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Vitiligo: Is It Grace or Curse?

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SUMMARY Vitiligo is a common skin disease, affecting approximately 0.5% of the general population. It is characterized by milky white macules and patches, which are a psychological burden to many patients. Although this disease has been known for a long time, the etiology is still under debate. Since melanin is a unique light absorbing and ultraviolet filtering pigment, it is generally accepted that its main function resides in the protection of skin cells against the deleterious effect of ultraviolet rays (UVRs). The occurrence of skin cancer in long lasting vitiligo is rare despite multiple evidence of DNA damage. The aim of this study was the immunohistochemical detection of p53 and Mdm2 in depigmented and "normal" pigmented skin of vitiligo patients to demonstrate the possible role of these proteins in the protection of vitiligo patients against actinic damage and non-melanoma skin cancer. Using standard immunohistochemical techniques, we examined 34 patients with vitiligo and 30 age- and sex-matched patients with noduloulcerative basal cell carcinoma as a control group. Both patients and control subjects had outdoor occupations. Skin biopsies were obtained from each case (from depigmented and "normal" pigmented UVR-exposed skin) and control subjects (from perilesional healthy skin). Both p53 and Mdm2 were strongly expressed in depigmented as well as "normal" pigmented skin of vitiligo patients. This expression involved the epidermis, skin adnexa and blood vessels, with significant differences between cases and controls. Both proteins showed nuclear and nucleo-cytoplasmic pattern of expression. Intense p53 and Mdm2 expression was in favor of generalized vitiligo. These results suggested that the over-expression of p53 and Mdm2 proteins in both depigmented and "normal" pigmented skin of patients with vitiligo could contribute to the decreased occurrence of actinic damage and non-melanoma skin cancer in these patients.

KEY WORDS: immunohistochemistry, p53, Mdm2, vitiligo, non-melanoma skin cancer

INTRODUCTION

Vitiligo is an acquired pigmentary disorder characterized by the presence of well-circumscribed depigmented milky white macules and patches (1), resulting from the loss of melanocytes from the epidermis, mucous membrane, inner ears, eyes, hair and occasionally from the hair bulbs (2). Numerous

epidemiological studies have documented that people with fair skin, as well as affected individuals with albinism are significantly more sun-sensitive compared with darker skin races (3). Furthermore, these groups experience multiple sunburns during lifetime, together with a high risk of developing actinic skin damage and skin cancer (4).

There is a plethora of evidence that the entire epidermis of patients with vitiligo shows multiple signs of oxidative stress, including the presence of allantoin (5), massive amounts of hydrogen peroxide (H_2O_2) (6), low catalase (7) and other important antioxidant enzymes including thioredoxin reductase/thioredoxin, glutathione peroxidase, glutathione reductase, superoxide dismutases and the repair enzymes methionine sulfoxide reductases A and B (8).

In addition, there is systemic oxidative stress, including the presence of DNA damage in peripheral blood lymphocytes and alteration of mitochondria in blood mononuclear cells (9).

In vitiligo, H_2O_2-mediated oxidation affects many proteins and peptides, yielding altered or even complete loss of functionality (10).

This scenario leads to many consequences, including the loss of functioning melanocytes and lipid peroxidation with the formation of vacuoles in epidermal melanocytes and keratinocytes (1). Consequently, it has been speculated that vitiligo patients are more vulnerable than normal population to photodamage, premature aging, malignant melanoma and non-melanoma skin cancer (NMSC) (11).

However, there is no significantly increased risk of actinic damage or NMSC even in long standing disease (5). Even more surprising is that the skin of these individuals is significantly younger compared with age-matched healthy individuals (10). Therefore, the question arises of which protective mechanism could be involved in the skin of these patients preventing the initiation of these cancers (12)?

Clearly, under these conditions, DNA damage would be an expected must. In this context, it is noteworthy that these patients exhibit persistently high levels of functioning wild type p53 protein in their skin (10).

p53 is considered the cellular gatekeeper for growth and division. The p53 gene product is a nuclear protein (wild type p53 protein), which binds to specific DNA sequences and functions as a transcription factor. It regulates the expression of genes that control cell cycle progression, induction of apoptosis, DNA repair, and functions involved in cellular responses to stress. These functions occur through mediating arrest of mammalian cells at one of two major cell cycle checkpoints, in G1 near the border of S phase or in G2 before mitosis. p53 modulates DNA repair process through the arrest of cell cycle progression, which may provide time for the repair of DNA damage. However, in some circumstances of irreversible damage, stress signaling will initiate p53 dependent apoptosis, thus preventing the fixation of DNA damage as mutations (13).

In dividing cells, the central regulator of p53 function is the oncogene Mdm2, which binds to and thereby regulates the activity of the p53 protein. It is a ubiquitin ligase which mediates ubiquitination of p53, thereby targeting it for degradation. In this way, p53 levels are kept low in normal cells (14).

The aim of this study was the immunohistochemical detection of p53 and Mdm2 in depigmented and "normal" pigmented skin of vitiligo patients to demonstrate the possible role of these proteins in the protection of vitiligo patients against actinic damage and NMSC.

MATERIALS AND METHODS

Study subjects

Our case-control study included 64 subjects, 34 patients with vitiligo (localized and generalized) and 30 age- and sex-matched patients with nodulocerative basal cell carcinoma (BCC; representative of NMSC) as a control group. Both cases and controls had outdoor occupations with excessive sun exposure and were selected randomly from Dermatology Outpatient Clinic, Menoufiya University Hospital, Menoufiya Governorate, Egypt during the period from April 2010 to October 2010. Cases and controls had skin phototypes 3 and 4. Written consent forms approved by Committee of Human Rights in Research of Menoufiya University were obtained from study cases and control subjects.

Each of the selected cases was subjected to complete history taking, complete general and dermatological examination. Clinical data describing patient demographics (age and sex) as well as the clinical variables (site of lesions and disease duration) were all documented. In vitiligo patients, biopsies were obtained from depigmented as well as "normal" pigmented skin. In control subjects, biopsies were obtained from normal perilesional skin. To avoid the possible effect of NMSC on the examined proteins, biopsies were taken 6 mm away from the border of BCC nodules (more than the tumor safety margin). To avoid the effect of sun exposure on the examined proteins, biopsies from normal skin (from both cases and controls) were taken from UVR-exposed sites.

Skin biopsy samples were taken under local anesthesia. All specimens were fixed in 10% neutral-buffered formalin and subjected to routine tissue processing that ended with paraffin-embedded blocks ready for sectioning at the Pathology Department, Faculty of Medicine, Menofiya University, Egypt. Examination of hematoxylin and eosin-stained sections was done to evaluate and verify epidermal and dermal pathologic alterations.

Immunohistochemical staining

Five-µm-thick sections were cut from the paraffin-embedded blocks with subsequent steps of deparaffinization and rehydration in xylene and a graded series of alcohol, respectively. Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6.0) for 20 min, followed by cooling at room

Table 1. Clinical and histopathologic data of study patients

Variable	X± SD	n	%
Age (yrs): 9-57	36±2	-	-
Duration (yrs): 8 months-34 years	5 ±2	-	-
Sex: male female		 20 14	 58.8 41.2
Distribution of vitiligo lesions: generalized acrofacial focal		 18 9 7	 52.9 26.5 20.6
Associated diseases with vitiligo: ocular manifestations (present) acoustic manifestations (present) diabetes mellitus (present) alopecia areata (present)		 6 3 2 1	 17.6 8.8 5.9 2.9
Family history of the disease: positive negative		 8 26	 23.5 76.5
Past history of melanoma or non-melanoma skin cancer: negative positive		 34 0	 100.0 0.0
Thickness of the epidermis: atrophy normal		 20 14	 58.8 42.2
Structure of stratum corneum: basket weave mixed compact		 22 4 8	 64.7 11.8 23.5
Parakeratosis (present)		8	23.6
Sunburn cells (present)		28	82.4
Presence of stratum granulosum		34	100
Basement membrane: absent focal thickening		 17 17	 50 50
Solar elastosis		4	11.5
Vacuolated keratinocytes		14	41.2
Dermal papillae: normal atrophy		 4 30	 11.8 88.2
Inflammatory infiltrate: mild moderate excessive		 13 13 8	 38.2 38.2 23.6

X± SD = mean ± standard deviation; yrs = years

Table 2. P53 expression in study groups

		Vitiligo patients				Control		χ^2	*P*-value
		Normal skin		Depigmented skin		No	%		
		No.	%	No.	%				
Epidermis	Mild	6	7.7	-	-	21	70	127.1	<0.001*
	Moderate	28	82.3	4	12	6	20		
	Strong	-	-	30	88	3	10		
Dermis	Mild	-	-	-	-	21	70	127.1	<0.001*
	Moderate	31	91	9	26.5	6	20		
	Strong	3	9	25	73.5	3	10		

χ2: chi-square; *significant

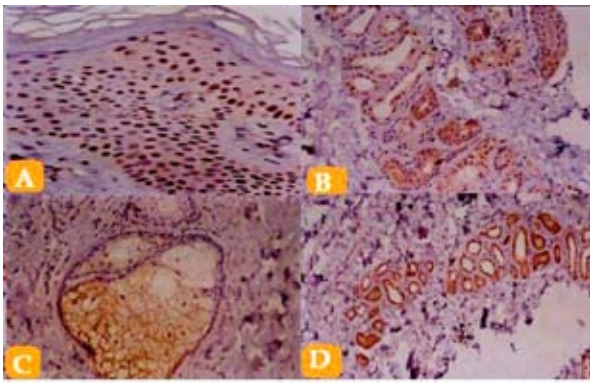

Figure 1. p53 expression in depigmented and "normal" pigmented patients' skin: (A) strong p53 immunostaining in basal and suprabasal layers in depigmented skin (immunoperoxidase, x400); (B) strong staining of sweat glands of depigmented skin (immunoperoxidase, x100); (C) strong staining of sebaceous glands of depigmented skin (immunoperoxidase, x400); and (D) moderate staining of sweat glands, hair follicles and endothelium of the blood vessels in the 'normal' pigmented skin (immunoperoxidase, x200).

temperature. The slides were incubated overnight at room temperature with {Mouse monocolonal anti-p53 antibody (1:50) (Catalogue No. # MS–738-R7-LabVision/Neomarkers-USA) and Rabbit polyclonal anti-Mdm2 antibody (1:50) (Catalogue No. # RB-9218-LabVision/Neomarkers-USA), respectively}. The Envision (Dako, Glostrup, Denmark) method was used for detection of p53 and Mdm2 binding. The reaction was visualized by an appropriate substrate/chromogen (diaminobenzidine) reagent with Mayer's hematoxylin as a counter stain.

Immunostaining interpretation

Positive immunostaining was considered when >5% of epidermal, dermal or adnexal cells showed brown nuclear staining (15).

p53 and Mdm2 expression was evaluated by using Quickscore, which considered both the intensity of staining and percentage of positive cells in a semi-quantitative pattern according to the following formula:

the intensity of immunostaining (I) was scored as 0: negative, 1: weak, 2: moderate, and 3: strong. The percentage of positive cells (P) scored as 1: 0%-4%, 2: 5%-19%, 3: 20%-39%, 4: 40%-59%, 5: 60%-79%, and 6: 80%-100%.

Quickscore was then calculated by multiplying the intensity by the percentage of positive cells (I x P) with a final score ranging from 0 to 18, where more than 10 was assigned as strong, 6-10 as moderate, 1-5 as weak and 0 as negative (16).

Statistical analysis

Data were collected, tabulated and statistically analyzed using the Statistical Package for Social Sciences (SPSS) version 11. Data were statistically described in terms of range, mean ± standard deviation (±SD), frequencies (number of cases) and relative frequencies (percentages) when appropriate. Chi square and Fischer exact test were used to study differences between two qualitative variables. Differences were considered statistically significant at $P<0.05$.

RESULTS

Table 1 summarizes clinical and histopathologic data of the studied patients.

Immunohistochemical staining

(A) p53 (according to intensity of immunostaining)

1) Control skin: immunoreactivity ranged from mild to strong (Table 2).

2) Vitiligo patients

- Depigmented skin: the epidermis showed strong positive nuclear immunostaining in the majority of cases and moderate immunostaining in few cases

Table 3. Mdm2 expression in study groups

		Vitiligo patients				Control		X^2	*P*-value
		Normal skin		Depigmented skin		No.	%		
		No	%	No	%				
Epidermis	Mild	4	12	-	-	30	100.0	156.0	<0.001*
	Moderate	30	88	9	26.5	-	-		
	Strong	-	-	25	73.5	-	-		
Dermis	Mild	-	-	-	-	30	100.0	156.0	<0.001*
	Moderate	34	100	7	20.5	-	-		
	Strong	-	-	27	79.5	-	-		

χ2: chi-square; *significant

(Fig. 1a, Table 2). In the dermis, skin appendages and endothelium of the blood vessels showed strong positive nuclear and nucleo-cytoplasmic immunostaining (Fig. 1b, c, Table 2).

Table 4. Correlation between Quickscore evaluation for p53 and Mdm2 expression and clinical data

	Quick score for P53				
	Moderate		Strong		*P*-value
	n	%	No.	%	
Age (yrs) <20 >20	 8 6	 57.2 42.8	 11 9	 55 45	FE test *P*=0.1
Sex: male female	 5 9	 35.7 64.3	 15 5	 75 25	FE test *P*= 0.7
Type: generalized acrofacial focal	 7 3 4	 50 21.4 28.6	 11 6 3	 55 30 15	FE test *P*=0.03*
	Quick score for Mdm2				
	Moderate		Strong		*P* value
	n	%	n	%	
Age <20 >20	 7 11	 38.8 61.2	 9 7	 56.25 43.75	FE test *P*=0.23
Sex: male female	 9 9	 50 50	 11 5	 68.75 31.25	FE test *P*=0.096
Type: generalized acrofacial focal	 4 9 5	 22.2 50 27.8	 14 0 2	 87.5 0.0 12.5	FE test *P*=0.01*

FE test: Fisher exact test; *significant

- "Normal" pigmented skin: the epidermis showed mild to moderate nuclear and cytoplasmic immunostaining (Table 2), while the dermis showed moderate nuclear and nucleo-cytoplasmic immunostaining of sweat glands, endothelium of the blood vessels, hair follicles and sebaceous glands (Fig. 1d, Table 2).

(B) Mdm2 (according to intensity of immunostaining)

1) Control skin: mild immunoreactive nuclear staining in the epidermis and skin adnexa was found in all study subjects (Table 3).

2) Vitiligo patients:

- Depigmented skin: both the epidermis and dermis showed strong nuclear immunostaining in most of the cases and moderate immunostaining in some cases (Fig. 2a, b, c, Table 3).

- "Normal" pigmented skin: the epidermis showed moderate nuclear and nucleo-cytoplasmic staining in most of the cases and mild staining in few cases (Fig. 2d, Table 3), while the dermis showed moderate staining of sweat glands, sebaceous glands, hair follicles and endothelium of the blood vessels in all cases (Table 3).

By applying Quickscore formula, positive p53 expression was moderate in 14 cases and strong in 20 cases. Regarding Mdm2 expression, Quickscore formula showed moderate staining in 18 cases and strong in 16 cases (Table 4).

DISCUSSION

Vitiligo is an acquired depigmentation disorder affecting 0.1%-2% of the world population. The disease is characterized by the loss of melanin pigment due to the partial or complete absence of functioning melanocytes in the affected areas (17).

Over the past decade, it has been extensively demonstrated, *in vivo* and *in vitro,* that the entire

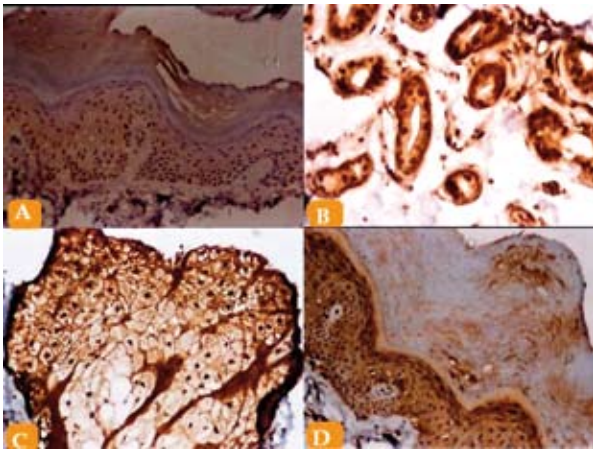

Figure 2. Mdm2 expression in depigmented and "normal" pigmented patients' skin: (A) strong nuclear and cytoplasmic staining of basal and suprabasal layers in depigmented skin (immunoperoxidase, x200); (B) strong staining of sweat glands of depigmented skin (immunoperoxidase, x400); (C) strong staining of sebaceous glands of depigmented skin (Immunoperoxidase, x400); and (D) moderate staining of the basal and suprabasal layers of "normal"pigmented skin (immunoperoxidase, x200).

epidermis of patients with vitiligo houses severe H_2O_2-mediated oxidative stress due to the accumulation of this reactive oxygen species in the millimolar range. Due to the lack of protecting pigment in the skin of affected individuals, increased susceptibility to solar rays as well as development of sun-induced skin cancer and premature aging of the skin would be highly expected (11). Surprisingly, this is not the case.

Schallreuter *et al*. (18) demonstrated that p53 protein markedly increased in the epidermal cell nuclei following UVR and H_2O_2 accumulation (19). As accumulation of the genomic aberrations is the key carcinogenic mechanism, the rapid induction of p53 activity in response to genomic damage thus serves to ensure that cells carrying such a damage are effectively taken care of (20).

Mdm2 is a key player in the regulation of p53. Mdm2 binds to p53 and leads to complete elimination of p53 through proteolytic degradation (21).

The aim of this study was the immunohistochemical detection of p53 and Mdm2 in depigmented and "normal" pigmented skin of vitiligo patients to demonstrate the possible role of these proteins in the protection of vitiligo patients against actinic damage and NMSC.

The present study showed that the expression of both p53 and Mdm2 proteins was significantly higher in "normal" pigmented and depigmented skin of vitiligo patients than in control subjects. The expression of both proteins extended also to the skin adnexa such as sweat glands, sebaceous glands, hair follicles as well as to the endothelium of the blood vessels. The intense expression of both proteins was in favor of generalized vitiligo.

There are few reports of NMSC in vitiligo skin. Lisi (22) stated that these cancers considered anecdotal since so far, only a few cases have been reported in the literature. Lassus *et al*. (23) reported two patients, one with squamous cell carcinoma (SCC) and BCC each, but it is not known whether these tumors were located on vitiliginous areas or not. Saarinen *et al*. (24) reported a case of SCC and actinic keratoses in a patient with generalized vitiligo. The patient was administered high doses of systemic corticosteroids for his chronic obstructive pulmonary disease. It remains uncertain whether the prolonged course of oral corticosteroids administered to this patient sufficiently compromised his immune system to permit the development of premalignant and malignant skin lesions. It is widely accepted that there is an increased incidence of skin cancers in immunocompromised individuals (25).

Park *et al*. (26) reported a case of SCC in vitiligo lesion after long-term PUVA therapy. Although PUVA has been used to treat vitiligo since 1976 (27), no skin cancers related to PUVA therapy in vitiligo patients were reported until 1996 (28). Squamous cell carcinoma after long-term PUVA therapy is much rarer in vitiligo patients than in psoriasis patients (26).

The significant over-expression of epidermal p53 protein in both "normal" pigmented and depigmented skin in vitiligo patients, in the present study, agreed with Schallreuter *et al*. (15) and Salem *et al*. (29). The authors concluded that there are increased levels of wild-type p53 expression in keratinocytes in the skin of patients with vitiligo. This tumor suppressor may protect against actinic damage and development of keratinocyte cancer. Schallreuter *et al*. (15) discovered that p53 in vitiligo exhibited the wild-type status with normal functionality. It has been speculated that epidermal p53 in vitiligo could be induced *per se* by the continuous H_2O_2 stress in this compartment (30). Salem *et al*. concluded that the constant up-regulated, activated, stabilized wild-type p53 could serve as a major protection mechanism for DNA repair in vitiligo. p53 expression remains high even after epidermal H_2O_2 reduction with pseudocatalase. Going with that, Teulings *et al*. (31) showed that vitiligo patients had a threefold decreased probability of developing NMSC during their lifetime due to p53 over-expres-

sion in their skin. However, Hexsel *et al*. (32) reported that there is an increased or equal risk of NMSC in Caucasian patients with vitiligo.

From another point of view, Saarinen *et al*. (24) have explained the rarity of cancer in vitiligo skin by the tendency of patients to avoid sun exposure because it accentuates the contrast of skin color due to the lesions, and consequently, chronic sun exposure may be reduced in vitiligo patients. However, in the current study, all patients had outdoor occupations with chronic sun exposure.

The current study showed that the expression of p53 protein extended also to the skin adnexa such as sweat glands, sebaceous glands, hair follicles as well as to the endothelium of the blood vessels. There was no comment on this phenomenon in previous similar studies. However, it can be explained by the accumulation of genomic aberrations due to DNA damage, and oxidative stress in adnexal cells, which leads to rapid induction of p53. Further larger scale studies are needed for more clarification of this observation.

In the present study, p53 expression was significantly higher in cases of generalized vitiligo than in cases of localized (focal and acrofacial) vitiligo. This observation was not discussed in previous similar studies. It can be explained by the longer duration of disease in generalized cases. It is established that the development of NMSC is merely a matter of disease duration and intensity of sun exposure (26).

The significant over-expression of epidermal Mdm2 protein in both "normal" pigmented and depigmented skin in vitiligo patients, in the present work, agreed with Salem *et al*. (29). Chen *et al*. (33) suggested that the increased expression of Mdm2 is the result of over-expression of p53 (because p53 binds specifically to the Mdm2 gene and stimulates its transcription leading to the production of Mdm2 protein). Mdm2 protein binds to p53 and inactivates it. Interestingly, experimental evidence also indicates that Mdm2 over-expression causes G1 arrest in normal cells (34). So the levels of both proteins are elevated in the cell and in this case, Mdm2 has a supportive role to p53 as it can stop the cell cycle in G1 phase (35).

On the other hand, our finding conflicted with what has been mentioned by Schallreuter *et al*. (15), who reported that Mdm2 protein remains unchanged in both depigmented and "normal" pigmented skin with no significant difference between vitiligo cases and normal controls. The authors reported that the lack of a regulatory increase in Mdm2 in response to elevated P53 could be based on the continuous oxidative stress in vitiligo epidermis resulting in increased phosphorylation of p53 protein in both N-terminal and C-terminal regions. It is known that post-translational modification is one of the most important ways to protect p53 from Mdm2 by destabilizing the interaction between the two proteins, leaving p53 in its transcriptionally active form.

Mdm2 expression was significantly higher in cases of generalized vitiligo than in cases of localized (focal and acrofacial) vitiligo. There was no comment on this phenomenon in previous similar studies. As Mdm2 follows p53, so the increased expression of p53 in generalized vitiligo, as mentioned above, could explain the increased Mdm2 in generalized vitiligo (33).

CONCLUSIONS

Vitiligo patients have protective effects against UVR-induced DNA damage. This could be due to over-expression of p53 and Mdm2 proteins in their depigmented and "normal" pigmented skin. This may explain the rarity of actinic damage and NMSC in this category of patients.

Further studies are needed on larger numbers of patients with different skin phototypes, with different ethnic backgrounds, in different climatic conditions and with different clinical varieties of vitiligo (segmental and universal). Long-term follow up, retrospective and preferably prospective studies in different populations of vitiligo patients taking PUVA therapy are also recommended to detect the incidence of NMSC and actinic damage in these patients. Large studies are needed to assess the risk of UVB-induced skin cancer development in patients with vitiligo.

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