



## Original Research Article

# ***TAP1* and *LMP7* Gene Polymorphisms Associated with Vitiligo in Saudi Community**

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## **A B S T R A C T**

### **Keywords**

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*LMP7*.

Vitiligo is a depigmenting complaint of the skin caused by selective obliteration of melanocytes. Vitiligo affects about 1–4% of the world population without any ethnic, gender, or regional differences in prevalence. The current research was performed on Saudi Vitiligo population to determine the prevalence of *TAP1* and *LMP7* in these patients. Samples were collected from forty one patients in several hospitals and medical centers. The study was case/control study on *TAP1* and *LMP7* using *restriction fragment length polymorphism* (RFLP) to determine if these genes are associated with vitiligo in Saudi patients. Genotypes were determined by RFLP analysis of the PCR products. High association was found in *TAP1* and no association of *LMP7* was found with the disease,

## **Introduction**

Vitiligo is an acquired disorder characterized by depigmented macules in the skin resulting from the loss of functional melanocytes and of melanin from the cutaneous epidermis (Kemp et al., 2007). It has been estimated that about 50 million persons around the globe are affected (Talsania et al. 2010), fifty percent of cases appear before the age of 20 years, and the disfigurement results in psychiatric morbidity in 16 to 35% of those affected. (Alzolibani, 2009). Little is known of its pathogenesis and various hypotheses have been suggested.

These hypotheses propose autoimmune, oxidant-antioxidant, neural, or genetic susceptibility (Halder and Chappell, 2009). However, as none of the three major hypotheses is sufficient to fully explain the mechanisms of vitiligo, the convergence theory is proposed stating that stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment and impaired melanocyte migration and proliferation can all contribute to vitiligo etiopathogenesis in varying proportions (Kingo et al., 2008).

Addressing psychosocial factors is an important aspect of the management of vitiligo, particularly in patients from communities where the disease is greatly stigmatizing (Pahwa et al., 2013), other studies have documented the importance of physical appearance in psychological adjustment and the impact of the physical disfigurement caused by depigmentation (Firooz et al., 2004).

There may in fact be two coexisting modes of inheritance for vitiligo depending on age of onset (Arcos-Burgos et al., 2002). In patients with early onset vitiligo (before the age of 30), vitiligo inheritance most closely followed a dominant mode of inheritance with incomplete penetration. However, a predisposition to vitiligo resulting from a recessive genotype and exposure to certain environmental triggers appeared to explain the inheritance pattern of late-onset vitiligo (Huggins et al., 2005). (after 30 years of age). Several genes might be associated with vitiligo disorder. Some of these genes are *TAP/LMP* loci, *AISI*, and *AIRE* genes.

Due to the suspected autoimmune origin of vitiligo, many HLA linkage disequilibrium studies have been done and have consistently found a significant association between the HLA system and a predisposition to vitiligo (Passeron and Ortonne, 2005). Case/control analyses reveal association of vitiligo and the gene encoding *TAP1*, whereas a family-based association method (TDT) revealed biased transmission of specific alleles from heterozygous parents to affected offspring for the *TAP1* gene, as well as for the *LMP2* and *LMP7* genes (Casp et al., 2003).. Changes in *LMP* and *TAP* expression and/or function might influence the peptide repertoire presented by vitiligo patient antigen-presenting cells, by

affecting antigen processing by the immunoproteasome or by preferential transport of peptides for MHC class I presentation. Future studies of immunoproteasome and *TAP* expression and function in vitiligo patient antigen-presenting cells are needed to further define a role for these genes in vitiligo susceptibility (Casp et al., 2003).

The design of the candidate-gene approach is simple; the fundamental requirements are the identification of a gene that is involved in the disease phenotype, a polymorphic marker within that gene and a suitable set of subjects to genotype for that marker. Identification of the potential candidate genes is the main stumbling block (Zhang et al., 2005; Halder and Chappell, 2009).. There are two main types of candidate that are generally considered in such studies: functional and positional. Functional candidate-gene studies in vitiligo have focused on many genes whose products participate in the immune and/or epidermal melanocyte barrier function. Positional candidates are genes that are identified because they lie within genomic regions that have been shown to be genetically important in linkage or association studies or by the detection of chromosomal translocations that disrupt the gene. Positional candidate-gene studies are not currently being undertaken (Zhang et al., 2005). More than 120 genes regulate the mammalian pigmentation. Each of them as well as those regulating the immune system represents potential candidate genes for vitiligo and underlines the importance of the research work that remains to be done (Passeron and Ortonne, 2005).

The aim of this study is to determine if Saudi vitiligo patients have high association of reported polymorphisms in

other populations for *TAP1* gene and/or *LMP7* gene by comparing DNA extracted from blood samples of patients with control DNA extracted from blood of non-vitiligo affected people using restriction fragment length polymorphism technique (RFLP).

## **Materials and Methods**

### **Study Design and Sampling**

The study was case-control study, which was performed on Saudi vitiligo population (male/female). Forty three samples were collected from vitiligo patients from several hospitals/medical centers. These hospitals include AL Dammam Medical Center (26 samples), Hera'a General Hospital (11 samples), King Abdul-Aziz Specialist Hospital in Taif (5 samples) and AL Noor specialist hospital (one sample), the average age was 24 years. And Forty three healthy controls samples were collected with 21 years average age. Intravenous blood samples were drawn in 3.5-cm<sup>3</sup> sterile tubes containing ethylene diaminetetraacetic acid (EDTA) solution. The blood samples were transferred to the molecular lab using boxes with ice blocks in temperature around (1-10°C) and stored in a freezer at -20°C till use.

### **DNA Extraction**

The blood samples were left to thaw and DNA extraction were performed using QIAamp spin column kit (QIAGEN, Maryland, USA). Proteinase K was prepared by dissolving the lypholyzed powder in 1.2ml protease solvents (nuclease free water).

A 20 µl of proteinase K solution was pipetted to the bottom of an Eppendorf

tube (1.5 ml). Whole blood specimen (200 µl) was added to the proteinase K, followed by lysis buffer (200 µl). The blood mixture were vortexed for 15 seconds using a vortex mixer (LabTech, LVM-202, Korea) and incubated for 15 min at 56°C (Eppendorf AG, Thermomixer Compact, GmbH, Germany) for complete hemolysis of RBCs.

Absolute ethanol (200 µl) was added to the lysed solution. The digested solution was mixed by vortexing and transferred into the QIAamp spin column. The spin column was centrifuged at 8000 rpm for 1 min, and the filtrate was discarded. AW1 washing buffer (500 µl) was added to column, and the mixture was centrifuged at 8000 rpm for 1 min, and the filtrate was discarded. AW2 washing buffer was added (500 µl) to the column, centrifuged at 14,000 rpm for 3 min, and the filtrate was discarded and the centrifugation process was repeated.

The QIAamp spin column was placed on a 1.5-ml Eppendorf tube, and AE elution buffer (200 µl) was added to column. The column was incubated at room temperature for 5 min, and centrifuged at 8,000 rpm for 1 min. The pure DNA was eluted and then refrigerated at 4°C until use. For longer storage period, the DNA was kept at -70°C for an infinite time.

### **DNA Recognition**

Gel electrophoresis was used to determine the quality of DNA using 2% agarose (Promega, USA) in 100 ml 1X of Tris-borate-EDTA (TBE), pH 7.8 (TBE buffer, Gel-Mate Mix, Gibco-BRL). The mixture was boiled in the microwave oven till dissolution, and 20 µl of ethidium bromide dye (Promega, USA) was added. The dissolved agarose was then poured in the

cast tray gel for 1 h containing 1X TBE buffer, as the electrophoretic buffer. The DNA solution sample (8 µl) was mixed with 2 µl with loading blue dye (Promega, USA), mixed and applied onto the well of the gel. The DNA sample was electrophoresed at constant voltage (100 V/cm) using an electric power supply (Thermo EC, UK) for a 45 minutes. This step established to confirm the presence and quality of DNA isolation products. The gel was removed from TBE buffer and placed under UV-trans-illuminator (G-Box Syngene, UK).

### **Polymerase Chain Reaction (PCR)**

For *TAPI* G/A exon 10, the thermal cycler program was 35 cycles of: denaturing at 94°C for 30 sec; annealing at 60°C for 30 sec, and extension at 72°C, followed by a 2 min final extension. For *LMP7* G/T intron 6, the same conditions were used with an annealing 58°C for 30 sec.

PCR of the DNA performed on 0.2 ml PCR tubes. A 10 µl of premix *Taq* enzyme (Promega, USA) containing 2.5 µl PCR buffer, 0.5 µl dNTPS, 0.5×2, 10µl *Taq* polymerase, 14 µl distilled water, and 10 µl of the isolated DNA in each PCR tube. The PCR tubes placed on the thermocycler (Dyad, Biorad, USA). The PCR buffer used contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1.5 mM MgCl<sub>2</sub>.

PCR products mixed with 2µl loading dye and then electrophoresed on 2% agarose-ethidium bromide (Promega, USA) using suitable size marker (50 bp ladder) in a TBE, pH 7.8. After electrophoresis, the gel was viewed under UV-trans-illuminator (G-Box Syngene, UK). The gel was photographed using gel documentation and analysis system. The PCR product showed fragment at 165-bp for *TAPI* G>A

exon 10 and at 252-bp for *LMP7* G/T intron 6 characterizing the amplified regions.

### **Restriction Fragment Length Polymorphism (RFLP)**

Genotypes were determined by RFLP analysis of the PCR products. Following restriction digestion of PCR products under conditions specified by the enzyme supplier (Fermantas, GmbH), restriction fragments were separated on 2% MetaPhor® agarose gel/ethidiumbromide dye (Promega, USA) in TBE buffer.

The expected product sizes for the *TAPI* (exon 10, G>A) and *LMP7* (exon 3, G>A) polymorphisms. The *TAPI* G>A alleles were differentiated by *AccI* restriction digestion (G-allele, 165- bp; A-allele, 136-29-bp), and the *LMP7* (G/T) alleles were differentiated by *HhaI* restriction digestion (G-allele, 212-, 40-bp; A-allele, 252bp) Table -1.

For detection of the genotypes of the samples in *TAPI* G>A exon 10 the amplified PCR product was incubated with 10 units of buffered restriction enzyme *AccI* at 37°C for overnight as recommended by the manufacturer and to detect the genotypes of the samples in *LMP7* G/T intron 6. The amplified PCR product was incubated with 10 units of buffered restriction enzyme *HhaI* at 37°C for overnight as recommended by the manufacturer.

Digested PCR products were separated on 2% MetaPhor agarose gel /ethidiumbromide dye (Promega, USA). The Gel was viewed under UV-trans-illuminator (G-Box Syngene, UK). The data analyzed to identify the genotypes for *TAPI* G/A exon 10 and *LMP7* G/T intron 6.

## Data Analysis

For case/control association studies, the significance of observed differences in allelic or genotypic frequencies between vitiligo patient and control populations was determined using standard  $X^2$  test.

## Results and Discussion

Patients with vitiligo and healthy controls were selected from Saudi subjects. The present study has tested 43 patients and 43 controls. As regard to the distribution of vitiligo lesion; legs (we consider foot as leg) were affected in 44%, face in 32%, knee in 27%, hand (we consider the fingers as hand) in 51%, and we have 3/43 are generalized lesion, hands was the most affected area in Saudi vitiligo patients (Fig. 1).

Family history of similarly affected person was present in 38%; the affected members may be daughters, sons, or cousin. The mean age of vitiligo case was 24 years (range 4–58 years) while the highest vitiligo case was in patients between eleven and twenty years (Fig. 2). The duration of the disease according to age of patients was various (Fig. 3).

The single-nucleotide polymorphism (SNPs) used in this study as genetic markers focused on the *LMP7* (intron6) and *TAP1* (exon10) loci. Preliminary case/control analyses of patient population consisting 43 unrelated Saudi Vitiligo patient and 43 healthy control revealed in significant difference in allele and/or genotype frequencies. The G/A SNP in exon 10 (D637G) of the *TAP1* gene displayed an excess of the A allele in patient VS control group ( $p$ -value = 0.005) means highly significant. While the G/T SNP in intron6 of the *LMP7* gene showed no significant difference ( $p$ -value= 0.586) Table -2.

The G/A SNP in exon 10 of *TAP1* gene (D637G) exhibited a significant excess ( $p$ -value= 0.001) of the AA genotype allele in vitiligo patients (48.82%) compared to controls (9.52%), while the G/T SNP in intron 6 of *LMP7* gene showed no significant difference ( $p$ -value= 0.543) Table-3.

Vitiligo is devastating to patients and to family members. The sense of being stigmatized or being different from others is a common reaction and may affect a person's interpersonal and social behavior (Borimnejad et al., 2006). To date, however, no treatment provides constant satisfactory results (Passeron and Ortonne, 2006).

The nature of the genetic association may vary according to different ethnic backgrounds. In this study, we investigated the role of *LMP7/TAP1* genes in the pathogenesis of vitiligo Saudi patients.

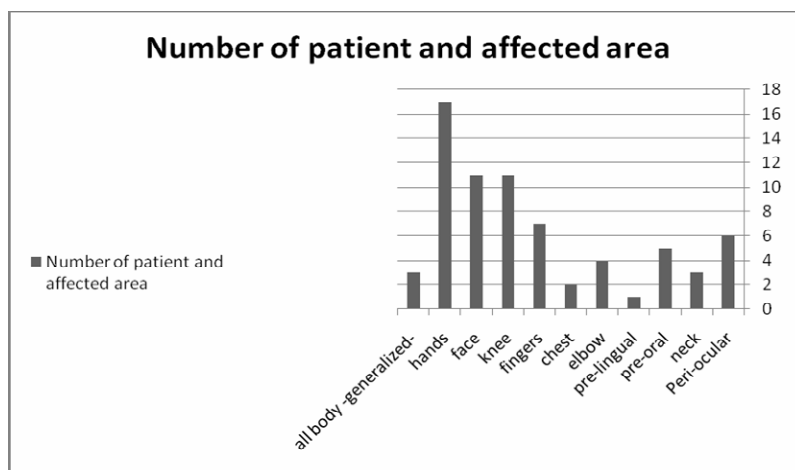
The mechanisms leading to the loss of pigment cells are not yet fully understood (Passeron and Ortonne 2005). Several theories have been proposed to explain the pathogenesis of vitiligo including autoimmune, oxidant-antioxidant, neural, genetic susceptibility and convergence theory (Halder and Chappell 2009). One of the most important tools underlying the revolution in medical genetics is the ability to visualize sequence differences directly in DNA. When studied in the context of a population, these differences in DNA sequences are called polymorphisms; they may occur in coding regions or noncoding regions of genes (Turnpenny and Ellard, 2007).

The current study is focusing on *LMP7/TAP1* genes polymorphisms in Saudi vitiligo patients. The *LMP7/TAP1* genes

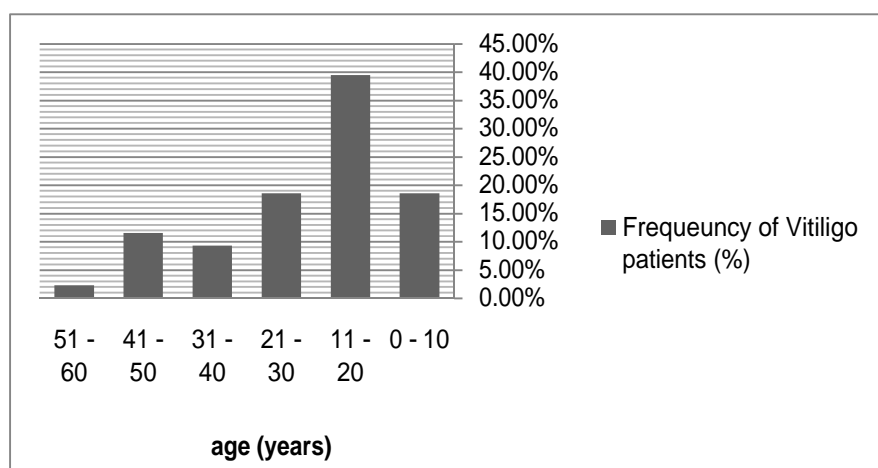
**Table.1** Primers used for *LMP/TAP* genotyping

Gene	Polymorphism	PCR Primers	Annealing temp	Restriction enzyme	Fragment sizes (bp)	
					Uncut	Cut
<i>LMP7</i>	G/T intron 6	TTG ATT GGC TTC CCG GTA CTG	58°C	<i>HhaI</i>	252	212,4
		TCT ACT ACG TGG ATG AAC ATG G	58°C	<i>HhaI</i>	252	212,4
<i>TAP1</i>	G/A exon 10	CTC ATC TTG GCC CTT TGC TC	60°C	<i>AccI</i>	165	136,29
		CAC CTG TAA CTG GCT GTT TG	60°C	<i>AccI</i>	165	136,29

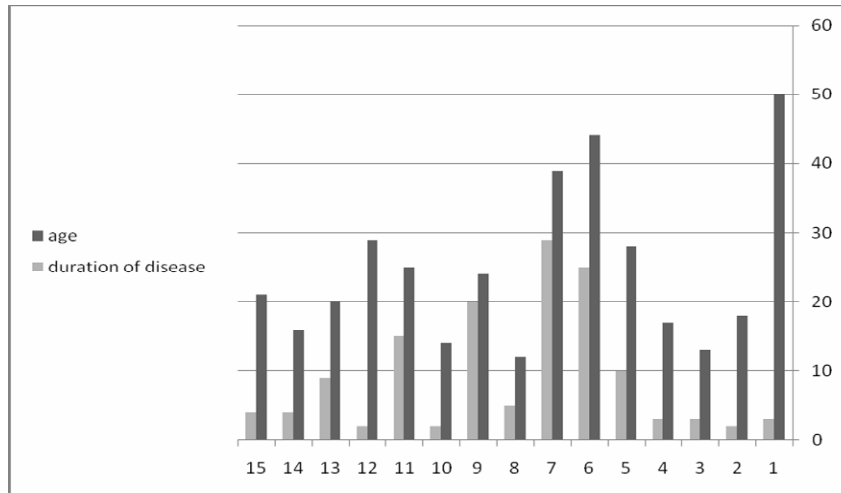
**Figure.1** A graphical representation showing that the hands was the most affected area in Saudi vitiligo patients



**Figure.2** Graphical representation showing the onset of the vitiligo disease ranged in 11-20 years among Saudi patients



**Figure.3** The duration of disease in vitiligo patient



**Table.2** Allele frequencies *TAP1* / *LMP7* candidate genes in Saudi vitiligo patient and control

Gene	Polymorphism	Allele	Vitiligo patients		Controls		P-value
			Count	%	Count	%	
<i>TAP1</i>	G/A exon 10 (D637G)	A	64	74.42%	45	53.57%	0.005
		G	22	25.58%	39	46.43%	
<i>LMP7</i>	G/T intron 6	A	35	40.7%	27	36.5%	0.586
		G	51	59.3%	47	63.5%	

**Table.3** Genotype frequencies *LMP7/TAP1* candidate genes in Saudi vitiligo patient and control

Gene	Polymorphism	Genotype	Patients		Controls		P-value
			Count	Percentage	Count	Percentage	
<i>TAP1</i>	G/A exon 10 (D637G)	AA	21	48.82%	4	9.52%	0.001
		GA	22	51.16%	37	88%	
		GG	0	0%	1	2.38%	
<i>LMP7</i>	G/T intron 6	AA	5	11.62%	5	13.5%	0.542
		GA	25	58.2%	17	46%	
		GG	13	30.22%	15	40.5%	

play important roles in the regulation of MHC class I antigen processing and presentation, but the functional consequences of *LMP7/TAP1* polymorphisms are unknown. Many polymorphisms have been reported for *LMP7/TAP1*.

*TAP1* translocate peptides from the cytosol to awaiting MHC class I molecules in the endoplasmic reticulum. The *TAP* genes are polymorphic and due to their essential involvement in class I antigen presentation might represent additional susceptibility to vitiligo. Association disequilibrium between patients and controls in exon 10 (D637G) of *TAP1* was detected which may indicate high relation with vitiligo disease in Saudi patient similar results were found by Caspet al.(2003) and contrasting with the results obtained by Seif Eldin et al., (2006) in vitiligo Egyptian patients.

Comparing patient and control genotypes for intron 6 of *LMP7* showed no significant difference between was detected. The same study revealed low association of *LMP7* intron 6 G/T with Caucasian vitiligo patients (Casp et al. 2003) and no significant was found in vitiligo Egyptian patients (Seif Eldin et al., 2006).

Until functional data are found to confirm associations with autoimmune disease suggested by genetic studies, definitive conclusions about genetic association in this region will remain controversial, as associations with autoimmune disease may be due to linkage disequilibrium with other genes in the MHC. It has also been suggested that the action of several MHC genes, including the *LMP/TAP* genes, might contribute to autoimmune disease pathogenesis (Fu et al., 1998).

Vitiligo is a polygenic disorder, several theories have proposed for vitiligo pathogenesis including autoimmune, oxidative stress, neural, and genetic theory. Several candidate genes have been studied to determine which gene has high effect on vitiligo patient (Alkhateeb and Qaraz 2010). Regarding to our study we have been found high association of *TAP1* on Saudi Vitiligo patients.

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