Effect of Dickkopf1 on the senescence of melanocytes: in vitro study

Seema Rani1 · Rakhee Chauhan1 · Davinder Parsad2 · Ravinder Kumar1

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Abstract
Fibroblasts secrete several growth factors which are important for the regulation of skin pigmentation. Dickkopf1 (DKK1) is also secreted by fibroblasts which inhibit the growth and function of melanocytes. Therefore, the study was designed to check the role of DKK1 in vitiligo pathogenesis. This study confirmed the higher expression of DKK1 in lesional skin of vitiligo patients. In vitro effect of DKK1 on cultured melanocytes revealed decrease in the melanocytes proliferation and pigmentation. In vitro effect of DKK1 was then checked on the melanocytes senescence and found that DKK1 induced senescence in the treated melanocytes. Expression of senescence markers was significantly higher in DKK1 treated melanocytes. This study suggests that higher expression of DKK1 in the dermis induced senescence in melanocytes that may lead to hypopigmentation and play role in vitiligo pathogenesis.

Keywords Vitiligo · Dermis · DKK1 · Melanocytes

Introduction
Skin pigmentation is regulated by melanin pigment secreted by the melanocytes and a defect in the pigmentation mechanism or death of melanocytes leads to the hypopigmentary disorder known as vitiligo [26]. Vitiligo is an acquired skin pigmentation disorder characterized as depigmented macules, whose pathogenesis is still unclear [14]. The melanocytes present in the epidermis are affected by external factors such as ultraviolet radiation and by the internal factors, which are secreted by keratinocytes in the epidermis and the fibroblasts present in the dermis [21, 26]. Dermal fibroblasts exert a regulatory role on the skin pigmentation by secreting various soluble growth factors such as hepatocyte growth factor (HGF), stem cell factor (SCF) and basic fibroblasts growth factor (bFGF) [3, 4, 13]. Regarding skin pigmentation, Dickkopf1 (DKK1) secreted by dermal fibroblasts in the palms and soles is thought to be responsible for the lighter color of these areas via its inhibitory effect on the growth of melanocytes and transfer of melanosomes [22, 24]. DKK1 is an inhibitor of Wnt signaling pathway and inhibits the growth of normal human melanocytes by reducing the Wnt/β-catenin signaling pathway [5, 23]. DKK1 treatment also reduced the uptake of melanin pigment by the keratinocytes, inducing a less pigmented skin phenotype [18]. Therefore, this study has been designed to check the role of DKK1 in vitiligo patients and assays for any effect on the culture of melanocytes.

Methods
Clinical evaluation of patient’s data
For this study nine lesional punch biopsies (eight male + one female; age, 30.11 ± 10.22 years) and seven non lesional punch biopsies (four male + three female; age, 21.28 ± 3.35 years) were collected from the vitiligo patients with their informed consent. Seven healthy controls (five male + two female; age 34.42 ± 10.11 years) were enrolled for this study. The duration of disease was 7.77 ± 4.96 years for lesional punch biopsies and 8.14 ± 5.55 years for non lesional punch biopsies. No family history was reported in case of lesional punch whereas one patient bears family history for non lesional punch. The study was approved by institutional ethics committee.
Gene expression studies by qRT-PCR

RNA isolation

Epidermis was separated from the dermis of punch biopsies with surgical blade. Total cellular RNA was then extracted from lesional dermis, non-lesional dermis of vitiligo patients and healthy control by TRI Reagent (Sigma Aldrich, USA) using manufacturer’s guidelines.

cDNA synthesis

Synthesis of cDNA was performed using 2 µg of total cellular RNA using random hexamer primers and cDNA synthesis kit (Thermo Fisher scientific, USA).

Primers

Primers for DKK1, p16, p21 and hp1 were obtained from Eurofins Genomics (India) and primers for β-actin, Tyrosinase (TYR), Microphthalmia-associated transcription factor (MITF), Dopachrome tautomerase (DCT) and c-KIT were obtained from sigma Aldrich, USA (Table 1).

qRT-PCR

SYBR Green master mix was used for Real-Time PCR System (LightCycler 480, Roche Molecular Diagnostics, Inc., USA). Expression of mRNA was calculated by Pfaffl analysis method and amplification of β-actin was used for the normalization of genes.

Immunohistochemistry

Skin sections from lesional skin, non lesional skin of vitiligo patients and controls were used for immunofluorescence study following the protocol described by Rani et al. [16]. Primary antibody for DKK1 was obtained from R&D system, USA. FITC labeled secondary antibody was obtained from Santa Cruz Biotechnology, Inc., USA and DAPI was obtained from Sigma Aldrich, USA. The expression was checked by confocal fluorescent microscope and measured by IMAGE J software.

Melanocyte treatment with DKK1

Melanocyte culture was established by following the procedure of Kumar et al. [8]. Melanocytes after second passage were used for the experiment. Melanocytes (5 × 10^3/well) were seeded in 96 well plates and treated with two concentrations of DKK1 (50 and 100 ng/ml) (Bio-vision, USA) for different time intervals (24, 48, 72 h). After treatment cell proliferation assay, melanin content assay, tyrosinase assay and mushroom tyrosinase activity assay were performed in triplicates of three experiments and expression of MITF, TYR, DCT, c-KIT was checked by qRT-PCR.

Proliferation assay

Proliferation of cells was checked by MTT assay. Cells with MTT solution (0.5 mg/ml in PBS) were incubated at 37 °C for 4 h. After incubation DMSO was added and absorbance was measured at 560 nm in ELISA plate reader (Tecan infinite M200 Pro).

Melanin content assay

Melanin content assay was performed by following the procedure of Lei et al. [9]. Treated cells were lysed with 1N chilled NaOH and homogenized by pipetting. Cell

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
<th>Annealing Tm (℃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5′-CATGTACGTGTTGCTATCCAGGC-3′</td>
<td>5′-CTCTTTAATGTCAGCGCACGAT-3′</td>
<td>254</td>
<td>58</td>
</tr>
<tr>
<td>DKK1</td>
<td>5′-AGGTTTCTGTGTGCTCCGCT-3′</td>
<td>5′-GAATTACGCCAGACAGCCT-3′</td>
<td>188</td>
<td>58</td>
</tr>
<tr>
<td>MITF</td>
<td>5′-TGAGCTTGGCCATGTCGAAAAC-3′</td>
<td>5′-ACGCTCTGAATGCTGAGTTC-3′</td>
<td>122</td>
<td>58</td>
</tr>
<tr>
<td>TYR</td>
<td>5′-GACTTTTCTTTTGTGGCGTG-3′</td>
<td>5′-GAATAATGCTCGGGCTGAT-3′</td>
<td>131</td>
<td>58</td>
</tr>
<tr>
<td>DCT</td>
<td>5′-GCAATTGTTACCTGGCCACC-3′</td>
<td>5′-ATCACACCTGCTCTCCCAG-3′</td>
<td>124</td>
<td>58</td>
</tr>
<tr>
<td>c-KIT</td>
<td>5′-TCTTGCTGCTCCAGAAATTTT-3′</td>
<td>5′-TCAGAGGATCGCCAGTCT-3′</td>
<td>156</td>
<td>58</td>
</tr>
<tr>
<td>p16</td>
<td>5′-CTCTCTGGACAGCAGCTTGTG-3′</td>
<td>5′-ATGTTACTGGCGCTCTGTC-3′</td>
<td>160</td>
<td>58</td>
</tr>
<tr>
<td>p21</td>
<td>5′-GGGACTGGATGCGGCTAATG-3′</td>
<td>5′-GAAGTACAGGCTGCGGAGCAGG-3′</td>
<td>141</td>
<td>58</td>
</tr>
<tr>
<td>hp1</td>
<td>5′-TGGGCAACGATTCTGTC-3′</td>
<td>5′-TGATGCGCATGTCATCTCCT-3′</td>
<td>148</td>
<td>58</td>
</tr>
</tbody>
</table>
homogenate was kept at room temperature for 30 min. Absorbance was measured at 405 nm in ELISA plate reader.

**Tyrosinase assay**

Treated cells were lysed with ice-cold PBS (0.1M pH-7.4) containing 0.1% triton at room temperature for 30 min and L-DOPA (10 mM) was added to each well and incubated at 37 °C for 2–3 h. Absorbance was measured at 475 nm.

**Mushroom tyrosinase activity assay**

Mushroom tyrosinase activity assay was done by following the procedure of Lim et al. [10]. DKK1 (50 ng/ml and 100 ng/ml), L-dopa (8.3 mM) and mushroom tyrosinase (120U) were mixed and incubated at 37 °C for 30 min. The inhibitory activity was measured at 490 nm using ELISA plate reader.

**β-Galactosidase staining**

The staining of β-galactosidase was performed in melanocytes treated with DKK1 using β-galactosidase staining kit and following manufacturer’s guidelines. The formation of blue colour in cells was observed under the light microscope (Radical: RTC-7). For quantitative measurement of β-galactosidase activity, treated cells were lysed with lysis buffer and cells lysate was incubated with X-gal for overnight at 37 °C. The absorbance was measured at 405 nm using ELISA plate reader.

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**Fig. 1** Relative mRNA expression and protein expression of DKK1 in control dermis, non lesional dermis and lesional dermis of vitiligo patients. a qRT-PCR of DKK1 in the control dermis, non lesional dermis and lesional dermis of vitiligo patients. b Measurement of protein expression of DKK1 in control, non lesional and lesional dermis of vitiligo patients by IMAGE J software (*p < 0.05: a = comparison with control, b = comparison between vitiligo patients). c Immunohistochemistry of DKK1 in control, non lesional and lesional skin of vitiligo patients. (i) Control skin, (ii) non lesional skin of vitiligo patients, (iii) lesional skin of vitiligo patients (magnification ×400) (scale bar 20 µm)
Statistical analysis

A statistical comparison was done using one way ANOVA by SPSS software. All data were expressed as mean ± SD. *p value < 0.05 was considered as significant.

Results

Expression of DKK1 in vitiligo patients and control

The expression of DKK1 was checked in the vitiligo patient’s lesional dermis, non lesional dermis and healthy control dermis. The result found that the relative mRNA expression of DKK1 in the lesional dermis was significantly increased (1.77 ± 0.2) as compared to the non lesional dermis of vitiligo patients and healthy control dermis (Fig. 1a). There was no significant difference observed in DKK1 expression between non lesional dermis and healthy control dermis. Protein expression of DKK1 was also found significantly increased in lesional dermis of vitiligo patients as compared to the non lesional dermis and healthy control dermis (Fig. 1b, c).

Effect of DKK1 on melanocytes proliferation, melanin content, tyrosinase content and mushroom tyrosinase activity assay

Melanocytes were treated with two concentrations of DKK1 (50 and 100 ng/ml) for 24, 48 and 72 h. We observed decrease in the proliferation of melanocytes (Figs. 2, 3a), Melanin content (Fig. 3b) and tyrosinase content (Fig. 3c) with both concentrations of DKK1 as compared to non treated cells. The mushroom tyrosinase activity assay also confirmed that DKK1 inhibited the activity of tyrosinase

Fig. 2 Representative pictures showing proliferation of melanocytes in control and melanocytes treated with 50 ng/ml DKK1. **a** Control 24 h, **b** treatment 24 h, **c** control 48 h, **d** treatment 48 h, **e** control 72 h, **f** treatment 72 h (magnification ×200)
Effect of DKK1 on melanocytes specific genes (MITF, TYR, DCT, c-KIT)

Melanocytes specific gene expression studies were performed with 50 ng/ml of DKK1 and results indicated significant decreased relative expression of MITF, TYR, DCT and c-KIT as compared to the melanocytes without DKK1 treatment (Fig. 3e).

Fig. 3 Bar diagram showing proliferation, melanin content and tyrosinase activity in control and DKK1 (50 and 100 ng/ml) treated melanocytes. a Proliferation of melanocytes, b melanin content of melanocytes, c tyrosinase activity of melanocytes, d Mushroom tyrosinase activity assay to check the effect of DKK1 on tyrosinase enzyme with both concentrations as compared to the control (Fig. 3d).

Effect of DKK1 on the melanocytes senescence

β-Galactosidase staining

β-Galactosidase staining was performed with 50 ng/ml DKK1 concentration to check the senescence and we observed high frequency of blue pigment positive cells in DKK1 treated cells as compared to the non treated melanocytes. The number of senescence cells was increased after every interval i.e. 24, 48 and 72 h (*p<0.05: a=comparison with control, b=comparison between treated groups).
confirmed higher expression of senescence in DKK1 treated cells as compared to control (Fig. 4j).

**mRNA expression of senescence markers p16, p21, hp1**

Further the senescence was confirmed at gene expression level and total RNA was isolated from melanocytes treated with DKK1 (50 ng/ml). The relative mRNA expression of p16 and hp1 was found to be significantly increased after treatment as compared to the non treated cells. Whereas, p21 expression was found to be insignificantly increased after treatment with DKK1 (Fig. 4k).
Discussion

Fibroblasts secreted factor, DKK1 inhibits the melanocyte proliferation and pigment production [2]. DKK1 is secreted by dermal fibroblasts in the palms and soles and is reported to be responsible for the lighter color of these body sites [22, 24]. Survey of literature showed the inhibition of Wnt signaling pathway by DKK1 dramatically inhibits the melanogenic pathway in melanocytes. It also inhibits the growth of normal human melanocytes by suppressing the expression level of melanocytes specific markers including DCT, MART1, TYR, GP100/Pmel17 and MITF. This effect of DKK1 in the melanocytes is mediated by the Wnt/β-catenin/MITF signaling pathway as DKK1 down-regulated the expression of β-catenin and glycogen synthase kinase (GSK) [17, 19, 23, 25]. DKK1 also acts on keratinocytes and decrease the expression of proteinase-activated receptor-2 (PAR-2), thus inhibiting the process of melanin transfers [15, 23]. Transgenic mice with Keratin 14-DKK1 lack trunk pigmentation because melanocytes were not present in the inner-follicular epidermis [1, 6, 25]. Our results also confirmed higher expression of DKK1 in lesional dermis of vitiligo patients as compared to the control. DKK1 treatment showed decrease in melanocyte proliferation and pigmentation. In DKK1 treated melanocytes, the relative mRNA expression of MITF, TYR, DCT, and c-KIT was found to be significantly decreased. Bastonini et al. [2] and Oh et al. [12] also showed the increased expression level of DKK1 in lesional dermis of vitiligo patients.

The key finding of this study is that DKK1 induced senescence in the melanocytes. DKK1 treated melanocytes showed senescent cells such as morphology and expression of senescence marker was found to be increased after the DKK1 treatment. Senescence has also been reported in the lesional fibroblasts of vitiligo patients in our previous study [16]. Induction of DKK1 has been strongly associated with tissue cellular injury, inflammation and cellular stress [7, 20]. High extracellular level of DKK1 is reported to inhibit epithelial cell growth in vitro by inducing cell cycle arrest via cellular senescence in esophagitis patients. DKK1 expression is directly correlated with p16 expression and SA-β-Gal activity [11].

This study showed a definite correlation between higher DKK1 expression and melanocytes senescence and its possible role in melanocyte loss in vitiligo lesions. Further studies with higher number of vitiligo patients are required to check the role of DKK1 in the repigmentation process and vitiligo pathogenesis.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interests to declare.

Ethical approval Skin punch biopsy was collected after informed consent of the patients from Pigmentary Clinic of the Department of Dermatology, Postgraduate Institute of Medical Education and Research, Chandigarh. The study was approved by ethical committee of the institute.

References
