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TGIF2 promotes cervical cancer metastasis by negatively regulating FCMR

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Abstract. – OBJECTIVE: We aimed at studying the correlation between TGIF2 expression and clinicopathological features of cervical cancer (CCa). The relationship between TGIF2 and FCMR and its influence on the proliferation and metastasis of tumor cells were investigated using molecular biology techniques, so as to reveal the pathogenesis of CCa and provide a new target for clinical treatment.

PATIENTS AND METHODS: TGIF2 expression in 60 pairs of cervical tumors and paracancerous tissues samples collected from CCa patients of our hospital was studied by quantitative real-time polymerase chain reaction (qPCR) analysis, and the association between TGIF2 expression and the clinical indicators or prognosis of CCa patients were analyzed. CCa cells with TGIF2 knockdown were constructed using transfection technology. Changes in the biological phenotypes (proliferation, migration, invasion) of CCa cells C33-A and HeLa after TGIF2 knockdown were determined by Cell Counting Kit-8 (CCK-8) and transwell assays. In addition, the effects of TGIF2/FCMR axis on CCa metastasis were further explored in nude mice *in vivo*.

RESULTS: Our data revealed a significant increase in TGIF2 mRNA expression in CCa tissue specimens compared to adjacent ones, and the increasing degree was positively correlated with the incidence of lymph node or distant metastasis of CCa patients. The results of CCK-8 and transwell suggested that knocking down TGIF2 effectively attenuated the proliferative ability and invasiveness of CCa cells. Luciferase assay confirmed that TGIF2 can directly bind to the DNA promoter of its target gene FCMR. Simultaneous transfection of sh-TGIF2 and sh-FCMR partially reversed the inhibitory effect of single transfection of TGIF2 knockdown on the malignant progression of CCa. Experiments in nude mice also suggested that TGIF2 could promote CCa tumorigenesis through the modulation of FCMR expression.

CONCLUSIONS: In summary, TGIF2 can promote the migration and proliferation ability of cervical cancer cells *via* down-regulating FCMR. Our study provides a new therapeutic target for the clinical treatment of cervical cancer.

Key Words:
TGIF2, FCMR, Cervical cancer, Metastasis.

Introduction

Cervical cancer (CCa) is one of the most common female genital tract malignancies worldwide. At present, the incidence rate of CCa ranks the first in China, with its onset age getting younger and younger and its incidence rate increasing year by year[1-3]. Many people die of CCa each year, 80% of which occur in less developed developing countries. As many as 50,000 people die of cervical cancer each year in China. World Health Organization (WHO) points out that the death rate of CCa will increase by 25% in the next 10 years if effective measures are not taken as soon as possible, which has aroused great concern all over the world[4,5]. CCa causes severe physical and mental damage to patients, which forces people to study its pathogenesis as soon as possible to reveal its biological characteristics and explore these novel treatment meth-

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ods[6,7]. Pelvic lymph node metastasis is one of the important factors affecting the prognosis of CCa patients; therefore, exploring the molecular mechanism of lymphatic metastasis of CCa, may provide theoretical basis for the diagnosis and treatment of cancers[8,9].

Transcription factor TGIF2 (TGF-B inducing factor homologous box 2) belongs to the TALE homologous domain protein family, including TGIF, TGIF2, TGIF2LX/Y[10,11]. TGIF2 has four transcripts but share one coding protein[11-13]. The protein encoded by TGIF2 is a DNA-binding transcription factor with 237 amino acids and a molecular weight of 25878 Da[13]. TGIF2 can be involved in tumor regulation in a variety of ways, and its expression levels have been reported to be upregulated in skin cancer, medulloblastoma, multiple myeloma, and rectal cancer[12,14-17]. Therefore, this study focuses on the influence of TGIF2 expression on the progression of CCa. Bioinformatics site analysis suggests that FCMR is a potential target gene of TGIF2. However, there are no reports on the regulation of interaction of TGIF2 and FCMR on the biological functions of CCa.

In this research, we studied TGIF2 expression in CCa tissues and cell lines and the correlation between TGIF2 and the clinicopathological features of CCa. We investigated the relationship between TGIF2 and FCMR, as well as its biological characteristics such as the regulation of proliferation and metastasis of CCa cell lines. We characterized a novel axis involving TGIF2, FCMR, and the metastasis that governs the progression of CCa.

Patients and Methods

Patients and Cervical Carcinoma Samples

A total of 60 pairs of CCa tissues and paracancerous tissues were collected January 2018 to January 2020 in our hospital. All patients did not undergo any treatment such as radiotherapy and chemotherapy before surgery. The collected specimens were diagnosed by pathology and staged and were stored in the RNA specimen preservation solution within 5 minutes of excision to prevent RNA degradation. CCa pathological classification and staging standards are implemented in accordance with the international union against cancer (UICC) CCa staging standards. Inclusion criteria of ESCC patients were as follows, (1) no severe diseases in other organs; (2) none of patients had preoperative chemotherapy/radiotherapy or molecular targeted therapy. In addition, the exclusion criteria of ESCC Patients were as follows, (1) mental disease; (2) myocardial infarction; (3) heart failure or those previously exposed to radioactive rays. Patients and their families had been fully informed in this study and signed informed consent. Our research was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University.

Cell Lines and Reagents

CCa cell lines (HCC94, MEG-01, HeLa and C33-A) and normal human cervical epithelial cells (HaCaT) provided by American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in McCoy's 5A medium (HCC94, MEG-01) or high-glucose Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) (HeLa and C33-A) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) in an incubator at 37°C with 5% CO_2.

Transfection

Lipofectamine reagent was mixed with TGIF2 knockdown sequence (GenePharma, Shanghai, China) and then added into cells when cell density reached to 30-50%. 48 hours later, cells were collected for analysis.

Transwell Assay

50 uL of Matrigel Matrigel was spread (Matrigel: culture medium=1: 8, no Matrigel for migration experiments) at the bottom of the transwell chamber (Corning, Corning, NY, USA). Cells were prepared into cell suspensions 24 h after transfection and seeded upper transwell chamber (10,000 cells/well) supplemented with serum-free medium, and then 10% FBS medium was added to the lower compartment. The migrated cells were counted and observed after stained by crystal violet under a microscope.

Cell Wound Healing

After 48 hours of transfection, cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5×10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the

cells reached 90% or more the next day. After the stroke, cells were rinsed gently with phosphate-buffered saline (PBS) for 2-3 times and observed again after incubation in low-concentration serum medium for 24 h.

Quantitative Real Time-Polymerase Chain Reaction (qPCR)

Real-time fluorescence quantitative PCR method was used to detect TGIF2 expression, FCMR, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in CCa tissues and cells. 1 mL of TRIzol was used to lyse the tissues and cells to extract total RNA. Real-time PCR was performed according to the instructions of SYBR® Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan), with GAPDH as internal reference. Primers used in the qPCR reaction: TGIF2: forward: 5'-GCTCCGCACAAAGTTTACCCA-3', reverse: 5'-GGTGCAAGTACAGCCAGTCC-3'; FCMR: forward: 5'-GGTTCCTGAGTAAGCAGCGT-3', reverse: 5'-TGATGGCTCGTATTCTGGCA-3'; GAPDH: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

Transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 × g for 15 minutes at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Immunoblotting was carried out using primary antibodies against TGIF2 and FCMR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the horseradish peroxidase-labeled secondary antibody (Genscript, Nanjing, China). The intensity of protein image was determined using alpha SP image analysis software.

In Vivo Xenograft Model

In vivo nude mice tumorigenesis experiments were approved by The Animal Ethics and Use Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University. Ten 8-week-old male nude mice were purchased from the animal center and randomly divided into 2 groups (5 in each group). The CCa cell line C33-A was co-transfected with TGIF2 and FCMR knockdown vectors and injected subcutaneously into the axilla of mice. The tumor size was monitored every 7 days; then the mice were sacrificed after 6 weeks. The volume of all samples is calculated using the following formula: tumor volume = (width 2 × length)/2.

Dual-Luciferase Reporter Assay

CCa cells were co-transfected with FCMR/NC and pMIR Luciferase reporter plasmids using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours after transfection, the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) was used to normalize the reporter Luciferase activity to the control firefly Luciferase activity.

Statistical Analysis

Measurement data were compared using *t*-test and presented as X±SD (standard deviation). Categorical variables were analyzed using χ^2-test or Fisher's exact probability method. The survival analysis probability was analyzed by Kaplan-Meier curves and evaluated using the log-rank test. p less than 0.05 was considered statistically significant.

Results

TGIF2 Has an Increased Expression in CCa

QPCR results showed that TGIF2 expression in CCa tissue specimens was remarkably increased compared with adjacent ones (Figure 1A). According to TGIF2 expression, we divided the 60 pairs of tissue samples into high and low expression group. Table I shows that the high expression of TGIF2 was positively correlated with the incidence of CCa lymph node or distant metastasis, but not with age and tumor pathological stage (Figure 1B). Meanwhile, Kaplan-Meier survival curve indicated that the high expression of TGIF2 was remarkably relevant to the poor prognosis of CCa patients ($p<0.05$; Figure 1C). In addition, compared with HaCaT, TGIF2 was remarkably overexpressed in CCa cell lines (Figure 1D).

Silencing TGIF2 Inhibits the Proliferation and Migration of CCa Cells

We, then, constructed TGIF2 knockdown models in HeLa and C33-A cells, respectively, and verified the transfection efficiency through Western blotting analysis (Figure 2A). It was found that knockdown of TGIF2 markedly attenuated the proliferation rate and migration capacity of CCa cells, measured by CCK8 (Figure 2B) and transwell experiment (Figure 2C).

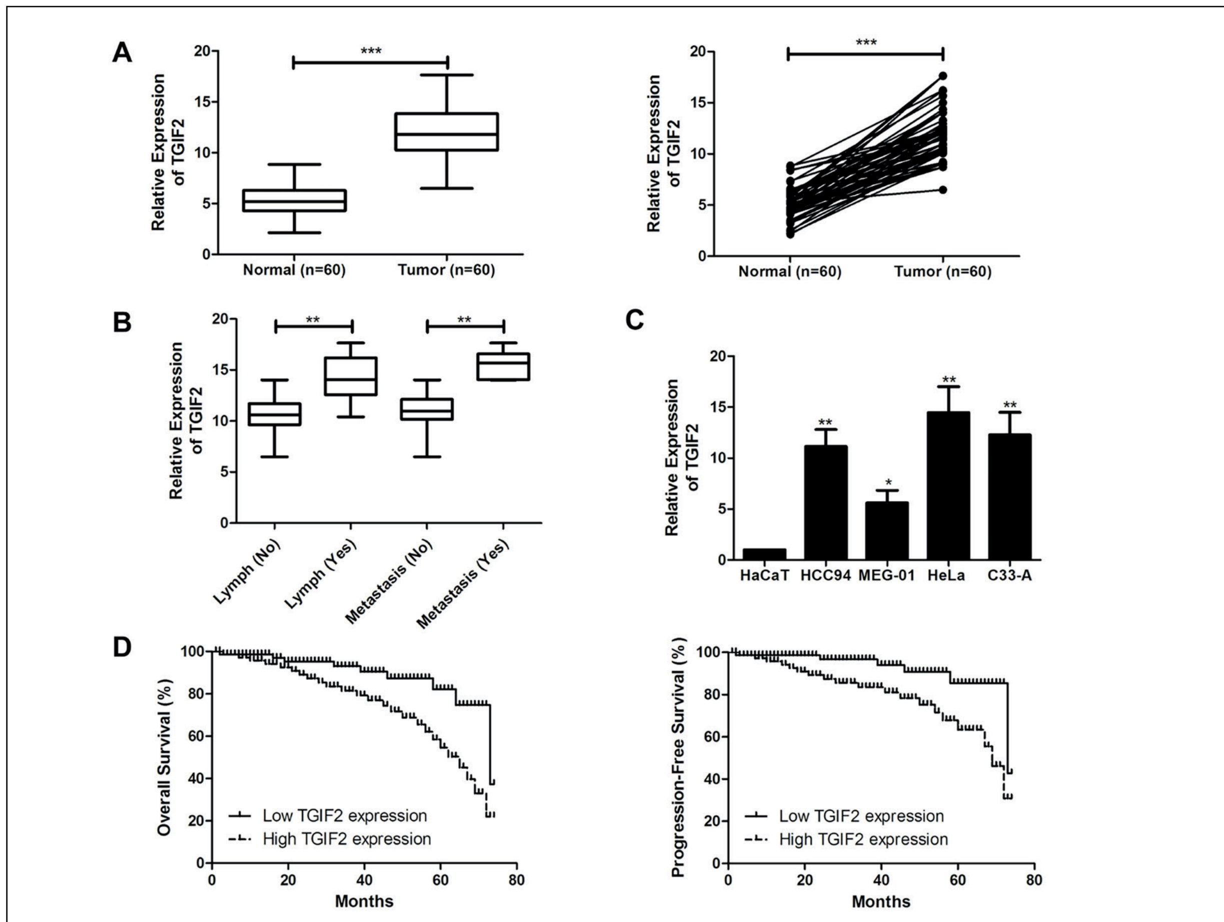

Figure 1. TGIF2 is highly expressed in cervical cancer tissues and cell lines. **A,** qRT-PCR was used to detect the difference in the expression of TGIF2 in cervical cancer tumor tissues and non-tumor tissues adjacent to cancer. **B,** qRT-PCR was used to detect the expression level of TGIF2 in lymph node and distant metastatic cervical cancer tissues. **C,** qRT-PCR was used to detect the expression level of TGIF2 in cervical cancer cell lines. **D,** Kaplan Meier survival curve of progression-free survival and overall survival of cervical cancer patients based on TGIF2 expression. Data are presented as average ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table I. Association of TGIF2 expression with clinicopathologic characteristics of cervical carcinoma.

Parameters	No. of cases	TGIF2 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.162
< 60	24	17	7	
≥ 60	36	19	17	
Tumor size				0.672
< 4 cm	33	19	14	
≥ 4 cm	27	17	10	
T stage				0.593
T1-T2	35	22	13	
T3-T4	25	14	11	
Lymph node metastasis				0.033
No	37	25	10	
Yes	23	11	14	
Distance metastasis				0.006
No	46	32	14	
Yes	14	4	10	

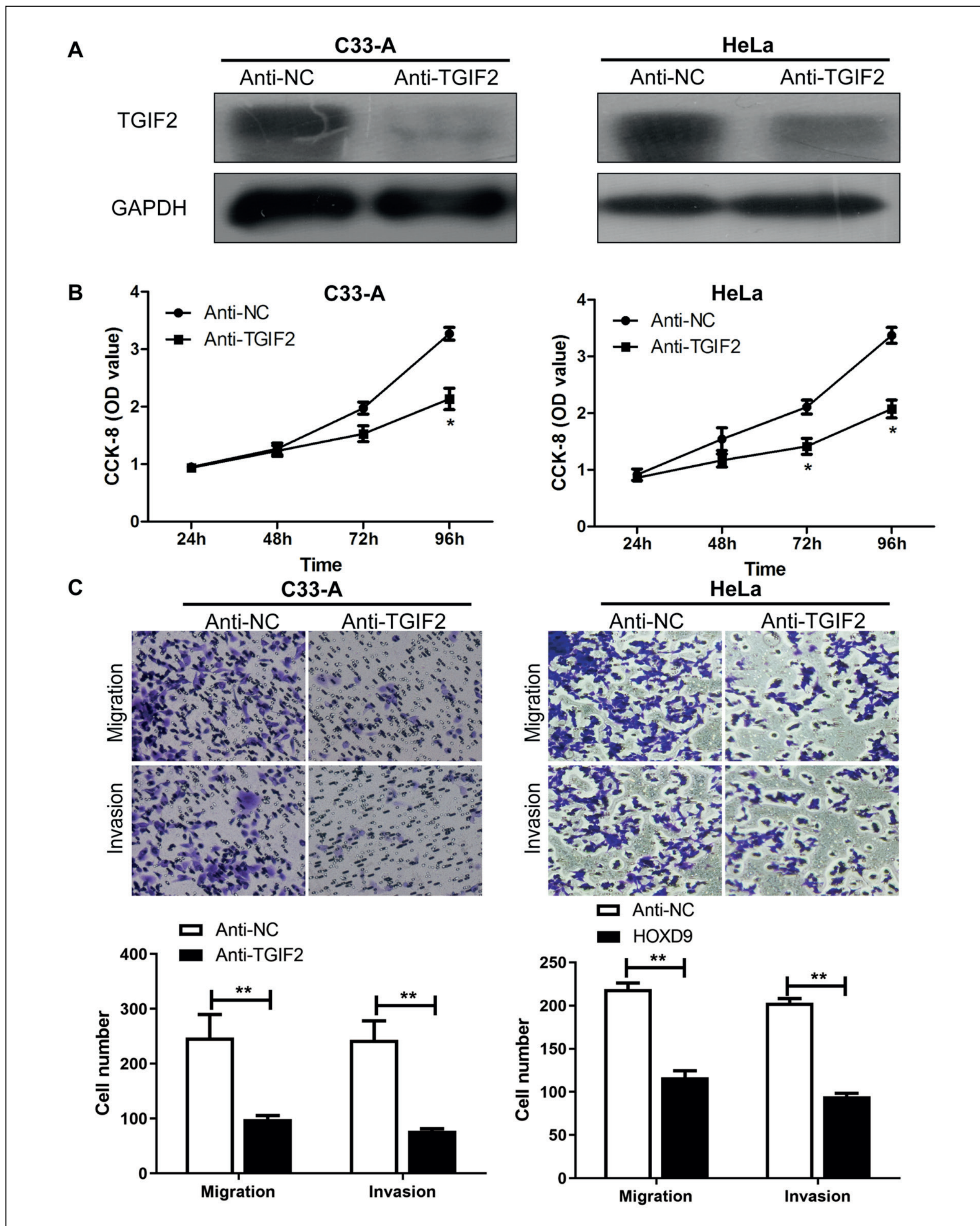

Figure 2. Silencing TGIF2 can inhibit the proliferation and invasion and migration of cervical cancer cells. **A,** Western blot experiment verified the efficiency of TGIF2 knockdown in cervical cancer cell lines HeLa and C33-A. **B,** The CCK-8 test was used to detect the ability of cell proliferation after transfection of TGIF2 knockdown vector in cervical cancer cell lines HeLa and C33-A. **C,** Transwell migration and invasion test was used to detect the ability of cell invasion and migration after transfection of TGIF2 knockdown vector in cervical cancer cell lines HeLa and C33-A (magnification: 40X). Data are average ± SD, * $p<0.05$, ** $p<0.01$.

Targeted Combination of TGIF2 and FCMR

To further explore through which TGIF2 regulates FCMR to promote CCa malignant progression, we conducted luciferase assay to confirm that TGIF2 as a transcriptional factor can directly bind to its target gene FCMR (Figure 3A). In addition, FCMR protein expression in CCa cells was remarkably up-regulated in sh-TGIF2 group in comparison to the sh-NC group (Figure 3B). Meanwhile, Figure 3C shows a negative correlation between the mRNA expression levels of TGIF2 and FCMR in CCa tissues (Figure 3C).

Silencing FCMR Reverses the Inhibitory Effect of TGIF2 Knockdown on Malignant Progression of CCa Cells

To further clarify the mutual regulation between TGIF2 and FCMR, we simultaneously transfected TGIF2 and FCMR knockdown vectors into HeLa and C33-A cell lines. As a result, in comparison to single transfection of TGIF2 knockdown vector, TGIF2 expression was upregulated after co-transfection of TGIF2 and FCMR knockdown vectors, indicating the negative correlation between the two genes (Figure 4A). Meanwhile, knocking down FCMR counteracts the suppressing effect of TGIF2 downregulation on the proliferation ability of CCa cells (Figure 4B) and reverses the reduction in the number of transcervical CCa cells in the transwell chamber induced by knockdown of TGIF2 (Figure 4C).

TGIF2 Promote CCa Tumorigenicity by Modulating FCMR In Vivo

C33-A cells transfected with TGIF2 and FCMR knockdown vectors were inoculated into each nude mouse and injected into the left armpit. By measuring the volume and weight of tumor-forming tissue in nude mice weekly, we found that both indicators of nude mice in co-transfection group were higher than those in single TGIF2 knockdown vector group (Figure 5A, 5B). In addition, simultaneous downregulation of the above two proteins increased the inhibited expression of TGIF2 induced by TGIF2 knockdown (Figure 5C). Finally, Western blot results suggest that knocking down FCMR reversed the promoting effect of knocking down TGIF2 on FCMR expression (Figure 5D). Taken together, the above data confirmed that TGIF2 can promote the tumorigenic ability of CCa cells in nude mice *via* negatively regulating FCMR.

Discussion

Cervical cancer as one of the most common malignant tumors in women, with its incidence

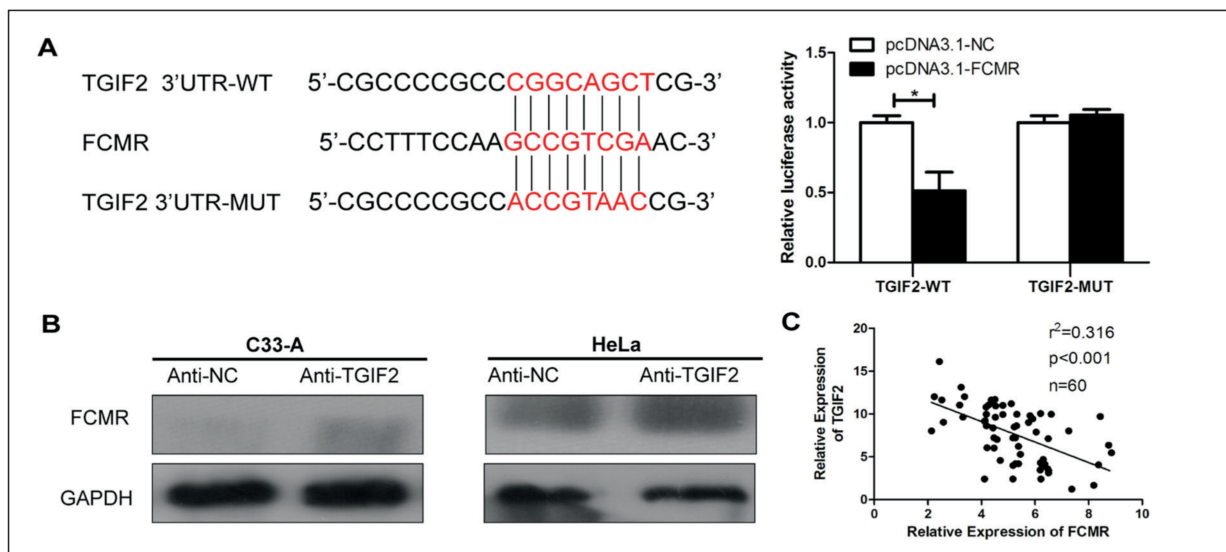

Figure 3. TGIF2 directly bind to its target gene FCMR. **A,** Luciferase reporter gene experiments verified that TGIF2 can target combine with the promoter of FCMR. **B,** Western blot detected the expression of FCMR after transfection of TGIF2 knockdown vector in cervical cancer cell lines HeLa and C33-A. **C,** The expression levels of TGIF2 and FCMR in cervical cancer tissue were significantly negatively correlated. Data are average ± SD, * $p<0.05$.

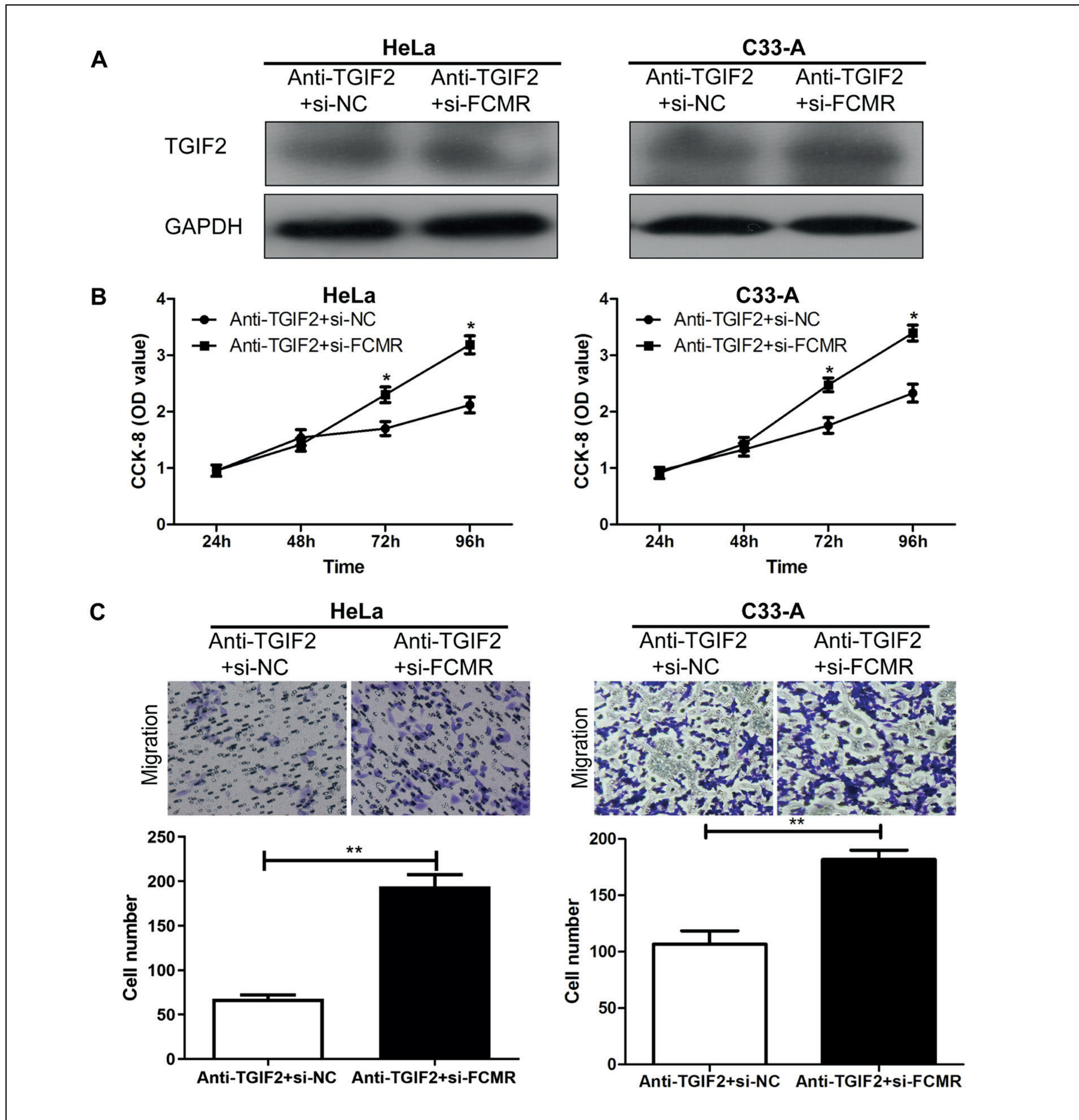

Figure 4. Mutual regulation of TGIF2 and FCMR promotes malignant progression of cervical cancer cells. **A,** Western blot detected the expression level of TGIF2 after co-transfection of TGIF2 and FCMR knockdown vector in cervical cancer cell lines HeLa and C33-A. **B,** CCK-8 test was used to detect the ability of cell proliferation after cervical cancer cell lines HeLa and C33-A after co-transfection of TGIF2 and FCMR knockdown vector. **C,** Transwell migration test was used to detect the ability of cell migration after co-transfection of TGIF2 and FCMR knockdown vectors in cervical cancer cell lines HeLa and C33-A (magnification: 40x). Data are average ± SD, * $p<0.05$, ** $p<0.01$.

only second to breast cancer, has become a serious threat to women's health. Its occurrence and development are a gradually evolving process, namely, cervical intraepithelial neoplasia (CIN) -- carcinoma *in situ*-early invasive cancer-invasive cancer[2-5]. Previous studies have indicated that persistent human papillomavirus (HPV) infection serves as a high-risk factor for CCa[18]. However, the morbidity and mortality of CCa have decreased with the continuous updating of screening methods and the popularization of vaccines[18,19]. Due to the uneven distribution of

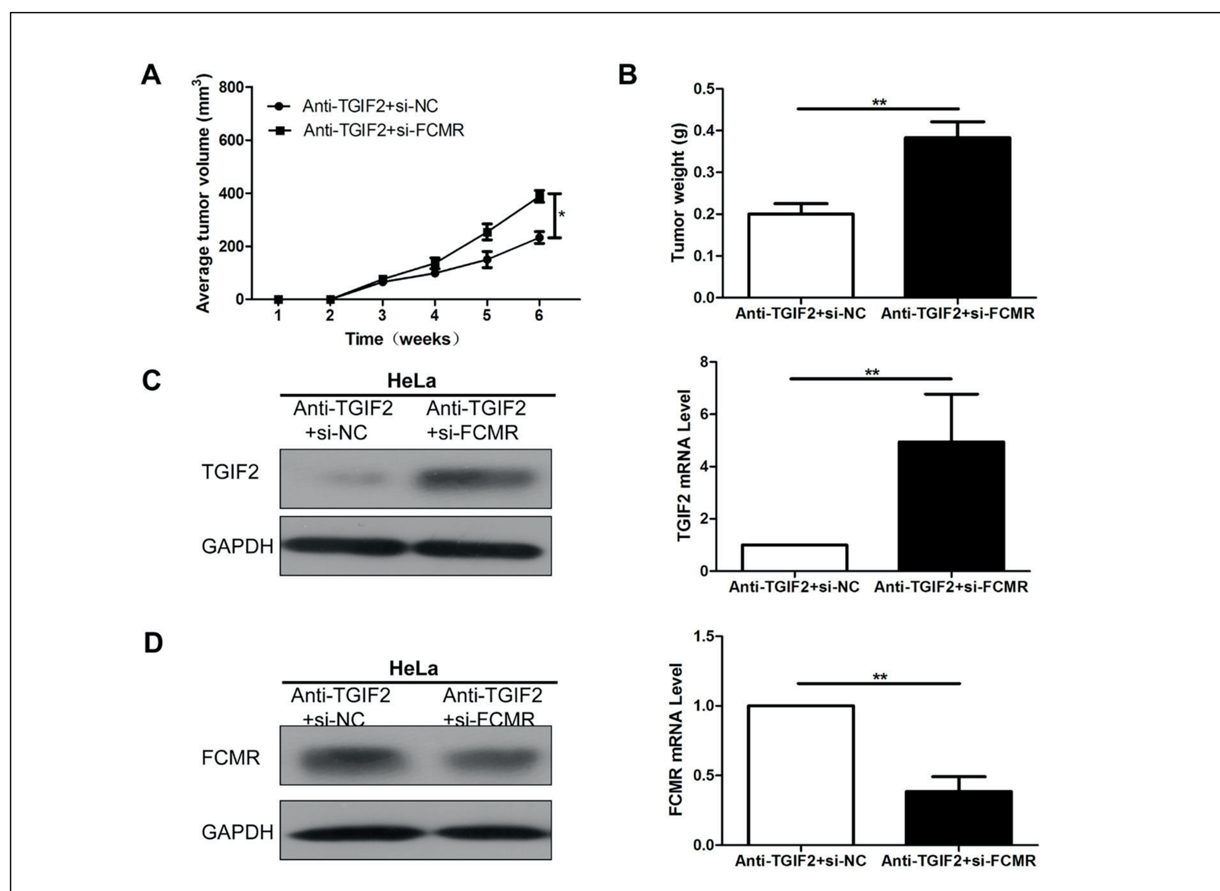

Figure 5. TGIF2 promotes the tumorigenic ability of nude mice injected with cervical cancer cells through negatively regulating FCMR. **A,** After injection of cervical cancer C33-A cells co-transfected with TGIF2 and FCMR knockdown vectors, the growth curve of tumor volume was calculated in different nude mouse groups. **B,** After injection of cervical cancer C33-A cells co-transfected with TGIF2 and FCMR knockdown vectors, respectively, the difference in tumor weight between different nude mouse groups was calculated. **C,** Western blot was used to detect the expression level of TGIF2 in the tumor tissue of cervical cancer nude mice injected with TGIF2 and FCMR knockdown vectors respectively. **D,** Western blot was used to detect the expression level of FCMR in tumor tissues of cervical cancer nude mice injected with TGIF2 and FCMR knockdown vectors. Data are average ± SD, * $p<0.05$, ** $p<0.01$.

resources, the incidence rate in developing countries is on the rise, and the trend becomes younger[1-3]. Nowadays, with the in-depth study of tumor molecular biology, a host of genes are discovered to be engaged in the development process of CCa, so the search for new molecular targets is of far-reaching significance for the prevention and treatment of this cancer[5-7].

TGIF is a TALE superfamily molecule that binds to Smads to inhibit the transcription of TGF-p[8,9]. TGIF can be phosphorylated by molecules of the Ras/MAPK pathway to promote cell proliferation and differentiation as well as inhibit cell apoptosis, affecting the occurrence of various tumors[9,10]. Notably, TGIF2 is highly expressed in ovarian tumor cell lines, suggesting that TGIF2 may play an essential part in the progression of tumors[12,14-17]. However, the association about TGIF2 and CCa is unclear. In this study, we detected that the expression of TGIF2 in CCa tissues was significantly higher than that in para-cancer normal ones. We also demonstrated a positive correlation between TGIF2 expression and the incidence of metastasis, suggesting that TGIF2 may act as an oncogene in the progression of CCa. *In vitro* experiments in CCa cell lines also revealed a consistent result. We revealed by CCK-8 and tranwell experiments that TGIF2 is able to enhance the migration ability and proliferation rate of CCa cells; however, the specific molecular mechanism still remains elusive.

Current methods for searching for target genes are mainly to predict possible target genes through bioinformatics analysis, then to verify the relationship between the original gene and the target gene through Dual-Luciferase assay[20,21]. In this study, we demonstrate that TGIF2 can directly bind to its target gene FCMR. After knocking down TGIF2, the expression of FCMR protein in CCa cells was markedly upregulated. Meanwhile, we also found a negative correlation between the mRNA expression levels of TGIF2 and FCMR in CCa tissues. In addition, *in vivo* and *in vitro* co-transfection experiments have shown that knocking down FCMR can upregulate the inhibited TGIF2 protein expression induced by single transfection of shRNA-TGIF2, and further enhance the invasion of CCa cells, achieving a similar effect to the overexpression of TGIF2. To sum up, our findings revealed that FCMR could reverse the promotive effects of TGIF2 on the proliferative and metastatic abilities of CCa.

Conclusions

Taken together, we identify TGIF2/FCMR axis as a critical promoting node in the development of cervical cancer, which may provide a new therapeutic target for the clinical treatment of CCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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