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Footprints of Stress in Vitiligo: Association of the *5-HTR2C* rs6318 Variant

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Abstract

Vitiligo is a chronic, progressive autoimmune dermatological disease, and stress is known to have an impact on the development of vitiligo. However, the effect of the serotonin pathway and its impact have not been clearly explained for disease progression. Thus, this study aimed to clarify the stress induced serotonin receptor *5-HTR2C* rs6318 variant and its association with vitiligo pathogenesis.

Case-control study was conducted with 108 vitiligo patients and 107 age-sex matched, unrelated healthy control group. Real Time-PCR analysis was used for genotyping the *5-HTR2C* variation. Genotype and allele frequencies, genotype distributions, Hardy-Weinberg Equilibrium (HWE) and vitiligo-related risk measurements were examined. Genotype correlations of the variant were also analyzed based on gender difference, age onset, Koebner phenomenon history, triggered with stress, clinical subgroups, treatment types, the presence of other autoimmune diseases, vitiligo presence in family members and other autoimmune diseases in relatives.

Statistical differences in 5HT-R2C receptor genotypes and allele frequencies between patients and controls were not detected. Genotype frequencies were not in agreement with Hardy-Weinberg Equilibrium in the patients' group ($p<0.00001$). The frequency of the risk allele (allele C) was not significantly different between the patient and control groups ($p=0.1392$). However, in the clinical subgroup analysis, the risk allele presence was detected to be significantly higher for early age onset (<40 years) vitiligo development ($p=0.035$, OR=Infinity, RR=1.391) and lower in Koebner phenomenon history ($p=0.0276$, OR= 0.219, RR=0.325).

In conclusion, although there was no association between the *5-HTR2C* variant rs6318 and vitiligo, current results indicate that there is an association between the *5HTR2C* rs6318 variant C allele and early onset vitiligo development.

Keywords: Case-control study; polymorphism; stress; vitiligo; *5-HTR2C* rs6318

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Introduction

Vitiligo is a chronic, progressive autoimmune disease characterized by depigmented macules and patches that develop due to loss of melanocytes [1]. It occurs equally in men and women [2]. Although it may develop at any age, most cases are observed before the age of 30 [3,4]. Disease prevalence varies from 0.5% to 2%, depending on the geographical area [5-7].

Vitiligo can occur anywhere on the body, such as face, dorsal side of hands, areola mamma, axilla, umbilicus, sacrum, inguinal and anogenital regions, knees, elbows, fingers and flexor surfaces of the wrist, dorsal ankles and legs. Furthermore, disease may develop due to repetitive traumatic surfaces on bony prominences. Vitiligo is categorized as generalized, segmental, focal and acrofacial [8-10]. Moreover, active disease stage is evaluated according to new lesion emergence or growth within an existing lesion for 12 moths [9]. As pharmacological treatments; topical corticosteroids, calcineurin inhibitors, vitamin D3 analogs, phesudocatalase/superoxide dismutase, MTX, janus kinase (JAK) inhibitors and systemic corticosteroids are commonly used. In addition, phototherapies (phototherapy narrow-band UVB, PUVA, laser therapy) are applied as physical local therapies [11]. The etiology of vitiligo is unknown but the Koebner

phenomenon, different diseases, physical trauma, sunburn, pregnancy and emotional stress were detected as associated with vitiligo development [12, 13].

The serotonin receptor 5-HTR2C is observed critical for the activation of stress induced hypothalamic-pituitary-adrenal (HPA) axis [14]. X-linked single nucleotide polymorphism (SNP) on the *5-HTR2C* gene causes a G to C switch at base pair 68 and results in cysteine synthesis instead of serine at codon 23 (Cys23Ser) [15]. Furthermore, this variation (rs6318) was also found to be associated with the induction of stress related physiological and psychological disorders. For instance, individuals with *5HTR2C* Cys23Ser gene variation had depressive behaviors under stressful life events [16]. Moreover, late on set Alzheimer patients who had the 5HTR2C C allele saw more visual hallucinations and developed more psychotic symptoms [17, 18]. In addition, individuals with the *5HTR2C* Cys23Ser gene variation had higher blood cortisol levels, central obesity, insulin resistance and higher blood pressure so these individuals had a tendency to develop diabetes and cardiovascular disorders [19]. Recently, in our previous study with childhood allergic asthma, risk allele (allele C) was detected as significantly associated with the development of atopy-related asthma [20]. Furthermore, the relevance of *5HTR2C* Cys23Ser variation and stress related dermatological diseases was investigated with psoriasis patients and although no significant difference was detected for allele frequencies compared to the control group, the *5-HTR2C* rs6318 variant was only found to be associated with treatment methodology because variation was significantly observed in patients treated with conventional therapy [21].

According to all the points mentioned in terms of vitiligo, recent studies have shown the importance of neurotransmitters (dopamine, acetylcholine, norepinephrine (NE) or epinephrine (EP), gamma-aminobutyric acid (GABA), glutamate and serotonin (5-hydroxytryptamine, 5-HT)) for the development, regeneration, and function of melanocytes [22]. Patients with pigment abnormalities were detected with reduced tryptophan metabolism and 5-HT serum levels [23]. In literature, there was no evidence about the impact of the *5-HTR2C* variant (rs6318) on vitiligo. Thus, this study aims to clarify the possible correlation between *5-HTR2C* rs6318 G>C polymorphism and its clinical importance in vitiligo, so this study may provide a better understanding of the underlying mechanisms.

Materials and Methods

In Patients and healthy controls

108 patients with clinical diagnosis of vitiligo who are followed in Bursa Uludag University Faculty of Medicine, Department of Dermatology and Venereal Diseases, and 107 age-sex matched, unrelated healthy control group were included in this study. Blood samples were collected in sterile tubes containing 0.1% ethylenediaminetetraacetic acid (EDTA) tetrasodium. The detailed information regarding patients' age and sex distribution, age of onset of vitiligo, possible triggering factors, clinical types of vitiligo, presence in relatives and other autoimmune diseases provided in Table 1. The control group was composed of biologically unrelated healthy volunteers (who do not have any family history of vitiligo) and were matched with the patient group in terms of age, sex and ethnicity. This study was approved by the Bursa Uludag University Ethical Committee and informed consent was obtained from all subjects. (Approval number: 2014-24/26).

Table 1. General and clinical characteristics of patients with vitiligo

Total n	**108**
Sex(n)	
Female	57
Male	51
Age	
Mean±SD	41.68±16.49
Median; min-max	43.5; 12-87
Age onset	
Mean±SD	28.09±16.38
Median; min-max	29.5; 2 months-65
Early onset n (<40 years old) (n)	81
Late onset n (≥ 40y ears old) (n)	27
Triggered with stress (n)	
Yes	17
No	91
Koebner phenomenon (n)	
Yes	90
No	18
Clinical Type (n)	
Generalized	59
Focal	17
Acral/Acrofascia	32
Active form of vitiligo (n)	
Yes	52
No	56
Vitiligo family history (n)	
Yes	28
No	80
Other autoimmune diseases (n)	
Yes	39
No	69

Molecular genotyping analysis

Blood-Animal-Plant DNA Preparation Kit was used according to the manufacturer's instructions for the isolation of genomic

Table 2. Genotype distribution and genotype frequencies of *5-HTR2C* rs8318 SNP in the two different groups according to sex

Sex	Group	5-HTR2C rs6318 Genotype						
		G/-	f(G/-)			C/-	f(C-)	*p* value
Male	Control	22	0.76			7	0.24	0.8668
	Vitiligo	43	0.84			8	0.16	
		GG	f(GG)	GC	f(GC)	CC	f(CC)	*p* value
Female	Control	55	0.70	21	0.27	2	0.03	0.3129
	Vitiligo	42	0.74	11	0.19	4	0.07	
		GG	f(GG)	GC	f(GC)	CC	f(CC)	*p* value
Total	Control	77	0.72	21	0.20	9	0.08	0.1392
	Vitiligo	85	0.79	11	0.10	12	0.11	

DNA from peripheral blood samples (Jena Bioscience, Germany). 5-hydroxytryptamine receptor 2C (rs6318) single nucleotide polymorphism was determined by the TaqMan System (Applied Biosystems, Life Technologies), Primer Prob Mix and qPCR Probes Master (Jena Bioscience, Germany, PCR-360). The interested sequence of the rs6318 variant was CTAATTGGCCTATTGGTTTGGCAAT(G/C)TGATATTTCT GTGAGCCCAGTAGCA. G allele was labeled with reporter dye VIC, and C allele was labeled with FAM reporter dye. The total reaction volume per well was adjusted to 20 µl. 10 µl qPCR Probes Master, 1 µl Primer Prob Mix, 7 µl PCR Grade water and 2 µl sample DNA were added to each well. The reaction was carried out on the Biorad CFX machine and cycling conditions were set as: 95°C, 2 minutes, 1 cycle followed by 95°C, 15 seconds, 40 cycles and 60°C, 1 minute, 1 cycle. SNP was determined as a result of fluorescence measurements of the reporter dyes at the end of the RT-PCR reactions.

Statistical analysis

Genotype distribution and genotype frequencies were considered depending on gender for both control and patient groups. Allele frequencies and genotype distributions for the Hardy-Weinberg Equilibrium (HWE) were analyzed. Vitiligo-related risk measurements for different genotype combinations were conducted. Genotype correlations of the 5-HTR2C gene rs6813 G>C variant were also considered for clinical subsets based on gender difference, age onset of vitiligo, Koebner phenomenon, clinical subgroups, treatment types, presence of other autoimmune diseases, vitiligo and other autoimmune diseases in relatives.

Descriptive statistics were presented as mean ± standard deviation and median (minimum-maximum). Frequency and percentage calculations were performed for the qualitative variables. The distributions of the quantitative variables were assessed using the Shapiro Wilk test of normality. The Pearson Chi-Square test and Fisher's Exact test were used to compare genotype and allele frequency differences between groups. The odds ratio (OR) and relative risk (RR) with a 95% confidence interval (95% CI) were calculated. The level of statistical significance was accepted as 0.05 for the whole study. All statistical calculations were performed using a commercial GraphPad Prism 8 software program (Graphpad Software, Inc. California, USA).

Results

The total number of subjects who were enrolled in this study was 215 (108 patients and 107 healthy volunteers). The vitiligo group consists of 57 females and 51 males with a mean range of 41.68±16.49 (min-max:12-87), and the control group consists of 78 females and 29 males with a mean age of 44.08±11.13 (min-max:12-98) healthy volunteers.

It is known that the *5-HTR2C* gene is located on the X chromosome, so the homozygote G allele was determined as most common allele for both the vitiligo and control groups (79% and 72%, respectively). There was no significant difference between genotype distributions among control and patient groups in terms of sex (for males p=0.8668, for females p=0.3129) as demonstrated in Table 2.

The allele and genotype frequency distributions of the *5-HTR2C* rs6318 G>C polymorphism among vitiligo and control groups were analyzed. Both allele and genotype frequencies showed no significant difference between the control and vitiligo patients (p=0.89 and p=0.1392 respectively) (Table 3). However, a statistically significant difference was observed in terms of the genotype distribution of the alleles in both the control and vitiligo patient groups (HWE p=0.001 and p<0.0001, respectively) (Table 3). Different genotype combination risk score analysis were conducted, and there was no statistical association between the *5-HTR2C* gene rs6318 C allele and vitiligo (Table 4). Furthermore, genotype correlations of the *5-HTR2C* gene rs6813 G>C variant between clinical subsets considered in terms of gender, age onset, Koebner phenomenon, triggered with stress, clinical subgroups, treatment types, treatment responses, other autoimmune diseases, vitiligo and other autoimmune diseases in family members. The *5-HTR2C* gene rs6318 C allele was observed to be statistically higher and associated with early age onset compared to late onset (p=0.035, OR=Infinity, RR=1.391) (Supplementary Table 1) and statistically lower in patients that

Table 3. Allele frequencies and amounts of genotype expected and observed among the studied groups

		Control	Vitiligo	
		n – frequency	n – frequency	*p* value
Allele	G	153 – 0.83 (p)	138 – 0.84 (p)	0.89
	C	32 – 0.17 (q)	27 – 0.16 (q)	
Genotype	HWE equation	Expected and observed	Expected and observed	*p value*
GG	p2	73.19 and 77	75.55 and 85	0.1392
GC	2pq	30.61 and 21	29.56 and 11	
CC	q2	3.20 and 9	2.89 and 12	
HWE χ^2, df and *p* value		χ^2=13.72 df=2 p=0.001	χ^2=41.55 df=2 p<0.0001	

Table 4. Vitiligo-related risk measures of different genotype combinations for rs6813

Genotypes	Odds Ratio (95% Confidence Interval)	Relative Risk (95% Confidence Interval)	*p* Value
GG vs GC & CC	0.695 (0.369-1.269)	0.840 (0.642-1.142)	0.271
CC vs GC & GG	0.735 (0.308 -1.730)	0.848 (0.477-1.297)	0.647
GC vs GG & CC	2.153 (1.011- 4.683)	1.396 (0.997-1.813)	0.057

had Koebner phenomenon history (p=0.0276, OR= 0.219, RR=0.325) (Supplementary Table 2). Moreover, there was no significant association between the *5-HTR2C* gene rs6318 G>C variant and other clinical subsets (Table 5).

Discussion

Recent studies have declared that people with vitiligo have depression and impaired general health compared to healthy individuals [13]. Studies, which considered psychological symptoms, reported the prevalence of depression in 2071 and prevalence of anxiety in 866 vitiligo patients [24]. Therefore, our study tried to clarify stress induced serotonin receptor 5HTR2C Cys23Ser gene variation and vitiligo pathogenesis.

In addition, the development of vitiligo is commonly observed in children compared to adults [5] and in late onset vitiligo (≥40 years) stress is detected as associated with the development of the disease [25, 26]. Physical and environmental stressors (sun burns, chemicals and skin trauma) and psychological stressors (death of relatives, work and financial problems) were determined as factors for the development of vitiligo [27-29]. The exact mechanism of stress impact on vitiligo development still needs to be clarified, but it is known that stress and stressors by themselves have an impact on the initiation of autoimmune responses [30]. Furthermore, in vitiligo patients, norepinephrine levels were higher compared to healthy control groups. Also in these patients, due to stress, cytokine imbalances occurred, and this situation caused oxidative stress [31]. According to several studies, due to oxidative stress; accumulations of free radicals, reactive oxygen species and hydrogen peroxide in the epidermis are detected, and this situation is found in relation to apoptosis or degradation of melanocytes, which leads to the development of vitiligo [32-34]. In vitiligo patients, polymorphisms in oxidative stress related genes are associated with the development of diseases such as glutathione S-transferases (GSTs), nuclear factor erythroid 2-related factor 2 (NRF2-ARE), heme oxygenase (HO), antioxidant catalase enzyme (CAT) and superoxide dismutase (SOD) [35].

Furthermore, it is known that stress responses induce HPA axis induction, which interacts with innate and adaptive immune cells and also causes activation of keratinocytes [36]. The serotonin receptor 5-HTR2C had also been found to be associated with stress induced hypothalamic-pituitary-adrenal (HPA) axis [14] and 5-HT receptors were highly observed in mammalian melanocytes and dermal fibroblasts [37]. Patients with pigment abnormalities and vitiligo had reduced tryptophan metabolism and 5-HT levels [23, 38]. Moreover, in a chronic mental stress exposed depigmented mouse model, 5-HT levels in serum and skin decreased as observed in vitiligo patients, and 5-HT receptors (HTR1A, HTR1B, HTR1D, HTR2A, HTR5A, and HTR5B) were detected as reduced in the skin of mice [39, 40]. Reuptake inhibitors of serotonin and receptor agonists could promote melanin production under *in vitro* conditions [41-43]. However, in previous studies, the effects of the 5-HT2C receptor and its genetic variants on vitiligo pathogenesis were not considered a risk factor.

In the *5-HTR2C* rs6318 variant, SNP occurs in codon 23, causes the G allele to switch to the C allele, which results in the substitution of cysteine for serine [15], and individuals with

Table 5. Genotype correlations of the 5-HTR2C gene rs6813 G>C variant between clinical subsets of the patient group.

Clinical Subsets		G Allele	No G Allele	*p* Value
Gender	Male	43	8	0.221
	Female	53	4	
Age onset	< 40 years	69	12	0.035*
	≥ 40 years	27	0	
Koebner phenomenon	Yes	13	5	0.028*
	No	83	7	
Triggered with stress	Yes	13	4	0.756
	No	72	19	
Clinical Subgroups	Generalized	51	7	0.769
	Focal/Acrofascial/Segmental	45	5	
Treatment Types	Local	83	12	0.353
	Systemic	13	0	
Treatment Responses	No Response	54	3	0.063
	Response	42	9	
Autoimmune diseases	Yes	35	4	>0.9999
	No	61	8	
Vitiligo presence in family	Yes	24	5	0.298
	No	72	7	
Autoimmune diseases in family	Yes	45	9	0.123
	No	51	3	
		C Allele	**No C Allele**	
Gender	Male	8	43	0.240
	Female	15	42	
Age onset	< 40 years	21	60	0.056
	≥ 40 years	2	25	
Koebner phenomenon	Yes	7	11	0.060
	No	16	74	
Triggered with stress	Yes	1	16	0.687
	No	11	80	
Clinical Subgroups	Generalized	13	45	>0.9999
	Focal/Acrofascial/Segmental	11	39	
Treatment Types	Local	21	74	0.731
	Systemic	2	11	
Treatment Responses	No Response	12	45	>0.9999
	Response	11	40	
Autoimmune diseases	Yes	8	31	>0.9999
	No	15	54	
Vitiligo presence in family	Yes	7	22	0.791
	No	16	63	
Autoimmune diseases in family	Yes	13	41	0.639
	No	10	44	
		Hemizygous/Homozygous	**Heterozygous**	
Age onset	< 40 years	72	9	0.727
	≥ 40 years	25	2	
Koebner phenomenon	Yes	16	81	>0.9999
	No	9	2	
Triggered with stress	Yes	14	3	0.3743
	No	3	83	
Clinical Subgroups	Generalized	52	6	>0.9999
	Focal/Acrofascial/Segmental	44	6	
Treatment Types	Local	86	9	0.619
	Systemic	11	2	
Treatment Responses	No Response	49	2	>0.9999
	Response	48	9	
Autoimmune diseases	Yes	35	4	>0.9999
	No	62	7	
Vitiligo presence in family	Yes	27	2	0.724
	No	70	9	
Autoimmune diseases in family	Yes	50	4	0.527
	No	47	7	

this variant represent more depressive behaviors under stress [16]. The present study was also conducted as a case control study, which considered 108 vitiligo patients and 107 healthy controls in terms of the 5-HTR2C rs6318 variant impact on vitiligo. Moreover, there were no statistically significant differences among the studied groups in terms of sex and age.

As known, the *5-HTR2C* rs6318 variant is an X linked SNP. Depending on that knowledge, genotype analysis was considered for all the subjects separately according to their gender. The homozygote G allele was the most common allele for both the vitiligo and control groups (79% and 72% respectively). There was no significant difference between the genotype distributions of the control and patient groups in terms of gender (for male p=0.8668, for female p=0.3129). Moreover, there were no statistical differences for allele and genotype frequencies among the studied groups (p=0.89 and p=0.1392). On the other hand, statistically significant differences were observed within the control and vitiligo groups in terms of genotype distribution (HWE p=0.001 and p<0.0001, respectively). This means that both control and patient groups were not in accordance with Hardy-Weinberg Equilibrium (p<0.001) because of the possible dominance of certain genotypes due to mutations, the possibility of an isolated population or the contribution of different genetic backgrounds in population [44].

Furthermore, risk score analysis was conducted for the different genotype combinations and there was no association between the *5-HTR2C* gene rs6318 C allele and vitiligo. Also, clinical subsets such as gender, age onset, presence of the Koebner phenomenon, triggered by stress, clinical subgroups, treatment types, treatment responses, other autoimmune diseases, vitiligo and other autoimmune diseases in family members were considered interms of any association of the *5-HTR2C* rs6318 variant. C allele substitution was statistically observed higher in early onset compared to late onset (p=0.035, OR=Infinity, RR=1.391) patients. Although, as mentioned, the *5-HTR2C* rs6318 variant was detected as associated with stress [16] and the development of late onset vitiligo stress was determined as a contributing factor [24], our findings were not consistent with these data, so psychiatric clinical diagnosis needs to be conducted with higher numbers of patients in terms of depression and stressful life events for early onset *5-HTR2C* rs6318 variant carriers and other subjects. Moreover, the *5-HTR2C* rs6318 variant was detected statistically lower in patients that had the Koebner phenomenon (p=0.0276, OR= 0.219, RR=0.325). It is known that the Koebner phenomenon may occur due to physical trauma, mechanical stress, chemical stimulations, and some other diseases (inflammatory dermatosis, cryoglobulinemia, leukocytoclastic vasculitis and infections) [45]. Although there was no certain association between Koebner phenomenon and psychological stress in the literature, since this phenomenon is commonly detected in stress associated dermatological diseases such as psoriasis and vitiligo, there might be an association between Koebner phenomenon and stress, but in our findings, the unexpectedly stress induced *5-HTR2C* rs6318 variant was observed significantly lower in patients who had Koebner phenomenon history.

Additionally, in our previous study with psoriasis patients, although there was no significant difference in terms of the *5-HTR2C* variant (rs6318) genotype and allele frequencies, a significant difference was observed for the arthritis development and psoriasis treatment methodology [20]. As with psoriasis, there were no significant differences in terms of *5-HTR2C* variant (rs6318) genotype and allele frequencies for vitiligo patients; however, statistical differences were observed for age onset vitiligo development and patients who had Koebner phenomenon history. This situation may be compatible with the research that determined vitiligo patients were less depressive, and their anxiety was lower compared to psoriasis patients [23].

Our study is the first to investigates the potential association between stress induced *5-HTR2C* rs6318 variant and vitiligo. Although there was no association between the *5-HTR2C* variant rs6318 and vitiligo development, current results indicate that there is an association between the *5HTR2C* rs6318 variant C allele and early onset vitiligo development. Thus, these findings may provide early onset disease detection and personalized treatment options. For further studies, the study sample size needs to be increased to clarify the *5-HTR2C* rs6318 variant and its importance on vitiligo pathogenesis and variation impact on melanocyte differentiation pathways, possible stress induced biochemicals and cytokine profiles need to be investigated.

Ethical statement

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Bursa Uludag University and the approval number was 2014-24/26. Informed consent was obtained from all participants included in the study.

Authors' contributions

S.G.T. conceptualized the project and designed the experiments. Material preparations and clinical evaluation of the patients performed by S.Y., E.B.B, K.A. Genotypical and statistical analysis and their clinical relevance performed by I.Y., E.B.B. and S.G.T. Data were interpreted by I.Y., S.Y., M.C.E., E.B.B., H.B.O., K.A., SG T. The first draft of the manuscript was written by I.Y. and S.G.T. All authors commented and approved the final manuscript.

Conflicts of Interest

The authors declare no competing interests.

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Supplementary Table 1. Age onset-related risk measures of different genotype combinations for rs6813

Genotypes	Odds Ratio (95% Confidence Interval)	Relative Risk (95% Confidence Interval)	*p* Value
GG vs GC & CC	0.229 (0.503-0.921)	1.251 (0.645-0.987)	0.056
CC vs GC & GG	Infinity (1.317- Infinity)	1.391 (1.043-1.577)	0.035*
GC vs GG & CC	1.563 (0.3570-7.581)	1.102 (0.6969-1.366)	0.727

Supplementary Table 2. Koebner phenomenon-related risk measures of different genotype combinations for rs6813

Genotypes	Odds Ratio (95% Confidence Interval)	Relative Risk (95% Confidence Interval)	*p* Value
GG vs GC & CC	0.219 (0.059-0.748)	0.325 (0.154-0.797)	0.028*
CC vs GC & GG	2.943 (1.058-8.483)	2.352 (1.016-5.133)	0.060
GC vs GG & CC	0.889 (0.190-4.420)	0.9072 (0.302-3.403)	>0.9999

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