Expression changes in the intracellular melanogenesis pathways and their possible role the pathogenesis of vitiligo

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KEYWORDS
Vitiligo; Pigmentation; Melanocytes; Biopsy

Summary

Background: Main pathway in human melanocytes through which signal from the melanocortin system reaches the melanogenesis enzymes is cAMP/PKA pathway and it is modulated by Wnt and MAPK pathways. In our previous study we established significant increase of melanocortin receptor expression in unaffected skin of vitiligo patients compared to healthy subjects.

Objective: The aim of this study was to assess the gene expression profile of the intracellular signalling pathways linking melanocortin system with enzymes involved in melanogenesis.

Methods: Using QRT-PCR method, mRNA expression levels of eight genes related to signal transduction from the melanocortin system to melanogenesis enzymes was measured in lesional and non-lesional skin of vitiligo patients and in the skin of healthy control subjects. Following genes were analyzed in the study: MITF, CREB1, p38, USF1, PIK3CB (PI3K), RPS6KB1, LEF1 and BCL2.

Results: The mRNA levels of MITF, LEF1, p38, PIK3CB and RPS6KB1 were decreased in lesional skin of vitiligo patients compared to skin of healthy control subjects. We also found increased expression of USF1 and BCL2 in non-lesional skin of vitiligo patients compared to skin of healthy control subjects. mRNA levels of MITF and BCL2 were decreased in lesional skin of vitiligo patients compared to non-lesional skin of vitiligo patients.

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1. Introduction

Vitiligo is an acquired cutaneous disorder characterized by declining melanocyte functions and depigmentation. To explain the dysfunction of melanocytes in epidermis during the disorder different hypotheses have been proposed [1—3]. These hypotheses propose autoimmune mechanisms, auto-cytotoxic mechanism or abnormality in melanocytes or in surrounding keratinocytes leading to the decreased function of melanocytes [4,5]. However, as none of the three major hypothesis is sufficient to fully explain the mechanisms of vitiligo, the convergence theory is proposed stating that stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment and impaired melanocyte migration and proliferation can all contribute to vitiligo etiopathogenesis in varying proportions [2].

In our previous study we showed significant alterations in the expression of genes of the melanocortin system in the skin of vitiligo patients [6]. We established reduced expression of melanocortin receptors and melanogenesis enzymes in lesional skin of vitiligo patients. Somewhat surprisingly we found that in non-lesional skin of the patients melanocortin receptors were significantly up-regulated. Increased expression of melanocortin receptors was accompanied by the up-regulation of the genes of enzymes involved in melanin synthesis [6,7]. As receptors and enzymes were altered to same direction, we concluded that this finding may possibly reflect the systemic compensatory changes to restore normal pigmentation in the lesions. To verify these findings further, we decided to analyze gene expression pattern in the intracellular signalling system linking melanocortin receptors with enzymes involved in melanin synthesis.

Different pathways modulate the melanogenesis in humans. Main pathway in human melanocytes through which signal from the melanocortin system reaches the melanogenesis enzymes tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1) and dopachrome tautomerase (DCT) is cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway and it is modulated by Wnt and MAPK (mitogen-activated protein kinase) pathways. In addition, inositol phosphate/protein kinase C (IP3/PKC) and nitric oxide/protein kinase G (NO/PKG) pathways are able to modulate the melanin synthesis [3,8].

As cAMP/ PKA is the most important pathway of pigmentation regulation, our study focused on this [9]. The melanocortin receptors activate adenylate cyclase (AC) leading to the rise of the intracellular cAMP concentration [10]. cAMP activates protein kinase A (PKA) [11]. PKA activates cAMP responsive element binding protein 1 (CREB1) through phosphorylation that raises the expression level of microphthalmia-associated transcription factor (MITF) [12]. MITF is a positive regulator of the expression levels of TYR, TYRP1 and DCT and increases the transcription of these enzymes [13—15]. MITF is the main gene responsible for the control of melanocyte differentiation; ectopic expression of MITF converts fibroblasts to cells with melanocyte characteristics [16]. In addition to other functions, MITF up-regulates the expression of the antiapoptotic factor B-cell lymphoma 2 (BCL2), the deletion of MITF in melanocytes results in an extensive apoptosis of these cells [17]. LEF1 (lymphoid enhancer-binding factor 1) is a transcription factor that participates in the Wnt signalling pathway [18]. In melanocytes, LEF1 acts as a pigmentation regulator, exerting its effects on MITF in two ways: firstly, LEF1 is an activator of the MITF gene transcription; secondly, MITF together with LEF1 can activate its own promoter [19]. The interaction of MITF and LEF1 also takes place in the regulation of the expression of DCT [20]. USF1 (upstream transcription factor 1) is a transcription factor that similarly with MITF belongs to the b-HLH-zip family [21]. In melanocytes, USF1 regulates pigmentation: when phosphorylated by 38 kDa MAP kinase (p38, ERK1/2), it binds to the promoter of TYR and activates its transcription [22]. As well, promoter of DCT contains the USF1 binding element [23]. Finally, cAMP has an inhibitory effect on the phosphoinositide 3-kinase/70 kDa ribosomal protein S6 kinase (PI3K/p70(S6)K) pathway [24].

Taken together, in order to evaluate potential changes in the intracellular signalling system, we analyzed the expression of the following genes: MITF, CREB1, p38 (ERK1/2), USF1, PIK3CB (PI3K), RPS6KB1, LEF1 and BCL2.

Conclusions: Present study indicates increased expression of the genes of the intracellular melanogenesis pathway in the non-lesional skin of vitiligo patients. This finding suggests activation of melanogenesis pathway in the non-lesional skin of vitiligo.

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2. Results

Using QRT-PCR method, mRNA expression levels of eight genes related to signal transduction from the melanocortin system to melanogenesis enzymes were measured in lesional and non-lesional skin of vitiligo patients and in skin of healthy control subjects. We analyzed patients according to their subgroups based on the stage of progression of the disorder and based on the extent of involvement of pigmentation, but we did not find any significant difference related to subgroups. Therefore we decided to analyze patients as one vitiligo group.

MITF mRNA expression in lesional skin of vitiligo patients was 3.3-fold lower when compared with non-lesional skin of vitiligo patients \( (p < 0.0001; \) Fig. 1A) and 2.4-fold lower when compared with skin of healthy control subjects \( (p = 0.0001; \) Fig. 1A). LEF1 expression level in non-lesional skin of vitiligo patients was 1.7-fold higher than in lesional skin of the patients \( (p < 0.0005; \) Fig. 1B). USF1 mRNA expression level in non-lesional skin of vitiligo patients was 1.6-fold higher when compared with skin of healthy control subjects \( (p < 0.01; \) Fig. 1C).

No statistically significant differences in CREB1 expression levels were found between lesional and non-lesional skin of vitiligo patients and between the skin of vitiligo patients and healthy control subjects \( (p > 0.005; \) Fig. 1D). However, there was a tendency towards higher CREB1 expression in non-lesional skin of vitiligo patients when compared with lesional skin of vitiligo patients or skin of healthy control subjects. p38 mRNA expression was 1.6-fold lower in lesional skin of vitiligo patients when compared with the skin of healthy control subjects \( (p < 0.005; \) Fig. 2A).

PI3KCB mRNA expression in lesional skin of vitiligo patients was 1.3-fold lower when compared with the skin of healthy control subjects \( (p = 0.01; \) Fig. 2B). RPS6KB1 mRNA expression in lesional skin of vitiligo patients was 1.3-fold lower when compared with the skin of healthy control subjects \( (p < 0.05; \) Fig. 2C). BCL2 mRNA expression level in non-lesional skin of vitiligo patients was 1.6-fold higher when compared with lesional skin of the patients \( (p < 0.05; \) Fig. 2D). BCL2 expression in non-lesional skin of vitiligo patients was 2.3-fold higher when compared with the skin of healthy control subjects \( (p < 0.05; \) Fig. 2D).

![Gene expression levels of the MITF, LEF1, USF1 and CREB1 (relative to housekeeping gene HPRT mRNA level) in the skin from healthy controls (HCS), non-lesional vitiligo skin (NLS) and lesional vitiligo skin (LS). Bars indicate mean ± S.E.M. *p < 0.05, **p < 0.01 and ***p < 0.001 (compared to HCS sample); ’p < 0.05, ’’p < 0.01 and ’’’p < 0.001 (compared to NLS sample).](image-url)
Possible interactions between the expressions of the studied genes were examined by Spearman rank correlation (Tables 1 and 2). In addition, the correlations between the expressions of studied genes and the genes encoding enzymes of melanogenesis (TYR, TYRP1 and DCT) were examined (Tables 1 and 2). In the present study statistically significant positive correlations were found between the expression levels of p38 and PI3KCB, and p38 and RPS6KB1 both in skin of vitiligo patients (respectively, \( r = 0.75, p < 0.001 \) and \( r = 0.75, p < 0.001 \)) and in skin of healthy control subjects (respectively, \( r = 0.92, p < 0.001 \) and \( r = 0.82, p < 0.001 \)). The expression levels of MITF, PI3KCB and p38 were positively correlated with the mRNA levels of TYR, TYRP1 and DCT in skin of healthy control subjects \( (p < 0.05) \) but not in skin of vitiligo patients.

![Fig. 2](image)

**Table 1** Results of the correlation analysis of the expression of studied genes in the skin biopsy of healthy controls

<table>
<thead>
<tr>
<th></th>
<th>TYR</th>
<th>TYRP1</th>
<th>DCT</th>
<th>MITF</th>
<th>LEF1</th>
<th>USF1</th>
<th>p38</th>
<th>CREB1</th>
<th>PIK3CB</th>
<th>RPS6KB1</th>
<th>BCL2</th>
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<tbody>
<tr>
<td>TYR</td>
<td>1.00</td>
<td>0.80**</td>
<td>0.73***</td>
<td>0.50*</td>
<td>-0.14</td>
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<td>0.57*</td>
<td>-0.71</td>
<td>0.52*</td>
<td>0.45</td>
<td>-0.50</td>
</tr>
<tr>
<td>TYRP1</td>
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<td>1.00</td>
<td>0.82***</td>
<td>0.69**</td>
<td>0.54</td>
<td>0.60</td>
<td>0.76***</td>
<td>-0.14</td>
<td>0.75***</td>
<td>0.68**</td>
<td>-0.50</td>
</tr>
<tr>
<td>DCT</td>
<td>0.73***</td>
<td>0.82***</td>
<td>1.00</td>
<td>0.58*</td>
<td>0.31</td>
<td>-0.40</td>
<td>0.68***</td>
<td>-0.29</td>
<td>0.58*</td>
<td>0.51</td>
<td>-0.50</td>
</tr>
<tr>
<td>MITF</td>
<td>0.50*</td>
<td>0.69**</td>
<td>0.58*</td>
<td>1.00</td>
<td>0.14</td>
<td>0.09</td>
<td>0.71***</td>
<td>0.07</td>
<td>0.66***</td>
<td>0.71***</td>
<td>0.03</td>
</tr>
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<td>0.14</td>
<td>1.00</td>
<td>0.35</td>
<td>0.41</td>
<td>0.55*</td>
<td>0.24</td>
<td>0.58*</td>
<td>0.38</td>
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<tr>
<td>USF1</td>
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<td>0.60</td>
<td>-0.40</td>
<td>0.09</td>
<td>0.35</td>
<td>1.00</td>
<td>0.34</td>
<td>0.51</td>
<td>0.22</td>
<td>0.12</td>
<td>0.36</td>
</tr>
<tr>
<td>p38</td>
<td>0.57*</td>
<td>0.76</td>
<td>0.68**</td>
<td>0.71***</td>
<td>0.41</td>
<td>0.34</td>
<td>1.00</td>
<td>0.33</td>
<td>0.92***</td>
<td>0.82***</td>
<td>0.45</td>
</tr>
<tr>
<td>CREB1</td>
<td>-0.71</td>
<td>-0.14</td>
<td>-0.29</td>
<td>0.07</td>
<td>0.55*</td>
<td>0.51</td>
<td>0.33</td>
<td>1.00</td>
<td>0.19</td>
<td>0.31</td>
<td>0.35</td>
</tr>
<tr>
<td>PIK3CB</td>
<td>0.52*</td>
<td>0.75***</td>
<td>0.58*</td>
<td>0.66***</td>
<td>0.24</td>
<td>0.22</td>
<td>0.92***</td>
<td>0.19</td>
<td>1.00</td>
<td>0.85***</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Spearman rank correlation coefficient (Spearman \( r \)) was used.
- * \( p < 0.05 \).
- ** \( p < 0.01 \).
- *** \( p < 0.001 \).
In vitiligo, melanocytes are destroyed and production of melanin is blocked, resulting in shaped white patches on the skin. In the present study we assessed the importance of genes of the signal transduction pathways between the melanocortin system and the melanogenesis enzymes in the pathogenesis of vitiligo. Out of the genes included in this study, all except CREB1 had statistically significant differences in expression levels between the skin of vitiligo patients and control subjects.

Lowered expression levels of MITF (p < 0.0001) and LEF1 (p < 0.0005) in lesional skin of vitiligo patients when compared with non-lesional skin of vitiligo patients were detected. In addition, MITF expression in lesional skin of vitiligo patients was lower when compared with skin of healthy control subjects (p = 0.0001). The difference in MITF expression between lesional and non-lesional skin of vitiligo patients has already been established before [25]. However, to our knowledge, no comparisons of MITF mRNA levels in the skin of vitiligo patients and in the skin of healthy control subjects have been made so far. In the cases of both MITF and LEF1 there were statistically non-significant tendencies towards higher expression in non-lesional skin of vitiligo patients when compared with skin of healthy control subjects. The tendencies in the expression profiles of MITF and LEF1 are similar with the ones of melanocortin receptors and melanogenesis enzymes established in our previous studies [6,7]. As there were statistically significant positive correlations between MITF and TYR (r = 0.50; p < 0.05), TYRP1 (r = 0.69; p < 0.01) and DCT (r = 0.58; p < 0.05) expression levels in skin of healthy control subjects but not in skin of vitiligo patients, it can be assumed that in the case of vitiligo, MITF’s function of raising the transcription levels of melanogenesis enzymes is impaired.

p38 (MAPK14) expression level was lower in lesional skin of patients when compared with skin of healthy control subjects (p < 0.005). p38 is an activator of CREB1 and USF1 proteins [26], therefore lowered p38 level in the skin of vitiligo patients may reduce the positive regulation of melanogenesis by CREB1 and USF1. The statistically significant positive correlations (p < 0.001) between the expression levels of p38 and PI3KCB and the levels of p38 and RPS6KB1 probably imply regulatory connections between the mRNA levels of these gene pairs. The occurrence of statistically significant positive correlations between p38 and TYR (r = 0.57; p < 0.05), TYRP1 (r = 0.76; p < 0.001) and DCT (r = 0.68; p < 0.01) mRNA levels in skin of healthy control subjects and the lack of these correlations in skin of vitiligo patients may impair the regulation of the positive regulation of melanogenesis by p38 in vitiligo.

USF1 expression in non-lesional skin of vitiligo patients was higher when compared with skin of healthy control subjects (p < 0.01). As USF1 regulates the transcription of MC1R, TYR, and DCT [22,23,27], it might be assumed that elevated MC1R expression and the tendencies towards higher TYR and DCT mRNA levels in non-lesional skin of vitiligo patients observed by authors in previous studies [6,7] could be the result of heightened USF1 expression. However, no statistically significant Spearman correlations were found between the USF1 mRNA levels and the expression levels of MC1R, TYR and DCT.

PIK3CB expression in lesional skin of vitiligo patients was lower when compared with skin of

### Table 2: Results of the correlation analysis of the expression of studied genes in the involved skin biopsy of vitiligo patients

<table>
<thead>
<tr>
<th></th>
<th>TYR</th>
<th>TYRP1</th>
<th>DCT</th>
<th>MITF</th>
<th>LEF1</th>
<th>USF1</th>
<th>p38</th>
<th>CREB1</th>
<th>PIK3CB</th>
<th>RPS6KB1</th>
<th>BCL2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TYR</strong></td>
<td>1.00</td>
<td>0.74**</td>
<td>0.69***</td>
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<td>0.03</td>
<td>-0.30</td>
<td>-0.30</td>
<td>-0.33</td>
<td>-0.02</td>
<td>-0.47**</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>TYRP1</strong></td>
<td>0.74***</td>
<td>1.00</td>
<td>0.81***</td>
<td>-0.14</td>
<td>0.27</td>
<td>-0.11</td>
<td>-0.16</td>
<td>-0.30</td>
<td>0.11</td>
<td>-0.32</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>DCT</strong></td>
<td>0.69***</td>
<td>0.81***</td>
<td>1.00</td>
<td>-0.12</td>
<td>0.13</td>
<td>-0.16</td>
<td>0.01</td>
<td>-0.32</td>
<td>0.34</td>
<td>-0.08</td>
<td>0.24</td>
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<tr>
<td><strong>MITF</strong></td>
<td>-0.37</td>
<td>-0.14</td>
<td>-0.12</td>
<td>1.00</td>
<td>0.35</td>
<td>0.30</td>
<td>0.46**</td>
<td>0.43*</td>
<td>0.35*</td>
<td>0.35*</td>
<td>0.46</td>
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<tr>
<td><strong>LEF1</strong></td>
<td>0.03</td>
<td>0.27</td>
<td>0.13</td>
<td>0.35</td>
<td>1.00</td>
<td>0.57**</td>
<td>0.12</td>
<td>0.53**</td>
<td>0.04</td>
<td>0.03</td>
<td>0.62**</td>
</tr>
<tr>
<td><strong>USF1</strong></td>
<td>-0.30</td>
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<td>-0.16</td>
<td>0.30</td>
<td>0.57**</td>
<td>1.00</td>
<td>0.12</td>
<td>0.26</td>
<td>-0.04</td>
<td>-0.06</td>
<td>0.32</td>
</tr>
<tr>
<td><strong>p38</strong></td>
<td>-0.30</td>
<td>-0.16</td>
<td>-0.01</td>
<td>0.46**</td>
<td>0.12</td>
<td>0.12</td>
<td>1.00</td>
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<td>0.63***</td>
<td>0.69***</td>
<td>0.19</td>
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<td><strong>CREB1</strong></td>
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<td>-0.32</td>
<td>0.43*</td>
<td>0.53**</td>
<td>0.26</td>
<td>0.22</td>
<td>1.00</td>
<td>0.13</td>
<td>0.36</td>
<td>0.58**</td>
</tr>
<tr>
<td><strong>PIK3CB</strong></td>
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<td>0.11</td>
<td>0.34</td>
<td>0.35*</td>
<td>0.04</td>
<td>-0.04</td>
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<td>0.12</td>
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<tr>
<td><strong>RPS6KB1</strong></td>
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<td>-0.32</td>
<td>-0.08</td>
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<td>0.03</td>
<td>-0.06</td>
<td>0.69***</td>
<td>0.36</td>
<td>0.55***</td>
<td>1.00</td>
<td>0.01</td>
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<tr>
<td><strong>BCL2</strong></td>
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<td>0.46</td>
<td>0.62**</td>
<td>0.32</td>
<td>0.19</td>
<td>0.58**</td>
<td>0.12</td>
<td>0.01</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Spearman rank correlation coefficient (Spearman r) was used.

- p < 0.05
- p < 0.01
- p < 0.001

### 3. Discussion

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PIK3CB expression in lesional skin of vitiligo patients was lower when compared with skin of
healthy control subjects ($p = 0.01$). As PI3K has an antiapoptotic effect [28], lowered PI3K level in the lesional skin of the patients may cause increased susceptibility of skin cells to apoptosis. In addition to that, decreased PI3K level probably increases oxidative stress in lesional skin of vitiligo patients, because inhibition of PI3K results in reduction of the intracellular glutathione concentration [29]. These results fit the data according to which in the skin of vitiligo patients, there are increased levels of apoptosis [30—32] and oxidative stress [33,34]. Similarly with MITF and p38, the expression levels of PI3KCB correlated with the mRNA levels of melanogenesis enzymes in skin of healthy control subjects ($TYR$: $r = 0.52$; $p < 0.05$; $TYRP1$: $r = 0.75$; $p < 0.001$; $DCT$: $r = 0.58$; $p < 0.05$) but not in skin of vitiligo patients. According to this, it can be assumed that in the skin of vitiligo patients, there may be dysfunctions in the pathways through which PI3K exerts positive effects on melanogenesis.

$RPS6KB1$ ($p70(S6)K$) expression was lowered in lesional skin of vitiligo patients when compared with skin of healthy control subjects ($p < 0.05$). As one of the main functions of $p70(S6)K$ is to inactivate the translation inhibiting factor PDCD4 (programmed cell death-4) in ribosomes [35], the decrease in $p70(S6)K$ expression may cause inhibition of protein synthesis, reduction of cell growth and slowing of cell cycle in the skin of vitiligo patients. This fits the data according to which melanocytes that are derived from the skin of vitiligo patients have growth defects in cell culture [36,37]. In lesional skin of vitiligo patients there was a negative correlation between the mRNA levels of $RPS6KB1$ and $TYR$ ($r = -0.47$; $p < 0.05$). This correlation matches the data according to which the inhibition of $p70(S6)K$ with rapamycin increases the expression of tyrosinase [24].

$BCL2$ expression was higher in non-lesional skin of vitiligo patients when compared with skin of healthy control subjects ($p < 0.05$) and lower in lesional skin of vitiligo patients when compared with non-lesional skin of the patients ($p < 0.05$). However, in a study using flow cytometry [38], no significant difference in $BCL2$ expression between melanocytes from non-lesional skin of vitiligo patients and melanocytes from skin of healthy control subjects was found. It could be that the difference we see in our study comes from keratinocytes, but this needs to be verified.

In conclusion, decreased expression of genes of the intracellular signalling pathways linking melanocortin system with melanogenesis enzymes in lesional skin is not surprising, because this fits the loss of functional melanocytes—major cell type expressing these genes in the skin. Interestingly, we have previously found significant up-regulation of genes of melanocortin system and melanogenesis enzymes in the non-lesional skin of vitiligo patients. Present study indicates that also genes for intracellular pathways are up-regulated in non-lesional skin of vitiligo patients. Therefore, we propose that signalling pathways related to the induction of pigmentation are activated in patients’ skins. However, these issues need to be further addressed by histochemical methods which would allow to localize expression of studied genes to distinct cell types in the skin and to study the function of these genes on protein level. Till now we cannot disregard the influence of keratinocytes or fibroblasts to our finding.

4. Materials and methods

The Ethical Review Committee on Human Research of the University of Tartu approved the study protocols and informed consent forms. All participants signed a written informed consent.

The patients and control subjects in the study were Caucasians living in Estonia. Unrelated patients with vitiligo ($n = 39$; 26 female; 13 male; age range 22—77 years) from the Department of Dermatology, the University of Tartu, were included in the study. The mean age of vitiligo onset of the patients was 30.4 years and the mean duration of vitiligo was 19.0 years. Eight patients had a family history of vitiligo. None of patients included in the study had received specific therapy in the previous 6 months. The clinical signs on which the diagnosis of vitiligo was based on were: characteristic loss of skin pigmentation with typical localization and white colour on the skin lesions under Woods lamp. The type of vitiligo was based on the extent of involvement and the distribution of pigmentation. Focal vitiligo involves depigmentation in a localized, dermal and asymmetric distribution ($n = 7$; 5 (F); 2 (M)). Segmental vitiligo encompasses depigmentation of the dermal, asymmetric distribution ($n = 1$ (F)). Generalized vitiligo is characterized by bilateral, symmetric loss of pigmentation of the torso, face, neck, or extensor surfaces of the hands and legs ($n = 30$; 20 (F); 10 (M)). Universal vitiligo occurs as depigmentation of the entire body surface area ($n = 1$ (M)). The stage of vitiligo was based on the interval of manifestation of new areas of depigmentation or enlargement of the area of depigmentation. The patients were divided into two subgroups based on the stage of progression of the disorder: patients with progressive vitiligo (active vitiligo, in which new areas of depigmentation or enlargement of depigmentation were observed during the
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previous 3 months; \( n = 26; 18 \text{ (F); 8 (M)} \) and patients with stable vitiligo (inactive vitiligo, in which no new depigmentation or enlargement of depigmentation had been observed for more than 3 months; \( n = 13; 8 \text{ (F); 5 (M)} \)).

The control group consisted of healthy volunteers \( (n = 31; 22 \text{ female; 9 male; age range 22—67 years}) \) free from the positive family history of vitiligo and other chronic dermatoses. Control subjects were recruited from health care personnel, medical students and patients present at the dermatological outpatient clinic with either facial telangiectasia or skin tags.

Two skin biopsies (Ø 4 mm) were obtained from each patient with vitiligo: one from the central part of involved skin and another from non sun-exposed uninvolved skin. One skin biopsy (Ø 4 mm) from non sun-exposed skin was taken from healthy control subjects. The non sun-exposed skin was defined as the skin never exposed to UVR previously and definitely not exposed to natural UVR in the last 12 months. Biopsies from uninvolved skin of vitiligo patients and healthy controls were taken from the lower abdomen. All probands had skin phenotype II or III, Fitzpatrick classification. Biopsies were instantaneously snap-frozen in liquid nitrogen and stored at \(-80 ^\circ \text{C}\) until further use.

Total RNA was isolated from tissues using RNeasy Fibrous Tissue Mini Kit (QIAGEN Sciences, Maryland, USA) described in our previous study [6]. Gene expression levels were detected in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reactions were carried out in 10 µl reaction volumes in four replicates. The expression levels of genes under the study were detected applying TaqMan-QRT-PCR method using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). For the detection of the expression assay mixes (20 x, Applied Biosystems, Foster City, CA, USA). For the detection of the expression levels of studied genes we used TaqMan Assay-On-Demand FAM-labelled MGB-probe gene expression assay mixes (20 x, Applied Biosystems, Foster City, CA, USA). The assay mixes used were Hs00165156_m1 (MITF), Hs00178872_m1 (PIK3CB), Hs00177357_m1 (RP56KB1), Hs00176247_m1 (p38), Hs00608023_m1 (BCL2), Hs00122390_m1 (LEF1), Hs00608023_m1 (USF1) and Hs00231713_m1 (CREB1).

For quantification of mRNA we used comparative Ct method (ΔCt value), where the amount of target transcript was normalized according to the level of endogenous reference HPRT-1 (hypoxanthine phosphoribosyl-transferase-1). Adjustment to normal distribution was tested by the Kolmogorov—Smirnov test. The distribution of measurements of gene expressions by the applied method did not follow a Gaussian distribution. Mann—Whitney \( U\)-test and Kruskal—Wallis test were used to test for differences between the groups using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA). Correlation analysis was used to investigate relations between two parameters of one group. For measure of correlation the Spearman rank correlation was applied. For all tests the two-tailed \( p\)-value <0.05 was considered significant.

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References


[31] Lee AY, Youm YH, Kim NH, Yang H, Choi WI. Keratinocytes in the depigmented epidermis of vitiligo are more vulnerable to trauma (suction) than keratinocytes in the normally pigmented epidermis resulting in their apoptosis. Br J Dermatol 2004;151:995—1003.


