Smoking-mediated nicotinic acetylcholine receptors (nAChRs) for predicting outcomes for head and neck squamous cell carcinomas

Yujie Shen, Qiang Huang, Mengyou Ji, Chi-Yao Hsueh* and Liang Zhou*

Abstract

Background: As a human tumor disease, head and neck squamous cell carcinoma (HNSCC) is associated with a high mortality rate worldwide. Nicotinic acetylcholine receptors (nAChRs) are transmembrane receptor proteins and exert their biological effects following activation by nicotine. We aimed to construct a prognostic signature based on the expression of nAChRs among smokers with HNSCC.

Methods: The transcriptome profile of nAChRs was obtained from The Cancer Genome Atlas (TCGA). Following the integration of survival information, univariate Cox regression and least absolute shrinkage and selection operator (LASSO) analyses were performed to screen the prognosis-related nAChRs and construct a prognostic signature. Kaplan–Meier (KM), receiver operating characteristic (ROC), principal component analysis (PCA), and independent prognostic analysis were utilized to verify the predictive power of the nAChR-associated prognostic signature. The expression of α5 nAChR in clinical samples was verified by quantitative reverse transcriptase PCR.

Results: Subunits α2, α5, α9, and β4 were related to the prognosis. The prognostic signature comprised the expression of subunits α5, α9, and β4. The nAChR-associated signature showed high sensitivity and specificity for prognostic prediction and was an independent factor for overall survival. Based on the clinical variables and expression of nAChRs, a nomogram was constructed for predicting the outcomes of HNSCC patients who were smokers in the clinical settings. In clinical specimens, α5 nAChR showed high expression in HNSCC tissues, especially among smokers.

Conclusions: The nAChR-associated signature constructed in this study may provide a better system for the classification of HNSCC patients and facilitate personalized treatment according to their smoking habits.

Keywords: Smoking, nAChRs, HNSCC, Prognosis, Outcome

Background

Head and neck squamous cell carcinoma (HNSCC) frequently originates in the oral cavity, larynx, or pharynx, and is the most common histopathological type of head and neck cancer [1]. Unhealthy lifestyles, including smoking and alcohol consumption, are major risk factors for HNSCC, especially cancers of the oral cavity and larynx [1]. Oropharyngeal cancer is closely associated with human papillomavirus (HPV) infection [2]. Accumulating evidence links HNSCC to alcohol consumption and HPV infection; however, smoking remains the most prominent risk factor for head and neck cancer, accounting for 70% of the cases of HNSCC [3–5]. Surgery, radiotherapy, and cisplatin-based chemotherapy are common treatment strategies for HNSCC [6]. Despite the continuous improvement in the treatment strategies for HNSCC, the incidence and mortality rate increase annually [7]. Thus, there is a need to construct an effective signature

*Correspondence: hsuehchiyao@gmail.com; liang.zhou@fdeent.org
Department of Otorhinolaryngology-Head and Neck Surgery, Eye, Ear, Nose, and Throat Hospital, Fudan University, Shanghai, China
to assist in early diagnosis and identify high-risk HNSCC patients with poor outcomes, especially among smokers.

Nicotinic acetylcholine receptors (nAChRs) are transmembrane receptor proteins, important for neuronal signaling relying on the neurotransmitter, acetylcholine [8]. Tobacco burning releases nicotine which activates nAChRs and increases their permeability to calcium and sodium ions [9]. To date, 16 human nAChRs including subunits α1-α7, α9, α10, β1-β4, γ, δ, and ε have been discovered in muscles and neural tissues [10]. Subunits α1, β1, γ, δ, and ε show prominent expression in muscle while the expression of α2-α7, α9, α10, and β2-β4 is high in other non-neuronal tissues [11]. The impact of smoking on human body has been elucidated given the role of nAChRs.

Despite the excellent progress in our understanding of smoking-associated diseases over the years, the relationship between smoking-mediated nAChRs and HNSCC remains largely unclear. In the present study, we integrated the transcript profiles of nAChRs and constructed a novel nAChR-associated signature which was found to have high predictive power amongst HNSCC patients who were smokers.

Materials and methods

Acquisition and differential analysis of nAChR expression profile

To investigate the prognosis of the smoking population among HNSCC patients, the mRNA expression profiles of nAChRs for 410 HNSCC samples who were smokers, including 378 tumor and 32 adjacent normal tissues, were extracted from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov) [12] for differential expression analysis. The clinical information on the smoking population of HNSCC patients is provided in Supplementary Table 1. The clinical information of the 32 adjacent normal tissues is presented in Supplementary Table 2. The Wilcoxon Rank-Sum Test was used to assess differential nAChR expression between tumor and normal tissues, wherein significance was defined as *p < 0.05, **p < 0.01, and ***p < 0.001.

Protein–protein interactions and pathway enrichment analysis

To examine the criticality of nAChRs in protein interactions, we imported the nAChRs into the STRING database (https://string-db.org/) [13] and visualized the network on Cytoscape (Version 3.7.1). In the network, the size of the node represented the degree value while the thickness of the edge represented the confidence of the experimental evidence. Functional analysis was performed to review the biological processes of nAChRs using the Kyoto Encyclopedia of Genes and Genomes (KEGG) [14] algorithm.

Construction of a prognostic signature based on nAChR expression

Univariate Cox regression analysis was performed to screen the prognosis-related nAChRs among the smoking population in the HNSCC cohort. To avoid overfitting, least absolute shrinkage and selection operator (LASSO) analysis was performed, and a novel prognostic signature was constructed using the following formula: risk score = nAChR (A) expression * coef (A) + nAChR (B) expression * coef (B) + nAChR (i) expression * coef (i) [15]. Based on the expression of nAChRs, the risk of each of the patients was scored and they were divided into lower- and high-risk groups according to the median value. Kaplan–Meier (KM) method, receiver operating characteristic (ROC), and principal component analysis (PCA) were performed to verify the predictive power of the above-described prognostic signature.

Immune infiltration and drug sensitivity analysis

To investigate the relationship between nAChR-associated signature and immunity, the CIBERSORT algorithm [16] was used to calculate the abundance of 22 immune cell types between the risk groups. We also performed a single-sample gene set enrichment analysis (ssGSEA) to assess the immune activities between the risk groups [17]. The transcript expression profile of each patient was uploaded to the Tumor Immune Dysfunction and Exclusion (TIDE) website (http://tide.dfci.harvard.edu/) and TIDE scores were obtained to predict the immunotherapeutic responses of these patients. This set of scores was compared between low- and high-risk groups. Further, we screened the effective chemotherapeutic drugs targeting the smoking population in the HNSCC cohort using the Genomics of Drug Sensitivity in Cancer (GDSC) database (https://www.cancerrxgene.org/) [18].

Prognostic analysis for independence and construction of a nomogram

To determine the independent prognostic factors for the smoking population in the HNSCC cohort, a multivariate analysis was performed, considering all the significant prognostic factors obtained from the univariate analysis. Based on the nAChR expression and other clinical variables, a nomogram was constructed using the “rms” package in R software [19], to guide clinical decision-making for the smoking population in the HNSCC cohort. To assess the efficacy of the constructed nomogram, time-dependent calibration curves and ROC curves were plotted and analyzed.
Tissue specimen collection for quantitative reverse transcriptase PCR (qRT-PCR) analysis

In this study, 61 paired samples of HNSCC were collected from Eye and ENT Hospital, Fudan University between October 2020 and July 2021. These were stored at -80 °C until subsequent qRT-PCR analysis. The Ethical Committees of Eye and ENT Hospital, Fudan University approved the study design (2018036) and informed consent was signed by HNSCC patients. The clinical information of 61 paired samples is provided in Supplementary Table 3. We performed qRT-PCR according to the protocol described in our previous study [20]. We aimed to verify the expression of nAChRs in a different independent cohort and the sequences of all primers used in this study are listed in Supplementary Table 4.

Public databases

To validate the results of TCGA, we performed a survival analysis based on the expression of α5, α9, and β4 nAChRs in all HNSCC patients using the Kmplot database [21]. In addition, methylated sites of α5, α9, and β4 nAChRs in all HNSCC patients were investigated using the MethSurv database [22]. Also, we performed immune infiltration analysis based on the expression of α5, α9, and β4 nAChRs in HNSCC patients using the Tumor IMmune Estimation Resource (TIMER) database [23].

Statistical analysis

All statistical analyses were conducted using the R software (version: x 64 3.6.1) and GraphPad Prism 7 and a p-value < 0.05 was considered statistically significant.

Results

Expression of nAChRs and enriched pathways in the smoking population of the HNSCC cohort

Of all the nAChRs, α5, α6, α9, β2, β3, and β4 nAChRs were significantly differentially expressed in the smoking population of the HNSCC cohort (Fig. 1A). In addition, the expression profile of β3 nAChR is shown in Supplementary Fig. 1A. As shown in Fig. 1B, nAChRs interact closely with each other evidenced by experimental data, and were significantly overrepresented in neuroactive pathways (Fig. 1C). We analyzed the expression of nAChRs in smokers and non-smokers using univariate Cox regression analysis (Fig. 1D). The LASSO model for nAChRs (Fig. 1E) selected the same three nAChRs (α5, α9, and β4) as TCGA, and the LASSO deviance profile selected the same log lambda value as the minimum cross-validation error point (Fig. 1F).
ligand-receptor interaction, cholinergic synapse, chemical carcinogenesis-receptor activation, and nicotine addiction (Fig. 1C). These results indicated that nAChRs were strongly related to the carcinogenic process and may be potential novel biomarkers for the smoking population in the HNSCC cohort.

**nAChR-based prognostic signature for predicting outcomes in the smoking population of the HNSCC cohort**

Four nAChRs, namely α2, α5, α9, and β4, were screened from a univariate Cox regression analysis based on the significance criterion of \( p < 0.05 \) (Fig. 1D). To avoid overfitting, LASSO regression was performed, and three nAChRs were selected for constructing a prognostic signature (Fig. 1E-F) using the following formula:

\[
\text{risk score} = \alpha_5 \text{nAChR expression} \times 0.077 + \alpha_9 \text{nAChR expression} \times 0.035 + \beta_4 \text{nAChR expression} \times 0.029.
\]

The patients were classified according to their risk scores calculated based on nAChR expression into low- and high-risk groups. Patients in the high-risk group showed poorer overall survival (OS) and progression-free survival (PFS) as compared to those in the low-risk group (Fig. 2A-B). The values of area under the curve (AUC) for nAChR-based signature for three-, five-, and ten-year OS were 0.600, 0.646, and 0.687, respectively (Fig. 2C). The signature showed a great clustering ability according to PCA (Fig. 2D). High levels of expression of the three nAChRs and high mortality were observed in the high-risk group as compared to the low-risk group (Fig. 2E-G).

**Relationship between expression of the prognostic signature and clinicopathological factors**

We evaluated the expression of the signature between clinical groups. In the smoking population of the HNSCC cohort, high signature expression was observed in patients aged less than or equal to 65 years \( (p = 0.41, \text{Fig. 3A}) \) and males \( (p = 0.20, \text{Fig. 3B}) \). The expression increased significantly with increasing grade (G1-G2: \( p = 0.027 \); G1-G3: \( p = 0.0019 \); G2-G3: \( p = 0.12, \text{Fig. 3C} \)) and 7th edition AJCC TNM pathology stage (stage I-stage II: \( p = 0.1 \); stage I-stage III: \( p = 0.079 \); stage I-stage IV: \( p = 0.021 \); stage II-stage III: \( p = 0.99 \); stage II-stage IV: \( p = 0.41 \); stage III-stage IV: \( p = 0.36, \text{Fig. 3D} \)), indicating the potential cancer-promoting effects of the signature in
HNSCC. Consistent with our results, the expression of nAChRs was high in current smokers as compared to the reformed smokers \((p = 0.20, \text{Fig. 3E})\). However, no significant relationship between prognostic signature expression and smoking package years was observed (Fig. 3H). Similar findings were observed between alcohol drinkers and non-drinkers \((p = 0.47, \text{Fig. 3F})\). Interestingly, the expression of nAChRs was significantly higher in HPV-positive smokers with HNSCC, especially with other HPV types, relative to the HPV-negative smokers (Fig. 3G). We also performed a Kaplan–Meier survival analysis according to stratification by HPV status using the prognostic signature (Supplementary Fig. 1B-C).

**Immune contexts between groups**

The impact of smoking use on the immune microenvironment is well recognized. As part of our analyses, two algorithms were used to characterize the immune microenvironment between low- and high-risk groups. Through the CIBERSORT algorithm, macrophage M0 was found to be highly enriched in the high-risk group \((p < 0.05, \text{Fig. 4A})\). Based on the ssGSEA, the high-risk group was found to show weak immunoreactivity (Fig. 4B), which could account for the poor outcomes of the patients in this group. Based on low TIDE scores, we speculated that the patients in the high-risk group may be more sensitive to immunotherapy (Fig. 4C). For immunotherapy, using the pRRophetic algorithm, nine potential drugs specifically targeting the smoking population in the HNSCC cohort were screened (Fig. 4D-L).

**The nAChR-based prognostic signature is an independent prognostic factor for the smoking population in the HNSCC cohort**

As shown in Fig. 5A, TNM pathology stage, smoking status, and risk score according to the prognostic signature were robustly related to the prognosis of the smoking population in the HNSCC cohort. Further, TNM pathology stage and risk score were independent prognostic factors for OS (Fig. 5B). Time-dependent AUC values of the three prognostic variables, namely TNM stage, smoking status, and risk score, are shown in Fig. 5C-E and risk score showed better sensitivity and specificity for prognostic prediction as compared to TNM pathology stage and smoking status.

**A nomogram based on nAChR expression for prognostic prediction for the smoking population in the HNSCC cohort**

According to the clinical variables and nAChR expression, we constructed a nomogram for predicting outcomes in the smoking population of the HNSCC cohort in clinical settings (Fig. 6A). For the validation of this nomogram, calibration curves displayed extremely high concordance between the actual observation and the nomogram-based prediction (Fig. 6B),
and the time-dependent ROC curves indicated that the nomogram showed higher sensitivity and specificity for predicting one-, three-, five-, and seven-year OS as compared to other prognostic variables (Fig. 6C-F). To demonstrate the additional contribution to predictive accuracy using the nAChR-based signature, we plotted the time-dependent ROC curves without calculating signature expression (Supplementary Fig. 1D-G). For three-, five- and seven-year OS, the AUC values of the prognostic nomogram based on nAChR-associated signature were higher than the null model without calculating the signature expression.
All the above results indicated the critical role of nAChRs in HNSCC patients, especially among smokers. Following qRT-PCR analysis, we selected and tested the mRNA expression of α5 nAChR. Among the 61 paired HNSCC samples, α5 nAChR was highly expressed in HNSCC tissues as compared to the adjacent normal tissues (p = 0.0182, Fig. 6G). When stratified according to the smoking status, the same trend was observed in the smoking population (p = 0.029) and there was no difference in α5 nAChR expression between tumor and adjacent normal tissues of non-smoking patients (Fig. 6H).

**Discussion**

HNSCC is the sixth most common cancer worldwide, and nearly half a million deaths were reported in 2018 due to HNSCC [7]. It is mainly caused by exposure to tobacco through smoking, excessive alcohol consumption, and HPV infection. Despite the rising number of patients with HPV infection, smoking and alcohol abuse remain the most prominent risk factors for HNSCC, especially those originating from the oral cavity and larynx.

The link between smoking and deteriorating health has been confirmed. Annually, millions of premature deaths are caused globally due to smoking [24]. Tobacco comprises several carcinogens that can lead to cancer. Smoking leads to the generation of DNA adducts and mutations in multiple genes [25]. Furthermore, tobacco smoking leads to exposure to carcinogens such as nicotine-derived nitrosamine ketone (NNK) and nicotine, which exert their biological functions by binding to nAChRs [26]. Based on the underlying mechanisms, numerous studies have focused on the role of nAChRs in tumorigenesis and cancer development. In lung cancer, nicotine promotes migration and stemness of non-small cell lung adenocarcinoma (NSCLC) cells via the α7 nAChR/Yap1/E2F1 axis [27]. α5 nAChR contributes to epithelial-mesenchymal transition (EMT) and metastasis of NSCLC cells by mediating the Jab1/Csn5 axis [28]. In head and neck cancer, nicotine causes the phosphorylation of EGFR by regulating α7 nAChR, leading to lymph node metastasis and cetuximab resistance in HNSCC cells [29]. nAChRs may function as oncogenes.
and mediate oncogenic activities in cancer cells. Consistent with previous studies, nAChRs were highly expressed in head and neck tumors and enriched in neuroactive ligand-receptor interaction, cholinergic synapse, chemical carcinogenesis-receptor activation, and nicotine addiction pathways. The expression of the nAChR-based signature increased significantly with increasing grade and TNM stage, suggesting that it could mediate oncogenic activities in HNSCC.

Studies on smoking have mainly focused on lung-related diseases, while related reports on head and neck cancer are scarce. In this present study, for the first time, the expression of nAChR transcripts rather than a single nAChR was used for predicting the outcomes in the smoking population of the HNSCC cohort through bioinformatic analyses and molecular biology techniques. Differential expressed genes may have potential biological function and significance in specific diseases. As is shown in Fig. 7, we firstly identified differentially expressed nAChRs (α5, α6, α9, β2, β3 and β4) via Wilcoxon Rank-Sum Test. Meanwhile, genes associated with prognosis may have the potential to be effective biomarkers for a certain disease. Therefore, we further screened out prognosis-related nAChRs (α2, α5, α9, and β4) via univariate Cox regression analysis. Finally, least absolute shrinkage and selection operator (LASSO) analyses was performed to avoid overfitting in model calculations, α2 nAChR was filtered out and the other three nAChRs (α5, α9, and β4) were included in the prognostic signature which showed a good performance. Although the AUC value of the prognostic signature in the smoking population of the HNSCC cohort was low, the nAChRs-based signature could distinguish the patients into two groups
with significant differences in OS and PFS (Fig. 2A-B) and showed an excellent clustering ability (Fig. 2C).

As the most studied nAChR, the cholinergic receptor nicotinic alpha 5 subunit (α5 nAChR), encodes CHRNA5 protein and mediates ligand-gated ion channels when it is connected to the α3 and β4 subunits [30]. Some studies suggest that α5 nAChR promotes cell migration and invasion in lung cancer [28, 31], prostate cancer [32], and breast cancer [33]. Mechanistically, α5 nAChR mediates the Stat3-Jab1/Csn5 and TGF-β1/Smad signaling pathways to promote metastasis and EMT of lung cancer cells [28, 31]. Further, α5 nAChR activates AKT signaling, which in turn promotes metastasis and proliferation of prostate cancer cells [32]. α5 nAChR also promotes metastasis and stemness of hepatocellular carcinoma cells by regulating the Hippo signaling pathway [34]. Cholinergic receptor nicotinic alpha 9 subunit (α9 nAChR) is predominantly expressed in the outer hair cells of the cochlea [35], which plays a critical role in the regulation of the proteins of deafness genes. The expression of α9 nAChR is related to the regulation of pain [36] and polymorphisms in this gene increase the risk of lung cancer [37] and breast cancer [38]. For β4 nAChR, most studies have focused on the CHRNA5/CHRNA3/CHRNB4 cluster, wherein polymorphisms may be associated with an increased risk of death and incidence of cancer among smokers [39–43]. We performed a survival analysis based on the expression of α5, α9, and β4 nAChRs in all HNSCC patients using the Kmplot database. As shown in Supplementary Fig. 2A-C, patients with high α5 nAChR expression showed a significantly poorer OS (HR = 1.54, log-rank p = 0.0018); patients with high β4 nAChR expression showed a significantly poorer OS (HR = 1.51, log-rank p = 0.0076), and patients with high α9 nAChR expression showed a poorer OS (HR = 1.11, log-rank p = 0.44). In addition, methylated sites of α5, α9, and β4 nAChRs in all HNSCC patients were investigated using the MethSurv database as shown in Supplementary Fig. 2D-F.

In this study, α5, α9, and β4 nAChRs were highly expressed in HNSCC tissues and significantly related to poor outcomes in the smoking population of the HNSCC-TCGA cohort. Another cohort comprising 61 paired HNSCC samples showed high levels of α5 nAChR in HNSCC tissues as compared to the adjacent normal tissues, and differential expression was observed in 55 paired samples from patients who were smokers. These findings may shed light on the molecular mechanism underlying nAChRs among smokers with HNSCC.

Accumulating evidence suggests that HPV is critical for HNSCC, especially oropharyngeal cancer [44]. The major understanding of this relationship has come from studies on cervical cancer, a female-specific tumor closely related to HPV. However, different studies have shed different perspectives on this relationship. In this study, we observed that smoking-mediated nAChR expression was different among groups with distinct HPV infection statuses. Patients with other HPV infections showed...
the highest expression of nAChRs, whereas HPV+ p16+ HNSCC patients exhibited higher levels of nAChRs relative to HPV− HNSCC patients (Fig. 3G).

Smoking interferes with the immune system and depresses functional immune activity [45]. Moreover, sustained smoking induces autoimmunity to self-antigens, which may cause a range of deleterious effects on smokers or the people around them [45]. High-risk patients with higher nAChR expression showed weaker immunoreactivity than the low-risk patients. These findings may explain the reason for poor prognoses of patients at high risk. We performed immune infiltration analysis based on the expression of α5, α9, and β4 nAChRs in HNSCC patients using the Tumor Immune Estimation Resource (TIMER) database. As shown in Supplementary Fig. 2G-I, CHRNA9 correlated negatively with B cell (p = 1.94e-03) and CD8+ T cell (p = 3.27e-06) infiltration. CHRNB4 correlated positively with B cell (p = 9.40e-04), CD8+ T cell (p = 2.86e-02), CD4+ T cell (p = 7.26e-06), macrophage (p = 7.96e-04), and dendritic cell (p = 1.23e-03) infiltration. In the future, studies should focus on the role of smoking in the immune system of patients with head and neck cancer, which could contribute to the discovery and development of immune checkpoint-related drugs.

Our study, however, has some limitations. For clinical utility and translation, validation of the nAChR-based signature in independent HNSCC cohorts with a smoking population is necessitated. Selecting or overexpression of nAChRs both in vitro and in vivo should be performed to identify hub phenotypic features for head and neck cancer. The potential downstream mechanisms should be elucidated using protein mass spectrometry and/or transcriptome sequencing technologies following the manipulation of levels of nAChR expression.

Conclusion
In this study, we elucidated the role of nAChRs in HNSCC and constructed a nAChR-based signature that showed a better classification of the smoking population in the HNSCC cohort.

Abbreviations
HNSCC: Head and neck squamous cell carcinoma; nAChRs: Nicotinic acetylcholine receptors; TCGA: The Cancer Genome Atlas; LASSO: Least absolute shrinkage and selection operator; KM: Kaplan–Meier; ROC: Receiver operating characteristic; AUC: Area under the curve; PCA: Principal component analysis; HPV: Human papillomavirus; KEGG: Kyoto Encyclopedia of Genes and Genomes; CIBERSORT: Cell type identification by estimating relative subsets of RNA transcripts; ssGSEA: Single-sample Gene Set Enrichment Analysis; TIDE: Tumor Immune Dysfunction and Exclusion; GDSC: Drug Sensitivity in Cancer; qRT-PCR: Quantitative reverse transcriptase PCR; OS: Overall survival; PFS: Progression free survival; TNM: Tumor Node Metastasis; DNA: Deoxyribonucleic acid; NNK: Nicotine-derived nitrosamine ketone; NSCLC: Non-small cell lung adenocarcinoma; Yap1: Yes1 associated transcriptional regulator; E2F1: E2F transcription factor 1; EMT: Epithelial-mesenchymal transition; COPSS: COP9 signalosome subunit 5; EGFR: Epidermal growth factor receptor; CHRNAs: Cholinergic receptor nicotinic alpha 5 subunit; CHRNA3: Cholinergic receptor nicotinic alpha 3 subunit; CHRNb4: Cholinergic receptor nicotinic beta 4 subunit.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-10161-x.

Additional file 1: Supplementary Figure 1. Prognostic analysis of the nAChR-based prognostic signature. (A) Expression profile of β3nAChR; blue and red dots represent normal adjacent and tumor tissues, respectively. **p < 0.01; (B) Kaplan-Meier survival analysis of HPV− HNSCC patients using the prognostic signature; (C) Kaplan-Meier survival analysis of HPV+ HNSCC patients using the prognostic signature; ROC curves without calculating signature expression for (D) one-year OS, (E) three-year OS, (F) five-year OS, and (G) seven-year OS.

Additional file 2: Supplementary Figure 2. Survival analysis, methylated sites and immune infiltration analysis of hub nAChRs. Survival analysis based on the expression of (A) α5, (B)α9, and (C) β4 nAChRs in all HNSCC patients; Methylated sites of (D) α5, (E)α9, and (F) β4 nAChRs in all HNSCC patients; Immune infiltration analysis based on the expression of (G) α5, (H) α9, and (I) β4 nAChRs in HNSCC patients.

Additional file 3: Supplementary Table 1. Clinical information of smoking HNSCC cohort.

Additional file 4: Supplementary Table 2. Clinical information of adjacent normal samples.

Additional file 5: Supplementary Table 3. Clinical information of 61 paired samples diagnosed with HNSCC.

Additional file 6: Supplementary Table 4. The sequences of all primers used in this study.

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Not Applicable.

Authors’ contributions
Yuyue Shen performed the data curation and analysis. Qiang Huang and Mengyue Ji analyzed and interpreted the results. Liang Zhou and Chi-Yao Hsueh drafted and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets analyzed during the current study are publicly available in the TCGA repository (https://portal.gdc.cancer.gov/repository?facetTab=cases; accession date: 12/03/2020; accession number: phs000178).

Declarations
Ethics approval and consent to participate
All participants provided written informed consent. The protocols were authorized by the The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Clinical Research Ethics Committee of the Eye & ENT Hospital of Fudan University (2018036). Written informed consent was obtained from individual or guardian participants.

Consent for publication
Not Applicable.
Competition interests

The authors declare that they have no competing interests.

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