Cytotoxic T-Lymphocyte Antigen 4 *(CTLA-4)* Gene Polymorphism in Females with Recurrent Spontaneous Pregnancy Loss

Original Article

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ABSTRACT

Background: Merely 2% of pregnant mothers experience two consecutive miscarriages, and as many as 50% of patients suffering from Recurrent Pregnancy Loss (RPL) lack a definitive cause for their condition. The CTLA-4 gene is located in band q33 on chromosome 2 of the human genome, is approximately 6.2 kilobases long, and is composed of 3 introns and 4 exons. In decidual and peripheral dendritic cells, it is expressed on human placental regulatory T (Treg) cells. Human miscarriages were associated with a downregulation of Treg cells and CTLA-4 expression in decidual and peripheral lymphocytes. The purpose of our analysis was to determine whether the CTLA-4 +49A/G (rs231775) & (rs3087243) gene polymorphism and unexplained RPL were related.

Subjects and Methods: This case-control study included women with RPL were contrasted with healthy females at the age of motherhood. The study was conducted at the National Research Centre (Clinical Genetics Department -Human Cytogenetic Department - Molecular Genetics & Enzymology Department) in collaboration with Benha Faculty of Medicine (Clinical & Chemical Pathology Department).

Results: Considering AG is the reference haplotype, no significant association was found between CTLA-4 rs231775-rs3087243 haplotypes with number of abortions. rs231775 AG+GG was considered a protective predictor, while rs3087243 GA+AA was considered a risky predictor of susceptibility to spontaneous recurrent abortion in uni- and multivariable analyses.

Conclusion: Our study revealed that a significant correlation was discovered between the CTLA-4 gene rs231775 AG, GG genotypes, G allele, rs231775- rs3087243 GG haplotype with protective effect against RPL. Whereas, a significant correlation was discovered between the CTLA-4 gene rs3087243 GA, AA genotypes, A allele AA haplotype and RPL risk.

Key Words: CTLA-4; Gene Polymorphism; Recurrent spontaneous abortion.

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INTRODUCTION

Recurrent pregnancy loss (RPL) is characterized as three or more consecutive spontaneously failed clinical pregnancies before 24 weeks of pregnancy, according to the Royal College of Obstetricians and Gynecologists (RCOG) (**Pourmasumi *et al*., 2018**).

There are several terms used to describe repeated pregnancy disruptions, including recurrent spontaneous abortion (RSA), recurrent miscarriage (RM), and RPL. Numerous studies have revealed that roughly one in every 100 women encounter RPL (**Turesheva *et al.*, 2023**).

2 to 5% of RPL cases are caused by genetic factors, which can include gene mutations or chromosomal abnormalities. An increasing number of research have concentrated on genetic variables, namely single nucleotide polymorphism (SNP) (**Abdi-Shayan *et al.*, 2016**).

The mother-fetus relationship is important for a successful pregnancy. Since the feotus and the mother

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do not share the same genetic makeup, this relationship is essential. It has been discovered that this feto-maternal connection is established by multiple pathways. One of the most significant of these is immunological mechanisms, which enable the mother immune system to develop tolerance toward the semiallogeneic feotus (**Mumusoglu *et al*., 2016**).

It is known that the tolerance of the feotus is influenced by negative co-stimulatory signalling pathways, such as CTLA-4/B7, which are expressed on Tregs and regulate effector T cell activation and proliferation (**Nasiri & Rasti, 2016**).

These signalling molecules function by inhibiting immune-reactive cells, and it has been demonstrated that reduced CTLA-4 expression contributes to human foetal loss (**Tripathi & Guleria, 2015**).

The CTLA-4 gene comprises four exons and more than 100 polymorphism sites and it corresponds to human chromosome 2q33. Of these, CTLA-4 +49A/G (rs231775) was examined in great detail and has been linked to a heightened risk of developing several human diseases, including Gravis diseases, RPL, and Type 2 diabetes mellitus (Rasti & Nasiri, 2016).

The study's objective was to assess the relationship of unexplained RPL with CTLA-4 +49A/G (rs231775) & (rs3087243) gene polymorphisms (SNPs).

SUBJECT AND METHODS

Subject

This case-control study included women with RPL were contrasted with healthy females at the age of motherhood. The study was conducted at the National Research Centre(Clinical Genetics Department- Human Cytogenetic Department - Molecular Genatics & Enzymology Department) in collaboration with Benha, Faculty of Medicine (Clinical & Chemical Pathology Department).

Fifty women in the study group, ages 20 to 35, had undergone at least three spontaneous abortions prior to the 24-week gestational period. Fifty women who were of the same age as the study group, had given birth to at least one healthy child, and had no prior history of pregnancy loss comprised the control group.

We exluded any abnormality including US as uterine abnormalities, thrombophilia screening, immunological testing. any endocrine problem including the thyroid and Karyotyping for both couples was performed.

Controls and research subjects were matched with regard to all other conceivable characteristics (There were no users of oral contraceptives, hormonal medications, or any other dangerous drugs that could have impacted vital bodily functions).

Those with established RPL causes as uterine structural abnormalities, Immunological causes, Medical causes as hypertension, Diabetes mellitus and Chromosomal abnormalities are excluded from this study.

Every study sample was drawn from Clinical Genetics Department at the National Research Centre from May 2018 to October 2019 and the control samples were recuruited from Obstetric and Gynacology Outpatient Clinic, Benha University Hospital, from May 2018 to October 2019.

ETHICAL COMMITTEE

The National Research Center's Ethical Medical Committee and Benha University approved the study protocol. Each and every participant in the study gave their informed consent.

Methods

The following was conducted on participants:

A thorough history and clinical evaluation paying attention to:

Age and history of recurrent pregnancy loss with no uses of oral contraception, hormonal or any serious medications.

Clinical assessment included abnormal and pelvic U/S.

Histoscope to detect normal uterine cavity.

Detailed pregnancy and obstetric history, medical history, family history, past history and consanguinity.

Cytogenetic analysis

G-banding karyotyping for peripheral blood chromosomes was performed for all patients to exclude any patient with chromosomal abnormality from the study Verma and Babu, (**VERMA & BABU, 1995**). Approximately 30 metaphases were analyzed and karyotyped for each patient following the International System for Human Cytogenomic Nomenclature [ISCN, 2020] recommendations (**MCGOWAN-JORDAN *et al.*, 2020**).

Molecular investigations

Sampling

Five millilitres of venous blood were collected in EDTA-containing tubes (violet tapped) under aseptic precautions and storage at -40°C to measure haemoglobin and identify SNPs utilizing Polymerase Chain Reaction-Restriction Fragment Polymorphism (PCR/RFLP).

Analysis of the polymorphism in CTLA4+49A/G (rs231775) and (rs3087243) by PCR-RFLP method with the following steps:

A. Extraction of genomic DNA from EDTA blood leucocytes.

B. Amplification of the isolated by sets of primers which detect the targeted polymorphism.

C. Detection of PCR amplified products using gel electrophoresis on 2% agarose gel with Tris borate EDTA buffer at 100 V and visualisation was done by ultraviolet (UV) light trans-illumination.

D. Digestion of PCR amplification products was done using the suitable restriction enzyme Endonucleas BbNI (Fermentase, Burlington, USA) specific for target polymorphism.

E. The past digestion products were examined by gel electrophoresis on 2% agarose gel comprising ethidium bromide and visualized by trans illumination for genotype calling.

Determination of CTLA-4+49A/G (rs231775) & (rs3087243) Extraction of DNA:

The purification of the genomic DNA of whole blood DNA was extracted from peripheral blood leucocytes using a Mini Kit employing a Wizard genomic DNA purification kit.

DNA amplification:

Enzymatic amplification was performed by PCR using PCR GOTaq polymerase enzyme (Madison, USA) and veriti thermal cycler (AB Applied Biosystem, Singapore).

Reagents:

- Green Master Mix(Madison, USA) contains i-TaqDA polymerase, reaction buffer, d NTPs, d CTP, d GTP, d TTP). MgCl2.
- CTLA4-49 (rs231775)&(rs3087243).

Protocol for PCR amplification:

For each reaction, the following were added to the Amico-centrifuge tube:

- DNA template……2 µL
- I-Star Taq DNA polymerase……..0.5 µL
- The exon 1 position 49 polymorphic restriction sites of the CTLA4 gene were genotyped via PCR amplification; the restriction fragment length polymorphism of the CTLA-4 gene was then amplified via PCR using the subsequent primers: (rs231775) forward TCCTGAAGACCTGAACACCG and (rs231775) reverse TGCCTTTGACTGCTGAAAC.
- (rs3087243) forward AGGCAGCAGGTGGCAGAAT and (rs3087243) reverse TAAGCAAGAATCACAGAGGGC.

The following parameters were utilized for the amplification: a 3-minute initial denaturation at 94, followed by 35 cycles of 45-second denaturation at 94.

Detection of the band of amplified material use trans-illuming UV nation:

- For the existence of the amplification band at 88 and 74 bp, the gel was observed through the filter region of an Alpha Innotech corporation transilluminator.

Gel was then photographed:

- Left lane: PCR marker (50-1000 bp Ladder).
- Other lanes: presence of the amplification band at 88 and 74 bp.

Digestion of the amplified products by restriction enzyme:

CTLA-4 (restriction enzyme) (Biolab, NewEngland) used for digestion of the amplified targeted DNA to study rs231775 & rs3087243.

Detection of the CTLA-4 gene polymorphism:

The CTLA-4 polymorphism was detected using 2% agarose gel electrophoresis.

Statistical methods:

The data that had been gathered was reviewed, coded, and tabulated utilizing the Statistical Package for the Social Sciences (IBM Corp., 2017). Version 25.0 of IBM SPSS Statistics for Windows (Armonk, NY: IBM Corp). Data were shown, and appropriate analysis was carried out based on the kind of data found for each parameter.

3.RESULTS

Cytogenetic analysis revealed normal outcomes for all patients; 46,XX (Figure 1).

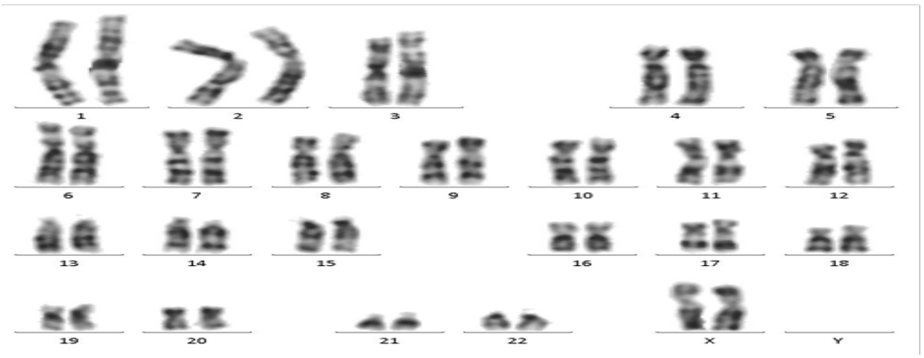


Figure 1: showing normal female karyotype ; 46,XX.

The current molecular investigation involved 50 cases of unexplained recurrent abortions. Their mean (±SD) age was 23.8 (±2.6) years. Furthermore, 50 age-matched, healthy control females (p>0.05) (Table 1).

Table 1: Comparison of age between studied groups:

	Control *N*=**50**	**Cases** *N*=**50**	***P***
	Mean±SD	Mean±SD	
Age (years)	24.8±2.7	23.8±2.6	0.881

SD: standard deviation; Numerical data are expressed as mean±SD, compared by t test.

Considering AA and A are the reference genotype and allele respectively, AG, GG, AG+GG genotypes, G allele exhibited a significantly reduced incidence of in contrast to control groups (p<0.05 for each), and had a protective effect against recurrent unexplained abortions (OR<1 for each) (Table 2).

Table 2: Association of CTLA-4 rs231775 genotypes and alleles with studied groups:

		Control N=50 N (%)	**Cases N=50 N (%)**	***p***	**OR (95% CI)**
rs231775	AA	10 (20 %)	21 (42 %)	-	Reference
	AG	31 (62 %)	24 (48 %)	0.047*	0.438(0.194-0.988)
	GG	9 (18 %)	5 (10 %)	0.032*	0.538(0.305-0.947)
	AG+GG	40 (80 %)	29 (58 %)	0.018*	0.516(0.299-0.892)
	A	51 (51 %)	66 (66 %)	-	Reference
	G	49 (49 %)	34 (34 %)	0.031*	0.677(0.475-0.966)

Data is presented as frequency (percentage), *significant as *p-value* <0.05, OR, odds ratio; CI: confidence interval. Logistic regression analysis was used. AA is the reference genotype and A is the reference allele; AG+GG is the dominant model.

Considering GG is the reference genotype and G is the reference allele, GA, AA genotypes, dominant model and A allele had significantly greater proportions in cases in contrast to control group (p<0.05 for each), with risk to develop recurrent abortions (OR>1 for each). (Table 3).

Table 3: Association of CTLA-4 rs231775-rs3087243 haplotypes among studied groups:

rs231775-rs3087243	Control	Cases	*p*	OR (95% CI)
	Frequencies	Frequencies		
AG	0.459	0.387	-	Reference
GA	0.259	0.237	0.721	1.072(0.576-1.995)
GG	0.231	0.103	0.015*	0.656(0.315-0.967)
AA	0.051	0.273	<0.001*	2.854(1.287-6.324)

OR: Odds ratio; CI: Confidence interval. Logistic regression analysis was used.

Haplotype is a group of alleles that are inherited together from a single parent. The rs231775 and rs3087243 SNPs are spaced about 6 kilo base pairs apart. The rs231775-rs3087243 haplotypes were calculated among each group, AG haplotype showed the highest frequency among cases (38.7%), and controls (45.9%), GG haplotype displayed the least frequency among cases (10.3%), while AA haplotype displayed the least frequency among controls (5.1%). Considering AG is the reference haplotype, GG showed lower frequency in cases when compared to controls (p=0.015), with protective effect against recurrent abortion development (OR<1), while AA showed greater frequency in cases in contrast to controls (p<0.001), with susceptibility risk for recurrent abortion (OR>1). GA haplotype was not associated significantly with recurrent abortion (Table 4).

Table 4: Association of CTLA-4 rs231775-rs3087243 haplotypes with number of abortions:

rs231775-rs3087243	3 abortions	>3 abortions	*p*	OR (95% CI)
	Frequencies	Frequencies		
AG	0.404	0.333	-	Reference
GA	0.220	0.233	0.559	1.285 (0.892-3.343)
GG	0.113	0.117	0.879	1.256(0.934-1.476)
AA	0.263	0.317	0.954	1.462(0.958-1.739)

OR: Odds ratio; CI: Confidence interval. Logistic regression analysis was used.

Considering AG is the reference haplotype, no significant association was found between CTLA-4 rs231775-rs3087243 haplotypes with number of abortions (Table 5).

Table 5: Regression analysis for prediction of susceptibility of spontaneous recurrent abortion:

	Univariable		Multivariable	
	p	OR (95% CI)	*p*	OR (95% CI)
rs231775 AG+GG	0.018*	0.516(0.299-0.892)	0.001*	0.288(0.141-0.587)
rs3087243 GA+AA	0.009*	2.098(1.202-3.661)	<0.001*	3.596(1.797-7.195)

*: significant as *P-value* <0.05, OR: Odds ratio; CI: Confidence interval. Logistic regression was used for analysis.

Regression analysis was done in order to predict spontaneous recurrent abortion susceptibility utilizing rs3087243, rs231775 genotypes as covariates. rs231775 AG+GG was considered a protective predictor, while rs3087243 GA+AA was considered a risky predictor of susceptibility to spontaneous recurrent abortion in uni- and multivariable analyses (Table 6).

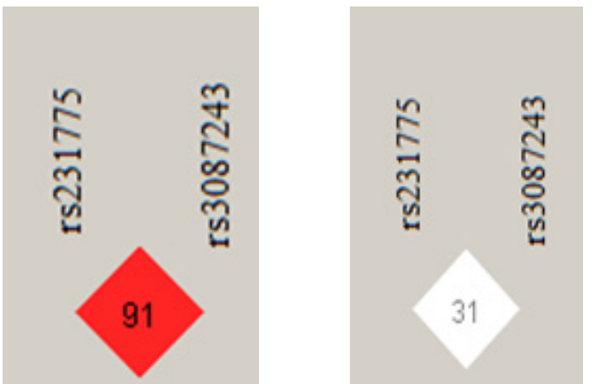
Table 6: Regression analysis for prediction of susceptibility of spontaneous recurrent abortion:

	Univariable		Multivariable	
	p	OR (95% CI)	*p*	OR (95% CI)
rs231775 AG+GG	0.018*	0.516(0.299-0.892)	0.001*	0.288(0.141-0.587)
rs3087243 GA+AA	0.009*	2.098(1.202-3.661)	<0.001*	3.596(1.797-7.195)

*: Significant as *P-value* <0.05; OR: odds ratio; CI: confidence interval. Logistic regression was used for analysis.

When alleles at two or more loci in a population are not randomly associated, it is referred to as LD. The value of D′ ranges from 0 (no disequilibrium) to 1. (maximum disequilibrium). Among our studied control group, D'=0.91; while among our studied cases, D'=0.31.Our results in control group agreed with those who demonstrated a strong linkage disequilibrium between rs231775, and rs3087243 (Figure 2).

When alleles at two or more loci in a population are not randomly associated, it is referred to as linkage disequilibrium (LD). The value of D′ ranges from 0 (no disequilibrium) to 1. (maximum disequilibrium). Among studied control group, D'=0.91; while among studied cases, D'=0.31 (Figure 2).



A B

Figure 2: LD plot in (A): control and (B): cases for studied SNPs. Squares correspond to D' values with numerical estimates given within the squares.

DISCUSSION

RPL refers to two or more consecutive clinically unsuccessful pregnancies that end before 20 full weeks of gestation, as confirmed by histology or ultrasound. It can also mean losing three or more early pregnancies in a row. Merely 2% of pregnant mothers experience two consecutive miscarriages, while as many as 50% of RPL patients lack a definitive cause (**Pillarisetty & Mahdy, 2023**).

According to the current study, there is a protective effect against unexplained recurrent abortions for CTLA-4 rs231775 AG, GG, AG+GG, and G allele genotypes, which demonstrated significantly lower frequency in cases in contrast to control groups. Our findings were supported by a meta-analysis of publications from the Chinese and PubMed databases. A total of 2607 controls and 2405 cases were included in the six case-control studies. The search criteria used to determine the abortion susceptibility associated with the CTLA-4 +49 G/A polymorphism, there was significantly protective association between G allele and RPL in non-Chinese population, while the reverse was found in Chinese populations (**Song *et al.*, 2019**).

rs231775 may reduce the risk of RPL; that is, people with the G-allele may have a lower association with RPL, or the G-allele is a protective factor for RPL risk; conversely, the A-allele is a potential risk factor, as the A-allele may rise the expression of CTLA-4 protein, which is protective in promoting RPL and feotus toleration and this agreed with another study conducted on Han Chinese population who suggested that conduction of genetic analysis for CTLA-4 rs231775 for RPL Patients could successfully direct efficient therapy and good parenting (**Fan *et al.*, 2018**); as well as other studies which were conducted on Iranian population (**Rasti & Nasiri, 2016**).

The National Center of Biotechnology Information's reports on the minor allele frequency (MAF) of rs3087243 for the primary global populations were examined. East Asian, 0.18; European, 0.45; African, 0.22; and South Asian, 0.60. The MAF in our analysis was 0.31 in the control group, which is slightly greater than that reported in African and East Asian populations and lower than that reported in European and Asian populations.

The polymorphism rs3087243, which is found downstream of the poly (A) termination point, may be crucial for the mRNA stability of soluble CTLA-4. If CTLA-4 function is compromised, this could result in heightened T-cell activation and an increased risk of autoimmunity (**Pang *et al.*, 2022**).

Our research showed that the CTLA-4 rs3087243 genotypes and alleles were associated with the groups under investigation. Specifically, we discovered that the dominant model, GA, AA, and A alleles had significantly higher proportions in cases in contrast to the control group, which was at risk of developing recurrent abortions. In agreement with other studies in Indian Misra *et al*. (**Misra *et al.*, 2014**) and Chinese population Fan *et al*. (**Fan *et al.*, 2018**). However, other studies reported non significant association between rs3087243 alleles and RPL in Tunisian Messaoudi *et al*. (**Messaoudi *et al.*, 2014**) Japanese Hayashi *et al.* (**Hayashi *et al.*, 2018**); and Kazakh Svyatova *et al.* (**Svyatova *et al.*, 2021**), northwestern Iran Rasti and Nasiri, (**Rasti & Nasiri, 2016**); northern India Mojarrad *et al*. (**Mojarrad *et al.*, 2013**).

In the present investigation, we noticed non significant correlation between rs231775 and rs3087243 genotypes with the number of previous abortions. Moreover, no significant association was found between CTLA-4 rs231775-rs3087243 haplotypes with number of abortions. In consistence with others (**Fan *et al.*, 2018**).

Haplotype is a group of alleles that are inherited together from a single parent. Haplotypes contribute important insights into the evolutionary history of humans and could help develop more effective methods for identifying genetic variations that raise a person's risk of developing diseases (**Niu, 2004**).

The rs231775 and rs3087243 SNPs are spaced about 6 kilo base pairs apart. In the present study, the rs231775-rs3087243 haplotypes were calculated among each group, AG haplotype showed the highest frequency among cases (38.7%), and controls (45.9%), GG haplotype displayed the least frequency among cases (10.3%), while AA haplotype displayed the least frequency among controls (5.1%). With AG serving as the reference haplotype, GG displayed a lower frequency of cases compared to controls, offering a protective effect against the development of recurrent abortions, whereas AA displayed a higher frequency of cases in contrast to controls, indicating a susceptibility risk for recurrent abortions. GA haplotype was not associated significantly with recurrent abortion.

Our results were in agreement with others who reported GG haplotype was associated with reduced susceptibility to RPL, while AA haplotype appeared to remarkably increase the RSA risk (**Fan *et al.*, 2018**).

While we agreed in part with Misra and his colleagues who found that the rs231775 - rs3087243 haplotype GA carriers showed higher frequencies in RPL when compared to control groups, with risky effect to have RPL (**Misra *et al.*, 2014**).

Variations in sample sizes, inclusion and exclusion criteria, and ethnicities could all contribute to discrepancies in results between research. Conversely, the variability of a disease can impact the degree and kind of genetic susceptibility factors involved.

It is widely recognized that the expression of an immune-suppressing enzyme can be induced, especially in the early periods of pregnancy, by CTLA-4 expressed on Treg cells in decidual and peripheral dendritic cells. In addition, maternal-fetal tolerance is promoted by elevated levels of the immune-suppressing enzyme (**Sasaki *et al.*, 2004**). Furthermore, multiple in vivo investigations found that human miscarriages were associated with down-regulated expression of Treg cells and CTLA-4 in peripheral and decidual lymphocytes (**Jin *et al.*, 2009**).

One of our study's limitations is that interactions among several polymorphic loci of the same CTLA-4 may alter the risk of RPL; these interactions should be taken into account in subsequent investigations and analyses. In addition, the sample size was insufficient to provide a higher test power. Although a number of factors influence the development of PRL, the current study did not find any evidence of interactions between the CTLA4 gene and other genes or environmental factors. Furthermore, an assay was not conducted to measure the impact of polymorphism on the serum level of CTLA-4.

CONCLUSION

Our study revealed that a significant correlation was discovered. between the CTLA-4 gene rs231775 AG, GG genotypes, G allele, rs231775- rs3087243 GG haplotype with protective effect against RPL. Whereas, a significant correlation was discovered. between the CTLA-4 gene rs3087243 GA, AA genotypes a allele AA haplotype and RPL risk.

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