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Original Article

Expression levels of MIF, NLRP1 and FOXP3 genes along with biomarker levels in patients with active form of non-segmental generalized vitiligo: A study in South Indian population

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Abstract

Vitiligo, the most widespread hypopigmentary syndrome, is considered to be a multifactorial disease in which the active melanocytes are lost. Vitiligo has been studied in a variety of ways, and several genes have been implicated. In this study we focused on investigating biomarker levels of TNF-α, MDA and TAS by using ELISA, and along with that mRNA expression levels of MIF, NLRP1 and FoxP3 genes were quantified with qRT-PCR. In expression studies, Non-segmental Generalized Vitiligo (NSV) subjects had a substantial (P <.05) fold change in MIF gene expression compared to healthy subjects, with a 0.7 change in expression level. In FoxP3 and NLRP1, however, there was just an 0.3-fold change in expression. However, there was a substantial increase (P <.01) in TNF-α and (P <.0005) in TAS levels, but no difference in Malondialdehyde levels (MDA). The current study is a baseline study suggesting that MIF, with a 0.7-fold change, may play a key role in the pathogenesis of active NSV subjects, while changes in NLRP1 and FoxP3 mRNA expression levels were weaker. We also found a significant increase (P <.01) in TNF-α and (P <.0005) in TAS levels, which may be noted as a key feature in Vitiligo subjects.

Keywords: vitiligo, MIF, NLRP1, FoxP3, TNF-α, MDA, TAS

1. Introduction

Vitiligo is a skin condition that manifests as white macules and patches due to a loss of functional epidermal melanocytes. Vitiligo affects 1% of the global population (Whitton *et al.*, 2006), while it has been stated that the prevalence in India is as high as 3-4 percent (Sehgal & Srivastava, 2007). Researchers from all over the world have looked into the possibility of common susceptibility genes in Vitiligo and other autoimmune disorders, as well as for additional genes that could mediate Vitiligo.

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Dermatologically, Vitiligo is characterized by the loss of pigment in the skin and hair follicles (Rodrigues, M., Ezzedine, K., Hamzavi, I., Pandya, A. G., & Harris, J. E. 2017). Many independent investigations have found a link between Vitiligo and a variety of genes, including MIF, NLRP1, and FOXP3. MIF is one of the immune-stimulatory cytokines involved in macrophage and T cell activation (Macrophage migration inhibitory factor), a pro-inflammatory cytokine that has lately been linked to an increased risk of vitiligo (Farag, Habib, Kamh, Hammam, & Elnaidany, 2018; Ma *et al.*, 2013; Serarslan *et al.*, 2009).

Another molecule and one of the principal controllers in the innate immune system is nucleotide oligomerization domain-like receptor, which has been

demonstrated to have high levels in the immune system (NLRP1, previously known as NALP1). It is a member of the NLR family (nucleotide oligomerization domain-like receptors). The role of NLRP1 in the general Vitiligo is examined in a study on polymorphism and subsequent activation of NLRP1 mRNA in patients with a susceptible genotype (Dwivedi, Laddha, Mansuri, Marfatia & Begum, 2013). FOXP3 (forkhead box P3) is a member of the FOX protein family and a Master Regulator Protein (transcription factor) in the development and function of regulatory T cells, according to some studies (Zheng *et al.*, 2007).

According to one report, there is a negative link between FOXP3 and Vitiligo patients, with FoxP3 levels in the peripheral blood being much lower in patients than in controls (Elela, Hegazy, Fawzy, Rashed & Rasheed, 2013). TNF is involved in apoptosis in a variety of cell types by activating the receptor-mediated apoptosis pathway (Gupta & Gollapudi, 2006).

Keratinocytes are responsible for the production of cytokines such as TNF. Interleukins IL-1, IL-6, and Transforming Growth-Factor (TGF) are paracrine melanocyte prolife and melanogenesis inhibitors. They also impact the apoptotic pathway in melanocytes, and their levels may be important in the development of vitiligo. TNF may also influence the development of melanocyte stem cells (Alghamdi, Khurrum, Taieb & Ezzedine, 2012). Oxidative stress is a sort of skin attack that was the original source of vitiligo cases, causing melanocyte death (Laddha *et al.*, 2013).

A few studies have demonstrated that antioxidant pathways are important in the pathogenesis of global Vitiligo (Koca, Armutcu, Altinyazar, & Gürel, 2004). Malondialdehyde (MDA), the end product of lipid peroxidation (LPO), causes cross-linking in lipids, proteins, and nucleic acids as a result of free radical degradation of polyunsaturated fatty acids.

In the epidermis and blood of Vitiligo patients, oxidant/antioxidant disorders such as hydrogen peroxide accumulation (H2O2), elevated Malondialdehyde (MDA) levels, and decreased catalase (CAT) levels have recently been discovered (Schallreuter *et al.*, 1999, 2001). The goal of this study was to determine the role of antioxidants in Vitiligo pathogenesis as well as to support the proposed etiology, which suggested that increased oxidative stress causes melanocyte destruction in Vitiligo, triggering an autoimmune response. In order to determine the involvement of MIF, NLRP1, and FOXP3 in generalized Vitiligo, we evaluated the mRNA levels of these three genes in the blood of non-segmental generalized subjects who were in the active form and compared them to our people's safe checks.

We investigated the anti-inflammatory function and antioxidant systems by measuring TNF-, total antioxidant status, and MDA levels because the cumulative effect of anti-inflammatory and antioxidants present in serum and bodily fluids may provide more meaningful biological information. Although many independent studies using various approaches have revealed a link between these genes and Vitiligo, to our knowledge, this is the first study in which the mRNA levels of the above-mentioned genes were analysed in the same set of samples from Vitiligo patients with a specific type of Vitiligo (active NSV). Aside from that, measuring the levels of oxidative stress associated with the specific form of Vitiligo was also of interest to us.

Earlier, it was stated that the current study was based on baseline samples. In order to determine the role of genes in the pathogenesis of vitiligo in broader way additional studies with larger sample sizes, including follow-up studies, should be considered, with as many genes in the same set of samples.

2. Materials and Methods

2.1 Chemicals, reagents and kits

For the current study chemicals like Reverse transcriptase kit, RNA isolation kit and Nuclease free water were purchased from Qiagen. Syber green dye, dNTPs, primer mix, and Taq polymerase were purchased from Kappa biosystems. PAX gene RNA tubes were purchased from BD Paxgene Company. Primers were designed by using serial cloner software and purchased from Bio-serve Company. Elisa kits like Human TNF-α came from Bio-legend, and MDA and TAS Elisa kits were purchased from Sigma-Aldrich.

2.2 Subjects

All Vitiligo and control subjects of the study were enrolled at National Research Institute of Unani Medicine for Skin Disorders. (NRIUMSD), Hyderabad, India. 30 Vitiligo patients (18 males and 12 females) were enrolled in this study. All vitiligo cases had non-segmental widespread vitiligo with progressive active vitiligo in which new depigmentation areas were found, depigmentation area was expanding, or both, during the last month condition, i.e. with VIDA ranking. The vitiligo subjects were excluded if they had any clinically significant abnormality identified, any systemic disease, other skin disease, were pregnant or lactating women; and the subjects should not have received any other investigational product within the last 4 weeks. The control group consisted of 30 healthy subjects (18 males and 12 females). The ages and gender ratios were well matched in the two groups.

All the subjects enrolled in this study gave written informed consent for the study. The Ethics Committee of NRIUMSD approved the study and the study is registered at Clinical Trials Registry- India (CTRI) with registration number CTRI/2015/10/006275.

2.3 Methods

2.3.1 RNA isolation

Blood was taken from all subjects (Vitiligo and Healthy) after an overnight fast, in the morning. For isolation of RNA, 2.5 ml collected blood was transferred to Pax gene blood RNA tubes and was stored at -20 ºC in a refrigerator until further use. Total RNA was isolated using Pax gene blood RNA isolation kit following manufacturer's instructions. The quality of the RNA samples was assessed by inspecting the 28S and 18S bands on agarose gel electrophoresis. Further 1.9 to 2.0 levels of 260/280 absorbance ratio for each RNA sample were observed. Extracted RNA was dissolved in RNase-free water and stored at -80 ºC until cDNA synthesis.

2.3.2 cDNA conversion and real time PCR evaluation

qRT-PCR was used to evaluate the specificity of primers used in the expression investigations. To do this, 500 ng of RNA was converted to cDNA by following the specified program, which included further incubation steps by adding RT enzyme, RT buffer, and RT primer mix, resulting in a final volume of 20µl. By detecting bands on a 1% agarose gel after electrophoresis, the presence of cDNA was also established. dNTPs, buffer, Taq polymerase, and forward and reverse transcriptase kits were used to test primer specificity. A CFX96 qRT-PCR from Bio-Rad was used to perform real-time RT-PCR. The primers (Table 1) were designed by serial cloner software and synthesized by Bio serve Company (India). All the reactions were carried out in 20 µl reaction volume each, in triplicates. Each gene's delta Ct was obtained by subtracting GAPDH, which acted as an internal control gene, and a melting curve graph was recorded for each gene.

2.3.3 Biochemical parameters of the study subjects

Liver function indicators (Bilirubin, ALT, AST and ALP) and renal function indicators (Urea, Creatinine) were also analysed by using an Erba Auto Analyser. A 5 ml venous blood sample was collected from the Vitiligo patients and control subjects in a metal-free sterile tube, between 8 and 9 am after overnight fasting.

At room temperature, the blood was then allowed to coagulate for 30 minutes, and the serum was centrifuged at around 5000 rpm for 15 minutes. The serum was collected and placed in an Eppendorf tube and stored at -80°C until analysis. Results are presented in Table 3.

2.3.4 Measuring of TNF-α, MDA, and TAS levels

Serum levels of TNF-α, MDA, and TAS levels in patients with Vitiligo and Healthy subjects were measured by enzyme-linked immunosorbent assay (ELISA) using the kit purchased from Bio-legend and Sigma-Aldrich. Following step by step manual instructions the serum samples were incubated and respective kit reagents were added, and the reaction was terminated by using a stop solution. Absorbances were taken at 450 nm for TNF-α, 532nm for TAS, and MDA was measured at 570nm using a multimode plate reader (Techan infinite 200 pro). The results are displayed in Table 2.

2.3.5 Statistical analysis

Data were analysed statistically by using SPSS 16.0 for Windows. For biochemical parameters and ELISA, mean and SD were calculated and Student's t-test was performed, whereas for Real-time PCR experiments ΔCp values were measured along with fold change. The required level of significance was set at $P \leq .05$ for measurements performed in triplicates.

3. Results

3.1 Real time PCR validation

In the gene expression analysis, we determined mRNA levels of MIF, NLRP1 and FOXP3 in non-segmental vitiligo subjects and in healthy controls. The specificity of the MIF, NLRP1, FOXP3 and internal control gene GAPDH products were checked by melting curve analysis and agarose gel electrophoresis. The PCR product sizes of MIF, NLRP1 and FOXP3 was 110bp, 112bp and 85bp with melting temperatures of 79°C, 78.5°C and 81°C respectively (Table 1).

3.2 Relative mRNA expression levels of MIF gene

mRNA expression levels were measured by using Syber green method. RNA was isolated from Vitiligo subjects and Healthy control subjects followed my mRNA expression determination for MIF gene. Levels of mRNA were normalized with GAPDH expression as internal control. ΔCp and mRNA folds were calculated for Vitiligo and Healthy subjects and results showed 1.71 and 1.7-fold changes in Vitiligo subjects, whereas Healthy subjects had 2.37 and 1.04-fold changes. The comparison of the findings showed statistically significant ($P < .05$) changes in expression levels of MIF in non-segmental active vitiligo subjects in comparison to healthy subjects (Figure 1). To interpret the above findings, the MIF gene was expressed more in Vitiligo subjects than in healthy controls, and with an inverse relation of ΔCp value with expression fold change.

Table 1. Primer sequences for MIF, NLRP1, Foxp3, and GAPDH

Gene	Sense	Antisense	Size (bp)
MIF	5´-ACCAGCTCATGGCCTTCG-3´	5´-CTTGCTGTAGGAGCGGTT-3´	110
NLRP1	5´-GGCAGCACAGATCAACATGGA-3´	5´-CAGGTTTCTGGTGACCTTGAGGA-3´	112
FoxP3	5´-GAGAAGCTGAGTGCCATGC-3´	5´-AGCCCTTGTCGGATGATG-3´	85
GAPDH	5´-ACCCACTCCTCCACCTTTGA-3´	5´-CATACCAGGAAATGAGCTTGACAA-3´	75

Table 2. Serum concentration levels of TNF-α, Lipid peroxidase, and TAS activity of Vitiligo patient and controls

Parameter	Control group(n=30)	Patients (n=30)	*P*-value
TNF-α (pg/ml)	3.821 ± 9.94	8.85 ±4.51	< .01
Lipid Peroxidation (MDA) (n mole/µL)	9.77 ± 1.22	9.891 ± 2.30	< 0.801
TAS activity (mM/ml)	0.103± 0.030	0.05 ± 0.057	< .0005

Data are expressed as Mean ± SD, P<=0.05 was considered statistically significant

Table 3. Biochemical investigation parameters of Vitiligo patients and Healthy controls subjects

Parameter	Reference Range	Controls	Vitiligo patients	*P*-value
Age		31.73 ± 5.9	32.8 ± 8.7	< 0.579
BMI		23.5 ± 4.3	24.6 ± 8.0	< 0.5097
Liver function tests				
Bilirubin(mg/dl)	0.1-1.2	0.725 ± 0.37	0.72 ± 0.271	< .98
ALT (IU/L)	0.5-40	22.63 ± 11.5	23.2 ± 6.65	< .81
AST (IU/L)	0.5-42	24.06 ± 8.10	22.8 ± 5.72	< .49
ALP (IU/L)	30-111	67.66 ± 21.7	86.23 ± 18.75	< .005
Urea (mg/dl)	13-45	22.24 ± 6.6	24.86 ± 5.00	< .09
Creatinine(mg/dl)	0.7-1.4	1.06 ± 0.15	1.037 ± 0.176	< .48

P<=0.05 is considered significant, Values are in Mean ± SD, *P*-value by student's t- test, BMI: body mass index, ALP: Alakaline phosphatase, ALT: Alanine amino transferase, AST: Aspartate amino transferase

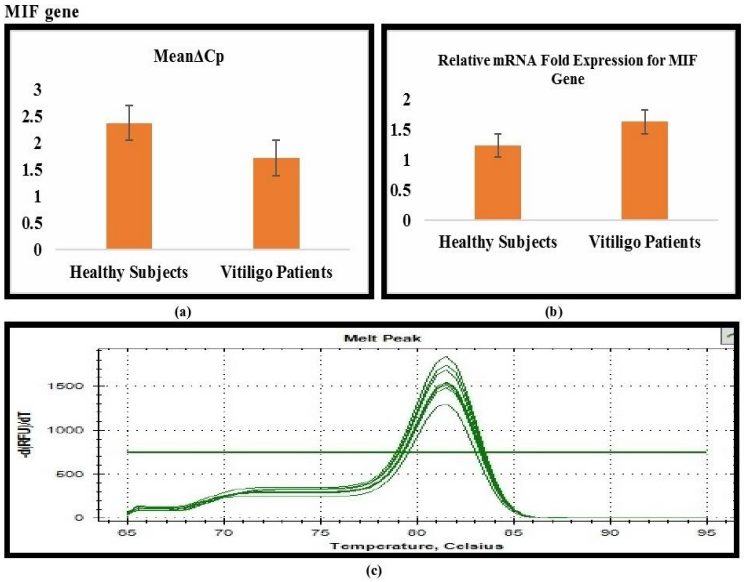

Figure 1. Relative expression of the gene MIF in controls and patients with non-segmental active vitiligo. (a) Expression of MIF mRNA as indicated by mean ΔCp, and (b) fold expression of MIF mRNA as determined by the $2^{\Delta\Delta Cp}$ method. Vitiligo subjects showed significant difference ($P < .05$) in MIF mRNA expression compared with controls. (c) Single peak from each melting curve shows that the designed primers were specific to MIF.

3.3 Relative mRNA expression levels of NLRP1

With the Syber green method, the levels of mRNA expression were determined. Subjects with Vitiligo had their RNA isolated, and healthy controls had their mRNA expression monitored for NLRP1 gene. The GAPDH gene was used as an internal control to normalize mRNA levels. In Vitiligo subjects, Cp and mRNA folds were found to be 3.33 and 1.68 while the Healthy subjects had a Cp of 3.59 and a 1.4-fold change. The comparison of the findings showed statistically non-significant (P > .05) change in expression levels of NLRP1 in non-segmental Vitiligo subjects in comparison to with the Healthy subjects (Figure 2). In the above results the Healthy subjects had slightly decreased expression level when compared with Vitiligo subjects.

3.4 Relative mRNA expression levels of FOXP3

To determine mRNA expression, the Syber green method was used. Isolation of the RNA from Vitiligo patients and Healthy subjects was performed. The expression of the gene FoxP3 in healthy controls was also monitored along with Vitiligo subjects. It was decided to normalize the mRNA levels using the GAPDH gene. Cp and mRNA folds were found to be 8.53 and 1.8 respectively, in Vitiligo patients. There were 8.51 and 1.54 -fold changes for healthy subjects. Comparison of the findings showed statistically non-significant (P > .05) change in expression levels of FOXP3 in non-segmental Vitiligo subjects in comparison to the Healthy subjects (Figure 3). From the findings it was observed that Foxp3 gene had increased expression when compared with GAPDH gene.

3.5 Other biochemical parameters

The age and BMI distributions were more or less similar in Vitiligo patients as in the controls. The liver function indicators (Bilirubin, ALT, AST and ALP) and those of renal function (Urea and Creatinine) for Vitiligo patients and controls were within normal limits and there were no significant differences (Table 3).

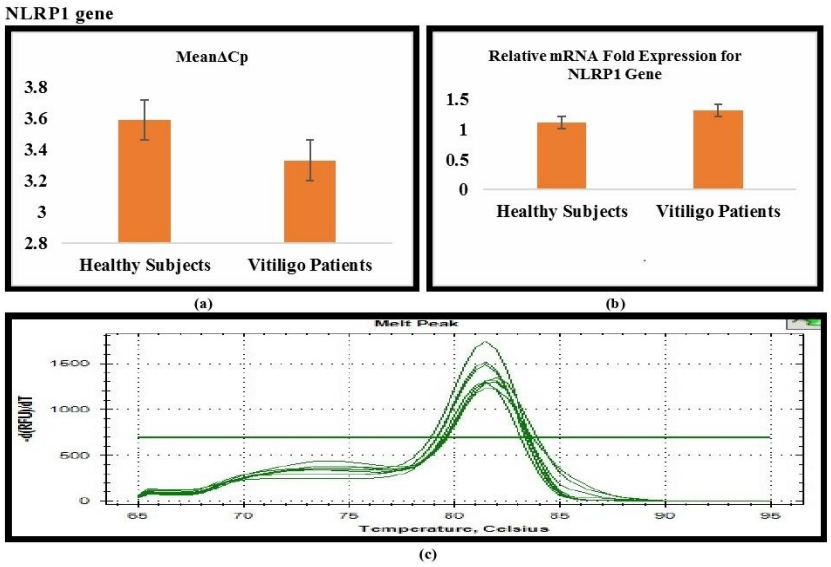

Figure 2. Relative expression of the gene NLRP1 in controls and patients with non-segmental active vitiligo. (a) Expression of NLRP1 mRNA as indicated by mean ΔCp whereas, (b) Fold expression of NLRP1 mRNA determined by the $2^{\Delta\Delta Cp}$ method. No significant differences (*NS*) in NLRP1 expression were observed between the two groups. (c) Single peak from each melting curve shows that the designed primers were specific to NLRP1.

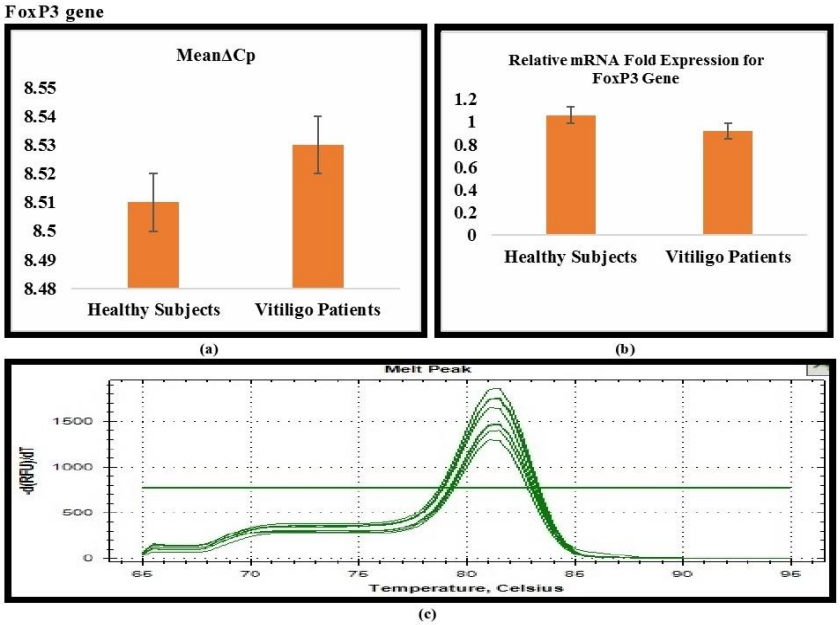

Figure 3. Relative expression of the gene FOXP3 in controls and patients with non-segmental active Vitiligo. (a) Expression of FOXP3 mRNA as indicated by mean ΔCp, and (b) fold expression of FOXP3 mRNA as determined by the $2^{\Delta\Delta Cp}$ method. No significant differences (*NS*) in FOXP3 expression were observed between the two groups. (c) Single peak from each melting curve shows that the designed primers were specific to FoxP3.

3.6 Levels of TNF-α, MDA and TAS in Vitiligo patients and Healthy Subjects

Serum samples were collected at the time of sample extraction and stored at -80 °C deep freeze. Following the respective kit protocols, ELISA was performed for both Vitiligo and Healthy subjects, followed by analysis of serum levels with respective kit. From the results the mean serum TNF-α concentration was increased in the patient group as compared to the control group, 8.85 ± 4.51 vs. 3.821 ± 9.94, and there was a significant difference between the means of the two groups. On the other hand, there was no significant difference in the levels of MDA serum levels between Vitiligo patients and the Healthy control groups (9.891 ± 2.30 vs. 9.77 ± 1.22). The mean serum level of TAS in the patient group was significantly lower than in the control group, 0.05 ± 0.057 vs. 0.103 ± 0.030. The above ELISA data suggest that cytokine TNF-α has a key role in depigmentation, while the

other cytokines in the current study had weaker level differences.

4. Discussion

For non-segmental Vitiligo (90 % of total vitiligo), the primary cause is functional loss of melanocytes (melanocyte dysfunction). Vitiligo is nowadays considered a multifactorial phenomenon that can lead to the loss of functional melanocytes in a variety of conditions.

Vitiligo treatment plans can last for months or even years, but they can still produce poor results. It's clear that, in addition to the current clinical selection criteria, there is a pressing need for biomarkers to track adverse immune events and predict vitiligo progression, as well as to monitor treatment responses.

As an example of vitiligo, we have evaluated a single disease subtype that is not segmented and has a progressive course. For this reason, the 2011 International Pigment Cell Conference (IPCC) emphasized the importance of careful consideration when studying this type of skin disease (Ezzedine *et al.*, 2012). MIF, FoxP3, and NLRP1 have all been implicated in Vitiligo.

These genes have been studied independently, but to our knowledge this is the first study to examine the relative expression of all three genes in the same group of non-segmental generalized Vitiligo patients, in a progressive or active state. Atopic dermatitis, systemic sclerosis, bullous pemphigoid, psoriasis, and Vitiligo have all been linked to MIF in previous studies (Asano *et al.*, 2008; Shimizu, 2005). As compared to healthy subjects, active NSV subjects had a significantly higher relative expression of MIF mRNA.

Our findings are consistent with one previously published study, which found that the MIF, in collaboration with other cytokines, causes local inflammatory and immunological responses in Vitiligo vulgaris-associated depigmentation. MIF could also be used as a measure of the severity and effectiveness of Vitiligo vulgaris. Furthermore, macrophages are implicated in melanocyte clearance, as macrophage infiltration and an increase in their quantity are seen in Vitiligo's perilesional skin (Kim *et al.*, 2008; Serarslan *et al.*, 2010).

MIF has also been demonstrated to cause an increase in the production of inflammatory cytokines, such as IL-6, IL-8, and TNF-, which have a role in melanocyte cytotoxicity and decreased pigmentation (Yu *et al.*, 1997). A further gene investigated in this study is NLRP1, which is implicated in inflammation, apoptosis, and melanocyte death mediated by apoptosis, most likely via cytokines (Dwivedi, Laddha, Mansuri, Marfatia & Begum, 2013). We could not find any significant difference in the expression of NLRP1.

However, an earlier study found that vitiligo patients' NALP1 (now known as NLRP1) gene expression was lower than normal, despite the fact that expression was evaluated semi-quantitatively with a smaller sample size (Deo, Bhagat & Shah, 2011; Huang, Nordlund & Boissy, 2002). In individuals with GV, the expression of NLRP1 mRNA was found to be significantly higher than in healthy controls in one investigation (Dwivedi, Laddha, Mansuri, Marfatia & Begum, 2013). Although the preceding study was conducted on Indian people, we believe that larger studies with a larger sample size are needed to determine whether there is a difference in relative expression of NLRP1 in the blood of vitiligo patients compared to healthy controls as we could not establish any correlation between active NSV and NLRP1 expression.

We found no significant difference in relative expression of FOXP3 mRNA in blood cells of active NSV subjects compared to healthy subjects ($P > .05$). Some genome-wide investigations, particularly one from the Indian subcontinent, have linked the FoxP3 gene polymorphism to vitiligo (Birlea *et al.*, 2011). Elela, Hegazy, Fawzy, Rashed, and Rasheed (2013) found a negative association between FoxP3 expressions in vitiligo patients in an earlier investigation. However, we were unable to find any link between FOXP3 expression in blood cells and the presence of active NSV in our cohort.

For the first time, we demonstrate that NSV induced considerably greater TNF-protein levels than controls, indicating that autoimmunity is involved in NSV condition. Studies on cytokines such as TNF- reveal that these inflammatory indicators play an essential role in apoptosis in a variety of cell types by activating the receptor-mediated apoptosis pathway (Venters *et al.*, 1999). Apoptosis can be activated by cytokines such as IFN-γ and TNF-α, leading to autoimmunity and melanocytic death (Huang, Nordlund & Boissy, 2002). TNF- also has the ability to prevent melanogenesis by inhibiting tyrosinase and tyrosinase-related proteins. (Huang, Nordlund & Boissy, 2002; Martinez-Esparza, Jimenez-Cervantes, Solano, Lozano & Garcia-Borron, 1998)

MDA is a byproduct of reactive oxygen species-induced lipid peroxidation (ROS). It is a good measure of oxidative stress since it is well associated with the degree of lipid peroxidation (Yildirim, Baysal, Inaloz & Can, 2004). The current study identified no significant differences in MDA levels in serum between NSV patients and the control group (Picardo *et al.*, 1994). Normal MDA serum levels in mixed vitiligo forms were consistent with this observation (Picardo *et al.*, 1994). Despite the fact that there are studies demonstrating more serum MSDs in patients than in controls (Yildirim, Baysal, Inaloz, & Can, 2004), there are also studies revealing less serum MSDs than in controls (Yildirim, Baysal, Inaloz, & Can, 2004). (Dammak *et al.*, 2009; Yildirim, Baysal, Inaloz & Can, 2004) discovered statistically significantly elevated levels of MDA, which represent oxidative stress conditions, in Vitiligo patient tissues as compared to the control group.

TAS levels in NSV patients were found to be lower than in controls. This suggests that either an increase in oxidative stress causes a depletion of TAS reserves in Vitiligo patients, or these Vitiligo patients were unable to neutralize the melanocyte-damaging oxidants due to a lack of TAS material.

MIF polymorphisms were linked to NSV susceptibility. MIF levels in the blood were higher in patients with active NSV and correlated negatively with the number of years of evolution. MIF was found in high concentrations in the depigmented skin of patients with active Vitiligo (Garcia-Orozco A, Martinez-Magaña IA, Riera-Leal A, Muñoz-Valle JF & Martinez-Guzman MA *et al.*, 2020)

We conclude that in Vitiligo, the antioxidant system has been weakened, resulting in free radical-mediated death of melanocytes or deregulation of melanogenesis, which can lead to an autoimmune reaction. Antioxidants can potentially be

used as a therapy for vitiligo. Researchers elsewhere have discovered similar results (Akoglu *et al.*, 2013; Jalel & Hamdaoui, 2009; Khan, Satyam, Gupta, Sharma & Sharma, 2009).

Finally, this study discovered that active NSV subjects' blood cells expressed three separate genes: MIF, NLRP1, and FoxP3. Among the three genes tested, we only found a significant (P <.05) difference in MIF mRNA expression in active NSV subjects compared to healthy subjects.

The current study reveals that MIF plays a role in the etiology of the non-segmental generalized active type of Vitiligo, but that NLRP1 and FoxP3 have lesser functions. TNF- also affects melanocyte apoptosis and may play a role in Vitiligo pathogenesis, and there is a deficiency in the Vitiligo antioxidant system, which contributes to the abnormality of free radical levels, which cause melanocyte destruction or dysregulation of melanogenesis, as well as an autoimmune response. As a result, antioxidants may be effective in the treatment of Vitiligo. The current study's strength is that three genes' expression levels were assessed in the same set of samples from active NSV patients. More research with a larger sample size, including follow-up studies, and with the same/specific type of Vitiligo, should be pursued, with as many genes in the same set of samples as possible, to determine their functions in disease development.

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