MiR-217 is down-regulated in psoriasis and promotes keratinocyte differentiation via targeting GRHL2

Haigang Zhu, Liyue Hou, Jingjing Liu, Zhiming Li*
Department of Dermatology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, 325000, China

ABSTRACT

MiR-217 is a well-known tumor suppressor, and its down-regulation has been shown in a wide range of solid and leukemic cancers. However, the biological role of miR-217 in psoriasis pathogenesis, especially in keratinocyte hyperproliferation and differentiation, is not clearly understood. In this study, we found the expression of miR-217 was markedly down-regulated in psoriasis keratinocytes of psoriatic patients. In addition, overexpression of miR-217 inhibited the proliferation and promoted the differentiation of primary human keratinocytes. On the contrary, inhibition of endogenous miR-217 increased cell proliferation and delayed differentiation. Furthermore, Grainyhead-like 2 (GRHL2) was identified as a direct target of miR-217 by luciferase reporter assay. The expression of miR-217 and GRHL2 was inversely correlated in both transfected keratinocytes and in psoriasis lesional skin. Moreover, knocking down GRHL2 expression by siRNA enhanced keratinocyte differentiation. Taken together, our results demonstrate a role for miR-217 in the regulation of keratinocyte differentiation, partially through the regulation of GRHL2.

1. Introduction

Psoriasis, which affects 2–3% of the world’s population, is a long-lasting autoimmune disease characterized by patches of abnormal skin [1]. Psoriasis is generally thought to be a genetic disease which is triggered by environmental factors [1]. Psoriasis skin lesions are characterized by hyperproliferation and aberrant differentiation of keratinocytes and infiltration of inflammatory cells, such as lymphocytes, macrophages into the dermis and epidermis [2]. However, the underlying mechanisms regulating these epidermal defects and immunological dysfunction remain largely unknown.

MicroRNAs (miRNAs) are a class of 18–22 nt long single-stranded noncoding RNAs that regulate target genes expression at the post-transcriptional level through imperfect with the 3’ untranslated region (3’UTRs) of specific target miRNAs [3]. Increasing evidence has shown that miRNAs regulate almost 60% of protein-coding genes in humans and have been shown to participate in the regulation of almost every cellular process, including keratinocyte proliferation and differentiation [4,5]. Previous studies have identified a distinct miRNA expression profile in psoriasis skin compared with healthy skin [6,7]. Several of these deregulated miRNAs have been shown to regulate keratinocyte proliferation and differentiation, such as miR-125b [8], miR-31 [9,10], miR-205 [11], miR-330-3p [12] and miR-378b [13]. Although a large number of miRNAs have been identified, their roles in the biology of psoriasis and the underlying mechanisms have not been fully explored.

MiR-217 is a well-known tumor suppressor, and its down-regulation has been shown in a wide range of solid and leukemic cancers, including pancreatic cancer [14], hepatocellular carcinoma [15], renal cell carcinoma [16], gastric cancer [17], osteosarcoma [18] and chronic myelogenous leukemia [19]. It is also becoming increasingly recognized that miR-217 plays an important role in alcoholic hepatitis [20], acute pancreatic injury [21] and metabolic disorders [22]. Recently, it was found that miR-217 promotes endothelial senescence and decreases angiogenesis through targeting silent information regulator 1 [22]. However, the biological role of miR-217 in psoriasis pathogenesis, especially in keratinocyte hyperproliferation and differentiation, is not clearly understood.

In this study, we identify a function for miR-217 in the context of psoriasis. We found the expression of miR-217 was markedly
down-regulated in psoriasis keratinocytes of psoriatic patients. Overexpression of miR-217 in primary human keratinocytes inhibited the proliferation and promoted differentiation. On the contrary, inhibition of endogenous miR-217 increased cell proliferation and delayed differentiation. Grainyhead-like 2 (GRHL2) was identified as a direct target of miR-217 by luciferase reporter assay. The expression of miR-217 and GRHL2 was inversely correlated in both transfected keratinocytes and in psoriatic skin. Knocking down GRHL2 expression by siRNA enhanced keratinocyte differentiation. Taken together, our results demonstrate a role for miR-217 in the regulation of keratinocyte differentiation, partially through the regulation of GRHL2.

2. Materials and methods

2.1. Patients and specimen collection

Patients with moderate or severe chronic plaque psoriasis were recruited from Department of Dermatology, The First Affiliated Hospital of Wenzhou Medical University. All patients were categorized according to disease severity using the psoriasis area severity index (PASI). Punch biopsies (6 mm) were taken from non-lesional (n = 30) or lesional (n = 38) skin of psoriasis patients, and from noninflamed, nonirritated skin of healthy individuals (n = 34). The psoriasis patients enrolled in the study had not used any local therapy for 2 weeks and had not received any systemic treatment for at least one month before the skin biopsy. This study was approved by the Ethics Committee of our institute. Informed consent was signed by the participants.

2.2. RNA extraction and quantitative real-time PCR

Skin biopsies were homogenized in liquid nitrogen and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. RNA concentration was assessed by measuring absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA). For the detection of GRHL2 mRNA, first strand complementary DNA was synthesized from 1 μg of total RNA in the presence of oligo-dT (12–18) primer and M-MLV reverse transcriptase according to the manufacturer’s instructions (Life Technologies Inc., Gaithersburg, MD, USA). Human GAPDH RNA was amplified in parallel as an internal control. For the detection of miR-217, 1 μg of total RNA was used in the reverse transcriptase reaction with stem-loop RT primer for miR-217. Human U6 small nuclear RNA was amplified as the internal control. Gene-specific amplification was performed with SYBR Green PCR Master Mix using Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). All primer sequences used for GRHL2 mRNA and miR-217 detection are listed in Supplementary Table 1. Relative gene expression was quantified using the comparative delta CT (2^ΔΔCt) method.

2.3. In situ hybridization

Skin biopsy specimens treated with proteinase K (2 μg/ml) at 37 °C for 10 min, washed with diethylpyrocarbonate-treated PBS and subsequently fixed with 4% paraformaldehyde. After prehybridization, hybridization was performed at 51 °C overnight with digoxigenin-labeled human mature miR-217 probes (Exiqon, Vedbaek, Denmark). Slides were then washed at 51 °C and incubated with alkaline phosphatase-conjugated digoxigenin antibody for 1 h at room temperature. Sections were visualized by adding BM purple AP substrate (Roche) according to the manufacturer’s instructions. The slides were then independently scored by at least two pathologists as negative, weak positive or strongly positive.

2.4. Cells cultures and transfections

Human neonatal epidermal keratinocytes (Cascade Biologics, Portland, OR) were cultured in Epilife™ serum-free keratinocyte growth medium with Human Keratinocyte Growth Supplement at a final Ca²⁺ concentration of 0.06 mM (Cascade Biologics) at 37 °C in a humidified incubator containing 5% CO2.

The miR-217 mimics, miR-217 inhibitor and negative control (NC) were designed and synthesized by GenePharma (Gene-Pharma, Shanghai, China). The oligonucleotide sequences of used for transfection are listed in Supplementary Table 2. For transfection, keratinocytes at a density of 30–40% were transfected with miR-217 mimics, miR-217 inhibitor or negative control (NC) using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The mixture was added to cells at a final concentration of 10–30 nM. After incubation in an environment with 5% CO2 at 37 °C for 4–6 h, the serum-free medium was removed, and cells were maintained in growth medium.

2.5. Cell proliferation assay

Cell proliferation was evaluated by flow cytometry using a CellTrace™ carboxyfluorescein diacetate, succinimidyl ester (CFSE) Cell Proliferation kit (Invitrogen, Carlshad, CA, USA) according to the manufacturer’s protocol. Briefly, 24 h after transfection, cells were harvested, washed, and labeled with a 5 μM CellTrace™ CFSE in PBS with 2% FCS for 10 min at 37 °C. The labeling reaction was quenched by addition of cold RPMI 1640 medium with 10% FCS, and cells were washed twice with PBS with 2% FCS to remove excess CFSE. The proliferation of keratinocytes was evaluated 72 h later with CFSE dilution by flow cytometry (BD FACS Calibur, BD Biosci,ence, San Diego, CA, USA). The proliferation index was calculated using FlowJo software (FlowJo, Ashland, OR, USA). The experiment was independently performed for three times.

2.6. Western blotting

The cells were lysed in RIPA buffer with protease inhibitor at indicated time, protein concentrations were determined by BCA protein assay kit (Beyotime, Shanghai, China). Total protein was separated by 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride membranes. Primary antibodies used are cytokeratin 1 (E12), cytokeratin 10 (RKSE60), Filaggrin (AE21), Loricrin (C-13), GRHL2 (A-15), and β-actin (C-11) (All from Santa Cruz Biotechnology, CA, USA). The protein levels were visualized by ECL system (Millipore, Billerica, WI, USA) using horseradish peroxidase (HPR)-conjugated antibodies (Beyotime, Shanghai, China).

2.7. Immunohistochemistry

Frozen skin biopsy specimens were fixed with 10% neutral formalin and embedded in paraffin, and 4-μm-thick sections were prepared. The expression of cytokeratin 10 was analyzed using mouse anti-human cytokeratin 10 antibody (RKSE60, Santa Cruz Biotechnology, CA, USA). As a negative control, sections were incubated with normal mouse IgG. After being incubated with the primary antibodies, the sections were then incubated with horse-radish peroxidase (HRP)-labeled anti-mouse IgG at 37 °C for 30 min, followed by visualization with 3, 3-diaminobenzidine (DAB). Desired color reaction was observed when monitored with the microscope.

2.8. 3’UTR luciferase reporter assay

The 3’-UTR of GRHL2 containing the putative miR-217 binding
performed in situ hybridization using miR-217-specific locked nucleic acid-modified probes on skin sections from healthy individuals (n = 14), non-lesional (n = 10), and lesional skin (n = 12) from psoriasis patients. As shown in Figs. 1B and S1A, miR-217 was mainly detected in the basal cell layer of the epidermis, and was high expressed in both healthy skin and psoriasis non-lesional skin. However, its expression was decreased in all epidermal layers in psoriasis lesions. We further separated epidermis from dermis by dispase treatment and measured the expression of miR-217 by qRT-PCR, the data showed that miR-217 expression was significantly decreased in epidermis from psoriasis lesional skin compared with healthy epidermis and psoriasis non-lesional skin (Fig. 1C). Taken together, these results demonstrate that miR-217 is down-regulated in keratinocytes in psoriasis skin lesions.

3.2. MiR-217 inhibits the proliferation and promotes differentiation of keratinocytes

We then investigated the biological role of miR-217 in keratinocytes. We first transfected human primary keratinocytes with miR-217 mimics (to overexpress miR-217) or miR-217 inhibitor (to inhibit endogenous miR-217) and analyzed the cell proliferation by CFSE dilution assay using flow cytometry. Transfection efficiency was evaluated by real-time PCR at day 1, 2, 3, 4, and 7 after transfection with miR-217 mimic or inhibitor as well as their respective controls (Fig. S2A and B). As shown in Fig. 2A and B, the percentage of cells that underwent cell division was reduced in the cells overexpressing miR-217 compared with the cells overexpressing control miRNA. In contrast, inhibition of the endogenous miR-217 increased the cell proliferation compared with the cells transfected with scrambled miRNA inhibitor (Fig. 2C and D). These results suggest that miR-217 might affect the proliferation of keratinocytes.

Psoriasis is usually characterized by hyperproliferation and abnormal differentiation of keratinocytes [1]. To determine whether decreased expression of miR-217 in psoriasis keratinocyte affects its differentiation, we analyzed the differentiation status of keratinocytes at day 1, 2, 3, 4, and 7 after transfection with miR-217 mimic or inhibitor as well as their respective controls. We measured the expression of both early and late differentiation markers such as cytokeratin 1 (CK1) and cytokeratin 10 (CK10), filaggrin (FLG) and Loricrin (LOR). As shown in Fig. 2E and G, overexpression of miR-217 significantly increased CK1 and CK10 expression at both mRNA and protein levels, whereas inhibition of endogenous miR-217 decreased their expressions (Fig. S1A). Similar results were observed for the mRNA and protein expression of two late differentiation markers FLG and LOR in the keratinocytes with overexpressed or inhibited miR-217 (Fig. 2F–G and Fig. S1B).

**Fig. 1.** MiR-217 is down-regulated in keratinocytes in psoriasis lesions. (A) MiR-217 expression was analyzed in healthy (n = 34), psoriasis non-lesional (n = 30) and lesional skin samples (n = 38) using qRT-PCR. ***P < 0.001. (B) MiR-217 expression on skin sections from (a) healthy individuals (n = 14), psoriasis (b) non-lesional (n = 10), and (c) lesional skin (n = 12) was examined by in situ hybridization using miR-217-specific locked nucleic acid-modified probes or (d) scrambled probe. The purple color indicates miR-217 expression. Bar = 50 μm. (C) MiR-217 expression was analyzed in epidermis of healthy (n = 14), psoriasis non-lesional (n = 10) and lesional skin samples (n = 12) using qRT-PCR. Data shown are mean ± SD. ***P < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 2. MiR-217 inhibits the proliferation and promotes differentiation of keratinocytes. Primary human keratinocytes were transfected with miR-217 mimics or miR-217 inhibitor. (A–D) Cell proliferation was examined by CFSE dilution assay using flow cytometry. Data shown are the representative flow cytometry histograms gated on liver propidium iodide (PI)-negative cells keratinocytes. (E–F) The expression of early and late differentiation markers cytokeratin 1 (CK1) and cytokeratin 10 (CK10), filaggrin (FLG) and Loricrin (LOR) on keratinocytes at day 1, 2, 3, 4, and 7 after transfection were analyzed by qRT-PCR (E–F) and western blotting (G). Data shown are mean ± SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Collectively, our data indicate that miR-217 promotes keratinocyte differentiation.

3.3. Grainyhead-like 2 (GRHL2) is a direct target of miR-217 in keratinocytes

In order to further investigate the mechanisms responsible for the effect of miR-217 on keratinocytes proliferation and differentiation, we next sought to identify the molecular targets of miR-217. Targetscan and miRanda were used to screen the potential target gene of miR-217. In the hundreds of predicted target genes, Grainyhead-like 2 (GRHL2), a developmental transcriptional factor that affects epithelial barrier function and keratinocyte differentiation [23,24], was identified as a potential target gene of miR-217, with the predicted binding site at the base from positions 2653 to 2659 (Fig. 3A).

To validate whether GRHL2 is a functional target of miR-217, a dual-luciferase reporter system was employed. We cloned 3’UTR of GRHL2 containing wild type or mutated binding site of miR-217 into the pMIR-Report vector, respectively, and co-transfected with the miR-217 mimics or control into keratinocytes. Data from the luciferase assay showed that over-expression of miR-217

Fig. 3. Grainyhead-like 2 (GRHL2) is a direct target of miR-217 in keratinocytes. (A) Sequence alignment of wild-type (WT) and mutated (Mut) putative miR-217-binding sites in the 3’-UTR of GRHL2. (B) Relative luciferase activities of plasmids carrying WT or mutant GRHL2 3’UTR in keratinocytes co-transfected with miR-217 mimics or miR-NC. (C and D) The expression levels of GRHL2 in keratinocytes were examined by qRT-PCR and western blotting after co-transfection. (E and F) GRHL2 expression was analyzed in healthy (n = 34), psoriasis non-lesional (n = 30) and lesional skin samples (n = 38) using qRT-PCR and immunohistochemistry, Bar = 50 μm. (G) The correlation between miR-217 and GRHL2 mRNA levels in psoriasis lesional skin was analyzed using Spearman’s correlation analysis. Data shown are mean ± SD. **P < 0.01, ***P < 0.001.
remarkably reduced the luciferase activity of the reporter gene with the wild-type construct but not with the mutant GRHL2 3'UTR construct in keratinocytes (Fig. 3B). In addition, overexpression of miR-217 resulted in a reduction of GRHL2 mRNA and protein expression in keratinocytes (Fig. 3C and D). Moreover, GRHL2 was found up-regulated in psoriasis skin lesions compared with healthy skin ($P < 0.001$) and psoriasis non-lesional skin ($P < 0.001$) (Fig. 3E and F). In addition, correlation analyses revealed that there was a significant inverse correlation between miR-217 and GRHL2 expression levels in psoriasis skin lesions (Fig. 3G). Therefore, our results suggest that GRHL2 is a direct target of miR-217 in keratinocytes.

3.4. Silencing of GRHL2 promotes keratinocyte differentiation

To determine whether the observed effects of miR-217 on keratinocyte proliferation and differentiation are mediated by repressing the expression of GRHL2, we analyzed the effects of silencing of GRHL2 expression on keratinocyte proliferation and differentiation. As shown in Figs. 4A and S2C, GRHL2 expression in keratinocytes was significantly decreased by siRNA at both mRNA and protein levels all along the 7 days of differentiation we analyzed. CFSE dilution assay showed that inhibition of GRHL2 had no effect on the proliferation of keratinocytes (Fig. 4B and C). However, GRHL2 silencing increased the expression of CK1 and CK10 at both mRNA and protein levels in keratinocytes (Fig. 4D and E). In addition, a significant induction of both mRNA and protein expression levels of FLG and LOR were noticeable after several days of differentiation, in conditions where GRHL2 was silenced in keratinocytes (Fig. 4D and E). Taken together, our findings demonstrated that the regulation of keratinocyte differentiation, but not proliferation, by miR-217 is mediated through targeting GRHL2.

4. Discussion

In this study, we investigated the biological role of miR-217 in the pathogenesis of psoriasis. We found significant down-regulation of miR-217 in psoriatic lesional epidermis compared with healthy skin and psoriasis non-lesional skin. Additionally, functional studies demonstrated that miR-217 suppressed the proliferation and promoted differentiation of keratinocytes through targeting the GRHL2. Altogether, our results demonstrate for the first time that miR-217 play a critical role in the regulation of keratinocyte proliferation and differentiation.

MiR-217 is a multifunctional miRNA that plays essential roles in many physiological and pathological processes. In particular, miR-217 has been implicated in carcinogenesis and function as a tumor suppressor in various tumors, including cancer [14], hepatocellular carcinoma [15], renal cell carcinoma [16], gastric cancer [17], osteosarcoma [18] and chronic myelogenous leukemia [19]. However, several reports showed that miR-217 may also function as an oncogene in human breast cancer [25,26] and B-cell lymphomas [27], suggesting that miR-217 plays different roles in different cancer types. To date, nothing is known about the function of miR-
217 in healthy or inflamed skin. In this study, we found significant downregulation of miR-217 in a nonmalignant hyperproliferative disease, psoriasis. Our results also showed that miR-217 inhibits keratinocyte proliferation, but promotes its differentiation, suggesting that the downregulation of miR-217 in the psoriatic epidermis may contribute to hyperproliferation and altered differentiation of keratinocytes, our study implicates that miR-217 may serve as a potential therapeutic target for psoriasis.

In this study, GRHL2 was selected as an experimentally verified target gene of miR-217 based on its particular functions and expression patterns. It had not been reported whether miR-217 could directly target GRHL2 in psoriasis. GRHL2, along with GRHL1 and GRHL3, belongs to Drosophila Grainyhead (GRH) family, which is involved in epithelial morphogenesis [28]. GRH is a developmental transcriptional factor that affects epithelial barrier function [29,30]. In mammalian cells, GRHL proteins orchestrate development of epithelial impermeability through transcriptional activation of genes required for cell adhesion [31]. Previous studies showed that GRHL2 was upregulated in chronic skin lesions, such as atopic dermatitis and psoriasis [23]; additional studies demonstrated that GRHL2 could inhibit keratinocyte differentiation through transcriptional inhibition of the genes clustered at the epidermal differentiation complex (EDC) [23]. In this study, we observed increased GRHL2 expression in psoriasis skin lesions, which was in line with previous studies. As GRHL2 is directly targeted by miR-217, increased GRHL2 levels in psoriasis may partially be attributable to the reduced expression of miR-217.

Are the observed effects of miR-217 on keratinocyte proliferation and differentiation are mediated by repressing the expression of GRHL2? In fact, GRHL2 silencing only repressed keratinocyte differentiation. However, inhibition of GRHL2 had no effect on the proliferation of keratinocytes, suggesting the anti-proliferation effects of miR-217 in keratinocytes cannot be explained by inhibiting GRHL2 expression. The regulation of keratinocyte proliferation by miR-217 is mediated through targets other than GRHL2. As we all known, miRNA can target hundreds of genes simultaneously. Thus, it is likely to assume that other targets may contribute to the effect of miR-217 on keratinocyte proliferation, in addition to GRHL2. In fact, several target genes for miR-217, which are involved in regulating cell proliferation, have been identified in other cell types. For instance, IGF1R in epithelial ovarian cancer [32], AEG-1 in colorectal cancer [33], and DACH1 in breast cancer [26]. Therefore, it would be interesting to study additional targets of miR-217 in keratinocyte and psoriasis skin, which may further explain the mechanisms of miR-217 in regulating keratinocyte proliferation and differentiation.

In summary, our results demonstrate that miR-217 inhibits keratinocyte proliferation and promotes differentiation. Promotion of keratinocyte differentiation by miR-217 is partially mediated by the inhibition of its direct target GRHL2. Therefore, miR-217 may represent a novel therapeutic target for psoriasis treatment.

Conflict of interest statement

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.01.157.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.01.157.