

INVESTIGATIVE REPORT

Autoimmune Diseases and Vitamin D Receptor *Apa-I* Polymorphism are Associated with Vitiligo in a Small Inbred Romanian Community

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Vitiligo has been associated with the host's genetic profile, metabolic abnormality and immunostatus. The purpose of this study was to investigate the association of vitiligo with autoimmune diseases for 31 out of 39 subjects with vitiligo and their first-degree relatives living in a small Caucasian inbred rural community. They were compared with healthy individuals. A 2.28% prevalence of vitiligo was calculated and the presence of consanguine marriages (72.3%) was noted for this community. Our results indicate an increased prevalence of thyroidopathies, diabetes mellitus and rheumatoid arthritis in families with vitiligo. We also show that the *Apa-I* polymorphism of the vitamin D receptor gene is associated with vitiligo. This is the first study of its kind performed in Romania suggesting that the vitamin D receptor gene might play a role in the aetiopathogenesis of skin depigmentation. Key words: vitiligo; autoimmunity; associated diseases; gene polymorphism; vitamin D receptor.

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Vitiligo is a circumscribed acquired leucoderma, characterized by complete but selective melanocyte loss from interfollicular epidermis (1, 2). Three main theories have been put forward to explain the aetiopathogenesis of this disease: immune (3), neural (4) and biochemical (5). The presence of T-cell-mediated melanocyte destruction (6) and the association of vitiligo with autoimmune conditions, such as Hashimoto's and Addison's diseases, pernicious anaemia, type 1 diabetes and systemic lupus erythematosus (7, 8), represent two major strands of evidence that support the autoimmune hypothesis. Furthermore, the family aggregation, described in 6–38% of patients, strongly supports the involvement of the genetic factors in vitiligo (9). The interaction between susceptibility genes and environmental factors (e.g. infections, sun exposure, chemicals) and/or psychological

factors (e.g. stress) has been suggested to play a pivotal role in the onset and evolution of the disease (10, 11). Despite these numerous and important recent findings, convincing evidence encompassing epidemiological and clinical data is still lacking.

The discovery of a Caucasian community exhibiting the characteristics of an isolated population, with a vitiligo prevalence of 2.28% prompted us to investigate further and to evaluate the inherited aetiopathology that may be associated with the autoimmune status of the patients.

MATERIALS AND METHODS

Population and epidemiological analysis

The present study was conducted between 2002 and 2004 on Ciocotis village, a small Romanian rural community of 1710 inhabitants. The survey was initiated by documenting the whole community using a questionnaire stating the names, addresses and medical histories of the population and the presence of white patches on the skin. The skin examination, performed by two independent observers (SB and RC), was repeated systematically within this period of time. Subjects from studied groups were also evaluated for autoimmune diseases, such as thyroidopathies, rheumatoid arthritis (RA) and type 1 diabetes.

Thirty-one out of 39 patients with clinically confirmed vitiligo and 49 out of their 105 first-degree relatives gave their consent to enter the study. These 31 patients formed the vitiligo group of our study (V group) and were compared with 33 unrelated inhabitants without vitiligo from Ciocotis village representing the control group (C_v group) and with 33 unrelated subjects from a close geographic region with no consanguinity (C_u group).

The family members (F group) in number 49 were compared with 51 healthy controls from the community (C_f group). The subjects from these groups were matched for ethnicity, age and sex.

Monitored parameters

Sex, age, age of onset, factors related to the onset and the type of vitiligo were recorded carefully. For the profile of the group of interest other parameters characterizing vitiligo were also taken into consideration.

Clinical examinations and laboratory tests

Besides the routine laboratory tests, several specific sets of analysis were performed for the studied groups; (i) thyroid hormones which were measured by ELISA assay (using kits from Diagnostic System Laboratories, Webstex, TX, USA

and Adaltis, Bologna, Italy, respectively): thyroid stimulating hormone (normal range 0.44–3.45 $\mu\text{U l}^{-1}$) and free thyroxin (normal range 10.3–25.7 pmol l^{-1}); (ii) anti-thyroid peroxidase antibodies were measured by immunochemiluminescence (using a kit from Randox, San Diego, CA, USA) (normal range < 34 IU ml^{-1}); (iii) anti-thyroglobulin antibodies, were measured by indirect immunofluorescence using a kit from DiaSorin (Stillwater, MN, USA) the results are presented as positive values obtained at different dilutions: 1/20, 1/40, etc. or as negative; (iv) rheumatoid factor was measured by nephelometric assay (Diagnosticum, Budapest, Hungary) (normal value < 20 mg dl^{-1}). All methods were performed according to the manufacturer's instructions. Endocrinological and rheumatological examinations were conducted to complete the clinical characterization of the vitiligo patients, their relatives and the controls, in order to explore the presence of thyroidopathies and RA. The diagnosis of RA was established on the basis of American Association of Rheumatology criteria, at least 4 out of 7 (12). Systemic lupus erythematosus was excluded based on the absence of the specific clinical and laboratory parameters in conformity with American College of Rheumatology criteria (13). We also noticed the presence of type 1 diabetes.

Genotype analysis

DNA was isolated from peripheral blood (2 ml on EDTA) of subjects from V and C_U groups, using a salting in protocol. The vitamin D receptor gene (VDR) *Apa-I*, *TaqI* and *FokI* polymorphisms were analysed by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method, as described previously (14); the primers used were: *Apa* and *Taq*-for 5'-CAG AGC ATC GAC AGG GAG CAA-3', *Apa* and *Taq*-rev 5'-GCA ACT CCT CAT GGC TGA GGT CTC-3', *Fok*-for 5'-AGC TGC CCC TGG CAC TGA CTC TGC TCT-3', *Fok*-rev 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3' (Sigma Genosys, Haverhill, UK). Amplifications were performed in a 15 μl final volume, using 2U of *Taq* polymerase (Fermentas, Hanover, MD, USA), 1.5 μl 10X *Taq* Buffer with KCl (Fermentas), 200 μM each dNTP (Fermentas), 1 μl DNA. The cycling reaction was performed in a thermocycler (Techne Progene USA) for 30 cycles. 5 μl of PCR products were digested with 6 U of restriction enzymes *Apa-I* and *TaqI* (Fermentas), or *FokI* (New England Biolabs, Hitchin, UK) for 3 h, on water bath, according to the manufacturer's instructions. Restriction products were separated by PAGE (8%) and were visualized with a UVP BioImaging System (Jencons PLS, USA) after ethidium bromide staining. Genotypes were assessed blindly and in concordance with previous publication, the alleles were noted with *A*, *a* (*Apa-I*), *T*, *t* (*TaqI*) and *F*, *f* (*FokI*), respectively; uppercase letters designate the absence of the restriction site and the lowercase letter the presence of restriction site.

The characterization of autoimmune profile of vitiligo families and the genotype distribution of vitiligo patients and controls were compared using χ^2 tests (SPSS Pearson χ^2 and Fisher exact tests); *p* values < 0.05 were considered to be statistically significant.

RESULTS

Epidemiological data

The community studied is geographically isolated from neighbouring villages and is located in the north-west part of Romania. It shows certain characteristics, such as consanguinity (marriages between cousins of II–V

degree) in 72.3% of families and the presence of three dominant origins. The prevalence of vitiligo in the community was high: 2.28% (39 cases identified so far out of 1710 inhabitants), which is more than nine times the 0–0.23% frequency in five surrounding communities.

Clinical aspects

As detailed in Table I, out of the 31 patients with vitiligo, 16 (51.6%) had the onset of vitiligo at an age of over 40 years. All cases presented generalized vitiligo and the majority of them (67.7%) had active forms of the disease. The other parameters recorded for this community - Koebner, leucotrichia, premature greying of the hair, mucosal involvement and family aggregation - were found in similar proportions with previous literature data (2).

Table II shows that autoimmune thyroidopathy was identified in over one-third of the patients (38.7%) and their relatives (38.8%), RA in 22.6% and 16.3% and type 1 diabetes in 9.6% and 4.1%, respectively. The simultaneous presence of vitiligo and other autoimmune conditions was noted in 15 (48.3%) patients. As detailed in Table II, the differences between V and C_V groups and between F and C_F groups were significant for thyroidopathy and for RA. It should be pointed out here that we have noted the presence of three dominant family origins in this community (over one-third belonged to family "D", over one-third to family "B" and one-fifth to family "G"). Fig. 1 details the distribution of vitiligo and autoimmune diseases in these families where the

Table I. Clinical details of the generalized vitiligo patients group (V group) (n=31)

Parameter of interest	n (%)
Sex	
Male	10 (32.3)
Female	21 (67.7)
Age (years) mean 53.0 \pm 17.1	
< 40	7 (22.6)
> 40	24 (77.4)
Age of onset (years)	
< 40	15 (48.4)
> 40	16 (51.6)
Evolution of the disease	
Stable	10 (32.3)
Active ^a	21 (67.7)
Skin surface affected	
< 20%	26 (83.9)
\geq 20%	5 (16.1)
Koebner phenomenon*	14 (45.1)
Leucotrichia*	7 (22.6)
Premature greying of the hair ^b *	11 (35.4)
Mucosal involvement*	4 (12.9)
Familial aggregation*	9 (29.0)

*n represents the number of patients out of the 31 of the vitiligo group showing the indicated parameter.

^aActive refers to expansion of existing lesions or the appearance of new lesions during the past year.

^bGreying of the hair in these patients started before 20 years of age.

Table II. Autoimmune diseases associated with generalized vitiligo

Associated disease	No. of affected individuals		p-value	F group (n=49)	C _F group (n=51)	p-value
	V group (n=31)	C _V group (n=33)				
Autoimmune thyroidopathy	12 (38.7)	4 (12.1)	0.022	19 (38.8)	7 (13.8)	0.006
Hashimoto's thyroiditis	7 (22.6)	2 (6.0)		3 (6.1)	2 (4.0)	
Subclinical autoimmune thyroid disease	5 (16.1)	1 (3.0)		5 (10.2)	3 (5.9)	
Autoimmune thyroidopathy with goiter	0	0		10 (20.4)	2 (4.0)	
Basedow disease	0	1 (3.0)		1 (2.0)	0	
Rheumatoid arthritis	7 (22.6)	0	0.004	8 (16.3)	2 (4.0)	0.039
Type 1 diabetes mellitus	3 (9.7)	1 (3.0)	0.259	2 (4.08)	0	0.149

V group =vitiligo group; C_V group =control group, vitiligo; F group =families' group; C_F group =control group, family.
Significant p values are shown in bold.

aggregation of vitiligo was observed (nine patients who belong to three families).

Genetic analysis

The *VDR Apa*, *Taq* and *Fok* polymorphisms were assessed in V and C_V groups; the results for *VDR Apa* are shown in Table III. The frequency of *aa* genotype is significantly higher in V group compared with C_V group (45.1% vs. 15.1%, *p* =0.029 OR – 2.034). The results remained significant when only patients with

vitiligo and other autoimmune diseases (53.3% vs. 15.1%, *p* =0.018) from V group were selected. The *a* allele was significantly more frequent in the subgroup with vitiligo and other autoimmune diseases compared with C_V group (73.3% vs. 45.4%, *p* =0.011).

The analysis of *Taq* polymorphism revealed only non-significant (*p* >0.12) variations of the *T/T* genotype (54.8% vs. 33.3%, *p* =0.217) and the *T* allele (69.3% vs. 56.0%, *p* =0.121) between V and C_V groups. Also, no significant differences between vitiligo patients and controls were observed for the *Fok* polymorphism in

- □ = Healthy individual
 - ■ = Vitiligo affected individual, otherwise healthy
 - ⊘ ⊚ = Deceased individual, not affected by vitiligo
 - ⊙ ⊛ = Deceased individual, vitiligo affected
 - ▨ = RA
 - ▩ = Hashimoto's thyroiditis
 - = Subclinical AI thyroidopathy
 - ⊕ = Vitiligo + Hashimoto's thyroiditis
 - ⊖ = Vitiligo + diabetes mellitus type 1
 - ⊗ = Vitiligo + RA + Hashimoto's thyroiditis
 - ⊠ = Vitiligo + RA + subclinical AI thyroidopathy
- "B", "G", "D" = the family dominant names

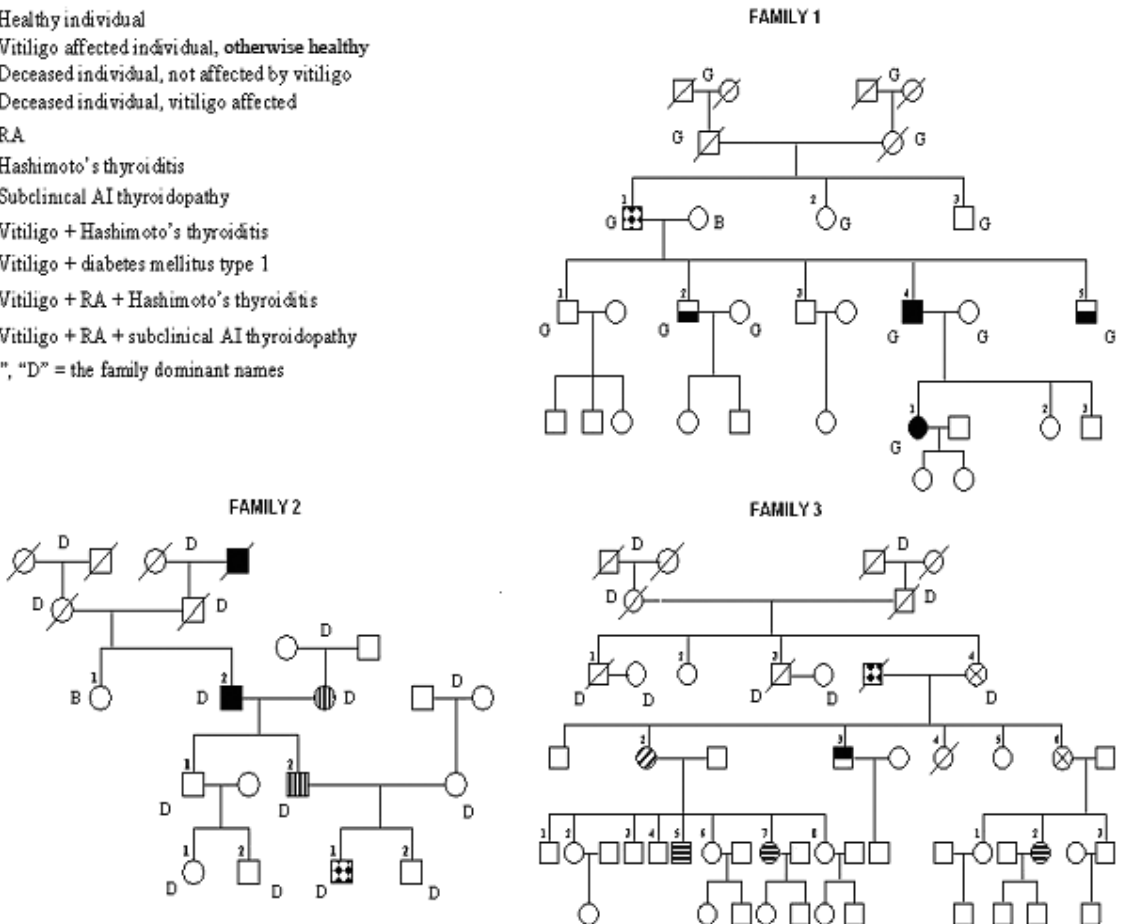


Fig. 1. Vitiligo familial aggregation (9 vitiligo patients) and the distribution of autoimmune diseases in the families with multiple vitiligo members. The pedigrees of the three families with three dominant names are shown. Couples with the same family name are third or fourth degree of cousins.

Table III. Distribution of alleles and genotypes for the *Apa-I* single nucleotide polymorphism in vitamin D receptor gene in the generalized vitiligo (V) and control (C_v) groups

Observed allele frequencies (%)			p-value ^c	Observed genotype counts (%)			p-value ^d	Combined genotype counts (%)		
	A	a		A/A	A/a	a/a		A/A and A/a	a/a	p-value ^e
Control (n=33)	36 (54.5)	30 (45.4)		8 (24.2)	20 (60.6)	5 (15.1)		28 (84.8)	5 (15.1)	
Vitiligo (V) (n=31)	23 (37.0)	39 (62.9)	0.048 ^a	6 (19.3)	11 (35.4)	14 (45.1)	0.029 ^a	17 (54.8)	14 (45.1)	0.009 ^a
Autoimmunity (n=15)	8 (26.6)	22 (73.3)	0.011 ^b	1 (6.6)	6 (40.0)	8 (53.3)	0.018 ^b	7	8	

^aGroup vitiligo (V) vs. group control (C_v).

^bGroup with other autoimmune diseases vs. controls (C_v).

^cWe used χ^2 test with (2x2 contingency table).

^dWe used χ^2 test with (3x2 contingency table).

either genotype (FF: 25.8% vs. 27.2%, $p=0.387$) or allele frequency (F: 51.6% vs. 43.9%, $p=0.614$).

DISCUSSION

Approximately 1% of the world population is currently affected by vitiligo, regardless of the gender or basic skin tone (10, 15). There are a few studies performed on Caucasians only; for example, a large survey performed on a Danish population from Bornholm showed a prevalence of vitiligo of 0.38% (16), i.e. lower than that described worldwide. The prevalence of 2.28% found by us in Ciocotis community is at least six-fold higher than that reported for the Bornholm population. These results are in good agreement with the observations that vitiligo clusters in families and that inbreeding increases its prevalence (17).

The results of our study also indicate a clear association between vitiligo and autoimmunity in vitiligo families. Thus autoimmune thyroidopathy, RA and diabetes had a higher frequency in the V and F groups than in the control groups. In other studies autoimmune thyroidopathy and diabetes were noted in vitiligo patients at frequencies of 30% (9) and 4.8–7.1% (18), respectively. By contrast, vitiligo-associated RA was only mentioned in a few previous studies. Spritz's group found in a large survey in the USA, a prevalence of 0.67%, not more than the frequency of RA in general population (7). In a more recent study, the same group identified an increased prevalence of RA (3.8%) and other vitiligo associated autoimmune diseases among 133 probands who belong to multiplex vitiligo families, indicating that such families have a greater genetic component of autoimmune susceptibility than do isolated cases (19).

Most of our patients present active evolution of depigmentation (Table I). Several studies showed that patients with generalized forms of vitiligo may have a high susceptibility to develop autoimmunity than those with more limited disease (9, 20). Vitiligo depigmentation frequently begins around the age of 20 years (21), although later onset is not uncommon. In our study, the onset of vitiligo occurred after the age of 40 years in 51.6% of patients which is unusual.

The etiology of vitiligo seems to be polygenic and plurifactorial (10). Therefore, genes associated with

vitiligo are currently explored, as they appear to support a role for the immune system in gradual depigmentation (22). The identification of two susceptibility genes for vitiligo and other autoimmune diseases, the AIS1 by Spritz's group (11) and *SLEVI* by Nath et al. (23), (confirmed by Spritz et al. (24)), prompted us to investigate other genes related to autoimmunity which could interfere with the mechanism of pigment loss. Moreover, the catalase gene was investigated as it appears to be a susceptibility gene in some vitiligo patients, further supporting the role of epidermal oxidative stress reactions in the aetiopathogenesis (25). It is possible that each of these genes or combinations of gene polymorphisms may predispose to the appearance of subsets of the disease. Besides the immune and genetic factors, the presence of a defective free radical defence, the accumulation of neurochemical substances, chemicals (4-tertiary butyl phenol), and an intrinsic defect of the function of melanocytes were mentioned among the possible causal factors; they could contribute in variable proportions to the destruction of pigmentary cells, as proposed in the convergence theory (26, 27).

The implication of *VDR* gene in vitiligo has not yet been explored, although a clear association between *VDR* polymorphisms and autoimmune conditions, e.g. Hashimoto thyroiditis, type 1 diabetes, and RA, has been demonstrated (28–30). We found in this study an association between the *VDR* – *Apa-I* polymorphism and vitiligo. The *aa* genotype of *Apa-I VDR* was significantly more frequent in patients with vitiligo; allelic frequencies showed a significant difference between vitiligo with other autoimmune diseases group and controls. Neither *Fok* nor *Taq* polymorphisms were significantly associated with vitiligo.

Genes for complex diseases as vitiligo are expected to be more easily identified in isolated populations than in a general outbred population (31). For example, the so-called "linkage disequilibrium mapping" approach was successfully used to map and subsequently identify the genes for Hermansky-Pudlak syndrome type 1 using isolated populations on the island of Puerto Rico and in the Swiss Alps (32, 33) and the gene for cleft lip/palate-ectodermal dysplasia syndrome utilizing an isolated population on Margarita Island (34). As a consequence of drift and founder effects, a large number of patients

in isolated populations probably inherited the disease susceptibility from a common genetic ancestor. Since adjacent markers on a chromosome are often transmitted together (linkage disequilibrium), patients who have inherited a susceptibility gene from a common ancestor are likely to share considerable stretches of DNA around the disease gene, leading to the association of multiple adjacent markers for the disease (35). The identification of the association between *VDR* gene on the chromosome 12 and vitiligo could be the first indication of a new susceptibility gene for vitiligo. In this respect, the study of other *VDR* polymorphisms, such as *BsmI* in vitiligo families from this community as well as the extension of the investigations on larger groups and other populations would be of a great interest. The presence of three dominant origins and consanguine marriages could explain the high vitiligo prevalence in Ciocotis community and the increased rate of autoimmune diseases, by the perpetuation of founder chromosomes.

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