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Original Article

Let-7g affects cell proliferation, migration and invasion in cervical squamous cell carcinomas via targeting collagen I

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Abstract: Cervical cancer is one of the most common types of malignant gynecological tumors worldwide. Let-7g and collagen I have been reported to be involved in the occurrence and development of various cancers, but the clinical significance and biological function of these proteins in cervical squamous cell carcinoma (CSCC) have not been reported. Therefore, this study aimed to determine the underlying roles of let-7g and collagen I in CSCC. Tissue chip, qRT-PCR and western blot assays were used to examine the expression of collagen I in CSCC patients. A dual-luciferase reporter system was used to demonstrate the interaction between let-7g and collagen I. CCK8 and Transwell assays were applied to examine the effects of let-7g and collagen I on cell proliferation, migration and invasion. The results showed that collagen I was obviously increased in CSCC patients. Moreover, it was confirmed that collagen I is the direct target of let-7g. Gain- and loss-of-function experiments, for let-7g and collagen I indicated that let-7g obviously inhibited the proliferation, migration and invasion abilities of CSCC by regulating collagen I. Collectively, these data suggest that let-7g suppresses tumor growth and metastasis by targeting collagen I; therefore, let-7g and collagen I can be considered novel potential therapeutic targets for CSCC.

Keywords: Cervical squamous cell carcinoma (CSCC), collagen I, let-7g, tumor growth and metastasis

Introduction

Globally, cervical cancer is the third most common type of gynecological malignant tumor, and cervical squamous cell carcinoma (CSCC) accounts for 90~95% of cervical cancer cases [1, 2]. Recently, an epidemiological survey revealed that approximately 275,000 women die from this disease every year, and its worldwide annual incidence is half a million; consequently, CSCC and its associated high rate of morbidity and mortality remain a serious threat to women's health [3]. Moreover, despite enormous improvements in the diagnosis and treatment of cervical cancer in the past 20 years, satisfactory approaches for curing cervical cancer are often not feasible, mainly due to the high recurrence rate [4, 5]. Hence, novel biomarkers are urgently needed to evaluate therapeutic effectiveness, optimize treatment strategies, and predict clinical outcomes for CSCC.

microRNA (miRNA) is a class of small endogenous non-coding functional RNAs that regulate various physiological and pathological processes, including cellular apoptosis, cell proliferation, cell differentiation, signal transduction, and cancer development, via mRNA degradation and/or translational repression [6]. Furthermore, abnormal miRNA changes contribute to the occurrence and development of many malignant cancers in humans [7]. For example, miR-204 inhibits the cell proliferation, migration and invasion of T cell acute lymphoblastic leukemia by down-regulating sex determining region Y-box 4 (SOX4) [8]; miR-21, which is markedly up-regulated in hepatocellular carcinoma, can promote tumor invasion and metastasis as well as tumor growth by targeting phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4) [9]; reduced miR-143 expression levels induce colorectal cancer development through derepressing Kirst-

Table 1. Detailed clinical characteristics of CSCC patients

Patient number	Age (years)	Pathological type	Pathological grade	Tumor size
P1	41	Squamous cell carcinoma	III	φ3
P2	35	Squamous cell carcinoma	II	φ2
P3	38	Squamous cell carcinoma	II-III	2.5×2.5×2
P4	37	Squamous cell carcinoma	III	φ3
P5	36	Squamous cell carcinoma	III	2×1×1
P6	41	Squamous cell carcinoma	II	φ2
P7	34	Squamous cell carcinoma	III	φ2
P8	37	Squamous cell carcinoma	II	2×3
P9	42	Squamous cell carcinoma	II	φ3
P10	43	Squamous cell carcinoma	II	φ3
P11	32	Squamous cell carcinoma	II	φ3
P12	37	Squamous cell carcinoma	III	φ3
P13	43	Squamous-cell carcinoma	II	4.5×4×3
P14	42	Squamous cell carcinoma	II	φ4
P15	39	Squamous cell carcinoma	II-III	φ2
P16	28	Squamous-cell carcinoma	II	2×1×1
P17	29	Squamous-cell carcinoma	II	φ3
P18	37	Squamous cell carcinoma	II	φ3
P19	27	Squamous cell carcinoma	II	φ3
P20	37	Squamous cell carcinoma	II	φ3
P21	28	Squamous cell carcinoma	II	φ3
P22	36	Squamous cell carcinoma	II-III	2.5×2×1.3
P23	32	Squamous cell carcinoma	II	2.5×1.5×1.3
P24	38	Squamous cell carcinoma	II-III	4.5×3.5×1.5
P25	32	Squamous cell carcinoma	II	φ4
P26	24	Squamous cell carcinoma	II-III	φ4.5
P27	40	Squamous cell carcinoma	II	φ4
P28	44	Squamous cell carcinoma	II-III	φ3
P29	43	Squamous cell carcinoma	II-III	φ3
P30	41	Squamous cell carcinoma	III	φ4
P31	37	Squamous cell carcinoma	II-III	φ3

en rat sarcoma viral oncogene homolog (KRAS) expression [10]; and miR-126, miR-96, miR-144, miR-657, miR-490-5p and miR-323-3p participate in multiple steps of the metastasis cascade by targeting metastasis-associated genes involved in CSCC progression [11]. In addition, miRNAs have been implicated in CSCC therapy-resistance in recent years. For instance, miR-181a enhances the chemoresistance of human cervical squamous cell carcinoma to cisplatin by targeting protein kinase C delta (PRKCD) [12]. Thus, miRNAs can be considered as new biomarkers for further exploring the underlying mechanisms of CSCC, which might provide a novel strategy for diagnosing or treating CSCC.

Collagens are an important class of proteins in bones, tendons and teeth [13]. More importantly, it has been reported that collagen is closely related to tumor cell adhesion, invasion and migration [14]. For example, collagen type I alpha 1 (COL1A1) is up-regulated in gastric cancer and plays a crucial role in cancer cell metastasis [15]. Moreover, it has been verified that COL1A1 is also involved in the radioresistance of solid tumors, such as breast cancer [16], hepatocellular carcinoma [17] and cervical cancer [18]. Additionally, previous studies have demonstrated that the expression levels of let-7g and COL1A2 are inversely correlated in hepatocellular carcinoma clinical specimens, which further supports that COL1A2 is a direct target of let-7g [19]. Nevertheless, let-7g has also been discovered to play anti-tumor roles in gastric cancer, non-small cell lung cancer, and esophageal carcinoma. Therefore, the aim of our study was to investigate the relationship between let-7g and collagen I and their effects on the growth and metastasis of CSCC.

Materials and methods

Patients and specimen collection

All fresh tumor and para-carcinoma tissues were obtained from Shanghai Outdo Biotech (Shanghai, China). Genomic DNA, total RNA, and tissue lysates were prepared and stored at -80°C until analyses. All experiments were performed in accordance with the approval of the Scientific Investigation Board of the Second Affiliated Hospital of Sun Yat-sen University and were conducted in accordance with the ethical principles of the Declaration of Helsinki. Detailed clinical characteristics of these patients are shown in **Table 1**. The collected specimens were used to examine the expression of let-7g and collagen I by tissue microarray (TMA)-immunohistochemical (IHC) staining, quantitative reverse transcription-polymerase chain reac-

Table 2. Primer sequences for miRNAs and genes

miRNA or gene	Primer sequences
Let-7g	RT primer: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTACAAAC-3' Forward primer: 5'-ACACTCCAGCTGGGTGAGGTAGTAGTTTGTAC-3' Reverse primer: 5'-CTCAACTGGTGTCGTGGA-3'
U6	RT primer: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAAATATGG-3' Forward primer: 5'-CTCGCTTCGGCAGCACA-3' Reverse primer: 5'-AACGCTTCACGAATTTGCGT-3'
Collagen 1	Forward primer: 5'-GACAAAGCAGAAACATCGGA-3' Reverse primer: 5'-GAACGAGGTAGTCTTTCAGC-3'
18S rRNA	Forward primer: 5'-CCTGGATACCGCAGCTAGGA-3' Reverse primer: 5'-GCGGCGCAATACGAATGCCCC-3'

tion (qRT-PCR) and western blotting (WB) assays.

TMA-IHC staining

Tissue arrays were obtained from Shanghai Outdo Biotech. Formalin-fixed and paraffin-embedded pathological specimens, including carcinoma and para-carcinoma tissues, from 31 CSCC patients were used for TMA as described by Chen et al. [20]. The EnVision+ detection system (Dako) was used per the manufacturer's instructions.

Cell culture

The human cervical cancer cell line HeLa was purchased from the American Type Culture Collection (ATCC) and grown in complete culture medium containing RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37°C and 5% CO_2 in a humidified incubator. For passaging, when HeLa cells were 90% confluent, they were washed twice with PBS and released by trypsinization (Trypsin, Sigma, USA).

293T cells, used for the dual luciferase reporter assay, were cultured in RPMI-1640 with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, and 1% penicillin/streptomycin at 37°C in a humidified 95% air/5% CO_2 atmosphere.

Total RNA extraction and qRT-PCR

Total RNA was isolated from the CSCC patient samples using TRIzol Reagent (TIANGEN, China) as recommended by the manufacturer. RNA quantity and quality were assessed by a BioPhotometer and agarose-formaldehyde gel electrophoresis, respectively. Complementary DNA (cDNA) synthesis was carried out using one microgram of RNA from each sample and an M-MLV reverse transcriptase Kit (Promega, USA) according to the manufacturer's guidelines. Then, PCR reactions were conducted using a SYBR Green qPCR SuperMix Kit (Invitrogen, USA) with a total reaction volume of 20 μl in an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, USA) in accordance with the manufacturer's instructions. The thermocycling parameters were as follows: All reactions were performed in triplicate on a 96-well optical plate with a minimum of three independent replicates, and the relative expression values of collagen I were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to 18S mRNA. The primers used in this study are listed in **Table 2**.

WB analysis

Radioimmunoprecipitation assay (RIPA) lysis solution (Beyotime, China) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, and a mixture of protease inhibitors was used to extract the total protein from CSCC patient samples; the samples were incubated for 20 min on ice. After centrifugation at 12000 rpm for 10 min at 4°C, the insoluble material was removed, and the total protein concentration in the supernatant was measured with a BCA protein assay kit (Beyotime, China). Samples containing equal amounts of protein (30 μg) per lane were separated by 8~10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes at a constant current of 200 mA for 60 min. After blocking with 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at room temperature, primary antibodies against

GAPDH (1:10000, as a reference control; Abcam, USA) and collagen I (1:1000; Abcam, USA) were allowed to react with the membranes overnight at 4°C. Following extensive washing with TBST three times, the membranes were probed with the corresponding secondary antibodies (including goat anti-mouse IgG and goat anti-rabbit IgG, 1:12000; Abmart, USA) for 1 h at 37°C with gentle agitation. Ultimately, the blots were detected with enhanced chemiluminescence reagents (Beyotime, China), visualized with X-ray film (Kodak, Japan) and quantified by densitometry using ImageJ analysis software (NIH, USA).

Dual-luciferase reporter assay

Fragments of the collagen I 3'-UTR that contain the putative target region and mutant region for let-7g were synthesized by Sangon Biotech (Shanghai, China) and were cloned into the psiCHECK2 basic luciferase reporter plasmid (Promega, USA) between the Xho I and Not I sites to generate the recombinant vectors WT-Collagen I and Mutant-Collagen I. The insertions were verified by sequencing. Subsequently, these constructed plasmids as well as the control psiCHECK-2 plasmid (Blank group), miR-155 mimics, miR-155 inhibitor, NC plasmid and NC inhibitor were transfected into 293T cells with the FuGENE® HD Transfection Reagent (Promega, USA) according to the manufacturer's recommendations. After a 6-h incubation, the media of the transfected cells was replaced with fresh media. Another 42 h later, the cells were split, and Firefly and Renilla relative luciferase activities were determined with the Dual-Luciferase Reporter Assay System (Promega, USA) in accordance with the manufacturer's instructions. The experiments were performed in triplicate. In addition, the expression levels of let-7g and collagen I were detected by qRT-PCR and WB assays as described above.

Cell transfection

HeLa cells were seeded at 1×10^5 cells/ml per plate in a 6-well plate 24 h prior to transfection with the plasmids, including the let-7g mimic, let-7g inhibitor, collagen I mimic and collagen I inhibitor, using the FuGENE® HD Transfection Reagent according to the manufacturer's protocols. The transfection efficiency was determined by qRT-PCR. The above plasmids were designed by and purchased from Sangon Biotech, Shanghai, China. After a 48-h transfection, the cells were harvested for the cell proliferation, migration and invasion assays.

Cell proliferation detection

Cell proliferation was determined by the colorimetric water-soluble tetrazolium salt method, and a Cell Counting Kit-8 (CCK8; Dojindo, Japan) was used according to the manufacturer's instructions. After a 48-h transfection, 0.25% trypsin was used to digest the HeLa cells into a single-cell suspension, which was plated in 96-well plates at a density of 5×10^3 cells per well. At the indicated time points, CCK8 reagent was added to each well; following a 4-h incubation, the absorbance at 450 nm was measured on an enzyme immunoassay analyzer (Bio-Rad, USA).

Cell migration and invasion assays

Cell invasion and migration assays were carried out using transwell inserts with 8.0-μm pores in 24-well plates (BD Biosciences, USA). For the cell invasion assays, approximately 2×10^5 of transfected HeLa cells with serum-free medium were seeded in the upper chambers on the 8-μm transwell inserts pre-coated with Matrigel basement membrane matrix (BD Biosciences, USA). Then, 600 μl of culture media containing 20% FBS was added to the bottom chamber as a chemoattractant. After a 24-h incubation at 37°C with 5% CO_2, the upper chamber was removed, the medium was discarded, and the non-invasive cells attached to the apical side of each transwell membrane were carefully scraped off with a sterile cotton swab. The invasive cells on the other side of the membrane were fixed in 4% paraformaldehyde at room temperature for 30 min, stained with 0.1% crystal violet solution for 10 min, rinsed with PBS three times and dried at 80°C for 30 min. Finally, the number of invasive cells that went through the Matrigel were photographed and counted in 4~5 random microscopic fields viewed under an inverted microscope (×200 magnification; Nikon, Japan). For the cell migration experiments, the concentration of the transfected HeLa cells in each group was adjusted to 1×10^5 cells/well, and they were similarly placed on top of the 8-μm transwell inserts; however, the inserts were not coated with Matrigel basement membrane matrix. The rest

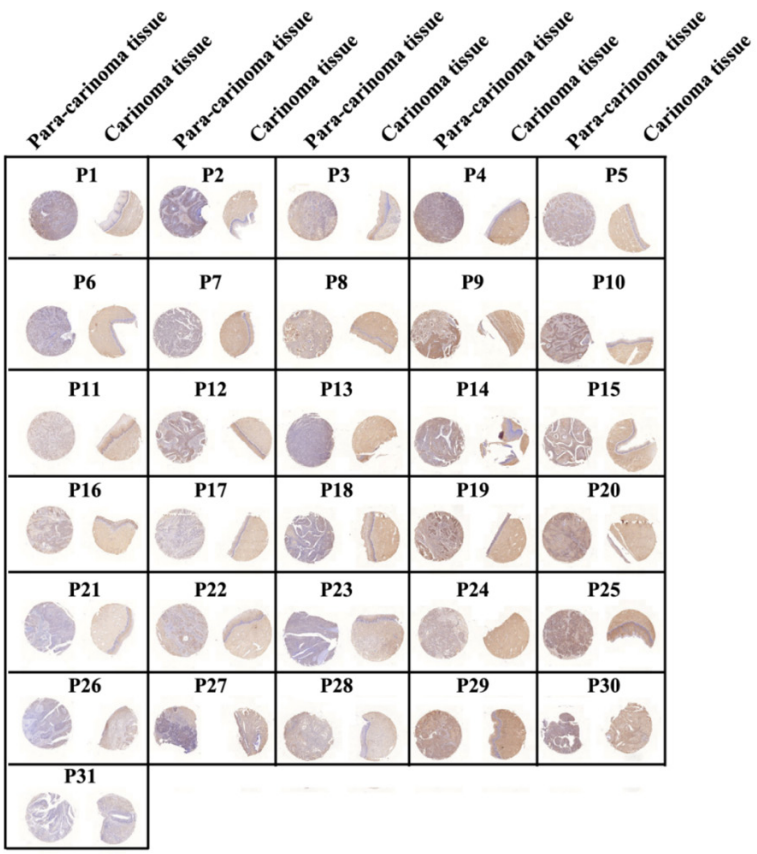

Figure 1. Collagen I expression was determined by TMA assay. "P" denotes patients.

of the procedure was similar to that of the invasion experiment.

Statistical analysis

The SPSS 18.0 statistical software package (IBM SPSS, USA) was used to analyze the data in the current study. All values are depicted as the mean ± standard deviation from at least three independent experiments with similar results. Unpaired, two-tailed Student's t-tests and one-way ANOVA were used for comparing data from two groups and from more than two groups, respectively. Differences were considered significant if $P < 0.05$.

Results

Collagen I expression levels in CSCC clinical specimens

As shown in **Figure 1**, the TMA results showed strong collagen I staining in carcinoma tissues compared to para-carcinoma tissues from 31 patients. Subsequently, we randomly selected 12 samples from CSCC patients to further examine the mRNA and protein expression levels of collagen I. We also found that collagen I expression was significantly higher in carcinoma tissues than in para-carcinoma tissues (**Figure 2A** and **2B**). Therefore, these data indicated that the changes in collagen I in CSCC patients might play an important role in the development of CSCC.

Collagen I is the direct target of let-7g

A dual-luciferase reporter assay was performed to confirm our hypothesis. First, the putative let-7g binding sites (named WT-Collagen I 3'-UTR) and the corresponding mutation (named Mutant-Collagen I 3'-UTR) are illustrated in **Figure 3A**. The relative luciferase activity was remarkably reduced after co-transfection with the WT-Collagen I 3'-UTR vector and the let-7g mimic plasmid, while it was markedly increased following co-transfection with the WT-Collagen I 3'-UTR vector and the let-7g inhibitor (**Figure 3B**). However, there were no changes in 293T cells co-transfected with the Mutant-Collagen I 3'-UTR vector and other plasmids ($P > 0.05$; **Figure 3B**). Moreover, qRT-PCR and WB results demonstrated that the overexpression and knockdown of let-7g (**Figure 3C**) could respectively inhibit and promote the expression of collagen I at the mRNA and protein levels (**Figure 3D** and **3E**). Thus, these findings revealed that there is a direct target interaction between let-7g and collagen I.

Let-7g suppresses cell proliferation by regulating collagen I in HeLa cells

The proliferation activity of let-7g and collagen I in HeLa cells was detected by CCK8 assay. The OD values were markedly lower in the let-7g group and the collagen I inhibitor group than in the cell group (**Figure 4A**). Hence, these data

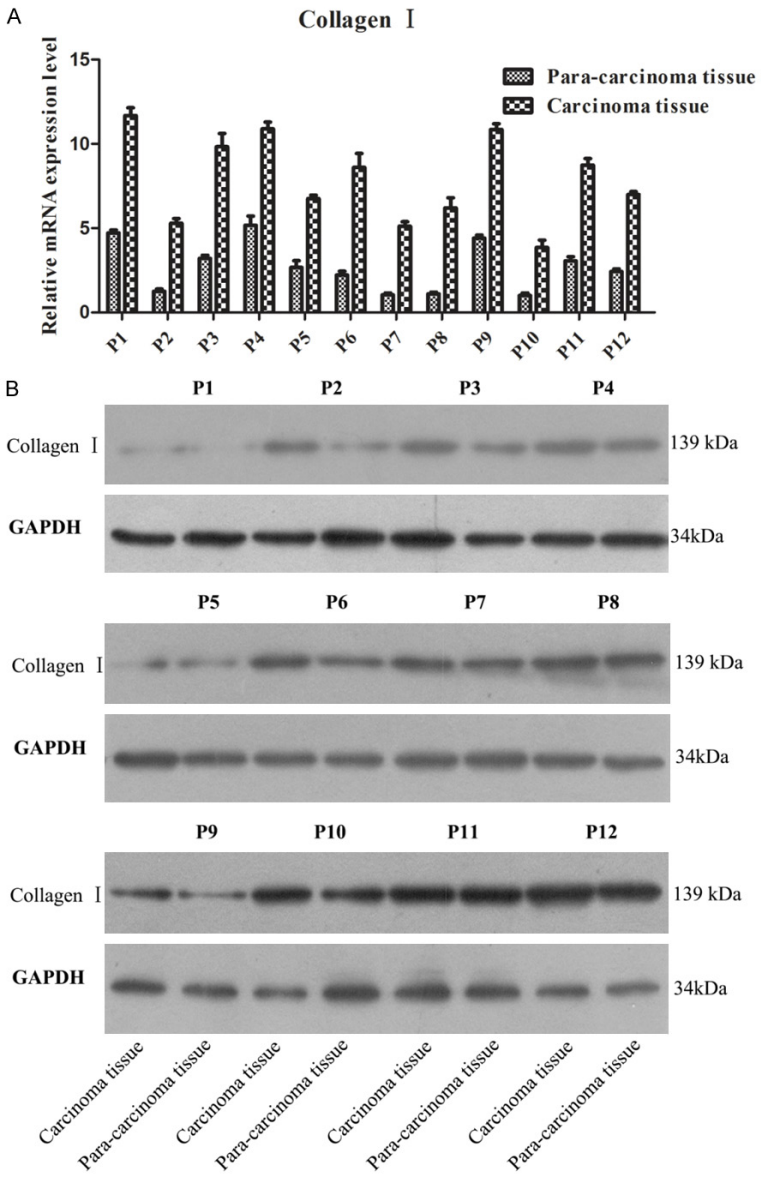

Figure 2. Let-7g is negatively correlated with collagen I in CSCC patient samples. A. Collagen I mRNA expression was measured by qRT-PCR. B. Collagen I protein expression was detected by WB. "P" indicates patients.

suggested that let-7g might inhibit tumor growth by regulating collagen I.

Let-7g limits the migration and invasion abilities of HeLa cells by targeting collagen I

To further evaluate the roles of let-7g and collagen I in the metastasis of HeLa cells, Transwell assays were applied. As shown in **Figure 4B** and **4C**, the numbers of migrated and invasive cells were dramatically lower in the let-7g group and the collagen I inhibitor group than in the cell group. Collectively, these results imply that the regulatory mechanism of let-7g in HeLa cell metastasis might be via targeting collagen I.

Discussion

CSCC remains a serious cancer burden that commonly affects females throughout the world [1]. Despite remarkable advances in therapeutic strategies in recent decades, the overall survival rates for CSCC patients have been limited due to frequent relapse and remote metastasis [3]. Recently, an increasing number of studies have demonstrated that CSCC is a multistage process resulting from the accumulation of genetic and epigenetic alterations, particularly ectopic miRNA expression [5]. For example, miR-138 is a potential biomarker and tumor suppressor of human cervical carcinoma that is reversely correlated with the transcription factor 3 (TCF3) gene [21]; miR-31 is an independent prognostic factor and functions as an oncomir in cervical cancer via targeting AT-rich interaction domain 1A (ARID1A) [22]; and miR-106b is involved in transforming growth factor β1 (TGF-β1)-induced cell migration via targeting disabled homolog 2 (DAB2) in cervical carcinoma [23]. In our study, we initially examined the expression of collagen I in clinical specimens of CSCC tissues and para-carcinoma tissues and found that collagen I was significantly up-regulated in the cancerous tissues compared to the non-cancerous tissues; these findings were confirmed by TMA, qRT-PCR and WB assays. However, previous studies have reported that collagen I in the tumor microenvironment substantially influences gastric cancer pathogenesis and progression and could serve as a valuable prognostic indicator [24]; moreover, it was

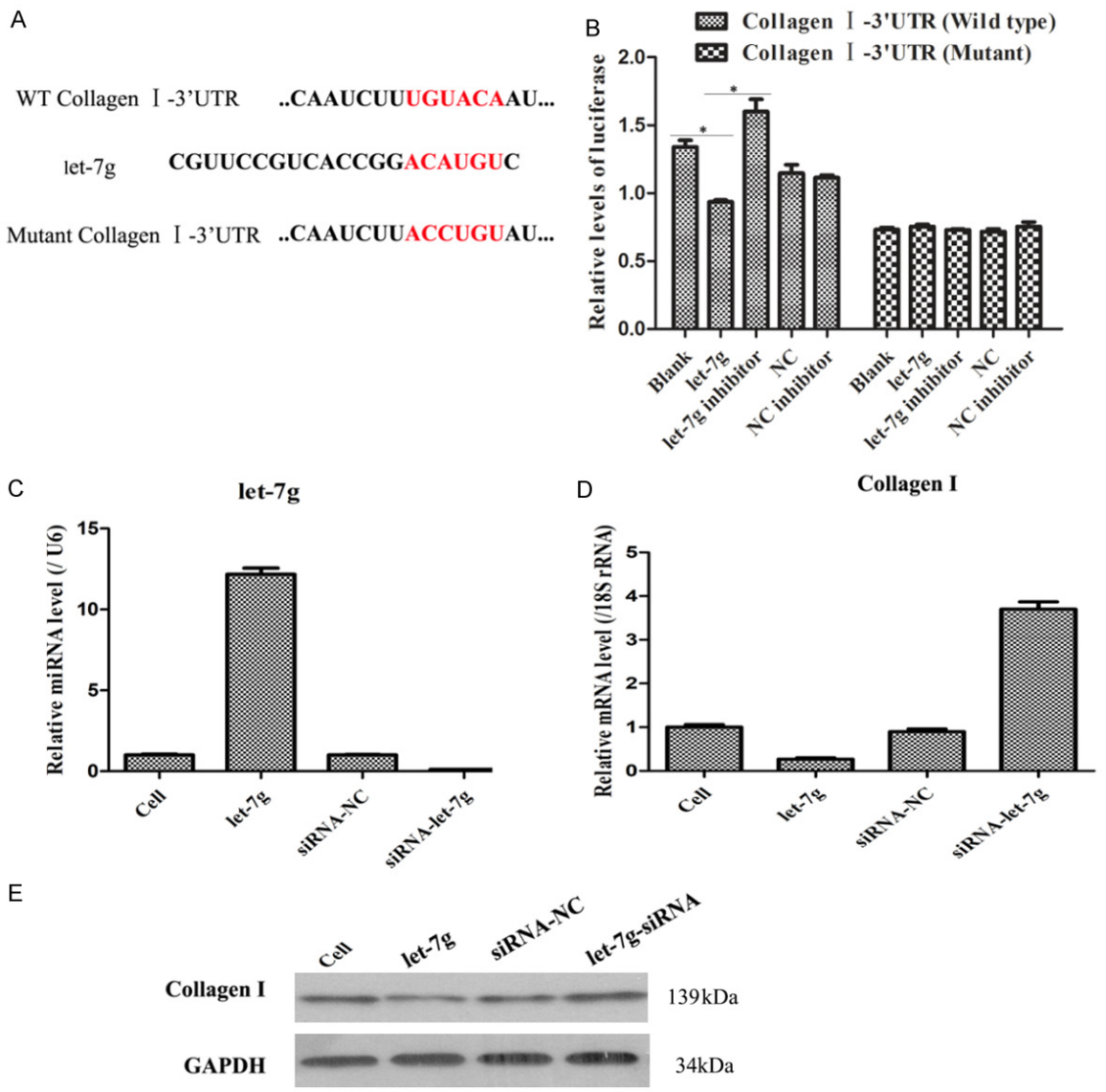

Figure 3. A dual-luciferase reporter assay was utilized to demonstrate the target interaction between let-7g and collagen I. A. The alignment between the predicted let-7g target site and the mutant site of the collagen I 3'-UTR region and let-7g. B. Relative luciferase activity in 293T cells co-transfected with WT Collagen I 3'-UTR or Mutant Collagen I 3'-UTR vectors and let-7g mimic/inhibitor or NC mimic/inhibitor plasmids. C. Let-7g expression was determined by qRT-PCR. D. Collagen I mRNA expression was detected by qRT-PCR. E. Collagen I protein expression was determined by WB in 293T cells transfected with let-7g mimic/inhibitor plasmids.

found that reduced let-7g expression plays a pivotal role in breast cancer invasion and metastasis [25]. Therefore, our data suggest that changes in collagen I and let-7g might play an important role in the progression of CSCC. Subsequently, based on the opposite expression patterns of collagen I and let-7g, we speculated that collagen I might be a direct target of let-7g; we confirmed this hypothesis with a dual-luciferase reporter assay.

To explore the biological functions of collagen I and let-7g in the growth and metastasis processes of CSCC, cell proliferation, migration and invasion abilities were determined by CCK8 and Transwell assays. Cell growth is strictly regulated by cell proliferation [26]. The dysregulation of cell proliferation originating from miRNA changes is implicated in the occurrence and development of cancer and is a dominant obstacle to cancer treatment [27]. For instance,

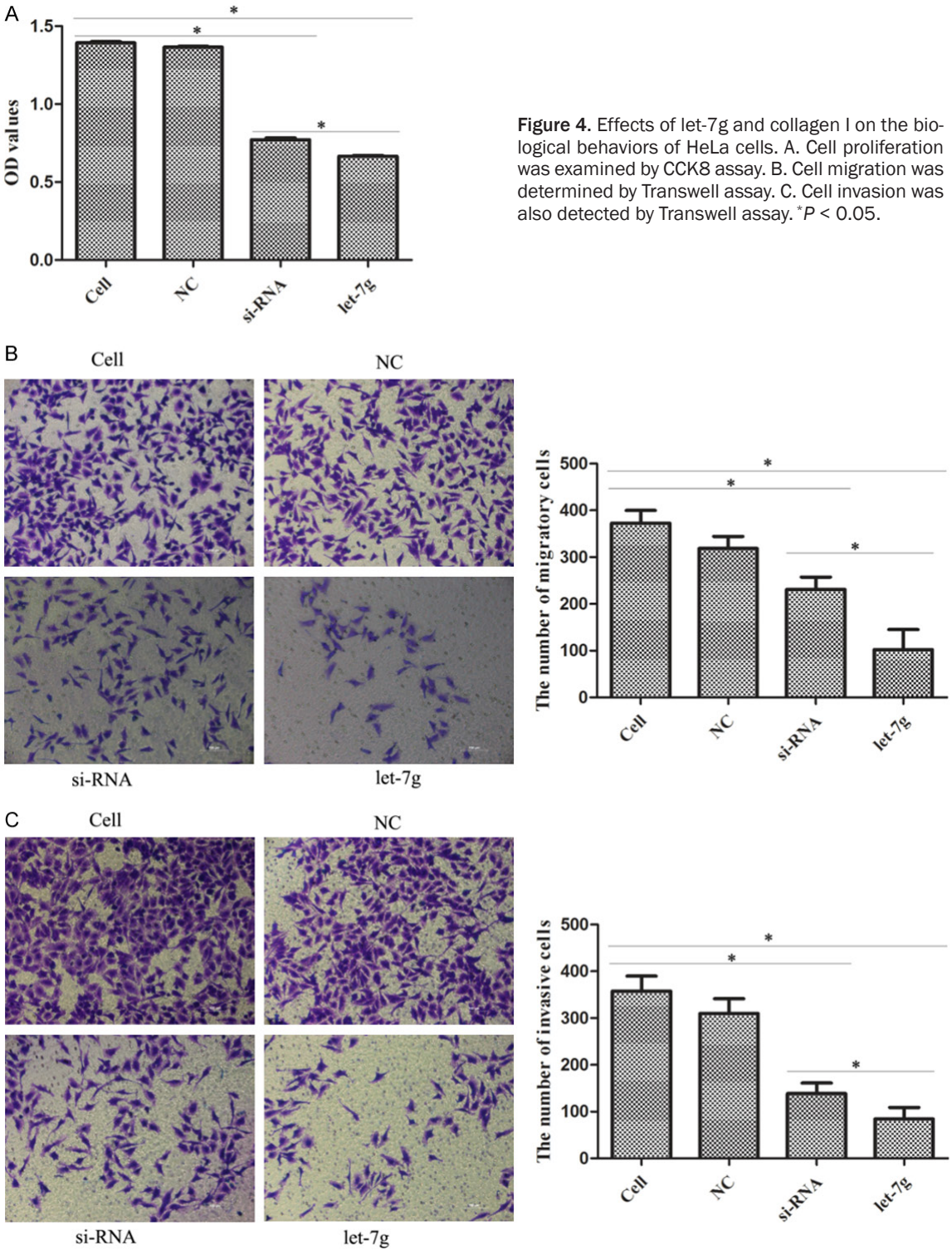

Figure 4. Effects of let-7g and collagen I on the biological behaviors of HeLa cells. A. Cell proliferation was examined by CCK8 assay. B. Cell migration was determined by Transwell assay. C. Cell invasion was also detected by Transwell assay. $^{*}P < 0.05$.

miR-543 suppresses breast cancer cell proliferation through directly repressing factors downstream of the mitogen-activated protein kinase/extracellular signal-regulated kinase 2 (MAPK/ERK2) pathway, which ultimately leads to rapid tumor growth [28]. In our study, both collagen I inhibition and forced let-7g expression negatively regulated HeLa cell proliferation, indicating that let-7g might suppress HeLa cell growth by targeting collagen I. Next, the

biological behaviors of tumor cell migration and invasion were investigated in HeLa cells transfected with a collagen I inhibitor and let-7g mimic. Cell migration and invasion, the key steps of tumor metastasis, are recognized as common markers of adverse prognosis for cancers, and these behaviors are intimately linked to miRNA regulation [29]. For example, miR-296 exerts anti-metastasis functions in colorectal cancer by suppressing epithelial-mesenchymal transition, migration and invasion in colorectal cancer cells [30], and miR-130a can enhance migration and invasion abilities in SiHa cells, which eventually facilitates the CSCC tumor metastasis [31]. Our results show that the knockdown of collagen I and the overexpression of let-7g hindered cell migration and invasion behaviors in HeLa cells, implying that let-7g negatively mediates CSCC tumor metastasis *in vitro* via collagen I.

Taken together, our study demonstrated for the first time two important points: 1) A target interaction between let-7g and collagen I was verified, and 2) let-7g affected cell proliferation, migration and invasion in CSCC via directly regulating collagen I. Thus, these findings may not only help us expand our knowledge of the mechanisms of let-7g and collagen I during CSCC growth and metastasis but also provide a new biomarker for the early detection of CSCC or a potential therapeutic target for CSCC.

Disclosure of conflict of interest

None.

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