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Original Research Article

Let-7c-5p/IGF2BP1 axis in Oral Squamous Cell Carcinoma represses progressions in vitro

Yan Wang, Hui Ren , Qing Wang , Shuang Li, Yanhua Lu , Wei Masu, Juan Zhang, Tao Wang*
The Second Central Hospital Dental Baoding City, Hebei Province, 072750, China

For correspondence:* ***Email:* vfj89895@126.com, ***Tel*****::** +86-17764436922

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Abstract

Purpose: *Oral squamous cell carcinoma (OSCC), a malignant tumor with high mortality, occurs in the head and neck. Let-7c-5p is involved in progression of many kinds of cancers. The purpose of this study was to determine the biological function of let-7c-5p in OSC.*
Methods: *RT-qPCR was performed to determine expressions of let-7c-5p and western blot measured protein expressions of IGF2BP1, E-cadherin, Vimentin and N-cadherin. Furthermore, dual-luciferase reporter test was used to evaluate the binding between IGF2BP1 and let-7c-5p. Moreover, MTT assay was employed to evaluate the cell viability while Transwell and Scratch assays were used to confirm cell metastasis. The cell apoptosis was validated using flow cytometry.*
Results: *Downregulation of let-7c-5p was recognized in OSCC cell lines. Let-7c-5p directly targeted and negatively modulated IGF2BP1. Other assays showed that let-7c-5p up-regulation had significant inhibitory roles on the cell viability, metastasis and tumor growth of OSSC cells. Additionally, EMT was also inhibited. Interestingly, upregulated let-7c-5p promoted apoptosis. Consequently, the abnormal expression of IGF2BP1 reversed functions of up-regulated let-7c-5p in OSCC cells.*
Conclusion: *These findings suggested that let-7c-5p behaves as an inhibitor regulating OSCC cell proliferation and migratory ability, which contributes to treatments by targeting IGF2BP1 in patients suffered from OSCC.*

Keywords: *Let-7c-5p, IGF2BP1, OSCC, Suppressor.*

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INTRODUCTION

OSCC is a common abdominal tumor with poor diagnosis due to persistent traits [1]. It is usually associated with differentiation and metastasis to lymph nodes [2]. OSCC is also a hostile tumor and its prognosis has shown less advancements in the recent 30 years [3]. It is frequently restricted to the tongue, but other common locations for tumor development are the lips and bottom of the mouth [4, 5]. This illness is amongst the leading causes of mortality and morbidity in South Central Asia, Melanesia, and Central and Eastern Europe [6], taking an approximated 90% of total oral neoplasms [7]. Beyond that, the incidence of OSCC has

increased rapidly among white female youths of the age 18 to approximately 44 years [8].

Despite advancements in chemotherapy, radiotherapy and surgery in treating oral cancer, the 5-year survival rate is lower than 0.5% in the past 3 decades, due to the high morbidity and death rate [9, 10]. Thus, in order to encourage a functional therapy of OSCC, the molecular mechanism during progression of OSCC is requested.

MicroRNAs (miRNAs) are small noncoding RNA molecules with roughly 22 nucleotides in length, which control expressions through permanent connecting to the 3' UTR of targeted mRNA and modulate the proliferation and metastasis of malignancies [11, 12]. Generally, miRNAs take part in many biological processes including proliferation and apoptosis [13, 14]. Scientist studies revealed that, abnormal expression of miRNAs may lead to the development and recurrence of certain diseases, mainly comprising of cancers [15]. Furthermore, miRNAs play vital roles in pathological and physiological processes of OSCC such as apoptosis, proliferation, metastasis, differentiation and metabolism[16, 17]. Let-7c-5p is immeasurably associated with the biological characteristics of tumor cells[18]. Furthermore, let-7c-5p expression is remarkably upregulated and interacted negatively with its target genes in lung cancer[19]. However, the system functionality and potential targets of let-7c-5p in OSCC remains unknown.

On the other hand, IGF2BP1 is an oncogene and a possible target to treat cancers [20]. Moreover, IGF2BP1 has been pointed out as a participant in the essential duties during proliferation and growth of cancer cells. Besides that, it also aids in the adhesion, apoptosis, migration, and invasiveness of cancer cells [21]. Additionally, IGF2BP1 is remarkably overexpressed in tumor cells in comparison with nonmalignant cells, implying that they may function as oncogenes in malignant diseases such as cancers [22]. IGF2BP1 has been regarded as a promising biomarker to treat cancers, and the use of inhibitors of IGF2BP1 might appear as a possible procedure for cancer therapy. Nevertheless, a few previous researchers have discovered its inhibitory duty in progressions of tumors [23, 24]. However, the effect of IGF2BP1 on OSCC remains unclear and misunderstood.

In the present study, expressions and roles of let-7c-5p were examined in OSCC cells. It was hypothesized that let-7c-5p functions as an inhibitor by silencing IGF2BP1 in OSCC.

METHODS

Cell Culture and transfection

SCC-9, SCC-25 and Cal27 cells (OSCC cell lines) and normal human OSCC cell line (HOK) obtained from ATCC (USA) were kept at constant in DMEM containing 10% fetal calf serum (Beyotime Shanghai China) and cultured in a moisture cylinder at 37℃ and 5% CO_2. SCC-9 and SCC-25 cells were transfected with let-7c-5p mimics, let-7c-5p inhibitor, miR-NC utilizing Lipofectamine 2000 (Thermo Fisher Scientific, US) based on the protocol of manufacturer.

Quantitative real time polymerization chain reaction (QRT-PCR)

Triazole reagent was utilized for removing all the RNA. Thereafter, complementary DNA was incorporated utilizing the Prime Script RT reagent kit (Abcam Cambridge UK). IGF2BP1 mRNA RT primer, forward sequence CGGTGAACGAGTT GCAGAAT, reverse sequence GCCTGGTTA CTCTGTCCCTT; GAPDH, forward sequence ACCCAGAAGACTGTGGATGG, reverse sequence TCAGCTCAGGGATGACCTTG; RT primers U6, forward sequence ACAGAGAA GATTAGCATGGC, reverse sequence TGGAC CATTTCTCGATTTGT and let-7c-5p RT primers, forward sequence GCGGCGTTGAGGT AGTAGGT, reverse sequence GTGCAGGGTC CGAGGTATTC[25] were evaluated with the SYBR Green Kit (Thermo Fisher Scientific, US). GPADH and U6 was internal controls of IGF2BP1 and let-7c-5p, respectively. The primers were outlined and harmonized by Gene Pharma. Expressions of let-7b-5p and IGF2BP1 were calculated by $2^{-\Delta\Delta Ct}$ method and the experiment was performed in groups of 3.

MTT Assay

Post transfection cells seeded onto 96-well plates were adjusted to 5000 cells per well followed by incubation for 5 days and addition of 15μl MTT (5mg/ml). Cells were cultured for 4h and the reaction was destroyed with 150μl DMSO. The measurement of absorbance was performed at 490nm utilizing a microplate reader. The experiment was run in triplicates.

Flow Cytometric Analysis (Apoptosis)

Determination of apoptosis, post-transfected cells were collected, rinsed and fixed by 70% ethanol for 24hours at −20℃. Subsequently, cells were cultivated 5µl Annexin V-FITC (Thermo Fisher Scientific, US) and 10µl propidium iodide (Abcam, Cambridge, UK). Cells were kept at

constant conditions for 30min at 25℃ and then evaluated by a FACS Canto II instrument and examined by Flow Jo.

Transwell invasion Assay

To evaluate the invasiveness of cells, transwell upper chambers were coated with matrigel. Basal chambers were added with DMEM containing 10% FBS. Cells in the lower compartment of the chamber were dyed by 0.5% crystal violet 24h after incubation. Numbers of cells from five randomly selected visual fields were calculated with the use of a light microscope.

Scratch Assay

OSSC cells transfected with MRNA-siRNAs or siRNA-NC were plated as a confluent monolayer in 8-well plates. When cells confluence reached to close to 80%, 'wounds' were formed using a pipette tip in the confluent cells. Cells were rinsed 3 times using PBS to eliminate the floating cells, then media with Chemokine ligand 12 (CXCL12) (40 ng/ml) (Beyotime, Haimen, China) was added to the cells placed at 37℃.

Dual-Luciferase Assay

The 3-UTR of IGF2BP1 with presumed let-7c-5p targeting sites (wild type) and haphazardly scrambled sequence (mutant type) were inlet into the pGL3 vector (Promega, USA). Wild-type or mutant type of IGF2BP1 with let-7c-5p mimics or miR-NC were co-transfected into cells. Dual-Luciferase Reporter System (Thermo Fisher Scientific, US) was used to evaluate the fluorescence after incubation for 48h.

Western Blot Analysis

All protein (Table 1) from post-transfected cells were segregated using RIPA, which was solved by 10% SDS-PAGE and then electro transferred onto PVDF at 60V for 1.5h. Membranes were sealed in 5% non-fat milk powder followed by the cultivation with primary and secondary antibodies listed in the table below at 4℃ for the whole night and examined by electrochemiluminescence kit. ImageJ software was applied for checking intensities of Bands.

Statistical Analysis.

All data were shown as mean ± SD and experiments were run in triplicate. Using SPSS 21.0 software, data were analyzed. Student's two-tailed t-test were used for comparisons between two groups while comparisons of groups over two were applied with one-way ANOVA. The significance was meaningful statistically when $P<0.05$.

Table 1: Protein antibodies

Examined Protein	Primary Antibody	Secondary Antibody
IGF2BP1	Anti-IGF2BP1/IMP1, (ab184305, Abcam, Cambridge, UK), Dilution rate 1:1000	Goat Anti-Rabbit IgG H&L (HRP) preadsorbed (ab7090, Abcam, Cambridge, UK) Dilution rate 1:900
E-Cadherin	Anti-E Cadherin (ab40772, Abcam, Cambridge, UK), Dilution rate 1:1000	
N-Cadherin	Anti- N Cadherin (ab76057, Abcam, Cambridge, UK), Dilution rate 1:1000	
Vimentin	Anti- Vimentin - Cytoskeleton Marker (ab92547, Abcam, Cambridge, UK), Dilution rate 1:1000	
GAPDH	Anti-GAPDH (ab8245, Abcam, Cambridge, UK), Dilution rate 1:2000	

RESULTS

Let-7c-5p is downregulated whilst IGF2BP1 is elevated in OSCC cells

Firstly, results of RT-qPCR revealed that let-7c-5p was greatly repressed in SCC-9, SCC-25 and Cal27 cells in comparison with the HOK cells (Figure 1a). Secondly, western blot assay examined the levels of IGF2BP1, revealing that IGF2BP1 was promoted dramatically in SCC-9, SCC-25 and Cal27 versus the HOK cells (Figure 1b). The results above showed that upregulated IGF2BP1 expression could also be part of the development of OSCC.

Let-7c-5p targets IGF2BP1 directly

Using Target Scan, binding sites between let-7c-5p and IGF2BP1 were shown (Figure 2a). We then determined the luciferase activity of let-7c-5p with IGF2BP1 (Figure 2b), indicating that the fluorescence in the wild type of IGF2BP1 with let-7c-5p mimics was decreased dramatically, whilst the fluorescence was insignificant in the transfection of mutant luciferase reporter constructs with let-7c-5p mimics. These results

suggested that let-7c-5p targeted the 3′-UTR of IGF2BP1 directly.

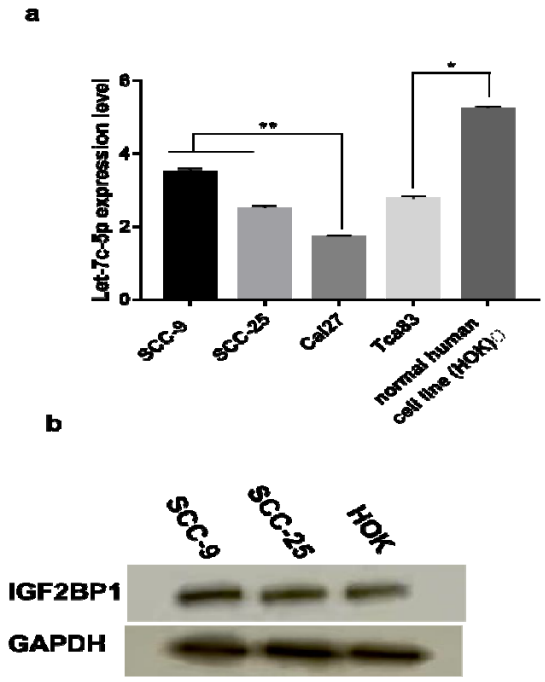

Figure 1: Let-7c-5p is downregulated whilst IGF2BP1 is upregulated in OSCC cell lines. (a) RT-qPCR analysis detected let-7c-5p expression in OSCC cells (SCC-9; SCC-25; Cal27; Tca83) **P < 0.01 vs normal human cell line, HOK. (**b**)Western Blot was applied to determined IGF2BP1 protein levels in SCC-9 and SCC-25 cells versus HOK cells

Let-7c-5p upregulation inhibits proliferation and metastasis of OSCC cells

Furthermore, functions of let-7c-5p were explored after upregulation. Results of RT-qPCR indicated that let-7c-5p expression was elevated greatly in OSCC cell lines (Figure 3a). Beyond that, MTT assay indicated that the cell viability of transfected OSCC cells was markedly reduced (Figure 3b). We continued to explore roles of the overexpressed let-7c-5p expression on regulating metastasis of OSCC cells. Results of transwell revealed that overexpressed let-7c-5p caused a lower cell invasion compared to the normal HOK cells (Figure 3c). Furthermore, Scratch Assay was used to examine the migratory ability, showing that compared to HOK cells, migratory capacities of OSCC cells were dramatically declined by let-7c-5p upregulation (Figure 3d). Results above showed that let-7c-5p played suppressive roles in biological functions of the OSCC cell lines.

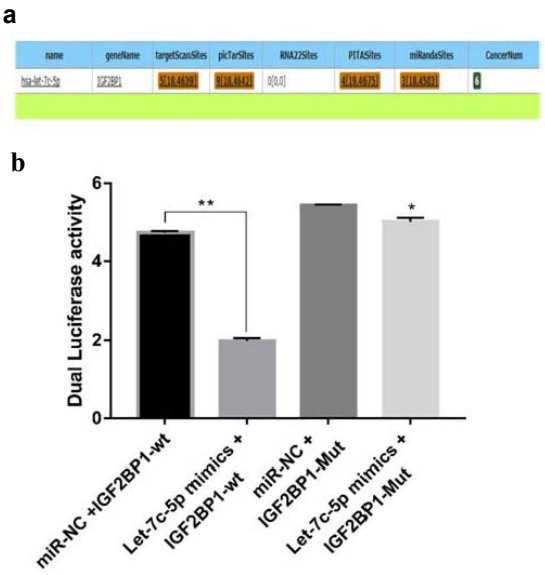

Figure 2: Let-7c-5p targets IGF2BP1 directly. (a) Targetscan Bioinformatics was used to determine binding sites between let-7c-5p and IGF2BP1. (**b**) Dual-luciferase reporter test was applied for detecting fluorescence in SCC-9 and SCC-25 cells (** P<0.01; * P < 0.05).

Upregulated let-7c-5p induces apoptosis and suppresses EMT in OSCC cells

To verify the apoptosis rate, flow cytometric method was utilized (Figure 4a). As expected, we observed an increased apoptosis rate in OSCC cells compared to miR-NC (HOK). The overexpressed let-7c-5p expression promotes Apoptosis. Thus, suggesting that overexpressing the let-7c-5p had positive effects on the apoptosis of the OSCC cells. We then evaluated the expression of EMT biomarkers in SCC-25; Cal27 cells via western blotting assay (Figure 4b). Improved E-cadherin and downregulated N-cadherin and Vimentin by overexpression of let-7c-5p were displayed in Figure 4b, suggesting that let-7c-5p inhibited the EMT.

Restoration of IGF2BP1 eliminates the effects of let-7c-5p initiation in OSSC

In order to explore further interactions between let-7c-5p and IGF2BP1, OSCC cells with IGF2BP1 transfection were to replenish let-7c-5p-depleted IGF2BP1. Furthermore, cell viability (Fig. 5a) measured via MTT assay was reduced. Subsequently, upregulated IGF2BP1 restored the invasiveness (Fig. 5b) and migratory capacity (Fig. 5c) of OSSC cells which were reduced in let-7c-5p expression levels. Lastly, expressions of the Vimentin and N-cadherin were elevated while the E-cadherin was reduced by overexpressed IGF2BP1 (Fig. 5d). In conclusion,

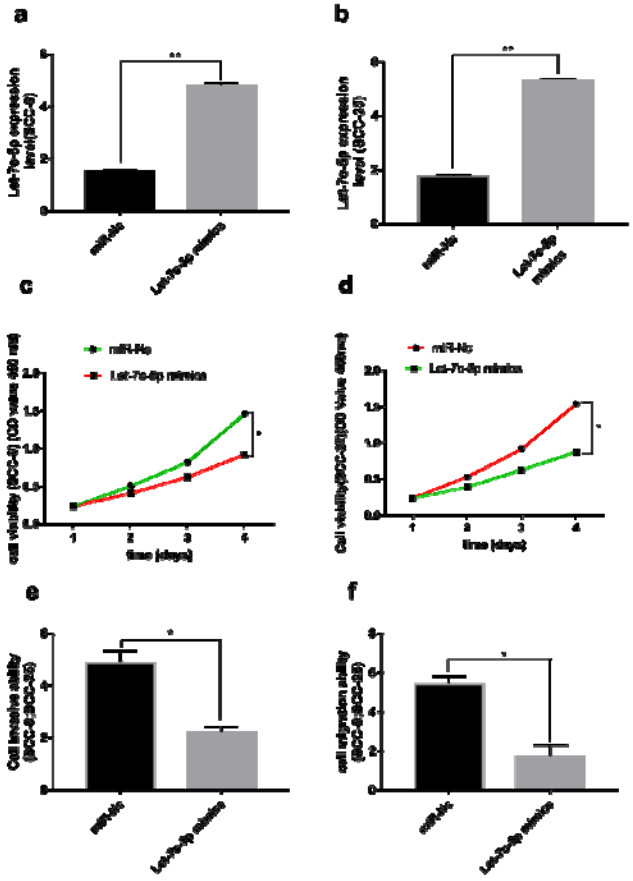

Figure 3: Overexpressed let-7c-5p restrains cell viability, invasion and migratory ability of OSCC cells. (a-b) Let-7c-5p expressions were measured by RT-qPCR. (**c-d**) The cell viability of the OSCC cells after miR-NC and let-7c-5p mimics transfection were examined by MTT, with an OD value at 450nm, *P<0.05. (**e-f**) OSCC cell invasive and migratory abilities were measured via Transwell invasion assay and Scratch assay, respectively (*P<0.05)

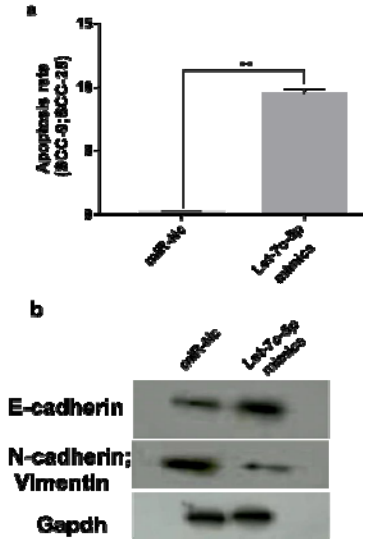

Figure 4: Let-7c-5p overexpression facilitated apoptosis and suppressed EMT. **(a)** apoptosis rates after let-7c-5p overexpression were determined by flow cytometry, ** P<0.01. (**b**) Western blot analyzed protein expressions of E-cadherin; N-cadherin and Vimentin

the compensatory effect of IGF2BP1 on hindering the let-7c-5p upregulation strongly verifies IGF2BP1 interacting with let-7c-5p in OSCC cells.

DISCUSSION

Up to date, numerous studies have pointed out the importance of let-7c-5p that it might a biomarker to diagnose or treat cancers. Recent research about members in let-7 family has demonstrated that they worked as tumor inhibitors in cancers by directly inhibiting the work of oncogenes[26, 27].The let-7c-5p upregulation diminished apoptosis and the increase of NLRC5 induced by EtOH[28]. The expression of *let-7c-5p* were delineated to be downregulated in inflamed human dental pulp cells[29]. Moreover, let-7c-5p were demonstrated to accelerate differentiation of osteogenesis in human stromal mesenchymal stem cells[30]. Furthermore, let-7c-5p also functions as a suppresser of breast cancer[31]. To understand the functions of let-7c-5p in OSCC, it is vital to

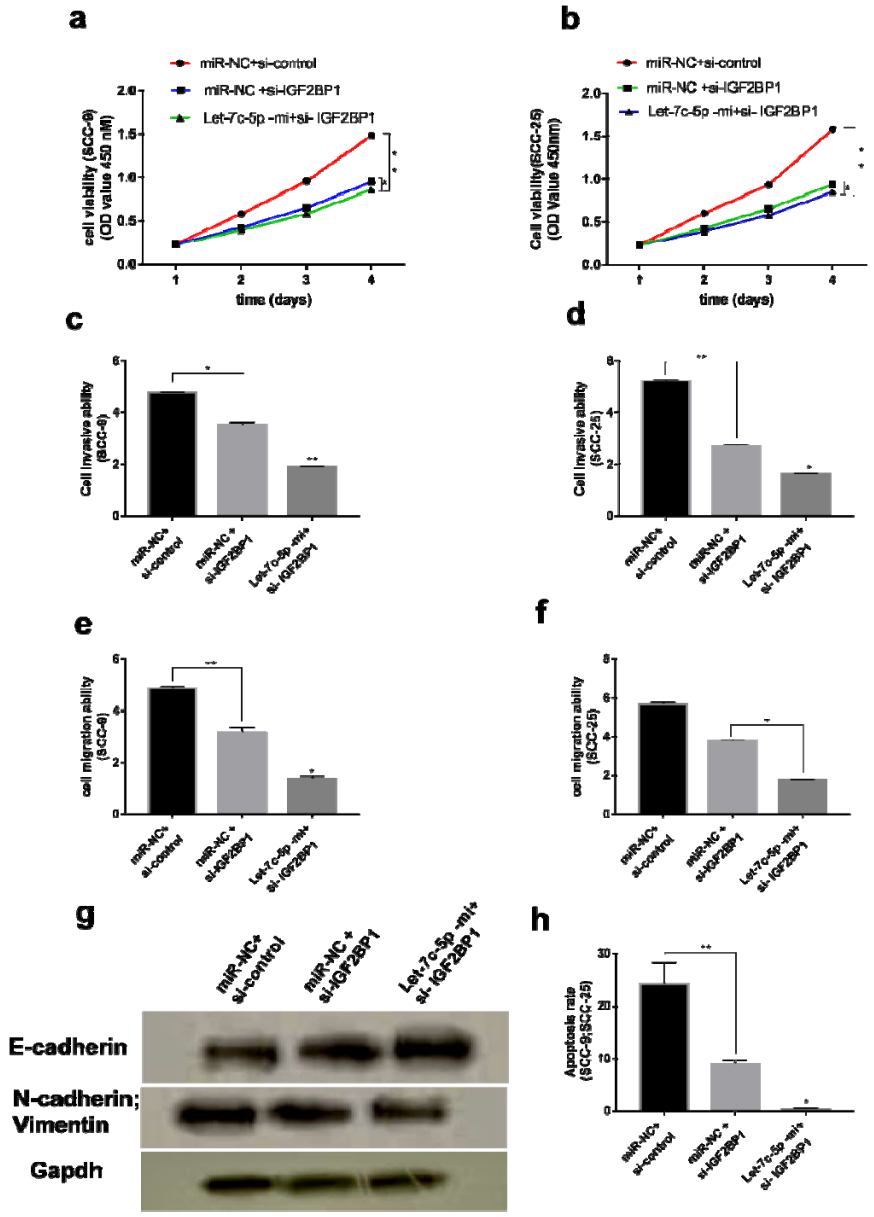

Figure 5: Restoration of IGF2BP1 eliminates roles of let-7c-5p in OSCC cells. **(a-b)** SCC-9 and SCC-25 cells were transiently transfected with miR-NC+si-NC, miR-NC+si-IGF2BP1 or let-7c-5p-mi+si-IGF2BP1, and cell viabilities were measured using MTT, **P<0.01, *P<0.05. (**c-d**) Cell invasion ability was measured by Transwell. (**e-f**) Scratch assay was employed to validate migratory ability, ** P<0.01; * P<0.05. (**g**) Western Blot analysis evaluated the EMT protein expressions in transfected OSCC cells. (**h**) Apoptosis of OSCC cells were evaluated using Flow cytometry (** P<0.01; * P<0.05)

recognize the object, which has been popularly verified that miRNAs control tumor processes by inhibiting expressions of targets [32].

Besides, IGF2BP1 prevents repression caused by miRNAs and modulates tumor progression[33], which was recognized as a target of various miRNAs including let-7c-5p [34, 35].However, the interaction between the two has not been researched in OSCC.

Here, in this present study, down-regulation of let-7c-5p and protein expressions of IGF2BP1 were distinguished. We utilized the Target Scan to detect putative target of let-7c-5p, revealing that IGF2BP1 was found to be the target with putative binding sites. Furthermore, the reduced luciferase activity of IGF2BP1-wt with IGF2BP1 indicated that let-7c-5p directly bound IGF2BP1. From these previous results, we gathered that the upregulation of let-7c-5p repressed cell viability, invasiveness and metastasis. Let-7c-5p

expression was up-regulated in OSCC cell lines after overexpressed transfection. Furthermore, the cell viability was decreased after let-7c-5p overexpression. In addition, transwell and scratch assays revealed that up-regulated let-7c-5p repressed the invasive and migratory abilities. The results suggested that let-7c-5p had negative migration and invasive effects on the OSCC cells during the 48hour period of experiments.

Based on the previous results, the apoptosis rate was promoted since the OSCC metastasis and cell viabilities were decreased. Flow cytometric method revealed that apoptosis rates of OSCC cells were dramatically accelerated by let-7c-5p upregulation. We assumed that let-7c-5p might regulate the development of OSCC cell by targeting IGF2BP1. During progressions of malignancies, epithelial markers were greatly downregulated and the intercellular junctions were diminished among cells in advanced stage, resulting in the lack of epithelial polarity and decreased intercellular adhesion. Therefore, the overexpression of let-7c-5p alleviated the EMT but induced apoptosis of OSCC cells.

The importance of the IGF2BP1 gene on the biological functions of let-7c-5p in the OSCC cells were analyzed. Western blot, MTT, Transwell invasion assay, Starch assay and flow cytometry method for the OSCC cells after transfections of siNC+miR-NC, siRNA, siRNA +let-7c-5p mimics groups suggested that IGF2BP1 played an inhibitory role by hampering let-7c-5p in OSCC cells. Hence, we discovered that IGF2BP1 is an important target that restrains functions of let-7c-5p in OSCC cells.

CONCLUSION

Let-7c-5p targeted IGF2BP1 directly and suppressed migration, invasion and viability of OSCC cells via downregulating IGF2BP1. Let-7c-5p is proposed to be an inhibitor that might serve as a biomarker for a new therapy for OSCC.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Tong Dong and Wen Jiang are co-first authors.

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