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Determination of BMPR2 Expression in Scleroderma Patients Via Real-Time PCR and its Association with the Clinical Course of Patients

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Abstract

Scleroderma is a rarely seen chronic collagen tissue disease which has been first described in the 18th century. It is indicated that the mutations in BMPR2 (*bone morphogenetic protein receptor type II*) gene affected the course of disease.

In this study; determination of BMPR2 gene expression levels via Real Time PCR and its association with the clinical course in scleroderma patients were revealed.

20 Scleroderma diagnosed and 29 healthy individuals were taken into this study. Performing the transformation of cDNA complementary to mRNA isolated from leucocyte cells, DNA amplification was carried out via Real-Time PCR. To determine the relative expression level of BMPR2 gene via Real-Time PCR, Beta actin gene was selected as the reference gene.

Mean expression levels of BMPR2 gene in patient group showed significant decrease in comparison with control group ($Cp<0,05$). Expression levels of patients decreased around 39,46% in comparison with control group. 4 of these 20 patients included into the study diagnosed with PAH (Pulmonary Arterial Hypertension) and gene expression levels of these patients were seen to increase.

Normalized data was analyzed by using mixed model and statistically different groups were determined by Minimal Important Difference (MID) test.

Consequently, in Scleroderma patients BMPR2 gene expression level showed changes from person to person but it decreased by comparison to healthy control group and in patients withPAH that was foundto be higher than the gene expression levels of other patient group.

Keywords: Scleroderma, BMPR2 gene, Beta actin gene, PAH

INTRODUCTION

Systemic sclerosis (SSc) takes the name of "sclerosis" and "derma"which means "hard" and "skin" in greek is characterized by accumulation of the collagen and the other components of matrix in skin and internal organs. This is a complex, heterogeneous, autoimmune connective tissue disease with an unknown source defined by excessive accumulation of other connective tissue macromolecules in skin and various organs [1, 2]. Scleroderma is a rare chronic disease which was first described in the 18th century. First known Scleroderma patient was diagnosed by Dr. Carlo Curzio in 1754, Napoli.

Pathogenesis of this disease is not clearly known. However, immune activation and vasculopathy have the important role for the pathogenesis [3,4]. Inflammatory cell infiltration prescribed notably T-lymphocytes, mast cells and macrophages in skin biopsy of the Scleroderma patients[5]. In addition, elevation of the serum levels of cytokines like tumor necrosis factor alpha (TNF-α), interleukin IL-2, IL-6, IL-8 and its correlation with the volume of skin involvement has been shown in Scleroderma patients [6].

Antibodies antagonistic to complex enzymes of RNA polymerase (RNA-p I,II, III) are also unique autoantibodies for Scleroderma. By comparison with Anti-Scl 70, anti-RNA polymerase antibodies are correlated with extensive skin involvement, more intensive skin thickening and renal involvement [7].

Pulmonary arterial hypertension (PAH) is frequently seen on Scleroderma patients and it is sign to a poor prognosis [8]. PAH occurs around 50% of a frequency on SSc patients. In this case blood pressure in lung can reach to dangerous levels. In some cases, pulmonary hypertension can cause a status named co-pulmonary.As a consequence, lung gets bigger and causes cardiac insufficiency by forcing the heart [9].

Bone Morphogenetic Protein Receptor gene (BMPR2) is an important gene which represses the cell growth in normal lung artery smooth muscle cells and stimulates apoptosis.

BMPR2 gene is located in 2q31-31 part of chromosome and has 13 exons. It has 4 functioned areas: N-terminal ligand binder area, transmembrane area, serine/threonine kinase and cytoplasmic area. Apart from Exon 5, 10, 13, mutation has been reported in all exons. Amino acid shifting, caused by point mutations,ruins receptor function.Generated insult relates to which section, extracellular, transmembrane, serine/threonine kinase or cytoplasmic tail, the mutation effects. Mutations cause interruption in BMP signal path by destroying kinase activity, inhibiting ligand binding and have an impact on heterodimeric dimer formation. BMP path contains the phosphorylation which begins inthe cell surface and extends over nucleus. BMP has two receptors: Type I and type II. Type II receptor phosphorylates type I receptors and type I receptor phosphorylates Smad family (Smad 1/5 or 8) activated by phosphorylation. This activated molecules effect target genes inthe cell nucleus. As a result, endothelium proliferation is inhibited, proteins which take part at control of cell cycle and angiogenesis get formed [10].

Genetic defects responsible for familial PAH which are related to BMPR2 gene mutations. It is a part of receptor TGF-β. More than 40 BMPR2 gene mutations are determined and all of them induce the removal of inhibition of cell proliferation. Besides BMPR2 abnormalities, mutations in different genes are alsoconsidered to have a role on progress of pulmonary hypertension [11].

The aim of this study is to reveal the clinical course of

disease by determining the expression levels of BMPR2 gene by Real Time PCR analysis in Scleroderma patients and its relationship with PAH.

MATERIAL and METHOD

Material

By determining the BMPR2 expression level in Scleroderma patients, association with clinical course of diseaseis aimed. 20 individuals diagnosed with Scleroderma and 29 healthy individuals were takeninto this study.

Method

7 mL of blood sample drew from patients with Scleroderma to a 9 mL of tube thatwas included EDTA. Leucocytes of the blood were separated by using erythrocyte splitting buffer (Red Blood Cell Lysing Buffer, Roche/Germany), from this samples mRNA isolation was performed by High Pure RNA isolation kit (Roche/Germany) by using RNA isolation procedure from blood. Afterwards, by using Transcriptor First Strand cDNA Synthesis kit (Roche/Germany) to convert mRNA to complementary DNA (cDNA),and Universal Probe Library Primer Probe design, analysis of the samples and BMPR2 (Bone morphogenetic protein receptor-2) expression carried out by Lightcycler 480 Real-Time PCR (Roche/Germany).

Real-Time PCR Process

BMPR2 and Beta Actin Real-Time ready catalog assay primer probe design (Roche/Germany) required for BMPR2 expression assay of Scleroderma patients was created over web-based Real-Time ready configurator web site (https://configurator.realtimeready.roche.com/assaysupply_cp/login.jsf). The study was executed with the master mix composition prepared using catalog assays and Lightcycler 480 probe master as master mix. For one reaction in Real-Time PCR; 4.0 µL of ddH_20, 10 µL of reaction enzyme (Probe master), 1.0 µL of Primer-probe mix was prepared and 15 µL was added to wells. After adding 5 µL of cDNA into the master mix composition, it was amplified in the device. Real-Time PCR protocol used for which the amplification is; denaturation at 95°C for 10 min, following 45 loop amplification; cooling at 95°C for 10 sec, at 60°C for 30 sec, at 72°C for 1 sec and at 40°C for 30 sec.

Evaluation was carried out at relative quantitation analysis part of the device. These methods and standard values used were optimized for each patient and based on the standards quantitative results and gene expression levels were estimated.

Analysis of Data

In all the experiments, evaluations, examinations and estimations during this study, housekeeping gene (reference gene, Beta actin) and target gene (BMPR2) assessments were executed with the samples from Scleroderma patients. Consideration of results were obtained by Real-Time PCR was performed using *LightCycler® 480 Software Version 1.5*. Concentration values were estimated based on crossing point (Cp) values of the samples and analyses were carried out according to these data.

Results were estimated with concentration identification method toward $2^{-\Delta\Delta CT}$ crossing point values. In this method, first, approximate concentration values of patient and control group samples were calculated separately for both beta actin and BMPR2. Then, by comparing mean value of expression level of BMPR2 gene to mean value of expression level of beta actin gene, concentration value of patient and control group and standard deviation of these were calculated separately.Beta actin gene expression levels were used as internal control and the results of patients were evaluated accordingly.

RESULTS

In the study 20 patient with Scleroderma and 29 healthy control group were assayed. Experiments were carried out by drawing full blood sample from patients. Blood samples drawn from patients were added to the test tube including EDTA and after required procedures the expression levels were examined by Real-Time PCR method.

According to literature findings, incidence of Scleroderma is much higher in women than men and the individuals included into this research are mostly women (18 women in 20 patients).

During Real-Time PCR process, reference gene (housekeeping gene) and BMPR2 expression levels were estimated for patient sample.

Standard sample was selected from patient samples for the quantitative analysis of gene expressions. This means, the sample amplified with Real-Time PCR was used as the standard. 1/10, 1/100, 1/1000 serial dilutions were prepared from standard sample (ST). Concentrations of standard and dilutions were expressed with the following units;

ST=1, **ST**-1=10, **ST**-2= 100, **ST**-3=1000 **ST**-4=10000 **ST**-5=10000 **ST**-6=100000

During amplification,Cp values of standards were defined and standard curve was obtained with assumed concentrations.

BMPR2 Gene Expression Results

In this study for both patient and healthy control group,BMPR2 gene was studied and concentration values differed in each samples. For patient group, the mean concentration value of BMPR2 gene was 20.044,45 and for healthy control group it was 39.362,07. According to these results, BMPR2 gene concentration value of the patient group was decreased approximately 39,46% in accordance with the healthy individuals. Decrease in BMPR2 gene expression level in Scleroderma patients is remarkable.

Comparison of BMPR2 Gene Expression Results with Reference Gene

To determine BMPR2 gene expression level,amplification curves and concentration values of reference gene (housekeeping gene) and BMPR2 gene area were compared. Hereby housekeeping gene was used as internal control. In case housekeeping gene was not working, thinking that it could be a problem in isolation stage or converting to cDNA stage, sample was not assessed.

Before the results of the samples were calculated, it was controlled whether amplification curves and crossing point values of each sample were in the required levels or not.Afterwards, concentration values were determined according to the crossing point results of the samples. To estimate $2^{-\Delta\Delta CT}$expression value, concentration values of target gene and reference gene were compared.For each sample concentration values imported from the device, reference gene and target gene were directly proportional. Following formula was used to evaluate the results:

$R = 2^{[CP\ sample - CP\ control]}$

$R = 2^{\Delta CP}$

$R = 2^{-[\Delta CP\ sample - \Delta CP\ control]}$

$R = 2^{-\Delta\Delta CP}$

In this study, defined concentration values of Beta actin gene and patient group showed 10% decrease in accordance with the healthy control group.Mean levels of gene expression$2^{-\Delta\Delta CT}$in control group was 102,50 and in patient group itwas determined as 62,05. When patient group and healthy control group were compared in terms of BMPR2 gene, patient group showed significant decrease as against control group.

As the samples included into the study at the same time, decrease of expression levels of patient and control groups was supposed to be approximately at the same ratio. However,expression level of BMPR2 gene in patient group decreased more than it was expected in comparison to control group.

To determine the levels of both the reference (Beta actin gene) and target gene (BMPR2) were studied 3 times. When the results were evaluated, they were observed to find out if the gene expressed itself and Crossing point was between the correct limits.

In our study reference gene was also used as an internal control. It helped us to understand if something went wrong during the experiment because we knew that the reference gene works only if DNA is present. Therefore, if it didn't react according to the data and the concentrations we knew the results from that patient samples wouldn't be taken into account. Otherwise it was counted as a normal sample.

The patients group and control group $2^{-\Delta\Delta CT}$ level average results were given as 62,05 and 102,50 respectively. It was seen that patients' expression level decreased %39,46 by comparison to control group. Control group was gathered from volunteers who did not have Scleroderma and high blood pressure. Both genes (Beta actin and BMPR2) were involved and studied at the same time in the study.

When patients' expression levels were observed in 16 patients the expression level of BMPR2 decreased in comparison to control group. In spite of these, expression levels in 4 women patients with pulmonary arterial hypertension were determined to be higher than other patients.

DISCUSSION

In 2003, Korniszewski et al. [12] found that BMPR2 mutations have a negative effect on natural growth, maturity, growth arrest and death of lymphocytes and they also reported that BMPR2 mutations partially results with PAH.

Also, Barreto et al. [13] showed that PAH was related with mutations of BMPR2 genes.

In a study Manes et al. carried out in 2003 [14], mutations in BMPR2 genes affected various biological functions such as; vascular malformation, cell proliferation and apoptosis.

Machado et al. in 2009 [15] defined missense, nonsense, frameshift mutation, RNA regulation inaccuracy and deletion in patients with PAH in a study with 24 patients and 196 healthy control group.

In 1997, Morse et al. [16] showed that PAH was autosomal genetic dominant in 10-20% ratio in some PAH cases. They found familial PAH in 6% of the cases.

In a study Roberts et al. carried out in 2004 [17] with 40 adults and 66 children with PAH/CHD, BMPR2 mutations was foundas 6% in PAH/CHD.

West et al. [18] found PAH in 20% of the patients with BMPR2 mutation in the study they carried out in 2008. It was reported that, 58% of the patients with BMPR2 mutation had familial PAH and 26% idiopathic PAH.

To determine BMPR2 mutation the PAH development was 6% by Morse at al. [16], 80% by Newman et al. [19], 6% Roberts et al. [17], 26% West et al. [18], McLaughlin et al. [20] found BMPR2 gene mutation in almost 10-20% of the PAH patients.

The sampling number is not sufficient in our study, yet, the ratio of the patients with PAH was 20% and we concluded that in all of them the BMPR2 gene expression level increased at the least.

In 2000, Thomson et al. [21] defined BMPR2 gene mutations as the main reason of genetic PAH. Besides these mutations were found in 9% of PAH patients related with fenfluramine in 26% of sporadic idiopathic PAH patients and in 70% of familial PAH patients. Yet, they revealed that BMPR2 which was a TGF family member was related with apoptosis and proliferation, but it was still unknown how the decrease in BMPR2 signal caused to PAH.

In the study Ozol and Erinc [22] found 6-10% of the PAH cases have familial liability. They found this mutation in 9 of the 19 cases with familial PAH. They reported that the functional loss in BMPR2 caused abnormal endothelium cell growth and proliferation, increasing apoptosis in some cells.

In a study on mothers and children in 2007, Zhang et al. [23] determined that the reason of the disease had resulted from BMPR2 gene mutation in half of the familial PAH patients and they put forward that the 25% of non-familial PAH has relationship with BMPR2 gene mutation.

Mark et al.[24], found out that the PAH patients were familial PAH and 50-90% of them have common BMPR2 gene mutation.

In our study, we determined the level of BMPR2 gene expression in the examined Scleroderma patients as 62,05 and in healthy control group it was 102,50. The expression level of the patients decreased around 39,46% in accordance with the healthy control group.

When patients' expression levels were examined one by one the level of expression in 16 patients was found to be close to the average, and in 4 women patients the level of expression was observed to have risen. These patients were observed to have PAH based on their clinical history. According to this result, in patients with pulmonary arterial hypertension the BMPR2 gene expression level rose in comparison to patients without PAH but compared to the healthy control group their expression level was less.

Besides 20 scleroderma patients and 29 healthy control group were included 18 of these patient were female and 2 male (female, male ratio 9:1) and all of the patients were middle aged (35-50 of age). 19 females and 10 males were included as control group. 10% of the patients were males and 90% were females. That the disease is seen in high ratio in women explains why the women included to the study.

In 20% of the women, PAH has been reported to develop. BMPR2 gene expression level in Scleroderma patients decreased compared to healthy individuals, but BMPR2 gene expression level in PAH patients has been found higher.

CONCLUSION

Scleroderma is a fibrotic autoimmune disease which is thought to arise as a result of complicated genetic and environmental factors and does not have a clear origin. Fibrosis in various tissues and organs that is the general characteristic of the disease was characterized with the excessive accumulation of paracellular matrix in connective tissue. The cause of constant and irregular expression of collagen proteins one of the paracellular matrix components in fibroblasts was not clarified yet.

In some of the Scleroderma patients we know that pulmonary arterial hypertension has developed and it is still a nightmare for its being an expensive, tiring and long term process which still forces the clinicians in practice. Due to the fact that there are various factors in its etiology and pathogenesis, (radioactive materials, associated diseases, genetic affinity, toxic materials etc.) handling the target points of etiopathogenesis increases the prospects of treatment.

Genetic diagnosis methods, mechanism put forward to subcellular level and developed medications increase the hope of success of treatment.

That there might be a genetic crossing over in PAH which was first mentioned by Dresdale in 1954. Hereditary crossing over is an autosomal dominant and shows variable and partial expression. It's observed more frequently in women and the ratio is reported to be 8:1. Liability in women reminds us that BMPR2 mutation has been influenced by other environmental factors like hormones. Only in 6% of the PAH cases there is familial anamnesis.

It is believed that idiopathic facts in variable mutations mask the familial transmission or "de novo" mutations could occur.

BMPR2 gene has a great importance for scleroderma patients. BMPR2 gene expression affects the clinical course and it changes in accordance with the patients and the course of the disease.

In this study BMPR2 gene expression levels have been put forward among the Scleroderma patients and control group. Reference gene, namely, Beta actin gene, expression levels have been determined to test the reliability and validity of the study.

According to the data after associating Beta actin gene expression level with BMPR2 gene the average expression level has been determined as 0,0007295126 in Scleroderma patients. When expression levels of Scleroderma patients were investigated it was observed that the expression levels seemed to decrease as the symptom and severity of the disease rose up and in patients with PAH these levels seemed to be lower than control group and higher than patient group.

The expression level changes according to the course of the disease. The expression levels in patients observed to be lower than the control group. But in some patients the expression level did not changed sightly.

To sum up, the average gene expression levels $2^{-\Delta\Delta CT}$ in control group are 102,50. This value $2^{-\Delta\Delta CT}$ has become 62,05 in patient group. The expression level of patients decreases to 39,46% compared to control group.

There has been increase in gene expression level of 4 PAH patients. All the patients with pulmonary arterial hypertension are females and BMPR2 gene expression level increased compared to non-PAH patients. The number of sampling is insufficient in this study but the PAH diagnosed patient from the 20% of all the examined patients (4 patients of 20 were diagnosed as PAH) and in all of them BMPR2 gene expression level increased.

The BMPR2 gene expression level in Scleroderma patients is low compared to control group in PAH patients it is low compared to control group but it is high when compared to patients group.

The reason for this study being important is in order to be a basis of follow-up studies in which more patients included, the expression level of each patient closely associated with the course of the disease, examination of blood pressure changes of expression level especially in patients with PAH and familial story. The next step must be to enhance the study with the experimental practices obtained in this field with more patients' participation.

Mutations and gene expression studies surely are not the primary effective parameters in treatment of the disease. However, the course of the disease, what the individual will come across in the future, early diagnosis in familial diseases, the contribution of clinician in diagnosing the type of treatment increase the importance of using molecular methods today. With studies of human genome project the identified genes are increasing day by day. Thus, through the molecular analysis it is possible to predict the prospects in the formation and course of diseases.

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