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Original Article

Genome-wide comparison of DNA methylation between sinonasal squamous cell carcinomas and paired normal mucosa tissues

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Abstract: Epigenetic regulation is an important player in the tumorigenesis, progression, and prognosis of various tumors, but its role in sinonasal squamous cell carcinomas has not been well addressed. The objective of this study was to investigate the genome-wide DNA methylation in the sinonasal squamous cell carcinoma tissues. DNA methylation in CpG islands and promoters was investigated in 7 sinonasal squamous cell carcinomas and paired normal mucosa tissues using a DNA Methylation CpG Island Plus Promoter Array. A total of 12207 differentially methylated sites were observed between tumor tissues and normal mucosa tissues. Sixteen sites containing 9 genes were significantly different at $P<0.01$. Of the 9 genes, 3 genes (PTPRN2, NLRP8, and GRIN3B) were hypomethylated and 6 genes (TACC2, LOC404266, RASL10A, HOXD9, OPCML, CNPY1) were hypermethylated in the sinonasal squamous cell carcinomas compared to the paired normal mucosa tissues. The categorizations, the gene network, and the protein interaction network of these 9 aberrantly methylated genes were further analyzed. This study was the first to investigate global DNA methylation in sinonasal squamous cell carcinomas. The identified genes might be hopeful to serve as diagnostic, prognostic, and predictive biomarkers in sinonasal squamous cell carcinomas.

Keywords: Sinonasal squamous cell carcinoma, methylation, CpG island, epigenetics

Introduction

Sinonasal tumors are rare malignancies that occur in the sinonasal cavities with an annual incidence of approximately 1 case per 100,000 people worldwide [1]. Sinonasal malignancies are often divided into those of epithelial origin; which include squamous cell carcinoma, adenocarcinoma, and adenoid cystic carcinoma; and those of nonepithelial origin, which include chondrosarcoma, olfactory neuroblastoma, and mucosal melanoma [2]. Sinus cancer is staged using the T-system where stage T1 tumor is confined to antral mucosa, stage T2 tumor is characterized by destruction of the structure, hard palate, and/or middle nasal meatus of the nose, stage T3 tumor is characterized by invasion, and stage T4 tumor is a massive tumor. Early stage sinonasal squamous cell carcinomas (SCCA) present non-specific symptoms, and more than half of all SCCA patients are diagnosed with stage T3 or T4 disease [3]. A combination of surgery and radiotherapy is the optimal treatment for resectable SCCA [4]. Adjunctive chemotherapy is used for larger tumors. The 5-year survival rate for SCCA is only 30-50% [5].

Although the etiology of sinonasal tumors is currently unclear, it is commonly thought to be associated with professions where there is exposure to wood and leather dust particles or other industrial compounds. Therefore, sinonasal tumors are recognized officially as occupational diseases [6]. These risk factors suggest that aberrant epigenetic changes may play a crucial role in the tumorigenesis of sinonasal tumors. Recently, the role of epigenetic regulation in tumorigenesis and cancer development has become a focus of cancer studies. Different mechanisms, such as DNA methylation, miRNA regulation, histone modification, and nucleo-

some remodeling, have been suggested to be involved in the epigenetic regulation of a variety of cancers [7]. Hypermethylation, particularly at the promoter CpG (C-phosphate-G) island, has been widely thought to contribute to carcinogenesis through induction of transcriptional silencing or downregulation of tumor suppressor genes. Currently, several hundreds of hypermethylated genes have been identified in cancers [8]. However, DNA methylation has been rarely investigated in sinonasal tumors. A study using the methylation-specific multiplex-ligation-specific polymerase assay revealed that aberrant hypermethylation in the promoter of several genes is involved in the pathogenesis of sinonasal papillomas [9]. In sinonasal squamous cell carcinoma tissues, low DLEC1 (deleted in lung and oesophageal cancer 1) expression is associated with promoter hypermethylation [10]. However, no study has investigat ed the methylation on the basis of the whole genome in sinonasal squamous cell carcinomas.

In this study, we used a comprehensive methylation profiling technique to investigate DNA methylation in CpG islands and promoters in 7 sinonasal squamous cell carcinomas and paired normal tissues using microarrays. Gene ontology analysis and network analysis were used to analyze gene function and construct gene and protein networks of aberrant methylated genes.

Materials and methods

Design of the study

This is a comparative study of DNA methylation between sinonasal squamous cell carcinomas and paired normal mucosa tissues.

Setting

This study was conducted at Beijing Tongren Hospital, Capital Medical University and Key Laboratory of Otolaryngology Head and Neck Surgery of Ministry of Education.

Participants

7 sinonasal squamous cell carcinomas and 7 paired normal mucosa tissues were obtained from 7 patients who underwent surgery for sinonasal tumor at Beijing Tongren Hospital from 2013 to 2014. No patient received chemotherapy, radiotherapy, or biological therapy before surgery. Of the 7 patients, 5 were male and 2 were female with a mean age of 51.3 years (range: 37-62 years). Cancer was staged according to the 7th edition of the American Joint Committee on Cancer Staging Manual [11]. All 7 patients had stage T1 and T2 tumors. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until use.

Ethics

This study was approved by the Ethics Committee of Beijing Tongren Hospital, Capital Medical University and signed informed consent forms were obtained from all subjects.

DNA extraction and microarray hybridization

Seven tumor and paired normal mucosa tissues were collected from patients and genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) by following the user manual. The purified DNA was bisulfite-treated with EZ-DNA Methylation Kit (Zymo Research, Orange, CA, USA) before hybridizing with the Illumina's Infinium Human Methylation 450 K Bead Chip (Illumina, San Diego, CA, USA) according to the manufactures' instruction. Image processing and methylation intensity analysis were performed using Illumina's Genome Studio Methylation module version 1.0 (Illumina, San Diego, CA, USA). The differences between each pair of sinus squamous cell carcinoma and normal mucosa tissue were analyzed for each probe at each methylation site. A differentially methylated site was identified when the Diffscore value was less than -20 or greater than 20 (corresponding to a p value of 0.01), while the Delta-Beta value was greater than 0.17 or less than -0.17 (corresponding to the degree of methylation above average) in tumor tissues compared to the paired normal tissue. Negative values indicate hypomethylation, while positive values indicate hypermethylation in tumor tissues compared with normal tissues.

Gene ontology analysis

The microarray data were subjected to gene ontology (GO) analysis as previously described [12]. GO stats package was used to test the over-represented GO terms and the hypergeo-

Table 1. Sixteen differentially methylated sites

Target ID	Diff Score	UCSC ref gene name	UCSC GENE location	Island name	CpG
cg04864538	-26.6	PTPRN2	Body	chr7:158301266-158301610	N_Shore
cg05926314	-26.6	PTPRN2	Body	chr7:157346613-157347069	S_Shore
cg14571622	-21.1	NLRP8	3'UTR	chr7:158766126-158767400	Island
cg26869412	-21.1	NLRP8	3'UTR	chr7:158766126-158767400	Island
cg07130601	-20.5	NLRP8	3'UTR	chr7:158766126-158767400	Island
cg08273362	-20.5	GRIN3B	Body	chr19:1004324-1005605	Island
cg19638968	-20.5	GRIN3B	Body	chr11:1215375-1215986	N_Shore
cg01198033	20.5	CNPY1	5'UTR	chr7:155302253-155303158	Island
cg03923934	20.5	OPCML	5'UTR, 1st Exon	chr6:28781857-28782302	N_Shelf
cg09797577	20.5	OPCML	5'UTR, 1st Exon	chr6:28781857-28782302	N_Shelf
cg11352083	20.5	LOC404266; HOXB6	Body	chr17:46675044-46675589	N_Shore
cg12493075	20.5	LOC404266; HOXB6	Body	chr12:113913615-113914322	Island
cg13158481	20.5	HOXD9	Body	chr2:176989337-176989587	N_Shore
cg12552771	21.1	RASL10A	Body,3'UTR	chr22:29709281-29712013	Island
cg22583148	21.1	LOC404266, HOXB6	Body	chr17:46673532-46674181	Island
cg25307318	21.1	TACC2	Body	chr10:123922850-123923542	Island

ID: Identification; Diff Score: a Diff score equal to 20, 30, or 40 corresponds to a significance *p* value of 0.01, 0.001, or 0.0001, respectively; UCSC: University of California, Santa Cruz; UTR: untranslated region; TACC2: transforming acidic coiled-coil containing protein 2; LOC404266: hypothetical LOC404266; RASL10A: RAS-like, family 10, member A; HOXD9: homeobox D9; OPCML: opioid binding protein/cell adhesion molecule-like; CNPY1: canopy 1 homolog; GRIN3B: glutamate receptor, ionotropic, N-methyl-D-aspartate 3B; NLRP8: NLR family, pyrin domain containing 8; PTPRN2: protein tyrosine phosphatase, receptor type, N polypeptide 2.

metric test was used to calculate the overlapping probabilities of differentially methylated region datasets [13, 14]. The differentially methylated genes were analyzed for three functions: 1) cellular components: the parts of a cell or its extracellular environment; 2) molecular function: the activities of a gene product at the molecular level; and 3) biological processes: operations or sets of molecular events with a defined beginning and end.

Gene network analysis

Gene networks were identified using the QIAGEN's ingenuity pathway analysis (IPA). The networks of genes were built according to the relationships among the genes in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [15]. The signal-net was used to examine a protein's relationship to an upstream or downstream protein within the KEGG pathway database.

Statistical analysis

Data analyses were performed using the Statistical Package for the Social Sciences, version 17.0 (SPSS 17.0, IBM, NC, USA). The GO categories were classified using Fisher's exact test, and *P* values were calculated for the GO terms for all differentially methylated genes. Fisher's exact test was also used to select significant pathways from the KEGG pathway database. A $p<0.05$ was considered statistically significant.

Results

Identification of aberrantly methylated genes

The methylated DNA was enriched from fragmented genomic DNA. The patterns of methylation at CpG islands and promoters in the DNA of 7 sinonasal squamous cell carcinomas and paired normal mucosa tissues were determined using Human DNA Methylation 3×720 K CpG Island Plus RefSeq Promoter Array. The differentially methylated site was determined by the Diffscore value. A total of 485577 methylation sites were measured and 12207 methylation sites showed 2-fold differences between normal mucosa and tumor tissues after log transformation (r^2 = 0.9596 r^2sel = 0.6206). Among the 12207 differentially methylated sites, 16 sites were significantly different at $P<0.01$. The 16 sites contained 9 genes: TACC2

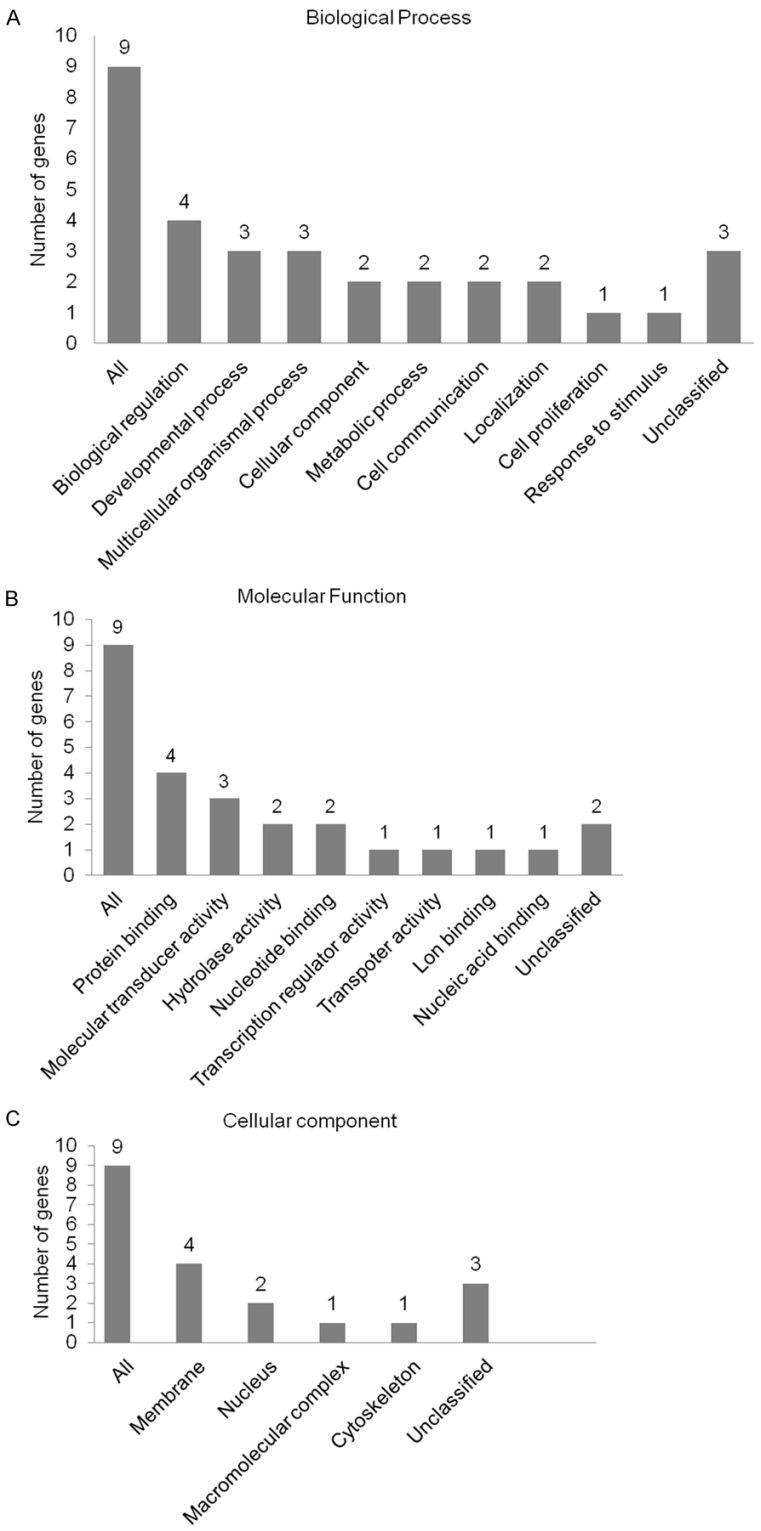

Figure 1. Gene ontology analysis of genes was abnormally methylated. (A) GO analysis by categories of biological process. (B) GO analysis by categories of molecular function, (C) GO analysis by categories of cellular components.

(transforming acidic coiled-coil containing protein 2), LOC404266 (hypothetical LOC404266), RASL10A (RAS-like, family 10, member A), HOXD9 (homeobox D9), OPCML (opioid binding protein/cell adhesion molecule-like), CNPY1 (canopy 1 homolog), GRIN3B (glutamate receptor, ionotropic, N-methyl-D-aspartate 3B), NLRP8 (NLR family, pyrin domain containing 8), and PTPRN2 (protein tyrosine phosphatase, receptor type, N polypeptide 2). Among the 9 abnormally methylated genes, PTPRN2, NLRP8, and GRIN3B genes were hypomethylated (**Table 1**).

Gene ontology analysis

Figure 1 summarized the categorizations of the 9 aberrantly methylated genes according to biological processes (**Figure 1A**), molecular function (**Figure 1B**), and cellular components (**Figure 1C**). The 9 genes were classified into 10 categories based on biological processes with the biological regulation (PTPRN2/HOXD9/TACC2/RASL10A), developmental process (HOXD9/TACC2/OPCML), and multicellular organismal process (HOXD9/TACC2/OPCML) being the 3 most over-represented biological processes. The 9 genes were classified into 9 categories based on the molecular function. Protein binding (RASL10A/NLRP8/GRIN3B/OPCML), molecular transducer activity (PTPRN2/OPCML/GRIN3B), hydrolase activity (OPCML/GRIN3B), and nucleotide binding (RASL10A/NLRP8) were 4 most frequent molecular functions. The 9 genes were finally classified into 5 categories based on cellular component with the membrane (PTPRN2/RASL10A/OPCML/GRIN3B) and nucleus (TACC2/RASL10A) being the most common cellular components.

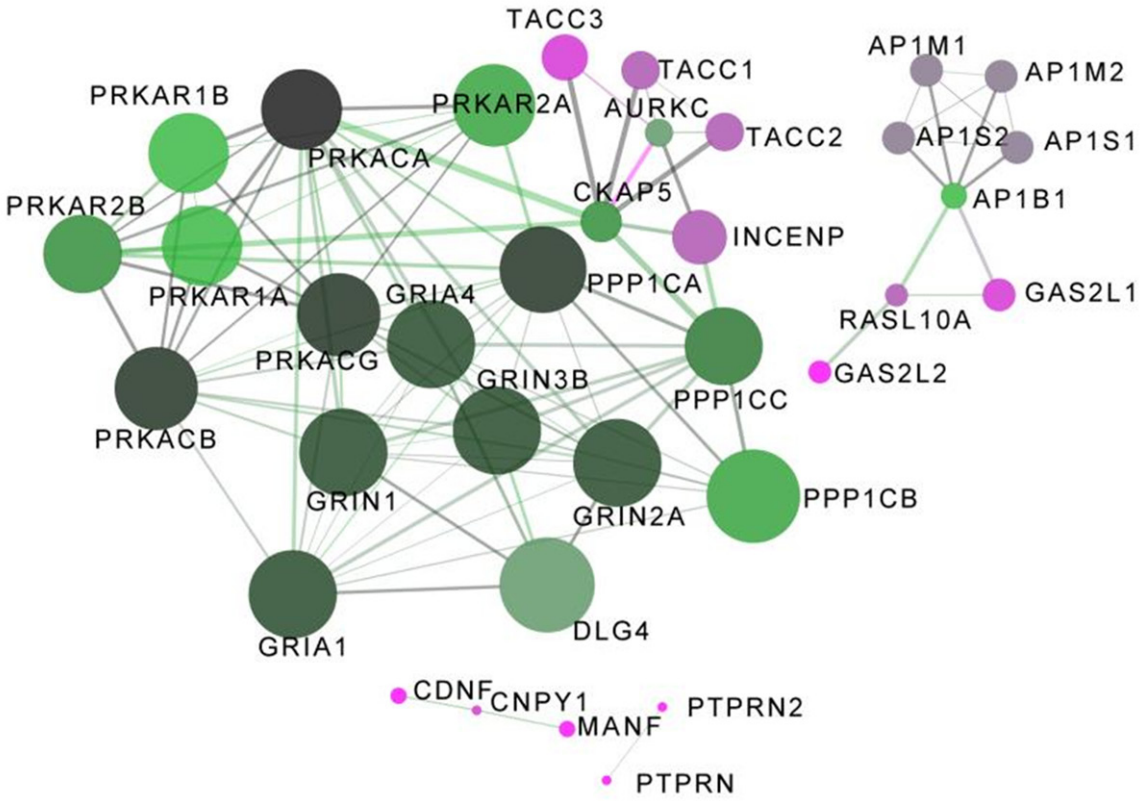

Figure 2. Kegg analysis of gene network among the 9 genes with significant abnormal methylation. Graph represents the known biological relationships of genes with abnormal methylation.

Network analysis

The interaction networks of genes (**Figure 2**) and proteins (**Figure 3**) were built according to the relationships among the genes and proteins in the KEGG database. QIAGEN's ingenuity pathway analysis and signal-net program were used to examine the relationship. The computed signaling network showed that TACC2, OPCML, and PTPRN2 were important genes in formatting this network (**Figure 2**). GRIN3B protein mainly interacts with other GRIN family members, while TACC2 protein had 4 interacting proteins (**Figure 3**).

Discussion

Epigenetic regulation has widely been demonstrated to be an important player in the tumorigenesis, progression, and prognosis of various tumors. In contrast, the roles of epigenetics in sinonasal squamous cell carcinomas have not been well documented. At present, only one study examined single gene methylation in sinonasal squamous-cell carcinomas. No study investigated global methylation in tumor tissue DNA of sinonasal squamous cell carcinomas. In this study, global DNA methylation in CpG islands and promoters was investigated in sinonasal squamous cell carcinomas and paired normal mocusa tissues using a comprehensive methylation profiling technique. This study found a total of 12207 differentially methylated sites between tumor tissues and normal mucosa tissues. Among them, 16 sites were significantly different at $P<0.01$. These 16 sites contained 9 genes, with 3 genes being hypomethylated and 6 genes being hypermethlated. The categorizations of these 9 aberrantly methylated genes were analyzed according to the biological processes, molecular function, and cellular components. Gene network and protein interaction network were analyzed. This is the first study that investigated global DNA methylation in sinonasal squamous cell carcinomas.

This study observed hypomethylation in the PTPRN2, NLRP8, and GRIN3B gene. Among these genes, PTPRN2 has traditionally been considered as a member of the protein tyrosine

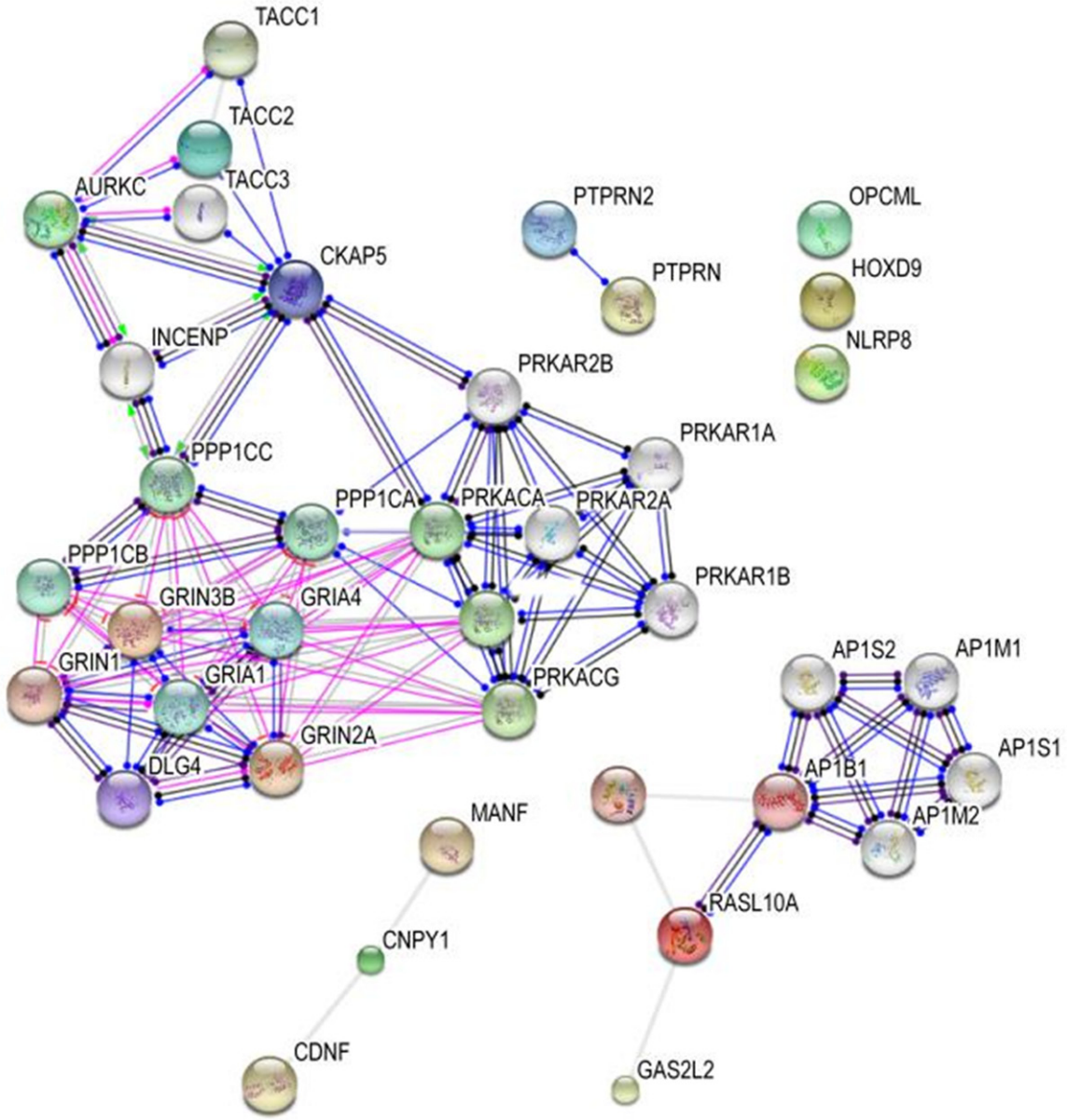

Figure 3. Kegg analysis of network among the 9 proteins with significant abnormal gene methylation. Graph represents the known protein interactions of genes with abnormal methylation.

phosphatase (PTP) family that is involved in a variety of cellular processes including cell growth, differentiation, and oncogenic transformation. A recent study demonstrated that PTPRN2 phosphorylates the lipid phosphatidylinositol and acts upon phosphatidylinositol 3-phosphate and phosphatidylinositol 4,5-diphosphate [16]. NLRP8 is an intracellular protein that is abundantly expressed in the ovaries, testes, and preimplantation embryos of mammals [17] and plays important roles in both mammalian innate immune systems and reproductive systems [18]. The hypomethylation of PTPRN2 and NLRP8 may result in high expression of these two genes, which may be involved in tumor cell growth, differentiation, and tumorigenesis or abnormal regulation of antitumor immune response. GRIN3B is a recently-discovered NMDA (N-methyl-D-aspartate) receptor gene [19]. It is unclear what could be the implications of the hypomethylation of the GRIN3B gene and its subsequent low expression in sinonasal squamous cell carcinomas.

This study also identified hypermethylation in 6 genes. Among them, 3 genes belong to the TACC family of proteins: TACC1, TACC2 and TACC3, and have been possibly implicated in tumorigenesis [20]. A previous study found overexpression of TACC1 and TACC3 in human cancers and downregulation of TACC2 expression in breast tumors, which is related to a more malignant phenotype [21]. This study showed that the DNA of TACC2 gene was hypermethylated. Network analysis revealed that TACC2 forms a signaling network with TACC1 and TACC3. RRP22 (RASL10A), a Ras-related protein on chromosome 22, has been suggested as a tumor suppressor in human cancers [22, 23]. Homeobox proteins are involved in the development and control of many cellular processes, including proliferation, apoptosis, and cell motion. HOXD9 is a member of homeobox family and has been revealed to contribute to cell proliferation and/or survival in glioma cells and cancer stem cells [24]. However, a study demonstrated that melanoma patients with HOXD9 hypermethylation had poorer disease-free and overall survival [25]. The opioid binding protein cell adhesion molecule (OPCML) is a broad-spectrum tumor suppressor gene, and its expression is frequently epigenetically inactivated in many tumors [26, 27]. This study observed hypermethylation in OPCML and RASL10A, two tumor suppressive genes. Although TACC2 and HOXD9 are generally thought to be oncogenes, downregulation of TACC2 expression due to hypermethylation was observed in breast tumors with malignant phenotype [21], while hypermethylation of HOXD9 gene had poorer survival in melanoma patients [25]. We also observed hypermethylation in TACC2 and HOXD9 genes in sinonasal squamous cell carcinomas. Canopy 1 is a novel regulator of FGF (fibroblast growth factor) signaling found in zebrafish [28], but its functions in mammals have not been addressed.

There were several limitations in the current study. First, the sample size was small and this may lead to bias. However, in this study, sinonasal squamous cell carcinomas were paired with normal mucosa tissues from the same patient. The 9 genes with abnormal methylation were identified at $P<0.01$. Thus, the information provided in this study may be reliable. Second, the expression pattern of these 9 genes was not further validated in sinonasal squamous cell carcinomas and compared with the normal tissues. However, this study paired tumor tissues with normal tissues. The difference in methylation level could reflect the gene expression level. Third, the clinical significance of these abnormally methylated genes has not been analyzed. Therefore, this is only a pilot study. Numerous studies should be conducted in the future.

In conclusion, 9 aberrantly methylated genes were identified in sinonasal squamous cell carcinomas in this study, suggesting that epigenetics may play a crucial role in the tumorigenesis. Most of these genes have been verified previously to participate in tumor cell proliferation, differentiation, apoptosis, or mitigation in a variety of tumors, but this study first highlighted their involvement in sinonasal squamous cell carcinomas. This study also suggests that genome-wide epigenetic studies will allow for identification of methylated changes in sinonasal squamous cell carcinomas and to provide new insights into their tumorigenesis. The aberrantly methylated genes might be hopeful to serve as diagnostic, prognostic, and predictive biomarkers in sinonasal squamous cell carcinomas.

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Disclosure of conflict of interest

None.

Abbreviations

DNA, Deoxyribonucleic acid; CpG, C-phosphate-G; SCCA, sinonasal squamous cell carcinomas; DLEC1, deleted in lung and oesophageal cancer 1; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SPSS, Statistical Package for the Social Sciences; TACC2, transforming acidic coiled-coil containing protein 2; LOC404266, hypothetical

LOC404266; RASL10A, RAS-like, family 10, member A; HOXD9, homeobox D9; OPCML, opioid binding protein/cell adhesion molecule-like; CNPY1, canopy 1 homolog; GRIN3B, glutamate receptor, ionotropic, N-methyl-D-aspartate 3B; NLRP8, NLR family, pyrin domain containing 8; PTPRN2, protein tyrosine phosphatase, receptor type, N polypeptide 2; NMDA, N-methyl-D-aspartate receptor.

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