GENETIC POLYMORPHISM OF TWO INTERLEUKINS GENES IN IDIOPATHIC RECURRENT PREGNANCY LOSS: CASE CONTROL STUDY.

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Abstract: Background: Recurrent pregnancy loss (RPL) is a multifactorial condition that may occur due to maternal or fetal causes. 50% of RPL are caused by well-known reasons and other 50% occurs as a result of unknown reasons (idiopathic). Interleukins (ILs) play critical roles in several developmental steps during embryogenesis. This study was conducted to investigate possible linkage between polymorphism of (IL)-6, (IL)-27 genes and idiopathic RPL in Egyptian women. **Methods**: This study detected the polymorphism in studied genes by means of RFLP-PCR procedure to compare 85 RPL cases with 60 healthy controls. **Results**: No significant differences were observed in age between cases and controls, all the members of case group were primary idiopathic RPL (i.e. no life birth at all). The frequency of polymorphic allele G of IL-6 -634 C/G in case group was 74.1%, while in control group it was 65.8% (p=0.036), But in IL-27 -964 A/G it was 39.4%, while in control group it was 25.8% which was significant (p=0.017). Our results suggested that -634 C/G and -964 A/G in (IL)-6 and (IL)-27 had significant impact on pregnancy outcome as indicated by increased incidence of presence of allele G, homozygous polymorphic genotype (GG) in (IL)-6 polymorphism and the heterozygous genotype (AG) in (IL)-27 with RPL patient respectively. The genotypes and allele frequencies of studied groups were also influenced due to the polymorphism of the two genes. **Conclusion**: Results suggested for the first time that single nucleotide polymorphism in the genes coding for (IL)-6, (IL)-27 are significantly related as possible etiological factors in idiopathic recurrent pregnancy loss in Egyptian women.
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Key words: Recurrent pregnancy loss, single nucleotide polymorphism, interleukins.

1. Introduction:

Recurrent pregnancy loss (RPL) is prevalent medical issues that influence nearly 15% of pregnant women **(Practice Committee of American Society for Reproductive Medicine 2013)**. RPL is determined as two or more sequential miscarriage before the 20th week of pregnancy **(Regan and Rai2000).** RPL is a multifactorial condition that may occur due to maternal or fetal causes. 50% of RPL are caused by multiple reasons, such as: metabolic or immunological defects, endocrine and uterus anatomical upsets, chromosomal aberrations or any infections. The rest 50% of RPL occurs as a result of unknown reasons **(Laird, *et al.*, 2003 and Rai and Regan, 2006).**

Interleukins, are proteins involved in cellular communication that produced by immune cells, and also they are also responsible for a variety of immunological, inflammatory functions and control of many infectious diseases **(Prins *et al.*, 2012).**Apart from aforementioned functions, critical roles inseveral developmental steps during embryogenesis, which are vital in successful pregnancy outcome,are played by interleukins **(Walia *et al.*, 2008).**

Two sub-types of T helper cells; namely T helper 1(Th-1) which is known as pro-inflammatory that block trophoblastic development. And the anti-inflammatory sub-type T helper 2 (Th-2) which produce interleukin antagonistic in action to the fore mentioned Th-1 dependent interleukins. These interleukins selectively enhance placental and early fetal development **(Medica *et al.*, 2009).**Successful pregnancy requires keeping adequate balance between the two T-helper cell sub-types. Several studies demonstrated that Th-1 is related to frequent involuntary miscarriage (**Medica *et al.*, 2009)**. While, the Th-2 is responsible for ordinary and successful pregnancy **(Kamali *et al.*, 2005 and Tanaka, *et al.*, 2014).**Few studies, have suggested that Interleukin (IL)-6, (Interleukin (IL)-17 and Interleukin (IL)-27 may have a role in the preservation of embryo throw pregnancy period **(Nematollahi *et al.*, 2015).**

(IL)-6 is a glycoprotein that has diverse functions, it also considered as pro and anti-inflammatory Interleukin. The gene that encodes for (IL)-6 is located at the seventh chromosome and consist of six coding regions i.e. exons **(Prins *et al.*, 2012).** The function of (IL)-6 in RPL is not identified. But, it was noticed that women with recurrent miscarriage, reflected a high level of this interleukin in their peripheral blood **(Pressman *et al.*, 2011).**

Bao *et al.* (2011), demonstrated that (IL)-17 is a pro-inflammatory interleukin produced by T helper cells. The Rate of Th17 in blood samples of aborted cases was higher than normal control cases **(Wang *et al.*, 2010).**

Recently, many studies demonstrated that (IL)-27 it is necessary for promoting T cells. It decreases inflammatory response in pregnancy. Also, it prevents the development of T-17 cells **(Stumhofer and Hunter, 2008)** and frustrates (IL)-12 and (IL)-23 activities **(Yoshimura *et al.*, 2006).** The encoding gene for (IL)-27 is located in the short arm of chromosome 16 and composed of five coding exons.

Single nucleotide polymorphism (SNP) is a change in one nucleotide that happens in a particular region of genome. SNPs lying in exon regions known as cSNP **(Collins *et al.*, 1998).** Polymorphisms in exonssequence may not cause a change in the code of amino acid in the protein, but this action may influence its function. On the contrary, when the Polymorphisms in exons sequence occur, it results in a change of the amino acid sequence in the protein **(Sauna *et al.*, 2007).**

Single nucleotide polymorphism in any interleukins mentioned earlier may affect their critical functions in pregnancy preservation and development. This study was conducted to detect the relation between polymorphism in (IL)-6 and (IL)-27 with RPL in Egyptian population. For our knowledge this is the first study to investigate possible link between polymorphism of (IL)-6, (IL)-27genes and idiopathic RPL in Egyptian women.

2. Materials and methods:

Materials:

A total of 85 RPL patients their ages ranged from (18 – 40), they suffered from more than two sequential spontaneous miscarriage without any successful pregnancy (primary RPL). Women with a diagnosed reason for pregnancy loss, such as an anatomical problems, chromosomal aberrations, an autoimmune diseases (i.e. antiphospholipid antibody syndrome or systemic lupus erythematosus), an alloimmunereasons (Existence of high natural killer cells), or endocrine abnormalities (such as hypothyroidism, hyperprolactinemia or diabetes) were excluded. Sixty women served as controls their ages ranged from 20 to 39 years, they have visited the health-care center in Zagazig University Hospital for annual comprehensive medical checkup with no health problems. They had two or more normal term deliveries after normal pregnancies and no history of idiopathic miscarriage. We have set controls from this general population to exclude the possibility that these participants were affected by RPL. The study was carried out from March 2015 to April 2016. The properties of cases and control groups are shown in Table (1).

The study protocol was reviewed and approved by the Institutional Review Board of the Zagazig University Hospitals. All cases and controls were Egyptians- Sharpie governorate. Written informed consent was obtained from all the subjects prior starting this study.

Table (1): properties of cases and control groups in the study

Variables	Controls	Cases		t	p-value
Range	20-39	18-40		1.342	0.152
Age (mean±SD)	30.5±7.6	32.7±10.9			
No of abortions		**No**	**%**		
2	0	71	83.5		
≥3	0	14	16.5		
No. of live births range	2-6	0			

Methods:

Blood samples collection:

5 ml Peripheral blood samples were collected individually in tubes containing EDTA as an anticoagulant agent.

DNA Extraction:

Genomic DNA was extracted using (BioFlux, whole blood genomic DNA extraction kit, Cat#BSC061). DNA concentration was adjusted using spectrophotometer at (0.05-0.10 ng/µL). Extracted DNA samples were stored at -20 until analysis.

Genotyping of IL-6 -634 C/G and IL- 27 (-964 A/G) SNPs:

Specific primer was designed to detect the IL-6-634 C/G and IL- 27 (-964 A/G) SNP region (Table2). PCR reaction mix was accomplished in final volume of 25 µLand PCR was conducted using (thermocycler) as shown in Tables (3) and (4).

All PCR products divided into two sections, the first: was electrophoresed on 1.5% agarose gel stained with ethidium bromide to evaluate their weights using 1k bp DNA ladder.

Second section was digested with the restriction enzyme (MbiI) for detecting IL-6-634 C/G SNP and the restriction enzyme (Ava1) for detecting IL-27-964

A/G SNP as follows: in a clean tube add 10 µL PCR products, 2 µL restriction enzyme and sterile water (up to 20µL). Then incubate the tubes at 37°C for one hour. To stop the digestion, raise the temperature to 65°C for 15 minutes. Restriction fragments were electrophoresed on 1.5% agarose gel stained with ethidiumpromid to estimate their weights using 50bp DNA ladder. All gels were visualized by U.V. transelemenator. Five samples were randomly selected and the whole procedure was repeated to ensure the results accuracy. Re-obtained results were similar to the initial experiments.

Table (2): IL-6 -634C/G and IL- 27 (-964 A/G) specific primers sequences.

Primer	Sequence
PrimerIL-6 -634C/G	
forward	5´-GAGACGCCTTGAAGTAACTG-3´
reverse	5´-AACCAAAGATGTTCTGAACTGA-3´
PrimerIL- 27 (-964 A/G)	
Forward	5´-AACTAGGGTCAGGGCTGGAT-3’
reverse	5´-CCTCGGCAGATGTTGGTATT-3’

Table3: PCR reaction mix for detecting IL-6-634 C/G and IL-27-964 A/G SNP

Component	Volume
Master mix	12.5 µL
Forward primer	1µL
Reverse primer	1µL
DNA template	1.5 µL
Water(ion free)	Up to 25

Table4: PCR protocol for detecting IL-6 -634 C/G and IL-27-964 A/G SNP

Operation	Temperature	Time	No. Cycles
Pre- denaturation	94^{o}C	5 min	
Denaturation	94^{o}C	30 sec	35
Annealing	57^{o}C (IL)-6 62^{o}C (IL)-27	45 sec	
Extension	72^{o}C	1 min	
Final extension	72^{o}C	5 Min	

Statistical analysis:

Risk was evaluated regarding to (OR) odds ratio with 95% (CI) confidence interval. Variations in allele and genotypes frequencies were calculated between studied groups by (t-test, p-value<0.05 significant) using SPSS version 14. The genotype and allele frequencies of IL-6-634 C/G and IL-27-964 A/G SNPs were calculated by direct counting.

3. Results and Discussion:

In case group the age of studied cases ranged from 18 to 40 years with mean of 32.7±10.9. While, in control group, the age of tested women ranged from 20 to 39 years with mean of age was 30.5±7.6 year. No significant difference was recorded in the age of both groups (p-value = 0.152). Properties of the control and cases groups are demonstrated in Table (1). All studied genotypes were in Hardy-Weinberg Equilibrium.

PCR amplification using IL-6 -634 C/G primer produced one amplified fragment weighted (180 bp) in all studied samples; controls and patients Fig (1). After digestion by the restriction enzyme (MbiI) that targeted mainly the polymorphic nucleotide in the enzyme’s restriction cite. Three different band sets were obtained. First, a set of one band weighted 180 bp, reflecting the homozygous wild genotype (CC) that was represented in 15% within control and 4.7% within cases.

Second set was distinguished by the appearance of three bands with molecular weights of 180bp, 120 bp and 60 bp announcing the heterozygous genotype (CG) which distributed as 38.33% in control group and 42.4% in cases group. The third set composed of two bands with molecular weights of 120 bp and 60 bp deducing the polymorphic homozygous genotype (GG). This genotype percentage was 47.67% and 52.9% in control and cases respectively (Fig. 1, 2 and Table 5).

PCR amplification using IL-27 -964 A/G primer produced one amplified fragment weighted (753 bp) in all studied samples (controls and patients). After digestion by the restriction enzyme (Ava 1) that targeted mainly the polymorphic nucleotide in the

enzyme's restriction cite. Amplified fragments had one main restriction site beside the additional sites that formed due to polymorphism in gene sequence. So, the restriction enzyme digested the fragment in many regions according to the number of restriction sites. Three different band sets were obtained. First, a set of two bands weighted 177bp and 576bp, reflecting the homozygous wild genotype (AA) that was represented in 60% within control and 35.3% with incases. Second set was distinguished by the appearance of four bands with molecular weights of 177 bp, 205 bp, 371 bp and 576 bp announcing the heterozygous genotype (AG) which distributed as 28.33% in control group and 50.6% in cases group. While, the third set consisted of three bands with molecular weights of 177 bp, 205bp and 371 bp deducing the polymorphic homozygous genotype (GG). This genotype percentage was 12.67% and 14.1% in control and cases respectively (Fig. 3, 4 and Table 6).

Analysis of IL-6 -634 C/G SNP showed that frequency of polymorphic allele G in case group was 74.1%, while in control group it was 65.8% (p=0.036). But in IL-27 -964 A/G SNP analysis the same allele G in case group was 39.4%, while in control group it was 25.8% which was significant (OR=1.86, 95%CI: 1.12 - 3.12, p=0.017) indicating possible associative effect of this allele in cases of idiopathic pregnancy loss.

Concerning genotypic frequency distribution in the two studied polymorphisms, the difference in distribution of the homozygous genotype GG was significant between cases and controls (p=0.047) of IL-6 -634 C/G SNP. On the other hand, only the distribution of heterozygous genotype AG was significant (p=0.003) in IL-27 -964 A/G SNP.

It was observed that the risk of disease markedly increase under the dominant pattern of allele G in each of the two studied polymorphisms GG+GC vs. CC(OR=3.57, 95%CI: 1.046-12.21, p=0.042) and GG+AG vs. AA (OR=2.75, 95%CI: 1.39-5.44, p=0.003respectively (Tables 5 and 6).

It is worthy note, that among the 145 studied participants, nine samples found to be positive for single nucleotide polymorphisms in the two genes IL-27 -964 A/G and IL-6 -634 C/G. out of these nine cases, 7 patients were within the case group. Different combinations of polymorphic genotypes were obtained. Moreover, it was observed that the most of these cases suffered from RPL in younger ages and has aborted in earlier gestational age. But the association between the combination of the two polymorphisms and the patient's age was non-significant. These results indicated that the combination of these two single nucleotide polymorphisms warrants farther detailed genomic association studies, in order to clearly document its possible association with idiopathic RPL.

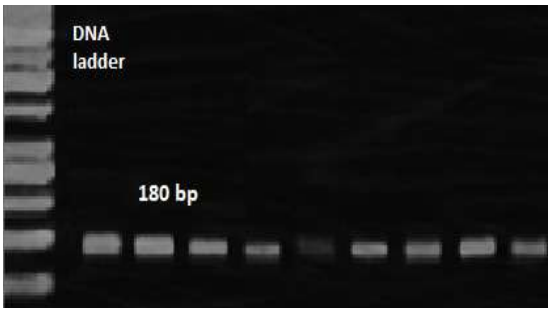

Fig. 1: PCR amplified fragment using IL-6 -634*C-G* primer in all studied groups, resulting in one common band weighted 180bp.

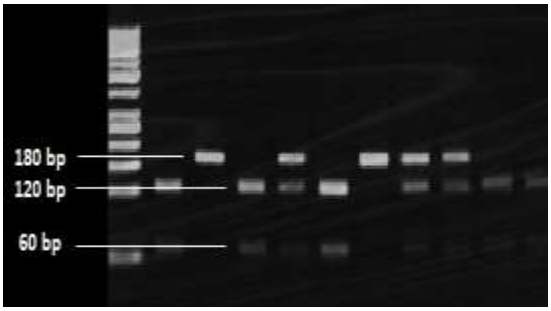

Fig. 2: Restriction fragment length polymorphism of PCR amplified fragment using IL-6 -634*C-G* primer and enzyme (MbiI) in the studied groups.

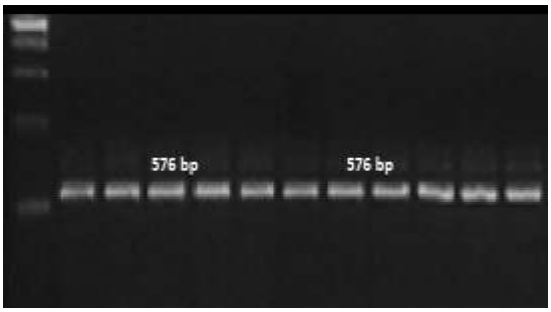

Fig. 3: PCR amplified fragment using IL-27 -964- primer and in all studied groups, resulting in one common band weighted 576 bp.

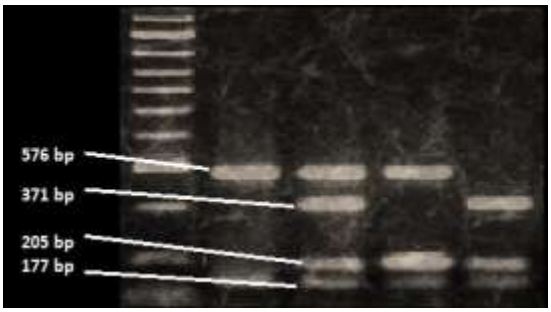

Fig. 4: Restriction fragment length polymorphism of PCR amplified fragment using IL-27 -964- primer and enzyme (Ava 1) in the studied groups

Table 5: Comparison between cases and controls of IL-6 -634 C/G polymorphism in genotypes, allelic frequencies and phenotype frequencies.

	Control (N=60)		Cases (N=85)		OR (95%CI)	p-value
	No	%	No	%		
Genotype						
CC	9	15%	4	4.7%	Reference	
CG	23	38.3%	36	42.4%	3.521 (0.970 – 12.778	0.055
GG	28	47.7%	45	52.9%	3.616 (1.016 – 12.861)	0.047
Allele						
C	41	34.2%	44	25.9	Reference	
G	79	65.8%	126	74.1%	1.486 (0.892 – 2.475)	0.128
Phenotype						
GG	28	46.7%	45	66%	1.285 (0.663 – 2.493)	0.457
CC+GC	32	53.3%	40	34%	Reference	
CC	9	15%	4	4.7%	Reference	
GG+CG	51	85%	81	95.3%	3.573 (1.046 – 12.211)	0.042

Table 6:Comparison between cases and controls of IL-27 -964 polymorphism in genotypes, allelic frequencies and phenotype frequencies in cases and controls.

	Control (N=60)		Cases (N=85)		OR (95%CI)	p-value
	No	%	No	%		
Genotype						
AA	36	60%	30	35.3%	Reference	
AG	17	28.3%	43	50.6%	3.035 (1.446 – 6.372)	0.003
GG	7	12.7%	12	14.1%	2.057 (0.719 – 5.882)	0.178
Allele						
A	89	74.2%	103	60.6%	Reference	
G	31	25.8%	67	39.4%	1.867 (1.119 – 3.115)	0.017
Phenotype						
GG	7	11.7%	12	14.1%	1.245 (0.459 – 3.373)	0.667
AA+AG	53	88.3%	73	85.9%	Reference	
AA	36	60%	30	35.3%	Reference	
GG+AG	24	40%	55	64.7%	2.750 (1.390 – 5.437)	0.003

Age, Stress, long standing endometritis and occupational or environmental exposures are the most common Risk factors for recurrent pregnancy loss (**Habbema *et al.*, 2015 and Sauer, 2015).** Many studies discussed the effects of different interleukins in the development of many serious diseases. Interleukins are biologically vigorous glycoproteins produced mostly by lymphocytes or macrophages. Massive information related to biochemical and biological features of interleukins has been acquired using different DNA technologies. The biological attributes of interleukins involve differentiation of T-lymphocyte and B lymphocyte **(Sharma *et al.*, 2015).**

Effects of polymorphism in IL-6 gene were discussed in many studies. Polymorphism of IL-6 gene affected the relation between diabetes and insulin reluctance **(Fernández *et al.*, 2000)**, also, it has a protective role for the existence and intensity of hip and knee osteoarthritis **(Fernandes *et al.*, 2015). Li, *et al.* (2015)** found a significant relation between bronchial asthma risk and IL-6 gene polymorphism. While a single nucleotide polymorphism in IL-6 was significantly related to Oral squamous cell carcinoma patients **(Singh *et al.*, 2015).** In respect of the association between RPL and single nucleotide polymorphism in IL-6 our results was in accordance with **Rasti *et al.* (2016),** who demonstrated risk of RPL was affected by polymorphism in coding gene of IL-6.

IL-6 is an anti –inflammatory cytokine, cytokines are divided into two major groups, pro- inflammatory; that is responsible for induction of T helper 1 cells. And anti- inflammatory; which is produces T helper 2 cells. T helper 1 cells are responsible for many gestational complications such as the prevention of trophoblast development. T helper 2, had a critical role in pregnancy maintenance and development, moreover it controls T helper 1 functions **(Kamali *et al.*, 2005).**

Polymorphisms in IL-6 gene might affect the formation of T helper 2 cells, and so this will disturb successes of regular pregnancy leading to idiopathic pregnancy loss.

IL-27 is another member of cytokines family that is plentiful existed in placenta during ordinary pregnancy, although its function has not yet been described **(Coulomb *et al*., 2007).**Polymorphism in IL-27 gene and its relation with development of many disorders were studied.

Ali *et al.* (2014), stated that Interleukin 27 gene polymorphism had no significant effect at the risk of Hepatitis B viral in Egyptian population. IL-27 gene is known an inhibitor of inflammation during pregnancy, IL-27 (−964 A / G) polymorphism may be a genetic factor that affect the accuracy of pregnancy.IL-27 is a critical genetic marker of RPL. It has been shown that IL-27 suppresses IL-17. Proportion of Th17 cells in the peripheral blood in the decidua of women with inevitable abortion was significantly higher than women with normal pregnancy.

Our results were disagreed with **Nematollahi *et al.* (2015),** they showed that IL-27 (−964 A / G) polymorphism was not represented as a risk factor for RPL in Iranian women. This difference in results may be due to the difference in the genotype of the Egyptian and Iranian population or due to geographical variations as well as limited sample size.

Medica *et al.* (2009) stated that single nucleotide polymorphisms in cytokine genes could be considered as risk factor for recurrent pregnancy loss. Interleukin genes polymorphism, have confirmed to be very critical to human health. They could modify response to particular drugs, tendency to environmental effects and excess the risk of improving many diseases. Interleukin genes single nucleotide polymorphisms considered as high-risk factors, they worth our awareness that may lead to more investigations to improve new treatment protocols.

4. Conclusion:

Results suggested for the first time that single nucleotide polymorphism in the genes coding for (IL)-6, (IL)-27 are significantly related as possible etiological factors in idiopathic recurrent pregnancy loss in Egyptian women. Further studies on Large-scale should be carried out to include Egypt's various governorates in order to arrive at a precise characterization of the role of these two unique SNPs in idiopathic RPL.

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