LncRNA TEX41 promotes the proliferation and migration of squamous cell carcinoma cells by upregulating Atg5 via sponging miR-153-3p

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ABSTRACT

This study was conducted to investigate if lncRNA TEX41 could have effects on SCC. The aim was to confirm that TEX41 could promote SCC progression by up-regulating Atg5 via miR-153-3p. TEX41, miR-153-3p, and Atg5 mRNA expression were examined by qRT-PCR. Western blot was used to examine Atg5 protein expression. CCK-8 assay and transwell assay were used to examine SCC cell proliferation and migration. Dual-luciferase reporter assay was used to forecast the binding site of miR-153-3p with Atg5 or TEX41. TEX41 expression was enhanced in SCC tissues and cells. TEX41 knockdown could reduce the SCC cell proliferation and migration. There was a binding site between TEX41 and miR-153-3p, and TEX41 negatively adjusted miR-153-3p in SCC cells. Atg5 was bonded with miR-153-3p, which was adjusted by TEX41. Our study revealed that TEX41 expression was increased in SCC cell lines and tissues. TEX41 could aggravate SCC progression through adjusting the miR-153-3p/Atg5 axis, providing a key target for SCC.

Introduction

As one of the seven malignant tumors (1), head and neck squamous cell carcinoma (HNSCC) has nearly a million cases new cases per year (2,3), being usually related to alcohol, tobacco, betel nut and HPV (4,5). The main treatment of HNSCC is radiotherapy (RT), chemotherapy (CT), monotherapy, and combination therapy with molecularly targeted drugs and surgery, but these treatments are highly toxic (6-8). It has already shown the drug resistance of these treatments in cases (6-8), and resistance to RT is one of the main reasons for the poor survival rate for HNSCC (6-8). To improve the effect of treatment and reduce toxicity and treatment expense, it is essential to explore new targets and drugs (6).

Long non-coding RNAs (lncRNAs) can transcript larger than two hundred nucleotides (9). It has been reported that lncRNAs play different roles by interacting with microRNA or binding to a variety of regulatory proteins, and are related to cell proliferation, differentiation, angiogenesis and apoptosis (9,10). In lots of studies, lncRNAs could as potential tumor suppressors or oncogenes (9,11-13) because they are easily detected in body fluids and stably expressed during cell differentiation, thus can help us to diagnose and determine prognosis in cancer (14,15). Such as, lncRNA FTX is up-regulated in CRC cells proliferation, migration and invasion (16). Besides, lncRNA KCNQ1OT1 was oncogenic in a number of cancers, and it could enhance retinoblastoma (RB) cell proliferation and migration via adjusting the miR-153-3p/HIF-1α axis (17). lncRNA testis expressed 41 (TEX41) is a novel discovered tumor-associated enhancer RNA (18) that can be involved in a variety of cancers through the uptake of different miRNAs (19,20). The expression of TEX41 was up-regulated in melanoma cells. TEX41 bound to miR-103a-3p and regulated C1QB, and TEX41 knockout inhibited the proliferation, migration and invasion of melanoma cells and induced apoptosis (9). However, the function of TEX41 for SCC is not yet certain.

As small non-coding RNAs (17,21), microRNAs (miRNAs) play a role in many cancers, such as melanoma, medullary thyroid cancer and so on. However, the effect on miR-153-3p in SCC has not yet been observed. Tanshinone IIA induced autophagy in OSCC in a multi-pronged manner by inducing the Beclin-1/Atg7/Atg12-Atg5 pathway and inhibiting PI3K/Akt/mTOR pathway (22), proving that Atg5 protein is involved in the progression of SCC.

In our study, it examined that TEX41 and miR-153-3p took part in promoting tumor, and demonstrated that TEX41 could adjust miR-153-3p. It also revealed that TEX41 can regulate the expression of autophagy-related protein 5 (Atg5) through miR-153-3p. The data revealed that miR-153-3p and TEX41 are new therapeutic targets for SCC.

Materials and Methods

Tissue samples

Human pharyngeal squamous cell carcinoma tissue samples and lateral normal tissue samples were extrac-
Cell culture

Human oral epithelial cell line HIOEC was bought from Shanghai iCell Bioscience Inc. (Shanghai, China) and exclusively saved in an incubator at 37°C and 5% CO2. The HNSCC cell line FADU, Cal27 and SCC25 were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and saved in RPMI medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 1% penicillin-streptomycin (Gibco BRL, Gaithersburg, MD, USA) and 5% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) in a humidified atmosphere with 5% CO2.

Cell transfection

FADU cells were transfected with Lipo2000 (Sigma-Aldrich, St. Louis, MO, USA) for small interfering RNA (siRNA) against TEX41 (si-TEX41) and overexpression vector plasmid (pcDNA-TEX41), and their negative control group (si-NC) or plasmid (pcDNA-NC), miR-153-3p mimics and miR-153-3p inhibitor (anti-miR-153-3p).

RNA extraction and qRT-PCR

Total RNA was extracted from tissues or cells using TRIZol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reverse transcribed into complementary DNA using reverse transcriptase. Besides, SYBR Premix Ex Taq TM II (TaKaRa, Tokyo, Japan) was used to prepare the qRT-PCR laboratory reaction system. It was used to analyze the relative expression of genes that the 2-ΔΔCt method. The primer sequences are shown in Table 1.

Cell counting kit-8 assay

The cells are seeded in 96-well cell plates. CCK-8 solution (Vazyme, Nanjing, China) was added at 0, 24, 48 and 72 h, respectively. Two hours later, the OD value was examined at 450 nm.

Transwell assay

The density of cell suspension was adjusted to 1×10^5 cells/mL. Next, 100 μL of it was added to a transwell chamber (Corning Costar, NY, USA) in a 24-well plate, 750 μL of the whole medium was added to each well, and the culture was incubated at 37°C. After 24 hours, non-migratory cells were removed from the upper surface of the filter. The cells on the lower surface of the filter were fixed with 4% paraformaldehyde, stained with crystal violet, dried and photographed for counting.

Dual-luciferase reporter assay

Wild-type and mutant-type luciferase vectors of TEX41/Atg5 were established. Next, vectors were transfected into FADU cells with pRL-TK vector (Promega, Madison, WI, USA), miR-153-3p mimics and its normal control mimics. After 48 h, 100 μL of supernatant was harvested to determine the Luciferase activity (Promega, Madison, WI, USA).

Western blot analysis

Total protein was extracted from collected tissues and cells with a Lysis mix. After denaturation, the samples were electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gel. Besides, proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) and then blocked with tris-buffered saline-casein containing Tween 20 (TBST; 10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.1% Tween20). The buffer was transferred to a solution containing 5% skim milk and nonspecific antigens were blocked for 30 min at room temperature. Then, primary antibody β-actin and anti-Atg5 (Abcam, Cambridge, MA) were added and incubated overnight at 4°C, then immersed in membrane TBST 3 times (15 min/time). The secondary antibody (1:1000) was added to the membrane which with incubated for 1 hour and washed 3 times (15 min/time) by TBST. At last, the bands were visualized using an ECL chemiluminescence solution (Beyotime Biotechnology, Shanghai, China).

Statistical analysis

GraphPad Prism 8.0 (La Jolla, CA, USA) was used for statistical calculation. One-way analysis of variance and student's t-test were used for multiple groups and two-group comparisons, respectively. The mean ± standard deviation (SD) was expressed in the findings. Each assay was performed in three independent biological replicates. P < 0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Sequence (5obilettal '3')</th>
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<tbody>
<tr>
<td>TEX41</td>
<td>Forward: TCATCTGTGAGGACCCTGTGAC GCCACAGGAGAAGCTGAGTT</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACACCTCCAGCTGGGTTGCATAGTCACAAAAGT</td>
</tr>
<tr>
<td>miR-153-3p</td>
<td>Forward: CTCGAATGGGATCTGCGAGTGGAGTCGGCAATTCAATG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AAATCCCTTCGACTGTCCCAAAAC</td>
</tr>
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<td>ATG5</td>
<td>Forward: TGAGGATGTCGTTT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGTTTCCGATTGATGGCCC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: CAC-CCTGAAAGTACCATG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTAGCAATGGTATGACCTG</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: CTCGGTTCGGCCGACACA</td>
</tr>
<tr>
<td></td>
<td>Reverse: AACGCTTCACGAATTTGCGT</td>
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Results

TEX41 was greatly enhanced in SCC tissues and cell lines

Primarily, we examined the expression of TEX41 in normal oral mucosa tissue samples and SCC tissue samples. Compared with normal tissues, we discovered that TEX41 expression was higher in SCC tissues (Figure 1 a1). Moreover, TEX41 in SCC cell lines expression was examined by qRT-PCR. Compared with normal cell lines, the findings displayed that TEX41 expression was significantly enhanced in SCC cell lines (Figure 1 a2).

Inhibition of TEX41 inhibited SCC cell proliferation and migration

Next, TEX41 knockdown after transfecting siRNA (si-TEX41) into FADU cells and detected cell proliferation and migration. The findings showed that si-TEX41 descended TEX41 expression (Figure 2a). Compared with the si-NC group, our data displayed that si-TEX41 obviously reduced SCC cell proliferation by CCK-8 assay (Figure 2b). Besides, si-TEX41 significantly reduced the SCC cell migration (Figure 2c, 2d). These findings pointed out that down-regulation of TEX41 would repress SCC cell proliferation and migration.

TEX41 targeted miR-153-3p in FADU cells

We forecasted that TEX41 was bonded with miR-153-3p (Figure 3a). Dual-luciferase reporter assay was used to detect the luciferase activity in FADU cells. (b) Results of miR-153-3p in SCC and normal tissues were examined by qRT-PCR. (c) Results of miR-153-3p expression in FADU, SCC25, and CAL27 were examined by qRT-PCR. (d) Si-TEX41 overexpression model (Figure 3f). According to Figure 3g, overexpression of TEX41 obviously inhibited miR-153-3p expression (Figure 3g). Above all, we conclude from the results that TEX41 and miR-153-3p acted upon each other, and TEX41 inhibited miR-153-3p expression in SCC.

Inhibited miR-153-3p reversed the effects of TEX41 knockdown on SCC cell proliferation and migration

We discovered that anti-miR-153-3p would obviously repress miR-153-3p in SCC by transfecting si-TEX41 and anti-miR-153-3p into FADU cells with si-TEX41 (Figure 4a). CCK-8 assay displayed that anti-miR-153-3p could obviously reverse si-TEX41-induced descent in FADU cell proliferation (Figure 4b). Besides, the transwell assay displayed that anti-miR-153-3p partially inhibited si-TEX41-induced FADU cell migration (Figure 4c, d). All these results...
suggested that TEX41 had taken effect on SCC by targeting miR-153-3p.

**miR-153-3p specifically modulated the expression of ATG5 in SCC cells**

In the SCC tissues and cells, our data displayed that ATG5 expression was enhanced (Figure 5a, b). And it displayed that miR-153-3p significantly regulated ATG5 expression in the picture (Figure 5c). What’s more, the TargetScan (http://www.targetscan.org/) was used to forecast that Atg5 was bonded with miR-153-3p (Figure 5d). Then, we could prove the relationship of prediction by Dual-luciferase reporter assay. Compared with the control group, the data was displayed that transfection of miR-153-3p mimics into FADU cells obviously descended the luciferase activity of the Atg5-wt group, but the luciferase activity of the Atg5-mut group had no obvious change (Figure 5e). It suggested that miR-153-3p overexpression could visibly descend both mRNA and protein of Atg5 expression in FADU cells (Figure 6a). To sum up, miR-153-3p would adjust ATG5 expression in SCC cells.

**TEX41 adjusted ATG5 in FADU cells via miR-153-3p**

Caused that miR-153-3p would regulate Atg5 and TXE41 also has a regulatory effect on miR-153-3p expression, we ulterior discussed if TEX41 would regulate miR-153-3p-mediated Atg5 expression. Our information indicated that the knockdown of TEX41 could significantly reduce Atg5 mRNA expression in FADU cells. This tendency could be reversed by transfection with an ti-miR-153-3p (Figure 7a). The above findings revealed that TEX41 could adjust Atg5 expression in SCC via miR-153-3p.

**Discussion**

HNSCC has a high mortality rate and the incidence of it has increased year by year (23). Surgery remains the mainstay of treatment for these diseases (6). Considering the inadequacy of surgery, we should explore new paths for better treatments.

More and more studies have shown that lncRNAs have been involved in the progression of various cancers. For SCC, FGD5-AS1 expresssi was obviously enhanced in OC and played an important role in promoting OC (24). LncRNA TFAP2A-AS1 overexpression promoted OSCC cell proliferation, migration and invasion (25). Besides, TEX41 plays a simulative role in various cancers, such as cervical cancer (26), compared with healthy subjects. Orlandella et al. (9) also discovered that lncRNA TEX41 was obviously overexpressed in pediatric B-cell acute lymphoblastic leukemia (9). Previous evidence confirmed that high TEX41 was related to poor
Figure 7. Atg5 expression was directly regulated by TEX41 in SCC cells. n=3 (a) Atg5 in FADU cells by transfection was tested through the qRT-PCR method. (P<0.05).

prognosis in skin cutaneous melanoma (SKCM), and it suggested TEX41 as a potential prognostic biomarker in SKCM (18,19). Yingna Zheng (20) et al. confirmed that TEX41 knockdown inhibited SKCM cell proliferation, migration and invasion by promoting apoptosis (20). Similarly, compared with normal cells and tissues, Mi Yang et al. (26) indicated that TEX41 had high expression in HNSCC, which suggested that TEX41 could promote tumours in HNSCC (26). Our data displayed that TEX41 expression was increased in SCC, TEX41 knockdown would significantly repress proliferation and migration in SCC cells. It is consistent with previous findings. It suggested that TEX41 was a potential therapeutic target of SCC. It has been reported that lncRNA can play a biological function as ceRNA of miRNA and affect the expression of miRNA target genes (17). It was that miR-153 has effects on many biological processes (especially in cancers), for example, inhibition of cancer development, therapeutic reaction, and resistance. The expression of miR-153 can also be adjusted by a variety of regulators, such as lncRNAs and circRNAs (27). In previous studies, up-regulation of miR-153-3p in tumor cells obviously enhanced T cell immunotherapy and tumor infiltration (28). Si-Schmachtenberg et al. (29) confirmed that miR-153-3p inhibition could reverse this trend. All of the findings demonstrated that TEX41 could regulate Atg5 expression of miRNA by bioinformatics and explored may downstream molecular mechanisms of TEX41 involved in SCC progression.

We forecasted that TEX41 was bonded with miR-153-3p. It was verified that miR-153-3p was directly targeted at TEX41 in SCC via the luciferase reporter assay. TEX41 regulated miR-153-3p expression in SCC. What counts was that repressed miR-153-3p could reverse the trend that si-TEX41-caused influenced SCC cell proliferation and migration. To sum up, TEX41 could inhibit miR-153-3p expression which was a molecular sponge in SCC. From our findings, we discovered that miR-153-3p would repress malignant expression in SCC by targeting Atg5. Atg5 can activate autophagy (30), playing a key role in tumor metabolism and immunity. Mostly, high expression of Atg5 was obviously connected with malignant behaviors (31). It was confirmed that autophagy could affect cell viability, migration, invasion, tumor cell dormancy and so on in a large number of studies (32). Autophagy promotes tumors in most cases (33). Qiong Zhang et al. (34) regulated gastric cancer (GC) through the effect of Atg5 on autophagy. In addition, it has been reported that lncRNAs could affect tumors by regulating autophagy. Qiong Wu et al. (35) proposed that autophagy promoted GC cell survival, migration, invasion and EMT, and their study revealed that lncRNA SnHG11 activated the Wnt/β-catenin pathway and aggravated GC progression via oncogenic autophagy (35-42). Epithelial-mesenchymal transition (EMT) plays a prominent role in SCC progression, and it is closely related to the proliferation and migration of cancer cells. Considering that EMT promotes the mobility of tumor cells and the formation of secondary tumors, this study revealed that TEX41 was high expression in SCC and TEX41 promoted SCC cell proliferation and migration, and it is reasonable to speculate that TEX41 can be a novel target of EMT in SCC.

In this study, compared with normal cells and tissue samples, Atg5 mRNA expression was up-regulated in SCC. It was roughly as same as the researches before. Besides, Atg5 expression was adjusted by miR-153-3p in SCC. It has been confirmed that Atg5 was bonded with miR-153-3p. What’s more, TEX41 knockdown could significantly reduce Atg5 expression, whereas miR-153-3p inhibition could reverse this trend. All of the findings demonstrated that TEX41 could regulate Atg5 expression in SCC via miR-153-3p, which is helpful in exploring the pathogenesis of SCC and provides clues to the diagnosis and treatment.

Conflict of Interests
The authors declared no conflict of interest.

References
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