MicroRNA-30e Functions as a Tumor Suppressor in Cervical Carcinoma Cells through Targeting GALNT7

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Abstract
Cervical cancer is the third most common cancer in women worldwide. However, the underlying mechanism of occurrence and development of cervical cancer is obscure. In this study, we observed that miR-30e was downregulated in clinical cervical cancer tissues and cervical cancer cells. Next, overexpression of miR-30e reduced the cervical cancer cell growth through MTT, colony formation, EdU, and Transwell assay in SiHa and Caski cells. Subsequently, UDP-N-acetyl-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 7 (GALNT7) was identified as a potential miR-30e target by bioinformatics analysis. Moreover, we showed that miR-30e was able to bind to the 3′UTR of GALNT7 by luciferase reporter assay. In addition, the mRNA and protein levels of GALNT7 in cervical cancer cells were downregulated by miR-30e. And we validated that downregulation of GALNT7 repressed the proliferation of SiHa and Caski cells by MTT, colony formation, and Transwell assay. We identified that the restoration of GALNT7 expression was able to counteract the effect of miR-30e on cell proliferation of cervical cancer cells. Furthermore, we found that the expression levels of GALNT7 were frequently upregulated and negatively correlated to those of miR-30e in cervical cancer tissues. In addition, we validated that restoration of GALNT7 rescued the miR-30e-suppressed growth of cervical cancer xenografts in vivo. In conclusion, the current results suggest that miR-30e may function as tumor suppressors in cervical cancer through downregulation of GALNT7. Both miR-30e and its novel target, GALNT7, may play an important role in the process of cervical cancer.

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Introduction
Cervical carcinoma is a malignancy with the second highest morbidity and mortality in women worldwide [1,2]. The incidence of cervical cancer in developing countries is high [3]. It is estimated that approximately 500,000 new cases of cervical cancer are reported annually and about 230,000 women die of cervical cancer annually [4]. Cervical cancer is a complex disease involving the abnormal expression of many oncogenes and tumor suppressor genes [5–8]. Previous studies have revealed several genes associated with human cervical cancer [9–13]. However, the detailed molecular pathogenesis of cervical carcinoma is obscure. Further studies to explore cervical cancer pathogenesis are needed.

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous small noncoding and double-stranded RNA molecules that function as posttranscriptional gene regulators [14]. MiRNA dysregulation
occurs in numerous human malignancies and is associated with altered malignant potential, affecting survival, proliferation, apoptosis, and invasion [15–17]. MiRNAs can bind to the 3′-untranslated region (3′UTR) of target messenger RNAs (mRNAs) and suppress their expressions. Recently, global profiling studies have enabled microRNA-based stratifications of cancer types and patient outcomes [18–20]. Aberrant miRNA expressions can be pathogenic and occur in various human malignancies including cervical cancer [21–23]. MiR-30e is abnormally expressed in several cancers, including lung cancer, breast cancer, and hepatocellular carcinoma [24–26]. However, the function and target genes of miR-30e in cervical cancer remain to be elucidated.

GALNT7, a member of the acetylgalactosaminyltransferase family, acts as a glycosyltransferase in protein O-GlcNAcylation. Aberrant glycosylation is a well-described hallmark of many human cancers and is associated with cell growth, differentiation, transformation, adhesion, metastasis, and tumor immune surveillance [27]. The O-glycosylation of these glycoproteins is considered to play a key role in determining tumor properties in many cases of cancer, such as cervical cancer, hepatocellular carcinoma, and esophageal squamous cancer [17,28,29]. It has been reported that GALNT7 was targeted by some of miRNAs, such as miR-214, miR-494, and miR-17, in different cancers [17,28,30]. However, the expression and the regulation of GALNT7 in cervical cancer remain obscure.

In the current study, we investigated the expression profile and correlation of miR-30e and GALNT7 in cervical cancer patient tissues. Furthermore, the cellular and molecular functions of miR-30e and one of its key targets, GALNT7, were elucidated. Our findings indicate that miR-30e may be an important biomarker and target for diagnosis and treatment of cervical cancer patients.

Materials and Methods

Patient Sample

Thirty cervical cancer tissue samples and their corresponding adjacent nontumorous tissues were obtained from Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) after surgical resection. Written consent approving the use of tissue samples for research purposes was obtained from patients. The information of patients with cervical cancer is presented in supporting materials. Transfection was performed using the Cell-Light TM EdU imaging detecting kit according to the manufacturer’s instructions (RiboBio, Guangzhou, China).

Analysis of Cell Proliferation

SihA and Caski cells were seeded into 96-well plates (1000 cells/well) for 24 hours before transfection, and 3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess cell proliferation every day from the first day until the third day after transfection. 5-Ethyl-2′-deoxyuridine (EdU) incorporation assay was carried out using the Cell-Light TM EdU imaging detecting kit according to the manufacturer’s instructions (RiboBio, Guangzhou, China).

Plasmid Construction

The CDS sequence of GALNT7 was amplified by PCR from the cDNA of SiHa using specific primers and was cloned into the pcDNA3.1 (+) vector. To construct luciferase reporter plasmids of GALNT7, the wild type or mutant of GALNT7 mRNA 3′UTR which was targeted by miR-30e was amplified by PCR and was inserted into the pGL3-Control vector to generate pGL3-GALNT7-wt and pGL3-GALNT7-mut, respectively. Primers used were as follows: 5′-GCTCTAGAGAAGACCTGTCGCAACTAT-3′, 5′-ACATTATCATCTCGAGTTACTAAAAG GAGACTGCG-3′, 5′-GCAGCTCTCCCTTGTAGTAAAGCTGATGATAATGT-3′, and 5′-GGGGGCC CGGCGGTAATCCTTCCAAAGAAGACGAC-3′.

Luciferase Reporter Gene Assay

Luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. Cells were transferred into 24-well plates at 3 × 10^4 cells per well. After 48 hours, the cells were transiently cotransfected with 50 ng/well of pRL-TK plasmid (Promega, Madison, WI) containing the Renilla luciferase gene used for normalization, pGL3-GALNT7-wt or pGL3-GALNT7-mut, respectively. Primers used were as follows: 5′-ACCAGGGAGGAGATTCCCAGAA-3′, 5′-GCACGCCATGCTGAAATGTAATGT-3′, and 5′-GGGGGCC CGGCGGTAATCCTTCCAAAGAAGACGAC-3′.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed with PrimeScript RT Reagent Kit (Perfect Real Time) (TaKaRa Biotechnology, Dalian, China). All steps were according to the manufacturer’s protocol. Primers were as follows: GALNT7 forward: 5′-TGTGGGTGGAAAATGTGGGAA-3′, GALNT7 reverse: 5′-GACACCGATCTGTTGATGTA AA-3′; MiR-30e forward: 5′-TGCATACACTCTGGAGAAGG-3′, MiR-30e reverse: 5′-GAGAGCTAGAGAGGGGACG-3′, U6 forward: 5′-CTCGTTCGGGCAGAGC-3′, U6 reverse: 5′-AAGCTGTCGAAGATCCCTG-3′, GAPDH forward: 5′-GGATGCCCCATGATGGAAAGG-3′, GAPDH reverse: 5′-ACACCCTGTAT TCCGGGTCAT-3′.

Western Blotting

Cancer cells were lysed in RIPA buffer at 4°C for 30 minutes and centrifuged at 12,000×g at 4°C for 15 minutes to obtain total protein lysates for immunoblot analysis. Cell lysates were separated by using 10% SDS-PAGE and transferred onto PVDF membranes. Proteins were then subjected to immunoblotting. All primary and secondary antibodies were diluted in PBS. Primary antibody incubation was performed at 4°C overnight, and secondary antibody incubation was performed at room temperature for 2 hours. The immunoreactive blots were visualized using an enhanced chemiluminescence reagent.
The anti-GALNT7 antibody mouse monoclonal antibody was obtained from Abcam, and anti-actin was purchased from Proteintech (Wuhan, China).

**Cell Invasion Assay**

Matrigel (1:5, 50 μl/well, BD Bioscience, San Jose, CA) was added to Transwell chambers in 24-well plate. A total of 2 × 10⁴ posttransfection SiHa and Caski cells in100 μl of serum-free DMEM medium were plated onto the upper chambers (24-well insert, pore size 8 μm, Corning), and the lower chambers were filled with 500 μl of DMEM medium with 10% FBS. After 24 hours of incubation, the membranes were fixed with methanol, stained by 0.1% crystal violet, and washed three times. Finally, the stained cells were visualized under a microscope and counted.

**Tumor Xenograft Experiment**

We obtained 18 BALB/c-nude mice (6-7 weeks old, female) from Beijing Huafukang Bioscience Co. Inc. (Beijing, China). The mice were housed in an SFP room that was maintained at a constant temperature (23°C-25°C) and humidity (40%-50%). The mice were randomly divided into three groups (each group, n = 6). SiHa Cells were harvested and resuspended at 2 × 10⁷ cells/ml. Then, 0.2-ml aliquots were injected subcutaneously into the flank of each mouse. Tumor growth was measured using a Vernier caliper after 6 days from injection and then every 6 days. At the end of experiment, mice were killed and tumors were weighed. The volume was calculated with the following formula: $V = \frac{\text{length} \times \text{width}^2}{2}$.

**Statistical Analysis**

All replicates are true biological replicates. The Student’s $t$ test was used to analyze the statistical significance of the results. *$P < .05$ and **$P < .01$ were considered statistically significant. Spearman correlation coefficients were used for relation statistics.

**Results**

**Downregulation of miR-30e in Cervical Cancer Tissues and Cervical Cancer–Derived Cell Lines**

We measured the expression of miR-30e in 30 human cervical cancer tissues and their counterparts using quantitative RT-PCR. MiRNA-30e exhibited apparently low level expression in cancer tissues relative to those of normal tissues (Figure 1A). Moreover, we tested the miR-30e expression in one normal epithelial cell line, Hacat, and three cervical cancer–derived cells, including HeLa, SiHa, and Caski. Our data showed that miR-30e was downregulated in cervical cancer cells relative to normal epithelial cell (Figure 1B). The results imply that miR-30e is reduced in cervical cancer tissues and may be serve as antitumor gene involved in cervical carcinogenesis.

**MiR-30e Inhibits Proliferation and Invasion Ability of SiHa and Caski Cells**

In order to test the effect of miR30e in cervical cancer, firstly, we transfected miR-30e mimics and control to SiHa and Caski cells and detected miR-30e level by real-time PCR. Our data showed that the miR-30e expression level was obviously increased in miR-30e mimics–transfected group compared to NC group (Figure 2A). Next, we used MTT assay to determine the effect of miR-30e on cell viability. Cells transfected with miR-30e had decreased cell viability (Figure 2, B and C). Consistent with this result, we confirmed the effect of miR-30e on the long-term proliferative capacity of SiHa and Caski cells with colony formation. The colony numbers of above-mentioned cells with miR-30e mimics were both decreased relative to control group (Figure 2D). Furthermore, EdU assay was used to analyze effect of miR-30e on the cell proliferation of SiHa and Caski cells. Overexpression of miR-30e resulted in a reduced number of SiHa and Caski cells compared to the NC group (Figure 2, E and F). In addition, we observed that invasion ability of SiHa and Caski cells was suppressed after overexpressed miR-30e (Figure 2, G and H). These results demonstrate that miR-30e can repress the growth of cervical cancer cells.

**MiR-30e Negatively Regulates GALNT7 Through Directly Targeting its 3′ UTR**

Next, we screened the target genes of miR-30e using Targetscan, Miranda, and Pictar software. Interestingly, we observed that GALNT7 might be a putative target gene of miR-30e (Figure 3A). Then, we cloned the 3′UTR of GALNT7 mRNA (or its mutants) into downstream of pGL3-control luciferase reporter gene vector named GALNT7 3′UTR and GALNT7 3′UTR mut, respectively (Figure 3B). The luciferase reporter gene assays revealed that miR-30e significantly suppressed the firefly luciferase activities of GALNT7 3′UTR in SiHa cells, whereas it failed to work when the seed sequence was mutated (Figure 3C). The inhibition of endogenous miR-30e by anti–miR-30e resulted in the increases of firefly luciferase activities of GALNT7 3′UTR in SiHa cells but not the mutant (Figure 3D).

![Figure 1](https://example.com/figure1.png)  
Figure 1. Expression of miR-30e mRNA in cervical tissues and cell lines. (A) qRT-PCR of miR-30e relative to U6 expression in cervical cancer and normal tissues. (B) qRT-PCR of miR-30e relative to U6 expression in immortalized cervical epithelial cell line and cervical cancer cell lines. *$P < .05$, **$P < .01$, Student’s $t$ test. The experiment was repeated at least three times.
Then, we performed the transient transfection of miR-30e in SiHa and Caski cells to evaluate the effect of miR-30e on GALNT7. Our data showed that the overexpression of miR-30e was able to suppress the expression of GALNT7 at the levels of mRNA and protein in the above cells (Figure 3, E and F). Taken together, we conclude that miR-30e negatively regulates GALNT7 through directly targeting its 3′UTR.

Knockdown of GALNT7 Inhibits Proliferation and Invasion Ability of SiHa and Caski Cells

It has been reported that GALNT7 was abnormally increased in human cervical cancer. Therefore, we detected the effect of GALNT7 knockdown on the growth and colony formation ability of SiHa and Caski cells. Both mRNA and protein levels of GALNT7 were decreased after treatment with si-GALNT7 in SiHa and Caski cells.
MTT assay showed that the cell proliferation was significantly decreased, compared with negative control transfection, in SiHa and Caski cells, respectively, when GALNT7 was silenced by siRNA (Figure 4, C and D). Transfection of si-GALNT7 also significantly decreased the colony numbers of SiHa and Caski cells (Figure 4, E and F). Moreover, we validated that the cell invasion ability was significantly decreased, compared with negative control transfection, in SiHa and Caski cells, respectively, when GALNT7 was silenced by siRNA (Figure 4, G and H). We conclude that knockdown of GALNT7 inhibits proliferation and invasion ability of SiHa and Caski cells.

**Restoration of GALNT7 Counteracts Effects of miR-30e Expression**

To confirm that the effects of miR-30e on the proliferation and colony formation of SiHa and Caski cells are mediated through GALNT7, we constructed a eukaryotic expression vector containing the GALNT7 CDS without the 3′UTR to avoid the influence of miRNAs. Transfection of SiHa and Caski cells with this eukaryotic expression vector construct reversed the negative effects of miR-30e on GALNT7 mRNA and protein levels (Figure 5, A and B). The MTT assay showed that overexpression miR-30e could inhibit the growth of SiHa and Caski cells. Conversely, the event caused by miR-30e was abrogated when co-transfected with the GALNT7 vector in SiHa and Caski cells (Figure 5, C and D). Moreover, colony formation assay showed a similar effect of the above mentioned in SiHa and Caski cells (Figure 5, E and F). In addition, we observed that overexpression of both miR-30e and GALNT7 could rescue the inhibition effect by miR-30e in SiHa and Caski cells (Figure 5, G and H). Thus, we conclude that restoration of GALNT7 counteracts effects of miR-30e expression in SiHa and Caski cells.

**Restoration of GALNT7 Can Rescue the miR-30e–Suppressed Growth of Cervical Cancer Xenografts In Vivo**

To further assess the effects of miR-30e on the proliferation of cervical cells through GALNT7. Nude mice were injected subcutaneously with pretreated SiHa cells. After 30 days, the mice were mercifully killed (Figure 6A). As shown in Figure 6B, the tumors formed by the miR-30e–transfected SiHa cells grew much slower than those formed by the negative control SiHa cells (Figure 6B). However, the event caused by miR-30e was rescued when co-transfected with the GALNT7
vector in SiHa cells (Figure 6B). At the termination of the experiment, the mice were sacrificed and the tumors were excised. Then, the tumors weights were recorded. The weights of the tumor obtained from miR-30e-transfected group were lower than those of negative control group (Figure 6C). The weights of the tumor due to the GALNT7-overexpressing cells were much heavier than those due to the miR-30e cells (Figure 6C). Moreover, the expression of Ki67, a well-known cell proliferation marker, was examined in the tumor xenografts tissues by immunohistochemical staining. As shown in Figure 6D, the expression of Ki67 in the tumor tissues formed by the miR-30e–co-transfected SiHa cells was decreased compared with the negative control cells. In contrast, many more Ki67-positive cells were found in the tumor tissues formed by the GALNT7 and miR-30e–co-transfected SiHa cells than in those formed by the miR-30e–transfected SiHa cells (Figure 6D). All these data suggest that GALNT7 can rescue the miR-30e–suppressed growth of cervical cancer xenografts in vivo.

**Figure 4.** Knockdown of GALNT7 restrains the cervical cancer cells proliferation and colony formation. (A) qRT-PCR analysis the GALNT7 mRNA expression level in SiHa and Caski cells after treatment with si-GALNT7. (B) Western blot shows the GALNT7 protein expression level in SiHa and Caski cells after treatment with si-GALNT7. (C and D) Cell growth was measured by MTT assay at different time intervals in SiHa and Caski cells, respectively. (E and F) Effect of si-GALNT7 on cell proliferation was determined by colony formation assays in SiHa and Caski cells, respectively. (G and H) Cell invasion. *P < .05, **P < .01, Student’s t test. The experiment was repeated at least three times. **MiR-30e is Negatively Correlated to GALNT7 in Human Cervical Cancer Tissues**

To further detect the level of GALNG7 in human cervical cancer tissues, we utilized qRT-PCR to detect the GALNT7 expression level...
in human cervical cancer tissues and adjacent normal tissues. We observed that expression levels of GALNT7 mRNA were significantly increased in all the 30 cervical cancer samples relative to their peritumor counterparts (Figure 7A). Then, we analyzed the data of GALNT7 expression level in human cervical cancer from OncoLnc (http://www.oncolnc.org) (Figure 7B), suggesting that high levels of GALNT7 are associated with poor survival of cervical cancer. Furthermore, we validated the relationship between miR-30e and GALNT7 mRNA in 30 clinical cervical cancer tissues using qRT-PCR analysis. As expected, we found that the levels of miR-30e exhibited a significant negative correlation with those of GALNT7 mRNA (Figure 7C). This result supports that GALNT7 is one of the target genes of miR-30e in cervical cancer. Taken together, we conclude that miR-30e is negatively correlated to GALNT7 in human cervical cancer tissues.

**Discussion**

Cervical cancer is the second women common malignancy worldwide, especially in developing countries, rank only second to breast cancer [3]. MiRNAs have been demonstrated to play important roles in multiple biological processes [31–33]. Various miRNAs have been reported to regulate cervical cancer through different mechanisms [34,35]. Some miRNAs modulate HCC by directly regulating the mRNA expression levels. Recent studies showed us that miR-30e was connected with proliferation, invasion, and migration of tumor cells because its dysregulation was found in various types of tumors [24]. However, the effect of miR-30e in cervical carcinoma has not been elucidated.

In this study, we found that the expression of miR-30e was significantly downregulated in tumor specimens compared to nontumor specimens. Moreover, qRT-PCR analysis was used to
test the expression level of miR-30e in cervical cancer–derived cells. We demonstrated that miR-30e might act as a cancer suppressor in cervical cancer.

Glycosyltransferases are a group of enzymes that catalyze the addition of monosaccharides to core proteins. In a previous report, GALNT7 is targeted by miR-214 in esophageal squamous cell cancer [17]. MiR-214 also suppresses growth and invasiveness of cervical cancer cells by targeting GALNT7 [36]. Our data showed that GALNT7 was a new target of miR-30e in cervical cancer. The expression level of GALNT7 was dramatically decreased in SiHa and Caski cells after overexpression of miR-30e. It has been reported that GALNT7 plays a role in carcinogenesis and metastasis in many common cancers [30,37]. Meanwhile, GALNT7 is found to be upregulated in cancers such as laryngeal carcinoma and cervical cancer [36,38]. Recent studies manifested that suppressing the expression of GALNT7 can inhibit cancer cell invasion and metastasis [17]. In addition, we tested whether the effects of miR-30e on the proliferation of cervical cells are mediated through GALNT7 using a xenografts mice model in vivo. Our results demonstrated that miR-30e could suppress the growth of cervical cancer xenografts in vivo. In our study, we also found that GALNT7 was upregulated in cervical cancer patient tissues and was positively correlated with poor survival time. Cervical cancer cells exhibited decreased growth and colony formation ability compared to control group when transfected with GALNT7 siRNA. Otherwise, GALNT7 could rescue the effect of miR-30e overexpression in SiHa and Caski cells.

Altogether, our study demonstrates that miR-30e expression is significantly decreased in cervical cancer tissues and cervical cancer cells. The miR-30e inhibits the proliferation and metastasis of cervical cancer cells. Furthermore, GALNT7 is a direct target of miR-30e in cervical cancer. In sum, miR-30e exerts its inhibitory effects on cervical cancer proliferation and metastasis, at least in part, by targeting GALNT7.

**Conclusions**

To conclude, we recognize miR-30e downregulation as a biomarker for cervical cancer. Moreover, miR-30e is able to suppress the proliferation and invasion ability of cervical cancer cells through targeting 3′UTR of GALNT7 mRNA. Our findings may provide insight into a novel mechanism of cervical carcinogenesis. MiR-30e
and its new target GALNT7 could be biomarker and therapeutic target for cervical cancer in the future time.

**Conflicts of Interest**
The authors declare that they have no competing interests.

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