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Long non-coding RNA ZFAS1 promotes nasopharyngeal carcinoma through activation of Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: To investigate the expression characteristics of long non-coding RNA (lncRNA) ZFAS1 in nasopharyngeal carcinoma (NPC) and to further investigate the role and mechanism of ZFAS1 in the occurrence and development of NPC.

PATIENTS AND METHODS: 76 surgical excision samples from newly diagnosed NPC patients and 76 cases of paracancerous tissues were selected. The expression of ZFAS1 was detected by qRT-PCR (quantitative real-time polymerase chain reaction), and the relationship between the expression of ZFAS1 and the clinical prognosis of patients with NPC was analyzed. After the knockdown, the expression of ZFAS1 in nasopharyngeal carcinoma cell line CNE-1 by small interfering RNA, the effect of ZFAS1 on the biological function of NPC cells was analyzed by cell counting kit-8 (CCK8), colony formation assay, flow cytometry and apoptosis assay. The effect of ZFAS1 on the Wnt/β-catenin pathway was analyzed by Western blot.

RESULTS: After analyzing the clinical data of enrolled patients, we found that ZFAS1 was overexpressed in NPC patients, which was closely correlated with tumor size and lymph node metastasis of NPC. Moreover, ZFAS1 was negatively correlated to the survival of NPC patients. After interfering with the expression of ZFAS1, the proliferation of NPC cells was significantly decreased, the cell cycle was inhibited and the apoptosis rate was increased. Western Blot showed that ZFAS1 promoted the occurrence of NPC by activating the Wnt/β-catenin pathway.

CONCLUSIONS: ZFAS1 was overexpressed in NPC and was significantly related to tumor size, lymph node metastasis and poor prognosis of NPC. ZFAS1 could promote NPC by activating Wnt/β-catenin pathway.

Key Words:

Long non-coding RNA; ZFAS1; nasopharyngeal carcinoma; prognosis; Wnt/β-catenin

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in clinic. The annual global incidence is about 25/100,000, and the incidence is increasing yearly worldwide[1,2]. Most NPC is sensitive to radiation, and radiation therapy is the preferred treatment. For NPC patients presented at an early stage, as the application of early diagnosis and accurate radiotherapy techniques advanced, the 5-year survival rate was up to 67%-80%. However, there were still some patients who presented local recurrence and distant metastasis. For those NPC patients presented distant metastasis when undergoing the initial treatment, the prognosis was worse[3-5]. Therefore, to clarify the occurrence and development mechanism of NPC and explore the related factors that promoted the proliferation and metastasis of which would be helpful to guide an individualized clinical treatment, and further improve the prognosis of NPC patients.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs without functions to encode proteins due to the lack of open reading frames, and are over 200 nt in length[6,7]. LncRNAs are involved in the biological function of tumors, mainly through the regulation of epigenetic, gene expression and translation under transcriptional and post-transcriptional levels[8,9]. Recent studies have indicated that lncRNAs are differentially expressed in various tumors[10]. Therefore, the role of lncRNA in tumorigenesis and development has attracted much attention.

Some lncRNA shave also been confirmed to be related to the carcinogenesis of NPC. For example, Linc00312 inhibited the proliferation, induced the apoptosis, but also stimulated the invasion of NPC cells[11]. Nie et al[12] found that HO-

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TAIR (HOX transcript antisense RNA) expression in NPC was high, the expression of which was related to the tumor size, clinical stage, and lymph node metastasis. Xie et al[13] found that MALAT-1 (metastasis associated lung adenocarcinoma transcript 1) expression in NPC cell lines was high with a highly metastatic potential, and CNE-1 cells overexpressing MALAT-1 significantly enhanced the invasion and metastasis. Yang et al[14] found that lncRNA may participate in the occurrence and development of NPC by interacting with adjacent coding transcripts or proteins. However, the specific molecular mechanism by which lncRNA regulated the development of NPC has not been clearly investigated. This study explored the expression characteristics of lncRNA ZFAS1 in NPC and investigated the effect and mechanism of ZFAS1 on the biological functions of NPC cells.

Patients and Methods

Collection and Processing of Samples

A fresh tissue of the lesion site was taken using a special clamp by a specialized physician under the guidance of the nasopharyngeal fiber laryngoscope, immediately washed with the diethyl pyrocarbonate (DEPC) water and put it into the freezing tube, marked and stored in the liquid nitrogen tank for freezing. Subsequently, related clinical data of patients from the Pathology Department and Medical Record Department was collected. All the above samples were collected for consent by the patient and approved by the local Ethics Committee.

Cell Culture

All cells were cultured in RPMI 1640 medium containing 10% inactivated neonatal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin (Gibco, Grand Island, NY, USA). Cells were cultured in an atmosphere at 37°C, relative humidity 90% and 5% CO_2 incubator. Cells were under regular observation and culture medium was replaced 3 times a week. Cells were passaged for 2 times a week with 0.25% trypsin (Gibco, Grand Island, NY, USA) combined 0.02% EDTA.

Cell Counting Kit-8 (CCK8)

Cells were incubated in 96-well plates with a density of 2.5-5 × 10^3 cells per well. Add 20 uL of CCK8 (Dojindo, Kumamoto, Japan) solution to each well. Cells were cultured in an atmosphere at 37°C and 5% CO_2 incubator for 4 hours, cell culture was stopped and culture medium was removed, absorbance values at 450 nm were detected by the microplate reader (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicate.

Cell Cycle Assay

Flow cytometry (Partec AG, Arlesheim, Switzerland) was used for detecting the cell cycle. Cells in logarithmic growth phase from each transfection group were inoculated into a 6-well plate with a density of 1 × 10^5/well. After cultured for 24 h, cells were harvested by trypsinization, washed three times with pre-cooled PBS (phosphate buffer saline), fixed with ethanol, incubated overnight at 4°C. Add propidium iodide (Abcam, Cambridge, MA, USA) for staining 25 min without light, cell cycle in each transfection group was detected using flow cytometry. Each experiment was performed in triplicate, the average value was calculated.

Apoptosis Assay

Flow cytometry was used for detecting the cell apoptosis. Cells in logarithmic growth phase were inoculated into a 6-well plate with a density of 1 × 10^5/well. After culturing for 24 h, cells were harvested by trypsinization and stained with Annexin V-FITC (purchased from Invitrogen, Carlsbad, CA, USA) for 15 min. The apoptosis of each transfection group was detected by flow cytometry. Each experiment was performed in triplicate, the average value was calculated.

Small Interfering Sequences Construction and Transfection

Small interfering RNA was purchased from (Invitrogen, Carlsbad, CA, USA) for gene synthesis. The sequence was: 5-GTGCATGTGGTAGGTTAGATT-3'. Cells were placed in RPMI 1640 medium supplemented with 10% fetal bovine serum and placed in a 37°C, 5% CO_2 incubator for 48 h. Cells were seeded into 6-well plates at 2 × 10^5 cells/well. After the cell density was grown to 70%-80%, the Lipofectamine™ 3000 reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect the cells to a final concentration of 100 nmol/L. Cells were divided into transfected si-Control nonsense sequence (negative control group) and transfected si-ZFAS1 group (experimental group). All plasmids were constructed by Invitrogen, Co, Ltd (Carlsbad, CA, USA).

qRT-PCR

Total RNA was extracted according to the instructions of TRIzol kit (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined to be 500 ng. After obtaining the reverse transcription volume, the extracted RNA was reverse transcribed into cDNA according to the instruction of the reverse transcription kit. The RNA was extracted according to SYBR Premix Ex Taq kit Configure (Invitrogen, Carlsbad, CA, USA) to prepare a 20 μL reaction system (SYBRII 10 μL, cDNA 2 μL, upstream primer 0.4 μL, downstream primer 0.4 μL, ultrapure water 8.0 μL). β-actin was used as an internal reference. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, followed by 95°C for 5 s and 60°C for 30 s, for 40 cycles; finally, 94°C for 90 s, 60°C 180 s for extension, draw the amplification curve. The ZFAS1 sequence was: F: ACGTGCAGACATCTACAACCT; R: TACTTCCAACACCCGCAT.

Colony Formation Assay

Cells were seeded in 6-well plates 24 to 48 hours after transfection, and 500 cells per well, 3 wells in each group, cultured in 10% fetal bovine serum (FBS) medium for 2 weeks. Cells were washed three times with phosphate buffered saline (PBS). Methanol was used to fix, 0.1% crystal violet (purchased from Sigma, St. Louis, MO, USA) was used to stain.

Western Blot

After transfection for 72 h, cells were collected and washed twice with PBS; 100 L cell lysate was added to lyse cells at 4°C for 30 min, centrifuged at 12 000 × g for 10 min, the supernatant was taken, and the concentration of protein was determined by Bradford method. The protein was electroblotted onto polyvinylidene difluoride (PVDF) membrane with 50 mg total protein per lane. Membranes were blocked with 5% skim milk for 1 h at room temperature, 1: 200 anti-human β-catenin, cyclin D1, c-myc, and β-actin antibody (Cell Signaling Technology, Danvers, MA, USA) were added and incubated overnight at 4°C. After the membrane was washed, IRDyeT-M700DX labeled secondary antibody (Cell Signaling Technology, Danvers, MA, USA) which was diluted 1:10000 was added and incubated at room temperature for 1 h in the dark. Membranes were completely washed, then put into the Odyssey two-color infrared laser imaging system for direct scanning imaging. After membranes were completely washed, Odyssey two-color infrared laser imaging system (LI-COR Bioscience, Lincoln, NE, USA) was utilized for detecting scanning imaging.

Statistical Analysis

Data were analyzed using statistical product and service solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA), the log-rank test was used for survival analysis, categorical data were analyzed by chi-square test, and *t*-test was used for measurement data. All measurement data were expressed as mean ± s. $p < 0.05$ was considered statistically significant.

Results

Expression of ZFAS1 in NPC

The clinical data of 76 NPC patients were collected from our center, it was found that ZFAS1 was highly expressed in NPC patients (Figure 1A), and there was a lower survival of patients with high expression of ZFAS1 than those with low expression of ZFAS1 (Figure 1B, C). Tumor grade in patients with high expression of ZFAS1 was higher compared to the low expression group (Table I). Further, it found that ZFAS1 was highly expressed in different NPC cell lines, and CNE-1 had the highest expression in NPC cell lines compared with normal cell line NP-69 (Figure 1D). Therefore, CNE-1 was selected for subsequent cell model.

Low Expression of ZFAS1 Inhibits Cell Proliferation

ZFAS1 expression was significantly reduced in the si-ZFAS1 transfected group by small interfering RNA transfection compared to the control group (Figure 2A). CCK8 assay found that low expression of ZFAS1 significantly reduced cell proliferation and this inhibitory effect could persist for more than 72 h (Figure 2B). We also found that cell proliferation was significantly inhibited by colony formation assay. Colony formation assay suggested that cell proliferation was significantly decreased by 60% after inhibiting ZFAS1 (Figure 2C).

Decreasing the expression of ZFAS1 intracellularly significantly increased apoptosis detected by flow cytometry (Figure 2D). Cell cycle analysis showed that cells in the low expression ZFAS1 group had a shortened S phase and prolonged G0/

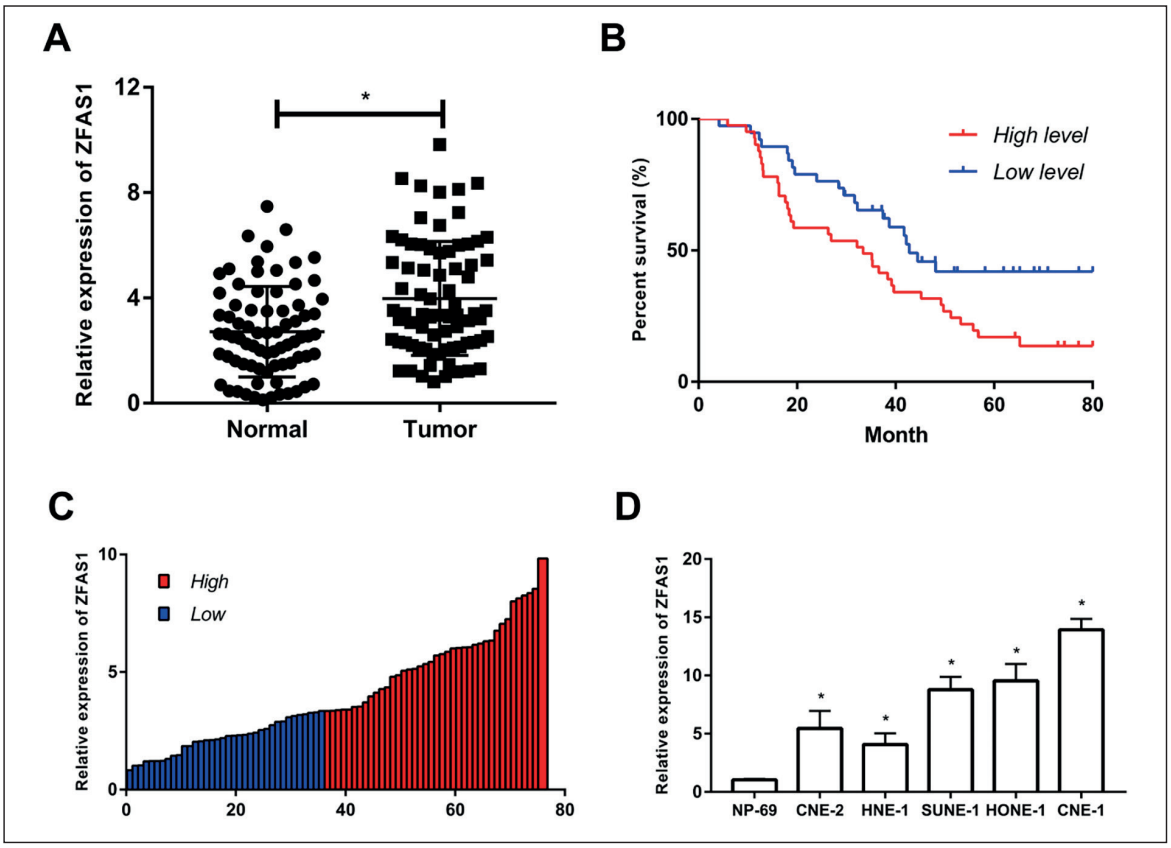

Figure 1. ZFAS1 expression in NPC patients and cells. **A,** ZFAS1 was significantly expressed in NPC patients. **B,** Survival of NPC patients with high expression of ZFAS1 was significantly lower than those with low expression of ZFAS1. **C,** ZFAS1 expression in NPC patients. **D,** ZFAS1 expression in NPC cell lines.

G1 phase (Figure 2E). This suggested that ZFAS1 may participate in tumor development by shortening G0/G1 phase. The above results showed that ZFAS1 may improve the malignant degree of NPC cells by regulating cell proliferation, cycle, and apoptosis.

Table I. Relationship between ZFAS1 and patients' clinical data.

Clinicopathologic features	Number of cases	LncRNAZFAS1 expression		*p*-value
		Low (n = 8)	High (n = 38)	
Age (years)		38	38	0.639
≤ 46	30	1	14	
> 46	46	22	24	
Gender				0.589
Male	58	30	28	
Female	18	8	10	
Tumor size				0.029*
T1-T2	37	25	12	
T3-T4	39	13	26	
N stages				0.001*
N0	34	24	10	
N1-N3	42	14	28	
Metastasis				0.105
Yes	33	13	20	
No	43	25	18	

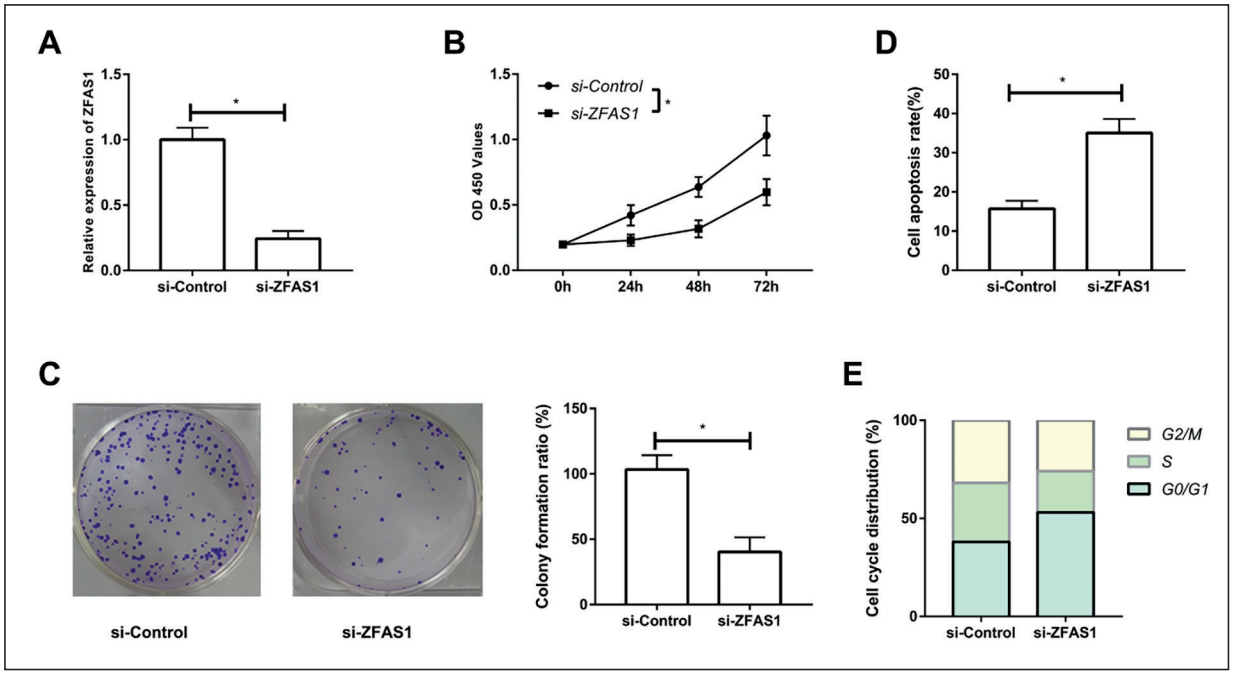

Figure 2. Low expression of ZFAS1 inhibits cell proliferation. **A,** After transfection of si-ZFAS1, ZFAS1 expression decreased significantly. **B,** Low expression of ZFAS1 inhibits cell proliferation. **C,** Low expression of ZFAS1 promotes apoptosis. **D,** Low expression of ZFAS1 blocks cells at GO/G1 phase. **E,** Low expression of ZFAS1 inhibits cell colony.

Low Expression of ZFAS1 Inhibits the Wnt/β-Catenin Pathway

Abnormal activated Wnt/β-catenin pathway has been reported to be involved in the development of NPC[15]. We found that expressions of β-catenin, cyclin D1, and c-myc in Wnt/β-catenin pathway both decreased significantly at the level of protein (Figure 3A) and mRNA (Figure 3B) after reducing the expression of ZFAS1 by small interfering RNA. It was suggested that ZFAS1 was involved in the regulation of Wnt/β-catenin pathway, which promoted the occurrence of NPC.

Discussion

Evidence demonstrated that lncRNAs were the most versatile functional ncRNAs that played a

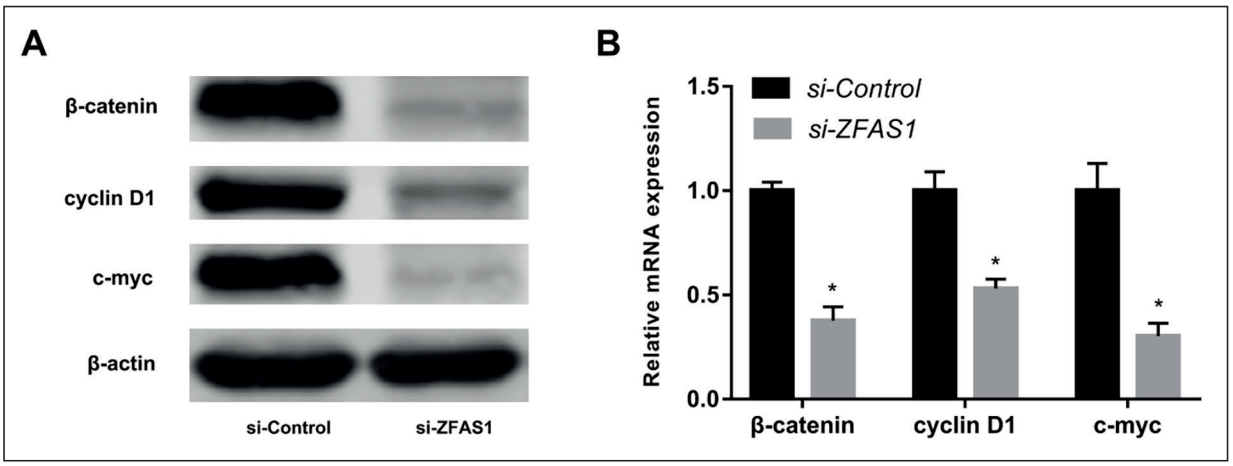

Figure 3. Regulation of Wnt/β-catenin pathway by ZFAS1. Low expression of ZFAS1 significantly reduced the protein **(A)** and mRNA expression **(B)** of β-catenin, cyclin D1 and c-myc.

regulatory and structural role in embryogenesis, stem cell multiplication, allele expression, regulation of protein-coding genes, apoptosis, cycle control, growth, differentiation, and senescence[16]. As lncRNAs were involved in a variety of significant physiological functions, dysfunction of which can have huge effects on homeostasis.

In this study, we aimed to find out the effect of ZFAS1 on the pathogenesis of NPC. After reducing expression of ZFAS1 in NPC cells, functions of lncRNAs through biological function experiments were explored. First of all, cell proliferation is the basis for the growth and development of the living body, tumor cells have the ability to proliferate indefinitely, and their proliferative capacity is also greatly enhanced with the increase of the degree of malignancy[17]. Therefore, alteration of the degree of malignancy could be evaluated by detecting the change of tumor cell activity. In this study, we found that ZFAS1 could promote the tumor cells proliferation *in vitro* and promote the malignant phenotype of NPC cells. In addition, the proliferation of eukaryotic cells is mainly mitosis, and mitosis is a cyclical, that is, the cell cycle, which is divided into two stages of interphase and mitosis, and the interval is classified into three periods, that was, Pre-DNA synthesis (G1), DNA synthesis (S) and DNA synthesis late (G2). Low expression of ZFAS1 in this study significantly blocked cells and GO/G1 phase. Apoptosis is another basic biological phenomenon of cells and influences the growth and development of tissues in maintaining the biological balance of the body as a regulatory role. Our results found that downregulated ZFAS1 could promote cell apoptosis, indicating that ZFAS1 is capable of reducing apoptosis and promoting tumorigenesis in NPC.

The classic Wnt signaling pathway is closely associated with the occurrence and development of tumors[18]. β-catenin is an essential protein in Wnt signaling pathway, which exists in the cell membrane, cytoplasm, and nucleus. When Wnt signaling was abnormally activated, β-catenin protein cannot be degraded by GSK-3β, resulting in the accumulation of β-catenin in the cytoplasm and its migration to the nucleus. β-catenin, therefore, bound to Tcf4 to form a transcriptional complex and initiates the transcriptional regulation of downstream target genes, thus regulating cell growth[19]. It has been demonstrated that the classical Wnt pathway was associated with the development and progression of a variety of malignancies, including glioma, colon cancer, lung cancer, and gastric cancer[20,21]. This study showed that ZFAS1 can participate in the development of NPC by regulating the Wnt/β-catenin pathway.

It is noteworthy that, in addition to neoplasia-infiltrating NPC cells, nasopharyngeal carcinoma biopsy samples are mixed with a larger number of non-tumor cells, including lymphocytes and interstitial cells, which could interfere the experimental results. Therefore, the purification of cancer cells in NPC tissue samples will be able to more accurately analyze the expression of ZFAS1 in NPC, which makes the result more accurate.

Conclusions

We found that ZFAS1 was overexpressed in NPC tissues. Overexpression of ZFAS1 may promote the occurrence of NPC by activating the Wnt/β-catenin pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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