Integrated analysis of lncRNA CTD-2357A8.3 expression and its potential roles in head and neck squamous cell carcinoma

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Abstract. Head and neck squamous cell carcinoma (HNSCC), one of the most common malignant tumors, endangers human health. Recently, the incidence of HNSCC has kept increasing: However, its prognosis has not significantly improved. Understanding the molecular mechanism underlying HNSCC development will therefore provide new strategies for therapy. The present study attempted to identify differentially expressed (DE) long non-coding (lnc)RNAs and investigated their functional role in HNSCC development. Expression profiles of HNSCC and normal samples were downloaded from The Cancer Genome Atlas (TCGA) database. DElncRNAs between the HNSCC and normal samples were highlighted and their potential functions were investigated through lncRNA-micro (mi)RNA-mRNA network by using Gene Expression Profiling Interactive Analysis, UALCAN, DIANA-LncBase v.2 and miRWalk 3.0 databases. A total of 343 dysregulated lncRNAs were identified. Among these DElncRNAs, CTD-2357A8.3 had the highest fold-change and was significantly associated with poor overall survival in patients with HNSCC. Furthermore, CTD-2357A8.3 was associated with 'signaling pathways regulating stem cell pluripotency', 'proteoglycans in cancer', 'transcriptional misregulation in cancer' and 'chemokine signaling pathway'. Further analysis demonstrated that CTD-2357A8.3 acted as a 'sponge' in order to competitively adsorb miRNA to regulate the expression of target gene caveolin 1 (*CAV1*) in HNSCC. In conclusion, CTD-2357A8.3 may be considered a promising diagnosis biomarker or a therapeutic target for the treatment of HNSCC.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of human malignancy in United States (1,2) and represents ~4% of all new cancer cases in United States (1,2). According to the latest statistics, >700,000 patients are affected every year worlwide, there are 358,000 cases of HNSCC-associated mortality cases and the incidence of HNSCC has significantly increased (3). Progress in clinical therapy technique has not significantly improved the survival rates of patients with HNSCC, and the 5-year survival rate of patients with HNSCC is only 40-50%. Investigating the underlying mechanism of HNSCC development may offer novel strategies for its prevention, diagnosis and treatment through the development of potential biomarkers or therapeutic targets (4).

Long non-coding RNAs (lncRNAs) are defined as transcripts of over 200 nucleotides in length that possess no protein-coding function (5,6). The human genome encodes tens of thousands of lncRNAs, which are associated with numerous physiological processes, including the regulation of transcription, translation, localization, and function of proteins (6). LncRNAs exert regulatory roles through different mechanisms. They can affect chromatin remodeling and methylation and be used as a functional sponge to competitively inhibit micro (mi)RNA and regulate the stability of protein complexes (7). It has been demonstrated that the abnormal expression of lncRNAs is associated with the occurrence of various types of human disease, including cancer (8). Furthermore, lncRNA has been reported to be involved in the regulation of HNSCC cell proliferation, differentiation and metastasis and to be associated with drug resistance (9,10). However, the role of lncRNA in the development of HNSCC remains unclear. In particular, strong

molecular markers that could be used to predict HNSCC prognosis are still missing.

The present study aimed therefore to identify differentially expressed (DE)lncRNAs between HNSCC and normal tissues via The Cancer Genome Atlas (TCGA) database. In addition, further analysis of TCGA will be performed to explore the prognostic value of these DElncRNAs. Eventually, the potential function and molecular mechanism of these DElncRNAs will be investigated through the lncRNA-miRNA-mRNA network.

Materials and methods

Data download and identification of DElncRNAs. The RNA sequence data (RNAseq) of HNSCC tissues and corresponding normal tissues were downloaded from TCGA database (https://www.cancer.gov/tcga). A total of 544 samples from 500 patients with HNSCC and 44 normal controls were collected in December 2018. The normal controls included normal tissues from the oral cavity, oral tongue, larynx, floor of the mouth and base of the tongue. The raw data were downloaded and limma package (version 3.2.5; https://www.r-project.org/) was used to screen DElncRNAs between HNSCC and normal tissues. A |logFC|>2 and adjusted P-value <0.05 were set as the cut-off criteria.

Kaplan-Meier survival analysis. In order to determine whether the screened DElncRNAs possessed prognostic value, Kaplan-Meier survival analysis and log-rank test were performed by using Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/) (11). In GEPIA, the 'Methods' and 'Group Cutoff' were set as 'overall survival' and 'Median', respectively. Regarding mRNAs, their prognostic value was also verified by conducting log-rank analysis in UALCAN database (http://ualcan.path.uab.edu/analysis.html) (12).

Construction of lncRNA-miRNA-mRNA regulatory network. The DIANA-LncBase v.2 (DIANA Tools) (13,14) was used to predict the target miRNAs of DElncRNA, and a score >0.95 was set as the screening threshold. The miRWalk 3.0 database (http://mirwalk.umm.uni-heidelberg.de/) was used to predict and screen the target mRNAs of the aforementioned miRNAs (15), and the filters were set as '0.95', '3UTR'and 'miRTarBase'. The Cytoscape software (version, 3.6.0; https://cytoscape.org/) (16) was used to visualize all the predicted results.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of target mRNAs. GO analysis (http://geneontology.org/) and KEGG pathway (https://www.genome.jp/) enrichment of differentially expressed genes (DEGs) were performed by using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; version 6.7; https://david-d.ncifcrf.gov/) to screen the potential biological processes and signaling pathways in which the mRNAs are involved (17). The resulting data were imported into Cytoscape software to conduct visual analysis. P<0.05 was considered to indicate a statistically significant difference.

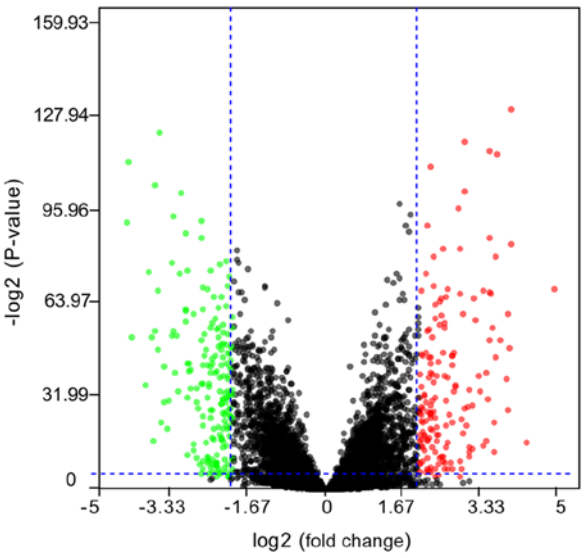

Figure 1. Differential expression of lncRNAs between HNSCC and normal tissues. Red points represent upregulated genes screened on the basis of |logFC|>2.0 and a corrected P-value <0.05. Green points represent downregulated genes screened on the basis of |logFC|>2.0 and a corrected P-value <0.05. Black points represent genes with no significant difference. FC, fold change.

Protein-protein interaction (PPI) network construction and hub genes identification. PPI networks were built by using the Search Tool for the Retrieval of Interacting Genes (STRING) software (version 11.0; http://string-db.org). The PPI network was visualized using Cytoscape software (18). By considering the node degree, Cytoscape CentiScaPe app (version, 3.6.0; http://apps.cytoscape.org/apps/centiscape) was used to screen hub genes in the network (19). Genes with a node degree >5 were considered to be candidate key genes.

Gene expression analysis. GEPIA was used to analyze the expression of DElncRNAs and mRNAs in HNSCC and normal tissues. Furthermore, the correlation between lncRNAs and mRNAs expression was analyzed using Pearson correlation in GEPIA (11). P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of DElncRNAs in HNSCC tissues. In the present study, RNAseq of HNSCC and corresponding normal tissue samples were downloaded from TCGA database. Following data screening, a total of 343 dysregulated lncRNAs were obtained, including 162 upregulated and 181 downregulated lncRNAs (Fig. 1).

Prognostic values of DElncRNAs. The association between the 343 DElncRNAs and the overall survival (OS) rate of patients with HNSCC was analyzed using GEPIA. The results demonstrated that the abnormal expression of 46 DElncRNAs was associated with the OS rate of patients with HNSCC (P<0.05). Furthermore, by applying more rigorous criteria (P<0.01), only 12 DElncRNAs were significantly associated with the OS rate of patients with HNSCC (Table I). CTD-2357A8.3, which was an lncRNA with the largest upregulation multiple, was

Table I. Information of 12 frequently dysregulated long non-coding RNAs in head and neck squamous cell carcinoma identified by The Cancer Genome Atlas.

Ensembl ID	Gene symbol	Fold change	P-value	Up or down regulation	Log-rank P-value
ENSG00000267123	CTD-2357A8.3	3.96	$3.43x10^{-26}$	Upregulated	0.0087
ENSG00000250874	CTC-480C2.1	3.62	$1.25x10^{-4}$	Upregulated	0.001
ENSG00000275216	RP11-54H7.4	3.46	$8.75x10^{-13}$	Upregulated	0.0023
ENSG00000233532	LINC00460	3.31	$6.37x10^{-11}$	Upregulated	0.0019
ENSG00000231131	LNCAROD	2.98	$3.56x10^{-09}$	Upregulated	0.0061
ENSG00000277268	LHX1-DT	2.96	$1.18x10^{-06}$	Upregulated	0.0028
ENSG00000259692	RP11-499F3.2	2.36	$2.63x10^{-10}$	Upregulated	0.0029
ENSG00000251185	RP11-542G1.1	2.03	$8.57x10^{-08}$	Upregulated	0.0063
ENSG00000215386	MIR99AHG	-2.09	$1.57x10^{-13}$	Downregulated	0.00065
ENSG00000176728	TTTY14	-2.48	$6.02x10^{-09}$	Downregulated	0.0037
ENSG00000267709	AC024592.9	-2.60	$7.76x10^{-17}$	Downregulated	0.0068
ENSG00000228789	HCG22	-4.29	$1.99x10^{-28}$	Downregulated	0.00065

Table II. miRNAs targeting CTD-2357A8.3 predicted by LncBase Predicted v.2.

miRNAs	MirBase ID	Score
hsa-miR-7113-5p	MIMAT0028123	0.984
hsa-miR-6895-3p	MIMAT0027691	0.984
hsa-miR-3657	MIMAT0018077	0.965
hsa-miR-7856-5p	MIMAT0030431	0.964
hsa-miR-346	MIMAT0000773	0.96
hsa-miR-3119	MIMAT0014981	0.955
hsa-miR-4514	MIMAT0019051	0.955

miRNAs, microRNAs.

selected for further analysis. GEPIA analysis demonstrated that CTD-2357A8.3 expression in HNSCC tissues was significantly increased compared to adjacent normal tissues, and was associated with OS rate of patients with HNSCC (Fig. 2).

LncRNA-miRNA-mRNA regulatory network. One of the important regulatory mechanisms of lncRNA is its competitive adsorption of miRNA as a 'sponge', which can be used to regulate the expression of target genes. The LncBase v.2 from DIANA Tools was used to predict the target miRNAs of DElncRNAs. For CTD-2357A8.3, hsa-miR-7113-5p, hsa-miR-6895-3p, hsa-miR-3657, hsa-miR-7856-5p, hsa-miR-346, hsa-miR-3119 and hsa-miR-4514 were identified as candidate target miRNAs (Table II). In addition, miRWalk 3.0 was used to predict and screen the target mRNAs of these miRNAs. These mRNAs were eventually validated by using miRTarBase. A total of 213 mRNAs were identified as the target genes of the aforementioned miRNAs and were visualized by using Cytoscape (Fig. 3).

GO analysis and KEGG pathway enrichment of target genes. GO and KEGG pathway analysis of the 213 mRNAs were performed using DAVID database. The criterion was $P<0.05$. As presented in Fig. 4, the results from KEGG pathway enrichment demonstrated that mRNAs were associated with 'signaling pathways regulating pluripotency of stem cells' (hsa04550, P=0.003), 'proteoglycans in cancer' (hsa05205, P=0.02), 'transcriptional misregulation in cancer' (hsa05202, P=0.033) and 'chemokine signaling pathway' (hsa04062, P=0.049). In addition, the GO terms of biological functions were mainly associated with 'regulation of transcription' (GO:0006355, P=0.014), 'negative regulation of cell proliferation' (GO:0008285, P=0.006) and 'angiogenesis' (GO:0001525, P=0.001).

Caveolin 1 (CAV1) was identified as the target gene of CTD-2357A8.3. The PPI network of the candidate target mRNAs was constructed using STRING database and visualized by Cytoscape software. In total, 110 mRNAs were entered into the PPI network complex (Fig. 5). With node degree >5 as inclusion criteria, the Cytoscape CentiScaPe was used to screen candidate key genes in the network. A total of 13 genes met the criteria and will be further investigated (Table III). Pearson's correlation between CTD-2357A8.3 and these 13 genes was analyzed using GEPIA, and the results demonstrated that the expression of glycogen synthase kinase 3 β (GSK3B), CAV1, Rho GDP dissociation inhibitor α (ARHGDIA) and cyclin dependent kinase 9 (CDK9) was positively correlated with CTD-2357A8.3 (Table III and Fig. 6). In addition, GEPIA and UALCAN were used to assess the association between these hub genes and OS of patients with HNSCC. The results demonstrated that only CAV1 was significantly associated with OS in the two databases, and CAV1 expression in HNSCC tissues was significantly higher than that in normal tissues (Table III and Fig. 6). Therefore, according to 'ceRNA Hypothesis', messenger RNAs, transcribed pseudogenes and lncRNAs can 'talk' to each other using microRNA response elements as letters of a new language (20). So, CAV1 may therefore be considered as a potential target of CTD-2357A8.3 in patients with HNSCC.

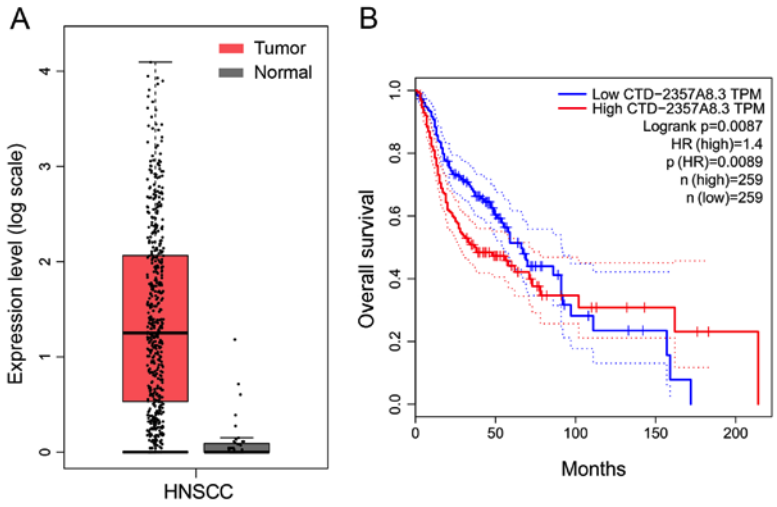

Figure 2. Expression level of CTD-2357A8.3 in HNSCC and its association with the OS of patients with HNSCC. (A) CTD-2357A8.3 was highly expressed in HNSCC tissues compared with normal tissues. Red represents tumor tissues and grey represents normal tissues. (B) Log-rank analysis demonstrated that the OS of patients with HNSCC and with high CTD-2357A8.3 expression was significantly reduced (P<0.05). HR, hazard ratio; OS, overall survival.

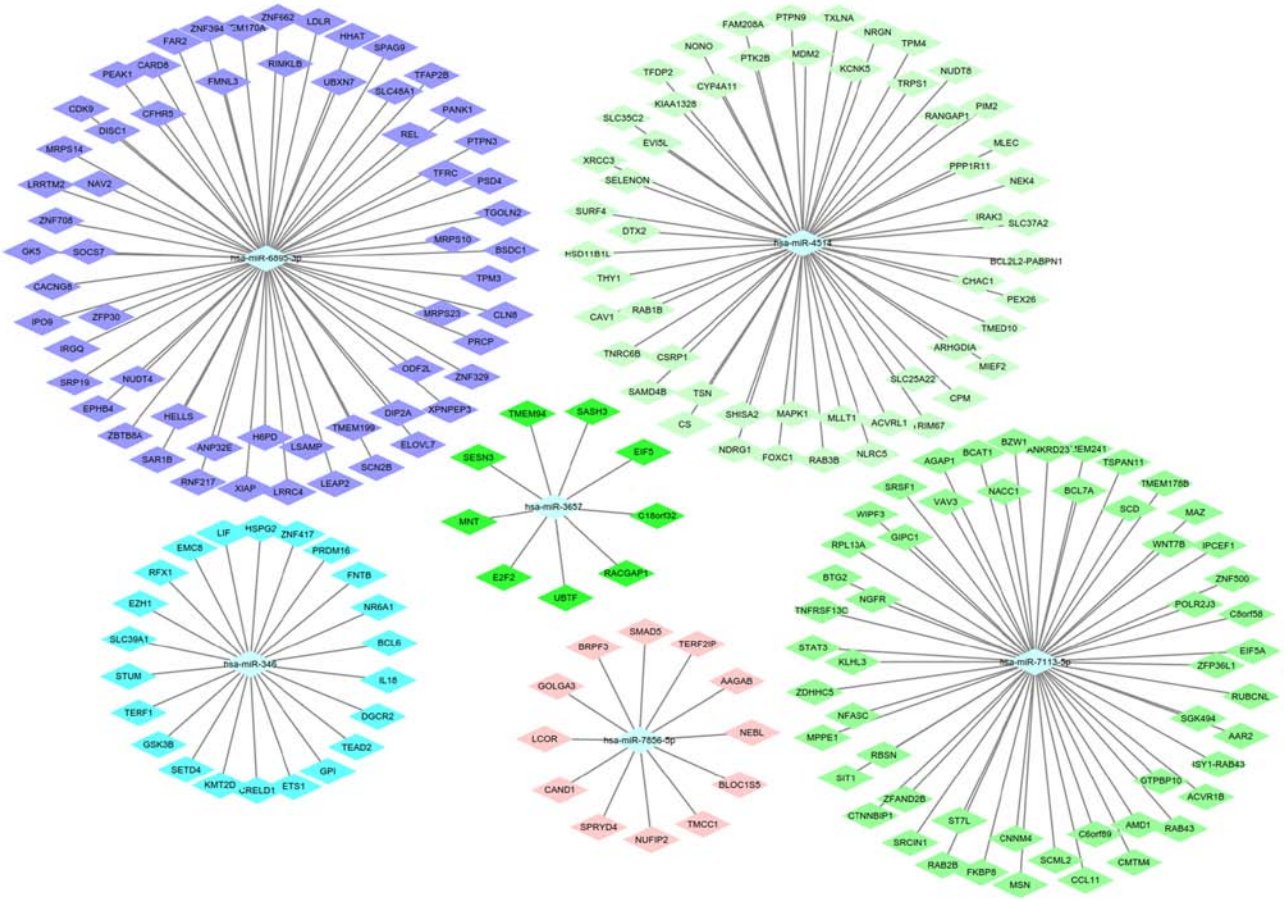

Figure 3. Construction of long non-coding RNA-miRNA-mRNA regulatory network. hsa-miR-7113-5p, hsa-miR-6895-3p, hsa-miR-3657, hsa-miR-7856-5p, hsa-miR-346, hsa-miR-3119 and hsa-miR-4514 were identified as the candidate target miRNAs. The target mRNAs of miRNAs were predicted using miRWalk3.0 and validated by at least one method using miRTarBase. miRNAs, mircoRNAs.

Discussion

HNSCC is one of the most common types of human malignancy. HNSCC is characterized by a rapid progression, high migratory capacity and high mortality rate; however, almost no biomarkers or targets exist for the diagnosis and therapy of HNSCC (2). The determination of DEGs between tumor and normal tissues may therefore help exploring the pathogenesis of HNSCC and providing potential novel biomarkers or targets for early diagnosis and therapy of HSNCC.

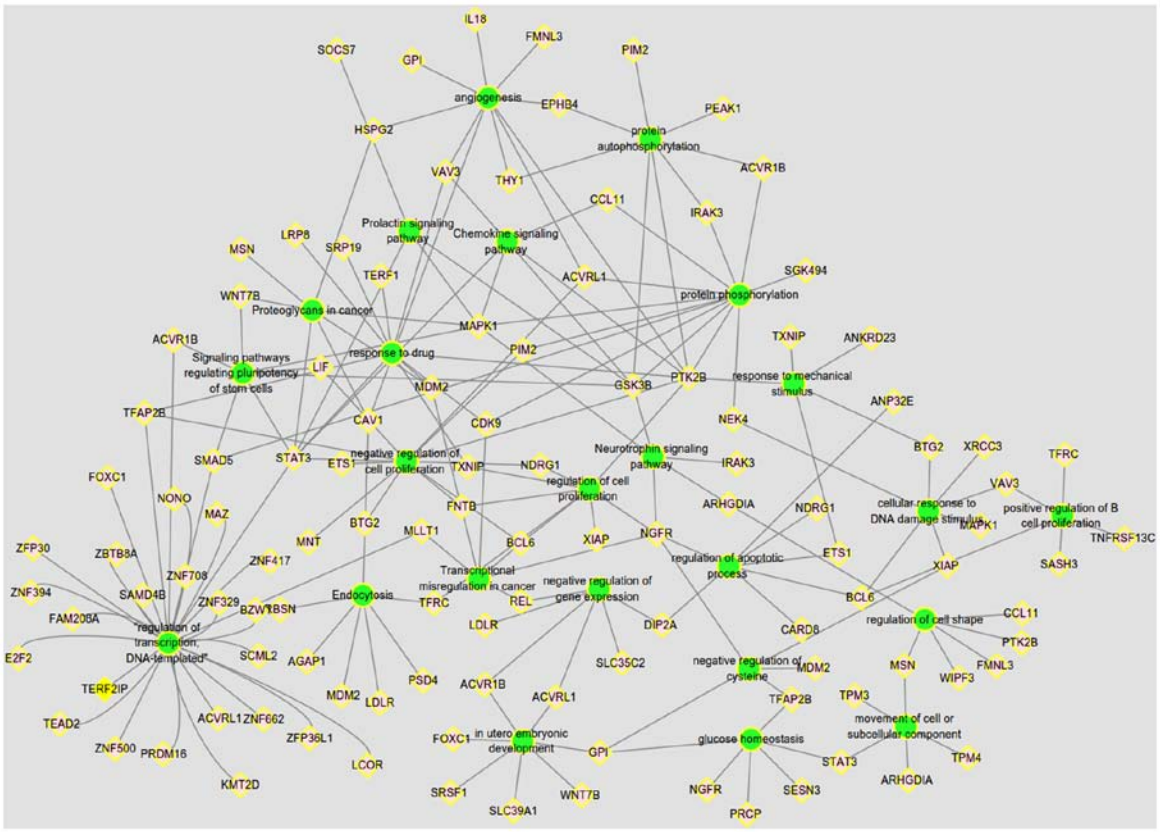

Figure 4. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis of the target genes of CTD-2357A8.3 in HNSCC tissues. Analysis of the 213 mRNAs was performed using the Database for Annotation, Visualization, and Integrated Discovery and visualized using Cytoscape. 'Signaling pathways regulating pluripotency of stem cells' (hsa04550, P=0.003), 'proteoglycans in cancer' (hsa05205, P=0.02), 'transcriptional misregulation in cancer' (hsa05202, P=0.033) and 'chemokine signaling pathway' (hsa04062, P=0.049) were the top four significantly enriched gene sets.

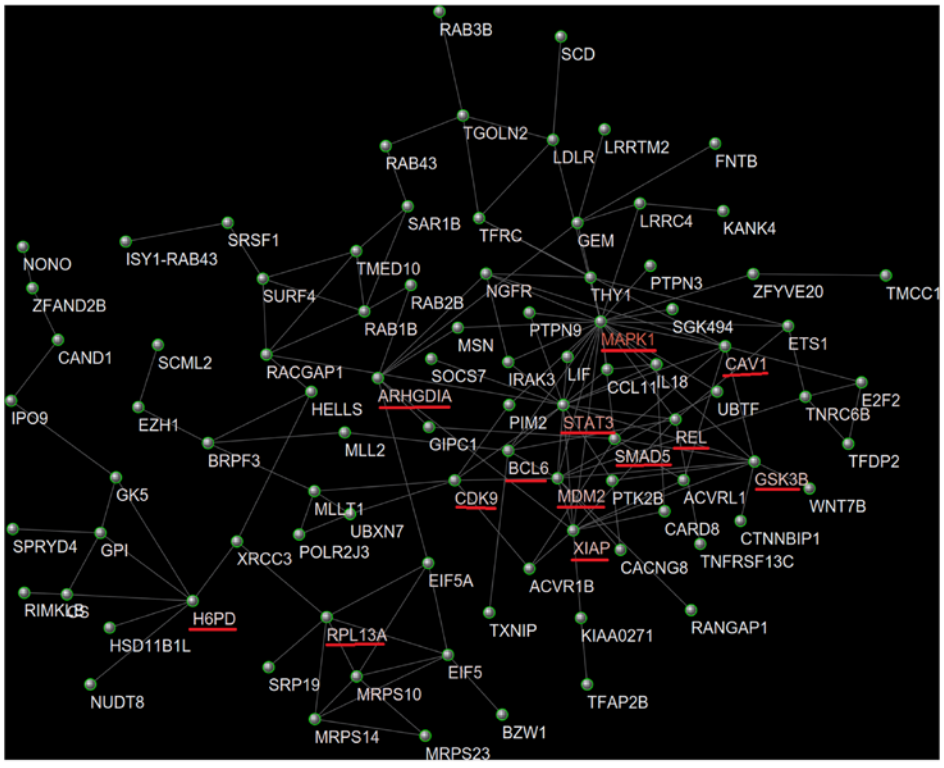

Figure 5. Identification of hub genes. The PPI network of the candidate target mRNAs was constructed using the Search Tool for the Retrieval of Interacting Genes software and visualized by Cytoscape. Confidence score≥0.4 was set as the cutoff criterion and total of 110 genes were filtered into the PPI network complex. The gene degree was further analyzed by CentiScaPe 2.2 plugin of Cytoscape. Genes with degree >5 were identified as hub genes. PPI, protein-protein interaction.

Table III. Pearson correlation analysis between CTD-2357A8.3 and 12 hub genes expressions and the log-rank analysis of these genes.

mRNA	r (Pearson)	P-value (Pearson)	Log-rank P-value (GEPIA)	Log-rank P-value (UALCAN)
MAPK1	0.029	0.49	0.54	0.8
STAT3	-0.13	0.002	0.1	0.12
MDM2	-0.11	0.0095	0.53	0.096
XIAP	0.075	0.076	0.53	0.66
GSK3B	0.14	0.0013	0.13	0.42
CAV1	0.42	0	0.0016	0.038
ARHGDIA	0.37	0	0.0028	0.26
H6PD	-0.021	0.61	0.44	0.038
RPL13A	-0.12	5.2x10-3	0.9	0.2
SMAD5	-0.11	0.013	0.3	0.96
CDK9	0.2	1.3x10-06	0.043	0.083
BCL6	-0.21	4.4x10-07	0.16	0.88
REL	-0.015	0.73	0.45	0.18

ARHGDIA, Rho GDP dissociation inhibitor α; BCL6, BCL6 transcription repressor; CDK9, cyclin dependent kinase 9; GEPIA, Gene Expression Profiling Interactive Analysis; GSK3B, glycogen synthase kinase 3 β; H6PD, hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase; MAPK1, mitogen-activated protein kinase 1; MDM2, MDM2 proto-oncogene; REL, REL proto-oncogene, NF-kB subunit; RPL13A, ribosomal protein L13a; SMAD5, SMAD family member 5; STAT3, signal transducer and activator of transcription 3; CAV1, caveolin 1; XIAP, X-linked inhibitor of apoptosis.

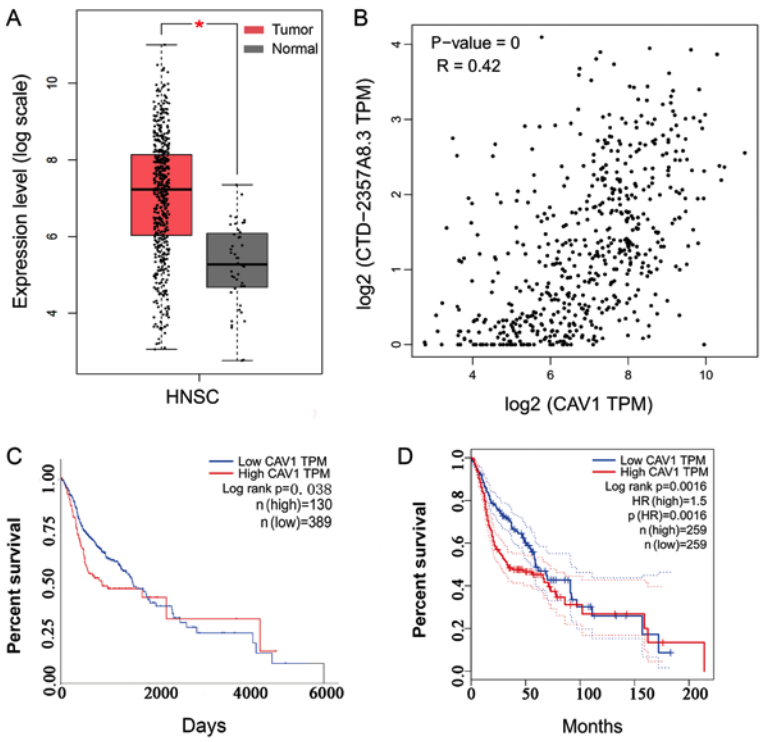

Figure 6. CAV1 was identified as the target gene of CTD-2357A8.3. (A) CAV1 expression level was increased in HNSCC tissue compared with normal tissues. Red represents tumor tissues and gray represents normal tissues. (B) Pearson correlation analysis of CAV1 and CTD-2357A8.3 expression in HSNCC tissues. (C) Kaplan-Meier survival analysis comparing the high or low CAV1 expression with overall survival of patients with HSNCC according to data from UALCAN database. (D) Kaplan-Meier survival analysis comparing the high or low CAV1 expression with overall survival of patients with HSNCC according to data from Gene Expression Profiling Interactive Analysis database. CAV1, caveolin 1; HSNCC, head and neck squamous cell carcinoma.

Recently, lncRNAs have attracted increasing interest. In cancer, some lncRNAs have been demonstrated to function as oncogenes, whereas others inhibit invasion and metastasis by participating in various cellular processes, including proliferation and differentiation (21). Some lncRNAs serve important role in the development and progression of HNSCC, and it

has been reported that these lncRNAs could be used as novel biomarkers and monitoring tools and as potential therapeutic targets in HNSCC treatment (22). Exploring the lncRNA differences between HNSCC and normal tissues could therefore provide a better understanding of the mechanism involved in the occurrence and development of HNSCC, and offer a basis for the development of novel diagnostic markers and therapeutic targets of HNSCC.

In the present study, the abnormally expressed lncRNAs in HSNCC were explored using TCGA database, and 343 dysregulated lncRNAs were identified, of which 162 lncRNAs were upregulated and 181 were downregulated. Following log-rank survival analysis, 12 lncRNAs were found to be significantly associated with the OS of patients with HNSCC. Among these lncRNAs, CTD-2357A8.3 had the largest |logFC| and was therefore further analyzed. The results demonstrated that CTD-2357A8.3 expression in HNSCC tissues was significantly increased compared to normal tissues and was associated with the OS rate of patients with HNSCC. However, following a detailed analysis of the literature, no relevant study on the role of CTD-2357A8.3 in HNSCC was found so far. Subsequently, it is really necessary and imperative to make further in-depth study investigating the specific role of CTD-2357A8.3 in HNSCC.

One of the important regulatory mechanisms of lncRNA is its competitive adsorption of miRNA as a 'sponge'. Subsequently, lncRNA serves a crucial role in regulating the expression of target genes. The candidate target miRNAs and mRNAs of CTD-2357A8.3 were therefore predicted in the present study. The GO and KEGG pathway analysis of the 213 target mRNAs reported that, 'signaling pathways regulating pluripotency of stem cells', 'proteoglycans in cancer', 'transcriptional misregulation in cancer' and 'chemokine signaling pathway' were the top significantly enriched gene sets and were all associated with cancer.

According to the mechanism of lncRNA, the expression of lncRNA and mRNA should be consistent. In the present study, the hub genes were identified by integrating centrality analysis with Pearson correlation and log-rank survival analysis. The results demonstrated that GSK3B, CAV1, ARHGDIA and CDK9 expression was positively correlated with CTD-2357A8.3. Furthermore, only CAV1 was significantly associated with the OS of patients with HNSCC according to GEPIA and UALCAN databases. These results suggested that CAV1 may be considered as a potential target gene for CTD-2357A8.3. CAV1 is a membrane-bound scaffold protein that can modulate signal transduction; however, the role of CAV1 in cancer is dependent on the tissue source (23). It has been reported that CAV1 inhibits cell migration and invasion by inhibiting epithelial mesenchymal transformation in pancreatic cancer (24). Furthermore, CAV1 has been reported to be highly expressed in colon cancer cells that have low metastatic ability, but not in colon cancer cells with high metastatic ability. These results suggest that CAV1 is involved in the negative regulation of tumor cell metastatic ability (25). Furthermore, in clear cell renal cell carcinoma and lung cancer, the increased expression of CAV1 was demonstrated to promote cell proliferation and invasion, and was positively correlated with poor prognosis (26,27). Further investigation is therefore required to verify whether CTD-2357A8.3 could regulate CAV1 expression through miRNA, and to explore the functional roles of CTD-2357A8.3 in the tumorigenesis of HNSCC. In addition, the present study used the GEPIA database to analyze the association between CTD-2357A8.3, CAV1 and the OS in HNSCC. This database contains RNA sequencing expression data of HNSCC from different primary sites, including the tonsil, oropharynx, oral cavity, larynx and hypopharynx. Further investigation is therefore required to determine whether CTD-2357A8.3 from different primary sites would remain positively correlated with the OS of patients with HNSCC. In addition, it may be also or even more important to consider the disease-free survival in HNSCC analysis.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

BoZ and XG downloaded the data, performed the identification of DElncRNAs and PPI network construction and drafted the manuscript. WZ and DW performed the survival analysis and constructed the lncRNA-miRNA-mRNA regulatory network. KX performed GO analysis and KEGG pathway enrichment analysis. DY was involved in the PPI network construction. ZM analyzed the association between the candidate genes and prognosis, and also participated the design of the study. BiZ was the major contributor in designing the present study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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