

# Role of microRNA-296-3p in the malignant transformation of sinonasal inverted papilloma

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Received March 24, 2016; Accepted March 3, 2017

DOI: 10.3892/ol.2017.6193

**Abstract.** Inverted papilloma (IP) is a benign tumor occurring in the nasal cavity and paranasal sinuses. It is reported that 5-15% of IPs undergo malignant transformation into squamous cell carcinoma (SCC), and the role of microRNAs (miRNA/miR) in this process remains to be elucidated. In the present study, whole miRNA profiles using samples of IP and SCC were investigated, in order to detect the function of miRNA in the carcinogenesis of IP. Samples from IPs (n=5) and SCC lesions (n=5), which arose from IPs, were used for miRNA analysis. A total of 200 miRNAs exhibited a >2-fold differential expression between IP and SCC. miR-296-3p was markedly upregulated in SCC with a 23-fold difference. Computational analysis indicated that miR-296-3p targeted PTEN, which regulates the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway and PTEN is involved in the carcinogenesis of SCC. miR-296-3p directly regulated PTEN expression in head and neck cancer cells, with PTEN protein levels decreased in 4/19 the SCCs (21.0%), as compared with those in the IPs (76.4%). Positive p21 staining was observed in 64.7% of IPs; this was a significantly increased rate compared with that for SCCs (26.3%,  $P=0.0086$ ). The results of the present study indicated that there were marked changes in the miRNA expression signature during the malignant transition. miR-296-3p may serve an important role in the malignant transformation of IPs via the regulation of PTEN, combined with the subsequent inhibition of the PI3K/Akt signaling pathway, and may be a novel agent for cancer prevention.

## Introduction

Inverted papilloma (IP) is a benign tumor occurring in the nasal cavity and paranasal sinuses (1-3). IPs typically arise from the lateral wall of the nasal cavity and may spread into the surrounding tissue, the ethmoid, the skull base and the orbita (3). Although surgical treatment has developed through the integration of skull base surgery, navigation systems and endoscopic surgical techniques (1), the management of this disease has remained challenging as IPs tend to recur following incomplete resection, and the malignant transformation to squamous cell carcinoma (SCC) occurs in 5-15% of cases (2,3).

MicroRNAs (miRNA/miR) are small non-coding RNAs of ~19-22 nucleotides that regulate gene expression through translational repression and mRNA cleavage (4). In total, >1,500 types of human miRNA have been detected and registered, with a number of them reported to be associated with carcinogenesis, differentiation, apoptosis and metastasis (5-7). In the head and neck field, miRNAs are aberrantly expressed in SCC tissue and are associated with carcinogenesis and drug-resistance (5-7).

High-risk human papilloma virus (HPV) infection is regarded as a potential etiological agent of IPs and their malignant transformation (3,8). Carcinogenesis resulting from HPV infection has previously been explained by the inhibition of tumor suppressors, including the p53 and retinoblastoma protein (pRB) signaling pathways (9). The E6 and E7 regions of high-risk HPV are transfected into the genes of epithelial basal cells following infection (10). E6 binds to p53 and promotes the ubiquitination and degradation of p53 itself. E7 also suppresses the cell cycle regulators of p21 and p27, and binds directly to pRB to promote aggressive cell growth (10). However, it is not always possible to detect high-risk HPV in sinonasal SCCs arising from IPs; the detection rate of high-risk HPV is ~37.8% (10). The rate of unapparent infection with HPV remains unknown, but a considerable number of such cases are speculated to exist (4). The majority of IPs require a lengthy period for malignant transformation, but the trigger underlying the malignant changes remains obscure (10). SCC arising from IP is one of the best models for studies, as certain tissue samples obtained via surgical resection may contain

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**Key words:** sinonasal inverted papilloma, microRNA 296-3p, malignant transformation, phosphatase and tensin homolog

normal epithelium, dysplasia and SCC (4). The present study aimed to identify the underlying mechanisms and roles of miRNAs in the malignant transformation of IPs.

## Materials and methods

**Samples and cell lines.** A total of 36 individual nasal and paranasal tumor specimens from 25 patients (19 males and 6 females) were obtained during surgery at Hokkaido University Hospital (Sapporo, Japan) between January 1993 and October 2012. All samples were obtained prior to radiotherapy and chemotherapy. The median age of the patients was 64.7 years (range, 39-93 years) at the time of diagnosis. The details of the pretreatment clinical and pathological characteristics are summarized in Table I. SCC25 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Written informed consent was obtained from all patients. The present study was approved by the Institutional Ethics Review Board of Hokkaido University Hospital.

**RNA isolation and miRNA TaqMan™ low density array (TLDA) assay.** Total RNA was isolated from the formalin-fixed tissue using a Recover All Total Nucleic Acid Isolation kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. Formalin-fixed tumor tissues were cut into 4-um thick sections, deparaffinized in xylene, rehydrated through graded ethanol, and stained with hematoxylin and eosin. These sections were examined for histopathological accuracy under a light microscope. Unstained sections for RNA isolation were cut into 8-um thick sections and macro-dissected to avoid contamination with other tissues. The quality and quantity of the RNA was determined using an Agilent RNA 6000 Nano LabChip kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Total RNA was used to run the ABI Megaplex™ protocol without pre-amplification (Applied Biosystems; Thermo Fisher Scientific, Inc.). A reaction was run using an ABI miRNA Reverse Transcription (RT) kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Megaplex™ RT Human Pool A primers (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cDNA from each sample was then transferred to a new tube and diluted with water and TaqMan™ Universal polymerase chain reaction (PCR) Master Mix (2X) without uracil-DNA glycosylase (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each sample was then loaded onto its own TLDA card. TLDA cards were queued into the ABI7900HT Real Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) and run according to the Applied Biosystems default TLDA protocol. Data were normalized based on the endogenous control on the TLDA card and analyzed using DataAssist™ v3.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.).

**Transfection of miRNA.** The miRNA mimics/inhibitor and negative controls were purchased from GE Healthcare Dharmacon, Inc. (Lafayette, CO, USA) and introduced into SCC25 cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) at 37°C according to the manufacturer's protocol. Following 6 h, the transfection medium was aspirated and Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck

Table I. Baseline characteristics of patients with IP and SCC.

A, Cases for microRNA analysis	
Characteristics	Patients, n
Total	8
Sex	
Male	7
Female	1
Age, years	
Range (median)	38-77 (54)
B, Cases for immunohistochemistry	
Total	25
Sex	
Male	19
Female	6
Age, years	
Range, median	32-84 (63)
Histology	
IP non-dysplastic	6
IP coexisting with SCC	12
SCC	7
IP, inverted papilloma; SCC, squamous cell carcinoma.	

KGaA, Darmstadt, Germany) with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) was added. Cells were lysed 48 h following transfection as described below. Transfection was confirmed using miRIDIAN miRNA Mimic Transfection Control with Dy547 (GE Healthcare Dharmacon, Inc.).

**Immunoblotting.** Cells were lysed with radioimmunoprecipitation assay lysis buffer (1 mM NaVO<sub>3</sub>, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA), phosphatase inhibitor cocktail and protease inhibitor cocktail mini tablet (Roche Diagnostics, Indianapolis, IN, USA) on ice. The samples were centrifuged at 10,000 x g for 4 min and the supernatant was collected. Protein concentration was quantified with a standard Bradford absorbance assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). Protein from each sample was fractionated on a 4-15% SDS-PAGE gel (Bio-Rad). The proteins were then transferred to nitrocellulose membranes and blocked with skim milk at room temperature for 1 h. Then, membranes were incubated for 12 h at 4°C with the appropriate primary antibodies phosphatase and tensin homolog (PTEN; cat no. 9559; dilution, 1:1,000), p21 (cat no. 2947; dilution, 1:1,000) (both Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (cat no. ab8226; Abcam, Cambridge, UK; dilution, 1:1,000); followed by incubation with secondary anti-rabbit or anti-mouse HRP-linked antibodies (cat nos., 7074 and 7076, respectively; Cell Signaling Technology, Inc., Danvers, MA,

Table II. Top 10 miRNAs that exhibited differential expression between SCC and IP.

Upregulated in SCC		Downregulated in SCC	
Name	Fold-change	Name	Fold-change
hsa-miR-206	37.74	hsa-miR-892b	183.83
hsa-miR-675	28.9	hsa-miR-874	144.4
hsa-miR-105	25.38	hsa-miR-371-3p	121.14
hsa-miR-296-3p	23.92	hsa-miR-16-2	116.27
hsa-miR-577	21.88	hsa-miR-129	85.26
hsa-miR-574-3p	12.94	hsa-miR-518f	70.78
hsa-miR-93	9.09	hsa-miR-431	59.55
hsa-miR-936	8.87	hsa-miR-593	49.27
hsa-miR-922	8.75	hsa-miR-1225-3p	45.06
hsa-miR-616	8.68	hsa-miR-92b	43.71

IP, inverted papilloma; SCC, squamous cell carcinoma; miR, microRNA; hsa, *Homo sapiens*.

USA) at the manufactures' indicated dilution ratio. Protein bands were visualized using ECL Western Blotting Detection reagent (GE Healthcare Life Sciences, Chalfont, UK) Signal intensities were determined using Image Gauge software (version 4.1; Fujifilm, Tokyo, Japan). Each gel was normalized to  $\beta$ -actin.

**Immunohistochemistry.** Paraffin-embedded tumor specimens from the primary sites were available from all 25 patients involved. These specimens were cut into 4- $\mu$ m thick sections, deparaffinized in xylene, rehydrated through graded ethanol and then placed in 0.1% hydrogen peroxide to quench any endogenous peroxidase activity at room temperature for 10 min. Following pretreatment in a 0.01 M citrate buffer (pH 6.0) for three cycles of 5 min at 750 W in a microwave, these sections were treated with 10% normal rabbit serum (Histofine® SAB-PO M kit; Nichirei Biosciences, Inc., Tokyo, Japan) at room temperature for 10 min to prevent any nonspecific binding of the antibody. The slides were then incubated with a specific monoclonal antibody against human PTEN or p21 (Cell Signaling Technology) in a humid chamber at 4°C overnight. The sections were then incubated with a biotin-labeled rabbit anti-mouse secondary antibody according to the manufacturer's protocol (Histofine SAB-PO M kit) for 30 min at room temperature, followed by incubation with a streptavidin-biotin horseradish peroxidase complex (Histofine SAB-PO M kit). The reaction products were observed by immersing the slides in a freshly prepared diaminobenzidine solution for 10 min and counterstaining with hematoxylin at room temperature prior to dehydration and mounting. Stained slides were scored based on the staining intensity and the percentage of positive cells. Staining intensity was scored using a 4-point system as previously described (11) (0+, negative; 1+, weak; 2+, moderate; 3+, strong). The positive cell percentage was scored using a 5-point system (0, 0%; 1+, 1-10%; 2+, 11-50%; 3+, 51-80%; 4+, >80%). PTEN expression was estimated by cytoplasmic staining, and p21 expression was estimated by nuclear staining. The expression of the two proteins was

scored semi-quantitatively using the immunoreactive score (IRS), which was calculated as follows: IRS=staining intensity score  $\times$  score of percentage of positive cells. Positivity for PTEN and p21 was defined as an IRS score  $\geq 3$ , according to the Nagata score (10). In all cases, the procedure was performed by two pathologists, who were blinded to the clinical outcomes. Unpaired t-tests were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA), and  $P < 0.05$  was considered to indicate a statistically significant difference.

Biological targets of miRNAs were predicted by searching for the presence of sites that match the seed region of each miRNA using the TargetScan 6.2 database (<http://www.targetscan.org>).

## Results

**Differential miRNA expression between IP and SCC cells.** Using a total of 10 samples (IP=5 and SCC=5), the role of miRNAs in the carcinogenesis of IP was examined. All five IP samples exhibited transformation to SCCs and all five SCC samples had arisen from IPs. A total of two IP and SCC samples were obtained from the same case (Table IA). miRNA expression analyses were performed using RT-PCR-based arrays to examine 754 unique miRNAs. Among the differentially-expressed miRNAs, the 10 that exhibited the greatest differential expression between IP and SCC are presented in Table II. miR-296-3p exhibited a 23-fold decrease in IP, compared with in SCC. miR-296-3p also demonstrated a 76-fold difference in expression between the IP and SCC from the same cases. A list of 66 target genes was obtained using TargetScan 6.2, including 66 conserved sites and 26 poorly conserved sites that are putatively targeted by miR-296-3p. PTEN, which was identified as a tumor suppressor and involved in the p53 and RB pathways, was included in the list of miR-296-3p target genes.

*PTEN is a direct target of miR-296-3p in head and neck SCC (HNSCC) cells.* Target prediction using TargetScan 6.2

revealed that miR-296-3p potentially targets PTEN, as seven nucleotides of the 5' seed region were complementary to the PTEN 3'UTR (Fig. 1A). It was established that miR-296-3p negatively regulates PTEN by adding a miR-296-3p mimic into SCC25 cells. The HNSCC cell line, SCC25, was transfected with a miR-296-3p mimic, miR-616 mimic, and a negative control. miR-616 mimic, which was upregulated in SCC cells but did not target PTEN or p21 *in silico*, was used as the negative control. Following transfection with the miR-296-3p, PTEN expression was markedly decreased, compared with that for the negative control (Fig. 1B). p21, a cyclin-dependent kinase inhibitor, is directly regulated by p53 and is involved in the carcinogenesis of HNSCC. p21 was also reported to be a target of miR-296-5p; therefore, p21 expression was investigated in HNSCC cells transfected with miRNA-296-3p. No significant difference was observed between the p21 expression following transfection with the miRNA-296-3p mimic and with the control (Fig. 1B).

**Expression of PTEN and p21 in IP and SCC, and its association with clinicopathological features.** Immunohistochemical staining for PTEN and p21 was performed for 17 IP and 19 SCC tissue samples. A total of 11 IPs coexisted with severe dysplasia or SCC, while the remaining six samples were non-dysplastic IPs. A total of 12 SCC samples were differentiated from IPs and the other 7 SCC samples did not coexist with IPs. One IP that coexisted with SCC samples was too small and badly damaged and, therefore, it was excluded from the present study. Representative images of staining for PTEN and p21 are presented in Fig. 2. PTEN expression was observed primarily in the cytoplasm of the tumor cells (Fig. 2C and D). Positive PTEN staining was observed in 13/17 IPs (76.4%), a rate significantly increased compared with that observed in SCCs (4/19, 21.0%;  $P=0.0005$ ; unpaired t-test with Welch's correlation; Table III). Positive p21 staining was observed in 11/17 IPs (64.7%), which was also a significantly increased rate compared with that in SCCs (5/19, 26.3%;  $P=0.0086$ ; Table III). No significant differences were observed in PTEN or p21 expression between non-dysplastic and dysplastic IPs, or between SCCs with IPs and SCCs without IPs. Double positive staining was observed in 12 of all the tissue samples, and double negative staining was observed in 15 tissue samples.

## Discussion

In the present study, it was revealed that the expression of numerous miRNAs was altered during the transition between sinonasal IP and SCC. In view of the multistep carcinogenic process, a large number of miRNAs are considered to be associated with the malignant transformation through the regulation of oncogenes (12). In the present study, number of miRNAs were detected that demonstrated differential expression between IP and SCC, but the majority were not expected to regulate the expression of oncogenes in *in silico* analysis. Regulation of PTEN by miRNA 296-3p may be one of the critical processes associated with multiple molecular alterations for transformation to invasive SCC.

PTEN is a well-known tumor suppressor gene that is associated with human carcinogenesis (13). PTEN transcription

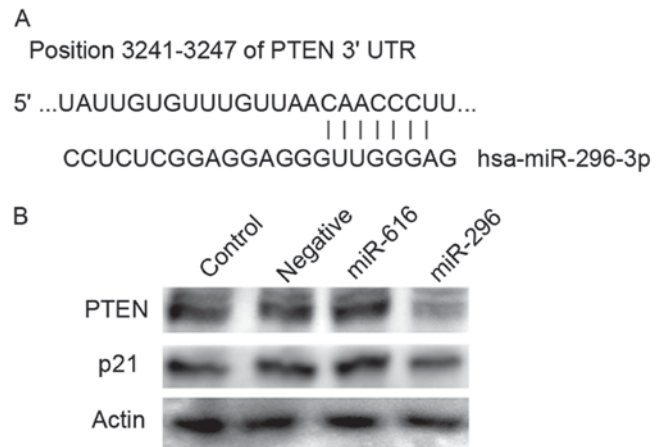


Figure 1. PTEN is a potential target of miR-296-3p. (A) The putative binding site of miR-296-3p in the PTEN 3'UTR site is indicated. (B) The protein levels of PTEN and p21 in SCC25 cells were assessed at 48 h post-transfection with miR-296-3p mimics, miR-616 and a negative control. PTEN, phosphatase and tensin homolog; UTR, untranslated region; miR, microRNA; hsa, *Homo sapiens*.

is activated by p53 and negatively regulates cellular survival by inhibiting the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway (13). The PI3K/Akt signaling pathway performs important roles in the carcinogenesis of HNSCC (14). However, a PTEN deficiency of  $\leq 30\%$  is observed in HNSCC (15). Recent genome-wide sequence analysis of HNSCC has revealed a large number of gene alterations, including the molecules involved in the PI3K/Akt signaling pathway (16,17). PTEN mutations were detected in only 5-10% of cases, and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit  $\alpha$  mutations in 8-10% of cases (16,17). Therefore, activation of the PI3K/Akt pathway due to PTEN deletion alone is insufficient for the induction of invasive HNSCC. Bian *et al* (18) promoted HNSCC via the deletion of transforming growth factor- $\beta$ 1 and PTEN. Squarize *et al* (19) demonstrated that mice harboring a PTEN deficiency developed multiple oral SCC lesions on exposure to a tobacco surrogate, whereas the control mice did not.

p21 is a major downstream molecular target of p53 that mediates the apoptotic effect of p53 through the suppression of DNA replication and G<sub>1</sub> stage cell cycle arrest (20). The results of the present study demonstrate that the presence of p21 was less common in SCC, as compared with in IP cells, but miRNA-296-3p may not regulate p21 expression in head and neck cancer cells. In cancer tissue, p21 expression is not always associated with p53 expression as p21 expression levels are low in tissues containing p53 harboring a functional mutation (20). The prognosis and aggressiveness of head and neck cancer were revealed to be associated with the expression of PTEN, but not with p53 and p21 (21,22). In the present study, PTEN and p21 expression were positively correlated with one another, and these results indicate that the trigger for the malignant transformation of IPs to SCCs may underlie the activation of the PI3K/Akt signaling pathway via the loss of PTEN and the subsequent degradation of the p53-p21 pathway.

Several studies have demonstrated the overexpression of miRNA-296 in breast cancer cells (23), glioma cells (24) and



Table III. The expression status of PTEN and p21.

Cancer type	Samples, n	PTEN-positive, n (%)	PTEN-negative, n (%)	P-value	p21-positive, n (%)	p21-negative, n (%)	P-value
Total	36						
IP non-dysplastic	6	5	1		3	3	
IP with SCC	11	8	3		8	3	
				n.s.			n.s.
SCC arise from IP	12	2	10		3	9	
SCC	7	2	5		2	5	
				n.s.			n.s.
All IP	17	13 (76.5)	4 (23.5)		11 (64.7)	6 (35.3)	
All SCC	19	4 (21.1)	15 (79.9)		5 (26.3)	14 (73.7)	
				0.0005			0.0086

IP, inverted papilloma; SCC, squamous cell carcinoma; n.s., not significant; PTEN, phosphatase and tensin homolog.

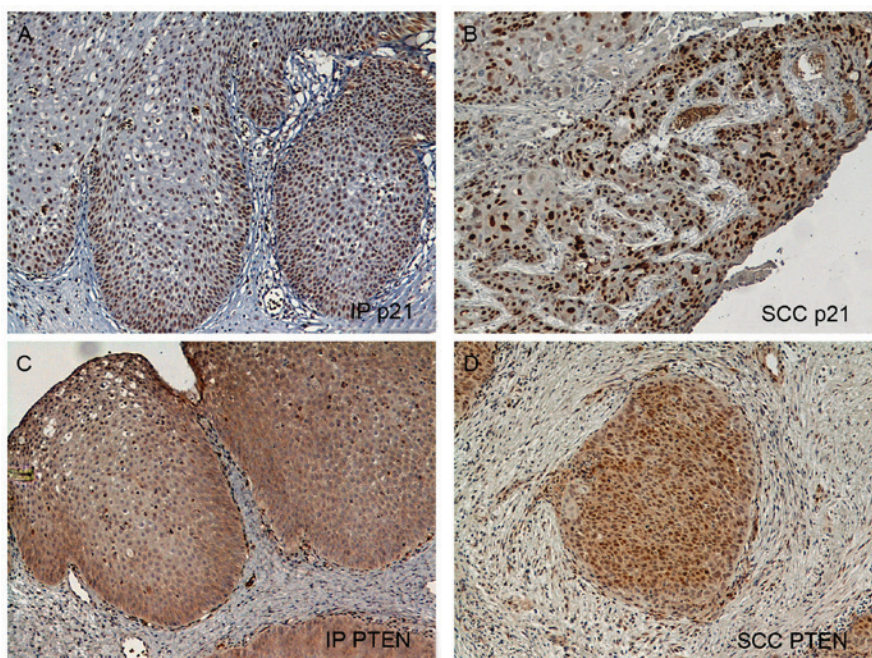


Figure 2. Immunoexpression of PTEN and p21 in IP and SCC. Original magnification, x200. (A) Positive p21 staining in IP. (B) Positive p21 staining in SCC. (C) Positive PTEN staining in IP. (D) Positive PTEN staining in SCC. PTEN, phosphatase and tensin homolog; IP, inverted papilloma; SCC, squamous cell carcinoma.

esophageal cancer (25). In these three studies, miR-296 was used to represent miRNA-296-5p+ (miRNA-296 from the 5' end), and its target genes were distinctly different from the targets of miRNA-296-3p. A previous study determined that miRNA-296-3p elevates and regulates intercellular adhesion molecule 1, in order to promote prostate cancer metastasis by possibly enhancing the survival of natural killer cell-resistant circulating tumor cells (26). *In silico* analysis has demonstrated miR-296-3p has 66 target genes, including carcinogenesis related-gene as zinc finger protein 629 and claudin-2. These genes may be involved in the attainment of invasion and metastases in the malignant transformation of IP. The present study focused on miR-296-3p, but the

expression of 200 miRNAs were revealed to vary by >2-fold during malignant transformation. Each miRNA exhibiting differential expression may perform a role in carcinogenesis; however, not all miRNAs are hypothesized to be associated with it. To identify the function of miRNAs in carcinogenesis, further comprehensive biological molecular studies are required. Recent developments in omics research as an analysis tool for the transcriptome and proteome have made it possible to identify cause and effect associations (27,28). Furthermore, exome studies using next-generation sequencers have been developed (15,16), and the association between genetic alterations and miRNA expression may now be investigated (16).

In conclusion, the present results indicate that miRNA expression varies during the malignant transformation of sinonasal IPs, and that miR-296-3p, which is overexpressed in SCCs, regulates PTEN expression. The findings of the present study have significant implications regarding the development of carcinogenesis biomarkers, as well as preventative and therapeutic strategies for SCCs arising from IP via the targeting of miRNAs, and the PI3K/Akt and p53-p21 signaling pathways.

## Acknowledgements

The present study was supported by a KAKENHI Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (grant no. 26462591), a Health and Labor Sciences Research Grant for Clinical Cancer Research from the Ministry of Health, Labor and Welfare of Japan (grant no. H26-141) and the National Cancer Center Research and Development Fund of Japan (grant no. 26-A-4).

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