

# Putative biomarkers of malignant transformation of sinonasal inverted papilloma into squamous cell carcinoma

Journal of International Medical Research

2019, Vol. 47(6) 2371–2380

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DOI: 10.1177/0300060519838385

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## Abstract

**Objective:** To compare genome-wide DNA methylation between samples of sinonasal inverted papilloma (SNIP) and squamous cell carcinoma (SCC) samples in order to identify aberrantly methylated genes that might be involved in malignant transformation.

**Methods:** Tissue samples were collected from patients. DNA methylation in C-phosphate-G islands and gene promoters was analysed using a DNA methylation microarray kit. The levels of mRNA or protein from aberrantly methylated genes were measured using real-time polymerase chain reaction or Western blot analysis.

**Results:** A total of 27 tissue samples were included in this study; 15 SNIP samples and 12 SCCs arising in SNIPs. A total of 11 201 nominally differentially methylated sites were observed between SNIP and SCC arising in SNIPs. Six sites were significantly different at  $P < 0.01$  and contained three genes (*MIR661*, *PLEC* and *OPA3*). These three genes were hypermethylated. In addition, the levels of mature miR-661 mRNA and PLEC protein were significantly upregulated in SCC tissues compared with SNIP samples. The levels of OPA3 protein were downregulated in SCC tissues compared with SNIP samples.

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**Conclusions:** This study demonstrated hypermethylation and abnormal expression of the *MIR661*, *PLEC* and *OPA3* genes, suggesting a role for their involvement in the malignant transformation of SNIP.

### Keywords

Sinonasal inverted papilloma, squamous cell carcinoma, methylation, C-phosphate-G islands, epigenetics

Date received: 27 September 2018; accepted: 26 February 2019

## Introduction

Sinonasal tumours are rare malignancies with an annual worldwide incidence of approximately 1 in 100000.<sup>1</sup> Histologically, sinonasal tumours are divided into sarcomas, olfactory neuroblastomas, mucosal melanomas and carcinomas. Over 55% of sinonasal tumours are carcinomas, which include squamous cell carcinomas (SCC), adenocarcinomas and adenoid cystic carcinoma.<sup>2,3</sup> Inverted papillomas (IP) are common benign tumours of the nasal cavity and paranasal sinuses. The malignant transformation rate of IPs is less than 27%,<sup>4</sup> but the histomorphology, immunohistochemistry and biological markers involved in their transformation remain unknown.<sup>5</sup> It is therefore important to explore the molecular mechanisms underlying the malignant transformation of IPs, which could help identify biomarkers for improved diagnosis of sinonasal tumours and better targeted therapy.

Epigenetic regulation, via DNA methylation and microRNA (miRNA) regulation, plays a critical role in a variety of cancers including head and neck SCC.<sup>6,7</sup> Hypomethylation and hypermethylation are thought to contribute to carcinogenesis by overexpressing tumour oncogenes and downregulating tumour suppressor genes.<sup>8</sup> A global methylation study revealed that

aberrant hypermethylation of gene promoters is involved in the pathogenesis of sinonasal papilloma.<sup>9</sup> A single gene methylation study showed that low *DLEC1* gene expression in sinonasal SCC tissues is associated with promoter hypermethylation.<sup>10</sup> However, no study has reported the effects of genome methylation on the malignant transformation of sinonasal IP (SNIP). The aim of this current study was to identify the different methylated genes between SNIP tissue samples and samples of SCC arising in SNIPs.

## Materials and methods

### Study design

This retrospective tissue analysis study compared DNA methylation and gene expression between tissue samples of SNIP and SCC arising in SNIP samples. A comprehensive methylation profiling technique was used to investigate DNA methylation in C-phosphate-G (CpG) islands and gene promoter regions in tissue samples of SNIP and samples of SCC arising in SNIPs. Gene ontology analysis and network analysis were used to analyse gene function and to construct gene networks of aberrantly methylated genes. Expression of these aberrantly methylated genes was further validated using real-time polymerase chain

reaction (PCR) or Western blot analysis in tissue samples of SNIP and samples of SCC arising in SNIPs. All of the SCCs arising in SNIP samples showed signs of malignant transformation of the SNIP based on histological analysis.

### Study participants

Tissue samples were collected from surgical patients at Beijing Tongren Hospital, Capital Medical University, Beijing, China between January 2013 and December 2016. None of the patients had received chemotherapy, radiotherapy or biological therapy before sample collection. None of the patients had other types of cancer. Cancer was staged according to the 7th edition of the American Joint Committee on Cancer Staging Manual.<sup>11</sup> All of the SCCs were arising in SNIPs and showed malignant progression. All tissues were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further use.

Written informed consent was obtained from all patients involved in this study, which was approved by the Ethics Committee of the Beijing Tongren Hospital, Capital Medical University, Beijing, China (no. TRECKY2014-027).

### DNA extraction, microarray analysis and analysis of aberrantly methylated genes

Genomic DNA from each cryopreserved tissue was extracted according to the user manual of the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). An EZ DNA Methylation<sup>TM</sup> Kit (ZymoResearch, Orange, CA, USA) was used to bisulfite the purified DNA. The methylation levels of over 485 000 individual cytosines were measured using an Infinium HumanMethylation450K BeadChip kit (Illumina, San Diego, CA, USA). The Infinium HumanMethylation450K BeadChip assay was performed according

to the manufacturer's instructions using an iScan<sup>TM</sup> SQ System (Illumina). The methylation level for each locus was designated as the  $\beta$  value, which was a quantitative measure of DNA methylation ranging from 0 (no cytosine methylation) to 1 (complete cytosine methylation) using Illumina GenomeStudio V2011.1 Methylation Module 1.9.0 (Illumina). Quality control was conducted by checking and removing the poorly performing probes or samples with the following exclusion criteria: (i) probes with a detection  $P$ -value above a certain cut-off ( $P < 0.05$ ); (ii) samples that failed in any of the array control probes; (iii) probes that had a significant detection  $P$ -value in  $>10$ – $25\%$  of the samples; (iv) probes for sex chromosomes, single nucleotide polymorphism sites and cross-reaction. A differential methylation analysis of the Infinium HumanMethylation450K BeadChip array data was conducted using Illumina GenomeStudio V2011.1 Methylation Module 1.9.0 (Illumina), with the following  $\Delta\beta$  and DiffScore calculations:  $\Delta\beta = \beta(\text{scc}) - \beta(\text{snip})$ ; and  $\text{DiffScore} = 10 \text{ sign}(\Delta\beta) \log_{10}(P)$ . The Illumina DiffScore is a transformation of raw  $P$ -values and no multiple adjustment is applied. The differentially methylated site was defined when the DiffScore value was  $< -20$  or  $> 20$  (corresponding to a  $P$ -value of 0.01), while the  $\Delta\beta$  value was  $> 0.17$  or  $< -0.17$  (corresponding to the degree of methylation of above the mean) between SNIP and SCC. Compared with SNIP, SCC tissues were identified as negative values for hypomethylation and positive values for hypermethylation, respectively.

### Gene ontology analysis

The microarray data were subjected to gene ontology (GO) analysis as previously described.<sup>12</sup> The over-represented GO terms were tested and the overlapping

probabilities of differentially methylated region datasets were calculated as previously described.<sup>13,14</sup> The cellular components, molecular function and biological processes of differentially methylated genes were analysed. *P*-values for GO term enrichment were calculated using Fisher's exact test for all differentially methylated genes.

### Gene network analysis

Qiagen's ingenuity pathway analysis was used to identify the gene networks. The Kyoto Encyclopedia of Genes and Genomes database was used to build the networks of genes.<sup>15</sup> Fisher's exact test was used to select significant pathways.

### Real-time PCR of miR-661 mRNA

Total RNA was extracted using TRIzol<sup>®</sup> reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription was performed using a One Step PrimeScript<sup>®</sup> miRNA cDNA Synthesis Kit (Takara Bio, Dalian, China). Real-time quantitative PCR was performed using SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II (Takara Bio).<sup>16</sup> The forward primers used for microRNA 661 (miR-661) mRNA amplification were the sequences of mature miR-661 and the reverse primers were provided by the kit.

### Western blot analysis of OPA3 and PLEC

Protein extracts were prepared from tissue samples using ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 32 mM 2-mercaptoethanol, 2% w/v sodium dodecyl sulphate [SDS], 10% glycerol) supplemented with ethylenediaminetetra-acetic acid-free complete protease inhibitors (Roche, Penzberg, Germany). Following lysis, samples were centrifuged at 12830 *g* for 20 min at 4°C and the supernatant was collected. Proteins were separated using SDS-polyacrylamide gel electrophoresis by adding 30 µg total extract to 12%

SDS-polyacrylamide gels for OPA3 and by adding 30 µg total extract to 5% SDS-polyacrylamide gels for PLEC. Separated proteins were then blotted onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk at room temperature for 1 h, then incubated with 1:2500 dilution goat antihuman OPA3 primary antibody or 1:5000 dilution goat antihuman plectin-1 primary antibody or 1:500 dilution goat antihuman  $\beta$ -actin primary antibody (all antibodies from Abcam, Cambridge, MA, USA) overnight at 4°C. Membranes were then washed three times with Tris-buffered saline Tween 20 (pH 8.0), followed by incubation with the secondary horseradish peroxidase-conjugated antigoat antibody (1:5000 dilution; Arigo, Taiwan, China) for 1 h at room temperature. Finally, the membranes were washed four times with Tris-buffered saline Tween 20 (pH 8.0).  $\beta$ -actin was used as the internal reference for relative quantification. Immunodetection was conducted with enhanced chemiluminescence (ECL; Engreen, Beijing, China) and exposed on X-radiography film. Immunoblots scanned by the densitometer were subjected to a grey value analysis using Quantity One software, version 4.6.2 (Bio-Rad, Hercules, CA, USA).

### Statistical analyses

All statistical analyses were performed using the SPSS<sup>®</sup> statistical package, version 17.0 (SPSS Inc., Chicago, IL, USA) for Windows<sup>®</sup>. The data on gene expression are presented as mean  $\pm$  SE. Statistical significance between groups were evaluated using Student's *t*-test or one-way analysis of variance. A *P*-value < 0.05 was considered statistically significant.

### Results

This study analysed 27 tissue samples that were collected from surgical

patients: 15 SNIP samples and 12 SCCs arising in SNIPs. The 15 SNIPs were collected from nine males and six females (age range, 35–61 years). The 12 SCCs were collected from eight males and four females (age range, 39–65 years).

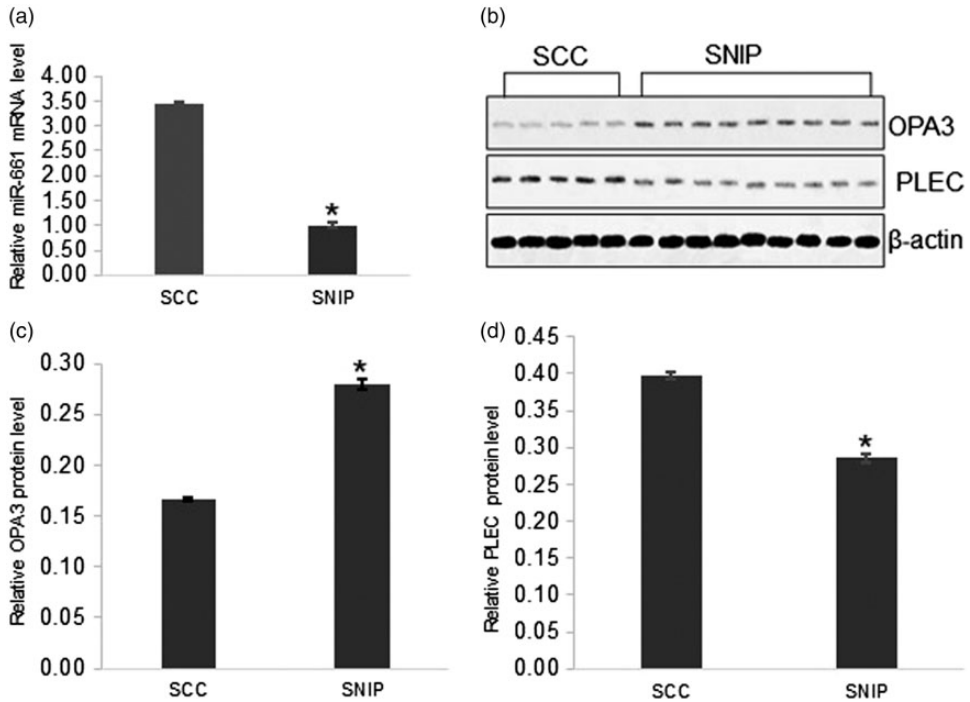
The methylated DNAs from six SNIP samples and seven SCC samples were profiled using the Infinium HumanMethylation450K BeadChip array. During the process, two SCC samples failed during analysis. Thus, six SNIP and five SCC samples were included in the final analysis. The differentially methylated sites were determined by the DiffScore value. A total of 42 9607 methylation sites were measured and 11 201 methylation sites showed 2-fold differences between SNIP and SCC tissues after log transformation ( $r^2 = 0.9606$ ,  $r^2_{sel} = 0.6311$ ). Among the 11 201 differentially methylated sites, six sites were significantly different between the SNIP and SCC samples at  $P < 0.01$ . The six sites contained three genes: outer mitochondrial membrane lipid metabolism regulator (*OPA3*), plectin (*PLEC*) and microRNA 661 (*MIR661*). All three abnormally methylated genes were hypermethylated in SCCs and not in the SNIPs (Table 1).

The expression levels of the aberrantly methylated genes were further validated using either real-time PCR to measure miR-661 mRNA levels or Western blot analysis to measure *OPA3* and *PLEC* protein levels in nine SNIP samples and five SCC arising in SNIP samples. Real-time PCR confirmed that miR-661 mRNA levels were significantly higher in SCCs compared with SNIPs ( $P < 0.001$ ) (Figure 1a). Western blot analysis demonstrated that the protein levels of *OPA3* were downregulated in SCCs compared with SNIPs (Figures 1b and 1c) ( $P < 0.005$ ), but the *PLEC* protein levels were significantly higher in SCCs compared with SNIPs (Figures 1b and 1d) ( $P < 0.005$ ).

**Table 1.** Six differentially methylated sites identified in six samples of sinonasal inverted papilloma (SNIP) and five samples of sinonasal squamous cell carcinoma arising in SNIPs.

Target identification	Differential score <sup>a</sup>	UCSC reference gene name	UCSC reference gene location	Island name	C-phosphate-G	Adjusted P-values
cg03537810	21.9	<i>PLEC</i>	Body	chr8:145018815-145019214	N_Shore	$P = 0.006418433$
cg04018533	22.6	<i>PLEC</i> ; <i>MIR661</i>	Body	chr8:145017868-145018444	S_Shore	$P = 0.005434417$
cg09235308	20.8	<i>PLEC</i> ; <i>MIR661</i>	Body	chr8:145017868-145018444	S_Shore	$P = 0.008295069$
cg10451724	21.9	<i>PLEC</i> ; <i>MIR661</i>	Body	chr8:145017868-145018444	S_Shore	$P = 0.005418433$
cg11956819	21.9	<i>PLEC</i>	Body	chr8:145018815-145019214	Island	$P = 0.006418433$
cg27391679	20.4	<i>OPA3</i>	Body	chr19:46056783-46057149	Island	$P = 0.009097289$

<sup>a</sup>A differential score equal to 20, 30 or 40 corresponds to a significant P-value of 0.01, 0.001 or 0.0001, respectively. UCSC, University of California, Santa Cruz; *PLEC*, plectin; *MIR661*, microRNA 661; *OPA3*, outer mitochondrial membrane lipid metabolism regulator.



**Figure 1.** Comparison of the expression levels of the aberrantly methylated genes as determined using either real-time polymerase chain reaction (PCR) or Western blot analysis in nine samples of sinonasal inverted papilloma (SNIP) and five samples of sinonasal squamous cell carcinomas (SCC) arising in SNIPs. (a) Real-time PCR of microRNA 661 (miR-661) mRNA levels in six SNIP samples and seven SCC samples. (b) Western blot analysis of outer mitochondrial membrane lipid metabolism regulator (OPA3) and plectin (PLEC) protein levels.  $\beta$ -actin was used as the internal control. (c) Relative OPA3 protein levels in Western blot analysis. (d) Relative PLEC protein levels in Western blot analysis. Data presented as mean  $\pm$  SE; \* $P < 0.001$  compared with SCC group; Student's  $t$ -test or one-way analysis of variance.

## Discussion

Even though the malignant transformation of SNIP into SCC is documented in the literature, the molecular mechanisms responsible for this transformation have not been elucidated. Aberrant methylation is an important mechanism that regulates the expression of tumour suppressive genes or oncogenes and is subsequently involved in the carcinogenesis and development of cancers.<sup>7</sup> The role of methylation in the malignant transformation of SNIP into SCC has not been currently addressed. In this current study, global DNA methylation in CpG islands and the promoter regions of

genes was compared between SNIPs and SCCs arising in SNIPs using a comprehensive methylation profiling technique. This current study found six methylation sites that were significantly different between the two groups at  $P < 0.01$ . These six sites contained three hypermethylated genes. The levels of miR-661 mRNA and PLEC protein were significantly higher in SCC arising in SNIP compared with SNIPs; and the levels of OPA3 protein were significantly downregulated in SCCs compared with SNIPs. To the best of our knowledge, this is the first study to compare global DNA methylation between SNIPs and SCCs arising in SNIPs. These current

findings suggest that the three genes that exhibited hypermethylation and abnormal levels of expression in SCC arising in SNIPs could be candidate biomarkers for the diagnosis of the malignant transformation of SNIP. These three genes might also serve as potential targets for developing treatment strategies in the future.

Previous studies have shown that miR-661 functions as a tumour suppressor in malignancy and acts as a tumour suppressor in breast cancer where it inhibits cell proliferation, cell motility and cell invasion.<sup>17</sup> There are some conflicting findings in breast cancer studies.<sup>17,18</sup> A recent study showed that low miR-661 expression was correlated with a poor outcome in patients with breast cancers expressing wild-type p53, whereas high miR-661 expression promoted invasion of tumour cells harbouring p53 mutations.<sup>19</sup> Thus, miR-661 may either suppress or promote cancer aggressiveness in the same type of tumour depending upon the status of p53 expression.<sup>19</sup> In glioma cells, miR-661 levels were downregulated and inhibited cancer cell proliferation, migration and invasion.<sup>20</sup> In this study, miR-661 could silence human telomerase reverse transcriptase (hTERT) by recognizing and specifically binding to the predicted site of the hTERT mRNA 3' untranslated region.<sup>20</sup> Another study indicated that hsa-circ-0012129 might act as a natural miR-661 sponge and expression of circ-0012129 could be suppressed by miR-661.<sup>21</sup> Several recent studies have shown that miR-661 may participate in the regulation of occurrence and progression in non-small cell lung cancer by directly targeting runt related transcription factor 3<sup>22</sup> or RB transcriptional corepressor 1<sup>23</sup> and interacting with adenocarcinoma predictive long intergenic non-coding RNA.<sup>24</sup> The expression of the *MIR661* gene and its role in sinonasal tumours remains unclear. In the current study, miR-661 mRNA levels were upregulated in SCCs compared with SNIPs.

Although the targets of miR-661 in sinonasal carcinomas have not been identified, the upregulation of miR-661 mRNA in SCC suggests a role in the malignant transformation of SNIP.

The OPA3 protein is an integral component of the mitochondrial outer membrane and mutations in the *OPA3* gene are associated with hereditary optic neuropathies.<sup>25</sup> A recent study in a mouse model showed that OPA3 is a novel regulator of mitochondrial function and controls thermogenesis and abdominal fat mass.<sup>26</sup> However, its role in tumour cells has not been reported. In this current study, the levels of OPA3 protein were significantly downregulated in SCCs compared with SNIPs, suggesting that OPA3 may be involved in the malignant transformation of SNIP into SCC.

Plectin is a giant multifunctional cytoskeleton protein that helps stabilize and orchestrate the intermediate filament network in cells.<sup>27</sup> Mutations in the human *PLEC* gene result in epidermolysis bullosa simplex associated with muscular dystrophy.<sup>28</sup> Only a few studies have explored the role of plectin in tumour cells. For example, a study screening potential biomarkers in oesophageal SCC detected upregulated plectin levels.<sup>29</sup> Another study suggested that plectin is either a biomarker for invasive and metastatic pancreatic ductal adenocarcinomas or serves as a marker for preinvasive precursor (PanIN III) lesions.<sup>30</sup> In addition, an earlier study demonstrated that stimulatory ascites affected the expression of the *PLEC* gene in epithelial ovarian cancer.<sup>31</sup> The expression of the *PLEC* gene in sinonasal tumours has not been reported. In this current study, PLEC protein levels were significantly upregulated in SCCs compared with SNIPs, suggesting that PLEC may be a marker for the malignant transformation of SNIP.

Recently, gene body methylation (GbM) was found to frequently occur in the transcribed regions of many oncogenic genes and

to be actively involved in multiple regulatory processes.<sup>32,33</sup> More detailed genome-wide studies have demonstrated that GbM may alter gene expression by silencing alternative promoters, affecting transcription elongation and regulating splicing.<sup>34-36</sup> Therefore, GbM could be serve as a novel biomarker or therapeutic target for human cancers.<sup>37</sup> In the current study, all hypermethylation occurred in the gene body and the expression of these three genes were different between SNIPs and SCCs, indicating that GbM might play a role in the malignant transformation of SNIP.

This current study had a number of limitations. First, the small sample size may lead to insufficient statistical power. The study only identified three genes with abnormal methylation at  $P < 0.01$ . Secondly, although the levels of mRNA and proteins from these three genes were validated in both the SCCs arising in SNIPs and SNIPs, their biological activities in sinonasal tumour cells were not studied further. Thirdly, the clinical significance of these three aberrantly methylated genes has not been analysed due to the small sample size. Finally, since these current results are very preliminary due to the small sample size, whether or not DNA methylation plays a crucial role in the malignant transformation of SNIP needs to be validated in future studies with larger sample sizes.

In conclusion, this current study identified three methylated genes that were differently expressed between SNIPs and SCCs arising in SNIPs. Although the roles of these three genes have not been previously shown to participate in tumorigenesis and progression, this study is the first to highlight their potential involvement in the malignant transformation of SNIP. This current study also demonstrates the ability of genome-wide epigenetic studies to identify potential biomarkers and thus, provide new insights into their involvement in tumorigenesis. Future research will focus

on the association of the methylation landscape with clinical outcome of patients with SNIP.


### Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

### Funding

This study was supported by grants from the Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (no. XMLX201507), the National Natural Science Foundation of China (no. 81670946), the Beijing Key Laboratory of Nasal Diseases, Capital Development Fund for Medical Research (no. 2011-2005-06), the Priming Scientific Research Foundation for Senior Researchers in Beijing Tongren Hospital, Capital Medical University (no. 2016-YJJ-GGL-001) and 2018-1-2052 Capital's Funds for Health Improvement and Research (CFH2018-1-2052).

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