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MiR-182-5p promotes proliferation and invasion by regulating Smad4 in vulvar squamous cell cancer

Xiuhua Yang[1], Xin Wu[2]

[1]Department of Obstetrics, [2]Department of Gynaecology, The First Hospital of China Medical University, Shenyang, Liaoning (China)

Summary

The purpose of this study was to investigate the potential mechanism of miR-182-5p and Smad4 in vulvar squamous cell carcinoma (VSCC). A431 cells were transfected with miR-182-5p mimics, a small interfering RNA targeting Smad4 (siR-Smad4) and their negative controls. The authors performed gene functional experiments in A431 cells. The protein levels of MMP-2, MMP-9, E-cadherin, N-cadherin, and vimentin were detected after transfection miR-182-5p mimics. The expression levels of Smad4, p21, plasminogen activator inhibitor-1 (PAI-1), Bax, and Bim were detected after transfection miR-182-5p mimics. In A431 cells, up-expression of miR-182-5p advanced the cell proliferation, migration, invasion, G1-S phase transition, and down-regulated Smad4; miR-182-5p also increased the expressions of MMP-2 and MMP-9 and influenced alterations in epithelial-mesenchymal transition (EMT) and suppressed downstream target genes of Smad4. The down-regulation of Smad4 by siRNA simulated the roles of miR-182-5p. The authors conclude that miR-182-5p plays an oncogenic role by its target gene Smad4 in VSCC.

Key words: Vulvar squamous cell carcinoma; miR-182-5p; Smad4; Epithelial-mesenchymal transition.

Introduction

Vulvar cancer is a relatively rare type of gynecologic cancer. However, more and more younger women suffer from vulvar cancer in recent years [1]. The majority of cases are vulvar squamous cell carcinomas (VSCCs). Radical operation is the advisable treatment for Stage IV patients with a high rate of complications (~40%), especially accompanied with chemotherapy or radiotherapy during the perioperative period. Exploring new molecular indicators for diagnosis is necessary, which could be used to offer individualized consultations and better treatments.

MicroRNAs (miRNAs) repress target gene expression and usually perform important functions in cancers. Using miRNA microarray analysis and RT-qPCR [2], we confirmed miR-182-5p was significantly increased in VSCC samples, but the biological mechanism remains obscure. Smad4 is considered as a regulator of TGF-β/Smad signal pathway, and it was identified down-regulated in VSCC tissues [2]. According to the target prediction program mirbase, Smad4 is one of the putative target genes for miR-182-5p, Hirata *et al.* [3] found miR-182-5p targets Smad4 by 3'UTR-luciferase assay in bladder cancer, the association between miR-182-5p and Smad4 in VSCC requires further investigation.

In this research, the authors performed functional studies to find the roles of miR-182-5p and Smad4 in VSCC. Smad4 was confirmed to be the target gene of miR-182-5p in VSCC.

Materials and Methods

The authors quantified the amplified products using the SYBR Green method with GAPDH as the internal control. The fold changes were quantified using the $2^{-\Delta\Delta Ct}$ method. The primers were as follows: GAPDH forward, AAGGTGAAGGTCGGAGTCAAC, and reverse, GGGTCATTGATGGCAACAATA; Smad4 forward, CGCTTTTGTTTGGGTCAACT, and reverse, CCCAAACATCACCTTCACCT; P21 forward, TGTCCGTCAGAACCCATG, and reverse, TGGGAAGGTAGAGCTTGG; plasminogen activator inhibitor-1 (PAI-1) forward, GAGACAGGCAGCTCGGATTC, and reverse, GGCCTCCCAAAGTGCATTAC; Bax forward, ATGGACGGGTCCGGGGAGCAG, and reverse, CATGATGGTTCTGATCAGTT; Bim forward, GCCTTCAACCACTATCTCA, and reverse, ATCCAGCTCGGTGTCTTCT. The RT-qPCR parameters were the following: 95°C for ten minutes followed by 40 cycles of 95°C for five seconds and 60°C for 34 seconds.

The total protein from cells was prepared for western blot analysis. Immunoblotting was performed with anti-Smad4 (1:5000), anti-E-cadherin antibody (1:500), anti-N-cadherin antibody (1:500), anti-vimentin antibody (1:500), anti-matrix metalloproteinase2 (MMP2) antibody (1:1000), anti-MMP9 antibody (1:1000) or anti-GAPDH antibody (1:10000). The membrane was washed and incubated with goat anti-rabbit (1:5000) or anti-mouse IgG (H+L)-HRP conjugate antibody (1:10000). The Image J software was applied to determine the relative protein expression level.

The A431 cell line was obtained from ATCC and cultivated in RPMI 1640 medium including 10% foetal bovine serum under standard conditions at 37°C and 5% CO_2 in a humid atmosphere. The cells were transfected with Dharmacon miRIDIAN miR-182-5p mimics (miR-182-5p) and the negative control at a final concentration of 100 nmol/l. A small interfering RNA targeting

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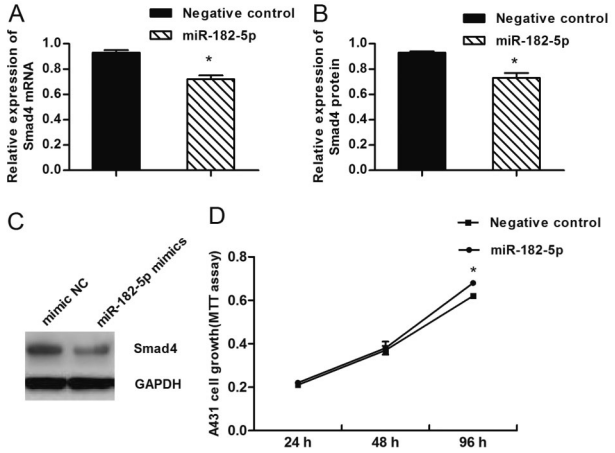

Figure 1. — miR-182-5p down-regulates the expression level of Smad4 and promoted cell proliferation. miR-182-5p inhibits the mRNA (A) and protein (B and C) expression levels of Smad4. (D) After 96 hours, miR-182-5p mimics increased cell proliferation. $*p < 0.05$.

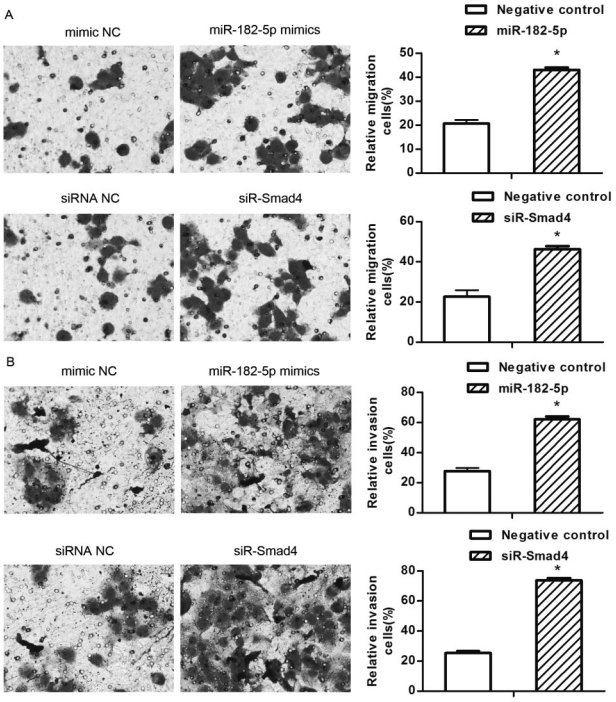

Figure 2. — miR-182-5p mimics and siR-Smad4 increases migration and invasion. (A) miR-182-5p mimics and siR-Smad4 increases migration. (B) miR-182-5p mimics and siR-Smad4 increases invasion. $*p < 0.05$.

Smad4 (siR-Smad4) was purchased. The cells were transfected by lipofectamine 2000.

The authors performed an MTT analysis to evaluate cell proliferation. A431 cells were plated in 96-well sample culture plates

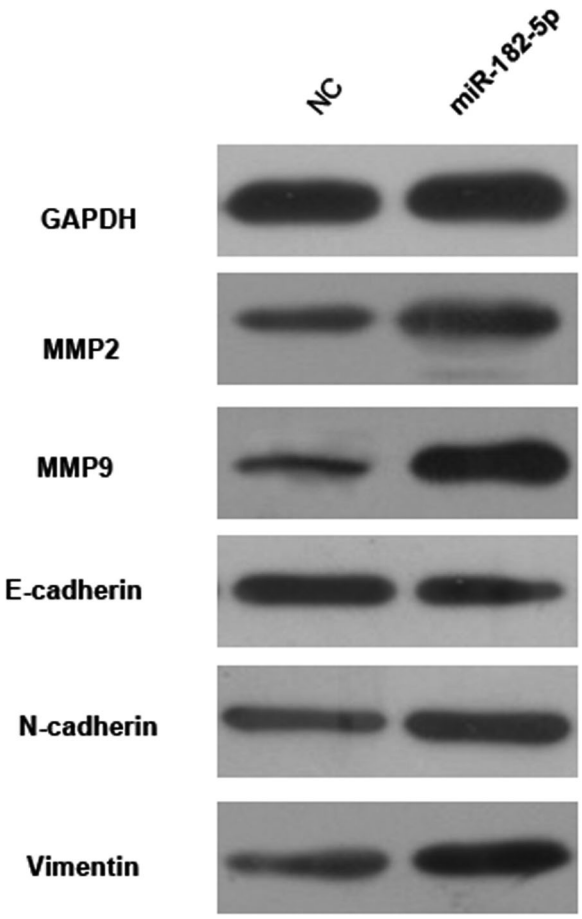

Figure 3. — Over-expression of miR-182-5p promotes MMPs and regulates EMT. Over-expression of miR-182-5p promotes the protein expression levels of MMP2 and MMP9, while the miR-182-5p mimics decreases E-cadherin but increases N-cadherin and vimentin.

at a density of 5×10^4 cells per well with the miR-182-5p mimics or siR-Smad4 and the corresponding negative controls. The cells were cultured for 48 and 96 hours, and the optical absorbance was read at 490 nm on a microplate reader. The experiments were conducted in triplicate.

A Transwell test was performed to examine cell migration. Forty-eight hours after transfection, 5×10^4 cells were placed in the upper chambers of transwell plates with an untreated membrane. After 24 hours of incubation, the chambers were treated with 4% paraformaldehyde and fixed with crystal violet. The cells that passed through the membrane were calculated. In invasion tests, the upper chamber was covered with matrigel for two hours. The other procedures were similar to the migration experiment. The migration and invasion assays were conducted in triplicate.

Cells transfected with miR-182-5p mimics or siR-Smad4 for 48 hours were collected and placed in ethanol (70%) for 24 hours. The cells were then treated with propidium iodide (40 μg/ml) for half an hour. Flow cytometry was used for the analysis. The experiments were conducted in triplicate.

The data are showed as the means ± SD, and the SPSS 19.0 software was used to analyse results. *P* values of less than 0.05 were considered to be statistically significant.

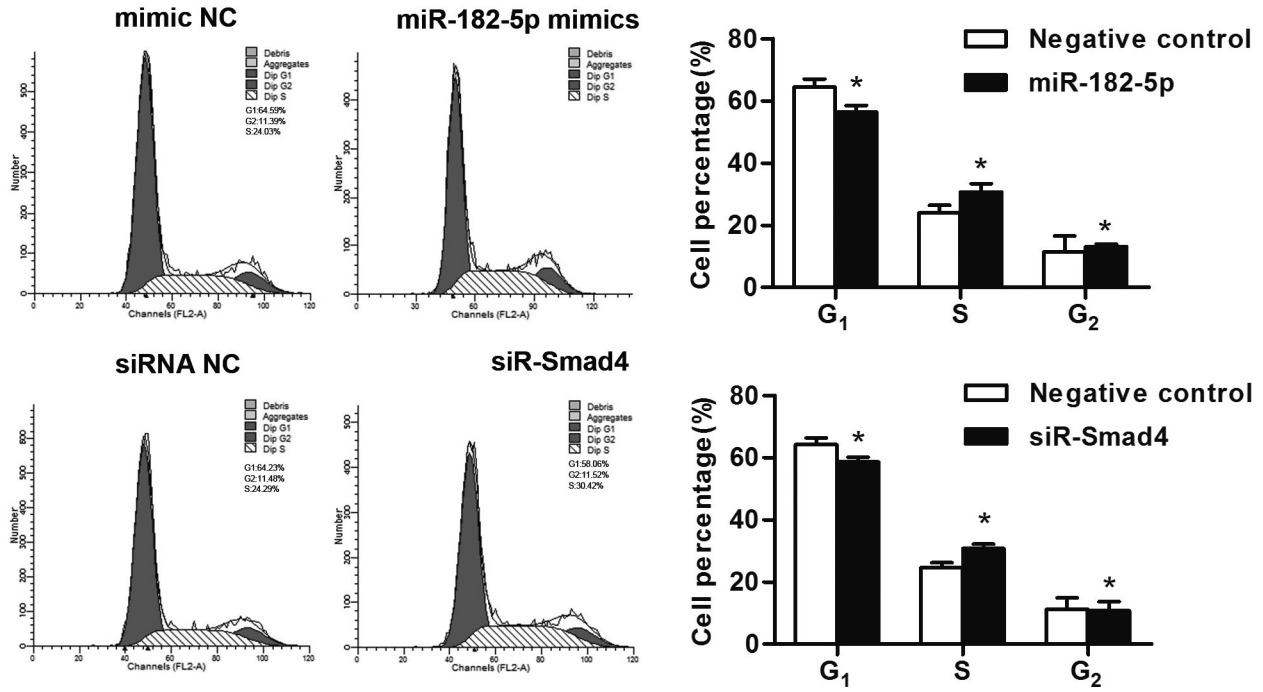

Figure 4. — miR-182-5p and Smad4 regulate the cell cycle. miR-182-5p mimics and siR-Smad4 promotes the G1-to-S transition. *$p < 0.05$.

Results

A431 cells were transiently transfected with the miR-182-5p mimics at a concentration of 100 nM. At 96 hours, the cell proliferation rate was markedly higher ($p = 0.002$) (Figure 1D). The migration rate was 22.33% higher ($p = 2.93\times10^{-5}$: Figure 2A) and the invasion rate was 23.66% higher ($p = 3.24\times10^{-4}$: Figure 2B). MMP-2 and MMP-9 were selected to test whether miR-182-5p could affect genes relevant to tumour invasion. MMP-2 and MMP-9 were increased in the miR-182-5p mimics group at 96 hours (Figure 3). Therefore, miR-182-5p was needed to promote invasion. An analysis of the cell cycle distribution showed that the cells in the G1 phase was significantly decreased from 64.63±2.63% before transfection to 56.33±2.18% at 48 hours after transfection ($p = 0.014$) and the cells in the S phase was remarkable improved from 23.95±2.47% before transfection to 30.61±2.77% at 48 hours after transfection ($p = 0.036$: Figure 4).

The authors used the siR-Smad4 to investigate the role of Smad4 in A431 cells. Forty-eight hours after the knockdown of Smad4, the mRNA expression level of Smad4 was reduced significantly ($p = 3.30\times10^{-4}$; Figure 5A), and the protein expression level of Smad4 was also reduced ($p = 9.03\times10^{-5}$; Figures 5B and 5C). Smad4 knockdown markedly promoted cell proliferation ($p = 0.009$: Figure 5D) and increased the migration ($p = 3.24\times10^{-4}$; Figure 2A) and invasion ($p = 2.65\times10^{-6}$; Figure 2B) of A431 cells. Smad4 knockdown also markedly decreased cells in the G1 phase from 64.19±2.15% before knockdown to 58.57±1.56% at 48 hours after knockdown ($p = 0.021$: Figure 4).

In A431 cells, Smad4 expression was significantly down-regulated after transfection miR-182-5p mimics 48 hours later: Smad4 mRNA expression was decreased ($p = 3.28\times10^{-4}$: Figure 1A) and Smad4 protein expression was also decreased ($p = 0.001$: Figures 1B and 1C).

The authors transfected the miR-182-5p mimics into A431 cells. Four days later, the shape of cells was not obviously changed; however the protein level of E-cadherin was down-regulated and the protein expressions of N-cadherin and vimentin were up-regulated (Figure 3).

The preset results displayed the mRNA levels of p21, PAI-1, Bax, and Bim were inhibited after transfection miR-182-5p mimics 48 hours later (Figure 6), indicating miR-182-5p could regulate Smad4-mediated signaling pathway.

Discussion

Nowadays, more and more researchers focus on the vital functions of miRNAs on the etiology of cancers. However, there are few studies in vulvar cancer. de Melo Maia *et al.* [4] identified 79 miRNAs that showed markedly different expression levels in vulvar cancer. They also confirmed a miRNA sponge which might be an effective method for diagnosis and treatment for this cancer [5]. In the present authors' previous study [2], miR-182-5p was remarkably increased in VSCC. Most studies showed miR-182-5p

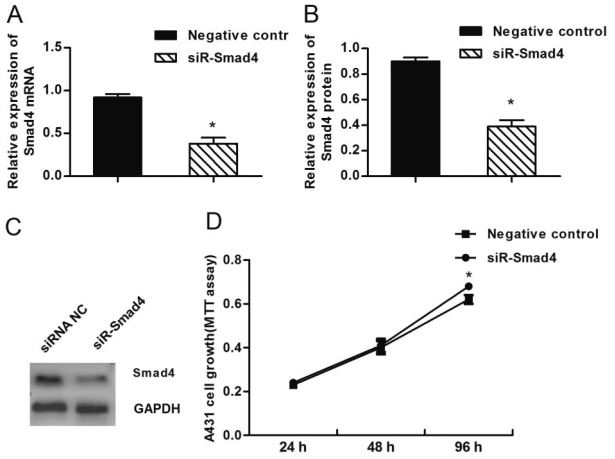

Figure 5. — siR-Smad4 suppresses the expression level of Smad4 and increases cell proliferation. siR-Smad4 decreases the mRNA (A) and protein (B and C) expression levels of Smad4. (D) Knockdown of Smad4 by siRNA increases the cell proliferation rate at 96 hours after transfection. *$p < 0.05$.

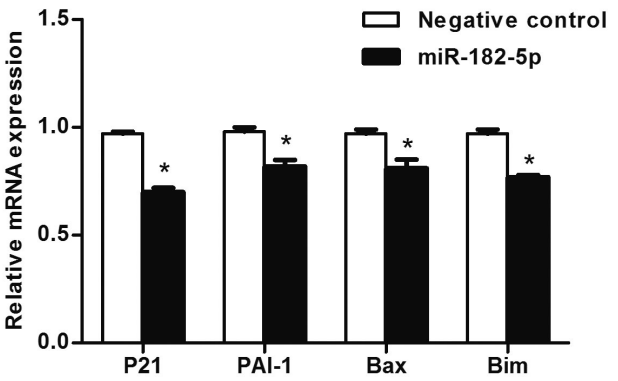

Figure 6. — The expressions of target genes of TGF -β/Smad4 pathway are reduced by miR-182-5p over-expression. The mRNA expression levels of p21, PAI-1, Bax and Bim are decreased with the miR-182-5p mimics. *$p < 0.05$.

worked as an oncogene, such as breast cancer, prostate cancer, liver cancer, cervical cancer, colon cancer, glioma, and ovarian cancer [6-12]. Some studies also indicated miR-182-5p inhibited the progression of cancer cells such as posterior uveal melanoma, renal cell carcinoma, and gastric adenocarcinoma [13-15]. In summary, the function of miR-182-5p is complex depending on the types of various tumours.

The present authors found that miR-182-5p promoted cell proliferation, migration, invasion, and the G1-to-S transition (Figures 1D, 2, and 4). MMPs have a vital role in tumour metastasis and invasion. The up-regulation of MMP-2 in vulvar cancer samples is associated with lower overall survival rate in patients [16]. MMP-2 and MMP-9 were increased with the miR-182-5p mimics (Figure 3). The findings show that miR-182-5p is involved in the invasion of VSCC. Recently, it is generally known that EMT has a key effect on the development of malignant tumours and might promote cancer cell metastasis. Some miRNAs are found to be associated with EMT [17–19]. E-cadherin expressions were declined with the miR-182-5p mimics (Figure 3). In contrast, N-cadherin and vimentin were increased (Figure 3). Therefore, the present author summarize that miR-182-5p might lead EMT to induce vulvar cancer metastasis. Besides, p21 [20], PAI-1 [21], Bax [22], and Bim [23] are the downscream targets of Smad4, the miR-182-5p mimics inhibited the mRNA expressions of p21, PAI-1, Bax and Bim (Figure 6). Further research will be required to clarify the detailed mechanism of miR-182-5p in the TGF-β/Smad signal pathway.

Smad4 is an important regulatory gene in the TGF-β signaling pathway [24, 25] and it is a vital regulator of EMT [26]. Smad4 suppressed the development of cancer cells including pancreatic carcinoma [27], head and neck squamous cell carcinomas [28], colorectal cancer [29], esophageal squamous cell carcinoma [30], lung adenocarcinoma [31], and breast cancer [32]. However, its role in VSCC has never been studied. The present authors confirmed silencing Smad4 phenocopied the functions of miR-182-5p over-expression (Figure 2, 4, and 5D), suggesting Smad4 acts as a tumour suppressor. The expression of Smad4 was also inhibited by miR-182-5p over-expression (Figures 1A, 1B, and 1C). So the present authors conclude Smad4 is the target gene of miR-182-5p in VSCC.

In conclusion, the results shows miR-182-5p works as an oncogene by targeting Smad4 in VSCC. MiR-182-5p might induce tumourigenesis and development in VSCC. Detecting the level of miR-182-5p or Smad4 in blood or samples of patients might contribute to the diagnosis or treatment of VSCC.

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Corresponding Author:
XIN WU, M.D.
The First Hospital of China Medical University
Department of Gynaecology
155 Nanjing North Street
110001 Shenyang/Liaoning (China)
e-mail: wuxin@cmu.edu.cn