Decreased expression of pseudogene PTENP1 promotes malignant behaviours and is associated with the poor survival of patients with HNSCC

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PTENP1, a pseudogene of PTEN, was previously reported to be a tumour suppressor in some cancer types. However, there was no evidence for the biological function and expression of PTENP1 in head and neck squamous cell carcinoma (HNSCC). Here, we evaluated the function and clinical implications of PTENP1 in HNSCC. Using RT-PCR and quantitative real-time PCR (qRT-PCR), we found that the level of PTENP1 was reduced in HNSCC specimens compared with adjacent tissues. A decrease in the PTENP1 copy number, but not in the PTEN copy number, was frequently observed in tumour cell lines (4 of 5 cell lines) by genomic real-time PCR. Decreased PTENP1 expression was significantly associated with a history of alcohol use (P = 0.034). Univariate and multivariate Cox regression analyses revealed that low expression of PTENP1 correlated with worse overall survival (OS, P = 0.005; HR:0.170; CI:0.049 to 0.590) and disease-free survival (DFS, P = 0.009; HR:0.195; CI:0.057 to 0.664) rates of HNSCC patients. Furthermore, ectopic PTENP1 expression inhibited the proliferation, colony formation and migration of HNSCC cells and the growth of xenograft HNSCC tumours. These results demonstrate that PTENP1 might play an important role in the initiation and progression of HNSCC.

LncRNAs are transcripts of more than 200 nucleotides without protein-coding function. The non-coding RNAs, which are not transcribed from protein-coding genes, constitute a large portion of the mammalian transcriptome. LncRNAs exist in many types of transcripts, including antisense RNA, small nucleolar RNA, enhancer RNA, endogenous RNA, intergenic transcripts and RNA overlapping the exon transcripts. Up to now, LncRNA has been described as a key address code, orchestrating the trafficking of protein complexes, genes, chromosomes and also RNAs to appropriate locations and ensuring that they are subject to proper activation or suppression. LncRNAs were reported to be involved in various biological processes and to exert great influence on disease development, especially cancers. The well-known LncRNA, metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), is highly expressed in early-stage non-small-cell lung carcinoma and could predict the metastasis and prognosis of these patients. Several LncRNAs were also found to be promising prognostic markers for other cancer types such as melanoma, prostate cancer, and kidney cancer. Recently, a landscape of LncRNAs expression has been identified to reveal the LncRNAs that are significantly differentially
expressed between the oral mucosa and oral premalignant lesions. Although a large number of human lncRNAs have been identified, the biological functions of these lncRNAs remain largely unknown, especially in cancers. LncRNA PTENP1 is the pseudogene of the PTEN, which is a tumour suppressor gene (TSG). It is highly homologous to PTEN, sharing 98% sequence identity with the PTEN mRNA sequence. An increasing number of studies have shown that PTENP1 functions as a competing endogenous RNA to suppress tumour progression. However, the expression and biological function of PTENP1 in HNSCC have not yet been elucidated. Here, we evaluated the possible function of PTENP1 and found that it acts as a potential tumour suppressor due to a reduction in the copy number, independent of PTEN, in HNSCCs and can serve as an independent prognostic factor in patients with HNSCC.

Results
The expression pattern and cellular sublocalization of lncRNA PTENP1 in HNSCC specimens and HNSCC cell lines. We first examined the expression of lncRNA PTENP1 in five HNSCC cell lines and oral mucosal epithelial cells by RT-PCR. The results showed a significant difference in PTENP1 expression between normal and tumour tissue specimens. We also examined the cellular location of PTENP1 in HN13 and HN30 cells. U2 was used as a positive control for nuclear RNA, and GAPDH was used as a positive control for cytoplasmic RNA. The Kaplan-Meier survival curve indicated overall survival (OS) and disease-free survival (DFS) by evaluation of the expression levels of PTENP1 in the cohort.
Table 1. Association Between PTENP1 RNA Level and Clinical Parameters. Abbreviations: SD, standard deviation; TNM stage, tumor-node-metastasis stage; ΔCT indicates the difference in the cycle number at which a sample’s fluorescent signal passes a given threshold above baseline (Ct) derived from a specific gene compared with that of GAPDH.

| Characteristics | PTENP1 (ΔCT) |  |  |
|-----------------|--------------|---|---|
|                 | No. | Mean ± SD | P  |
| Age, year       | 57  | 10.69 ± 1.99 | 0.832 |
| ≥60             | 34  | 10.80 ± 2.12 |
| <60             | 23  | 10.20 ± 1.99 |
| Gender          | 57  |             |    |
| Male            | 41  | 10.99 ± 2.05 | 0.183 |
| Female          | 16  | 10.16 ± 1.89 |
| Smoking history | 57  |             |    |
| Smoker          | 19  | 11.26 ± 2.19 | 0.167 |
| Non-smoker      | 39  | 10.47 ± 1.91 |
| Alcohol history | 57  |             |    |
| Drinker         | 16  | 11.63 ± 2.08 | 0.034 |
| Non-drinker     | 41  | 10.38 ± 1.91 |
| Disease site    | 57  |             |    |
| Oral cavity     | 49  | 10.67 ± 2.09 | 0.548 |
| Oropharynx      | 8   | 11.14 ± 1.64 |
| Tumor status    | 57  |             |    |
| Primary         | 50  | 10.70 ± 2.11 | 0.718 |
| Recurrence      | 7   | 11.00 ± 1.28 |
| Tumor size      | 52  |             |    |
| ≥2 cm           | 48  | 10.93 ± 1.92 | 0.123 |
| <2 cm           | 4   | 9.35 ± 2.17  |
| TNM Stage       | 53  |             |    |
| I–II            | 15  | 10.22 ± 2.02 | 0.177 |
| III–IV          | 38  | 11.02 ± 1.89 |
| Tumor grade     | 54  |             |    |
| I–II            | 47  | 10.77 ± 2.05 | 0.591 |
| III             | 7   | 11.20 ± 1.29 |
| Lymph node metastasis | 57 |             |    |
| Negative        | 28  | 10.57 ± 1.96 | 0.545 |
| Positive        | 29  | 10.89 ± 2.10 |

Table 2. Univariate Cox Proportional Hazards Regression Models for Estimating Overall Survival and Disease-Free Survival. Abbreviations: CI, confidence interval; HR, hazard ratio; TNM, tumor-lymph node-metastasis classification.
by RT-PCR and real-time PCR (Fig. 1A and B). Compared with normal oral mucosal epithelial cells, PTENP1 expression was reduced in all five HNSCC cell lines. We then detected the expression level of PTENP1 in 57 HNSCC tissues and 27 adjacent normal tissues (Fig. 1C). Consistent with the results in cell lines, PTENP1 expression was decreased in HNSCCs compared to adjacent normal tissues ($P < 0.01$).

To address the cellular sublocalization of PTENP1, we next examined the distribution of PTENP1 in two randomly selected HNSCC cell lines using two positive control genes: U2 small nuclear RNA, which is mainly found in nuclei and GAPDH mRNA, which is mainly present in the cytoplasm. In both HN13 and HN30 cells, PTENP1 mainly existed in the nucleus (Fig. 1D).

**Decreased PTENP1 expression was associated with a history of alcohol use and a worse clinical outcome.** Our findings showed that low PTENP1 transcript levels were significantly correlated with a history of alcohol use ($P = 0.034$) (Table 1) and a worse OS ($P = 0.005$) or DFS ($P = 0.012$) (Fig. 1E and F). However, there were no significant associations between the PTENP1 level and age, gender, smoking history, disease site, tumour status, tumour size, TNM stage, tumour stage or lymph node metastasis of HNSCCs patients (Table 1).

The univariate COX proportional Hazards regression analysis showed that the PTENP1 level was an independent predictor of the OS ($P = 0.005$; HR:0.170; Cl:0.049 to 0.590) and DFS ($P = 0.009$; HR:0.195; Cl:0.057 to 0.664) in patients with HNSCC (Table 2).

**Positive correlation between PTENP1 and PTEN in HNSCCs.** Because PTEN was reported to be the target gene protected by PTENP1, we also detected the expression of PTEN in tumour cells. We found that the PTEN expression was significantly decreased in all five HNSCCs cell lines as determined by real-time PCR (Fig. 2A). In a Western blot assay, PTEN showed lower expression in HNSCC cell lines compared with normal oral mucosal epithelial cells (Fig. 2B).
Copy number reduction in IncRNA PTENP1 from the genome in HNSCCs. Since genomic PTENP1 and PTEN were previously found to be decreased in terms of the copy number in human melanoma, we also examined the genomic status of these two genes in HNSCC cell lines as reported in the previous study. A genomic qPCR analysis of PTENP1 revealed complete deletion in HN4 cells, partial deletion in the HN6, HN13 and HN30 cell lines, and no deletion in the Cal27 cell line (Fig. 2C). An analysis of PTEN intron 1, intron 3 and exon 9 revealed no significant deletion in the HN4, HN6, HN30 or Cal27 cell lines. However, there was a partial deletion of PTEN intron 1 in the HN13 cells (Fig. 2D). These results suggest that deletion of the genomic PTENP1 is a frequent event in HNSCCs, but deletion of genomic PTEN is not common in HNSCCs.

PTENP1 was not sufficient to completely recover the level of PTEN in HNSCCs. There is high homology between PTEN and PTENP1. To better understand the potential functions of PTENP1, we established an over-expression plasmid for PTENP1. The plasmid was packed into a lentivirus, which was then transfected into the HN13 and HN30 cell lines. Cells carrying the empty vector plasmids were used as mock controls. We examined the expression of PTENP1 in stable cell lines by real-time PCR (Fig. 3A and B). Compared with the wild type control and PCMV mock control, the expression of PTENP1 was notably increased in HNSCCs. We also examined the expression of PTEN in these stable cell lines (Fig. 3C and D). Although both expression of PTENP1 and PTEN were concurrently observed in HNSCC cells with stable PTENP1 expression, the level of PTEN was still insufficient compared with that of the normal epithelial cells, suggesting that PTENP1 was not sufficient to fully recover the PTEN level in HNSCCs.

PTENP1 inhibits the growth and colony formation of HNSCCs. To decipher the influence of PTENP1 on the biological functions in HNSCCs, we evaluated the proliferation of tumour cells by the MTT assay. Compared with controls, there was a moderate decrease in cell growth on day 3 when PTENP1 was expressed in HN13 cells (Fig. 4A). In HN30 cells, PTENP1 expression led to a mild decrease in cell growth on day 3 (Fig. 4B).
In accordance with the findings of the proliferation assay, the colony numbers of PTENP1-expressing cells were remarkably decreased (Fig. 4C). Meanwhile, the sizes of the colonies of the PTENP1-expressing cells were smaller compared with the large and visible clones in control cultures. The colony formation rate of PTENP1 cells decreased sharply by approximately 33% and 50%, respectively, in HN13 and HN30 cells (Fig. 4D and E).

PTENP1 suppresses the invasion and migration of HNSCCs. To assess whether the invasion of HNSCC cells is influenced by PTENP1, the transwell assay was performed. The number of migratory cells was significantly reduced in the PTENP1-expressing HN13 and HN30 cells (Fig. 5A). The metastasis rate decreased to approximately 26% and 42%, respectively, in the HN13 and HN30 cells (Fig. 5B and C). To evaluate whether the migratory ability of HNSCC cells was influenced by PTENP1, the scratch wound assay was also performed. The HN13 control cells at the wound edge migrated into the wound space quickly and merged together at 48 h (Fig. 5D). When PTENP1 was over-expressed, the cells migrated into only about half of the wound space without merging. The HN30 cells at the wound edge crept across the wound space and merged together at 24 h (Fig. 5E). However, HN30 cells with PTENP1 migrated slowly without merging.

PTENP1 overexpression suppressed tumorigenicity in vivo. To confirm the tumorigenicity of PTENP1 in vivo, we established a xenograft model of nude mice using PTENP1-enriched HN13 cells and mock control cells. The control cells gradually grew into visible lumps on the flank of mice (Fig. 6A). However animals carrying cells presented expression of the PTENP1 did not show any visible lump (Fig. 6B). The tumorigenic ability was significantly reduced by PTENP1 (Fig. 6C). These data further indicated that PTENP1 could modulate HNSCCs progression in vivo.

Discussion
An increasing number of studies have suggested that dysregulation of lncRNAs in HNSCC. Maternally expressed gene 3 (MEG3) was identified as a lncRNA tumour suppressor in a series of cancer types, including tongue squamous cell carcinoma (TSCC) caused by genetic and epigenetic disorders. LncRNA UCA1 was found to be overexpressed in TSCCs and to promote tumour metastasis. LncRNA HOTAIR and MALAT-1 were confirmed to be detectable in the saliva of oral squamous cell carcinoma (OSCC) patients, suggesting that lncRNAs may be potential markers for diagnosing cancer.

Recently, lncRNA PTENP1 expression was found to be decreased in some cancer types, including Hodgkin's lymphoma, acute myelocytic leukaemia and nasopharyngeal carcinoma. PTENP1 3' UTR over-expression
resulted in the growth inhibition of cancer cells, suggesting that PTENP1 plays a tumour suppressive role in prostate cancers. In clear-cell renal cell carcinoma (ccRCC), PTENP1 could suppress tumour growth and migration. In endometrial cancer, patients with PTENP1-positive tumours exhibited a trend towards lower disease recurrence. However, the expression patterns and biological functions of PTENP1 in HNSCCs have not yet been uncovered. In the present study, we found that ectopic expression of PTENP1 leads to inhibition of the tumour growth, colony formation, migration and xenograft tumour growth of HNSCC.

In previous studies, the mechanisms underlying the reduced PTENP1 expression in cancers were mainly divided into two categories: DNA methylation of the PTENP1 promoter and copy number alterations of PTENP1 in the genome. PTENP1 shares 91% sequence identity with a 921 bp region of the PTEN CpG island. The hypermethylation of PTENP1 has been detected in lymphomas, colorectal cancers, ccRCC and non-small-cell lung cancers (NSCLCs). In sporadic colon cancer samples, copy number reduction was detectable at the PTENP1 locus (9p13), independent of nearby locus loss. In addition, partial or complete deletions of the PTENP1 copy number were also detected in 14.3% of melanoma cell lines and 20.9% of melanoma tissues. In this study, we confirmed that there was copy number reduction in the PTENP1 locus in 80% of HNSCCs cell lines (one line showed complete deletion, three showed partial deletion and one showed no deletion), suggesting that copy number alterations were one of the main factors affecting the expression of PTENP1 (Fig. 2).

**Figure 5. PTENP1 ectopic expression influences on tumour invasion migration and migration.** (A) The transwell assay for evaluation of the invasion of HN13 and HN30 cells, respectively. (B,C) The invasion rate based on the transwell assay. The value of the control was normalized to 100%. The data were presented as the means ± SD. *p < 0.05. (D,E) The scratch wound assay for evaluation of the migration of HN13 and HN30 cells, respectively.
PTENP1 was named as a pseudogene of PTEN, which remains the only definite target gene of PTENP1. PTEN is a tumour suppressor gene that is dysfunctional in many hereditary and sporadic cancers. Its expression level was positively correlative with that of PTENP1. Within the high homology region of the PTEN 3' UTR, PTENP1

**Figure 6.** Exogenous expression of PTENP1 inhibited the growth of xenograft tumour of HN13 cell lines. (A,B) Lumps of mice carrying Mock cells and PTENP1 expressing cells in xenograft tumour model, respectively. (C) Growth curve based on the xenograft tumour assay. The tumour size was calculated using the formula length * width * width / 2. The data were presented as the means ± SD.
had perfect conserved seed matches for the PTEN-targeting microRNA families, including miR-17, miR-21, miR-
214, miR-19 and miR-26 families. PTENP1 bound PTEN-targeting microRNAs, which protected PTEN mRNA,
preventing its degradation by microRNAs. PTENP1 acted as a decoy for miR-19b and miR-20a in prostate cancer
cells and for miR-21 in ccRCC cells. In the present study, a positive correlation between PTENP1 expression
and the PTEN mRNA level was found in the HNSCC cells as described above. However, the activation induced by
PTENP1 was not potent enough to recover PTEN to a normal level. As the deletion of PTEN was concomitant
with that of PTENP1 in some cancers, we also examined the genomic PTEN status in HNSCCs. There was only one
partial deletion at intron 1 of PTEN in the HN13 cell line. Based on these data, we hypothesize that: 1) PTENP1
is not a potent activator of PTEN in HNSCCs. As a decoy, its function of protecting PTEN from microRNAs is
limited. 2) There are other targets of PTENP1 in HNSCCs. Of note, even in the absence of PTEN, PTENP1 still
exhibited a tumour suppressive function. Some studies attribute this activity to PTENP1 acting as a decoy for other
miRNA-targeting tumour suppressors. We believe that PTENP1 is not restricted to serving only a decoy function.
As an indispensable partner of epigenetics, the lncRNA can work through multiple mechanisms: establishing gene
imprints, orchestrating chromosome conformation, recruiting factors to modulate DNA and histone modification.
The mechanisms of action of PTENP1 in cancers independent of PTEN still warrant further investigation.

Conclusions
In summary, the expression of PTENP1 was decreased in both HNSCC cell lines and clinical HNSCC specimens
compared with adjacent tissues due to a reduction in the PTENP1 copy number. Decreased PTENP1 expression
was significantly associated with a worse OS and DFS in HNSCC patients. Overexpression of PTENP1 signif-
ically inhibited the proliferation, colony formation and migration of HNSCC cells and xenograft tumour growth,
suggesting that that PTENP1 may be a novel therapeutic and prognostic target for HNSCC.

Methods
Cell culture. The human HNSCC cell lines (WSU-HN4, HN6, HN13, HN30 and Cal27) were cultured in
Dulbecco’s modified Eagle medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inac-
tivated FBS (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO2 atmosphere. Normal oral mucosal
epithelial cells were harvested and cultured as described in a previous study.

Clinical samples. Samples were collected from a cohort of 57 patients who were diagnosed with head and
neck squamous cell carcinoma between June 2008 and May 2010. All samples were obtained from the Department
of Oral Maxillofacial-Head and Neck Oncology, School of Stomatology, Shanghai Jiao Tong University School
of Medicine (Shanghai, China) and quickly frozen in liquid nitrogen upon resection until total RNA was extracted.
The clinicopathological characteristics of the study cohort are summarized in Table 1. All of the patients
provided written informed consent in accordance with the institutional guidelines and this study was approved
by Shanghai Ninth People’s Hospital Ethical Committee (No. 2016-172-T121). All methods were performed in
accordance with the relevant guidelines and regulations.

RNA extraction, DNA preparation, reverse transcription, RT-PCR, qRT-PCR and genomic qRT-PCR.
Total RNA was extracted from freshly frozen samples and cell lines using the TRIzol Reagent (Invitrogen,
Carlsbad, CA, USA), and total RNA was reverse-transcribed with the PrimeScript RT-PCR Kit (TaKaRaBio, Otsu,
Japan). Genomic DNA was extracted using a TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China).
Cytoplasmic and nuclear RNA was extracted using a Fisher BioReagents SurePrep Nuclear or Cytoplasmic RNA
Purification Kit (Thermo Fisher, Waltham, MA, USA). The RT-PCR was performed using Premix Ex Taq reagent
(TakaraBio, Otsu, Japan). The qRT-PCR and genomic qRT-PCR were performed using SYBR Select Master Mix
(Applied Biosystems, Irvine, CA, USA) and an ABI 7500 real-time PCR system (Applied Biosystems, Irvine,
CA, USA). The primer sequences used were as follows: forward 5'-GGGCTCAAATATGGGCTAGATG-3' and
and reverse 5'-TCTAAGAAACAACTAAGCCAAAGTC-3' for PTENP1; forward 5'-CTTA CAGTTGGGCCCTGTACCATTCC-3'
and reverse 5'-TTTGTATGCTGGGCAATACTCACTC-3' for PTEN; forward 5'-TCAAGTTTGAGTACAAACATGTGG-3'
and reverse 5'-TCACATACCTATACCTGCTGCC-3' for PTENP1; forward 5'-AACTGCAAGAATTCCTGGAGATGC-3'
and reverse 5'-TTAT GGCTCTAATATGGGCTAGTG-3' for intron 1 of PTEN; forward 5'-AGGTGTTGAGTATGATTGGGCAATCC-3'
and reverse 5'-GTGAAACCCAATTTATGTAGCGTAT-3' for UBE2E1; forward 5'-AGGTCGG GTGTAAGGAGATTGG-3'
and reverse 5'-TGAGACCATATGATGCTTCTTA-3' for GAPDH; and forward 5'-TTTGGGTCGTTGCTCTATCGGAGG-3'
for U2 nuclear RNA. The 2−ΔΔCT equation was used to calculate the relative expression levels.

Western blot analysis. Cells were rinsed twice with PBS and incubated with RIPA lysis buffer (Millipore,
Billerica, MA, USA) on ice for 30 min. Cell extracts were harvested and centrifuged at 13,000 g for 30 min at
4 °C. Then, the lysates were quantified and denatured. Protein samples (10 μg) were separated by sodium dode-
cyl sulfate–polyacrylamide gel electrophoresis in 10% (wt/vol) polyacrylamide gels and transferred to polyvi-
nylidene fluoride membranes. After blocking, the membrane was incubated with 0.15 μg/ml PTEN antibody
(Abcam, Cambridge, MA, USA) overnight at 4 °C or with a β-actin antibody for 1 h (Sigma-Aldrich, St. Louis,
MO, USA). The membrane was then incubated with a secondary antibody conjugated to a fluorescent tag
(Invitrogen. Carlsbad, CA, USA) overnight. The band signals were visualized by the Odyssey Infrared Imagining
System (LI-COR) at an 800 channel wavelength.

Plasmid construction. The 3′ UTR of PTENP1 was cloned using KOD-Plus-Neo DNA polymerase
(TOYOBO, Osaka, Japan) from the genomic DNA of normal oral mucosal epithelial cells. The primers used
for cloning were as follows: forward 5′-CGCCTACTACGGGATTCGAGGGCTGCTTTACATCGGAGG-3′ with
a BamHI site and reverse 5′-ACTACTACGGATCCCTCGTCGATGGTACGTTTCC-3′ with an EcoRI site. Then, the 3′ UTR of PTENP1 was inserted into the MCS of the pCDH-CMV-MCS-EF1-Puro lentivirus vector (eukaryotic expression plasmid, PCMV for short) to generate the PCMV-PTENP1 plasmid (System Biosciences, Palo Alto, CA, USA), and the PCMV with no insertion was called PCMV-Mock used as a control.

**Lentivirus package.** When 293 T cells had grown to 70% confluence on a 10 cm disc, 30 μl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) incubated with Opti-MEM I reduced serum medium (Gibco, Carlsbad, CA, USA) were added, together with 3 μg PCMV-PTENP1 plasmid or PCMV-Mock plasmid, 3 μg pMD2.G plasmid and 6.0 μg PsPax plasmid, and the cells were incubated for 6h. The medium was then replaced with 10 ml of fresh medium. The supernatants of cells were collected at 48h and 72h. The resulting viruses were filtered through a 0.45-μm cellulose acetate filter, concentrated with Amicon Ultra-15 Centrifugal Filter Units (Millipore, Billerica, MA, USA) and then stored in several aliquots in a −80 °C freezer. HN13 and HN30 cells were seeded at 1.0 × 10^5 cells per well of six-well plates. One day later, virus (PCMV-PTENP1 or PCMV-Mock) was added into the medium containing 10 ng/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). After 48h, the medium was replaced with fresh medium containing 4 μg/ml puromycin (Invivogen, San Diego, CA, USA), and cells were incubated for at least 2 weeks. Colonies were selected and expanded for further analyses. Stable cell lines with PTENP1 expression were termed “PTENP1 cells” and cells with PCMV-Mock were termed “Mock cells”.

**Proliferation assay.** The MTT assay was used to detect the proliferative rates of the indicated cell lines. A total of 2,000 PTENP1 or Mock cells in 100 μl of medium were seeded per well in 96-well plates. At 0h, 24h, 48h and 72h, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The cells were lysed with 100 μl of dimethylsulfoxide 4h later with gentle shaking. The optical density was detected with a microplate reader at 490 nm. The absorbance values were normalized to the values of the 0-hour sample. The normalized value was set as the growth index.

**Colony formation assay.** Five hundred PTENP1 or Mock tumour cells were seeded per well in 6-well plates and cultured in complete medium for 10 days. Colonies were then stained with 0.25% crystal violet and colonies of more than 50 cells were counted under a dissecting microscope. The data are shown as the means (±SD) from at least three independent experiments.

**Transwell assay.** Ten thousand PTENP1 or Mock tumour cells were suspended in 400 μl of the appropriate medium supplemented with 2% FBS and were seeded into the upper compartments of a 24-well transwell system with 8-μm pore size polycarbonate filters (Millipore, Billerica, MA, USA). The lower compartments contained 900 μl of medium with 10% FBS. On the second day of incubation, the upper transwells were stained with 0.25% crystal violet. The inner sides of the transwells were scrubbed away and the outer sides were photographed. The crystal violet was washed with 100 μl of 33% acetic acid. The absorbance values of the liquid were detected with a microplate reader at 630 nm.

**Wound-healing assay.** PTENP1 or Mock cells were seeded in 6-well plates with complete medium until they reached full confluence. A wound was then made by using a 200 μl pipette tip to scratch along a line. The cells were rinsed 3 times with PBS to remove suspended cells. Then, the cells were cultured in medium without FBS and the width of the wound was photographed at 0h, 24h and 48h.

**In vivo xenograft model.** All animal experiments were approved by the Shanghai Jiao Tong University School of Medicine Animal Ethics Committee and this study were conducted following the Shanghai Jiao Tong University School of Medicine animal policy. Stable PTENP1 or Mock cells were harvested and washed twice with PBS. The cells (5 × 10^6) were injected subcutaneously into the right flanks of 4-week-old male nude mice in 100 μl of PBS (six mice for each group). The growth of the tumours was observed once a week, and the length and the width of the tumours were measured until four weeks after injection. After euthanasia, the images of mice were stored and the growth curve of tumours was calculated using the formula length*width*width/2.

**Statistical analysis.** All of the experiments in our study were performed in triplicate at least. The log-rank test was used to assess the univariate associations between the PTENP1 transcript level and the Overall survival (OS) and Disease-free survival (DFS). The hazard ratios with corresponding 95% confidence intervals (CIs) and P values are reported. For the real-time PCR analysis, the associations between the PTENP1 mRNA levels and patient characteristics were evaluated using the Kruskal-Wallis test. All tests were two-sided, and P values < 0.05 were considered to be statistically significantly. All analyses were conducted using the SPSS software program (standard version 18.0; SPSS Inc., Chicago, Ill).

**References**
1. Chen, W. et al. Cancer statistics in China, 2015, CA: a cancer journal for clinicians 66, 115–132 (2016).
2. Cabanillas, R. et al. P53 expression in squamous cell carcinomas of the supraglottic larynx and its lymph node metastases: new results for an old question. Cancer 109, 1791–1798 (2007).
3. Rinn, J. L. & Chang, H. Y. Genome regulation by long noncoding RNAs. Nature reviews genetics 7, 447, 466–473 (2005).
4. Consortium, E. P. et al. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447, 799–816 (2007).
5. Pasman, E. et al. Characterization of a germline deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of ANRIL, an antisense noncoding RNA whose expression coclusters with ARF. Cancer research 67, 3963–3969 (2007).
PTENP1 pseudogene expression was decreased in oral squamous cell carcinoma tissue compared to normal mucosa. This suppression might contribute to the biological and clinical characteristics of oral squamous cell carcinoma, and could help identify possible therapeutic targets.

### Author Contributions
W.C. and C.P.Z. conceived the study; J.N.L. and Y.X. performed the experiments and drafted the manuscript; W.T.C. discussed and revised the manuscript. All authors read and approved the final manuscript.

### Additional Information

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