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Assessment of Angiotensin-Converting Enzyme Gene in Idiopathic Pulmonary Fibrosis

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ABSTRACT

Background: *Interstitial pulmonary fibrosis (IPF) is a progressive fibrotic interstitial lung disease with a distinct histopathological form referred to as usual interstitial pneumonia (UIP). Evidence has indicated that a local renin-angiotensin system is present in distal lung parenchyma. Expression of the component of this system is present in a number of fibrotic lung diseases. In this study, we assessed the association of Insertion/Deletion (I/D) polymorphism of angiotensin-converting enzyme (ACE) gene in IPF.*

Materials and Methods: *By using semi-nested PCR, we determined the I/D polymorphism of ACE gene in 23 paraffin-embedded open lung biopsy specimens from patients having clinical and imaging findings of IPF and pathologic diagnosis of UIP at National Research Institute of Tuberculosis and Lung Disease (NRITLD). Afterwards, we compared the results with I/D polymorphism of ACE gene in a healthy control group (n= 88).*

Results: *The frequency of I allele was 71.7%(33 out of 46) and the frequency of D allele was 28.3% (13 out of 46). The frequent genotype was I/D (56.5 %) which was statistically significant comparing with healthy group (27.3 %). We had no D/D genotype .There was a difference in the distribution pattern of ACE genotype between patients and controls (P<0.05).*

Conclusion: *Our study revealed an association between carriage of I allele and I/D genotype in IPF.* ***(Tanaffos 2007; 6(2): 20-26)***

Key words: *Angiotensin-converting enzyme, Interstitial lung disease, Pulmonary fibrosis, Usual interstitial pneumonia.*

INTRODUCTION

A wide range of acute and chronic pulmonary disorders, with known and unknown etiologies are capable of affecting lung parenchyma with variable amounts of inflammation and fibrosis. The mechanism of destruction and repair of lung parenchyma by the process of fibrosis has centered on various enzymes and inflammatory mediators (Figure1) (1, 2). Genetic variation in these enzymes and mediators may alter the repair process of the lungs toward more production of fibrogenic cytokines eventually leading to fibrosis of lung

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interstitium (3).

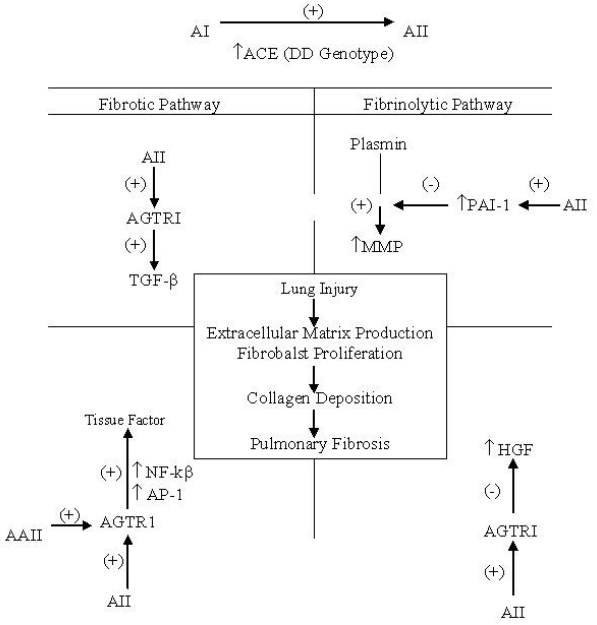

Figure 1. Summary of the interaction of ACE and AII with the proposed fibrotic and fibrinolytic pathway of pulmonary fibrosis. Interactions with up-regulation of TGF-β and tissue factor are involved in the fibrotic pathway and interactions with down-regulation of plasmin, and matrix metalloproteinases. HGF is involved in the fibrinolytic pathway. AAII, angiotensin II autoantibodies; AP-1, activator protein 1; MMP, matrix metalloproteinases; PAi-1, plasminogen activator inhibitor 1; NF-κB, nuclear factor –κB and NO, nitric oxide have not been shown in models of lung injury or pulmonary fibrosis.(1)

Evidence has indicated that a local intrinsic rennin-angiotensin system (RAS) is expressed in distal lung parenchyma. Expression of the components of this system and elevation of angiotensin-converting enzyme (ACE) in lung parenchyma in a number of fibrotic lung diseases suggest that ACE may play a role in the pathogenesis of pulmonary fibrosis (4, 5). In addition, transforming growth factor beta 1 (TGF-ß1) which is a cytokine that plays a significant role in fibrotic patterns of the lungs is increased in lung parenchyma due to production of local angiotensin II (A II) via the action of ACE (1- 3, 6-8).

Several studies have shown that approximately 50% variability of ACE production among individuals is due to insertion (I) or deletion (D) of a 287 – base pair (bp) sequence of DNA in intron 16 of ACE gene on chromosome 17(6,7); such that any person can be defined as I/I, I/D or D/D. Based on several studies, the frequency of D and I alleles of ACE gene differs in various ethnic groups (9-12).

Several reports have indicated the association of I/D polymorphism of ACE gene in sarcoidosis (13-15). It is possible that this association may also be present in idiopathic pulmonary fibrosis (IPF). IPF is currently recognized as both a prototype of interstitial lung disease associated with pulmonary fibrosis, and a distinct histopathological form referred to as usual interstitial pneumonia (UIP) (16).

In this study, due to the availability of tissue specimen from patients suffering from pulmonary fibrosis with histologic patterns of UIP, and also availability of a healthy control group, we subjected them to an ongoing investigation by evaluating their ACE gene polymorphism in DNA extracted from paraffin-embedded lung tissue.

MATERIALS AND METHODS

Case selection

The ethics committee of the National Research Institute of Tuberculosis and Lung Disease (NRITLD) approved this study to investigate the archival paraffin-embedded blocks. All open lung biopsy specimens with a diagnosis of pulmonary fibrosis were selected from the pathology archive during the period of 1999- 2004. All lung biopsies were reviewed by two pathologists, independent of clinical and imaging findings. Cases with histopathologic diagnosis consistent with UIP were included (16).

Clinical information, including onset of symptoms, progression degree, pulmonary function test (PFT), imaging findings and laboratory tests, were reviewed.

Patients with a history of acute onset disease, occupational exposure, history of connective tissue disease (CTD) or positive serologic tests for

autoimmune markers were not included. Radiation history or known exposure to asbestos, drugs or other fibrogenic and toxic agents, were also reasons for exclusion from the study.

Radiographic findings of all patients were reviewed and cases with diffuse basilar interstitial pattern were enrolled in the study.

Controls

The healthy control group included 88 healthy individuals who were kidney donors (70 males, 18 females, mean age 30.7 yrs). Their ACE genotyping was performed on DNA extracted from 5 cc EDTA containing blood, after obtaining consent.

PCR Method

We used archival material from formalin-fixed paraffin-embedded blocks for DNA extraction. This was based on an investigation carried out by Arima et al. (Hisayama Study; they confirmed the accuracy of ACE I/D genotyping from paraffin-embedded tissues) (17).

DNA EXTRACTION: DNA was extracted from 3µm tissue sections of paraffin blocks by the standard phenol-chloroform procedure after treatment with proteinase K.

To avoid cross-contamination of samples, the microtome blade was carefully cleaned with xylene between sectioning of blocks and the scalpel blade was changed for each case. Paraffin was removed with two rounds of warm xylene extraction (60˚C) followed by two 90% 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% sodium dodecyl sulfate (SDS), 0.5%Tween 20, 0.2mg/ml proteinase K) . Proteinase K was inactivated at 95˚C for 10 minutes, and an 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 the aqueous phase by an equal volume of 100% ethanol in the presence of 40 IU 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 and stored at -20˚C. Contamination monitoring was performed by using blank and water controls.

Integrity of purified DNA and absence of inhibitors of Taq DNA polymerase were assessed by the human beta globin gene specific primer. Semi-nested polymerase chain reaction (PCR) assay was chosen due to the presence of inhibitor in most of the DNA samples.

At initial phase of PCR two primers were used: ACE 1 (5'-cat cct ttc tcc cat ttc tc-3') as forward primer (TIB MOL BIOL, GER) and ACE 3 (5'-att tca gag ctg gaa taa aat t-3') as reverse primer to amplify outside of the insertion sequence of ACE intron 16. In the second phase, the primer ACE 2 (5'-tgg gat tac agg cgt gat aca -3') as forward primer and ACE 3 as reverse primer, were used to detect the insertion (I) or deletion (D) alleles in inner fragment of the ACE gene. The lengths of amplified PCR product were 65 bp and 84 bp for allele I and D, respectively. PCR was performed in a final reaction volume of 25 ul containing 5 ul of isolated DNA, 16.6 mM (NH4)2SO4, 66.7mM Tris-HCL (pH: 8.8), 2.0 mM Mgcl2, 200 uM each deoxynucleoside triphosphate, 10 pmol of each of forward and reverse primers, and 1 u of Taq DNA polymerase (Roche, GER). For the second step of PCR, the reagents were the same except for the primers. The genotype was determined from amplified products.

Conditions and thermocycling parameters were identical for both the first and the second amplification. After heating at 95˚C for 5 minutes the mixture was subjected to 40 cycles of PCR amplification (denaturation at 95˚C for 1 minute, annealing at 53˚C for 1 minute, extension at 72˚C for

3 minutes) .The last cycle was followed by an additional 5-minutes of extension at 72˚C. The amplified fragments were separated by electrophoresis on 10% acrylamide gel and stained with ethidium bromide and visualized on a UV transilluminator (Uvitec, UK).The results were photographed and stored in a computer (Figure 2).

STATISTICAL METHODS

The difference in allele and genotype frequencies between control subjects and patients was tested by the chi-square test without Yates' correlation, Fisher's exact test, the student's t-test and the Mann-Whitney U test. For all tests, a P-value of 0.05 or less was considered significant. All statistical analyses were performed by the use of SAS statistical software (version 9.01, SAS Institute, Cary, N.C)

RESULTS

Patient Information

Out of 96 cases with the diagnosis of lung fibrosis, only 23 cases with definite histologic and clinical criteria of UIP were selected. The average age of the patients was 51.78 years with a range of 30 to 71 years and median of 53 years. There were 10 (43.48%) women and 13 (56.52%) men in the population under investigation. Five (21.7%) cases were current or former smokers. Duration of symptoms from the onset of disease to the time of open lung biopsy averaged 12.34 months for all patients, with a range of 1 to 24 months and a median duration of 12 months. All patients enrolled in the study had restrictive patterns on pulmonary function test (PFT) and the imaging findings were consistent with the pathologic diagnosis.

PCR Findings

The frequencies of I allele and D allele in patients were 71.7% (33 out of 46) and 28.3% (13 out of 46) respectively.

In the healthy control group (88 cases), the frequencies of I allele and D allele were 51.1% (90 of 176) and 48.9% (86 of 176) respectively.

The frequencies of the ACE genotype and alleles for I/I, I/D and D/D in patients and controls are shown in Table 1.

Table 1. Genotype and allele frequencies of the ACE gene in patients with pulmonary fibrosis and the healthy control group

		I/I Genotype	I/D Genotype	D/D Genotype	I Allele	D Allele
Case	No	10	13	0	33	13
	%	43.5	56.5	0	71.7	28.3
Control	No	33	24	31	90	86
	%	37.5	27.3	35.2	51.1	48.9

The frequent genotype for patients was I/D (56.5%) which was statistically significant as compared with healthy control group (27.3 %)(P value <0.05).

There was no correlation between age, gender and smoking with the genotype.

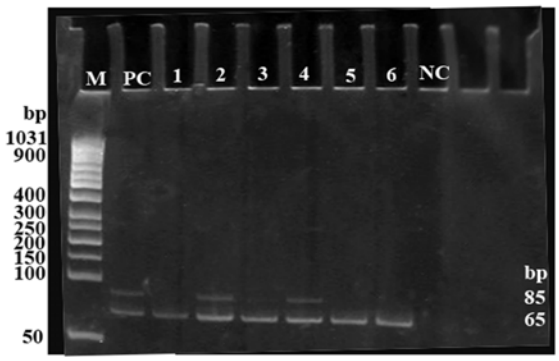

Figure 2. Acrylamide gel electrophoresis (ethidium-bromide-stained) for I/D gene polymorphism in paraffin-embedded specimens by semi-nested PCR. NO. 1, 3, 5, and 6 represent individuals with II genotype. NO. 2 and 4 are for ID genotype. M, PC and NC stand for marker, positive control and negative control, respectively.

DISCUSSION

This study assessed the association of I/D polymorphism of the ACE gene in interstitial pulmonary fibrosis. There are few studies about the association of ACE gene polymorphism and IPF due to the rarity of disease (18).We found that the frequent allele in the patient group was I (71.7%) which was statistically significant as compared to healthy controls ($P< 0.05$, OD = 2.426).This is in contrast to Morrison's finding in the U.S. population who detected 69% D allele in 24 cases of pulmonary fibrosis.(1)The frequent genotype for our patient group was I/D (56.5 %) as compared to a healthy control group (27.3 %) which was also statistically significant ($P< 0.05$). The frequency of this genotype is similar to Morrison's study group (54%). The important difference between these two studies is the absence of D/D genotype in ours, while its frequency was 31% in Morrison's study.

Technical problems may explain some of the controversies in published genetic reports. There are few reports regarding the presence of mistyping of ACE I/D polymorphism using conventional genotyping method. All of these reports addressed the mistyping of D/D genotype (19, 20, 21). Ueda et al. in their investigation reported that from 335 cases with D/D genotype, 10.5% (35 cases) were I/D using nested triple primer PCR (19). To overcome any technical problem, we also used semi-nested triple primer PCR (19, 20). In our study we did not have D/D genotype so this form of mistyping is not considered in our results and the presence of D/D genotype in Morrison's study was confirmed by a second independent PCR step using insertion-specific primer. If we rule out undetectable technical problems in both studies we may suggest racial difference present in ACE genotype polymorphism among IPF patients, which requires further study using a precise design to assess genetic influence of ACE polymorphism on IPF in different races.

IPF may still be considered as a heterogeneous group of diseases with unknown etiology and the same diagnostic findings. (18) Although the criteria of patient selection in these two studies are similar, it may not be definitely identical.

In this study, the control subjects were selected from the kidney donors whose DNA extracts were available. The strength of using this type of control group is that they do come from the same population as the cases and their genotype frequencies are therefore representative of the general population as a whole. Population of donor controls have been used in many genetic studies of respiratory disease, including the major study of IPF by Pantelidis et al. (3, 22). 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 representatives of the general population as a whole.

Although the mean age of the cases in this study was higher than controls, as long as the aim of study was to determine the genotype which is constant through 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 can be disregarded (23). However, the lack of strict age matching may have contributed to variations in genotype frequency; although, in the large number of studies performed to date, no association has been observed between ACE genotype and age (24).

The main limitation of these two studies is the small number of cases which may possibly account for some controversial results observed, but this is

inevitable due to rarity of the disease and death of some known cases.

CONCLUSION

Our study revealed an association between carriage of I allele and I/D genotype in lung fibrosis. Further studies are required to design an effective trial to delineate the precise mechanism of I/D polymorphism involved in the pathogenesis of IPF.

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