CHAPTER II

EVALUATION OF AUTOIMMUNE HYPOTHESIS IN VITILIGO PATIENTS

2.1 INTRODUCTION

Vitiligo is an acquired depigmenting disorder characterized by the loss of melanocytes from epidermis. The mechanism of melanocyte loss from epidermis to cause vitiligo is not yet clearly understood (Taieb *et al.*, 2000). The three major hypotheses that are proposed to explain the pathogenesis of vitiligo are autoimmune, neurochemical, and oxidative stress hypotheses (Ortonne and Bose, 1993). Vitiligo is considered to be an autoimmune disease because of its association with several other autoimmune diseases such as diabetes, pernicious anemia, thyroid diseases, Addison's disease and alopecia areata (Kemp *et al.*, 2001). In addition, various circulating antimelanocyte and antikeratinocyte antibodies are found in vitiligo patients (Kemp *et al.*, 2001). A study performed on 2624 vitiligo probands from North America and UK confirmed significant increase in the frequencies of six autoimmune disorders in vitiligo probands and their first degree relatives: vitiligo itself, autoimmune thyroid disease (particularly hypothyroidism), pernicious anemia, Addison's disease, systemic lupus erythematosus and inflammatory bowel disease (Alkhateeb *et al.*, 2003). Association of these diseases with vitiligo indicates that vitiligo disorder shares common genetic etiologic link with other autoimmune diseases (Passeron and Ortonne, 2005). Also the immune suppressive effect of a number of repigmenting therapies (steroids) indirectly supports the autoimmune mediated process of depigmentation (Ongenae *et al.*, 2003).

The aim of the present study was to evaluate autoimmune hypothesis in vitiligo patients by estimating antimelanocyte antibody levels in patients and unaffected controls. The antimelanocyte antibody levels were also analyzed based on the onset of disease and disease progression.

2.2 MATERIALS AND METHODS

2.2.1 Study subjects:

The study group included 300 vitiligo patients [223 generalized (including acrofacial vitiligo and vitiligo universalis) and 77 localized vitiligo cases] comprised of 138 males and 162 females who referred to S.S.G. Hospital, Vadodara and Civil Hospital, Ahmedabad, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based

on the presence of depigmented patches on the skin and patients had no other associated autoimmune disease. A total of 400 ethnically sex-matched unaffected individuals (188 males and 212 females) were included in the study (Table 1). None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. Importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 300)	(n = 400)
Average age (mean age ± SD)	31.24 ± 12.13 yrs	27.54 ± 13.26 yrs
Sex: Male	138 (46.0%)	188 (47.00%)
Female	162 (54.0%)	212 (53.00%)
Age of onset (mean age ± SD)	21.96 ± 14.90 yrs	NA
Duration of disease (mean ± SD)	8.20 ± 7.11 yrs	NA
Type of vitiligo		
Generalized	223 (74.33%)	NA
Localized	77 (25.67%)	NA
Active vitiligo	215 (71.67%)	NA
Stable vitiligo	85 (28.33%)	NA
Family history	41 (13.66%)	NA

2.2.2 Estimation of antimelanocyte antibody levels:

In the present study, plasma from vitiligo patients was examined for the reactivity with the human melanoma cell line (SK Mel 28) to find the levels of antimelanocyte antibodies in vitiligo patients compared to controls. We have analyzed plasma samples of 300 vitiligo patients and 400 controls for the presence of antimelanocyte antibodies by ELISA. 5 ml venous blood was collected from the patients and healthy

subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, NJ 07417, USA) and plasma was extracted. Human melanoma cell line SK Mel 28 was obtained form NCCS, Pune and grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 50 mg/L gentamycin at 37°C under 5% CO_2 in a humidified atmosphere (Hann and Kim, 1995).

2.2.3 Enzyme linked immunosorbent assay (ELISA):

Cells were harvested by scraping and lysed by adding lysis buffer (HEPES 20 mM, EGTA 1 mM, PMSF 1 mM, $MgCl_2$ 1.5 mM, NaCl 150 mM, $CuSO_4$ 1mM, 1%,Trition –X 100, 1%, Glycerol). Protein estimation in the lysate was done by Lowry's method (Lowry *et al.,* 1951). 50 µl of the cell lysate containing 1 µg protein was loaded in each well of the microtiter plate and kept at 4° C overnight. Excess antigen was discarded, washed 3 times with PBS, blocked with 1% BSA in PBS and incubated for 1 hour. Excess blocking reagent was discarded and washed 3 times with PBS. 50 µl of plasma (1:10,000 diluted in PBS) was added to the microtiter plates and incubated for 2 hours at room temperature. Excess plasma was discarded and washed 3 times with PBS containing 0.2% Tween-20. Then 50 µl of 1: 2000 diluted secondary antibody was added (Rabbit anti human IgG HRP conjugate, Bangalore Genei, India) and incubated for 1 hour. Excess antibody was discarded and washed 3 times with PBS containing 0.2% Tween 20. 50 µl substrate (TMB-H_2O_2) was added and incubated for 5 minutes for the color development. The reaction was stopped by adding 200 µl of 1N H_2SO_4 and OD was read at 405 nm.

2.2.4 Statistical analysis:

Antimelanocyte antibody levels were plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). *p*-values less than 0.05 were considered as statistically significant.

2.3 RESULTS

2.3.1 Antimelanocyte antibody levels in vitiligo patients and controls:

Our results suggest significant increase in antimelanocyte antibody levels in vitiligo patients as compared to controls ($p<0.0001$; Figure1 A). We found that 75% of

vitiligo patients had antimelanocyte antibodies in their circulation suggesting that autoimmunity may play an important role in vitiligo pathogenesis.

2.3.2 Antimelanocyte antibody levels in different types of vitiligo patients:

Antimelanocyte antibody levels were also compared between generalized and localized vitiligo patients. Generalized vitiligo patients showed significant increase in antimelanocyte antibody levels as compared to localized vitiligo patients (p=0.001; Figure 1B). Moreover, when antimelanocyte antibody levels were compared between active and stable cases of vitiligo, active vitiligo patients exhibited higher levels of antimelanocyte antibody as compared to stable vitiligo patients (p<0.0001; Figure 1C).

2.3.3 Gender biasness in antimelanocyte antibody levels in vitiligo patients:

Furthermore, when antimelanocyte antibody levels were compared based on the gender of patients, female patients showed significantly increased levels of antimelanocyte antibodies as compared to male patients (p=0.003; Figure 1D).

2.3.4 Antimelanocyte antibody levels in different age of onset groups of vitiligo:

When effect of antimelanocyte antibody levels on vitiligo onset was analyzed between different age of onset groups, we found that patient group with an early age (1-20 yrs) of onset had significantly higher levels of antimelanocyte antibodies in their circulation as compared to the age groups 21-40 yrs (p=0.0004), 41-60 (p<0.0001) and 61-80 yrs (p<0.0001) (Figure 1E).

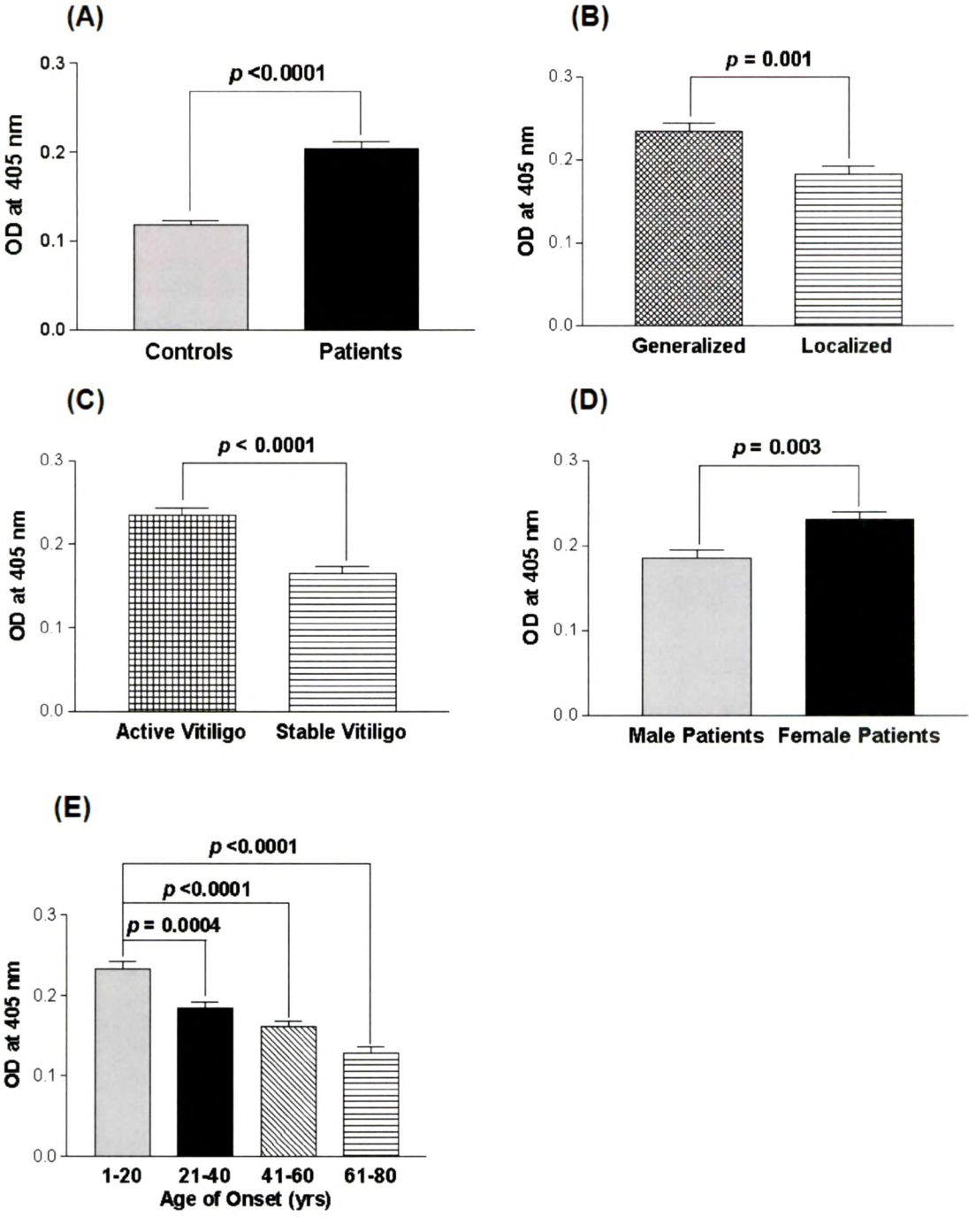

Figure 1. Antimelanocyte antibody levels in vitiligo patients and controls:

(A) Antimelanocyte antibody levels in 300 vitiligo patients and 400 controls. Patients showed significantly higher levels of antimelanocyte antibody as compared to controls (Mean ± SEM: 0.2029 ± 0.0080vs 0.1186 ± 0.0042; $p<0.0001$).

(B) Antimelanocyte antibody levels in 223 generalized and 77 localized vitiligo patients. Patients with generalized vitiligo showed significantly higher levels of

antimelanocyte antibody as compared to localized vitiligo (Mean ± SEM: 0.2338 ± 0.0105 vs 0.1830 ± 0.0090; p=0.001).

(C) Antimelanocyte antibody levels in 215 active and 85 stable vitiligo patients. Patients with active vitiligo showed significantly higher levels of antimelanocyte antibody as compared to stable vitiligo (Mean ± SEM: 0.2350 ± 0.0086 vs 0.1653 ± 0.0079; p<0.0001).

(D) Antimelanocyte antibody levels in 138 male and 162 female vitiligo patients. Female patients showed significantly higher levels of antimelanocyte antibody as compared to male patients (Mean ± SEM: 0.2306 ± 0.0096 vs 0.1853 ± 0.0099; p=0.003).

(E) Antimelanocyte antibody levels with respect to different age groups in 300 vitiligo patients. Vitiligo patients with the age of onset 1-20 yrs showed significantly increased antimelanocyte antibody levels as compared to the age groups 21-40 yrs (Mean [redacted] ± SEM: 0.2323 ± 0.0089 vs 0.1830 ± 0.0091; p=0.0004), 41-60 (Mean [redacted] ± SEM: 0.2323 ± 0.0089 vs 0.1606 ± 0.0076; p<0.0001) and 61-80 yrs (Mean [redacted] ± SEM: 0.2323 ± 0.0089 vs 0.1280 ± 0.0084; p<0.0001).

2.4 DISCUSSION

Vitiligo is strongly associated with other autoimmune conditions, such as type 1 diabetes, pernicious anemia, Addison's disease, systemic lupus erythematosus, alopecia areata, rheumatoid arthritis, and polyglandular autoimmune disease. Histological evidence further supports an autoimmune etiology. Michelsen (2010) has proposed antibody-based dominant mechanism in generalized vitiligo as a contributory factor for precipitation of autoimmune vitiligo. Antibodies (mainly IgG) against melanocyte antigens are detected in the sera of vitiligo patients and a correlation exists between the level of melanocyte antibodies and disease intensity in vitiligo patients (Harning *et al.*, 1991). Further, a correlation between the presence of antibodies and the extent of disease was established, as antibodies are present in more than 90% of patients with greater depigmentation and in 50% with minimal lesions (Abu Tahir *et al.*, 2010).

Interestingly, deposits of IgG have been observed in the basal membrane zone of lesional skin in relation to increased intensity of disease, which correlates well with

the observation of IgG binding to cultured melanocytes. Further studies have shown even the presence of anti-melanocyte IgA antibodies associated with disease manifestation (Ongenae et al., 2003, Kemp et al., 2007). These findings clearly suggest a vitiligo associated link with alterations in immunoglobulins.

The results of present study indicate that 75% of Gujarat vitiligo patients exhibited antimelanocyte antibodies in their circulation. Vitiligo patients showed significant increase in the levels of antimelanocyte antibodies in their circulation compared to controls (Figure 1A). Naughton *et al.*, (1983a) reported that 82% of vitiligo patients showed antimelanocyte antibodies in their circulation. In another study, 12/12 vitiligo patients and 0/12 controls were reported to have antimelanocyte antibodies in their circulation (Naughton *et al.*, 1983b). However, Grimes *et al.*, (1983) reported that 19% of Black vitiligo patients showed antimelanocyte antibodies in their circulation. Farrokhi *et al.*, (2005) also showed the presence of antimelanocyte antibodies in 30.9% of Iranian vitiligo patients. These reports suggest the involvement of antimelanocyte antibodies in pathogenesis of vitiligo.

Further we have analyzed antimelanocyte antibody levels in different groups of vitiligo to find whether antimelanocyte antibodies play a significant role in a particular type of vitiligo. Interestingly, we found that generalized vitiligo patients have significantly increased levels of antimelanocyte antibodies compared to localized vitiligo. This finding also supports the fact that generalized vitiligo has an autoimmune origin and is now considered as 'Autoimmune Vitiligo' (Nordlund *et al.*, 2006). Furthermore, our analysis based on progression of vitiligo suggested increased antimelanocyte antibodies levels in active cases as compared to stable cases suggesting the crucial role of antimelanocyte antibodies in disease progression. Interstingly, patients with an early onset of vitiligo exhibited significantly higher levels of antimelanocyte antibodies as compared to late onset patients. This suggests that antimelanocyte antibodies play an important role in early onset of the disease.
In addition, anaysis based on gender suggested higher antimelanocyte antibodies levels in female patients as compared to male patients indicating that females have increased susceptibility towards vitiligo as compared to males, implicating gender biasness in the development of autoimmunity (Whitacre, 2001; Panchanathan and Choubey, 2012; Afshan *et al.*, 2012).

The exact role of antimelanocyte antibodies in the pathogenesis of vitiligo remains unresolved. Autoantibodies against pigment cells might result from a genetic predisposition to immune dysregulation at the T cell level (Kemp *et al.,* 2001). Alternatively cross-reacting antigens expressed either on other target cells or infecting microorganisms could elicit their production. Autoantibodies could also result from an immune response to melanocyte antigens released following damage to pigment cells by other mechanisms, and these antibodies might then exacerbate the condition. The selective destruction of melanocytes might result from antibody reactivity directed to the antigens preferentially expressed on pigment cells (Kemp *et al.*, 1997) or from an antibody response against antigens expressed on a variety of cell types (Cui *et al.*, 1992) that might selectively destroy melanocytes because they are intrinsically more sensitive to immune mediated injury than other cells (Norris *et al.*, 1988).

Tyrosinase is the principal antigen recognized by these antibodies (Fishman *et al.*, 1993; Song *et al.*, 1994). The other melanocyte antigens recognized by autoantibodies are gp100/Pmel 17 (a melanosomal matrix glycoprotein), and tyrosinase related proteins 1 and 2 (TRP 1 and TRP 2) (Kemp *et al.*, 1998b). These cell differentiation antigens are localized primarily to melanosomes (Nordlund *et al.*, 1999). The transcription factors SOX9 and SOX10 are also identified as melanocyte autoantigens (Hedstrand *et al.*, 2001). Also autoantibodies against HLA Class I molecules are reported in vitiligo (Ongenae *et al.*, 2003). *In vitro* studies showed that autoantibodies are able to destroy melanocytes by complement mediated damage and antibody dependent cellular cytotoxicity (Gilhar *et al.,* 1995; Norris *et al.,* 1988; Cui *et al.*, 1993).

Moreover, autoantibodies could result from a failure to kill an autoreactive cell or by inducing autoimmunity against apoptotically modified cellular constituents. Therefore, the process of apoptosis may provide a source of cellular antigens to drive the autoantibody response and provide antigens. Reports have suggested that apoptosis is abnormal in autoimmune diseases and may play a role in the induction of autoimmunity. It is worth noting that efficient clearance of apoptotic cells is crucial for the avoidance of autoimmune responses to intracellular antigens (Casiano and Pacheco, 2006).

In conclusion, our results suggest significant increase in antimelanocyte antibody levels in vitiligo patients as compared to controls. However, it is unclear whether antimelanocyte antibodies are the cause or effect of the disease.

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