divergent results. (Chaili, 2010 and Naderi, 2014) have reported that there is a significant association between *CTLA-4* "-318 C/T" polymorphism and RPL [17, 10]. (Messaoudi et al., 2014) however, did not replicate such an association [20].

As for *CTLA-4* "-1661 A/G" SNP, although the difference was not significant between the control women and the RPL patients, the A/G genotype of this SNP was higher in the RPL patients relative to the control group (45%, 39.5%, respectively). In harmony with our results, (Chaili, 2010) showed that *CTLA-4* "-1661 A/G" polymorphism is not associated with RPL [17].

For, *CTLA-4* "-1722 T/C" SNP, the comparison of genotype and allele frequencies in the case and control groups did not show significant differences. Similar results were reported by (Chaili, 2010) who also showed a lack of association between *CTLA-4* "-1722 T\C " polymorphism and RPL [17]. Moreover, the CC homozygote genotype of this SNP was not encountered in any of the study subjects. The explanation for the absence of the CC genotype is mainly due to the uncommon occurrence of the C-allele (MAF ~ 0.08) in our population.

Contradictory results are a common place in genetic association studies performed on different populations. Possible explanations for discrepant results include one or more of the following: differences in the ethnicity (genetic background), the sample size, the inclusion and exclusion criteria of the study subjects, presence of nucleotide polymorphism(s) somewhere else in the examined gene, epigenetic alterations, linkage disequilibrium to other sequence variants in the vicinity of the studied locus, and prevailing environmental conditions.

Lack of association of the investigated SNPs does not exclude the importance of *CTLA-4* in RPL. Research is ongoing to examine other *CTLA-4* polymorphisms and correlate those polymorphisms with the level of CTLA-4 in RPL women.

References

[1] Rasti, Z., & Nasiri, M. (2016). Association of the +49 A/G Polymorphism of *CTLA-4* Gene with Idiopathic Recurrent Spontaneous Abortion in Women in Southwest of Iran. *J Reprod Infertil.* 17(3): 151–156.

[2] Li, T. C., Makris, M., Tomsu, M., Tuckerman, E., & Laird, S. (2002). Recurrent miscarriage: aetiology, management and prognosis. *Human reproduction update*, *8*(5), 463-481.

[3] Riella, L. V., Dada, S., Chabtini, L., Smith, B., Huang, L., Dakle, P., Mfarrej, B., D'Addio, F., Adams, L. T., Kochupurakkal, N., Vergani, A., Fiorina, P., Mellor, A. L., Sharpe, A. H., Yagita, H.,& Guleria, A. (2013). B7h (ICOS-L) maintains tolerance at the fetomaternal interface. *The American journal of pathology*, *182*(6), 2204-2213.

[4] Jain, N., Nguyen, H., Chambers, C., & Kang, J. (2010). Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. Proceedings of the National Academy of Sciences, 107(4), 1524-1528.

[5] Gupta, R., Prakash, S., Parveen, F., & Agrawal, S. (2012). Association of CTLA-4 and TNF-α polymorphism with recurrent miscarriage among North Indian women. *Cytokine*, *60*(2), 456-462.

[6] Bonyadi, M., Parsa, S., Taghavi, S., & Zeinalzadeh, N. (2014). Studies on the relationship of CTLA-4+ 49A/G gene with Recurrent Miscarriage in Northwest of Iran. *Molecular and Biochemical Diagnosis (Journal), 1*(3), 171-176.

[7] Dias, F. C., Medina, T. D. S., Mendes-Junior, C. T., Dantas, R. O., Pissetti, C. W., Junior, V. R., Joviliano, R. D., Marin-Neto, J. A., Gutierrez, R. S., Moreau, P., Donadi, E. A., & Silva, J. S. (2013). Polymorphic sites at the immunoregulatory *CTLA-4* gene are associated with chronic chagas disease and its clinical manifestations. *PloS one*, *8*(10), e78367.

[8] Dehaghani, A. S., Doroudchi, M., Kalantari, T., Pezeshki, A. M., & Ghaderi, A. (2005). Heterozygosity in *CTLA-4* gene and severe preeclampsia. *International Journal of Gynecology and Obstetrics, 88*(1), 19-24.

[9] Wang, X., Lin, Q., Ma, Z., Hong, Y., Zhao, A., Di, W., & Lu, P. (2005). Association of the A/G Polymorphism at Position 49 in Exon 1 of CTLA-4 with the Susceptibility to Unexplained Recurrent Spontaneous Abortion in the Chinese Population. *American Journal of Reproductive Immunology*, *53*(2), 100-105.

[10] Naderi, F., Kazemi, T., Fatemi, R., Zarei, S., & Idali, F. (2014). P-212: Association between Polymorphisms of *CTLA-4* Gene and Unexplained Recurrent Spontaneous Abortion in An Iranian Population. *Cell j (Yakhteh)*, *7*(3), 223-223.

[11] Jääskeläinen, E., Toivonen, S., Keski-Nisula, L., Paattiniemi, E. L., Helisalmi, S., Punnonen, K., & Heinonen, S. (2008). *CTLA-4* polymorphism 49A–G is associated with placental abruption and preeclampsia in Finnish women. *Clinical Chemical Laboratory Medicine, 46*(2), 169-173.

[12] Tsai, A. F., Kaufman, K. A., Walker, M. A., Karrison, T. G., Odem, R. R., Barnes, R. B., Scott, J. R., Schreiber, J. R., Stephenson, M. D., & Ober, C. (1998). Transmission disequilibrium of maternally-inherited CTLA-4 microsatellite alleles in idiopathic recurrent miscarriage. *Journal of reproductive immunology, 40*(2), 147-157.

[13] Ghaderi, A. (2011). CTLA4 gene variants in autoimmunity and cancer: a comparative review. *Iranian Journal of Immunology*, *8*(3), 127-127.

[14] Sun, T., Hu, Z., Shen, H., & Lin, D. (2009). Genetic polymorphisms in cytotoxic T-lymphocyte antigen 4 and cancer: the dialectical nature of subtle human immune dysregulation. *Cancer research, 69*(15), 6011-6014.

[15] Wang, X. B., Pirskanen, R., Giscombe, R., & Lefvert, A.K. (2008). Two SNPs in the promoter region of the *CTLA-4* gene affect binding of transcription factors and are associated with human myasthenia gravis. *Journal of internal medicine*, *263*(1), 61-69.

[16] Pastuszak-Lewandoska, D., Domanska, D., Rudzinska, M., Bossowski, A., Kucharska, A., Sewerynek, E., Czarnecka, K., Migdalska-Sęk, M., & Czarnocka, B. (2013). *CTLA-4* polymorphisms (+ 49 A/G and-318 C/T) are important genetic determinants of AITD susceptibility and predisposition to high levels of thyroid autoantibodies in Polish children-preliminary study. *Acta Biochim Pol*, *60*(4), 641-646.

[17] Chaili (2010). Association of *CTLA-4* gene polymorphisms with recurrent spontaneous abortion, (Unpublished Master Thesis). Ningxia medical university-China, Accessed March 10, 2016 from dissertation topic website, http://www.dissertationtopic.net/doc/83153

[18] Pendeloski, K. P., Sass, N., Torloni, M. R., Mattar, R., Moron, A. F., Franchim, C. S., & Daher, S. (2011). Immunoregulatory gene polymorphisms in women with preeclampsia. *Hypertension Research*, *34*(3), 384-388.

[19] Hayashi, Y., Matsukawa, Y., Kitaori, T., Katano, K., Ozaki, Y., & Sugiura, M. (2015). Genotyping analyses for polymorphisms of PD-1 gene and CTLA4 gene in patients with recurrent pregnancy loss. *Human Reproduction, 30*, 191-191.

[20] Messaoudi, S., Houas, I., Yaseen, K., Dandana, M., & Mahjoub, T. (2014). CTLA-4 gene polymorphisms and risk of idiopathic recurrent pregnancy loss in a Tunisian population. *BMC Genomics*, *15*(Suppl 2), P11. doi: 10.1186/1471-2164-15- S2-P11.