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Original Research

LncRNA SNHG1 promotes the development of oral cavity cancer via regulating the miR-421/HMGB2 axis

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Abstract: Oral cancer (OC) is a common malignant tumor in oral surgery, which is prone to metastasis and the prognosis is not optimistic. Long-non-coding RNA (lncRNA) is a kind of endogenous transcripts with more than 200bp in length, lack of specific and complete open reading frame, and does not have the function of protein-coding. Studies have found that it can regulate gene expression at many levels, such as epigenetic level, transcriptional level and post-transcriptional level, thus affecting the occurrence and development of diseases. Recent studies have shown that the occurrence, development, of oral cancer, are associated with lncRNA. In this research, we found that lncRNA SNHG1 was up-regulated in oral cancer. Knockdown of lncRNA SNHG1 would inhibit the proliferation of oral cancer cells. Then we revealed a new mechanism that lncRNA SNHG1 regulated the growth of oral cancer via controlling the miR-421/HMGB2 axis, which provided new therapy for patients with oral cancer.

Key words: Oral cancer; lncRNA SNHG1; miRNA; Proliferation.

Introduction

Oral cancer (OC) is a kind of malignant tumor with high incidence, which is mainly treated by surgery and combined with radiotherapy and chemotherapy(1, 2). Oral squamous cell carcinoma (oral squamous cell carcinoma, OSCC) accounted for more than 90%. With the continuous development of basic research and clinical treatment technology, the 5-year mortality rate of patients has been significantly reduced, but the mortality rate is still at the forefront of malignant tumors, which is one of the main diseases leading to the death of cancer patients(3). Exploring new biomarkers and therapeutic targets are of great significance for the clinical diagnosis and treatment of oral cancer.

For a long time, lncRNA has been regarded as genomic transcriptional "noise" without any biological function(4). With the deep study of genomics, a large number of studies have shown that lncRNA has an indisputable regulatory effect on the tumor(5). LncRNA is a kind of non-coding RNA, which is more than 200bp in length and has no protein-coding function. It has the characteristics of many types, abundant quantity and various modes of action(6). According to the position of coding genes in the genome, LncRNA, as an important regulator in the human genome, regulates histone modification, DNA methylation or chromosome remodeling through epigenetic regulation, transcriptional or post-transcriptional level regulation, translation or post-translation level regulation and so on. Silencing or activating genes, and then dynamically controlling disease-related gene changes(7). The expression of lncRNA HOTAIR is significantly increased, which is related to the degree of metastasis, tumor stage and differentiation of oral cancer(8, 9). LncRNA MALAT1 induced cell migration, invasion, epithelial-mesenchymal transformation and inhibited apoptosis by regulating the Wnt/β-catenin signaling pathway in oral cancer (10, 11).

It was reported that lncRNA SNHG1 (SNHG1) could induce the progression of NSCLC by increasing MTDH through targeting miR-145-5p(12). SNHG1 was predicted as a promoter and induced hepatocellular carcinoma tumorigenesis(13). Downregulation of SNHG1 prevented the cell cycle via inhibiting the Notch-1 signaling pathway in pancreatic cancer(14). Xu *et.al* found that SNHG1 controlled colon cancer cell development via binding with miR-154-5p(15). SNHG1 induced tumor growth by regulating the transcription of genes(16).

In this study, we observed that lncRNA SNHG1 was abnormally up-regulated in oral cancer, recovered the level of lncRNA SNHG1 would prevent the proliferation of oral cancer. Further, we found that lncRNA SNHG1 could target miR-421 and regulate the expression of HMGB2. Our results uncovered an underlying therapeutic target in OC.

Materials and Methods

Clinical samples

Tumor tissue samples were collected from 12 OSCC patients at Jinan Central Hospital. All of the patients or their guardians provided written consent, and the Ethics Committee of Jinan Central Hospital approved all aspects of this study.

Cell culture

NHOK, SCC9, SCC15, Ca9-22 and HSU3 cell lines were purchased from the Science Cell Laboratory. Cells were cultured in DMEM/F12 (GIBCO, USA) supplemented with 10 % FBS(Cromwell, USA) and 100 μL/mL penicillin and streptomycin (Sigma-Aldrich, USA) and placed at 37℃ with 5% CO2.

Cell transfection

ShRNA was constructed for downregulating lncRNA. A scrambled shRNA was used as negative control (sh-NC). About 5×10^5 cells per well were cultured in 6 well plates, transfection of shRNA into the cells was performed using lipo2000 (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. Cells were transfected with 20 nmol/L siRNA for 48 h, and then siRNA transfection in the best condition was performed.

Real time-PCR

Total RNA was isolated from tissues and cells according to a standard protocol. And then, the purity and concentration of RNA were detected and all the samples were converted into cDNA using a reverse transcription kit. We used SYBR Green (Thermo Fisher Scientific) system to perform the qRT-PCR. The primer sequences: SNHG1, forward: 5′-AGGCTGAAGT TACAGGTC-3′ and reverse: 5′-TTGGCTCCCAGTGTCTTA-3′; GAPDH, forward: 5′-TGCCATCAATGACCCCTTC-3′ and reverse: 5′-CATCGCCCCACTTGATTTTG-3′

Western blot

Total protein was isolated from tumor and cells with RIPA lysis Mix. Briefly, 50 μg protein extraction was loaded *via* SDS-PAGE and transferred onto nitrocellulose membranes (Life Sciences, Mexico), the membranes were incubated in 5% non-fat milk blocking buffer for 2 h. Then incubated with primary antibodies for 2 h at room temperature, then plated at 4 °C overnight, After incubation with secondary antibodies, the membranes were readied using an Odyssey, and data were analyzed with Odyssey software (LI-COR, USA). The primary antibody brand and usage concentration were as follows. E-cadherin (20874-1-AP,1:1000), MMP2 (10373-2-AP, 1:500), MMP7 (10374-2-AP), MMP9 (10375-2-AP, 1:500) were purchased from Proteintech; N-Cadherin (#14215) was purchased from CST HMGB2 (Proteintech, 15605-1-AP, 1:500) and GAPDH (Proteintech, 60004-1-Ig, 1:2000) was used as an internal control.

Wound-healing assay

The wound-healing assay was carried out on SCC9 cell. 5×10^5 cells were cultured in transwell plates and then the cells were gently scratched with a pipette tip. The fresh medium was changed. After 48 h treatment, the scratched spaces on the plate were read by microscopy.

Matrigel invasion assay

Cells in the logarithmic growth phase were adjusted to 2×10^5 cells/well of medium (without serum) and plated 1μg/μl Matrigel into the upper chamber. The lower chamber was added with 500 μL of the medium, and then incubate the plate at 37℃ for 48 h. Then the invading cells were visualized by the crystal violet and inverted microscope.

In *vivo* tumor growth assay

Nude mice were purchased from the Beijing Charles river. The nude mice were injected with treated-SCC9, SCC9 cells (5×10^6) were subcutaneously injected in the right lower limb of the nude mice. Tumor sizes were detected every 3 days. After another 15 d of injection, the tumor was removed for follow-up study.

Cycle assay

Cells were collected with 1ml trypsin for 2min, suspension the cell with 5ml PBS, centrifuge at 1000 RPM for 5 min at 4℃. 10ml PBS buffer was used to the re-washed and dropping medium, Then the cells were fixed with 70% ethanol overnight. The next day, the cell medium was filtered with a 300-mesh sieve, centrifuged at 1000 RPM at 4℃ for 5min, and the supernatant was discarded. The cells were avoided light and fixed with 1ml PI solution and stated at 4℃ for 30 min. A flow cytometer was used to evaluate the cell cycle.

Luciferase Assay

HEK293T cells were co-transfected with 20 mmol/L miRNA mimic or miR-NC together with SNHG1-WT or SNHG1-mutation. Luciferase activity was calculated with Dual-Luciferase Reporter Assay Kit (Transgene, China) on GloMax20/20 at 48 hr after the transfection.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical significances were measured by Student's t-test and ANOVA A two-tailed value of $P < 0.05$ was indicated as a statistically significant difference. Data statistics were used the GraphPad 7.0.

Results

LncRNA SNHG1 expression was an upregulation in OSCC tissues and cell lines

RT-PCR was employed to explore the expression of SNHG1 in OSCC patients and plasma. We observed that the expression of SNHG1 was upregulated in OSCC (Fig.1A and 1B). Then we detected the expression of SNHG1 in OSCC cell lines (SCC9, SCC15, Ca9-22, HSU3), and NHOK was indicated as a control. We got similar results *in vitro* (Fig.2C), which would be a new therapeutic target in OSCC.

Knockdown of LncRNA SNHG1 knockdown inhibited OSCC growth *in vitro*

To further explore the role of SNHG1 in OSCC, we constructed the shRNA to silence the SNHG1 (sh-SNHG1), and sh-NC was identified as a negative control. The transfection efficiency of SNHG1 was performed by RT-PCR (Fig. 2A). Next, sh-SNHG1/sh-NC were transfected into SCC9 cells. CCK8 assays revealed that SNHG1 downregulation could inhibit the growth in SCC9 cells (Fig. 2B). Subsequently, we used flow cytometry to detect the effect of SNHG1 on the cell cycle. Knockdown of SNHG1 reduced the number of cells in the G0/G1 phase to the S phase (Fig. 2C). Meanwhile,

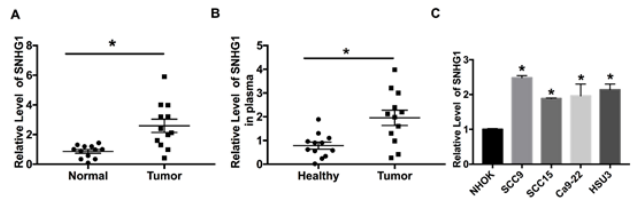

Figure 1. LncRNA SNHG1 levels in OSCC tissues and cell lines. A. RT-PCR detected the mRNA level of SNHG1 in OSCC patients tissues. n=12, **P* <0.05. B. RT-PCR detected the mRNA level of SNHG1 in OSCC patients and healthy volunteers plasma. n=12, **P* <0.05. C. The mRNA level of SNHG1 in pulmonary adenocarcinoma cell lines (SCC9, SCC15, Ca9-22, HSU3), and NHOK was indicated as a control. n=12, **P* <0.05.

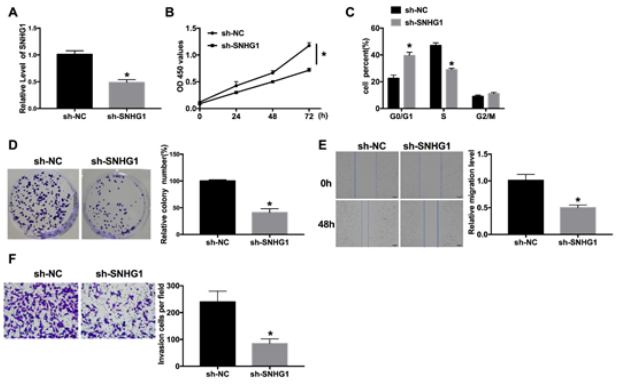

Figure 2. Downregulation of SNHG1 inhibits proliferation and metastasis in SCC9 cells. A. The level of SNHG1 was measured by RT-PCR. n=6, **P* <0.05. B. CCK8 assay was performed to detect the proliferation ability. n=4, **P* <0.05. C. The effect of SNHG1 on cell cycle. n=5, **P* <0.05. D. Colony formation was measured n=5, **P* <0.05. E. Representative images (left) and histogram (right) from wound-healing assays using A549 cells. n=4, **P* <0.05. F. Cell invasion ability was detected in SCC9 after shRNA transfection. n=6, **P* <0.05.

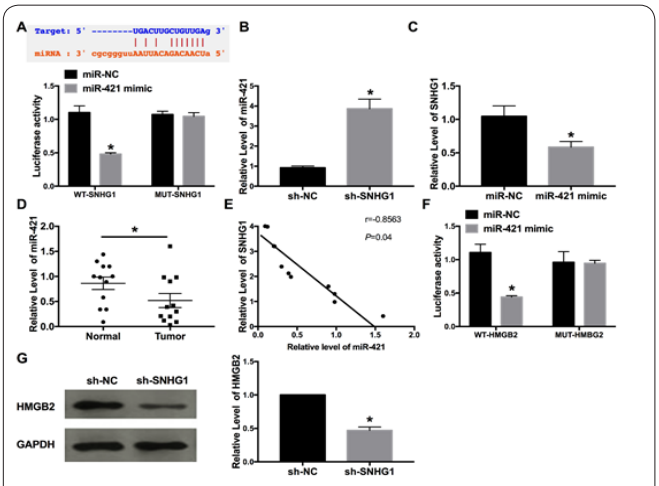

Figure 3. LncRNA SNHG1 interacted with miR-421 and regulated HMGB2. A. Bioinformatics prediction binding sequence and luciferase report. n=4, **P* <0.05. RT-PCR was employed to detect the level of SNHG1 (B) and miR-421 (C). n=4, **P* <0.05. D. The level of miR-421 in OSCC patients. n=12, **P* <0.05. E. Correlation Analysis of SNHG1 and miR-421. F. Luciferase assay was performed to verify the relationship between miR-421 and HMGB2. n=4, **P* <0.05.

the clone formation experiment showed that sh-SNHG1 prevented clone formation (Fig. 2D). By performing a wound-healing assay, we found that sh-SNHG1 inhibited the migration of SCC9 cells (Fig. 2E), and matrigel invasion assay revealed that knockdown of SNHG1 blocked the invasion of SCC9 cells (Fig. 2F). In summary, downregulation of SNHG1 would prevent proliferation and metastasis of OSCC cells.

LncRNA SNHG1 regulated miR-421/HMGB2 and in OSCC

Most human genes can be transcribed, but most of the products are non-coding RNA (ncRNA). Partial ncRNA can regulate life activities by affecting genes and epigenetics. lncRNA and miRNA are the two most important types, which play an important role in a variety of life activities(17, 18). At present, it has been found that lncRNA regulates miRNA. miRNA mainly by acting as the precursor of miRNA, competitively combining mRNA with miRNA and "sponge effect". It can form a complex regulatory network by directly combining lncRNA or indirectly regulating lncRNA. LncRNA and miRNA through intermediate factors. Through the prediction of the bioinformatics website, we found that there was a binding sequence between lncRNA SNHG1 and miR-421. Luciferase assay reported that miR-421 could combine with WT-SNHG1, but not with mutation-SNHG1 (Fig. 3A). After transfection with sh-SNHG1/sh-NC, we measured the expression of miR-421, which was increased in the sh-SNHG1 group (Fig. 3B), and the expression of SNHG1 was decreased after miR-421 mimic transfection (Fig. 3C). In OSCC patients tissues and plasma, we both observed the decreased level of miR-421 (Fig. 3D). Correlation analysis showed that there was a negative correlation between SNHG1 and miR-421 (Fig. 3E). As Fig. 3F shown, HMGB2 was a target of miR-421 and decreased in SCC9 cells after sh-SNHG1 transfection (Fig. 3G). Taken together, SNHG1 would regulate miR-421/HMGB2.

LncRNA SNHG1/miR-421/HMGB2 axis involved in the development of OSCC

To detect the role of LncRNA SNHG1 /miR-421/HMGB2 pathway in OSCC, we co-transfected sh-SNHG1 with miR-421 inhibitor/HMGB2 plasmid into SCC9 cells. RT-PCR assay performed that co-transfected with miR-421 inhibitor/HMGB2 plasmid recovered the level of miR-421 induced by knockdown of SNHG1 (Fig. 4A). The upregulation of HMGB2 was also blocked by miR-421 inhibitor and HMGB2 plasmid (Fig. 4B), which revealed the regulation relationship among SNHG1, miR-421 and HMGB. Then we performed a CCK8 assay for cell proliferation. The results showed that the inhibition growth effect of SNHG1 was abolished by miR-421 inhibitor and HMGB2 (Fig. 4C). Similarly, miR-421 inhibitor and HMGB2 had the same effect on the cell cycle (Fig. 4D). Downregulation of miR-421 or overexpression HGMB2 both recovered clone formation, which was prevented by silencing SNHG1 (Fig. 4E). Wound healing and transwell assays showed that downregulation of miR-421 or overexpression HGMB2 would promote migration and invasion by blocking sh- SNHG1 function (Fig. 4F&G).

LncRNA SNGH1 knockdown prevented OSCC growth *in vivo*

In order to further verify the results, we constructed

a stable SCC9 cell line with low expression of SNHG1, and a normal expression of SNHG1 SCC9 cell was described as control. The cells were subcutaneously injected into the right lower limb of the mice. After another 15 d of injection, the tumors were removed and calculated (Fig. 5A). Sh-SNHG1-SCC9 injection prevented the development of the tumor (Fig. 5B&C). Then we measured the level of SNHG1 and miR-421 in the removed tumor by performing RT-PCR. The decreased SNHG1 and increased miR-421 was revealed in sh-SNHG1-SCC9 injection mice tumor (Fig. 5D&E). We also found the down-regulated of HMGB2 in sh-SNHG1-SCC9 injection mice tumor by employed western blot (Fig. 5F). Further, we detected that the metastasis-associated protein (E-cadherin, N-cadherin, MMP2, MMP7 and MMP9) in tumors, we observed that the expression of E-cadherin was up-regulated, and the expression of N-cadherin, MMP2, MMP7 and MMP9 were down-regulated (Fig. 5G). In conclusion, lncRNA SNHG1 controlled the growth and development of OSCC via regulating miR-421/ HMGB2.

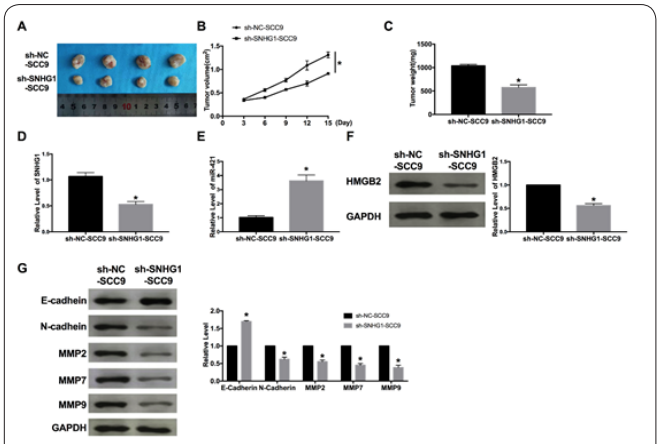

Figure 5. LncRNA SNHG1 regulates tumorigenesis *in vivo.* A. Effects of SNHG1 knockdown on the size of SCC9 xenograft tumors in nude mice. B&C. The average tumor volume and weights of the tumors. n=5, **P*<0.05 D&E. The expression level of SNHG1 and miR-421 in tumor tissue. n=6, **P*<0.05. F. The protein level of HMGB2 was detected by western blot. n=5, **P*<0.05. G. The protein level of metastasis-associated protein (E-cadherin, N-cadherin, MMP2, MMP7 and MMP9) were detected in tumor tissue. n=5, **P* <0.05.

Discussion

In this article, we revealed the function of lncRNA SNHG1 in OSCC for the first time. Downregulation of lncRNA SNHG1 would prevent the proliferation, migration and invasion of OSCC cells, meanwhile, we got similar results *in vivo.* Further, we performed a new mechanism that lncRNA SNHG1 regulated the development of OSCC through the miR-421/ HMGB2 signal pathway.

OSCC is the most common malignant tumor in Southeast Asia. In China, the incidence of OSCC is about 3.6 million per 100000 people(19, 20). OSCC is the eighth leading cause of cancer death each year in the United States. OSCC is highly invasive and often causes morphological loss and dysfunction in patients. Up to 60% of OSCC patients are in the late stage of clinical progress (stage III, stage IV), OSCC's five-year survival rate is only 10%-50% (21, 22). Therefore, how to detect and diagnose OSCC, early to achieve targeted therapy is the focus of current research. The development of OSCC is regulated by the changes of genome and epigenome. However, the relevant mechanism remains to be clarified.

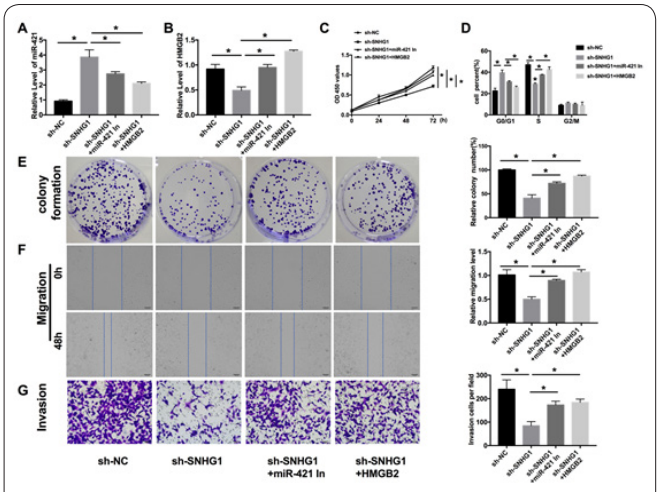

Figure 4. LncRNA SNHG1/miR-421/HMGB2 axis involved in the development of SCC9 cells. RT-PCR was employed to detect the expression of miR-421 (A) and HMGB2 (B) in SCC9 cells after transfection. n=5, **P* <0.05. C. CCK8 assay was performed to detect the proliferation ability. n=4, **P* <0.05. D. The effect of SNHG1 on cell cycle. n=5, **P* <0.05. E. Colony formation was measured n=5, **P* <0.05. F. Representative images (left) and histogram (right) from wound-healing assays using A549 cells. n=4, **P* <0.05. G. Cell invasion ability was detected in SCC9 after shRNA transfection. n=6, **P* <0.05.

There is growing evidence that the imbalance of lncRNAs expression may affect the progression of a variety of cancers, mainly at the epigenetic, transcriptional and post-transcriptional levels to induce and promote cancer progression, including oral cancer. Therefore, lncRNAs are expected to become a reliable biomarker and target for anti-tumor therapy. After knocking out lncRNA MALAT1, it was found that it could prevent the development of tumors in vivo and in vitro, such as inhibiting cell proliferation, promoting apoptosis and inhibiting the development of OSCC cells. Recently, it has been found that MALAT1 is increased in OSCC tissues compared with normal oral mucosa(23). Using the qPCR method, it was found that the level of MALAT1 in TSCC tissue was higher than normal tissue, and it was related to cervical lymph node metastasis(24). The down-regulation of MALAT1 expression by small interference RNA in OSCC cell lines can describe its role in keeping stable of EMT-mediated cell metastasis. Compared with the surrounding non-malignant tissues, the expression of lncRNA MEG3 has markedly decreased in tongue squamous cell carcinoma, suggesting that the prognosis of patients with TSCC is poor(25). The expression of LncRNA HOTAIR was closely related to the clinical stage, lymph node metastasis, tumor stage and differentiation of OSCC(26). The patients with high expression of HOTAIR had a poor prognosis. Further experiments showed that after knocking down HOTAIR by siRNA in vitro, the growth and clone formation of OSCC cells was inhibited, and the invasion and metastasis ability of OSCC cells was enhanced. There is a

significant negative correlation between HOTAIR and E-cadherin. The expression of E-cadherin is regulated by combining PRC2 complex unit EZH2 and histone H3K27me3 with E-cadherin promoter, so it is speculated that the expression of lncRNA HOTAIR is closely related to the occurrence, development and metastasis of OSCC and may be an important marker for predicting the survival rate of OSCC.

LncRNA SNHG1 plays a role as a pro-tumor factor in tumors and promotes the occurrence and development of tumors in a variety of ways. It has been found that SNHG1 can inhibit the level of the p53 gene and promote proliferation, invasion, and metastasis of tumor cells. In addition, through the different analyses of the tumor tissues of gastric cancer(27), liver cancer, lung cancer(28) and colorectal cancer(29) and the corresponding paracancerous tissues, it was found that SNHG1 was significantly up-regulated in these four kinds of malignant tumors. Through cell experiments, it is confirmed that SNHG1 can promote the proliferation of esophageal cancer cells and inhibit apoptosis(30). Further assays in vitro confirmed that the competitive binding of SNHG1 to miR-338, down-regulated the expression of miR-338 in esophageal cancer cell lines, and then promoted the expression of CST3(31).

Some achievements have been made in the study of SNHG1 in tumors. However, SNHG1 plays a role in promoting cancer through different signal transduction pathways in tumors. As a tumor-promoting factor, SNHG1 is closely related to tumor size, clinical stage, prognosis and so on. As a new research hotspot, the expression and regulation of SNHG1 in tumors need to be studied urgently. With the continuous progress of research techniques and methods, the exploration of the regulatory mechanism of SNHG1 on tumor will not only help to reveal the occurrence and development of tumor, but also provide new ideas for early diagnosis and therapy of the disease, and hopefully become a new prognostic marker and drug therapy target. In general gastric cancer has many factors and components that need to be carefully evaluated (32-34).

Tobacco use is the biggest risk factor for oral cancer. People who use these products are about 50 times more likely than the general population to develop cancers of the lips, gums and cheeks. Smokers and people who drink alcohol are also more likely to develop oral cancer than non-smokers and non-alcoholics. Sun exposure increases the risk of skin cancer. It can also increase the risk of oral cancer (35-40). HPV infection can lead to oral cancer. Suppressed immune systems increase the risk of oral cancer. Poor oral hygiene, which leads to gum disease (periodontitis), is known to contribute to the development of oral tumors. Even without the above risk factors, you may develop an oral tumor. This may not be due to a genetic risk factor. In this regard, the role of enzymes, proteins, genes, coding and non-coding regions of mRNAs should be considered (41- 46).

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