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miR-128 targets the CC chemokine ligand 18 gene (CCL18) in cutaneous malignant melanoma progression

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ABSTRACT

Background: The CC chemokine ligand 18 (CCL18) has a higher expression in some tumors, while the CCL18 level can be a marker of tumor progression and prognosis. We previously reported that the expression of *CCL18* gene was dramatically up-regulated in cutaneous malignant melanoma (CMM) and its expression levels were correlated with tumor thickness.
Objective: To investigate miRNAs which could target the *CCL18* gene so as to mediate CMM development and improvement.
Methods: The expression of miR-128 and *CCL18* in CMM were measured by qRT-PCR. The interaction of miR-128 with *CCL18* 3′UTR was verified by Luciferase reporter gene assay. The changes in expression of *CCL18* after miR-128 mimic transfection of A375 melanoma cells were determined by both qRT-PCR and Western-bloting. Cell viability was accessed by CCK8-assay. Flow cytometry was employed to detect the incidence of apoptosis. Clonogenic assay was used to detect the ability of colony formation. Cell migration was evaluated by Transwell migration study. The protein levels of epithelial-mesenchymal transition (EMT), such as E-cadherin, N-cadherin and β-catenin were analyzed by Western-bloting.
Results: The expression of miR-128 had negative relevance with *CCL18* in CMM. miR-128 could interact with *CCL18* 3′UTR. Transfected miR-128 mimic significantly reduced *CCL18* expression and this impairment of *CCL18* gene promoted apoptosis, inhibited migration and colony formation of A375 melanoma cells. Furthermore, the relative expression of N-cadherin was decreased.
Conclusion: CCL18 is a target gene of miR-128. Overexpression of miR-128 inhibits the oncogenic effect of *CCL18*.
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1. Introduction

Cutaneous malignant melanoma (CMM) is a highly malignant skin cancer, with the incidence constantly increased worldwide. Although CMM accounts for less than 5% of skin cancers, it is responsible for 65% of death [1]. Moreover, once CMM has metastasis, only less than 5%–10% patients survive for 5 years with an average survival time of 6–9 months [2]. In the past five years, with the approval of new targeted therapeutic agents as BRAF and MEK inhibitors or immunotherapy as CTLA-4 and PD-1 blocking antibodies, the overall survival of patients with advanced-stage melanoma has improved to at least 2 years [3]. Thus, it is of great significance to further understand the molecular mechanism driving the progress of CMM. Previous study showed that tumor microenvironment plays an important role in tumor progression and metastasis in a bidirectional manner with tumor cells through molecular signals such as chemotactic cytokines [4].

The CC (C-C motif) chemokine ligand 18 (CCL18) protein is a chemokine mainly produced by monocytes/macrophages and DC. Studies have confirmed that CCL18 is involved in many

Abbreviations: CCL18, CC chemokine ligand 18; CMM, cutaneous malignant melanoma; ALM, acral lentiginous melanoma; TAM, tumor-associated macrophages; EMT, epithelial-mesenchymal transition; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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inflammatory and tumor diseases [5]. As in tumors, it is predominantly expressed by tumor-associated macrophages (TAM), and it could promote the invasiveness of tumor cells [6,7]. Currently the up-regulation of CCL18 has been discovered in breast cancer, ovarian cancer, prostate cancer, bladder cancer, as well as in cutaneous T-cell lymphoma which accelerates progression and metastasis of tumor [8–12], while in gastric cancer it predicted a better survival [13]. However, the involvement of CCL18 in CMM has not been clarified. As acral lentiginous melanoma (ALM) is the most common subtype in Asian patients [14], we had discovered that *CCL18* gene was dramatically up-regulated in tumor tissues of ALM compared with that in paracancerous normal tissue of the same patient. Furthermore, the immunohistochemical staining of CCL18 in 58 CMMs and benign nevi and clinicopathologic significance analysis results showed that the expression of CCL18 in CMM had a significantly positive correlation with tumors' Clark level and Breslow depth [15]. in vitro, CCL18 can be produced by IL-4 (20 ng/ml) activated monocytes [16]. We also investigated the effects of CCL18 on a human melanoma cell line A375, which showed that CCL18 can promote the invasion ability of A375 cells and tumor angiogenesis [17]. All these results of our previous studies suggested that CCL18 plays a critical role in the progress of CMM.

MicroRNAs (miRNAs) are a class of small non-coding RNAs of 19–22 nucleotides that can negatively regulate the translation or degradation of target mRNAs. Altered expression of miRNAs involves in melanoma progression and metastasis [18]. In order to discover and identify the miRNAs targeting *CCL18*, in this study we predicted the possible microRNA 128 (miR-128) by means of computational algorithms; analyzed their expression correlation based on their expressions in fourteen CMM tissues by real-time PCR; proved the interaction of miR-128 with the 3′ untranslated region (3-UTR) of *CCL18*. In order to clarify the actual function of miR-128 on cell behaviour by target-regulating *CCL18*, in this study we further investigated whether the proliferation and invasion of melanoma cells could be partly influenced by ectopic expression of miR-128. Our results not only facilitate our deep understanding of the molecular mechanism of CMM, but also provide a promising candidate miRNA for the future CMM gene therapy.

2. Materials and methods

2.1. Patients and tissue samples

14 primary CMM patients (5 female and 9 male, mean age: 61.71 ± 9.81) were recruited from inpatient service in the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College. Local ethics guidelines were followed. All patients were diagnosed CMM, ALM subtype by clinical and pathological examination. Matched CMM and paracancerous normal tissues were obtained from surgery after removal of the necessary amount for routine pathology examination. These specimens were snap frozen in liquid nitrogen, and then stored in −80 °C.

2.2. Luciferase plasmid construction

Full-length (440 bp) wild-type (WT) and mutant (Mut-128) *CCL18* 3′UTR reporter plasmids were synthesized (Invitrogen, USA) to include the 3′UTR of *CCL18* (NM_002988.2) within the pGL3-Promoter Vector (E1761). Modification were made to the *CCL18* 3′UTR at the predicted miR-128 binding region by replacing the sequence "cactgtga" with "cTcAgAgT" (from the site of 433–440). miR-128 was inserted into the Vector pcDNA™6.2-GW/miR128 (Invitrogen, USA). All constructs were verified by DNA sequencing.

2.3. Luciferase reporter assays

CCL18 3′UTR luciferase reporter assays were performed by using miR-128 molecule vector pcDNA™6.2-GW/miR128 as shown in Supplemental Table 1. *CCL18* transcriptional activity was determined by a *CCL18* driven luciferase reporter or a negtive control reporter with pcDNA™6.2-GW/miR128.

Lysates were assayed for firefly and Renilla luciferase activities 48 h post-transfection with the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was first normalized to Renilla, then each condition expressed relative to the negative control reporter.

2.4. Cell culture

Human melanoma cell line A375 was cultured in DMEM medium (Gibco, USA) with 10% FBS (Gibco, USA) under 37 °C and 5% CO_2. PBMC (Peripheral blood mononuclear cell) was separated from 40 ml human preipheral blood by density gradient centrifugation with lymphocyte separation medium (Haoyang Biol. Co., Tianjin, China). PBMC was grown in 1640 medium (Gibco, USA) with 10% FBS. After cultured for 1 h, PBMC was repeatedly washed by FBS-free 1640 medium, and then the adherent cells were monocytes. Medium was added with IL-4 (Peprotech, USA) (at a final concentration of 20 ng/ml) to induce monocytes producing CCL18 for 48 h. A375 and monocytes were co-cultured in 6-well transwell cells of 0.4 um pore size membrane (Corning, USA) for 24 h.

2.5. Experimental design

Monocytes were divided into four groups as follows: (i) untreated control cells; (ii) cells induced by IL-4 (20 ng/ml) for 48 h; (iii) cells transfected with miR-128 mimic (adenovirus-coated) for 24–36 h and then induced by IL-4 (20 ng/ml) for 48 h; (iv) cells transfected with mimic-control (adenovirus-coated) for 24–36 h and then induced by IL-4 (20 ng/ml) for 48 h. Then monocytes of each group were co-cultured with A375 cells for next 24 h.

2.6. Adenovirus transfection

Monocytes were transfected with adenovirus containing a miR-128 mimic insert or the control miRNA vector without any insert (GenePharma, Shanghai, China). After the carried gene was transfected into host cell, the concomitant green fluorescence protein was expressed. Almost 1,000,000 cells were separated and cultured 24 h in 6-well plate before transfection. A total of 20 ul of the adenovirus (10^9 /ml) was added in, followed by 2 ul polybrene (5 ug/ml) (GenePharma, Shanghai, China). Monocytes were cultured in 2 ml medium for 24–36 h followed by changing complete medium under 37 °C and 5% CO_2.

Transfection effect was confirmed by green fluorescence generated by monocytes under microscope (Olympus, Japan). miR-128 and *CCL18* expression was confirmed by real-time PCR. The level of CCL18 protein was detected by western blotting.

2.7. Real-time PCR

The expression of miR-128 and *CCL18* mRNA in 14 CMM samples and paracancerous normal tissues were analyzed. Total RNA was extracted with TRIzol Reagent (Invitrogen, USA). Reverse transcription was performed to synthesize the cDNA for mRNA with Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and for miRNA with All-in-oneTM miRNA RT Detection Kit (Genecopoiea, USA). Then SYBR green real-time PCR

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