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Down-Regulation of *miR-148a* Promotes Metastasis by DNA Methylation and is Associated with Prognosis of Skin Cancer by Targeting *TGIF2*

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: MicroRNAs (miRNA) dysregulation has been considered to be significantly related to the occurrence and development of cancers. Several studies had proved that DNA methylation is an important cause of the abnormal expression of miRNAs. The purpose of this study was to investigate the methylation status of *miR-148a* and its effects on the metastasis and prognosis of skin cancer, as well as the interaction with *TGIF2* gene.

Material/Methods: According to the qRT-PCR analysis, the expression of *miR-148a* was down-regulated in tumor tissues compared with the adjacent tissues and healthy controls ($P<0.05$). *In vitro* cell metastasis assay revealed that *miR-148a* could inhibit cell metastasis and its down-regulation promoted metastasis. Luciferase reporter assay found that *TGIF2* gene was a target gene and its expression was suppressed by *miR-148a* in skin cancer.

Results: Methylation-specific PCR demonstrated that DNA methylation rate of *miR-148a* was higher in tumor tissues than in adjacent tissues and healthy tissues ($P<0.05$). *miR-148a* expression was proved to be epigenetically regulated after the demethylation of it by 5-aza-20-deoxycytidine treatment and qRT-PCR analysis. *miR-148a* methylation was significantly influenced by many clinicopathologic characteristics such as age ($P=0.000$), pathological differentiation ($P=0.000$), and lymph node metastasis ($P=0.000$). Besides, Kaplan-Meier analysis showed patients with *miR-148a* methylation lived shorter than those without that ($P<0.001$). Cox regression analysis manifested that *miR-148a* methylation (HR=0.053, 95CI%=0.005–0.548, $P=0.014$) could be serve as an independent prognostic marker for skin cancer.

Conclusions: Taken together, the expression of *miR-148a* was regulated by DNA methylation and targeted by *TGIF2*. Its methylation may be a potential prognostic indicator in skin cancer.

MeSH Keywords: Genes, Tumor Suppressor • Methylation • MicroRNAs • Prognosis • Skin Neoplasms

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Background

Skin cancers are one of the most common seen malignancies in human (1). Among them, non-melanoma skin cancer accounts for 10% of all cancer types and for less than 0.1% of cancer-related deaths [1,2]. The incidence of skin cancers has been increasing year by year and 2.75 million new cases in the worldwide annually [3,4]. Melanoma, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC) are the main types of skin cancers [5]. And there are many risk factors for the incidence and development of skin cancer such as ultraviolet light exposure, age, male gender, genetic susceptibility, phenotypic features and immunity have been reported [6,7]. However, the pathogenesis of skin cancer has not been fully elucidated. Epigenetic alterations in multiple genes are believed to play crucial roles in skin carcinogenesis. The study of the fundamental mechanism of DNA methylation may be helpful to understand the progression of skin cancer.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules with a length of 18–25 nucleotides. They have been confirmed to be related to progression of various cancers via acting as an oncogene or tumor suppressor and other roles. miRNAs also can regulate gene expression by specific sites within the 3'-Untranslated Regions (3'-UTR) of a target-mRNA at post transcriptional level [8]. Besides, they participate in many processes of diseases such as cell growth, cell cycle, apoptosis, migration and invasion [9]. The deregulated of miRNAs haven been confirmed to play vital role in various disease including cancers in previous studies. *miR-148a* had been considered to be a tumor suppressor in various of cancers including gastric, non-small cell lung, colorectal cancer, liver cancer, pancreatic cancer, breast cancer, osteosarcoma and so on [10–16]. Amaia et al., proved that *TGIF2* was a target gene of *miR-148a* [17]. Nevertheless, the interaction of *miR-148a* and *TGIF2* as well as their role in skin cancer had never been covered. Besides, DNA methylation has also been found to regulate the expression of *miR-148a* in several studies [18–20]. However, whether the expression of *miR-148a* in skin cancer is related to DNA methylation remains unknown.

In this study, we investigated the expression level of *miR-148a* and its methylation status in skin cancer. At the same time, we explored the relationship between *miR-148a* and *TGIF2*. In addition, the association between *miR-148a* and the prognosis of skin cancer was also detected. And these were expected to provide a new prognostic marker and therapy for this disease.

Material and Methods

Patients and samples

The current study was conducted in The General Hospital of Beijing Military Command and was permitted by the Ethics Committee of the hospital. 105 patients diagnosed as skin cancer were included. All the patients had never received any chemical treatment and physical therapy before sampling. Besides, 89 healthy people were taken as normal controls. Written informed consents were signed by each participator in advance.

Tumor tissues, adjacent tissues, and healthy tissues were collected from the patients with skin cancer and healthy people, respectively. Liquid nitrogen was used to immediately freeze the samples. Then the samples were stored at –80°C for later use. Clinicopathologic characteristics, such as age, sex, tumor thickness, tumor subtype, pathological differentiation, lymph node metastasis, and clinical stage, were recorded in a database. The follow-up was performed with a telephone interview or questionnaire for 5 years. The overall survival was defined as the day of surgery to the day of death. Patients who died from unexpected events or other diseases were excluded in our study.

Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from the patients with skin cancer and healthy controls with TRIzol (Invitrogen, Carlsbad, CA, USA). The reverse transcription was made to synthesize the first chain of cDNA with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then RT-PCR reaction was performed in the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA). *GAPDH* was taken as the internal control. The relative expression quantification of *miR-148a* at mRNA level was evaluated by comparative cycle threshold (CT) method.

Enzyme-linked immunosorbent assay (ELISA)

Total protein was isolated from the tumor tissues, adjacent normal tissues, and healthy tissues. Then the levels of TGIF2 were determined by a commercial reagent kit following the manufacturer's instructions.

Luciferase reporter assays

The wild- or mutant-type 3'-UTR of TGIF2 was inserted into the pcDNA3.1/HisC vector (Invitrogen, Carlsbad, CA). Cells were seed in 24-well plates for 24 h before transfection. Then the cells were transfected with the reporter vector (pcDNA3.1/HisC-TGIF2-WT-3'UTR or pcDNA3.1/HisC-TGIF2-MT-3'UTR) and *miR-148a* mimics or scrambled mimics using Lipofectamine 2000

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(Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, cells were collected and the luciferase activity was measured using a dual-luciferase reporter assay system according to the manufacturer's protocol (Promega). All experiments were performed in triplicate.

Lentivirus infection and siRNAs

Lentiviruses containing *miR-148a* (*Lv-miR-148a*) and negative control (Lv-NC) were purchased from GeneChem (Shanghai, China). When the concentration of cells reached to 70%, the *Lv-miR-148a* or negative control lentivirus were added. Six days later, the expressions of *miR-148a* and *TGIF2* were detected by qRT-PCR and ELISA, respectively.

Cell culture and cell metastasis assays

Human skin cancer cell lines A431 were obtained from the Pasteur institute of Iran and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The mediums were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell metastasis was assessed by wound-healing assay. A confluent cell monolayer without FBS was scratched artificially using sterile tips, and wound-healing images were taken at 24 and 48 h later.

DNA extraction and bisulfite modification

Genomic DNA was extracted from the tumor tissues, adjacent tissues and healthy controls with the DNA Mammalian Genomic Purification Kit purchased from Sigma-Aldrich Co. (St. Louis, MO). Then the DNA was treated with sodium bisulfite using the EZ DNA Methylation TM Kit (Zymo Research, Orange, CA) to conduct the conversion of cytosine to uracil. The modified DNA was resuspended in elution buffer and stored at –20°C.

Methylation-specific polymerase chain reaction (MSP)

The modified DNA was used to analyze the methylation status of *miR-148a* in skin cancer using methylation-specific polymerase chain reaction on the ABI7500 PCR system (ABI Co, Norwalk, Connecticut) with the SYBR Premix Taq ExTaq Kit (TaKaRa Co Ltd, Otsu, Shiga, Japan). The primer for the methylated *miR-148a* and unmethylated sequences were as follows: methylated, forward-5'-TAGGGGAGGTTTCGTAAAGC-3', reverse-5'-CACGAAAACGAATATTCGAAA-3'; unmethylated, forward-5'-TTTTAGGGGAGGTTTTGTAAAGT-3', reverse-5'-ACACAAAAACAAATATTCAAAACT-3'. The PCR amplification system contained 2 µl Modified DNA, 2.5 µl 10×PCR buffer, 2.5 µl $MgCl_2$ solution, 2.5 µl dNTPs, 2.5 µl forward and reverse primer respectively, 0.1 µl 5U/µl Taq Gold polymerase and sterile water which was supplied to total 25 µl. The procedure of MS-PCR was: initial denaturation at 95°C for 15 min, 45 cycles of denaturation for 30 s at 95°C, annealing at 55°C for 45 s, extension at 72°C for 30 s, and extension at 72°C for 5 min.

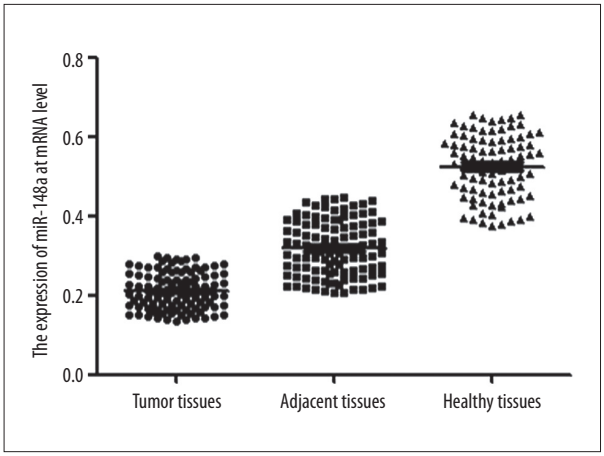

Figure 1. The expression of *miR-148a* in tumor tissues, adjacent tissues and healthy tissues. The expression was increasing successively in three kind of samples (P<0.05).

5-Aza-dC treatment

To analyze the influence of *miR-148a* methylation on the expression of *miR-148a* and TGIF2 protein, A431 cells were treated with 5-aza-20-deoxycytidine (5-Aza-dC, Sigma-Aldrich, USA) to do the demethylation study. The dose of 5-Aza-dC was 0.5 mmol/l and the time lasted for 4 days while the culture medium containing 5-Aza-dC was replaced every 24 h.

Results

The down-regulated of miR-148a in patients with skin cancer at mRNA level

The expression of *miR-148a* was detected by qRT-PCR at the mRNA level. The result demonstrated that its expression was significantly lower in tumor tissues than in adjacent normal tissues and healthy tissues (Figure 1, P<0.05). This revealed that *miR-148a* is a tumor suppressor in skin cancer.

miR-148a inhibits the cell metastasis

To further understand the function of *miR-148a* in skin cancers, we made a wound-healing assay to estimate its effects on cell metastasis. The outcome proved that the down-regulation of *miR-148a* induced the metastasis of skin cancer cells compared to the controls (Figure 2). In contrast, the cell metastasis was inhibited when the endogenous *miR-148a* was silenced. These results suggest that *miR-148a* might function as a tumor suppressor by promoting cell metastasis in skin cancer cells.

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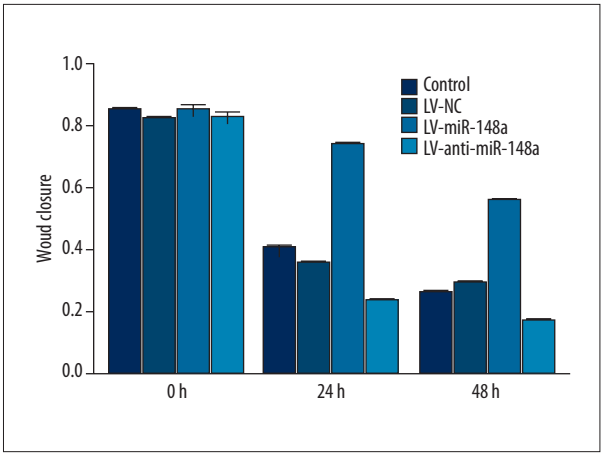

Figure 2. Cell metastasis assay in skin cancer cells. *miR-148a* inhibited the cell metastasis compared with the cells transfected with *anti-miR-148a* and controls ($P<0.05$).

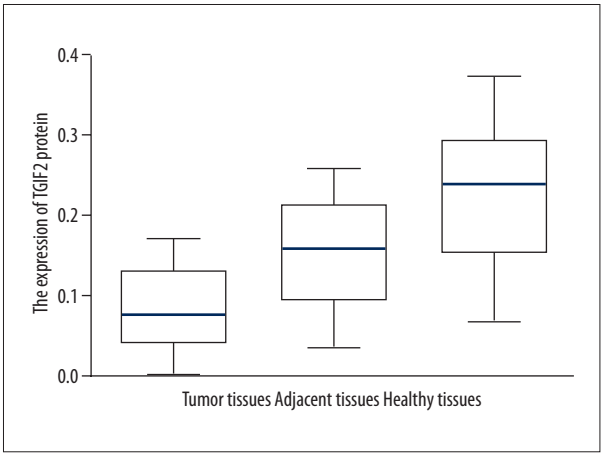

Figure 3. The expression of TGIF2 protein. Its expression was lower in tumor tissues than in adjacent tissues and healthy tissues ($P<0.05$).

miR-148a directly targets TGIF2 and inhibits its expression in skin cancer *in vitro*

To elucidate the molecular mechanism by which *miR-148a* exerts its inhibitory effect on skin cancer cells, we measured the expression of TGIF2 which was predicted as potential targets of *miR-148a*. ELISA assay showed that the expression of TGIF2 protein was down-regulated in tumor tissues compared with those in adjacent tissues and healthy tissues (Figure 3, $P<0.05$). Then, we estimated the correlation between the expression of *miR-148a* and TGIF2, a positive correlation was shown (Figure 4). A luciferase reporter assay was further performed to verify whether *miR-148a* directly targets TGIF2 gene in skin caner cells. The wild-TGIF2 and mutant-TGIF2 containing the predicted binding sites of *miR-148a* were constructed and cloned downstream to a luciferase reporter, respectively. Then these two vectors were co-transfected with *miR-148a*

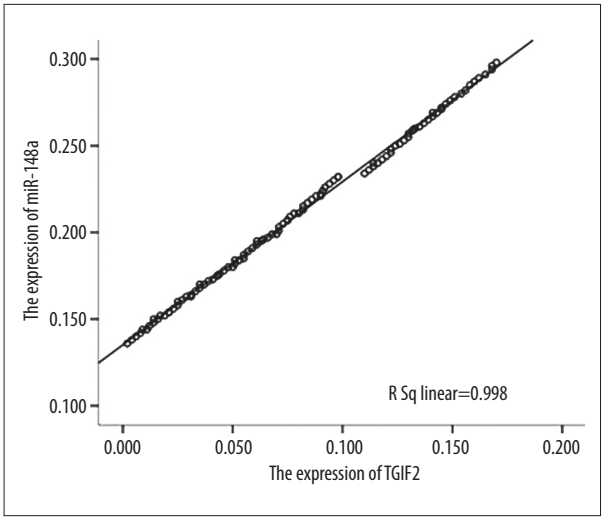

Figure 4. The correlation between the expression of *miR-148a* and *TGIF2* which manifested positive (r=0.998).

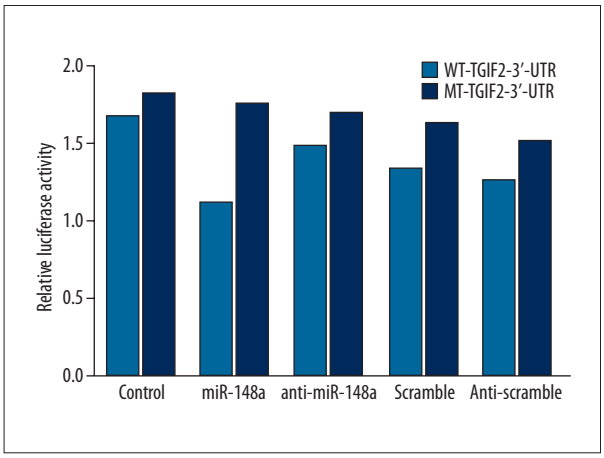

Figure 5. The repression of luciferase activity by 3'UTR of *TGIF2* was dependent on *miR-148a*. Mutated 3'UTR of *TGIF2* abrogated *miR-48a*-mediated repression of luciferase activity in A431 cells.

mimics, *miR-148a* inhibitor (anti-*miR-148a* mimics), or their respective scrambled controls into A431 cells. The result indicated that transfection of *miR-148a* mimics significantly decreased the luciferase activity of cells (Figure 5). Moreover, the luciferase activity which was mediated by *miR-148a* was suppressed by the mutant putative binding sites. These findings support the hypothesis that *TGIF2* is a direct target gene for *miR-148a* in skin cancer cells.

Knock-down of TGIF2 contributes to cell migration of skin cancer cells *in vitro*

The *TGIF2* was knocked-down to analyze its function in skin cancer by *TGIF2*-specific small interfering RNAs (si-*TGIF2*) and was confirmed by ELISA (Figure 6A). Then we performed

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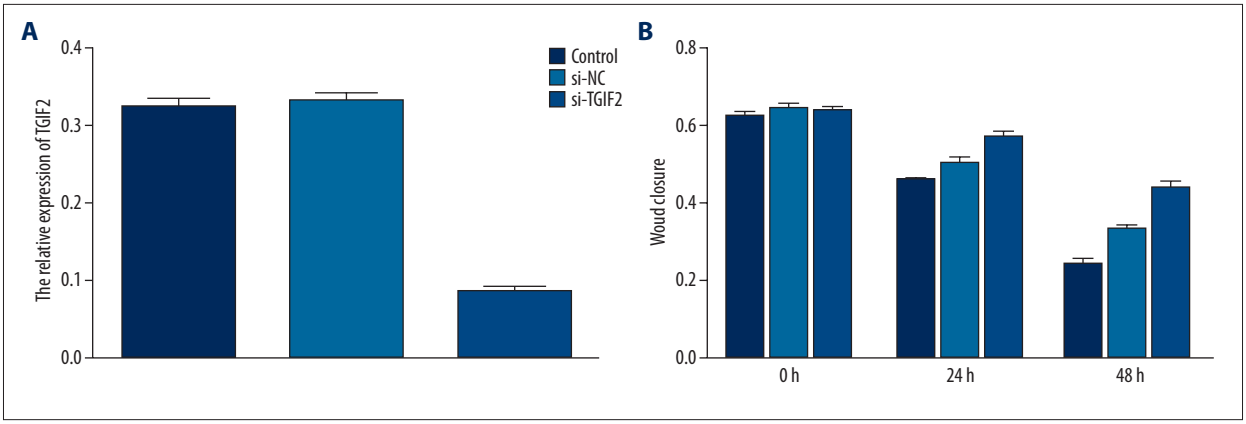

Figure 6. Knockdown of TGIF2 inhibits cell migration of skin cancer cells. (**A**) A431 cells were infected with TGIF2-specific small interfering RNAs (si-TGIF2) and negative control (si-NC), and the expression of TGIF2 was examined by ELISA. (**B**) The effect of TGIF2 knockdown on the cell metastasis was assessed by wound healing.

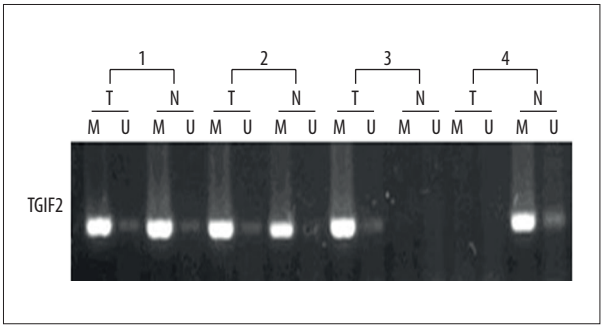

Figure 7. The methylation status of the DNA of *miR-148a* which was significantly higher in tumor tissues than in adjacent tissues and healthy tissues ($P<0.05$).

transwell assays with wound-healing assays, which clarified that knockdown of *TGIF2* in skin cancer cells could significantly inhibit cell migration ability (Figure 6B). This effect was as same as the down-regulation of *miR-148a*.

The expression of miR-148a is epigenetically regulated by DNA methylation in skin cancer cells

MSP was carried out to observe the methylation of *miR-148a* in skin cancer cells. The A431 cells were treated with 5-Aza-dC, a demethyltransferase inhibitor, showing that the expression of *miR-148a* was up-regulated after the 5-Aza-dC treatment compared with those without treatment). We found that DNA methylation in *miR-148a* frequently occurred in 85 tumor tissues (55 partially methylated and 30 completely methylated), while the adjacent tissues and healthy controls were all unmethylated (Figure 7). This suggests that epigenetic factors can affect the expression of *miR-148a,* and that DNA methylation might be an important mechanism in the function of *miR-148a* on skin cancer.

The relationship between miR-148a methylation and clinicopathologic characteristics

the above assays showed that *miR-148a* methylation might be a participator of the development of skin cancer. Therefore, we estimated the relationship between *miR-148a* methylation and clinicopathologic characteristics to prove our inference. As shown in Table 1, age ($P=0.000$), pathological differentiation ($P=0.000$), and lymph node metastasis ($P=0.000$) were considered to be vital influential factors to the methylation of *miR-148a,* which provided strong support for our view above.

The association between miR-148a methylation and overall survival

To make sure the prognostic value of *miR-148a* methylation in skin cancer, we concluded the follow-up information and analyzed its role in prognosis via Kaplan-Meier and Cox regression analysis, respectively. Kaplan-Meier analysis showed that the overall survival of patients with *miR-148a* methylation was shorter than in those without *miR-148a* methylation (Figure 8, log rank test, $P<0.001$). The clinicopathologic characteristics, such as tumor thickness (HR=0.359, 95CI%=0.150-0.861, $P=0.022$), lymph node metastasis (HR=0.364, 95CI%=0.086–0.810, $P=0.020$), and clinical stage (HR=0.417, 95CI%=0.186–0.935, $P=0.034$), were proved to be related to the prognosis of skin cancer as well as *miR-148a* methylation (HR=0.053, 95CI%=0.005–0.548, $P=0.014$) and they might be independent prognostic indicators in skin cancer (Table 2).

Discussion

Numerous reports had reported that miRNAs act as important regulator in about one-third of human coding genes at the post-transcription level and plays a crucial role in most tumor

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Table 1. The relationship between *miR-148a* methylation and Clinicopathologic Characteristics of patients with skin cancer.

Clinicopathologic characteristics	n	*miR-148a* methylation		P
		Methylated	Unmethylated	
Age				0.000
≤60	78	33	45	
>60	27	22	5	
Sex				0.757
Female	30	18	12	
Male	75	37	38	
Tumor thickness				0.057
≤2 cm	57	25	32	
>2 cm	48	30	18	
Tumor subtype				0.909
SSM	29	16	13	
LMM	42	21	21	
Others	34	18	16	
Pathological differentiation				0.000
Low	54	18	36	
High + moderate	51	37	14	
Lymph node metastasis				0.000
No	60	20	40	
Yes	45	35	10	
Clinical stage				0.310
I+II	45	21	24	
III+IV	60	34	26	

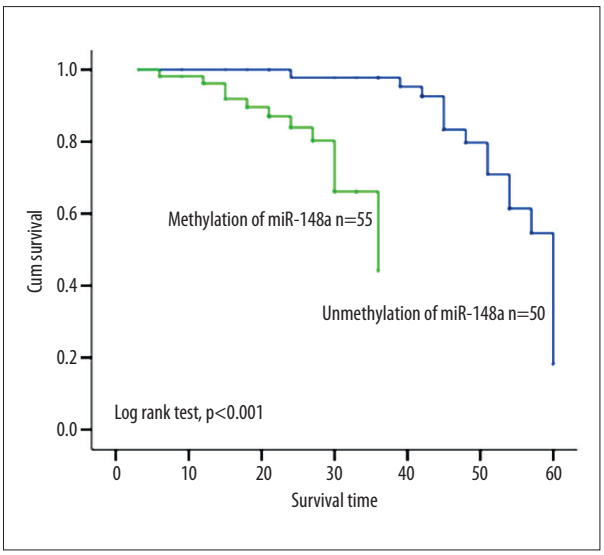

Figure 8. Kaplan-Meier analysis for patients with skin cancer and the *miR-148a* methylation.

formation and development [21]. *miR-148a* has been shown to have aberrant expression in several cancers and it is considered to be an important tumor suppressor in these cancers. It was also found to be down-regulated in melanoma [22]. In this study, we detected the expression of *miR-148a* in 105 patients with skin cancer and 89 healthy controls. The expression of *miR-148a* was significantly lower in tumor tissues compared with adjacent tissues and healthy tissues, which is consistent with the trend in melanoma. In addition, *TGIF2* was thought to be a target gene of *miR-148a*. Therefore we validated this view according to measure the expression of TGIF protein and the expression of *miR-148a* when the *TGIF2* knocked down. The experimental illuminated that *TGIF2* gene was one of the targeted gene and its expression was mediated by *miR-148a* which was agree with the point before [17].

Epigenetic modifications are a main cause of abnormal gene expression, including some miRNAs [23–25]. The aberrant DNA methylation of miRNA plays a significant role in cancer

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Table 2. Multivariate analysis for the prognostic factor of patients with skin cancer.

Parameter	Risk ratio	95% confidence interval	*P* value
Tumor thickness	0.359	0.150–0.861	0.022
Lymph node metastasis	0.264	0.086–0.810	0.020
Clinical stage	0.417	0.186–0.935	0.034
miR148a methylated	0.053	0.005–0.548	0.014
miR-148a unmethylated	–	–	–

progression [26,27]. To make determine the influential factors of the down-regulation of *miR-148a*, we detected the methylation status of *miR-148a* in skin cancer and analyzed whether its status could influence the expression of *miR-148a*. Firstly, we tested the methylation status of *miR-148a* by MSP and verified that *miR-148a* methylation rate was higher in tumor tissues than in adjacent tissues and healthy tissues. Then 5-Aza-dC was added into skin cancer cells for demethylation, and after that the expression of *miR-148a* was increased in tumor tissues compared with the expression before demethylation via qRT-PCR. These revealed that the methylation might be the mechanism of the down-regulation of *miR-148a*.

Metastasis is responsible for 90% of deaths in patients with solid tumors, especially lymphatic route of metastasis [28–30]. Several miRNAs had been confirmed to be linked with the metastasis of tumors such as miR-10b, miR-373, miR-126 and miR-335 in some cancers [31–33]. *miR-148a* was also found to be significantly associated with the appearance of lymph node metastasis in human cancer [17]. Our findings indicated that DNA methylation-associated down-regulation of *miR-148a* could contribute to the metastasis of skin cancer by targeting with *TGIF2* gene.

To explore the function of methylation in *miR-148a*, the relationship between the expression of this miRNA and clinicopathologic characteristics, as well as overall survival of patients with skin cancer, were estimated. The methylation of *miR-148a* was clearly impacted by age, pathological differentiation, and lymph node metastasis in our study. Kaplan-Meier analysis revealed patients with *miR-148a* methylation had a much shorter overall survival than those without this condition. Moreover, cox regression analysis adjusted for clinicopathologic characteristics demonstrated that tumor thickness, lymph node metastasis, clinical stage, and *miR-148a* methylation were independent prognostic factors in skin cancer.

Conclusions

The expression of *miR-148a* is decreased in patients with skin cancer by targeting with *TGIF2*. Its expression is a promoter for the metastasis of skin cancer cells. Meanwhile, the expression and function of *miR-148a* are regulated by DNA methylation, and its methylation can be an independent indicator in the prognosis of skin cancer. However, due to the limitation of the number of samples and experimental conditions, as well as other unfavorable factors, further studies are still necessary.

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