miR-375 inhibits cell growth and correlates with clinical outcomes in tongue squamous cell carcinoma

LINGFEI JIA1,2*, YIPING HUANG3*, YUNFEI ZHENG1*, MINGYUE LYU1, CHUNAN ZHANG1, ZHEN MENG1,2, YEHUA GAN1,2 and GUANGYAN YU1

1Department of Oral and Maxillofacial Surgery, 2Central Laboratory and 3Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing 100081, P.R. China

Received September 17, 2014; Accepted December 10, 2014

DOI: 10.3892/or.2015.3759

Abstract. miR-375 has been implicated in various types of cancers. However, its role in tongue squamous cell carcinoma (TSCC) remains unclear. This study aimed to investigate the effects of miR-375 on cell growth and the prognosis of TSCC patients. Using quantitative reverse transcription-polymerase chain reaction, we evaluated miR-375 expression in TSCC samples and TSCC cell lines. The results showed that miR-375 expression was significantly reduced in the TSCC tissues and cell lines. A low level expression of miR-375 in TSCC patients was related to poor of prognosis. Moreover, the effects of miR-375 overexpression on cell proliferation, the cell cycle and the expression of Sp1 and cyclin D1 were examined in TSCC cells. We demonstrated that overexpression of miR-375 significantly inhibited the cell proliferation and cell cycle progression in TSCC cell lines. Overexpression of miR-375 inhibited Sp1 expression by targeting the 3’ untranslated region of the Sp1 transcript. The knockdown of Sp1 expression resulted in the subsequent downregulation of cyclin D1. Taken together, our study suggests that miR-375 inhibits the cell growth, and its expression is correlated with clinical outcomes in TSCC.

Introduction

Squamous cell carcinoma of the oral cavity and oropharynx is the sixth most frequent solid cancer worldwide (1). Tongue squamous cell carcinoma (TSCC) is the most common types of oral carcinoma and is well-known for its rapid proliferation (2). Prognostic assessment is critical for making better therapeutic choices for patients, and the tumor-node-metastasis (TNM) staging system is the key prognostic determinant for TSCC patients in clinical practice (3). However, conventional prognostic factors based on clinicopathological features remain inadequate, and are unable to discriminate tumors at the same clinical stage but with distinct clinical outcomes. The lack of efficient diagnostic and prognostic biomarkers is responsible for the high mortality rates (4). Therefore, it is necessary to identify biomarkers that can provide additional prognostic information beyond the standard clinical prognostic system for TSCC patients (5).

Recent studies have shown that the expression of miR-375 is associated with various clinicopathological parameters in oral squamous cell carcinoma (OSCC) (11), and is correlated with clinical outcomes in head and neck squamous cell carcinoma (HNSCC) (12). However, these studies included heterogeneous groups of patients with cancers from different subsites of the head and neck, including tongue, gingival, buccal, lip, larynx and oropharynx carcinomas. Since the gene expression patterns in HNSCC at different subsites may not be equally associated with cancer prognosis (13,14), a study focusing on a specific anatomical subsite, such as the anterior body of the tongue, is likely to provide more accurate and clinically useful information on the prognostic significance of miR-375.

miR-375 frequently shows reduced expression in various cancers, and it may act as a tumor suppressor by targeting...
JAK-2 (15), PDK-1 (16), IGF1R (17) and ASCL-1 (18). However, the role of miR-375 and the underlying mechanism in TSCC remain to be explored. Sp1 is a target gene of miR-375 in cervical squamous cell cancer (19). Moreover, Sp1 upregulates cyclin D1 expression by binding to the cyclin D1 promoter (20) and is required for cell cycle progression through the G1 phase (21). Thus, it was reasonable to test whether miR-375 directly targets Sp1 and subsequently downregulates cyclin D1 to induce cell cycle arrest in TSCC.

In the present study, we examined the expression of miR-375 in 105 pairs of TSCC samples and matched adjacent normal tissues, and the association of miR-375 expression with the overall survival of TSCC patients. Moreover, we tested whether miR-375 downregulates Sp1 expression by targeting the Sp1 transcript and subsequently downregulates cyclin D1 to induce cell cycle arrest in TSCC cells. Our results suggest important roles for miR-375 in TSCC pathogenesis and support its potential application in the evaluation of patient prognosis.

Materials and methods

Clinical specimens. Paired primary TSCC samples and adjacent histologically normal tissues were obtained from 105 patients who were admitted to the Department of Oral and Maxillofacial Surgery of the Peking University School of Stomatology (Beijing, China) between 2008 and 2011. None of the patients received treatment prior to radical surgical treatment. Tumor tissues and matched nonmalignant tissues, at least 1.5 cm distal to the tumor margins, were snap-frozen in liquid nitrogen and then stored at -80°C until use. None of the TSCC patients had received adjuvant chemotherapy or radiotherapy before surgery. The clinicopathological characteristics of the patients are summarized in Table I. This study was approved by the Ethics Committee of the Peking University School of Stomatology, and all samples were obtained from patients who had signed informed consent forms.

Cell lines and culture. Primary normal human oral keratinocyte (HOK) cells were purchased and cultured in a keratinocyte growth medium (ScienCell Research Laboratories, San Diego, CA, USA) according to the manufacturer's instructions. Human TSCC cell lines, SCC-15 and CAL-27, were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Vector construction. A 417-base pair (bp) fragment of the 3' untranslated region (UTR) of the human Sp1 transcript containing the miR-375 binding site (19) was amplified by polymerase chain reaction (PCR). It was then cloned into a modified version of pcDNA3.1(+) containing a firefly luciferase reporter gene (a gift from Brigid L.M. Hogan, Duke University, Durham, NC, USA) (22) and named wild-type Sp1 3'UTR. The following primers were used to clone the wild-type Sp1 3'UTR: sense, 5'-GAA TGA TAG CCC AGT TGT TAA AGA CAA CTG GGC TAT CAT TC-3' and antisense, 5'-ACA AGA TTT CTT TAA AGA CAA CTG TTC TTG TAT TTG TGG GCC GCC CTT TC-3'. Site-directed mutagenesis of the miR-375 binding site in the Sp1 3'UTR was performed using a Site-Directed Mutagenesis Kit (SBS Genetech, Beijing, China). The product was named mutant Sp1 3'UTR.

The following primers were used to clone the mutant Sp1 3'UTR: sense, 5'-GAA TGA TAG CCC AGT TGT TAA AGA AAT CTT GT-3' and antisense, 5'-ACA AGA TTT CTT TAA AAT CTT GT-3' and antisense, 5'-ACA AGA TTT CTT TAA AAT CTT GT-3'. A chemically modified double-stranded miR-375 mimic and the corresponding miRNA mimic control were designed and purchased from RiboBio Co. (Guangzhou, China). A small interfering RNA (siRNA) targeting the human Sp1 transcript (siSp1) and the corresponding scrambled control were purchased from Integrated Biotech Solutions Co. (Shanghai, China). The miRNA and siRNA sequences are listed in Table II.

Table I. Relationship between expression of miR-375 and the clinicopathological factors in the 105 TSCC patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No.</th>
<th>miR-375 (T/N) (mean ± SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>49</td>
<td>0.194±0.245</td>
<td>0.132</td>
</tr>
<tr>
<td>Female</td>
<td>56</td>
<td>0.131±0.169</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>65</td>
<td>0.172±0.243</td>
<td>0.424</td>
</tr>
<tr>
<td>≥60</td>
<td>40</td>
<td>0.142±0.138</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>67</td>
<td>0.189±0.232</td>
<td>0.041</td>
</tr>
<tr>
<td>T3-T4</td>
<td>38</td>
<td>0.111±0.153</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>45</td>
<td>0.168±0.257</td>
<td>0.896</td>
</tr>
<tr>
<td>Moderate</td>
<td>47</td>
<td>0.159±0.178</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>13</td>
<td>0.137±0.122</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>59</td>
<td>0.175±0.211</td>
<td>0.419</td>
</tr>
<tr>
<td>III-IV</td>
<td>46</td>
<td>0.142±0.208</td>
<td></td>
</tr>
<tr>
<td>Node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>62</td>
<td>0.178±0.247</td>
<td>0.266</td>
</tr>
<tr>
<td>Yes</td>
<td>43</td>
<td>0.135±0.136</td>
<td></td>
</tr>
<tr>
<td>Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surviving</td>
<td>60</td>
<td>0.197±0.235</td>
<td>0.028</td>
</tr>
<tr>
<td>Deceased</td>
<td>45</td>
<td>0.111±0.158</td>
<td></td>
</tr>
</tbody>
</table>

T, tumor; N, nonmalignant tissue; T1-T4, T stage of the TNM classification system.
Transient transfection. Cells were plated into 6-well plates before transfection. After reaching 80% confluency, the cells were transfected with 100 nM miRNA mimic or siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s procedure. The cells were harvested 48 h after transfection.

RNA isolation and quantitative reverse-transcription (qRT)-PCR. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's procedure, and then reverse-transcribed into complementary DNA (cDNA) using a cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was conducted with the ABI Prism 7500 real-time PCR system (Applied Biosystems). The following thermal settings were used: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The primers used for miR-375, Sp1, cyclin D1, U6 (internal control for miRNAs) and β-actin (internal control for mRNAs and lncRNAs) are listed in Table II. The data was analyzed using the 2-ΔΔCt relative expression quantity as described previously (8).

Cell proliferation assays. Cell proliferation was determined using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). SCC-15 and CAL27 cells were plated into 96-well plates (2x10³ cells/well). After transfection with miR-375 mimic or the scrambled control, CCK-8 (10 µl) was added to each well at 24, 48, and 72 h, and the plates were incubated at 37˚C for 3 h. The absorbance at 450 nm was measured using a microplate spectrophotometer (Bio-Tek Instruments).

Colony formation assay. At 24 h after transfection, the cells were plated into 60-mm dishes at an initial density of 500 cells/dish and cultured until colonies were visible (10 days). Cell colonies were fixed with cold methanol for 20 min and stained with 0.25% crystal violet for 30 min. The number of cells that formed a clone was calculated.

Cell cycle analysis. At 48 h post-transfection, CAL27 cells were harvested by trypsinization and washed with phosphate-buffered saline. The cell cycle was analyzed with fluorescence-activated cell sorting (FACS) using FACScalibur flow cytometry (Becton Dickinson, Bedford, MA, USA) as described previously (8).

Dual luciferase reporter assay. Luciferase assays were performed as described previously (23). Briefly, SCC-15 and CAL27 cells grown in a 48-well plate were cotransfected with the miR-375 mimic or the miRNA mimic control (100 nM), the luciferase reporter plasmid (40 ng/well) and pRL-TK, a plasmid expressing Renilla luciferase (4 ng/well; Promega). Luciferase activity was measured 24 h after transfection using the Dual-luciferase reporter assay system (Promega).

Western blot analysis. Western blotting was performed as described previously (8). Primary antibodies against Sp1 (Cell Signaling Technology, Beverly, MA, USA), cyclin D1 and β-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted at 1:1,000. The intensities of the bands obtained by western blotting were quantified using ImageJ software (http://rsb.info.nih.gov/ij). The background was subtracted, and the signal of the target bands was normalized to that of the β-actin band.

Statistical analysis. Statistical analyses were performed using SPSS for Windows ver. 16.0 (IBM, Armonk, NY, USA). All data are expressed as the mean ± standard deviation (SD). Differences between groups were analyzed by the Student's t-test. A one-way ANOVA was used to analyze the relationship between miR-375 expression and the clinicopathological characteristics. Survival curves were constructed by the Kaplan-Meier method and were compared using the log-rank test. The Cox regression model was applied to simultaneously adjust all potential prognostic variables. Experiments with cell cultures were conducted at least in triplicate. A two-tailed

Table II. Sequences of RNA and DNA oligonucleotides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense strand/sense primer (5’-3’)</th>
<th>Antisense strand/antisense primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers for qRT-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-375 RT primer</td>
<td>GTCGTATCCAGTGCAAGGTCCTGAGGTAT</td>
<td>TCACGTGGATACGACTCACGC</td>
</tr>
<tr>
<td>miR-375</td>
<td>GTCGAGGGTCCCGAGGT</td>
<td>TCACGTGGATACGACTCACGC</td>
</tr>
<tr>
<td>U6</td>
<td>TCTGGTTCGCAAGCACACTA</td>
<td>AGCCGGTTCGAGGTTCGAGGT</td>
</tr>
<tr>
<td>Sp1</td>
<td>ACCAGAATAAGAAGGGAGG</td>
<td>GGTGTAATAAGAACCGCTGAA</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>GTCGTCGCAAGTGGGAGG</td>
<td>ATCCAGTGCCGAGGAG</td>
</tr>
<tr>
<td>β-actin</td>
<td>CGG GAA ATC GTG GTG GAC</td>
<td>CAG GCA GCT GTG AGC TCT T</td>
</tr>
<tr>
<td>miRNA mimic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-375 mimic</td>
<td>UUUGUUCGUGUCGGACGCAU</td>
<td>AAAACAGCAAGGCGAGCGCCACU</td>
</tr>
<tr>
<td>miRNA mimic control</td>
<td>UUUGUACUACACAAAGUACUG</td>
<td>AAACUAGUUGUGUUUCAAUGAC</td>
</tr>
<tr>
<td>siRNA duplexes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp1 siRNA</td>
<td>UGUAGAGUCUGCAACACUGACAGCACGTTT</td>
<td>GACAGGUCAGUUGGGCAGACACUCATG</td>
</tr>
<tr>
<td>siRNA control (scramble)</td>
<td>UUC UCC GAA GUC GUC AGC UTT</td>
<td>ACG UGA CAC GGU CGG AGA ATT</td>
</tr>
</tbody>
</table>

Transcript transfection. Cells were plated into 6-well plates before transfection. After reaching 80% confluency, the cells were transfected with 100 nM miRNA mimic or siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. The cells were harvested 48 h after transfection.
value of $P<0.05$ was considered to indicate a statistically significant difference.

**Results**

miR-375 expression is reduced in TSCC and is correlated with cancer progression. The expression of miR-375 was examined in 105 pairs of tongue cancer tissues and matched adjacent histologically normal tissues by qRT-PCR. The average expression level of miR-375 was significantly decreased in the tumor tissues when compared with the level in the normal tissues (Fig. 1A). Moreover, miR-375 expression was substantially reduced in the TSCC cell lines, SCC-15 and CAL27, compared with that in the primary normal human oral keratinocyte (HOK) cells. Data are shown as the mean ± SD (*$P<0.01$).

Decreased miR-375 expression is associated with poor overall survival in the TSCC patients. With the mean fold change of miR-375 expression (T/N) chosen as the cut-off point, the patients were divided into high and low expression groups. The patients with high miR-375 expression survived significantly longer than those with low miR-375 expression (Fig. 2). Moreover, we conducted multivariable Cox proportional hazards analysis to identify whether miR-375 is an independent prognostic covariate for TSCC patients. Our results showed that low miR-375 expression in TSCC was associated with a poor prognosis in terms of overall survival ($P=0.044$, relative risk =0.449), independent of the other clinical covariates (Table III).

Overexpression of miR-375 inhibits cell proliferation and cell cycle progression in TSCC cell lines. To investigate the biological function of miR-375 in TSCC, we transfected the miR-375 mimic into TSCC cells, and confirmed that miR-375 expression was significantly increased in the SCC-15 and CAL27 cells at 48 h post-transfection (data not shown). CCK-8 and colony formation assays showed that overexpression of miR-375 significantly inhibited the proliferation of the SCC-15 and CAL27 cells (Fig. 3A and B), and resulted in substantial accumulation of cells in the G1-phase (Fig. 3C).

miR-375 inhibits Sp1 expression by targeting its 3'UTR. According to a previous study, the human Sp1 transcript contains a putative miR-375 target site in its 3'UTR (19) (Fig. 4A). We constructed a luciferase reporter plasmid containing the human Sp1 3'UTR with the miR-375 target site intact (wild-type) or mutated. SCC-15 and CAL27 cells were co-transfected with the miR-375 mimic, the miRNA mimic control, and the reporter plasmid. Ectopic expression of miR-375 significantly suppressed the luciferase activity of the reporter with the wild-type Sp1 3'UTR, but not that of the reporter with the mutant Sp1 3'UTR, at 24 h post-transfection (Fig. 4B). Meanwhile, overexpression of miR-375 significantly decreased the mRNA and protein levels of endogenous Sp1 at 48 h post-transfection compared to the negative control (Fig. 4C and D).

Overexpression of miR-375 downregulates cyclin D1 expression by targeting Sp1. To explore whether miR-375 also regulates the expression of cyclin D1, we measured its mRNA and protein levels in SCC-15 and CAL27 cells transfected with the miR-375 mimic and the miRNA mimic control. Overexpression of miR-375 significantly downregulated the mRNA and protein levels of cyclin D1 (Fig. 5A and B). A previous study reported that Sp1 binding sites in the cyclin D1 promoter are involved in transcriptional activation of the gene (20). We constructed a luciferase reporter plasmid containing
Figure 3. Overexpression of miR-375 inhibits proliferation, colony formation, and cell cycle progression. (A) Inhibition of cell proliferation by overexpression of miR-375. SCC-15 and CAL27 cells were transfected with the miR-375 mimic or the miRNA mimic control (negative control, NC). Proliferation was measured using CCK-8 assays. (B) Inhibition of colony formation by overexpression of miR-375. Representative results of colony formation by the SCC-15 and CAL27 cells transfected as in A are shown. (C) Inhibition of cell cycle progression by overexpression of miR-375. SCC-15 and CAL27 cells were transfected as in A. Cells were stained with propidium iodide (PI) at 48 h post-transfection and analyzed by FACS. Data are shown as the mean ± SD ("P<0.05, **P<0.01).
the cyclin D1 promoter with the Sp1 binding sites. When SCC-15 or CAL27 cells were co-transfected by the miR-375 mimic and the cyclin D1 promoter luciferase reporter plasmid, the luciferase activity level was significantly reduced compared with the control (Fig. 5C). Moreover, luciferase activity was markedly suppressed by the introduction of siRNA against the Sp1 transcript into the SCC-15 and CAL27 cells, which mimicked the effects of miR-375 overexpression (Fig. 5D). In addition, the transfection of Sp1 siRNA in the SCC-15 or CAL27 cells significantly reduced the protein expression levels of both Sp1 and cyclin D1, as expected (Fig. 5E).

Knockdown of Sp1 inhibits cell proliferation and cell cycle progression in TSCC cell lines. To test whether the downregulation of Sp1 is involved in the antitumor actions of miR-375, we knocked down Sp1 expression in the SCC-15 and CAL27 cells. The knockdown of Sp1 in the TSCC cells suppressed cell proliferation (Fig. 6A) and colony formation (Fig. 6B), and induced a G1 phase arrest (Fig. 6C).

Discussion

In the present study, we demonstrated that miR-375 expression was reduced in TSCC specimens and cell lines, and was correlated with tumor size and patient mortality. Moreover, our results suggest that low expression of miR-375 was correlated with shorter survival of TSCC patients, and served as a prognostic factor independent of other clinicopathological factors. Similarly, a study of 123 HNSCC patients, including patients with cancers of the tongue, gingival and buccal tissue, revealed that miR-375 expression was reduced and was correlated with clinical stage and tumor size (11). However, the prognostic implication of miR-375 in oral carcinoma was not analyzed. Variation in correlation with clinicopathological parameters and the prognostic significance of miR-375 in previous studies may be attributable to differences in sample size and the inclusion of tumors from different subsites of the head and neck. Thus, our study of 105 patients with HNSCC at a single anatomical site, the anterior tongue, is valuable for confirming the prognostic value of miR-375 in TSCC patients.

Previous reports have shown that low miR-375 expression is also related to poor survival and a poor therapeutic outcome in gastric cancer (24), esophageal squamous cell carcinoma (17,25), non-small cell lung cancer (26,27) and glioma (28). Thus, as a tumor suppressor, miR-375 seems to be a useful prognostic factor for various types of malignant tumors. However, upregulation of miR-375 was reported to correlate with a poor prognosis in pediatric acute myeloid leukemia (29). Variation in the prognostic significance of miR-375 in previous studies may be attributable to differences in cancer type.

The present study also demonstrated that overexpression of miR-375 in TSCC cell lines inhibited cell proliferation and cell cycle progression, consistent with previous studies of avian leucosis (30) and colorectal cancer (31). We focused on the biological effects of miR-375 on TSCC growth since miR-375 was significantly correlated with tumor size but showed no relationship with other clinicopathological parameters. Furthermore, we confirmed that Sp1 is a direct target gene of miR-375, consistent with a previous study of squamous
cervical cancer (19). Since Sp1 is an important transcription factor in cellular processes (32-34), it is reasonable that the downregulation of Sp1 contributes to the inhibition of TSCC cell growth.

We further demonstrated that overexpression of miR-375 in TSCC cells downregulated cyclin D1 via Sp1, leading to cell cycle arrest (Fig. 7). Cyclin D1 is a key protein involved in cell cycle control and is essential for G1 to S transition (35,36), and is regulated by Sp1 (20). More importantly, several studies have shown that the dysregulation of cyclin D1 contributes to HNSCC development (37-39) and our recent study also demonstrated that cyclin D1 plays an important role in cell growth and cell cycle progression in TSCC cell lines (8). Therefore, the fact that miR-375 inhibited cell proliferation and blocked G1 to S transition can at least partially be explained by the Sp1-dependent downregulation of cyclin D1.

However, as a ubiquitous transcription factor, Sp1 is also reported to regulate cell cycle progression by interaction with...
Figure 5. miR-375 downregulates cyclin D1 expression by reducing Sp1 expression. (A) Downregulation of cyclin D1 mRNA expression by miR-375 overexpression. SCC-15 and CAL27 cells were transfected with the miR-375 mimic or the miRNA mimic control (NC). The mRNA expression of cyclin D1 was quantified by qRT-PCR 24 h after transfection. (B) Downregulation of cyclin D1 protein expression by miR-375 overexpression. SCC-15 and CAL27 cells were transfected as in A. After 48 h, cyclin D1 and β-actin (internal control) were detected by western blotting. (C) miR-375 increased the cyclin D1 promoter luciferase reporter activity. SCC-15 and CAL27 cells were co-transfected with a firefly luciferase reporter plasmid containing the cyclin D1 promoter, the pRL-TK plasmid, and the miR-375 mimic or the miRNA mimic control (NC). After 24 h, firefly luciferase activity was measured and normalized to Renilla luciferase activity. (D) Sp1 knockdown upregulated cyclin D1 promoter luciferase reporter activity. SCC-15 and CAL27 cells were co-transfected with the firefly luciferase reporter plasmid containing the cyclin D1 promoter, pRL-TK, and siRNA control (scramble) or Sp1 siRNA. After 24 h, firefly luciferase activity was measured and normalized to Renilla luciferase activity. (E) Expression analysis of cyclin D1 after Sp1 knockdown. SCC-15 and CAL27 cells were transfected with siRNA control (scramble) or Sp1 siRNA. After 48 h, Sp1, cyclin D1 and β-actin (internal control) were detected by western blotting. Data are shown as the mean ± SD (**P<0.01).
Figure 6. Sp1 knockdown mimics the effects of miR-375 overexpression. (A) Sp1 knockdown suppressed cell growth. SCC-15 and CAL27 cells were transfected with the siRNA control (scramble) or Sp1 siRNA. Cell proliferation was measured by the CCK-8 assay. (B) Sp1 knockdown inhibited colony formation. Representative results of the colony formation assay in SCC-15 and CAL27 cells transfected as in A are shown. (C) Cell cycle arrest due to Sp1 knockdown. SCC-15 and CAL27 cells were transfected as in A. Cells were stained with propidium iodide (PI) at 48 h post-transfection and analyzed by FACS. Data are shown as the mean ± SD (*P<0.05, **P<0.01).
many other genes, including those encoding retinoblastoma protein (40,41), retinoblastoma-related protein p107 (42), the transcription factor E2F (43), p53 (44) and mdm2 (45). We cannot exclude the possibility that miR-375 inhibits TSCC growth by simultaneously regulating other cell cycle regulatory proteins via Sp1. Further study is needed to identify other possible signaling pathways downstream of miR-375 in the suppression of TSCC growth.

In conclusion, miR-375 expression was reduced in TSCC and its downregulation was correlated with a poor prognosis in TSCC patients. Overexpression of miR-375 in TSCC cells inhibited proliferation and induced cell cycle arrest through an Sp1-cyclin D1 signaling pathway. These results suggest that miR-375 inhibits cell growth and is correlated with clinical outcomes in TSCC.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81402255, 81472527), and the Foundation of the Peking University School and Hospital of Stomatology (PKUSS20140104).

References