Smyrna Tıp Dergisi ***Araştırma Makalesi***

FOXO3a gene polymorphism and serum FOXO3a levels in patients with chronic obstructive pulmonary disease and healthy controls: Effects of genetic polymorphism in chronic obstructive pulmonary disease

Kronik obstrüktif akciğer hastalığı olan hastalarda FOXO3a gen polimorfizmi ve serum FOXO3a seviyeleri: Kronik obstrüktif akciğer hastalığında genetik polimorfizmin etkileri

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Abstract
Objective: The aim of the study was to compare FOXO3a gene polymorphism and serum FOXO3a levels in patients with chronic obstructive pulmonary disease (COPD) and healthy controls.
Materials and Methods: A total of 87 COPD patients, admitted to the university, between March 2012 and March 2013, constituted the study group. Eighty-eight healthy subjects were used as controls. Two single-nucleotide polymorphisms (SNPs) (rs2253310 and rs4946936) in FOXO3a were selected for genotyping experiments using polymerase chain reaction-restriction fragment-length polymorphism technique. FOXO3a levels in plasma were measured with ELISA.
Results: As for genotype distributions of the FOXO3a gene, significant difference was found between the COPD group and the control group as for rs2253310 SNPs (p:0.019). When subgroups were analyzed, no significant difference was found between the COPD group and the control group as for G/G, G/C, C/C, T/T, T/C, and C/C. FOXO3a mean plasma level was 21.97±5.48 in the COPD group, and 26.72±10.56 in the control group (p<0.001). As for FOXO3a plasma concentrations, significant difference was found between the COPD group and the control group as for G/G, C/C, and T/T (p:0.033, p:0.005, and p:0.042, respectively).
Discussion: Although an association between rs2253310 SNPs and the risk of COPD was identified, no significant association was identified in subgroups of rs2253310 and rs4946936 SNPs.
Key Words: COPD mechanisms, FOXO3a gene polymorphism, rs2253310, rs4946936

Özet
Amaç: Bu çalışmanın amacı kronik obstruktif akciğer hastalığı (KOAH) olan hastalar ile sağlıklı kontrol grubundaki FOXO3a gen polimorfizmi ve serum FOXO3a seviyelerini karşılaştırmaktır.
Gereç ve Yöntem: Mart 2012 ile Mart 2013 tarihleri arasında üniversiteye Kabul edilen toplam 87 KOAH hastasıçalışma grubunu oluşturmaktadır. 88 sağlıklı kişi de kontrol grubu olarak kullanılmıştır. FOXO3a için, iki tekli-nükleotid polimorfizmi (SNPs) (rs2253310 ve rs4946936), polimeraz zincir reaksiyon-kısıtlı fragment-uzun polimorfizm tekniği kullanılarak genotiplemede uygulanmak üzere seçildi. Plazma FOXO3a düzeyleri ELİSA ile hesaplandı.
Bulgular: FOXO3a geni genotip dağılımı için, KOAH grubu ile kontrol grubu arasında rs2253310 SNPs açısından istatistiksel farklılık tespit edildi (p:0.019). Altgrup analizleri yapıldığında, KOAH grubu ile kontrol grubu arasında G/G, G/C, C/C, T/T, T/C, ve C/C açısından istatistiksel farklılık bulunmadı. FOXO3a ortalama plazma düzeyi KOAH grubu için 21.97±5.48 iken kontrol grubu için 26.72±10.56 bulundu (p<0.001). FOXO3a plazma konsantrasyonları için; KOAH grubu ile kontrol grubu arasında G/G, C/C, ve T/T açısından istatistiksel anlamlı farklılık saptandı (p:0.033, p:0.005, ve p:0.042, sırasıyla).
Tartışma: Her ne kadar rs2253310 SNPs ile KOAH riski arasında ilişki tespit edilmiş olsa da, rs2253310 ve rs4946936 SNPs alt grupları arasında istatistiksel ilişki tespit edilemedi.
Anahtar kelimeler: KOAH mekanizmaları, FOXO3a gen polimorfizmi, rs2253310, rs4946936

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Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease characterized by progressive bronchial obstruction (1). Peripheral muscle wasting is a feature of this debilitating disease in a significant number of afflicted patients. Skeletal muscle is the largest organ in the human body and its mass and composition are critical for movement, energy expenditure and glucose metabolism (2). In patients with COPD, peripheral muscle wasting is associated with exercise intolerance, poor quality of life and reduced survival (3). Only modest improvement in muscle mass can be obtained with exercise training, nutritional supplementation and anabolic drug supplementation in this population [4]. There is a need to develop more potent therapeutic strategies specifically designed to improve peripheral muscle mass in patients with COPD. This important goal will be achieved only with a better understanding of the mechanisms underlying the wasting process in this disease.

Differential expression screening studies have identified two genes whose expression is increased significantly in multiple animal models of skeletal muscle atrophy. These two genes MuRF-1 (muscle ring finger-1; TRIM63) and MAFbx (muscle atrophy F-box; FBXO32) (5), which is also known as atrogin-1 (6), encode E3 ubiquitin ligases (5). Apart from stimulating muscle hypertrophy pathways, activated Akt is also able to block muscle protein breakdown by downregulating MuRF-1 and atrogin-1 expression. This action is mediated by the inactivation of the FoxO (forkhead box O) class of transcription factors (7).

The aim of the present study was to compare FOXO3a gene polymorphism and serum FOXO3a levels in patients with chronic obstructive pulmonary disease and healthy controls.

Materials and Methods

Study Design:
This study was approved by the local Institutional Review Board (31.01.2012, B.30.2.MĞÜ.0.61.00.00-050.00.00.00-81/809). Written informed consent was obtained from all subjects and all procedures were performed according to the principles of The Helsinki Declaration.

A total of 87 COPD patients, admitted to the Department of Chest Diseases of Sıtkı Kocman University, between March 2012 and March 2013, constituted the study group.

Eighty-eight healthy subjects were used as controls. The control group included healthy, never-smokers with normal spirometry and no history of asthma, allergy or other pulmonary diseases. In both groups, subjects were excluded if they had prolonged bed rest or presented previous inflammatory or muscle disease.

SNP selection and genotyping:
Two single-nucleotide polymorphisms (SNPs) (rs2253310 and rs4946936) in FOXO3a were selected for genotyping experiments using polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) technique.

Venous blood samples were collected into vacutainer plastic tubes containing sodium/ potassium EDTA. Blood samples were centrifuged at 1000 ×g for 10 min. Plasma samples were separated and immediately stored at -20ºC. PCR was performed in a 50 µl volume with 20-100 ng DNA, 100 µm dNTPs, 20 pmol of each primer, 1mM MgCl2, 20µM Tris-HCl pH 8.6, 50µM KCl and 1 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). Amplification was performed on an automated thermal cycler (Techne-Genius, Princeton, NJ). PCR conditions were 3 min for initial denaturation at 94 ºC; 35 cycles at 94 ºC for 1min for denaturation, 1 min at 53 ºC for annealing and 1 min at 72 ºC for extension, followed by 7 min at 72 ºC for final extension. These genes polymorphisms were determined by fragment separation at 120 V for 40–50 minutes on a 3.5% Agarose gel containing 0.5 mg/mL ethidium bromide. A 100-bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat, Marne La Vallee, France) (Figure 1A and B)

Figure 1A. *The genotype analysis of rs 2253310 polymorphisms of FoxO3a gene*

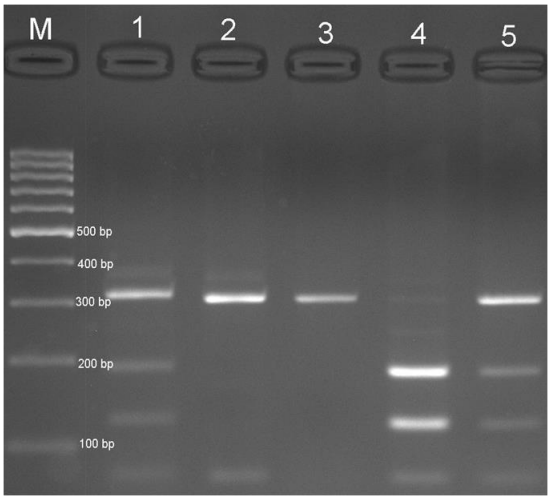
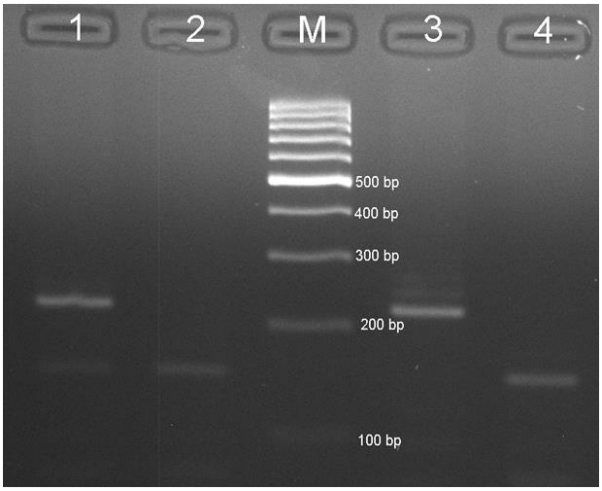


Figure 1B. *The genotype analysis of rs 4946936 polymorphisms of FoxO3a gene.*



Serum FOXO3a levels:
FOXO3a levels in plasma were measured with ELISA according to the instructions of the manufacturers (Cusabio Biotech, Co. Ltd., Newark, USA). The levels of FOXO3a were calculated from a standard curve and expressed in nanograms per milliliter (ng/mL).

Statistical Analyses:
Data were analyzed using the Statistical Package for Social Sciences (SPSS) software version 19.0 for Windows (SPSS Inc., Chicago, IL) and Medcalc® (Medcalc® Software, Mariakerke, Belgium) statistical software program. A normal distribution of the quantitative data was checked using Kolmogorov-Smirnov test. Parametric tests were applied to data of normal distribution and non-parametric tests were applied to data of questionably normal distribution. Independent-samples t-test was used to compare independent groups. Differences between independent groups were assessed by one-way ANOVA followed by Games-Howell post hoc test. The distribution of categorical variables in both groups was compared using Pearson chi-square test. Multivariate logistic regression analysis was used to determine the independent significance of certain risk factors. All differences associated with a chance probability of 0.05 or less were considered statistically significant. Continuous variables are presented as mean±SD.

Results

One hundred seventy-five patients met the eligibility criteria for the study. Of the 175 patients (107 males, 68 females) whose charts were reviewed, the average age was 52,33±3,67 (range 41 to 60) years.

PCR-RFLP conditions of polymorphisms (C/G transition at rs 2253310 and C/T transition at rs 4946936**)** of FOXO3a gene have been shown at Table 1.

Table 1. *PCR-RFLP conditions of polymorphisms of FOXO3a gene*

Gene name	SNP	Primers	PCR Tm	Restriction enzymes	PCR-RFLP products
FOXO3a	rs 2253310	F 5'-GAGCTTGCTTTGGAGATGCA-3' R 5' CCCAGTCACTCACATAGTCCT-3'	53 ^{0}C	DpnI	GG= 321 bp GC= 321, 127, 194 bp CC= 127, 194 bp
	rs 4946936	F 5'-GGGTCCTGAGAACTTCTGAGT-3' R 5'-GACATTCTGTAAGACATTCTGCCT-3'	53 ^{0}C	SfcI	TT= 224 bp TC= 224, 152, 72 bp CC= 152, 72 bp

SNP= Single nucleotide polymorphism; Tm= Temperature; RE= Restriction enzymes; PCR-RFLP= Polymerase chain reaction-restriction fragment-length polymorphism

As for genotype distributions of the FOXO3a gene, significant difference was found between the COPD group and the control group as for rs2253310 SNPs. No significant association was found for rs 4946936 SNPs (p:0.019 and p:432, respectively) (Table 2). When subgroups were analyzed, no significant difference was found between the COPD Group and the Control Group as for G/G, G/C, C/C, T/T, T/C, and C/C (Table 2).

Table 2. *Genotype distributions of the FOXO3a gene.*

		Genotypes n (%)				
Gene name	**SNP**		**COPD (%)**	**Control (%)**	***p* Value**	***p* Value**
FOXO3a	rs2253310	G/G	38 (43.7%)	57 (64.8%)	0.059	0.019
		G/C	42 (48.3%)	26 (29.5%)	0.047	
		C/C	7 (8.0%)	5 (5.7%)	0.550	
	rs4946936	T/T	18 (20.7%)	25 (28.4%)	0.304	0.432
		T/C	43 (49.4%)	42 (47.7%)	0.870	
		C/C	26 (29.9%)	21 (23.9%)	0.441	

SNP= Single nucleotide polymorphism; COPD= Chronic obstructive pulmonary disease

FOXO3a mean plasma level was 21.97±5.48 in the COPD group, and 26.72±10.56 in the control group. The difference was statistically significant (p<0.001). As for FOXO3a plasma concentrations, significant difference was found between the COPD group and the control group as for G/G, C/C, and T/T (p:0.033, p:0.005, and p:0.042, respectively) (Table 3).

As for the plasma levels of FOXO3a cut-off value of ≤29.31, the area under the curve (AUC) of the ROC curve was 0.650±0.0413 with a sensitivity of 95.4% and specifity of 42.05% for COPD cases (p<0.001) (Table 4 , Figure 2).

Table 3. *FOXO3a plasma concentration differences for G/G, C/C, and T/T.*

	FOXO3a plasma concentrations			
SNP		**COPD (mean±SD)**	**Control (mean±SD)**	***p* Value**
rs2253310	G/G	21.32±5.60	26.55±10.88	0.033
	G/C	22.84±5.47	25.79±10.49	0.770
	C/C	20.32±4.82	33.55±4.19	0.005
rs4946936	T/T	20.83±5.76	29.42±12.16	0.042
	T/C	21.89±5.31	25.46±9.15	0.252
	C/C	22.90±5.64	26.06±11.11	0.840

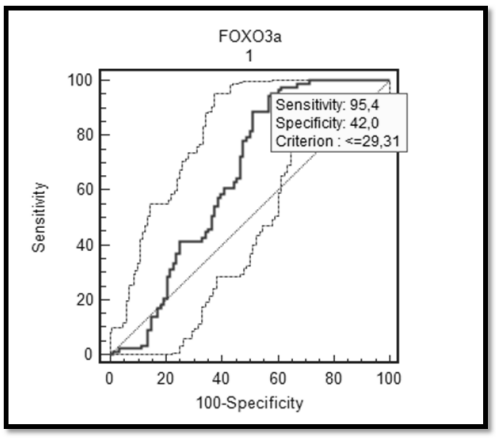
FOXO3a plasma concentrations between groups.
SNP= Single nucleotide polymorphism; COPD= Chronic obstructive pulmonary disease

Table 4. *FOXO3a levels evaluated for the diagnostic accuracy of chronic obstructive pulmonary disease.*

	Cut-off (U/L)	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**AUC±Sh**	**p Value**
FOXO3a	≤29.31*	95.4	42.05	-----	-----	0.650±0.0413	<0.001

PPV= Positive predictive value; NPV= Negative predictive value; AUC= Area under the curve

Figure 2. *The area under the curve of the ROC curve for patients with chronic obstructive pulmonary disease.*



Discussion

Peripheral muscle tissue homeostasis is an important issue in an increasing number of acute and chronic disorders where increased contractile protein degradation occurs, resulting in muscle atrophy. Muscle atrophy is known to take place in a significant number of COPD subjects jeopardizing their functional status, quality of life and survival. In order to broaden our understanding, a microarray experiment was undertaken.

Despite the relevance of skeletal muscle dysfunction in COPD, the pathogenic mechanisms of this phenomenon remain unclear. Several potential mechanisms have been related to peripheral muscle dysfunction/wasting in patients with COPD: a) protein synthesis/ breakdown

balance, b) nutritional abnormalities, c) muscle disuse, d) systemic corticosteroids, e) tissue hypoxia and hypercapnia, f) alterations in muscle remodelling, g) inflammation, h) oxidative/nitrosative stress; and, i) mitochondrial abnormalities (2,3).

Through phylogenetic analysis, forty-three human FOX family proteins are classified into 17 subfamilies (FOXA-FOXQ) (8). Among these families, the FOXO subgroup contains four members, FOXO1, FOXO3a, FOXO4 and FOXO6 (8,9). FOXO-1 and Atrogin-1/MAFbox mRNA levels were found to be elevated in peripheral muscles of patients with COPD compared to healthy controls (10). FOXO1 is phosphorylated by activation of the IGF-1 receptor via Akt/PI3K signaling pathway. Once unphosphorylated, FOXO1 and FOXO3 may suppress the rise in skeletal muscle mass by inhibiting IGF-1-mediated skeletal muscle cell proliferation or increase the degradation rate of skeletal muscle proteins by promoting the transcription of Atrogin-1/MAFbx (7,11). A recent study reported an increased Atrogin-1/MAFbx expression in the diaphragm of patients with COPD (12). FOXO-1 and Atrogin-1/MAFbox mRNA levels were assessed and a significant elevation in their expression in peripheral muscles of patients with COPD compared to healthy controls was found (10). In the present study, as for genotype distributions of the FOXO3a gene, significant difference was found between the COPD group and the control group as for rs2253310 SNPs. No significant association was found for rs 4946936 SNPs. As for FOXO3a mean plasma level, the difference between groups was statistically significant.

Conclusion

Although an association between rs2253310 SNPs and the risk of COPD was identified; no significant association was identified in subgroups of rs2253310 and rs4946936 SNPs. However, our results need to be confirmed in the other populations as our population is small in sample size.

Acknowledgements

SK designed the research study; SK and AZ found patients with COPD and took their peripheric blood samples; TGE isolated DNA from blood samples and did the molecular analysis (PCR-RFLP), UOT did the quantification of serum FOXO3a levels with ELISA technique; ESC contributed to molecular analysis of samples (PCR-RFLP) and HC did the statistical analysis of the research.

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