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The association of FOXO3A gene polymorphisms with serum FOXO3A levels and oxidative stress markers in vitiligo patients

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ABSTRACT

Vitiligo is an acquired epidermal pigment loss of the skin. Oxidative stress is one of the major theories in the pathophysiology of vitiligo. FOXO3A is the forkhead members of the class O (FOXO) transcription factors, and plays an important role in cell cycle regulation, apoptosis, oxidative stress, and DNA repair. The aim of our study was to investigate FOXO3A gene polymorphisms and FOXO3A protein levels, activities of superoxide dismutase (SOD) and catalase antioxidant enzymes in vitiligo patients and healthy controls. Moreover, the level of plasma advanced oxidation protein products (AOPP) in subjects was evaluated to understand the possible role of protein oxidation in disease etiology. Study groups included 82 vitiligo patients and 81 unrelated healthy controls. FOXO3A polymorphisms were determined using polymerase chain reaction–restriction fragment length polymorphism method. FOXO3A levels and catalase activity were measured by ELISA whereas AOPP levels and SOD activity was measured by spectrophotometric analysis. We found a significant relationship between rs4946936 polymorphism of FOXO3A gene and vitiligo/active vitiligo patients ($p = 0.017$; $p = 0.019$ respectively), but not for rs2253310 ($p > 0.05$). SOD activity and AOPP levels of vitiligo patient were increased compared with control group, whereas FOXO3A levels and catalase enzyme activity of vitiligo patient were decreased compared with control group ($p < 0.05$). Our study indicates that rs4946936 of FOXO3A gene may associate susceptibility of vitiligo, especially active vitiligo. Moreover, our results confirm that oxidative stress may play a role in the pathophysiology of vitiligo. Further studies with larger samples are required to elucidate the role of FOXO3A in vitiligo.

1. Introduction

Vitiligo is an acquired depigmenting disease characterized by milky white patches of skin due to local loss of the epidermal melanocytes (Halder and Chappell, 2009; Huang et al., 2002). It occurs with a frequency of 0.1–2% worldwide (Ortonne, 2008). Although its pathophysiology is still unknown, diverse theories have been proposed, including autoimmune, neural, oxidative stress, apoptosis, and genetic factors (Passeron and Ortonne, 2005). Recent studies have suggested that oxidative stress might play a prominent role in the pathogenesis of vitiligo (Maresca et al., 1997; Schallreuter et al., 1991, 1994, 1999). Oxidative stress is defined as a disruption of the delicate balance between the formation of reactive oxygen species (ROS) and the antioxidant defense system (Sies, 1991). ROS, especially superoxide anions and hydrogen peroxide (H_2O_2), can cause lipid peroxidation and protein and DNA oxidation, as well as alter apoptotic pathways, thereby inducing cellular injury. It has been suggested that some enzyme defects during melanogenesis might cause oxidative stress, with a consequent accumulation of H_2O_2 (Jimbow et al., 2001). Several clinical studies have demonstrated increased H_2O_2 levels and decreased antioxidant enzyme activities in vitiligo patients (Beazley et al., 1999; Hazneci et al., 2005; Koca et al., 2004; Schallreuter et al., 1991); high levels of H_2O_2 are thought to trigger melanocyte degeneration in patients with

Abbreviations: FOXO3A, Forkhead box class O 3A; SOD, Superoxide dismutase; AOPP, Advanced protein products; ROS, Reactive oxygen species; UV, Ultraviolet; H_2O_2, Hydrogen Peroxide; PCR-RFLP, Polymerase chain reaction–restriction fragment length polymorphism; SNP, Single nucleotide polymorphism, ELISA, Enzyme-linked immunosorbent assay; PTPN22, Protein Tyrosine Phosphatase, Non-Receptor Type 22; NLRP1, NLR Family, Pyrin Domain Containing 1; ESR1, Estrogen Receptor 1; FOXP1, Forkhead Box P1; CAT, Catalase; COMT, Catechol-O-Methyltransferase; TLR-2, Toll-Like Receptor 2; TLR-4, Toll-Like Receptor 4; HWE, Hardy Weinberg Equilibrium; OR, Odds ratio; CI, Confidence Interval; HOCl, Hypochlorous acid; MnSOD, Manganese superoxide dismutase; mRNA, Messenger Ribonucleic Acid.

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vitiligo. It has been suggested that ROS such as H_2O_2 stimulate apoptosis through activation of signaling molecules, including protein kinase C and c-Jun-N-terminal kinase (Essers et al., 2004).

Genetic factors are also thought to have an important role in the etiology of vitiligo (Imran et al., 2012). Recently, single-nucleotide polymorphisms (SNPs) of several candidate genes, including PTPN22, NLRP1, ESR1, FOXP1, CAT, COMT, TLR-2, and TLR-4, were reported to be associated with vitiligo susceptibility (Casp et al., 2002; Imran et al., 2012; Karaca et al., 2013; Shajil et al., 2006). Forkhead box class O (FOXO) proteins are a subclass family of the Forkhead box (FOX) transcription factors, including FOXO1, FOXO3A, FOXO4, and FOXO6, which play a crucial role in diverse cellular processes such as cell cycle regulation, apoptosis, oxidative stress and DNA repair (Brunet et al., 1999; Furukawa-Hibi et al., 2005). Studies have shown that FOXO3A have important roles in the regulation of oxidative stress. Activation of FOXO3A has been shown to induce gene expression of manganese superoxide dismutase (MnSOD) and catalase antioxidant enzymes (Kops et al., 2002; Nemoto and Finkel, 2002), as well as prevent cell integrity against genotoxic agents H_2O_2 and UV irradiation (Brunet et al., 2004; Klagge et al., 2011). The human FOXO3A gene is located at chromosomal position 6q21 and is comprised of three exons and two introns (Anderson et al., 1998). Thus far, functional SNPs of FOXO3A have been found to be associated with longevity, obesity, and bipolar disorders (Anselmi et al., 2009; Flachsbart et al., 2009; Kim et al., 2006; Li, 2008; Magno et al., 2001; Willcox et al., 2008). However, to the best of our knowledge, there have been no reports regarding the relationship between FOXO3A genetic variants and the risk of vitiligo.

Taking into account the possible effects of oxidative stress and apoptosis in the pathophysiology of vitiligo, we hypothesized that FOXO3A genetic variants might be associated with risk of vitiligo. For this reason, we investigated two functional SNPs (rs2253310, rs4946936) in the FOXO3A gene, as well as its protein levels and superoxide dismutase (SOD) and catalase antioxidant enzyme activities in vitiligo patients and healthy controls. In addition, plasma advanced oxidation protein product (AOPP) levels were evaluated in an effort to understand the possible role of protein oxidation in the etiology of vitiligo.

2. Methods

This study was approved by the Ethics Committee of the Bulent Ecevit University Faculty of Medicine (protocol no; 2011-63-17/05) and performed in accordance with the guidelines of the Declaration of Helsinki; written informed consent was obtained from all the subjects. Eighty-two vitiligo patients and 81 matched healthy controls, all recruited from the Department of Dermatology at Bulent Ecevit University Hospital, were included in the study. The healthy controls displayed no clinical evidence of vitiligo or any autoimmune disorder, systemic disorders, or family history of vitiligo (including first-, second-, and third-degree relatives). Demographic and clinical information was obtained from all the subjects through the use of questionnaires (Table 1). The activity status of vitiligo in the clinical diagnosis was classified as active or stable. In our study, active vitiligo was defined as appearance of new lesions, spreading of existing lesions or the presence of the Köbner phenomenon (depigmentation induced by trauma) in the last 6 months. Other patients were classified as stable status.

Venous blood samples were collected in plastic Vacutainer tubes, either without additives or containing EDTA. Whole blood samples were separated and stored at −20 °C for genomic DNA extraction. The blood samples in EDTA were centrifuged at 1000 ×*g* for 10 min, and the plasma was separated and immediately stored at −20 °C until used to measure AOPP levels and catalase activities. In addition, serum samples were separated and immediately stored at −20 °C until used to measure FOXO3A levels and SOD activities.

Table 1
Clinical characteristics of vitiligo patients and healthy controls.

Clinical characteristics	Vitiligo (n = 82) Median (Min.–Max.)	Control (n = 81) Median (Min.–Max.)	p-Value
Age	36.68 (32.96–40.40)	34 (18–80)	0.919
	n (%)	n (%)	
Gender, female	37 (0.45)	43 (0.53)	
Family history			
Yes	64 (0.78)	–	
No	18 (0.22)	–	
Vitiligo types			
Generalize	39 (0.48)	–	
Segmental	2 (0.02)	–	
Localize	23 (0.28)		
Acrofacial	13 (0.16)		
Vulgaris	2 (0.02)		
Focal	3 (0.04)		
Stability			
Stable	17 (0.21)	–	
Active (unstable)	65 (0.79)	–	
Age onset			
0–15	17 (0.21)	–	
16–30	29 (0.35)	–	
31–45	16 (0.20)	–	
46–70	20 (0.24)	–	
Duration			
<1	31 (0.38)	–	
1–3	17 (0.21)	–	
>3	34 (0.41)	–	

2.1. Genomic DNA isolation and genotype analysis

Genomic DNA was isolated from peripheral blood lymphocytes by standard procedures using GeneJet DNA purification kits (Fermentas, USA). The FOXO3A genotypes were determined using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. The primer sequences [26], annealing temperatures, and restriction enzyme are shown in Table 2. The amplified products were digested using one unit of *Dpnl* (rs2253310; C/G) and one unit of *Sfcl* (rs4946936; C/T). The digested fragments were separated on 2% agarose gel by electrophoresis. The alleles were identified according to their fragment sizes (Table 2).

2.2. Measurement of serum FOXO3A levels

Serum FOXO3A levels were measured with a human enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech Co., Newark, NJ, USA) according to the manufacturer's instructions. The levels of FOXO3A were calculated from a standard curve and expressed in nanograms per milliliter (ng/mL).

2.3. Measurement of serum SOD activity

Serum SOD activity was measured using the method of Sun et al. (1988), based on the inhibition of nitroblue tetrazolium, using the xanthine–xanthine oxidase system as a superoxide generator. The serum samples were mixed with 3/5 (v/v) chloroform and ethanol. This mixture was centrifuged at 10,000 ×*g* for 1 h, and the supernatant was used to determine SOD activity. One unit of SOD was defined as the amount of protein that inhibits the rate of nitroblue tetrazolium reduction by 50%. SOD activity was expressed as units per milliliter (U/mL).

2.4. Measurement of plasma catalase activities

Plasma catalase activity was measured with a human ELISA kit (BenderMed Systems, Biosource International, Inc., Camarillo, CA, USA) according to the manufacturer's instructions. The activities were calculated on a standard curve and expressed in U/mL.