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Idiopathic Pulmonary Fibrosis and Mutation of TGF-beta Gene, Codon 10

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ABSTRACT

Background: *Idiopathic pulmonary fibrosis (IPF) is associated with histological appearance of usual interstitial pneumonia. These fibrotic changes in lung interstitium are mostly attributed to cytokine production such as TGFβ which stimulate migration and differentiation of fibroblast to myofibroblasts. The polymorphism of TGFβ gene was found to be associated with development of IPF. We investigated whether TGFβ1 gene polymorphism in codon 10 is associated with interstitial pulmonary fibrosis in Iranian population.*

Materials and Methods: *The different genotypes of TGFβ1 at (+ 870) position (in codon 10) was studied in41 cases and 83 control subjects. The allele specific PCR method was used for genotyping.*

Results: *In the patient group, the frequency of T allele (NO: 58) was 70.7% and C allele (NO: 24) was 29.3%. The frequency of TT genotype (NO: 20) was 48.8%, followed by T/C (NO: 18) 43.9% and CC (No. 3) 7.3% while in the control group, the frequency of T allele (N:117) was approximately 70.5% and C allele (NO: 49) was 29.5%. The frequency of TT genotype in control group (NO: 41) was 49.4%, followed by T/C (NO: 35) 42.2% and C/C (NO: 7)8.4%*

Conclusion: *In comparison with the control group, there was no association between TGFβ1 codon 10 T/C polymorphism in our cases with IPF.* ***(Tanaffos 2009; 8(1): 23-28)***

Key words: *Idiopathic pulmonary fibrosis,TGF-beta, Gene*

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic and poorly understood disorder which is associated with histological appearance of usual interstitial pneumonia (1, 2). Cumulative effects of several cytokins and growth factors expression determine the lung phenotype in IPF (1). The main pathologic mechanisms in IPF include epithelial cell microinjuries and activation, fibroblast migration and differentiation to myofibroblasts. Fibroblast and myofibroblast aggregation disturbs alveolar reepithelialization, epithelial apoptosis and finally

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results in exaggerated matrix accumulation. These changes are mostly attributed to cytokines and growth factor. TGFβ is one of the cytokines involved in this process which stimulates differentiation of myofibroblast, a hallmark of fibrotic disorder and epithelial mesenchymal transition (3). TGFβ has implicated as a key point in this process and is expressed at high levels in lung tissue of patients suffering IPF (4). It is also reported that the polymorphism of TGFβ gene has been associated with development of disease (1). The human gene encoding TGFβ1 is located on chromosome 19 (5). Eight polymorphisms in the TGFβ1 gene have been identified (5, 6), one of them is T→C transition at +870 nucleotide in the region encoding the signal sequence which results in a Leu-to –Pro substitution at amino acid 10 of exon 1 (7). This polymorphism in codon 10 (T→C) affects the amino acid-coding sequence and modulates TGFβ1 production (7, 8). Recent reports demonstrate that genetic polymorphism leads to inter-individual differences in cytokine expression (9). While it was reported that the polymorphism of TGFβ1 gene was found to be associated with concentration of TGFβ1 and development of idiopathic pulmonary fibrosis (8, 10), Xaubet et al. did not find TGFβ1 gene polymorphism in this disease instead they suggested that polymorphism of this gene may affect disease progression (9).

In this background we investigated whether TGFβ1 gene polymorphism in codon 10 is associated with interstitial pulmonary fibrosis in Iranian population.

MATERIALS AND METHODS

TGF beta gene polymorphism was studied in 41 cases and 83 control subjects. The ethic committee of "National Research Institute of Tuberculosis and Lung Disease" approved this study.

Case selection

Cases were selected according to the following criteria: bilateral crackles on auscultation, presence of typical features on chest high-resolution computed tomography (HRCT), a restrictive pulmonary function test and/ or reduced gas transfer measurements. All known cases of lung fibrosis or those with conditions associated with lung fibrosis (occupational exposure, connective tissue disease, positive serologic test, and autoimmune markers) were excluded from the study. 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 fixed paraffin embedded blocks.

Control selection

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

DNA Extraction

5 milliliters of whole peripheral blood from 15 cases of IPF patients were collected in EDTA content tube and then stored at -4°C until DNA extraction. In the remaining 26 cases we used paraffin embedded block of open lung biopsy specimens with histological findings of usual interstitial pneumonia. We used standard phenol-chlorophorm procedure for extraction of DNA from whole blood (11). 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 block and scalpel was 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 (50mM Tris-HCL pH: 8.5 1mM EDTA, 1% SDS, 5% Tween 20, 0.2 mg/ml Proteinase-K). Proteinase-K was inactivated at 95°C for 8 minutes, and 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 precipitated from aqueous phase by an equal volume of 100% ethanol in the presence of 40 UI of 3 molar Na-acetate (PH: 5.2). The precipitated DNA was washed with 70% ethanol to remove the salt. The plates were air-dried for 10 minutes and then dissolved in deionized distilled water. Integrity of purified DNA and absence of inhibitors of Taq polymerase were assessed by the human beta-globin gene specific primer.

Identification of polymorphisms in the TGFβ1 gene

Polymorphisms in codon 10 of the TGFβ1 gene were determined according to the method of Suthanthiran M and coworkers (12). Genomic DNA was purified from peripheral leukocytes by phenol chloroform technique. Fragment of TGFβ1 gene containing codon 10 region was amplified by allele specific polymerase chain reaction. Two complimentary reactions were used for each allele, consisting of target DNA, allele specific ARMS primer, and the common primer (12, 13). Primers are used in a way that the 3 base is at the site of a particular mutation. Therefore, amplification of this product only occurs in the presence or absence of this mutation. The test can distinguish between individuals who are homozygote or heterozygote for all mutations (14). Sequences used as oligonucleotide primers for detection of codon 10 polymorphism by ARMS PCR were consisted of: Sense (common) primer 5'-CACTGCGCCCTTCTCCCTGTG-3', Antisense (ARMS) C-primer (pro 10 allele): 5'-ACAGCAGCGGTAGCAGCAGGG-3' and Antisense (ARMS) T-primer (Leu 10 allele): 5'-CACAGCAGCGGTAGCAGCAGGA-3'. These primers were employed to amplify a 200 fragment containing the variable (+870) nucleotide of human TGFβ1 codon 10 (12). 50 ng of genomic DNA was amplified with 1u DNA polymerase enzyme (boiline Led, London, UK) in 25 µl of 70 mM tris-hydroxymethyl-amino methane (Tris)-Hcl containing 20 mM KCL, 2mM MgCl2, 200 µM of each deoxyribonucleoside triphosphate (dNTP) and 50pM of each primer. PCR contamination was checked by the inclusion of negative control subjects. Extracted DNA of cases and controls were initially denatured at 94°C for 3 minutes and subjected to 35 cycles of amplification with 20 seconds of denaturing at 94°C, 20 seconds of annealing at 67°C and 20 seconds of extension at 72°C. PCR products were fractioned in 2% polyacrylamide gels and visualized by ultraviolet fluorescence after ethidium bromide staining. The pro allele in codon 10 is defined by the presence of a 200-bp fragment after amplification by C-primer, and the leu allele in codon 10 is detected by a 200-bp fragment after amplification by T-primer.

RESULTS

Forty-one patients with the diagnosis of IPF were studied including 21 males and 20 females with the mean age of 55.6±13.2 years (range 30-78 years). The control group consisted of 83 kidney donors, 63 males and 20 females with a mean age of 30±7 years.

The allele frequencies of codon 10 TGFβ1 gene polymorphisms (+870) in the IPF population were compared with those in control subjects to assess the difference between the two groups. In IPF patients, the frequency of allele T and allele C was 70.7% (n:58) and 29.3% (n:24), respectively. Table 1 shows distribution of alleles in IPF cases and controls. There was no significant difference between the patient and the control group ($p>0.05$). Table 2 shows frequencies of genotyping in the IPF cases and the control group. The frequent genotype in the

patient group was T/T. we did not find statistically significant difference between the patient and the control groups. Pattern of genotype distribution (T/T, T/C, C/C) was also approximately the same in the patient and the control groups.

Table 1. Transforming growth factor-β1 allelic frequencies in Iranian population (%).

Allele (codon 10)	Control Subjects (n=83)	IPF (n=41)
T(Leu)	70.5(117/166)	70.7(58/82)
C(Pro)	29.5(49/166)	29.3(24/82)
Total	166 allele	82 allele

Table 2. Transforming growth factor β1 genotype frequencies in Iranian population (%).

Genotype (codon 10)	Control Subjects (n=83)	IPF (n=41)
T/T	49.4(41/83)	48.8(20/41)
T/C	42.2(35/83)	43.9(18/41)
C/C	8.4(7/83)	7.3(3/41)
Total	83	41

DISCUSSION

Researchers are still working on identifying genetic susceptibility of interstitial lung disease (3). There are several areas that require precise designing for genetic studies on diffuse lung disease. One of them is an approach that involves association of genetic polymorphism with disease by comparison with controls (15). There have been a few studies on association of genetic polymorphism with IPF. Researchers addressed the association of INFα gene polymorphism with IPF (16, 17, 18). There were also reports about association of polymorphism of IL-6 with this disease (15). The approach to genetic predisposition to diffuse lung disease encountered with a number of problems because several genetic loci may contribute to developing disease in variable strength (15). In this study we observed no significant difference in the allele and genotype frequencies of codon 10 in the TGFβ1 gene between patients and healthy subjects. Considering the certain role of TGFβ1 in induction and establishment of fibrosis in the interstitium of lung, it seems that there will be a number of different genes polymorphisms at one or more genetic loci, rather than single polymorphism affecting TGFβ1 expression (15). For example, pigment epithelium derived factor which is up-regulated in IPF is a TGFβ1 target gene. It was demonstrated that this factor had strong angiostatic properties and is co-localized with TGFβ1 within fibrotic interstitium and within alveolar epithelium overlying the fibroblastic foci in IPF (3). There is also reported that TGFβ1 is increased in lung parenchyma due to local production of angiotensin II via the action of angiotensin converting enzyme. Polymorphism of this gene was reported by two researchers (19, 20). Ideally, large number of patients and control subjects should be studied for evaluation of gene polymorphism (15). A variety of reasons including small number of cases and careful case selection may influence in the result of genetic evaluation in IPF. Our under-study cases (41 patients) although low in number but were about the same or even higher than other studies on gene polymorphism in IPF patients (19). The reason for low number of cases in all of these investigations is the rarity and fatality of IPF (21). Defining of clinical phenotype is also an important prerequisite for identification of genetic determinants associated with disease. Although the criteria of patient selection in our study are similar to those of others, it may not be definitely identical (21). A number of considerations must be addressed in this study:

First: we used DNA extraction of blood specimens of kidney donors as controls. This type of donor control has been used in many genetic studies (22, 23). One important point in using this type of controls is that their genotype frequencies are representative of the general population.

Second: the mean age of cases in this study was higher than controls, but since the aim of this study was to determine genotype which is constant all through the life, this difference in age between the two groups appears to be insignificant. Another 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 significant (24).

Third: Using two types of specimen (formalin fixed paraffin embedded blocks of open lung biopsy in 26 cases and blood specimen in 15 cases) for DNA extraction does not seem to affect the result because the genotype content in different types of cells in one person is the same.

CONCLUSION

There was no association between TGFβ codon 10 T/C polymorphism with IPF in our cases. We suggest using age-matched control group for evaluation of gene polymorphism in IPF. Due to the rarity of disease, for precise evaluation of influence of gene polymorphism on IPF close collaboration of centers for increasing the number of cases is recommended.

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