

VİTİLİGODA MELANOKORTİN-1 RESEPTÖR GENİ POLİMORFİZMLERİ

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Amaç: Vitiligo; her yaş grubunda, herediter veya kazanılmış olarak ve sıklıkla görülen, ilerleyici, bir deri pigmentasyon bozukluğudur. Melanokortin 1 reseptör (MC1R) geni insan pigmentasyonunda major bir belirleyicidir. Bizim çalışmamızda; vitiligolu olgularda MC1R genindeki polimorfik farklılıklar DNA seviyesinde araştırılmaktadır.

Hastalar ve Yöntemler: Bizim çalışmamızda; MC1R genindeki polimorfik farklılıklar en az üç kuşaktır Türkiye'nin Trakya bölgesinde yaşayan, başka bir diğer sistemik veya dermatolojik bir hastalığı olmayan, 60 vitiligolu olguda ve 60 gönüllü sağlıklı bireyde DNA seviyesinde araştırılmıştır.

Bulgular: Kontrol ve olgu gruplarının her birisinde toplam 5 adet SNP bulunmuştur. Val60Leu (G178T), Val92Met (G274A), Arg151Cys (C451T), Arg160Trp (C478T) and Arg163Gln (G488A). Genotip sıklıkları yoluyla her iki grup karşılaştırıldığında istatistiki olarak anlamlı bir fark bulunmadı ($p>0.05$). Ancak allel sıklıkları yoluyla değerlendirildiğinde kontrol grubunun lehine Arg163Gln (G488A) allelinde istatistiki olarak anlamlı bir fark bulundu($p<0.05$).

Sonuç: Bundan dolayı, bizim çalışmamıza göre MC1R geni Arg163Gln (G488A) alleli vitiligo için koruyucu bir faktör olabilir.

Anahtar Kelimeler: Vitiligo; MC1R; melanocortin-1 receptor geni; polimorfizm

33 **POLYMORPHISMS IN THE MELANOCORTIN-1 RECEPTOR (MC1R) GENE IN**
34 **VITILIGO**

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41 **ABSTRACT**

42 **Objectives:** Vitiligo is a progressive skin pigmentation disorder, which may be acquired or
43 hereditary, seen frequently, and may influence every age group. The melanocortin 1 receptor
44 (MC1R) gene is a major determinant of human pigmentation. In our study, polymorphic
45 differences of the MC1R gene at DNA level has been searched in patients with vitiligo.

46 **Patients and Methods:** In our study, polymorphic differences of the MC1R gene at DNA
47 level has been searched in 60 patients with vitiligo, who have been living in the Thrace
48 region of Turkey for at least three generations; the 60 volunteer healthy individuals have no
49 other systemic and dermatological disease..

50 **Results:** Totally, five types of SNP were found in each case and control groups: Val60Leu
51 (G178T), Val92Met (G274A), Arg151Cys (C451T), Arg160Trp (C478T), and Arg163Gln
52 (G488A). Comparing both groups in terms of genotype frequencies, no difference which is
53 meaningful statistically was detected ($P>0.05$). However, assessing in terms of allele
54 frequencies, a meaningful difference was found in the Arg163Gln (G488A) allele statistically
55 in favor of the control group ($P<0.05$).

56 **Conclusion:** Therefore, it has been found in our study population that MC1R gene
57 Arg163Gln (G488A) allele may be a protective factor for vitiligo.

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59 **Keywords:** vitiligo; MC1R; melanocortin-1 receptor gene; polymorphism

60 INTRODUCTION

61 Vitiligo is a pigmentation disorder which is characterized by color fading, as a result of the
62 loss of melanin pigment which gives color to the skin. The disease, which may be observed at
63 every age group, progresses in various sizes and localizations with depigmented, strict
64 bordered, and generally symmetric macules (1–3).

65 The melanocortin 1 receptor (MC1R) gene has been specified as the pigmentation gene, with
66 a first-degree role in the determination of human skin color phenotype in the human
67 pigmentation system, and in the reaction to the ultraviolet radiation (UVR) by coding MC1R,
68 which is the key control point in the melanogenesis (3–7).

69 Detection of family story at the rate of 20–40% in vitiligo etiology, other than neural,
70 autoimmune, autotoxic theories, and the role of free oxygen radicals, has caused the necessity
71 to consider the genetic role of this disease as well (1–3,8).

72 Reduction in contact sensitization, changes in T lymphocyte subgroups, Langerhans cells,
73 and natural killer cells suggest existence of a disorder in the cellular immune system. Those
74 with the active disease have been found with increased helper T cell/suppressor T cell ratio
75 (9). Vitiligo may also develop, based on the regulation disorder of the molecules arranging
76 apoptosis. It is suggested that in vivo melanocyte apoptosis may be induced by autoreactive T
77 cells or macrophages in vitiligo (10).

78 HLA associations in vitiligo have been examined by some researchers in various populations.
79 It has been suggested that there is an association between vitiligo and HLA-DR4 in the
80 American Whites and African Americans (11). Association with HLA-D or HLA-DR
81 antigens may be considered as evidence that a disease has both a hereditary and an
82 autoimmune aspect.

83 Studies on the connection between HLA and vitiligo in different populations do not show
84 similarity. Although Vijlder et al. (12) found a positive relation with HLADR4 and
85 HLADR53, they could not show any relation between HLADR3 and vitiligo.

86 A considerable increase has been found between HLAB21, HLACw6, and HLADR53 allele
87 frequencies, in a study carried out in Kuwaiti. In the same study, a significant decrease has
88 been found in HLA A19 and HLADR52 allele frequencies as well (13).

89 A decrease has also been observed in HLAA30, HLACw6, and HLADQw3 allele
90 frequencies, in a study conducted in those with vitiligo in North Italy (14). Zamani et al. (15)
91 concluded in their study that there might be a connection between HLADRB4*0101 and
92 HLADQB1*0303 alleles and vitiligo.

93 MC1R gene is the basic pigmentation defined by human pigmentation (4–6). It is suggested
94 that sequencing of the human genome and their functional genomic analysis would make
95 clear etiopathogenesis of the vitiligo in the near future (4).

96 Based on our knowledge, there are only two studies researching the relation between MC1R
97 gene polymorphisms and vitiligo. Na et al. reported in the study they conducted with a
98 Korean population that they could not find a statistically significant relation between MC1R
99 gene polymorphisms and vitiligo. However, Szell et al. suggested in the study they conducted
100 with a Hungarian population that Arg160Trp allele of MC1R gene might be protective
101 against vitiligo (16).

102 In our study, the MC1R gene which encodes the MC1R, the control point in the human
103 pigmentation system, has been examined in vitiligo which is a pigmentation disorder, by
104 making a DNA sequence analysis, and polymorphic differences of the MC1R gene at DNA
105 level have been searched. This study is the third research analyzing the relation between
106 MC1R gene polymorphisms and vitiligo.

107 It is thought that this study will be useful in the research of vitiligo susceptibility in
108 individuals and in its therapy by lighting genetic etiology of the disease.

109 **PATIENTS AND METHODS**

110 Cases and controls were grouped by their skin phototypes. The possible connections between
111 vitiligo and pigmentation were analyzed in vitiligo patients and healthy individuals grouped
112 by their skin phototypes. However, those who had a second chronic disease accompanied
113 with vitiligo, those who were relatives, or those who voluntarily wanted to leave were
114 excluded from the study.

115 One hundred and twenty individuals were involved in this study (60 vitiligo patients and 60
116 control individuals). The study included patients who applied to the Trakya University
117 School of Medicine Department of Dermatology Polyclinic with depigmented macular
118 lesions on their body, for whom we diagnosed with vitiligo by clinical and Wood light
119 examination.

120 Ages and genders of the cases, who have no relationship with each other and who have been
121 living in Thrace for at least three generations, have been specified. Although the clinical form
122 of the patients has been determined according to the vitiligo clinical classification, their skin
123 types have been identified based on the Fitzpatrick skin phototypes (Table 1.)

124 It has been considered, specifically, to ensure that our patients have no systemic and
125 dermatological disease accompanied with the vitiligo. Therefore, the patients have been
126 subject to an anamnesis in terms of the diseases accompanied with vitiligo, and necessary
127 systemic and dermatological examinations of the patients have been implemented. As we
128 applied routinely to all patients diagnosed with vitiligo other than clinical and anamnestic
129 data in terms of diabetes mellitus, thyroid diseases (hyperthyroidism, hypothyroidism,
130 Hashimoto's thyroiditis), pernicious anemia which is one of the diseases accompanied by
131 vitiligo most frequently, fasting blood glucose level, blood biochemistry, full blood count and

132 blood level of folic acid, vitamin B12 and thyroid-stimulating hormone, triiodothyronine,
133 thyroxine, thyroid autoantibodies (anti-TPO, anti- troglobulin) level have been observed.
134 The control group has been formed by healthy volunteers who have been residing in Thrace
135 for at least three generations. The questions asked included their ages, genders, occupations,
136 diseases and operations which they had up to that time, and the medicine taken.

137 **Isolation of DNAs:** Peripheral venous blood of 2.5 cc was taken from the patients with
138 vitiligo and normal control cases in the EDTA tubes of 5 cc. Genomic DNA was isolated
139 from the peripheral blood leucocytes with the E.Z.N.A. DNA isolation kit was based on the
140 protocol of the kit (E.Z.N.A. Blood DNA kit II; D.3492-01 Lot# GA/T070203).

141 Segment of the MC1R gene of which polymorphism would be searched (AF_153431;
142 complete cds; Genbank Database) was reproduced in two parts by DNA sequence analysis.
143 Sequences were analyzed using BioEdit program (Ibis Therapeutics, Carlsbad, CA). The
144 same PCR protocol was used for both parts. The entire coding region of the MC1R gene
145 (GenBank accession number AF153431) was split into two fragments, respectively, using
146 specific primers (Forward Primer 1 (F₁): 5'-gga ggc ctc caa cga ctc ctt c-3'; R_{ewers} Primer 1
147 (R₁): 5'-cac gtg gcc gtc ctg ctg tg-3'; Forward Primer 2 (F₂): 5'-gct cca tgc tgt cca gcc tc- 3').
148 Thermocycling was performed in a 96-well Genius (Techne, U.S.A) and GeneAmp PCR
149 system 9700 (Applied Biosystems, U.S.A) thermocyclers. Initial denaturation of DNA at
150 94°C for 2 minutes was followed by 40 cycles of denaturation at 94°C for 1 minute, primer
151 annealing at 65°C for 1 minute, and primer extension at 72°C for 90 seconds. A final
152 elongation step was performed at 72°C for 5 minutes. Cycle sequencing was conducted in a
153 96-well GeneAmp PCR system 9700 thermocycler (Applied Biosystems, U.S.A) with the
154 following program: initial denaturation of DNA at 94°C for 2 minutes was followed by 30
155 cycles of denaturation at 94°C for 15 seconds, primer annealing at 60°C for 3 minutes, and
156 primer extension at 72°C for 90 seconds.

157 The protein area, consisting of approximately 20 amino acids in front and behind the amino
158 acid which changed due to mutation, was assessed by CLC Main Workbench 5.1 software
159 trail program in order to compare antigenicity of mutant and wild proteins. Respective
160 graphic curves were drawn showing respective antigenicities for wild and mutant proteins.
161 Then, both graphic curves were compared on a single graph.

162 **Statistical analysis:** Statistical analysis of data was carried out by using S0064 Minitab
163 Release 13 package program (License No: wcp 1331.00197). Definitive statistics of the
164 vitiligo and control groups were calculated. In the statistical assessment, consistency of age
165 and disease starting age variables with the normal distribution was examined with the
166 Kolmogorov–Smirnov test, and *t* test was used in independent groups in the comparisons
167 because they were consistent with the normal distribution. Chi-square test was used in
168 comparison of the groups based on the qualitative data. As a result of the assessments carried
169 out, $P < 0.05$ was accepted as statistical significance limit.

170 **RESULTS**

171 Sixty patients with vitiligo, living in Thrace for at least three generations and who did not
172 have any other systemic and dermatological disease, and 60 healthy volunteers were included
173 in our study. Forty-one patients had vitiligo vulgaris, 9 had acrofacial vitiligo, 3 had
174 segmental vitiligo, 1 had vitiligo universalis, and 6 had focal vitiligo.

175 Thirty-two members of the patient group were women (53.3) and 28 were men (46.7%).
176 Their ages were between 5 and 77, and the average age was 33.5 ± 15.5 . Thirty-one members
177 of the control group, however, were women (51.7%) and 29 were men (48.3%). Their ages
178 were between 19 and 76 and the average age was $34.4 (\pm 10.2)$. No significant difference was
179 found between vitiligo and control group regarding age ($P > 0.05$). Skin phototypes, according
180 to Fitzpatrick, of vitiligo patients and healthy controls are shown in Table 2. No significant
181 difference was found between vitiligo and control group regarding skin phototypes ($P > 0.05$).

182 In our study, there was no significant difference between both the vitiligo cases and control
183 group with respect to the number of nucleotide changes ($P>0.05$) (Table 3).
184 When SNPs of both groups were compared in terms of their genotype frequencies, no
185 statistically significant difference was found ($P> 0.05$) (Table 4).
186 Assessing both groups regarding allele frequencies, there was no statistically significant
187 difference between the vitiligo and control groups in terms of allele frequencies in the 178th,
188 274th, 451st, and 478th nucleotides ($P>0.05$). In the 488th nucleotide, however, wild type G
189 allele frequency was found as 92.55% and mutant A allele frequency was found as 7.5% in
190 the vitiligo cases; wild type G allele frequency was found as 81.7% and mutant A allele
191 frequency was found as 18.3% in the control group; and a significant difference was found
192 between the vitiligo and control groups in terms of mutant A allele frequency statistically in
193 favor of the control group ($P<0.05$) (Table 5). Conversion of the 488th nucleotide in MC1R
194 gene, from Guanine to Adenine upon mutation, gives rise to conversion of the 163rd amino
195 acid in MC1R protein from arginine to glutamine. This amino acid change causes a reduction
196 in antigenicity of MC1R protein (Fig. 1).

197 **DISCUSSION**

198 It is suggested that vitiligo, which is a pigmentation system disorder, may be caused by
199 MC1R gene variations (17).
200 According to the literature survey we conducted, no significant relation is specified in the
201 case-control study by Na et al. (17), which consists of 114 Korean vitiligo cases and 11
202 healthy volunteers; we identified this as the first study which searches the relation between
203 MC1R gene polymorphisms and vitiligo. It has been stated in this study in which MC1R gene
204 variants have been identified by the method of DNA sequence analysis that such SNPs
205 consisting of Val60Leu (G178T) rs1805005, Val92Met (G274A) rs2228479, Arg151Cys
206 (C451T) rs1805007, Arg160Trp (C478T) rs1805008 and Arg163Gln (G488A) rs885479 were

207 observed. Most frequently observed SNPs, however, are specified as Val92Met (24% in the
208 case group, 15% in the control group) and Gln163Arg (34% in the case group, 32% in the
209 control group). Na et al. observed in the same study a SNP (g.8818A->G) in 3'UTR region
210 (untranslated region) on the fourth exon of agouti signaling protein (ASIP) gene. MC1R gene
211 expression was regulated by its agonist (α -MSH) and its antagonist (ASIP). They could not
212 find a statistically significant relation between vitiligo and ASIP SNP. However, they found
213 that the individuals with MC1R G274A SNP in addition to ASIP SNP were more prone to be
214 vitiligo. A second similar study was conducted by Szell et al. in a Hungarian population (16).
215 Szell et al. suggested that C478T SNP might be a protective factor against vitiligo.

216 In our study, MC1R gene coding the melanocortin receptor, which is the control point in
217 human pigmentation system in vitiligo, was analyzed by performing a DNA sequence
218 analysis. Five types of SNP were found in each case and control groups: Val60Leu (G178T),
219 Val92Met (G274A), Arg151Cys (C451T), Arg160Trp (C478T), and Arg163Gln (G488A).
220 When both groups were compared regarding genotype frequencies, no statistically significant
221 differences were found ($P>0.05$). However, regarding allele frequencies, a statistically
222 significant difference was observed in Arg163Gln (G488A) allele in favor of the control
223 group ($P<0.05$). Therefore, MC1R gene Arg163Gln (G488A) allele was determined likely to
224 be a protective factor against vitiligo in our study population (Fig. 2).

225 Difference between our conclusion and results of the study of Na et al. is considered as the
226 genetic polymorphic differences between the populations. However, the reason for the
227 conflictive results between Arg163Gln (G488A) polymorphism and vitiligo may be explained
228 as general characteristics of the multifactorial hereditary disorders. There is a cumulative
229 effect of multiple genes with the environmental factor in the multifactorial heredity. Specific
230 combination of multiple alleles may create a stronger or weaker effect rather than a single
231 allele (18). The conclusion drawn by Szell et al. and our conclusion, however, agrees and

232 suggests that certain MC1R polymorphisms may be protective against vitiligo. However,
233 Szell et al. determined Arg160Trp (C478T) allele as the protective factor unlike Arg163Gln
234 (G488A) allele found by us. Szell et al. suggested that a reduction occurred in antigenicity of
235 the affected peptide epitope of Arg160Trp (C478T) allele. A similar reduction in antigenicity
236 of the affected epitope was found the same way in our study as well (Fig. 1). Surprisingly, the
237 epitope region with reduced antigenicity is the sequence between the 155th and 165th amino
238 acids in the study conducted by Szell et al. In our study, however, it is the sequence between
239 the 158th and 165th amino acids. Therefore, reduction in antigenicity of the epitope region
240 which overlaps in both studies (the sequence between the 158th and 165th amino acids) may
241 lead to a protective effect in vitiligo. Correspondingly, vitiligo may be caused by the
242 antibodies which occurred against MC1R in vitiligo development. It was shown in many
243 studies that changes occurred both in humoral and cellular immune activity in the patients
244 with vitiligo (3, 19, 20). Among organ-specific antibodies, antithyroid, gastric antiparietal
245 cell, and antinuclear antibodies were found to be high.

246 Again, it was reported in many studies that antimelanocytic antibodies were found in patient
247 serums (8, 21, 22, 23). Most studies suggested that antimelanocytic antibody titres are
248 parallel with the severity of vitiligo, and that there is a relation between antibody titres and
249 prevalence of depigmentation. It is reported in these studies that the level of antibodies
250 decreases when the disease becomes inactive. (20, 22, 23). Considering results of these
251 studies and results of our study together, it may be said that antigenicity of the structures
252 which play a role in pigmentation development takes an important part in vitiligo.

253 It is thought that future research on this subject with different populations, and to cover not
254 only MC1R gene but also other genes which are associated with the human pigmentation,
255 would facilitate our understanding of genetic etiology, genotype–phenotype, and genotype–
256 environment relation of the vitiligo as well. We think that this information will serve as a

257 guide in the determination of a new protocol for molecular diagnosis, therapy, and prognosis
258 of vitiligo.

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332 Table 1. Fitzpatrick skin phototypes

Skin Phototype	Skin color	Sunburn and suntan anamnesis
I	White	Always burns, never suntans
II	White	Always burns, sometimes suntans
III	White	Minimally burns, minimally suntans.
IV	Light brown	Burns, always suntans.
V	Dark brown	Rarely burns, shows dark brown pigmentation
VI	Black	Never burns, shows black pigmentation.

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356 Table 2. Skin phototypes according to Fitzpatrick of vitiligo patients and healthy controls

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Skin phototypes	Vitiligo Group	Control Group
Type 1	3 (5%)	9 (15%)
Type 2	31 (51.7%)	30 (50%)
Type 3	24 (40%)	17 (28.3%)
Type 4	2 (3.3%)	4 (6.7%)
Total	60	60

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382 Table 3. Distribution of number of nucleotide changes according to the vitiligo and control groups.

	Vitiligo n=60 (%)	Control n=60 (%)	p
With nucleotide change	46.7	55	p>0.05
Without nucleotide change	53.3	45	

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415 Table 4. Comparison of genotype frequencies of the vitiligo and control group SNPs.

MC1R Gene SNPs	Genotype	Vitiligo n=60 frequency (%)	Control n=60 frequency (%)	P
Val60Leu (G178T)	G/G	50 (83.3)	51 (85)	p>0.05
	G/T	8 (13.3)	8 (13.3)	
	T/T	2 (3.3)	1 (1.7)	
Val92Met (G274A)	G/G	53 (88.3)	51 (85)	p>0.05
	G/A	4 (6.7)	3 (5)	
	A/A	3 (5)	6 (10)	
Arg151Cys (C451T)	C/C	56 (93.3)	56 (93.3)	p>0.05
	C/T	3 (5)	2 (3.3)	
	T/T	1 (1.7)	2 (3.3)	
Arg160Trp (C478T)	C/C	57 (95)	58 (96.7)	p>0.05
	C/T	3 (5)	1 (1.7)	
	T/T	-	1 (1.7)	
Arg163Gln (G488A)	G/G	55 (91.7)	48 (80)	p>0.05
	G/A	1 (1.7)	2 (3.3)	
	A/A	4 (6.7)	10 (16.7)	

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436 Table 5. Comparison of allele frequencies of the vitiligo and control group SNPs.

MC1R Gene SNPs	Allele	Vitiligo 2n=120 frequency (%)	Control 2n=120 frequency (%)	P
Val60Leu (G178T)	A	-	-	p>0.05
	C	-	-	
	G	108 (90)	110 (91.7)	
	T	12 (10)	10 (8.3)	
Val92Met (G274A)	A	10 (8.3)	(12.5)	p>0.05
	C	-	-	
	G	110 (91.7)	105 (87.5)	
	T	-	-	
Arg151Cys (C451T)	A	-	-	p>0.05
	C	115 (95.8)	114 (95)	
	G	-	-	
	T	5 (4.2)	6 (5)	
Arg160Trp (C478T)	A	-	-	p>0.05
	C	117 (97.5)	117 (97.5)	
	G	-	-	
	T	3 (2.5)	3 (2.5)	
Arg163Gln (G488A)	A	9 (7.5)	22 (18.3)	p<0.05
	C	-	-	
	G	111 (92.5)	98 (81.7)	
	T	-	-	

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467 Figure 1. Antigenicity plot of the 40 amino acid long epitope affected by the Arg163Gln allele

468 Figure 2. Sequencing of PCR amplified DNA (Case no 11. MC1R gene, Arg163Gln G488A-

469 Homozygot)