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TNFα Gene Polymorphism in Idiopathic Pulmonary Fibrosis

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ABSTRACT

Background: *Idiopathic pulmonary fibrosis (IPF) is characterized by a chronic inflammatory process and abnormal wound healing. Tumor Necrosis Factor alpha (TNFα) is considered to play a key role in fibroblast proliferation and increased collagen synthesis. It appears that there is a genetic predisposition to IPF. The genetic associations of TNF-α with IPF have been reported in different cohorts and revealing conflicting results. This study was conducted to evaluate the association of TNF- alpha-308 G/A polymorphism with IPF in Iranian patient by PCR-RFLP method.*

Materials and Methods: *TNFα gene polymorphism at position 308 G/A was examined on DNA extract of 41 cases with IPF defined clinically, radiologically and histologically and compared with 82 unrelated healthy controls who were kidney donors.*

Results: *The understudy population included 20 males and 21 females with the mean age of 50.4 yrs. Data showed that the frequencies of G allele (NO:72) and A allele (NO:10) were 87% and 12% respectively. The frequency of G/G genotype (NO:31) was 76%, followed by G/A (No:10) being 24%.we had no A/A genotype.*

Conclusion: *There was no association between TNF alpha-308 G/A polymorphism and IPF in Iranian patients.* ***(Tanaffos 2006; 5(1): 25-30)***

Key words: *TNF alpha, IPF, Gene, Polymorphism*

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic interstitial lung disease characterized by abnormal wound healing in the lung, in response to multiple microscopic sites of alveolar epithelial injury and activation (1). Although the recent theories about pathogenesis of IPF is crosstalk between epithelial and mesanchymal cells, there is still a strong link between the expression of cytokines and development of pulmonary fibrosis (2, 3, 4, 5). Among numerous cytokines, TNF alpha is thought to play a key role as mediator of inflammation and cellular immune response. This pluripotent cytokine stimulates fibroblasts toward proliferation and increased collagen synthesis (6-9). It was one of the earliest cytokines implicated in the pathogenesis of fibrous lung disease and polymorphism of this cytokine has been significantly associated with increased risk of developing lung fibrosis (10, 11). Recent reports demonstrate that genetic polymorphism leads to inter-individual differences in cytokine expression (12). It is also

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suggested that about 60% of variations in TNF alpha production is considered to be genetically determined (13). The gene coding for TNF alpha is located on chromosome six in the class III region of the major histocompatibility complex. Several biallelic polymorphisms of this gene are known, including the TNF-α-308 G/A gene polymorphism, which is the first discovered TNF-α gene polymorphism, the TNF-α-376 G/A and the-238 G/A gene polymorphisms (14-17).

Against this background, we have evaluated associations between IPF and TNF-α genes polymorphism at location-308 G/A in Iranian patients.

MATERIALS AND METHODS

TNF alpha gene polymorphism was studied in 41 cases and 82 control subjects. The ethic committee of National Research Institute Tuberculosis and Lung Disease approved this study.

Case selection:

Cases were selected according to the following criteria: bilateral crackles on auscultation; exclusion of all known causes or associations with lung fibrosis (occupational exposure, connective tissue disease, positive serologic test, autoimmune markers), presence of typical features on chest high- resolution computed tomography (HRCT); and a restrictive pulmonary function test and/ or reduced gas transfer measurements. The diagnoses of all selected patients were histologically approved. To assess the DNA extract, we used peripheral blood in 15 cases and in the remaining 26, we used DNA extract of formalin fix paraffin embedded blocks.

Control selection:

Controls were unrelated kidney donors who did not have any abnormalities regarding their physical examination, family history, chest x ray, urine analysis, and routine laboratory blood tests.

DNA Extraction:

5 milliliters of whole peripheral blood form 15 cases of IPF patients were collected in EDTA content tube then stored at -4°C until DNA extraction. In the remaining 26 cases, we used paraffin block of open lung biopsy specimens with histological findings of UIP. We used standard phenol-chloroform procedure for extraction of DNA from whole blood (18), for paraffin block DNA was extracted from 3μm tissue sections. To avoid cross contamination of samples the microtome blade was carefully cleaned with xylene between sectioning of blocks and scalpel changed for each case. Paraffin was removed with two rounds of warm xylene extraction (65°C) followed by two 100% ethanol washes. After high speed centrifugation, samples were rehydrated by 70% and 50% ethanol, then while shaking, incubated overnight at 56 °C with digestion buffer (50 mM Tris-HCL PH: 8.5, 1mM EDTA, 1% SDS, 0.5% Tween 20, 0.2mg/ml proteinase K). proteinase K inactivated at 95°C for 8 minutes, then equal volume of phenol-chloroform (1:1) was added mixed gently and centrifuged. The aqueous phase was mixed with an equal volume of chloroform. DNA was precipitate from aqueous phase by an equal volume of 100% ethanol in the present of 40UI of 3 Molar Na-acetate (pH: 5.2). the precipitated DNA was washed with 70% ethanol to remove the salt.. the pellets were air-dried for 10 minutes. Dissolved in deionized distilled water. Integrity of purified DNA and absence of inhibitors of Taq DNA polymerase were assessed by the human beta globin gene specific primer.

PCR and RFLP

A pair of primers 5'-AGGCCATAGGTTTTGAGGGCCAT-3 as forward primer and 5'-TCCTCCCTGCTCCGATTCCG-3' as reverse primer (synthesized by Sinagene Company) were employed to amplify a107 bp fragment containing the variable-308 nucleotide of the humans

TNF-α promoter.

The sense primer was modified to incorporate the polymorphic site into an NCOI restriction site, as previously described by other workers (14). One microliter of genomic DNA was amplified with 1U superTaq DNA polymerase (superTaq company, England) in 25µl of 70nM tris-hydroxymethyl-amino methane (Tris)-HCL containing 20mM KCL,2mM $MgCl_2$, 200µM of each deoxyribonucleoside triphosphate, dNTP (Fermantase) and 50 pmol of each primer. Amplification was done for 35 cycles with denaturation at 94°C for 1 min, annealing at 60° for 1 min and extension at 72° for 1 min. Ten microliters of the product was digested with 5 U NCOI (Gene Craft) for 4h, then subjected to electrophoresis on 3.5% agarosis, sample of them was run on 10% acryl amide gel stained with ethidium bromide. The TNF1(G) allele gives two fragments of 87bp and 20bp and the TNF2 (A) allele gives a single 107bp fragment. Therefore a homozygote for the TNF1 allele (TNF1/1) (G/G) gives two bands whereas a homozygote for the TNF2 allele (TNF2/2) (A/A) gives one band. The heterozygote (TNF 1/2) (G/A) gives three bands (figure 1 and 2).

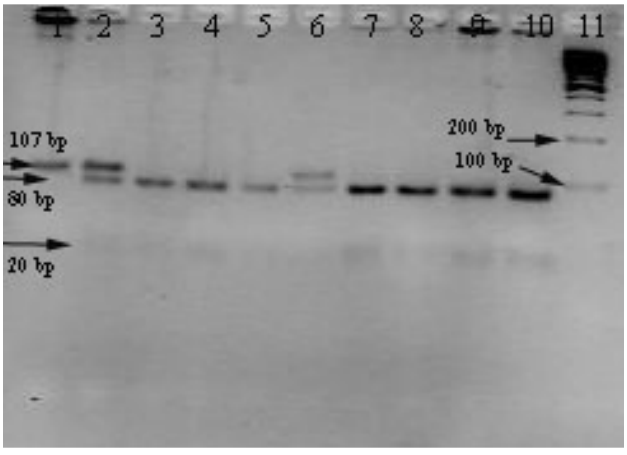

Figure 1. Result of digestion sample on electrophoresis on 3.5% agarosis: 1) uncut sample 107 bp, 2) positive heterozygote sample (107bp, 80 and 20 bp-3,4,5,7,8,9,10) cut homozygote sample (80 bp and 20 bp-11) marker 100 bp ladder.

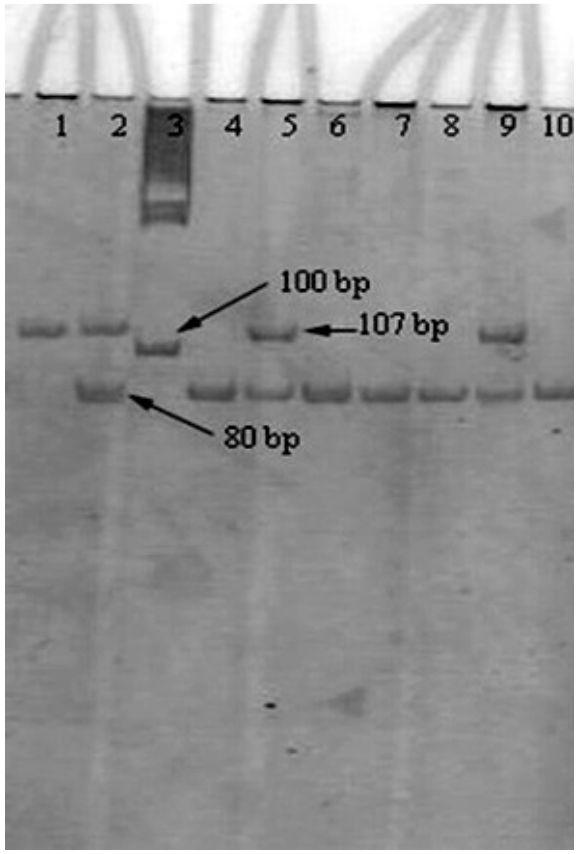

Figure 2. Result of digestion sample on electrophoresis on 10% acryl amide: 1) uncut sample 107 bp, 2) positive heterozygote sample 107 bp, 80 and 20 bp-4,6,7,8,10) cut homozygote sample (80 bp and 20 bp-11) marker 100 bp ladder.

Statistical analysis:

The allele and genotype frequencies were determined in both the patient and the control groups.

We performed the fisher's exact test to compare the allelic frequencies of the two groups. Chi-square test with Yates' correlation was used to compare the genotype frequencies of the two groups.

Data were analyzed using two-tailed test. P-value less than 0.05 was considered statistically significant.

RESULTS

Forty-one cases with the above mentioned criteria were studied. There were 20 males and 21 females with the mean ±SD age of 55.6±13.2 years (range 30-

78 years). The control group consisted of 82 kidney donors, (63 males, 19 females) with a mean ±SD age of 30±7 years. The allele frequencies of TNF alpha gene polymorphism codon (-308) in the IPF population were compared with those in control subjects in order to access difference between the two groups. In IPF patients the frequencies of G allele and A allele were 87% (n:72) and 12% (n:10) respectively. Table 1 shows distribution of alleles in IPF cases and control group. There was no significant difference between the patient and the control groups.

Table 1. TNFα allele's frequency in -308 promoter gene in case and control.

Group	(G) n(%)	(A)n(%)	P (pcorr)
Control (n=82)	149(91)	15(9)	-
IPF (n=41)	72(87)	10(12)	<0.05 NS

NS: Not Significant

The frequent genotype in the patient group was G/G (n:31). When compared with the genotype frequency in control group (table 2), we did not find any statistically significant difference between the patient and the control groups [CI95%: {-0.25-0.06}]. Pattern of genotype distribution was the same in both patient and control groups.

Table 2. TNFα genotype frequency in -308 promoter gene in case and control.

Group	(G/G)	(G/A)	(A/A)	P (pcorr)
Control (n=82)	67(82)	15(18.3)	0(0)	-
IPF (n=41)	31(76)	10(24)	0(0)	<0.05 NS

NS: Not Significant

DISCUSSION

In this study the prevalent allele in IPF patients was G and the frequent genotype was G/G. We did not find any difference regarding allele or genotype frequencies in patient and control groups. This was also in accord with the results of Pantelidis et al. and Whyte et al. studies (8, 13). In contrast to our finding, Riha et al working on 22 patients with IPF demonstrated a significant association between TNF and IPF (10). Precise clinical phenotyping is essential for assessing the genetic influence on diffuse lung disease (19). The possible explanation for these different findings in various reports may be the fact that IPF is a heterogeneous group of disease and criteria of patient selection may not be definitely identical. Ideally, the large number of patients and control subjects should be studied for evaluation of gene polymorphism. Another explanation for this discrepancy between different studies might be the small number of cases in all studied groups which may account for controversial results, but this fact is inevitable due to rarity and fatality of IPF.

Population donor controls have been used in many genetic studies (10, 13). In this study, the control subjects were selected from the kidney donors whose DNA extracts were available. The strength points of using this type of control group are as follows:

1) These subjects are healthy and have been thoroughly evaluated.
2) These subjects come from the general population of the community; therefore, their genotype frequencies are the representatives of the general population as a whole.

Although the mean age of cases in this study was higher than controls, as long as the aim of study was to determine the genotype which is constant all through the life, this difference in age between the two groups appears to be insignificant.

The second issue in this regard is the probable risk of occurrence of pulmonary fibrosis in older ages in our controls. Considering the prevalence rate of this disease as 1 to 5 in 100/000, this risk might be

less considered (20).

Due to rarity of this disease or death of some known cases, we used formalin fixed paraffin embedded blocks of open lung biopsy specimens in 26 cases. Since the aim of this study was determining the genotype, using two types of specimens (lung biopsy specimen in 26 cases and blood specimen in 15 cases) for DNA extraction does not seem to affect the results. But in using paraffin embedded blocks risk of PCR-inhibitors should be considered. To overcome this problem integrity of purified DNA and absence of inhibitors of Taq DNA polymerase were assessed by the human beta globulin gene specific primer.

We used PCR-RFLP technique for determination of genotyping of TNF alpha which was similar to the technique used in all previous studies.

CONCLUSION

- There was no association between TNF alpha-308 G/A polymorphism and IPF in Iranian patients in this study.
- We suggest selecting age-matched control group for evaluation of this polymorphism in IPF patients.

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