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Cover Page Footnote

The present study was possible by technical support of genetics lab, school of biological sciences university of the Punjab and subjects and controls were provided by Allama Iqbal Medical College, Jinnah hospital Lahore, Pakistan.

TUMOR NECROSIS FACTOR ALPHA SNP VARIANT IN PROMOTER REGION G308A, CAUSE PREECLAMPSIA DURING PREGNANCY IN PAKISTANI WOMEN, A CASE CONTROL STUDY

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ABSTRACT

Preeclampsia (PE) is a very common critical condition during pregnancy. As PE is a high-risk condition during pregnancy, occurring in 25% of all pregnancies, worldwide. In women with PE there is an increase in hypertension and albuminuria. Elevated blood pressure can be life-threatening after 20th week of pregnancy. Single nucleotide variation in gene sequence can be disease causing, among these pathogenic SNPs, a variant in *TNF-α*, G308A is analyzed in many studies as a causative variant to cause preeclampsia. In this case control study fifty patients and fifty healthy individuals were enrolled for analysis of TNF-α promoter region SNP G308A from Jinnah hospital, Lahore, Pakistan. The genotyping of TNF-α (G308A) rs1800629 polymorphisms was performed by PCR-RFLP method. Data analysis was performed by using SNPStats, statistical tool. The mean age of all patients and controls were calculated, 24.4 ± 6.6 and 25.1 ± 5.3 years, respectively. The frequency of G308A polymorphism was more prevalent in the case group, in association with control group ($p<0.001$). There was a significant correlation between inflammation promoting genotypes of TNF-α and PE. It can be warily concluded that: TNF- α (G-308A) polymorphism can be reflected as a marker of predisposition to preeclampsia in our population.

Key words: Preeclampsia, SNP, tumor necrosis factor (TNF), interleukin 1, PCR-RFLP.

Abbreviations: PE: Preeclampsia, SNP: Single Nucleotide Polymorphism, TNF: Tumor Necrosis Factor, IL-1: Interleukin 1, MHC: Major Histocompatibility complex, RFLP: Restriction Fragment Length polymorphism, EDTA: Ethylenediaminetetraacetic acid, PCR: Polymerase Chain Reaction, G: Guanine, A: Adenine, dNTPs: Deoxyribonucleotide triphosphate

INTRODUCTION

A well-known human pregnancy specific syndrome called Preeclampsia (PE), which characterizes the occurrence of hypertension and significant proteinuria in formerly healthy females on or after the 20th week of gestation (Moodley, 2004; Ghulmiyyah et al., 2012). It is a severe problem during pregnancy in human with prevalence 2-10% worldwide (Molvarec et al., 2008). The diagnostic criteria of this syndrome are the beginning of hypertension, with systolic blood pressure more than 140 mmHg, diastolic blood pressure is more than 90 mmHg), and proteinuria more than 300 mg/24 h (Brichant et al., 2014). Pregnant female with minor preeclampsia usually are asymptomatic however but the women with severe preeclampsia (usually Blood pressure about 160/110 mmHg) have complications like perturbed renal function, liver disease, neurological

disorders and hematological disturbances (Brown et al., 2000). Preeclampsia is a clinical condition which causes vascular endothelial dysfunction and narrowing the arteries that usually happens after twenty weeks of gestation and this condition persist up to four to six weeks postpartum. The clinical presentation is by hypertension, in which with pathologic edema can either be present or not. It is thought that there is controlled maternal systematic inflammation occur during normal pregnancy during which particular proinflammatory cytokines are circulating, such as Tumor Necrosis Factor-α, Interleukin-6 and Interleukin-1 etc. The source of these cytokines is appearing to be maternal peripheral blood leukocytes and trophoblast (Rusterholz et al., 2007).

Healthy normal pregnancy is related to a controlled inflammatory procedure however, in PE, it gets worse in response to excessive placental stimuli (C. Redman et al., 2003). It has been demonstrated that various cytokines are released by white blood cells like lymphocytes and those which are linked to immune system during pregnancy. Many cytokines has been found to be elevated in pregnant females with PE which could be the possible reason for the development of this disorder (Benyo et al., 2008). Levels of TNF-*α* in serum are higher in preeclamptic pregnant female then normal pregnant females (Benyo et al., 2008). The high levels of TNF-*α* in serum causative of growth, succession, and complications of atherosclerosis. It affects the synthesis of endothelial nitric oxide synthase (eNOS) which results in nitric oxide (NO) production resulting endothelial function undermined. Physiological induction of TNF-α is defensive, but increase in production cause direct damage to vascular endothelial cell & reduction of regional blood flow (Hunt, 1989).

The genomic position of *TNF-α* is on human chromosome 6p21.3, located near the cluster within the class III region of major Histocompatibility complex (MHC). Many different microsatellite and single nucleotide polymorphism (SNPs) are important in inflammatory response causing preeclampsia. Among all SNPs within the *TNF-α* promoter from which, the 308 G to A polymorphism has been the studied frequently (Hajeer et al., 2001). This study, is designed to analyze the polymorphisms within one of the candidate gene for preeclampsia i.e. *TNF-α* G-308A SNP variant in the promoter region. This is a case control study in which polymorphism in pregnant females with PE has compared with pregnant females without PE.

MATERIALS & METHODS

A total of 100 blood samples of pregnant women were included in this study, in which fifty blood samples were having severe PE while other fifty were randomly selected having no sign of PE, from Jinnah hospital, Johar Town, Lahore. The control group of 50 women blood sample has no history of hypertension and proteinuria during pregnancy and they have given birth to healthy fetus of normal size and weight also having healthy full term gestational age. These samples were collected from September 2016 to March 2018. The Blood samples (5ml) were collected from each patient in EDTA vials and these were analyzed for all baseline diagnostic tests including complete blood count (CBC), PTT APTT, (Partial thromboplastin time and Activated partial thromboplastin time), Hepatitis B and C screening in the diagnostic lab of Jinnah Hospital, Allama Iqbal Medical College, Lahore.

Extraction of Genomic DNA was done by using salting out method (Helms et al., 1990). The polymorphism *TNF-α* (G-308A) in the promoter region was screened by polymerase chain reaction (PCR)-based methods. The set of primers used for this purpose is as follows:

Forward primer:
5'AGGCAATAGGTTTTGAGGGCCAT-3',
Reverse primer:
5'ACACTCCCCATCCTCCCGGCT-3
(Farnaz et al., 2012)

The PCR reaction of 25 µl holds in PCR buffer $(NH_4)_2SO_4$, dNTPs 2.5mM, $MgCl_2$ 25mM, each primer 10µM, Taq DNA polymerase 0.1 µl and remaining volume made up with distilled water. The followed PCR cycle is: denaturation at 95°C for 2 min, elongation follows 35 cycles at 95for 1 min, 62 °C for 1 min, and finally extension at 72°C for 1 min and 72for 5 min. The fragment of 117bp was obtained after amplification, the 4µl of PCR product is mixed with 2µl bromophenol blue (dye) prior to loading on gel then it was analyzed on 2.0% agarose gel stained with ethidium bromide. The gel was documented by using Gel Doc System (Dolphin). Then the gene clean from agarose gel was performed by using, DNA recovery kit (Vivantis). For RFLP (Restriction fragment length polymorphism), the PCR product of TNF-α (117 bp) were digested with NcoI endonuclease (New England biolabs, Ipswich, MA, USA).

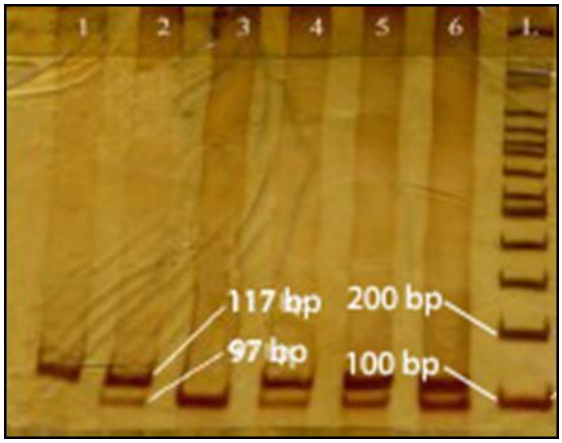

Figure 1: RFLP results, Analysis of TNF-α G-308A promoter polymorphism in patients with preeclampsia; Lane 6: Homozygote for Allele G (G/G); Lane 5, 3, 3 & 1: Heterozygote g (G/A); M: Ladder 50bp. 17% polyacrylamide gel stained with silver nitrate

The protocol of digestion is as follows: total reaction of 50µl was prepared, for this purpose 5µl 10X NEB buffer was added in an eppendorf then added 1 µl of restriction enzyme i.e. NcoI, then added 10µl of PCR product obtained after gene recovery from agarose gel and final volume of 50 ul was made up by double distilled water. The vial containing restriction digestion reaction was incubated at 37°C for 30 minutes. The polyacrylamide gel (17%) was prepared and then resulting digested fragments were subject to electrophoresis and then stained with silver nitrate.

RESULTS AND DISCUSSION

Preeclampsia (PE) is the most common serious syndrome during pregnancy and is the most common cause of pregnancy-induced hypertension and proteinuria after the 20th week of gestation. During PE, the immune system of mother shift towards T-helper type 1, immunity where in result, T-lymphocytes produce proinflammatory cytokines (Jonsson et al., 2006).

TNF-α is a proinflammatory cytokine, for this purpose, this study was designed to find out the relation between *TNF-α* polymorphism and preeclampsia in 50 preeclamptic females of Pakistan. This was case control study in which subjects are pregnant females with preeclampsia and controls included females having normal health conditions during pregnancy. The subject group is with elevated blood pressure having mean value 160.5±5.2 mmHg (systolic blood pressure) and in control group was 120.5±6.5 mmHg. The gestational age of females with preeclampsia was 35±1 weeks and control group were 36±4 weeks. Polymorphism of G308A, in the promoter region of the TNF-α gene, was notably associated with pre-eclampsia (*p* -value is 0.002). The TNF-*α* gene, at position 308 in promoter region i.e. the variant genotype (GG + AG) is more ubiquitous in

the patient group (52.0, 48.0%) compared with controls (86.0, 14.0 %) $P < 0.001$. The frequency of the TNF-α 308-GA allele was significantly higher in patients with preeclampsia than in control subjects as shown in tables below:

Table 1: Genotype frequencies of TNF-α (G308A) polymorphism.

SNP variant *TNF-α* (G308A) rs1800629	**Control group** (females with normal gestational period) N=50	**Patients group** (females with severe symptoms of preeclampsia) N=50	**Control group** (%age)	**Patient group** (%age)	***p*-value**
GG (Normal risk)	43	26	86	52	0.001
GA (High risk of certain disease)	07	24	14	48	
AA (Higher risk of certain disease)	0	0	0	0	

Table 2: Allele frequencies of G308A polymorphism, rs1800629.

TNF- α **SNP variant in promoter region G308A)**	**Preeclampsia Patients (subjects) N= 50**	**Allelic Frequency of preeclampsia patients (% age)**	**Females with normal gestational period (Controls) N= 50**	**Allelic Frequency of control group (%age)**	***p*-value**
Allele A	19	23.9	7	7	0.002
Allele G	81	76.1	93	93	

The study described, increased maternal serum levels of inflammatory cytokines, TNF-α in females having PE compared with healthy pregnant women as mentioned in table 1 and Table 2. Due to endothelial damage and endothelial dysfunction which results in excessive maternal inflammatory response to pregnancy by which there was increase in serum proinflammatory molecules, cytokines, and adhesion molecules which were considered as key pathophysiological parameters for PE (Redman et al., 2005; Laskowska et al., 2007).

According to our findings, there is a significant correlation between inflammation promoting genotypes of TNF-α and PE. The result of this study showed the high levels in serum of TNF-α related with the severity of PE. Complete blood count in Women with PE can have higher WBCs counts and also high level of neutrophils in complete blood picture in comparison to normal pregnant group (Jaramillo et al., 2001). One of the possible mechanisms in PE is the endothelial dysfunction in pregnant women due to increased production of TNF-α which, in turn, result in abnormal activation of neutrophils & monocytes. A research work in this regard by Sacks *et al.*, 1998 wires the elevation in leukocytes counts.

Another study has been done on twenty four subjects has shown that high levels of TNF-α in plasma of mother has high value in contrast with women suffering from PE. Another statistical analysis based on 160 patients with preeclampsia compared to 100 normal patients described that TNF-α (G308A) allelic frequency are appreciably high in women with PE. These findings hold up this present study.

CONCLUSION

In this study beside of limitations, that is undersized sample size which influences the precision of this study, it can be warily concluded that: TNF- α SNP variant G308A in promoter region can be used as a marker of tendency to PE in our population.

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