miR-3147 serves as an oncomiR in vulvar squamous cell cancer via Smad4 suppression

XIU-HUA YANG1 and FENG GUO2

1Department of Obstetrics, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001; 2Department of Emergency, Shengjing Hospital of China Medical University, Shenyang, Liaoning 110004, P.R. China

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Abstract. The incidence of vulvar squamous cell carcinoma (VSCC) has increased annually over the last decade. MicroRNAs (miRNAs/miRs) serve an important role in tumor progression and development. Our previous microarray studies have revealed that miR-3147 was overexpressed in VSCC. However, its function and underlying mechanism in VSCC remain unknown. In the present study, it was confirmed by reverse transcription-quantitative polymerase chain reaction that the expression of miR-3147 was markedly upregulated in VSCC tissues. The increased expression of miR-3147 was positively associated with the depth of invasion. The overexpression of miR-3147 resulted in the promotion of vulvar cancer cell proliferation, migration, invasion, G1/S progression and invasion-associated gene expression. miR-3147 may participate in the process of epithelial-mesenchymal transition and reduce the expressions of downstream target genes in the transforming growth factor-β/Smad signaling pathway in A431 cells. The knockdown of Smad4 by small interfering RNA promoted malignant behaviours in A431 cells. In addition, miR-3147 regulated Smad4 by directly binding to its 3' untranslated region. In conclusion, the results indicated that miR-3147 may serve an oncogenic role in VSCC by targeting Smad4. miR-3147 may represent a novel potential therapeutic target marker for VSCC.

Correspondence to: Dr Xiu-Hua Yang, Department of Obstetrics, The First Affiliated Hospital of China Medical University, 155 Nanjing North Street, Shenyang, Liaoning 110001, P.R. China

E-mail: yangxihuajing@hotmail.com

Abbreviations: VSCC, vulvar squamous cell carcinoma; miRNA, microRNA; EMT, epithelial-mesenchymal transition; WHO, World Health Organization; FIGO, International Federation of Gynecology and Obstetrics; UICC, Union for International Cancer Control; cDNA, complementary DNA; MMP-2, matrix metalloproteinase 2; DMSO, dimethylsulfoxide; NCCN, National Comprehensive Cancer Network; OS, overall survival; HNSCC, head and neck squamous cell carcinomas

Key words: vulvar squamous cell carcinoma, microRNA-3147, Smad4, EMT, transforming growth factor-β

Introduction

Malignant tumors of the vulva represent a very small number of female genital malignancies, 80-90% of vulvar cancers are vulvar squamous cell carcinomas (VSCCs). There will be about 6020 new patients in 2017, and 1150 people are expected to die. The incidence is increasing by 0.6% annually in the last 10 years (1). Women with distant metastases (5%) and local diffusion (31%) have bad prognosis. The 5 year survival rates were 17 and 57%, respectively (1). Thus it is of important clinical essentiality to research the occurrence and development of VSCC. In the present case, many scholars have begun to focus on the microRNAs (miRNAs).

miRNAs prevent translation or promote mRNA degradation (2). miRNA plays a key role in the carcinogenesis and evolution by accommodating epithelial-mesenchymal transition (EMT), oncogenic signaling pathways and metastasis (3). miRNAs repress target gene expression and usually perform important functions in cancers.

Based on our previous findings (4), miR-3147 was markedly increased in VSCC tissues, but the underlying mechanism is still unknown. Smad4 is responsible for regulating the TGF-β/Smad signaling pathway and it was downregulated in VSCC samples (4). Based on the target prediction program miRanda, Smad4 is a target gene for miR-3147, but the association between miR-3147 and Smad4 in VSCC requires further investigation. In the research, we performed functional studies to find the roles of miR-3147 and Smad4 in VSCC. Smad4 was confirmed to be a target gene of miR-3147.

Materials and methods

Experimental samples. Twenty VSCC and adjacent non-dysplastic tissues were obtained from the patients admitted to the Department of Gynaecology at our hospital between 2010 and 2017. Every sample of VSCC was negative in HPV, and Linear Array HPV Genotyping (Roche Applied Science, Pleasanton, CA, USA) was used for the approach HPV testing. According to the standard of World Health Organization (WHO), the diagnosis in histopathology was conducted. The grade of tumor was determined according to the new version of the International Federation of Gynecology and Obstetrics (FIGO) system published in 2010; the stage of tumor was identified by the 7th TNM categorization of the Union for
International Cancer Control (UICC). For the stages of tumors that diagnosed before 2009, they were identified again based the latest version. The frozen specimens were microscopically identified by two pathologists. Clinico-pathological data were retrospectively collected. Patients without preoperative radiotherapy or chemotherapy were chosen. The research was performed based on the Helsinki declaration. Prior to surgery, all subjects provided written informed consent to participate in the study. The Ethics Committee of the First Affiliated Hospital of China Medical University (Liaoning, China) approved the present study.

Cell culture and transfection. Acquisition of the A431 cell line was made from ATCC, in addition to culturing in RPMI 1640 with 10% fetal bovine serum at a temperature of 37˚C and a humid atmosphere of 5% CO₂. Using the Dharamcon miRIDIAN miR-3147 mimics (miR-3147), the transfection of the cells was carried out, together with the negative control (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at an eventual amounting to be 100 nmol/l. A small interfering RNA targeting Smad4 (siR-Smad4; sc-29,484) was attained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Transfections were conducted with the help of Lipofectamine 2000 in accordance with the protocol of the manufacturer.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA extraction was conducted by making use of TRIzol reagent (Takara Bio, Inc., Shiga, Japan) in accordance with the guidelines of the manufacturer. Stem-loop RT-PCR was performed for the purpose of testing the miR-3147 expression (5). Complementary DNA (cDNA) synthesis was conducted according to a Gene Amp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.). We calculated the mRNA expressions with the 2^−ΔΔCq approach. Furthermore, the primers for miRNAs and mRNAs are shown (Table I).

Western blotting. Cells were harvested, together with the calculation of protein concentration through the use of a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Three days subsequent to the transfection with the miR-3147 mimics or siRNA-Smad4, the total protein was obtained (6). In respect of immunoblotting, the membrane was incubated with antibodies against E-cadherin (1:500, ab15148; Abcam, Cambridge, UK), N-cadherin (1:500, ab18203; Abcam), Vimentin (1:500, ab24525; Abcam), matrix metalloproteinase 2 (MMP-2; 1:300; BIOSS, Beijing, China), MMP-9 (1:300; BIOSS) or GAPDH (1:10,000, ab18602; Abcam). Rinsing of the membrane was performed, followed by incubation with anti-mouse or anti-rabbit IgG (H+L)-HRP conjugate (1:10,000; Invitrogen; Thermo Fisher Scientific, Inc.) antibody. We made use of the Image J software (National Institute of Health, Bethesda, MD, USA) in order to analyze the results.

MTT test. Cell proliferation assay was assessed by MTT test. Ninety-six well plates were seeded with 5x10⁴ cells per well. At the appointed time after transfection (0, 48, and 96 h), 20 μl MTT (Sigma-Aldrich, St. Louis, MO, USA) was put into the well, followed by the incubation of the cells at a temperature of 37˚C for a period of 4 h. The liquid was abandoned and then we put 150 μl dimethylsulfoxide (DMSO; Sigma-Aldrich) to lysis the formazan. Measurement of the optical density was also taken at 490 nm by using a microplate spectrophotometer.

Cell migration and invasion. For the purpose of testing cell migration, a Transwell test was conducted. 48 h of time following the transfection, addition of 5x10⁴ cells was made into the above chambers of plates by an untreated membrane. Subsequent to 24 h of incubation, addition of 4% paraformaldehyde was made into the chambers, followed by a fixation using the crystal violet. We carried out the calculations of the cells, which passed all across the membrane. As for the invasion experiments, Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used on the above chamber. The other mechanisms possessed similarities with the migration experimentation. All the tests were repeated in triplicate.

Distribution of cell cycle. More than 1x10⁴ cells were trypsinized, collected, washed twice, as well as positioned in 70% ethanol for a time period of 24 h. The cells were washed twice with PBS, following the incubation using 400 μl RNase (0.25 mg/ml) at a temperature of 37˚C for 1 h, proceeding to treatment with propidium iodide (40 μg/ml). Determination of the cell cycle distribution was made through flow cytometry. The experiments were conducted for three times.

Luciferase reporter assay. The fragment of the human Smad4 with (wild-type) or without (mutant) the miR-3147 binding site at the 3'-untranslated region (3'-UTR) was cloned and inserted into the pGL3-basic luciferase report plasmid (Promega Corporation, Madison, WI, USA) to generate luciferase reporter vectors, Smad 4 3'‑UTR-wt and Smad 4 3′-UTR-mut. The 293T cells were treated in 96-well plates at 5,000 cells per well and permitted to stay for 24 h prior to transfection. Then, transfection of the miR-3147 mimics or miR-NC was performed into the 293T cells with the use of Lipofectamine 2000 with 100 ng of Smad 4 3'-UTR-wt or Smad 4 3′-UTR-mut. The 293T cells were treated in 96-well plates at 5,000 cells per well and permitted to stay for 24 h prior to transfection. Then, transfection of the miR-3147 mimics or miR-NC was performed into the 293T cells with the use of Lipofectamine 2000 with 100 ng of Smad 4 3'-UTR-wt or Smad 4 3′-UTR-mut, in addition to 10 ng of pRL-TK Renilla plasmid (Promega Corporation). Following the incubation for a time period of 48 h, determination of the luciferase activities was performed with the help of a Dual Luciferase Reporter system (Promega Corporation).

Statistical method. The presentation of the data has been made as the means ± SD. Furthermore, we made use of the SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA) for the purpose of analyzing the findings. A P-value of <0.05 was thought to be statistically significant. Analysis of the significance of differences in the mean values was carried out with the application of Student's t-test. The use of a two-sided Fisher's exact test was made for the purpose of determining the association between the expression level of miR-3147 and clinico-pathological data.

Results

RT-qPCR analysis. A RT-qPCR analysis of miR-3147 in VSCC as well as surrounding non-dysplastic tissues was conducted. The expression of miR-3147 was significantly increased in VSCC tissues (P=8.67x10⁻⁴; Fig. 1).
Relationship between miR-3147 and clinicopathological parameters. The 75th percentile of the $2^{-\Delta\Delta Ct}$ values was considered to be the cut-off value for tissues with low or high expressions of miR-3147 ($\Delta$). The expression level of miR-3147 was not related to age, tumor differentiation, vascular invasion, FIGO stage, tumor size and lymph node metastasis, but was positively related to the depth of invasion ($P=0.023$; Table II).

The results showed that the upregulation of miR-3147 contributes to the invasion of VSCC.

Effects of miR-3147 on vulvar carcinoma in vitro. In comparison with the negative control, the proliferation rate of the cells that had undergone transfection with the miR-3147 mimics amounted to be significantly higher at 96 h ($P=0.007$; Fig. 2A). Selection of MMP-2 as well as MMP-9 was made for the purpose of testing if miR-3147 could affect the genes associated with tumor invasion. MMP-2 and MMP-9 were increased after transfection ($P=0.007$; Fig. 2B). For the fact that Smad4 is termed as a crucial mediator in TGF-$\beta$ signal pathway, the inhibition of Smad4 by miR-3147 is most likely to decrease the expressions of downstream target genes in this pathway. Our results displayed that miR-3147 transfection resulted into the inhibition of the mRNA levels of p21 ($P=0.007$), PAI-1 ($P=0.011$), Bax ($P=0.007$) and Bim ($P=0.002$; Fig. 2C), indicating miR-3147 could regulate Smad4-mediated signaling pathway. The miR-3147 mimics markedly increased the migration ($P=0.002$; Fig. 2D) and invasion ($P=0.015$; Fig. 2E) of A431 cells. Cell cycle results revealed the fact that the cells in the G1 phase were notably dropped from 62.34±1.62% to 57.87±1.78% ($P=0.032$; Fig. 2F). In addition, the cells in the S phase were remarkably added from 24.84±1.07% to 27.70±0.94% at 48 h ($P=0.004$; Fig. 2F).

The effect of miR-3147 in EMT. The transfection of the miR-3147 mimics into A431 cells was performed. After 4 days, no obvious change in the shape of cells was observed. miR-3147 transfection downregulated E-cadherin protein expression, in addition to upregulating the protein expressions of N-cadherin as well as Vimentin (Fig. 2B). The findings revealed the fact that miR-3147 could take part in the mechanism of EMT in vulvar cancer.

miR-3147 targets Smad4. We searched the target prediction website miR and a (http://www.microrna.org/microrna/home.do) and predicted that the miR-3147 promoter contains an Smad4 binding site (Fig. 4A). miR-3147 transfection reduced Smad4 mRNA expression by 33.0±7.0% after 48 h ($P=0.001$; Fig. 4B). The change of Smad4 protein was consistent with that.

Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.

| Gene       | Forward primers (5'-3') | Reverse primers (5'-3') |
|------------|-------------------------|-------------------------|
| U6         | CTCGCTTCGGCACAGCA       | AACGCTTCACGATTTGCAGT    |
| miR-3147   | GGUGUGGCCAGUGAGGUGUG    | TACGAGTGGTGCGAGGAGGGTG  |
| Smad4      | CGCTTTTGGTGGTCACT       | CCCAAACATCACCTCACCTC    |
| E-cadherin | TGCTTCTGCGGTTCTGTGCGG   | CTCTCCTGGATTCTCTCCTCT  |
| N-cadherin | TGGGAGGGTGTTAAAAGTGTCC  | AAGAACAGGGGCCACCCCTTT  |
| Vimentin   | CGGTTGAGACGAGAGATGGA    | TGGTTGACTGCACTGGTGG     |
| P21        | TGCTCCGTCAAGACCCATG     | TGGGAGGTAGGCCGCTTG     |
| PAI-1      | GAGACAGGGAGCTCGGATTC    | GGCTCTCCAAATGTCATTAC   |
| Bax        | ATGGACGGGTCCGGGGACAG    | CATGATTTTCGATCAGT      |
| Bim        | GCCTTTCAACCATATCTCA     | ATCCAGCTCGGTGTCTTCT    |
| GAPDH      | AAGGTGAAGGTCCGGAGTCAAC  | GGGTCATTGATGGCAACATA   |

miR, microRNA; PAI-1, plasminogen activator inhibitor-1; Bax, B-cell lymphoma 2-associated X protein; Bim, B-cell lymphoma-2-like protein 11.
miRNA is widely studied in many fields of medicine including cancers. The identification of 79 miRNAs was made by de Melo Maia et al. (11), which revealed surprisingly varied expression levels in vulvar cancer in comparison with control specimens. In addition, they also explored some links between special miRNAs and the clinical pathological data (11). Furthermore, a miRNA sponge was also verified by them, which was most likely to appear as an efficient method for diagnosing and treating this cancer (12). It has been reported that miR-223-5p works as an oncogene by targeting p63 in vulvar cancer (13). miR-590-5p drives promotion of cellular malignant conduct with the help of the target gene TGFβRII in VSCC (4). In this study, we discussed the mechanism of miR-3147 in VSCC.

Discussion

VSCC is a relatively rare disease. Standard therapy for VSCC has been described in the recently developed National Comprehensive Cancer Network (NCCN) compendium for VSCC. Nonetheless, advanced VSCC carries a poor prognosis and it is less responsive to cytotoxic agents than other solid tumors. Patients with advanced vulvar carcinoma experience significantly shorter overall survival (OS) durations than those with other metastatic or recurrent solid tumors treated with novel phase I therapeutics (8). Because of this poor prognosis in advanced disease, the development of satisfactory improvements is warranted and necessary (9,10).
miR-3147 by transfection with the miR-3147 mimics in VSCC cell line A431. miR-3147 increased vulvar cancer cell proliferation, migration, invasion and G1/S progression (Fig. 2). These results reinforce that miR-3147 functions as an oncogene in vulvar carcinoma.

When classify the VSCCs, we can summarize two different kinds. The first one is HPV-associated, which accounts for 20 to 50% in the whole cases. The second one is HPV-independent. In epidemiology, clinics, pathology and molecular mechanism, all of them have different features. While A431 cells are HPV negative, we studied the expression of miR-3147 in HPV negative samples and A431 cells to avoid viral interference in miRNA pathways. As we know, no information about miR-3147 and the infection of HPV has been reported in other tumors. 25 different expressions of miRNAs have been found between HPV-positive vulvar cancers and HPV-negative vulvar cancers (11). By changing the expressions of miRNAs, the HPVs have oncogenic properties. This conclusion has been proved through some HPV-related researches such as cervical cancer. Researchers thought that there was a connection between cancer progression and the expressions of miR-92a and miR-378 in HPV-positive cervical cancer tissue samples (16). At the same time, there is a report showing that the overexpression of miR-155 was linked to the increasing danger of cervical cancer in HPV E6/E7 mRNA positive tissues (17). There are lot of factors that can distinguish HPV-positive head and neck squamous cell carcinomas (HNSCC) patients from HPV-negative HNSCC patients. For example, a panel includes miR-9, miR-134, miR-196b, miR-210 and miR-455 (18).

Smad4 poses to be a vital regulatory factor in the TGF-β/Smad signaling pathway (19), and the downregulation of Smad4 is also indicative of advanced tumor stage and a worse prognosis (20). Smad4 performs the function of a tumor suppressor gene in a great number of cancers, for instance pancreatic cancer (21), colorectal cancer (22), lung cancer (23), gastric adenocarcinoma (24), cervical cancer (25), HNSCC (26) and breast cancer (27). Smad4 was reported to be oncogenic in other cancers such as hepatocellular carcinoma (28), suggesting...
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that its role might be cell-context-dependent. Smad4 has been validated as a direct target of several oncogenic miRNAs, including miR-224 (29), miR-20a-5p (30) miR-1285 (31), miR-210 (25) and miR-130a/301a/454 (32). Consistent with these results, our study found the down-expression of Smad4 in A431 cells can simulate the oncogenic role of miR-3147 (Fig. 3), revealing that Smad4 acts as a tumor suppressor in VSCC. TGF-β/Smad signaling pathway as well as miRNAs participate in various cellular processes. However, the association between the TGF-β/Smad signaling pathway and miR-3147 in VSCC is not fully understood. We found the miR-3147 mimics could suppress the mRNA expressions of downstream target genes of Smad4, indicating that miR-3147 negatively regulates the TGF-β signaling pathway via the suppression of Smad4.

Cell migration and invasion are complicated biological processes that have key effects on the progression of cancer. EMT has a key role in the progression and metastasis of tumors. Cancer cells are capable of spreading and invading other tissues when they experience EMT (33). We increased miR-3147 in A431 and tested the expressions of key molecules in EMT. E-cadherin decreased but N-cadherin and Vimentin increased, revealing that the miR-3147 mimics could promote EMT (Fig. 2B). Current studies have confirmed that certain MMPs promote the progression of EMT. We also identify this view. We found that miR-3147 promoted MMP-2 and MMP-9 expression (Fig. 2B).

A prediction was validated by the application of the dual luciferase reporter assay, which suggested that the miR-3147 promoter sequence possesses a Smad4 binding site. The down-expression of Smad4 led to the promotion of miR-3147 mRNA expression (Fig. 4D). Furthermore, the miR-3147 mimics exhibited a decrease in the mRNA as well as protein expressions of Smad4 (Fig. 4B and C) that evidences the fact that miR-3147 targets Smad4 directly in VSCC.

In this article, we pointed out that miR-3147 can play as an oncomiR by promoting vulvar cancer cells invasion and metastasis. Cell migration and invasion are complicated biological processes that have key effects on the progression of cancer. EMT has a key role in the progression and metastasis of tumors. Cancer cells are capable of spreading and invading other tissues when they experience EMT (33). We increased miR-3147 in A431 and tested the expressions of key molecules in EMT. E-cadherin decreased but N-cadherin and Vimentin increased, revealing that the miR-3147 mimics could promote EMT (Fig. 2B). Current studies have confirmed that certain MMPs promote the progression of EMT. We also identify this view. We found that miR-3147 promoted MMP-2 and MMP-9 expression (Fig. 2B).

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be an equipment for inhibiting offense from the tumor. In addition, it might become a chance for the therapy of VSCC. Further studies should be followed in this direction. The goals are not only to examine if our findings can be applied in a wider range, but also to build \textit{in vitro} and \textit{in vivo} inhibition models for miR-3147 in VSCC.

In conclusion, we transfected the miR-3147 mimics and siR-Smad4 into A431 cells whereby we discovered that introducing an increase in the expression of miR-3147 and a decrease in the expression of Smad4 could result into promoting vulvar cancer cell proliferation, migration, invasion and G1/S progression. miR-3147 performs the function of an oncogene for playing a key biological function by targeting Smad4 in VSCC. As for the etiology and progression of vulvar carcinoma, miR-3147/Smad4 may be a new research direction. For example, making detection of the expression levels of miR-3147 or Smad4 in the blood or tissues of patients is likely to be used for early detection and prognostic evaluation. Therefore, our study may contribute to the diagnosis and treatment of VSCC in the future.

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\textbf{Availability of data and materials}

The datasets used during the current study are available from the corresponding author on reasonable request.

\textbf{Authors' contributions}

XHY analyzed and interpreted the patients' data, and was a major contributor in writing the manuscript. FG performed the histological examination of the tissues. All authors have read and approved the final manuscript.

\textbf{Ethics approval and consent to participate}

The present study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University. All subjects provided written informed consent to participate in the study.

\textbf{Consent for publication}

All subjects provided written informed consent.

\textbf{Competing interests}

The authors declare that they have no competing interests.

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