A novel onco-miR-365 induces cutaneous squamous cell carcinoma

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Introduction

Mutation or abnormal expression of micro-RNAs (miRNAs), which can function as either tumor suppressors or oncogenes, is implicated in various cancers (1–4). miRNAs act as an oncogene in tumors mainly via repressing tumor suppressors or inducing mutations of tumor suppressor genes (5). miRNAs are a family of ~22 non-protein-coding small RNA molecules that act as a class of RNA-interference agents (6). They negatively regulate the levels of their target proteins, mainly by annealing with 3’ untranslated region of the target genes (7,8), to regulate the cellular proliferation, differentiation and apoptosis (9,10). About 40% of the confirmed miRNAs in the genome have been found to be related to the occurrence or/and development of cancer. For example, miR-21 and miR-155 are overexpressed in breast cancer and diffuse large B-cell lymphoma, respectively (11–13). Let-7, on the other hand, can inhibit the proliferation of lung cancer cells by downregulating RAS expression and act as a tumor suppressor gene (14,15). Thus, there is great interest in determining the role of miRNAs in the pathology, classification, diagnosis and treatment of cancers (11,16,17).

miR-365 is located in the chromosome region 16p13.12, and the sequence of matured hsa-miR-365 is sheared from two precursors: hsa-miR-365-1 and hsa-miR-365-2. It has been reported that miR-365 is overexpressed in human breast cancer and downregulates interleukin-6 levels in cooperation with Sp1 and nuclear factor kappaB in HeLa cells (18,19). Moreover, miR-365 has been reported to be involved in signal transduction in the carcinogenesis of small cell lung cancer and colorectal cancer (20). In a prior study in which we analyzed the miRNA expression profile of the NIH 3T3 cell line after irradiation with ultraviolet B, we found that miR-365 was one of the most sensitive miRNAs to ultraviolet B irradiation. We, therefore, hypothesize that miR-365 may play an important role in the development of skin cancer.

In the present study, we investigated the function of miR-365 in cutaneous squamous cell carcinoma (SCC), the second most common skin cancer.

Materials and methods

This study was approved by the Institutional Review Board of our hospital, and all patients provided written informed consent for the use of surgical samples. All animals were treated according to standard guidelines for the use and care of laboratory animals.

Cell culture and tumor samples

Cutaneous SCC lines A431, Tca8113, SCC3/C, HSC-5 (China Center for Type Culture Collection and Cell Bank of the Chinese Academy of Sciences, Shanghai, China) and HSC-1 (Dongguang Biotech. Co., Ltd, Guangzhou, China) and human benign epidermal keratinocyte cell line HaCaT (China Center for Type Culture Collection, Wuhan, China) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and streptomycin (Invitrogen, Carlsbad, CA) and maintained at 37°C with 5% CO₂ in a humidified atmosphere.

Cutaneous SCC samples were obtained from patients diagnosed with cutaneous SCC from January 2009 to August 2011 in the departments of dermatology, pathology and oncology at Nanfang Hospital and Zhuhai Hospital, affiliated to Southern Medical University and The Third Affiliated Hospital to Sun Yat-sen University. A total of 108 specimens of cutaneous SCC were included in the analysis (69 samples from males and 39 from females). Biopsy specimens were collected from patients (fresh biopsies, n = 37), and formalin-fixed paraffin-embedded specimens were collected from the archive (n = 71). Approximately 74% of the patients were >50 years of age. Adjacent noncancerous tissue samples were used as normal control tissues. Fresh samples taken from the surgery were immediately frozen in liquid nitrogen for subsequent total RNA extraction and paraffin embedding. Tumors were classified according to the SCC Broders Pathological Classification: stage I (well differentiated) with 0–5% undifferentiated cells, stages II–III (moderately differentiated) with 25–75% undifferentiated cells and stage IV (poorly differentiated) with 75–100% undifferentiated cells. Of the 108 cases, 78 cases were well differentiated (72.22%), 26 moderately differentiated (24.07%) and 4 poorly differentiated (3.7%). The proliferative marker Ki-67 was used to confirm the differentiation status of the tumors and on average the expression was 80–90% positive. Tumor data are summarized in Table I.

Isolation of RNA and quantitative real-time-PCR

Total RNAs of the cutaneous SCC cell lines (2 × 10⁶ cells) and tissues (100 mg) were extracted with Trizol (Invitrogen) according to the manufacturer’s protocol. The RNA was quantified by absorption at 260 nm. The reverse-transcription reaction and quantitative real-time PCR (qRT-PCR) were performed with a TaqMan qRT-PCR kit (Takara Bio, Shiga, Japan) and TaqMan microRNA assay (Ambion, Austin, TX).

Primer sequences for pre-miR-365 were designed using Primer Express Software from Applied Biosystems. The following primers and reverse primers were used for amplification—pre-miR-365-1, forward: 5′-AGTCTCCCTTTGCTTACTG-3′ and reverse: 5′-GCTACGCGGAAGTTT-3′; pre-miR-365-2, forward: 5′-TCCCTGGCTCATCGG-3′ and reverse: 5′-TGCACCTTCTCGGACT-3′; GAPDH, forward: 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse: 5′-GATACGGTGATGGAGTTTC-3′ and mature miR-365 primer (Ambion). Reactions were...
performed on a Stratagene MX3005P instrument. Cycling parameters were 95°C for 10 min, 40 cycles of 95°C (15 s) and annealed/extended at 60°C for 1 min. The gene expression ΔΔCt values of miRNAs from each sample were calculated by normalizing with internal control U6 rRNA (Ambion). The ΔΔCt was calculated by subtracting the Ct of U6 rRNA from the Ct of the miRNA of interest. Fold change was calculated using the equation 2−ΔΔCt. All experiments were performed in triplicate.

miRNA-FISH

The formalin-fixed paraffin-embedded cutaneous SCC was used for miRNA hybridization. Specimen were deparaffinized and hydrated, followed by treatment with proteinase K and rehydration in 4% paraformaldehyde. After washing with phosphate-buffered saline and air-drying, the sections were hybridized with 10 nM DIG-labeled LNA-miR-365 probe that was prepared by a DIG-3′ and 5′-end labeling kit (Exiqon, Vedbaek, Denmark) following the manufacturer’s protocol (www.exiqon.com) before they were stained with the fluorescent isothiocyanate-labeled LNA-miR-365 probe. Nuclei were routinely stained using 4′,6-diamidino-2-phenylindole.

HaCaT

The pre-miR-365-2 cDNA was obtained from NCBi (NC_000017.10), and three cDNA fragments were amplified by PCR. A 971 bp fragment was first amplified with the forward pre-miR-365-2 (5′-CCGGATCTGGTCGGCCGCTTCATGCCTGAGTCG-3′) and the reverse pre-miR-365-2 (5′-CCGGATCTGGTCGGCCGCTTCATGCCTGAGTCG-3′) primers. The obtained DNA was then digested with BamHI and EcoRI and cloned in pBabe-puro vector. Next, the pBabe-puro-miR-365-2 plasmid was obtained by T4 DNA (TaKaRa) conjugation and DHTse transformation before all constructs were confirmed by DNA sequencing. Then retrovirus package pBabe-puro-miR-365-2 or empty vector in 293FT cell line was obtained by calcium phosphate precipitate. The HaCaT cell line was infected by the supernatant containing virus before the cells were screened by puromycin. The pre-miR-365-2 and miR-365 expressions in HaCaT pre-miR-365-2 and HaCaT pBabe-puro vector cells were confirmed by qRT-PCR.

Western blotting

Western blot analysis was performed as described by Ding et al. (21), with minor modifications. Briefly, total cellular proteins (20 µg) were electrophoresed through a 10% denaturing polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were probed with the anti-NFIB or anti-β-actin (Abcam, Cambridge, MA) and the bound antibody was detected using an ECL Plus kit (GE Healthcare, Piscataway, NJ). The blots were exposed to X-ray film and scanned. The Western blot, image analysis, and statistical analysis were done using ImageJ.

Results

miR-365 is overexpressed in cutaneous SCC

To understand the role of miR-365 in the development of cutaneous SCC, the expressions of miR-365 in 37 cutaneous SCC and 1 human benign epidermal keratinocyte cell line, HaCaT, and 5 cutaneous SCC cell lines, A431, HSC-1, Tca8113, SCC13 and HSC-5, were first examined by qRT-PCR. As shown in Figure 1A, miR-365 expression was elevated compared with the adjacent control in 72% of the clinical SCC cases. For miR-365 expression in cell lines, the results showed that compared with the control cell line HaCaT, the expressions of miR-365 were markedly increased in all five SCC cell lines, with a maximum increase of over 15-fold in A431 (15.67 ± 1.12, P < 0.001) and a minimum increase of almost 5-fold in Tca8113 (4.72 ± 0.85, P < 0.05) (Figure 1B). Next, using miRNA-FISH, we detected the expression of miR-365 in cutaneous SCC (37 fresh, 71 paraffin embedded). miR-365 positive expression was detected in 72.5% of the 37 fresh SCC samples (representative photos are shown in Figure 1C).

The association between miR-365 expression and differentiation status of the cutaneous SCC was determined, and the results showed that the positive index of miR-365 was remarkably higher (76.8% on average) in tumors that exhibited venous invasion than in those without venous
miR-365 as an onco-miRNA in SCC

**Fig. 1.** miR-365 is overexpressed in cutaneous SCC. (A) miR-365 expression in fresh cutaneous SCC compared with adjacent non-cancerous tissue samples by qRT-PCR \((n = 37); \) y-axis denotes fold change. (B) qRT-PCR analysis of miR-365 expression in different cutaneous SCC cell lines: A431, HSC-1, SCL-1, SCC13 and HSC-5, and human benign epidermal keratinocyte cell line HaCaT \(y\)-axis denotes fold change). Means ± SD are shown for all panels. *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\) \((n = 3)\). (C) A representative sample of hsa-miR-365 expression by in situ hybridization with mercury LNA™ micro-RNA. (D) The correlation of miR-365 positive expression with the cutaneous SCC histological differentiation \((n = 108)\).
invasion in which miR-365 was hardly expressed (Table I). Moreover, no overexpression of miR-365 was detected in the metastatic lymph nodes of cutaneous SCC (seven cases in all; Table I). There was an inverse correlation between the miR-365 expression level and differentiation of cutaneous SCC cells ($R = -0.76, P < 0.05$) (Figure 1D).

**Tumorigenicity of miR-365 in nude mice**

Stable cell lines with empty vector control (HaCaTpBabe-puro) and pre-miR-365 overexpression (HaCaTpre-miR-365-2) were constructed. As shown in Supplementary Figure 1A, available at Carcinogenesis Online, the expression of pre-miR-365-2 and miR-365 in the HaCaTpre-miR-365-2 cell line was increased by 64 and 37 times, respectively ($64.18 \pm 0.97, P < 0.001; 37.47 \pm 1.01, P < 0.001$). In addition, the HaCaTpre-miR-365-2 cell line featured increases in proliferation, migration and invasion in vitro, which are the characteristics of malignant phenotype of cancer cells, compared with the HaCaTpBabe-puro cell line (Supplementary Figure 1B–D, available at Carcinogenesis Online).

HaCaTpre-miR-365-2 was then used to induce subcutaneous tumors in 13 BALB/c-nu mice. Mice died 70–110 days after the tumor formation in their right back flank (Figure 2A). Resected tumors at 21 days of tumor formation are shown in Figure 2B. Approximately 50% of mice that received HaCaTpre-miR-365-2 survived to 90 days, whereas 100% of those that received HaCaTpBabe-puro survived to 90 days (Figure 2C).

**Antagomir-365 inhibits tumorigenesis in vitro and abolishes tumor growth in vivo**

To determine whether miR-365 played a role in the occurrence of cutaneous SCC, we further applied anti-miR-365 oligonucleotides (antagomir-365) to suppress the growth of cutaneous SCC both in vitro and in vivo. After transfection of anti-miR-365 oligonucleotides (antagomir-365) in A431 cells, $G_1$ phase cell arrest and an increased apoptotic rate were noted (Figure 3A). Cell proliferation was suppressed compared with the control with no antagomir-365 (Figure 3B). Decrease in cell migration and invasion was noted after transfecting anti-miR-365 oligonucleotides (antagomir-365) in A431 cells (Figure 3C and D).

To further examine antagomir-365 function in vivo, subcutaneous SCC tumors formed by injection of $2 \times 10^7$ A431 cells into nude mice were challenged with silencing of miR-365 using antagomir-365 treatment. After the tumor volume reached $>150\text{mm}^3$ (22) at 8 days after injection, intratumoral multidot injection of antagomir-365 was performed. The tumor volume and apoptosis of the SCC cells were analyzed 2 weeks later. The results showed that tumor growth was significantly suppressed compared with the control group that did not receive injection of antagomir-365 ($496.61 \pm 42.5$ versus $20.5 \pm 14.89, P < 0.001$) and was even abolished in one sample (Figure 3E). Apoptosis occurred in all A431 tumors treated with antagomir-365, with an
average apoptosis ratio of 85.6%, which was about 4-fold that of the control (0.856 ± 0.087 versus 0.167 ± 0.047, \( P < 0.01 \)) (Figure 3F).

Discussion

miR-365 has been reported to be involved in the carcinogenesis of small cell lung cancer and colorectal cancer (23). The present study, to the best of our knowledge, is the first to report overexpression of miR-365 in both fresh and paraffin-embedded samples of cutaneous SCC. In order to further identify its role in the differentiation of cutaneous SCC cells, as well as in the genesis and progression of tumors, we constructed a cell line HaCaT\textsuperscript{pre-miR-365-2} by overexpression of pre-miR-365-2, resulting in malignant cancer cell formation and induction of subcutaneous tumors in nude mice. Further research is needed to explore the underlying functional mechanisms of the cell line. These findings strongly suggest that miR-365 is an oncogene in cutaneous SCC.

miR-365 has rarely been studied in cutaneous cancers, though it was reported as an apoptosis-promoting agent and a growth inhibitor in malignant melanoma (24, 25). Our results, however, showed that miR-365 could act as an onco-miRNA. The discrepancy between our results and those of other studies may be due to the pre-miR-365-1 used in other reports (26) or the different types of skin cancers studied. In this study, we used pre-miR-365-2 to successfully construct a
new HaCaT-pre-miR-365-2 cell line, which expressed pre-miR-365-2 and the results indicated that miR-365 regulates NFIB protein expression (Supplementary Figure 2, available at Carcinogenesis Online).

NFIB is a member of the NFI gene family, which is composed of NFI-A, -B, -C, and -X in vertebrates and functions as a versatile transcriptional repressor of many promoters either through competition with other transcriptional factors for binding or through changes in the nucleosome structure (27). NFIB functions to enhance expressions of more than 100 genes in organs like the brain, lung, liver and intestine, and it regulates cell proliferation and differentiation in lung maturation (28,29). It has been reported that NFIB can react with miR-21 through a double-negative feedback loop (30) and is also a target gene of miRNAs-372/373 (31). The contradictions between our study and previous studies indicate the role of miR-365 in carcinogenesis may vary with its different precursors. Although so far there has been no research addressing the different roles of different precursors, we tentatively presume that the two different precursors of mature miR-365 may result in the different functions.

**Fig. 3.** Antagomir-365 inhibits tumorigenesis of A431 cells in vitro and abolishes A431 tumor growth in vivo. (A) The distribution of A431 cell cycles following treatment in the presence or absence of antagomir-365 by flow cytometry (n = 3). (B) Colony formation of the A431 cell line following mock transfection or transfection with antagomir-365 and then cultured for 14 days (n = 4). (C) Transwell migration assay of A431 cell line following mock transfection or transfection with antagomir-365 (n = 4). (D) Matrigel invasion assay of A431 cell line transfected with antagomir-365 and incubated for 24h (n = 4). Control was mock transfected. (E) Changes of tumor volume in the absence or presence of antagomir-365 treatment with intratumoral multitot injection. The time course of tumor treatment with antagomir-365 is shown (red, n = 7) in comparison with the untreated control (black, n = 3) (top right). Tumors from representative mice 21 days after treatment are shown in the right-side panels. (F) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay for apoptosis measurement of tumors with and without antagomir-365 treatment. *P < 0.05; **P < 0.01; Means ± standard deviation (SD) are shown for all panels.
As miRNAs can function as key molecules in oncogenes and suppressors, they have been targeted in target-based therapies for various cancers (32). For example, antagomir-17-5p abolishes the growth of therapy-resistant neuroblastomas (22). miR-335-specific inhibitor antagomir-335 strongly blocks the malignant glioma cells (33). Similarly, the antagonist-365 can be used to suppress the growth of cutaneous SCC. Coincidentally, we found that antagomir-365 can upregulate the NFIB expression (Supplementary Figure 2, available at Carcinogenesis Online).

The present study has certain limitations. First, to better characterize miR-365, more downstream target genes of miR-365 should be investigated, including the NFIB gene. Second, future studies should include an examination of miR-365 expression in clinical blood samples to better determine any association between miR-365 and metastasis of cutaneous SCC.

Conclusions

In this study, we demonstrated that miR-365 was overexpressed both in fresh SCC samples and paraffin-embedded tissues and that miR-365 may act as an onco-miR in cutaneous SCC both in vitro and in vivo. Overexpression of miR-365 induced tumorigenicity of HaCaT cells, and silencing of miR-365 using an antagonist-365 could reduce A431 tumor growth in vivo. These findings demonstrate that miR-365 may be a carcinogenic factor in cutaneous SCC and a potential target in cutaneous SCC therapy. The overexpression of miR-365 in cutaneous SCC and its role in differentiation of cutaneous SCC cells as well as in the venous invasion of the tumor suggest that the expression level of miR-365 can be used as a potential indicator both in the clinical diagnosis and treatment.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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References


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