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Factors affecting vitiligo response to treatment: do MiRNA 196a2C/T gene polymorphism and serum tyrosinase levels have any role?

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ABSTRACT
Background: Factors contributing to the pathogenesis of vitiligo and factors affecting its response to treatment are still a major area of debate.
Aim of the work: The study aimed to assess the serum levels of tyrosinase and Micro-RNAs (miRNAs) gene polymorphism in a sample of Egyptian vitiligo patients, and to determine factors affecting the response of vitiligo to treatment.
Subjects and methods: This prospective case-control interventional study included 212 non-segmental vitiligo patients and 96 control subjects. Before treatment, vitiligo was evaluated using Vitiligo Area Severity Index. Detection of miRNA 196a-2 polymorphism was done using PCR-RFLP and serum tyrosinase was measured using ELISA. After treatment, patients were reevaluated clinically and serum tyrosinase levels were re-measured.
Results: The tyrosinase levels were significantly elevated in patients. The TT genotype was the most prevalent one in the patients. The percentage of improvement showed a significant positive correlation with patients’ ages and age of the disease onset and a negative correlation with disease duration, baseline VASI scores and serum tyrosinase levels.
Conclusion: MiRNA 196a-2 C/T (11614913) gene polymorphism and the elevated serum tyrosinase levels might be related to the pathogenesis of vitiligo and may affect its therapeutic response.

Introduction
Vitiligo is a common autoimmune depigmenting disease affecting up to 1% of people worldwide. More than the cosmetic disfigurement, vitiligo can impair the psychological well-being of the patients and may affect all aspects of quality of life. The devastating stigma of this disease has encouraged scientists to find out preventive and curative options for this condition. However, this target has not been fulfilled so far. The response to the available lines varies greatly. Although, the complexity of vitiligo pathogenesis makes predicting the response to treatment too difficult, some immunological factors that may affect the response of vitiligo to treatment.

Micro-RNAs (miRNAs) are a group of small non-coding RNAs (about 22 nucleotides in length) that have a regulatory role on the messenger RNA (mRNA) cleavage or a translation repression effect, and on most of the human genes that encode proteins. Thus, many vital physiological, developmental and pathological processes are controlled by miRNAs. The miRNA-145 overexpression is associated with a defective pigmentation. It reduces the expression levels of the genes involved in pigment formation including the tyrosinase gene (which encodes the key enzyme in melanin synthesis; tyrosin-ase). It also reduces the expression of the genes involved in the transport of melanosomes to the cell periphery leading to a perinuclear aggregation of melanosomes. Serum and skin biopsies taken from vitiligo patients showed overexpression of miRNA-16 and 145, and the successful vitiligo treatment with NB-UVB was associated with a significant reduction in their expression.

The miRNA 196a-2 could target tyrosinase-related protein 1 (TYRP1) in melanocytes leading to an impaired eumelanin synthesis and melanocytes death due to their increased sensitivity to oxidative stress, which results from abnormal synthesis and processing of TYRP1. Huang et al. found that MiRNA 196a-2 C/T (11614913) gene polymorphism may be related to vitiligo development.

The current study aimed to evaluate serum levels of tyrosin-ase in vitiligo patients and to study miRNA 196a-2 C/T (11614913) gene polymorphism in a sample of Egyptian vitiligo patients. The study also aimed to determine factors that may affect the response of vitiligo to treatment.

Patients and methods
The Research Ethics Committee of the Faculty of Medicine, Benha University, approved this prospective case-control interventional study. The study purpose was discussed with the participants and informed consents were obtained from them before samples collection.
The study was conducted on non-segmental vitiligo patients, attending the phototherapy unit in Benha University Hospital during the period from January 2019 to December 2019. The study included 212 patients who did not receive any topical or systemic treatment for vitiligo for at least one month before samples collection. Patients with segmental vitiligo or with any contraindication to phototherapy were excluded. Ninety-six healthy, age and sex-matched individuals were included as a control group. Control subjects were apparently healthy sub-jects; free from vitiligo and have no family history of vitiligo. They were recruited from the nursing team and the relatives of admitted inpatients.

Patients’ evaluation

Before treatment, all patients were subjected to full history tak-ing, a general medical examination to detect the signs of other systemic diseases and local dermatological examination to evaluate the site, size, distribution and clinical type of vitiligo. The severity of vitiligo was assessed according to the Vitiligo Area Severity Index (VASI) (12). The miRNA 196a-2 polymorphism was detected by using PCR-REFLP technique while serum tyrosinase levels were measured using the ELISA. Patients received three weekly narrowband UVB treatment sessions. The tyrosinase levels were measured using Human Tyrosinase ELISA kit (supplied by SunRed Company, China, cataloque No 201-12-7682) according to the manufacturer protocol. Sensitivity was 0.77 ng/ml and the assay range was 0.8–200 ng/ml.

The amplified samples were then run in on 2% agarose gel in the presence of DNA markers using gel electrophoresis and visualized on a UV trans illuminator to detect the presence of amplified product and to type the genotype. This step was done by gel electrophoresis machine (Wealtec Corp.) (E5W0255). The power supply was programed to give 130 volts and 100 milliamperes for 30 min. The 3 expected genotypes would appear as follows: CC 125 bp, TT 149 bp, CT 125 bp. The PCR marker was loaded into one of the wells. The PCR reaction mixture was mixed well, then PCR reaction mixture was added to the PCR tube.

Gene amplification using polymerase chain reaction

Enzymatic amplification was performed by PCR using My Taq Red Mix polymerase enzyme (Intron Biotechnology) and Vereti thermal cycler (AB Applied Biosystem – USA).

The reaction mixture was prepared as follows: 2.5 ml of DNA template, 12.5 ml of My Taq Red, 0.5 ml of Forward Primer, 0.5 ml of Reverse Primer and 9 ml of Nuclease free water to reach a total volume of 25 ml per tube. The sequence of the primer was Forward: 5'-CCCCCCCTCCTTCTCCAGATA-3' Reverse: 5'-CGAAACCAGCTGATGAATCCCG-3'. This reaction mixture was added to the PCR tube.

The PCR reaction mixture was mixed well, then PCR reaction tubes were closed and placed inside the heating block in the DNA Vereti thermal cycler, and the cap was tightly closed. The computerized thermal cycler was programmed for the following conditions: initial denaturation at 95 C for 2 min, followed by 35 cycle of denaturation at 95 C for 30 s, annealing at 55 C for 30 s and extension at 72 C for 20 s.

Restriction digestion

Digestion was done by restriction enzyme (mspl). Samples were prepared for loading by adding 2 ml loading buffer to 7 ml of the PCR reaction mixture, and then were carefully loaded into the sample wells using an automatic micropipette. The PCR marker was loaded into one of the wells.

Gel electrophoresis

The amplified samples were then run in on 2% agarose gel in the presence of DNA markers using gel electrophoresis and visualized on a UV trans illuminator to detect the presence of amplified product and to type the genotype. This step was done by gel electrophoresis machine (Wealtec Corp.) (E5W0255). The power supply was programed to give 130 volts and 100 milliamperes for 30 min. The 3 expected genotypes would appear as follows: CC 125 bp, TT 149 bp, CT 125 bp.

Determination of serum tyrosinase levels using ELISA technique

Determination of serum tyrosinase levels was done by using Human Tyrosinase ELISA kit (supplied by SunRed Company, China, cataloque No 201-12-7682) according to the manufacturer protocol. Sensitivity was 0.77 ng/ml and the assay range was 0.8–200 ng/ml.

The kit used a double-antibody sandwich ELISA to assay the levels of Human Tyrosinase in the samples. Tyrosinase was added to the monoclonal antibody enzyme well which is pre-coated with human tyrosinase monoclonal antibody then incubation was carried out. Tyrosinase antibodies labeled with biotin was added and combined with Strepavidin-HRP to form an immune complex then incubation and washing were carried out again to remove the uncombined enzyme. When Chromogen Solution A & B were added, the color of the liquid was changed into blue, and at the effect of acid, the color finally became yellow. The chroma of color and the concentration of the Human Substance Tyrosinase of the sample were positively correlated.

Statistical design

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS), version 20 (IBM, Chicago, USA). The collected data were presented in suitable tables. Quantitative data were summarized in the form of mean and standard deviation, while qualitative data in frequency and percentage form. One...
way analysis of variance (ANOVA) test, paired and independent sample t-tests were used for comparing means between groups. The Chi-square (X²) test was used for the association between variables, when it is not suitable, Mont-Carlo Exact test (MCET) was used instead. The significant p-value was adopted to be <.05.

Results

There was an insignificant difference between patients and control groups regarding age (p = .17) and gender (p = .91) (Table 1). Thirty-six patients (17%) had associated autoimmune disease/s; thyroiditis (44.5%), rheumatoid arthritis (33.3%) and alopecia areata (22.2%). Patients presenting with vitiligo vulgaris represented 85%, while acrofacial vitiligo represented 15% only of our sample. The mean age of vitiligo onset in the studied sample was 21.64 ± 14.5 years. The mean baseline VASI score was 9.28 ± 6.7.

Serum levels of tyrosinase were significantly elevated in patients when compared to the controls (p < .0001). Both patients and control groups were in Hardy Weinberg equilibrium regarding the MiRNA 196a2C/T (11614913) genotypes (p > .05). The genotypes distribution in patients and control groups varied significantly. CC genotype was the most prevalent genotype in the control subjects (62.5%), while TT genotype was the most prevalent one in the patients group (56.6%) (Table 1).

After phototherapy, the response to treatment was variable in our patients. Twelve patients (5.7%) were very much improved (56.6%), followed by patients (69.43 ± 3.9% reduction in VASI score), 16 patients (7.5%) were much improved (34.42 ± 3.9% reduction in VASI score), 65 patients (26.4%) were improved (18.89 ± 3.6% reduction in VASI score), while 80 patients (37.73%) were minimally improved (5.93 ± 2.3% reduction in VASI score). On the other hand, 16 patients (7.5%) showed minimal worsening (the mean percentage of VASI score deterioration was 5.7 ± 3.12%). No changes in VASI score following treatment was detected in 32 patients (15.1%). Serum levels of tyrosinase showed significant reduction after treatment (88.75 ± 2.8 versus 88.04 ± 3.1, p = .0137).

Regarding the relations between the studied variable, TT genotype of MiRNA 196a2C/T (11614913) was significantly associated with higher baseline serum tyrosinase levels, higher base-line VASI scores, lower improvement percentages and earlier age of disease onset (p < .0001) (Table 2).

The percentage of improvement showed a significant positive correlation with the patients’ ages and with the age of the disease onset. On the other hand, the improvement percentage showed a significant negative correlation with the disease duration, the baseline VASI scores and the serum tyrosinase levels (Table 3).

There was insignificant difference in the degree of response between males and females (15.3 ± 20.3, 12.76 ± 15.18, p = .45). The genotypes distribution in patients and control groups were in Hardy Weinberg equilibrium (p = .91). The genotypes distribution in patients and control groups varied significantly. CC genotype was the most prevalent genotype in the patients group (33.3%) and the TT genotype was the most prevalent one in the control subjects (62.5%), while TT genotype was the most prevalent one in the patients group (56.6%) (Table 1).

Table 1. Baseline comparison between patients and control groups.

<table>
<thead>
<tr>
<th>The variables</th>
<th>Patients n = 212</th>
<th>Control n = 96</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Mean ± SD</td>
<td>37.28 ± 15.37</td>
<td>35.75 ± 6.7</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>n (%)</td>
<td>72 (34%)</td>
<td>32 (33%)</td>
</tr>
<tr>
<td>Female</td>
<td>n (%)</td>
<td>140 (66%)</td>
<td>64 (66.7%)</td>
</tr>
<tr>
<td>Serum tyrosinase (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>Mean ± SD</td>
<td>88.75 ± 2.8</td>
<td>8.42 ± 1.2</td>
</tr>
<tr>
<td>miRNA 196a2 polymorphism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>n (%)</td>
<td>92 (43.4%)</td>
<td>60 (62.5%)</td>
</tr>
<tr>
<td>CT</td>
<td>n (%)</td>
<td>0 (0%)</td>
<td>4 (4.2%)</td>
</tr>
<tr>
<td>TT</td>
<td>n (%)</td>
<td>120 (56.6%)</td>
<td>32 (33.3%)</td>
</tr>
</tbody>
</table>

Table 2. Relation between MiRNA 196a-2 C/T(11614913) genotypes and the study variables.

<table>
<thead>
<tr>
<th>MiRNA 196a2C/T(11614913)</th>
<th>CC n = 92</th>
<th>TT n = 120</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of disease onset</td>
<td>30.95 ± 13.4</td>
<td>14.5 ± 10.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Baseline serum tyrosinase (ng/ml)</td>
<td>8.76 ± 2.4</td>
<td>90.27 ± 1.9</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Baseline VASI</td>
<td>8.18 ± 5.6</td>
<td>10.14 ± 7.3</td>
<td>.034</td>
</tr>
<tr>
<td>Improvement percentage</td>
<td>25.8 ± 19.2</td>
<td>4.28 ± 5.9</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Discussion

In the present study, the serum levels of tyrosinase enzyme were significantly elevated in patients when compared to the control group. This comes in agreement with Cui et al. (15) study. The relation of elevated tyrosinase levels and vitiligo development may be attributed to the fact that tyrosinase enzyme has a vital role in melanogenesis by catalyzing the con-version of tyrosine to DOPA then to DOPA quinone (16). These reactions can create radical species that exert a cytotoxic effect on melanocytes. This is one of the currently accepted theories explaining the melanocytes loss in vitiligo (17).

Human miR-196a-2 gene (miR196a-2) is located within the homeobox (HOX) gene clusters region on chromosome 12q13.13 and the C allele is the ancestral one (18). In accordance with Huang et al. (11) and Cui et al. (15), our results demonstrated the predominance of TT genotype in patients and the CC genotype in the control group.

The presence of the C allele can provide a protective effect on melanocytes by reducing the Tyrp1 and Tyr expression in normal melanocytes leading to a subsequent reduction in the generation of the toxic free species inside the melanocytes (15). Moreover, Huang et al. (11) detected an association between the T allele and increased melanocytes apoptosis. This may explain the significantly higher disease severity (VASI scores) and the significantly elevated serum tyrosinase levels in the patients carrying TT genotype in our sample. This also can explain why patients carrying miR-196a-2 gene (miR196a-2) TT genotype and (or) having higher baseline serum tyrosinase
levels showed a lower clinical response to phototherapy in our sample.

It was suggested that older patients show lower response rates to phototherapy either due to the lower compliance to treatment sessions (19), or to the appearance of white hairs which means the loss of the main melanin reservoir (20). However, in our study, we found a significant positive correlation between the degree of vitiligo improvement and the patients’ age. The better response rates in older patients in our study may be based on the coping ability of different ages which determines the degree of stress created. The emotional and mental stability of older people (21,22) may alleviate the stress created by the diseases. On the other hand, having a dis-figuring disorder during young age may be associated with dev-astating psychological consequences (23). The higher levels of stress can decrease the response of vitiligo to different treat-ment lines (24).

In the current study, there was a significant negative correlation between the disease duration and the percentage of improvement indicating a better response to treatment in patients with shorter disease duration. This comes in agreement with the previous results of other workers (25–28). Hallaji et al. (29) also found a significantly better response to phototherapy in vitiligo patients with shorter disease duration and advised to start treatment as early as possible. This could be explained by the poor compliance to treatment with longer disease duration due to loss of trust in the treatment (20). Patients with a chronic illness will discover that their illness can’t be cured completely, so they only try to accept it and live with it (30).

Conclusion
MiRNA 196a-2 C/T (11614913) gene polymorphism and elevated serum tyrosinase levels may be related to vitiligo development and may also affect the response of the patient to treatment.

Disclosure statement
No conflict of interest to declare.

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