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ORIGINAL PAPER

Cisplatin and paclitaxel target significant long noncoding RNAs in laryngeal squamous cell carcinoma

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Abstract The objectives of this study were to identify specific long noncoding RNAs (lncRNAs) in laryngeal squamous cell carcinoma (LSCC) and to clarify the function of cisplatin and paclitaxel on the confirmed laryngeal cancer lncRNAs. Fifty-four pairs of laryngeal tumor and adjacent normal tissue were collected. Candidate lncRNAs were searched in authorized databases. The significant lncRNAs were identified and confirmed through high-output real-time PCR. Chemotherapy assay evaluated the influences of cisplatin and paclitaxel on the significant lncRNAs. Thirty-seven cancer-related candidate lncRNAs were selected. Three up-expressed and two down-expressed significant lncRNAs were identified and confirmed. The expressions of lncRNA CDKN2B-AS1, HOTAIR and MALAT1 were dramatically reduced with the increasing concentration of cisplatin and paclitaxel and also lengthening of the treatment duration. Cisplatin and paclitaxel have target function on significant lncRNAs in LSCC, which presents novel molecular targets to cure LSCC patients and also leads an orientation for developing new drugs.

Hui Chen and Yuan Xin have contributed equally to this study.

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Introduction

Laryngeal cancer is one of the most malignant cancers in head and neck. Despite profound improvements in the treatment of laryngeal carcinoma, some cases of laryngeal cancer remain incurable, which forces us to seek a new solution. With the rapid researches on long noncoding RNA (lncRNA), identification of significant lncRNAs of laryngeal cancer and clarifying the effects of chemotherapeutics on them is of great clinical value for the cure of laryngeal squamous cell carcinoma and for the development of new drugs.

The nonprotein-coding regions of the genome work not only as a substrate for DNA-binding proteins, but also as a template for the transcription of noncoding RNAs [1], which present sophisticated cell-specific and developmental dynamic expression patterns [2–4] and also being able to transact a wide repertoire of regulatory functions [5]. LncRNAs have crucial roles in gene expression control during both developmental and differentiation processes [6]. Long noncoding RNAs are found in sense or antisense orientation to protein-coding genes, functioning in cis through the act of the transcription and also in trans with the intrinsic RNA-mediated function [7]. Moreover, the dysregulation of lncRNAs has increasingly been linked to many human diseases, especially cancers [8]. Accumulating reports of dysregulated lncRNA expression in numerous cancer types imply that lncRNAs may act as potential onco- or tumor-suppressor RNAs [9, 10]. It has been reported that the pathogenesis of brain tumor was related to identify lncRNAs like anti-NOS2A [11] and MEG3 [12–14]. As to

breast tumor, lncRNAs like BC200 [15, 16], GAS5/SNHG2 [17], H19 [18], SRA-1/SRA [19], HOTAIR [20], Zfas1 [21], Kcnq1ot1 [22] and MALAT1 [23] play important role in its pathological process through various molecular mechanisms. In head and neck, published articles reported that lncRNA MEG3 was strongly reduced [24] and increased expression of lncRNA UCA1 had possible correlation with cancer metastasis [25] in tongue squamous cell carcinomas. More important, the newest researches reported that lncRNA HOTAIR contributed to cisplatin resistance of human lung adenocarcinoma [26] and lncRNA AK126698 had effect on cisplatin resistance in non-small cell lung cancer [27]. However, the expressions of lncRNAs in laryngeal squamous cell carcinoma (LSCC) and the influences of the chemotherapeutics on the significant lncRNAs have not been investigated.

In this study, we were the first to comprehensively identify significant lncRNAs in LSCC and also investigated the influences of cisplatin and paclitaxel on the lncRNAs. Differential expressions were identified for five lncRNAs by high-output real-time PCR, and the target function of cisplatin and paclitaxel on the significant lncRNAs was determined.

Materials and methods

Primary LSCC specimens

Fifty-four pairs of primary LSCC specimens and adjacent normal tissue were obtained from consenting patients, immediately after primary resection of laryngeal carcinoma. All procedures were approved by the Ethics Boards at the Affiliated Eye and ENT Hospital of Fudan University (Shanghai, China). The clinical and histological characteristics of the included patients are presented in Table 1.

Table 1 Clinicopathologic characteristics of 54 patients with laryngeal squamous cell carcinoma

Characteristic	No. of cases
Age (year)	
≦ 60	32
>60	22
Sex	
Male	52
Female	2
Stage	
I–II	14
III–IV	40
Primary tumor	
T1–T2	20
T3–T4	34
Lymph node metastasis	
N0	40
N1–N4	14
Location	
Supraglottic	24
Glottic	30
Histology	
SCC I–II	50
SCC II–III	4

RNA extraction

The tissue samples were ground into powder in liquid nitrogen. In total, 1 ml of Trizol per 50–100 mg tissue was added. Total RNA was extracted from tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA). In total, 100 μl absolute ethanol and sedimented RNA were added overnight at −20 °C, following centrifugation for 10 min with the speed of 12,000 rpm at 4 °C. The supernatant was pipetted out, and the residual was centrifuged for 5 min with 7,500 rpm at 4 °C after rinsing with 75 % ethanol. The sediment was dissolved with DEPC-ddH20 and quantified. The concentration of total RNA was measured by NanoDrop2000 software (Thermo Fisher, Waltham, MA). In total, 1 μl RNA sample was pipetted into the test head and quantified.

RNA reverse transcription

All the procedures were accorded to the manufacturer's protocol of M-MLV1 RT-PCR kit (Promega, Madison, WI). First, the annealing mixture was formulated. In total, 1 μl Oligo dT (0.5 μg/μl) (Sangon Biotech, Shanghai, China) and 2 μg total RNA were added into PCR tube and made up the final volume to 9 μl with RNase-free H_2O (Axygen, Tewksbury, MA). The sample tube was centrifuged after fine blended. After warm bathing for 10 min at 70 °C, the sample was immediately transferred into ice–water mixture at 0 °C and underwent ice bathing for 10 min, which made Oligo dT and the template anneal. Second, the reverse transcription reaction solution was formulated on ice, blended and centrifuged at 7,500 rpm for 5 min. The components of the reaction solution contained 4 μl 5 × RT buffer, 2 μl 10 mM dNTPs (Promega), 0.5 μl RNasin (Axygen), 1 μl Random Hexamers (Qiagen, Hamburg, Germany), 1 μl M-MLV-RTase (Promega) and 2.5 μl DEPC H_2O (Axygen). Third, the reaction solution was added into the annealing mixture to make up the final reverse transcription reaction system to the total volume of 20 μl. The reverse transcription reaction system underwent the reaction for 1 h at 42 °C in water bathing, followed by the reaction for 10 min at 80 °C, through which inactivated the reverse transcriptase and obtained the product of cDNA. The cDNA solution was stored at −80 °C.

Table 2 Sequences of all primers in the present research

Gene symbol	Primer sequence	
	Forward	Reverse
7SK	AAACAAGCTCTCAAGGTC	CCTCATTTGGATGTGTCT
BCAR4	GGACTCATTGTTGTTCTAC	ACCTATGGCTATCATTGTT
BCYRN1	CTGGGCAATATAGCGAGAC	TGCTTTGAGGGAAGTTACG
BOK-AS1	CTTGGCAGTTCTGATTGTG	TTGTCCGCTGTGGATAAG
C1QTNF9B-AS1	AGACACCTGAACATTCCT	CTGAGCAAGTTTCCTTCTT
CASC2	CTATTCCGAGTAAGAAGTG	TCTGTGTTGATGTTGATT
CBR3-AS1	CTTCTGGTTACAATGATTCTC	CACTTACTGCCTACATTAGA
CCAT1	TCACTGACAACATCGACTTTGAAG	GGAGAAAACGCTTAGCCATACAG
CDKN2B-AS1	TCATCATCATCATCATCATC	TGCTTCTGTCTCTTCATA
DNM3OS	ATAGAGCAAGTCTGGATT	GGATGAGGCAATAACATT
DSCAM-AS1	ACTAGCACAGATGGCATTC	CAGGAAGCATCGTGAACA
EPB41L4A-AS1	TAAGACAGTGAGGATGTGAAT	ATTATGGTGACAGCAGTGA
GAS5	CACAGGCATTAGACAGAA	AGGAGCAGAACCATTAAG
H19	CTCCACGACTCTGTTTCC	TCTCCACAACTCCAACCA
HIF1A-AS1	AATGTGTTCCTTGCTCTT	GTATGTCTCAGTTATCTTCCT
HOTAIR	AATAGACATAGGAGAACACTT	AATCTTAATAGCAGGAGGAA
IGF2-AS	CGCCACTGTGTTACCATT	TTGCCCATCCCAGATAGAA
KCNQ1OT1	GCATATCTGTCTTCCGTAT	CCTCTTCCTTCGTTCAAT
lincRNAp21	CCTGTCCACTCGCTTTC	GGAACTGGAGACGGAATGTC
LSINCT5	TAGACAACTTACTTAACCTCAT	TCCTTATCCACCTTATCCA
MALAT1	CCGCTGCTATTAGAATGC	CTTCAACAATCACTACTCCAA
MEG3	TGGCATAGAGGAGGTGAT	AGACAAGTAAGACAAGCAAGA
MIR31HG	ACTTCCACGATAGCAATG	GAATGAATCCTCTGTCCTC
PCA3	AATCATACTGGTCACTTATCT	TTAACAACTGGTCCTGAG
PCAT1	TAGAGCCTTGAAGATGAG	TCGTGTAGTTGTAAGATGA
PCGEM1	TAGTTAAGCAGATTCATAGA	GATGTCATAGTCCTCTTC
PRNCR1	TCTCAATGCCCAAGAGATGAGC	GAACTGTGAAGCTGTGAGTAACCATG
PVT1	CTTGAGAACTGTCCTTACG	CAGATGAACCAGGTGAAC
RRP1B	CAGTATATCTCAACTCAGT	TTCTTCTTCCTTCTTCTC
SRA1	TTACAGAGATTAGAACCACATT	GGCAAGTCAGAGTTACAAT
TDRG1	GATTCGTCTGGTTCCTTA	TTCCTCTTGACTGATTCTAA
UCA1	TTCCTTATTATCTCTTCTG	TCCATCATACGAATAGTA
WRAP53	CAATAGTGCTGATAACAT	CAGTAATCATAGATGGTAT
XIST	GAACCACCTACACTTGAG	TGCTATGCGTTATCTGAG
Yiya	TATCCTATTCTTAGCAACTG	ACATACCTGGCATATAGT
ZNFX1-AS1	CCAGTTCCACAAGGTTAC	GCAGGTAGGCAGTTAGAA
U6	CTCGCTTTGGCAGCACA	AACGCTTCACGAATTTGCGT

Screening of cancer-related lncRNAs

Collected the authorized lncRNA database, including LncRNADisease, NONCODE, lncRNAdb, LNCipedia and ChIPBase. The published lncRNAs were searched in the lncRNA databases, and the cancer-related lncRNAs were screened. All the screening procedures were performed by two researchers separately for three times. The candidate lncRNAs were obtained after verifying by both researchers.

Real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was used to evaluate lncRNA expression using the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), and ABI PRISM 9700 PCR cycler (Applied Biosystems) was used for gene amplification. Real-time PCR system was established with a 20 μl volume containing 6 μl dd H_2O, 10 μl SYBR Green Master Mix, 1 μl forward

primer of target gene with the concentration of 10 μM, 1 μl reverse primer of target gene with the concentration of 10 μM and 2 μl stored cDNA solution. Sequences of all primers are listed in Table 2. The condition of real-time PCR was 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min, in accordance with the manufacturer's manual. U6 gene was used as an internal reference marker. For calculation of differential gene expression, the $2^{-\Delta Ct}$ formula was used.

Prediction of microRNA–lncRNA interactions

Based on our previous research about microRNA expression profile in laryngeal squamous cell carcinoma [28], microRNA–lncRNA interactions would be of great value for both the cancer pathogenesis and pharmacological mechanism. starBase v2.0 (http://starbase.sysu.edu.cn/) was a newly developed database to provide the most comprehensive CLIP-Seq experimentally supported miRNA–mRNA and miRNA–lncRNA interaction networks to date [29, 30]. With searching in starBase v2.0, the identified microRNA–lncRNA interaction was exposed.

Cell culture

The Hep-2 cell line was established in 1952 from a 56-year-old male with laryngeal carcinoma. The AMC-HN-8 cell line was established by Kim [31] in 1997 from head and neck cancer patients with primary or metastatic cancer, which has been used in a few laryngeal cancer research [32, 33]. The cell lines were cultured in RPMI-1640 medium (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS, GIBCO) in a humidified incubator at 37 °C with 5 % CO_2.

Chemotherapy assay

Hep-2 and AMC-HN-8 cells in RPMI 1640 with 10 % FBS were plated in 24-microwell plates at 1×10^5 cells/well. Each cell line was divided into chemo-treated group and control group. In the chemo-treated group, Cisplatin (Nanjing Pharmaceutical Factory Co., Nanjing, China) and Paclitaxel (Anzatax, Mayne Pharma Pty Ltd., Melbourne, Australia) were utilized to treat the two cell lines, respectively, with the concentration of 1, 10 and 100 μM. The chemotherapy reagent should be added into six wells for each concentration with the volume of 100 μl. Same volume of 0.1 % DMSO was added into the control group wells. The culture plates were located into the incubator. We were also supposed to investigate the influence of chemotherapy reagents on the lncRNAs with different treating periods. After 3, 12 and 24 h, respectively, the plates of two cell lines were taken out of the incubator. In total, 1 ml Trizol was added into each well. Real-time PCR was performed to determine the effects of the chemotherapy reagents on the expression of lncRNAs. All experiments were performed three times.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD) for at least three independent experiments. Statistical differences were evaluated by independent-sample *t* tests in the identification of significant lncRNAs, analysis of variance and Kruskal–Wallis tests in the chemotherapy assay. Stata 12.0 (StataCorp LP, College Station, TX, USA) and Microsoft Office Excel 2003 were performed for data processing and statistical comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

Selection of candidate lncRNAs

Through carefully screening of the comprehensive lncRNA database, including LncRNADisease, NONCODE, lncRNAdb, LNCipedia and ChIPBase, and also the well-known paper databases such as PubMed and Web of Knowledge for cancer-related lncRNA reports, 37 cancer-related lncRNAs were selected and confirmed by two separated researchers. All of the chosen lncRNAs were published in authorized journals and proved that they had significant functions on the pathogenesis of different cancers. However, these identified lncRNAs, especially the relations with the chemotherapy agents, have not been investigated in laryngeal squamous cell carcinoma. The characteristics and genetic information of the selected lncRNAs are listed in Table 3.

Validation of lncRNAs by real-time PCR

The validation of lncRNAs was completed through two parts. The first part was identification of target lncRNAs of LSCC by large amount of real-time PCR. The second part was confirmation on more primary samples. Fifty-four pairs of fresh samples were collected, and the patient characteristics and cancer-related characteristics are presented in Table 1. High-output real-time PCR was performed on six pairs of the sample tissues, with T3–T4 stage LSCC. We discovered that five lncRNAs were differentially expressed between primary LSCC samples and adjacent normal tissues. The relative expressions of all lncRNAs are presented in manner of heatmap in Fig. 1. The deeper the color showed, the higher the expression would be. Among them, three lncRNAs were up-expressed in tumor specimens, including

Table 3 Reported cancer-related lncRNAs

Gene symbol	Related cancer	Chromosome locus	Start	End	Strand	Species	Aliases
7SK	Cancer	6p12.2	52995620	52995951	+	Human	RN7SK
BCAR4	Breast	16p13.3	11819830	11828832	−	Human	None
BCYRN1	Breast	2p21	47335315	47335514	+	Human	BC200; BC200a; LINC00004; NCRNA00004
BOK-AS1	Testicular, prostate	2q37.3	241544384	241559143	−	Human	BOKAS; NAToB; BOK-AS; NCRNA00151
C1QTNF9B-AS1	Prostate	13q12	23888889	23892103	+	Human	PCOTH
CASC2	Endometrial	10q26.11	118046821	118210153	+	Human	C10orf5
CBR3-AS1	Esophageal, prostate	21	36131767	36156308	−	Human	PlncRNA-1
CCAT1	Colon, gastric	8q24.21	127207382	127219268	−	Human	CARLo-5
CDKN2B-AS1	Breast, prostate, leukemia	9p21.3	21994791	22121097	+	Human	ANRIL; p15AS; PCAT12; CDKN2BAS; CDKN2B-AS; NCRNA00089;
DNM3OS	Ovarian	1q24.3	172136879	172144835	−	Human	MIR199A2HG
DSCAM-AS1	Breast	21	40383083	40385358	+	Human	M41
EPB41L4A-AS1	Lung	5q22.2	112160526	112162501	+	Human	TIGA1; C5orf26; NCRNA00219
GAS5	Prostate	1q25.1	173863901	173867987	−	Human	SNHG2; NCRNA00030
H19	Bladder, gastric	11p15.5	1995176	1997835	−	Human	ASM; BWS; WT2; ASM1; PRO2605; D11S813E; LINC00008; NCRNA00008
HIF1A-AS1	Kidney	14q23.2	61681041	61695823	−	Human	5′aHIF-1A; 5′aHIF1alpha
HOTAIR	Esophageal, melanoma, lung, breast	12q13.13	53962308	53974956	−	Human	HOXAS; HOXC-AS4; HOXC11-AS1; NCRNA00072
IGF2-AS	Renal, prostate	11p15.5	2140528	2148666	+	Human	PEG8; IGF2AS; IGF2-AS1
KCNQ1OT1	Hepatic, adrenal, colorectal	11p15	2608328	2699998	−	Human	LIT1;KvDMR1;KCNQ10T1;KCNQ1-AS2;KvLQT1-AS;NCRNA00012
lincRNAp21	Lung cancer	17 A3.3; 17 15.04 cM	29057474	29078961	−	Human	Gm16197;linc-p21;OTTMUSG00000031656
LSINCT5	Breast cancer	5p15.33	N/A	N/A	N/A	Human	N/A
MALAT1	Prostate, lung, bladder	11q13.1	65497762	65506469	+	Human	HCN; NEAT2; MALAT-1; PRO2853; mascRNA;LINC00047; NCRNA00047
MEG3	Bladder, glioma	14q32	100826108	100861026	+	Human	GTL2; FP504; prebp1; PRO0518; PRO2160; LINC00023; NCRNA00023
MIR31HG	Breast, lung	9p21.3	21454268	21559698	−	Human	None
PCA3	Prostate cancer	9q21.2	76764438	76787549	+	Human	DD3; NCRNA00019
PCAT1	Prostate	8q24.21	127013154	127021014	+	Human	PCA1; PCAT-1
PCGEM1	Prostate	2q32	192749845	192776899	+	Human	PCAT9; LINC00071; NCRNA00071
PRNCR1	Prostate, colorectal	8q24.21	127079874	127092595	+	Human	PCAT8
PVT1	Breast, colorectal, pancreatic	8q24	127890628	128101253	+	Human	LINC00079; NCRNA00079
RRP1B	Breast	21q22.3	43659551	43696079	+	Human	Nnp1; RRP1; NNP1L; KIAA0179
SRA1	Breast	5q31.3	140550067	140558093	−	Human	SRA; SRAP; STRAA1; pp7684
TDRG1	Testicular	6p21.2	40378424	40379892	+	Human	LINC00532
UCA1	Bladder	19p13.12	15828947	15836321	+	Human	CUDR; UCAT1; LINC00178; NCRNA00178
WRAP53	Ovarian, rectal	17p13.1	7686071	7703502	+	Human	DKCB3; TCAB1; WDR79
XIST	Breast, testicular	Xq13.2	73820651	73852753	−	Human	SXI1; swd66; DXS1089; DXS399E; LINC00001; NCRNA00001

Table 3 continued

Gene symbol	Related cancer	Chromosome locus	Start	End	Strand	Species	Aliases
Yiya	Breast, rectal, gastric	1q32.3	213924749	213926654	+	Human	LINC00538; PROX1UP
ZNFX1-AS1	Breast, hepatic, gastric	20q13.13	49278178	49289260	+	Human	HSUP1; HSUP2; ZNFX1-AS1; C20orf199; NCRNA00275

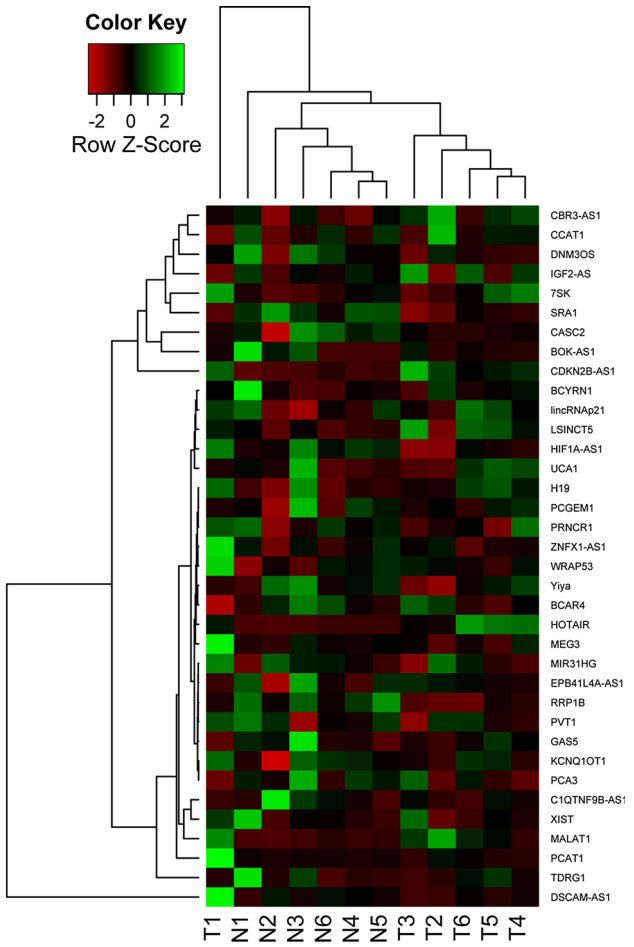

Fig. 1 The heatmap of 37 candidate lncRNAs through performing high-output real-time PCR in six pairs of laryngeal squamous cell carcinoma and adjacent normal tissue. *T* laryngeal tumor. *N* adjacent normal tissue

CDKN2B-AS1 (* $p < 0.05$), HOTAIR (** $p < 0.05$) and MALAT1 (*** $p < 0.05$), as indicated in Fig. 2a. More, two lncRNAs had significant down-expression, which were lncRNA RRP1B (* $p < 0.05$) and SRA1 (** $p < 0.05$), as presented in Fig. 2b. The characteristics and genetic information are shown in Table 4. Confirmation experiments were performed in 48 pairs of sample tissues through real-time PCR. The results presented the consistent finding, which indicated that lncRNA CDKN2B-AS1 (* $p < 0.05$), HOTAIR (** $p < 0.05$) and MALAT1 (*** $p < 0.05$) were relatively up-expressed and lncRNA RRP1B (* $p < 0.05$) and SRA1 (** $p < 0.05$) were relatively down-expressed in tumor samples. The Fig. 3 shows this confirmation.

Prediction of microRNA–lncRNA interactions

Since the function of microRNA has been widely investigated and we discovered the comprehensive microRNA profile in LSCC in our previous research [28], we supposed that there would be certain interaction between microRNAs and lncRNAs, which could play important role in cancer pathogenesis, also in the pharmacological mechanisms. Through searching in the starBase v2.0, we found that lncRNA CDKN2B-AS1 interacted with 20 microRNAs; lncRNA HOTAIR had interaction with 30 microRNAs, and lncRNA MALAT1 could interact with 113 microRNAs, as listed in Table 5. Most of them only had one target site, while several could interact at more target sites, even up to 6. The searching from published reports showed that the microRNA–lncRNA interactions had influences on several cancers such as breast cancer, acute myeloid leukemia and cutaneous melanoma. (data were not shown).

Cisplatin and paclitaxel influenced the expressions of lncRNAs in laryngeal cancer cell lines

In order to determine the function of cisplatin and paclitaxel on the significant lncRNAs, chemotherapy assay was performed. Cisplatin and paclitaxel were administrated into the Hep-2 cell line and AMC-HN8 cell line. First, we evaluated the influence of different concentration of the agents on the expression of lncRNAs. In Hep-2 cells treated with cisplatin, the expression of CDKN2B-AS1 decreased with the increase of the concentration, comparing with DMSO control group. There was statistical difference among 1, 10, 100 μM and control group (* $p < 0.05$). The expression of HOTAIR declined with the rising of cisplatin concentration, which presented statistical difference among the groups (** $p < 0.05$), and MALAT1 expression also went down due to adding concentration of the agent (*** $p < 0.05$) (Fig. 4ai). When treating Hep-2 cells with paclitaxel, the relative expression of CDKN2B-AS1 (* $p < 0.05$), HOTAIR (** $p < 0.05$) and MALAT1 (*** $p < 0.05$) all decreases with increasing the concentration of paclitaxel, with statistical significance (Fig. 4aii). In AMC-

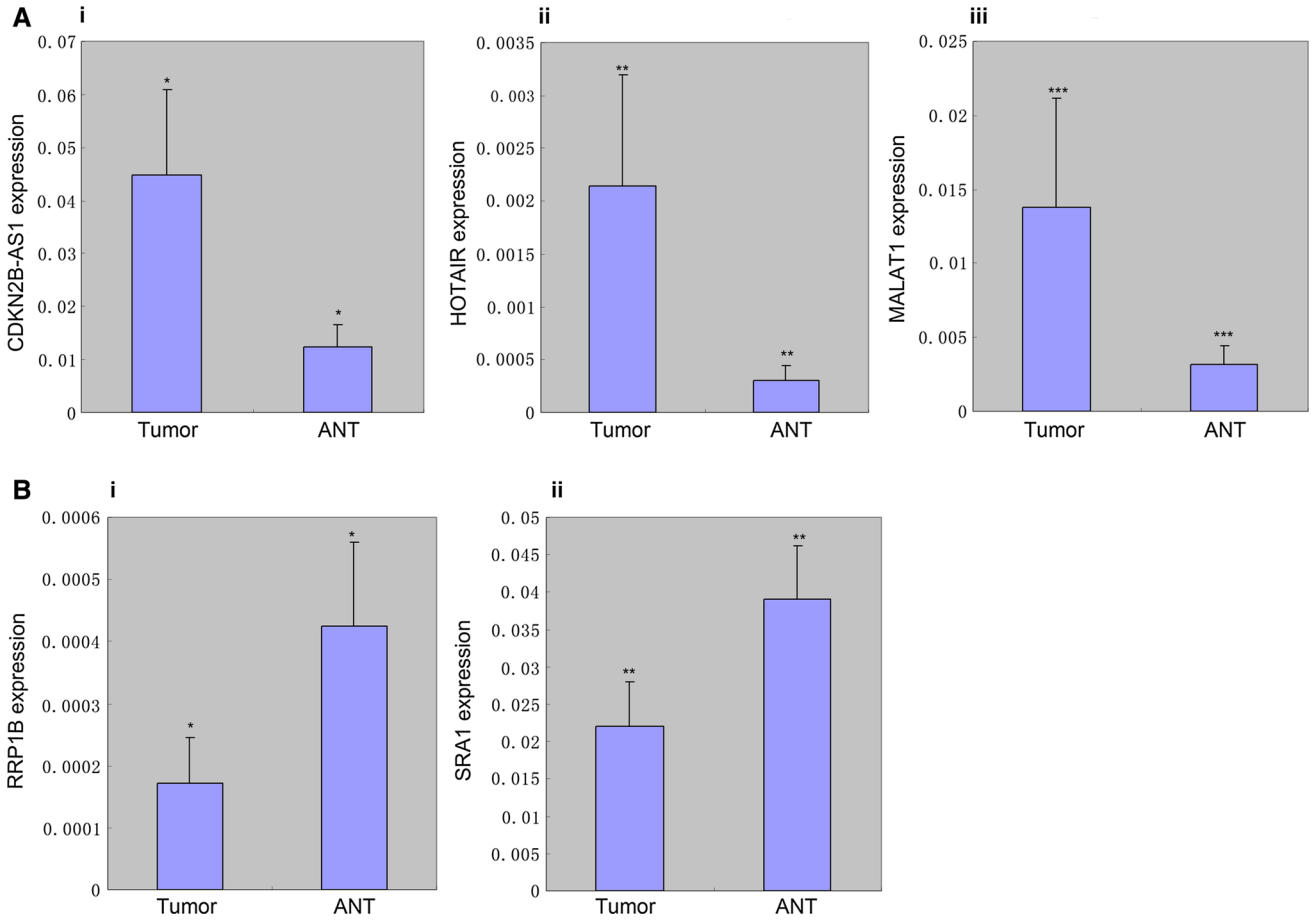

Fig. 2 The differentially expressed lncRNAs identified from candidate lncRNAs. **a** The up-expressed lncRNAs in laryngeal squamous cell carcinoma. (**i**) The relative expression of CDKN2B-AS1 comparing laryngeal tumor with adjacent normal tissue. Results were mean ± SD ($n = 6$; * $p < 0.05$). (**ii**) The relative expression of HOTAIR comparing laryngeal tumor with adjacent normal tissue. Results were mean ± SD ($n = 6$; ** $p < 0.05$). (**iii**) Relative expression of MALAT1 comparing laryngeal tumor with adjacent normal tissue. Results were mean ± SD ($n = 6$; *** $p < 0.05$). **b** The down-expressed lncRNAs in laryngeal squamous cell carcinoma. (**i**) The relative expression of RRP1B comparing laryngeal tumor with adjacent normal tissue. Results were mean ± SD ($n = 6$; * $p < 0.05$). (**ii**) The relative expression of SRA1 comparing laryngeal tumor with adjacent normal tissue. Results were mean ± SD ($n = 6$; ** $p < 0.05$)

Table 4 Differentially expressed lncRNAs in laryngeal squamous cell carcinoma

Gene symbol	Related cancer	Chromosome locus	*p* value
CDKN2B-AS1	Breast, prostate, leukemia	9p21.3	0.0035
HOTAIR	Esophageal, melanoma, lung, breast	12q13.13	0.0075
MALAT1	Prostate, lung, bladder	11q13.1	0.0155
RRP1B	Breast	21q22.3	0.0023
SRA1	Breast	5q31.3	0.0012

HN8 cells treated with cisplatin, the expression of CDKN2B-AS1 decreased with the concentration climbing, which reached statistical significance among each concentration group (* $p < 0.05$). As to the expression of HOTAIR, it declined when the concentration of the agent rose (** $p < 0.05$). MALAT1 expression also dropped, with rising of the concentration, which got significant difference (*** $p < 0.05$) (Fig. 4bi). Treating with paclitaxel, the expressions of CDKN2B-AS1 (* $p < 0.05$), HOTAIR (** $p < 0.05$) and MALAT1 (*** $p < 0.05$) descended, when increasing the concentration of paclitaxel (Fig. 4bii). Second, the influences of the treating duration on the lncRNA expression were assessed. In Hep-2 cells treated with cisplatin, the expression of CDKN2B-AS1 fell with lengthening of the treating duration, which had the statistical significance among 3, 12 and 24 h and control group (* $p < 0.05$). The expression HOTAIR also showed the trend of decline with lengthening of the period (** $p < 0.05$). MALAT1 expression presented descending with the extension of the treating duration, which reached statistical difference (*** $p < 0.05$) (Fig. 5ai). Treating Hep-2 cells with paclitaxel injection, the relative expression of CDKN2B-AS1 (* $p < 0.05$), HOTAIR (** $p < 0.05$) and

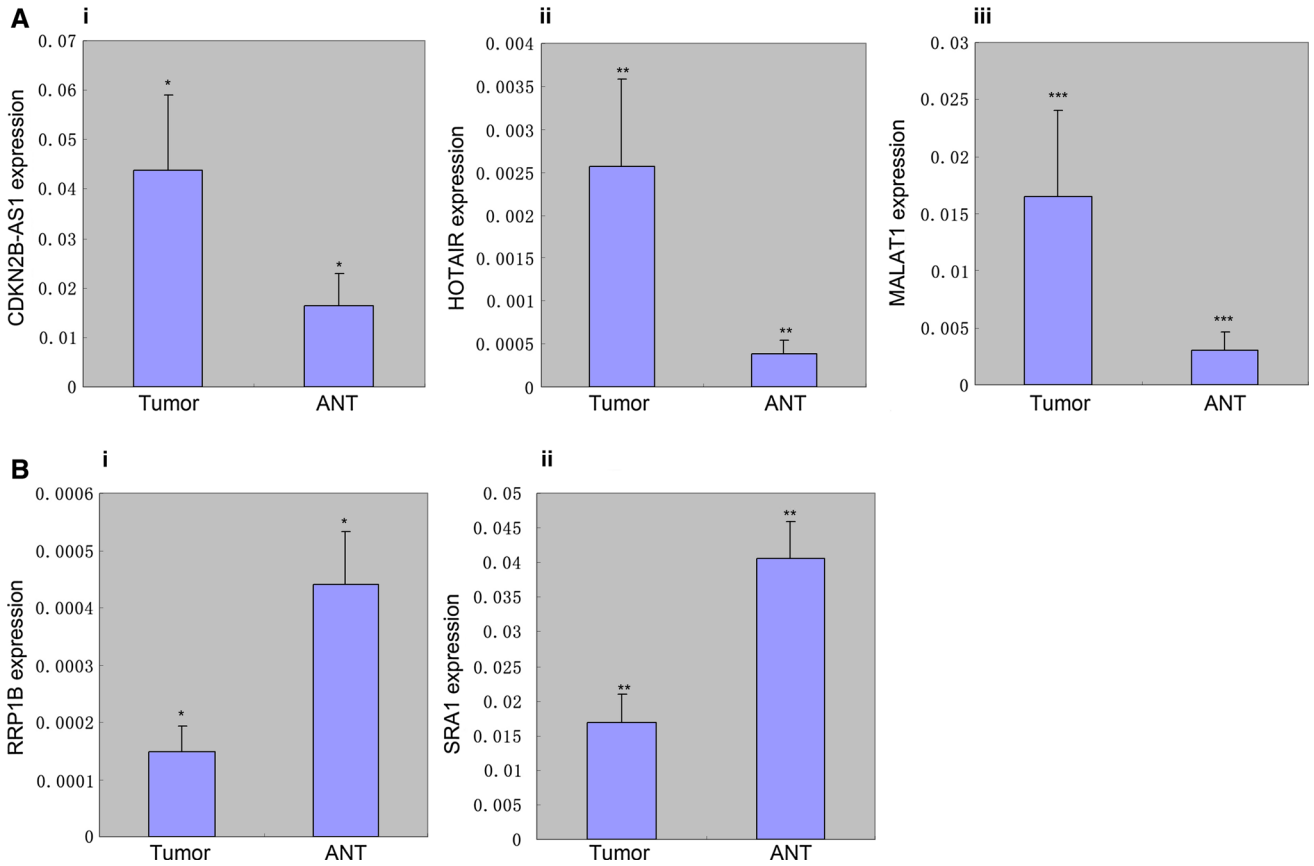

Fig. 3 The confirmation of significant lncRNAs through real-time PCR in laryngeal squamous cell carcinoma. **a** Significantly high-expressed lncRNAs in laryngeal squamous cell carcinoma. (**i**) The comparison of the expression of CDKN2B-AS1 between 48 primary laryngeal tumor samples and 48 adjacent normal tissues. Results were mean ± SD ($n = 48$; * $p < 0.05$). (**ii**) The comparison of the expression of HOTAIR between 48 tumor samples and 48 adjacent normal tissues. Results were mean ± SD ($n = 48$; ** $p < 0.05$). (**iii**) The comparison of the expression of MALAT1 between 48 tumor samples and 48 adjacent normal tissues. Results were mean ± SD ($n = 48$; *** $p < 0.05$). **b** Differentially low-expressed lncRNAs in laryngeal cancer. (**i**) The expression of RRP1B comparing tumor samples with adjacent normal tissues. Results were mean ± SD ($n = 48$; * $p < 0.05$). (**ii**) The expression of SRA1 comparing tumor samples with adjacent normal tissues. Results were mean ± SD ($n = 48$; ** $p < 0.05$)

MALAT1 (*** $p < 0.05$) all dropped off with prolonging the duration (Fig. 5aii). In AMC-HN8 cells treated with cisplatin, CDKN2B-AS1 expression reduced when the treating duration extended, which had statistical significance among each duration group (* $p < 0.05$). For HOTAIR expression, it also lessened with increasing the treating duration (** $p < 0.05$). The MALAT1 expression had a significant reduction when the duration prolonged (*** $p < 0.05$) (Fig. 5bi). When treating the AMC-HN8 cells with paclitaxel, the relative expression of CDKN2B-AS1 (* $p < 0.05$), HOTAIR (** $p < 0.05$) and MALAT1 (*** $p < 0.05$) all cut down with the duration extension (Fig. 5bii).

Discussion

With the development of whole genome and transcriptome sequencing technologies, lncRNAs have received more attention. LncRNAs can regulate gene expression in many ways, including chromosome remodeling, transcription and posttranscriptional processing. Further, the dysregulation of lncRNAs has increasingly been linked to many human diseases, especially in cancers. More importantly, the latest research reported the function of cisplatin on specific lncRNA in lung cancer, which brought us new hope of cancer treatment. However, so far, there has been no comprehensive lncRNA research on laryngeal cancer, and the influence of chemotherapeutics on lncRNA has not been investigated yet. In order to have a profound research of lncRNAs in LSCC and to have a better understanding of the chemotherapeutics' mechanisms, we started this project as the first step. Based on the published lncRNAs and authorized lncRNA databases, thirty-seven candidate lncRNAs were selected. All of them were related to cancer pathogenesis or function regulation. Performing high-output real-time PCR on six pairs of primary LSCC tissues and adjacent normal tissues, we discovered five differentially expressed lncRNAs, three of which were highly

Table 5 Prediction of microRNA–lncRNA interactions

Identified lncRNAs	Interacted microRNAs
CDKN2B-AS1	hsa-miR-181b-5p;hsa-miR-181a-5p;hsa-let-7a-5p;hsa-let-7i-5p;hsa-miR-4500;hsa-miR-10a-5p;hsa-miR-122-5p;hsa-miR-181c-5p;hsa-miR-181d-5p;hsa-let-7e-5p;hsa-miR-125a-3p;hsa-miR-4262;hsa-miR-10b-5p;hsa-let-7c-5p;hsa-let-7b-5p;hsa-let-7 g-5p;hsa-miR-4458;hsa-miR-339-5p;hsa-let-7f-5p;hsa-let-7d-5p;hsa-miR-98-5p
HOTAIR	hsa-miR-222-3p;hsa-miR-206;hsa-miR-221-3p;hsa-miR-1;hsa-miR-326;hsa-miR-148a-3p;hsa-miR-148b-3p;hsa-miR-17-5p;hsa-miR-20a-5p;hsa-miR-130a-3p;hsa-miR-19a-3p;hsa-miR-19b-3p;hsa-miR-330-5p;hsa-miR-93-5p;hsa-miR-106b-5p;hsa-miR-106a-5p;hsa-miR-761;hsa-miR-214-3p;hsa-miR-4295;hsa-miR-613;hsa-miR-152-3p;hsa-miR-454-3p;hsa-miR-301a-3p;hsa-miR-519d-3p;hsa-miR-217;hsa-miR-301b;hsa-miR-130b-3p;hsa-miR-3619-5p;hsa-miR-3666;hsa-miR-20b-5p
MALAT1	hsa-miR-503-5p;hsa-miR-197-3p;hsa-miR-92b-3p;hsa-miR-28-5p;hsa-miR-25-3p;hsa-miR-370-3p;hsa-miR-149-5p;hsa-miR-155-5p;hsa-miR-378a-3p;hsa-miR-23b-3p;hsa-miR-506-3p;hsa-miR-135b-5p;hsa-miR-129-5p;hsa-miR-200c-3p;hsa-miR-17-5p;hsa-miR-20a-5p;hsa-miR-203a;hsa-miR-1;hsa-miR-23a-3p;hsa-miR-181c-5p;hsa-miR-125a-3p;hsa-miR-216a-5p;hsa-miR-26b-5p;hsa-miR-185-5p;hsa-miR-206;hsa-miR-455-5p;hsa-miR-363-3p;hsa-miR-200b-3p;hsa-miR-200a-3p;hsa-miR-429;hsa-miR-30e-5p;hsa-miR-30c-5p;hsa-miR-181b-5p;hsa-miR-181a-5p;hsa-miR-205-5p;hsa-miR-146b-5p;hsa-miR-378c;hsa-miR-141-3p;hsa-miR-26a-5p;hsa-miR-135a-5p;hsa-miR-4306;hsa-miR-494-3p;hsa-miR-328-3p;hsa-miR-140-5p;hsa-miR-22-3p;hsa-miR-142-3p;hsa-miR-181d-5p;hsa-miR-217;hsa-miR-216b-5p;hsa-miR-425-5p;hsa-miR-143-3p;hsa-miR-145-5p;hsa-miR-146a-5p;hsa-miR-590-3p;hsa-miR-876-5p;hsa-miR-873-5p;hsa-miR-204-5p;hsa-miR-23c;hsa-miR-20b-5p;hsa-miR-106a-5p;hsa-miR-194-5p;hsa-miR-202-3p;hsa-miR-3167;hsa-miR-320d;hsa-miR-1297;hsa-miR-92a-3p;hsa-miR-485-5p;hsa-miR-154-5p;hsa-miR-211-5p;hsa-miR-124-3p;hsa-miR-1271-5p;hsa-miR-30a-5p;hsa-miR-93-5p;hsa-miR-106b-5p;hsa-miR-96-5p;hsa-miR-383-5p;hsa-miR-491-5p;hsa-miR-32-5p;hsa-miR-374b-5p;hsa-miR-374a-5p;hsa-miR-224-5p;hsa-miR-101-3p;hsa-miR-320b;hsa-miR-346;hsa-miR-708-5p;hsa-miR-613;hsa-miR-376a-3p;hsa-miR-376b-3p;hsa-miR-544a;hsa-miR-422a;hsa-miR-144-3p;hsa-miR-338-3p;hsa-miR-320c;hsa-miR-150-5p;hsa-miR-519d-3p;hsa-miR-499a-5p;hsa-miR-378b;hsa-miR-367-3p;hsa-miR-3139;hsa-miR-320a;hsa-miR-30b-5p;hsa-miR-30d-5p;hsa-miR-384;hsa-miR-378f;hsa-miR-4429;hsa-miR-4262;hsa-miR-378i;hsa-miR-378d;hsa-miR-378 h;hsa-miR-378e;hsa-miR-4465;hsa-miR-4644;hsa-miR-4770

expressed, including CDKN2B-AS1, HOTAIR and MALAT1, and two of which were lowly expressed, including RRP1B and SRA1.

CDKN2B-AS1, also named ANRIL, which is located as part of the 42 kb INK4b-ARF-INK4a locus on chromosome 9p21.3 [34]. The INK4b-ARF-INK4a locus has an important role in cell cycle control, cell senescence, stem cell renewal and apoptosis through P14ARF-MDM2-P53 and P16INK4a/p15INK4b-Cdk4/6-pRb pathways [35]. More, the INK4b-ARF-INK4a locus is subject to frequent deletion or hypermethylation in cancers, including leukemia, melanoma, lung and bladder cancers [36]. It has been reported an intriguing mechanisms for ANRIL-mediated silencing of the INK4b-ARF-INK4a locus, which was resulted from ANRIL binding two polycomb repressor complexes PRC1 and PRC2 [37]. In LSCC, the INK4b-ARF-INK4a pathway plays an important role as tumor suppressor; therefore, it is possible that lncRNA ANRIL has a significant function on tumor pathogenesis. In this research, we confirmed ANRIL was highly expressed in LSCC, which gave us a hope for further functional investigations.

HOTAIR is one of the most studied lncRNAs involved in genome modification. It has been reported that HOTAIR is significantly overexpressed in many cancers, including breast cancer, hepatocellular cancer and colorectal cancer [38, 39]. Due to the function on promoting invasion of breast cancer cells, significant high expression of HOTAIR is a powerful predictor of final metastasis and death [40]. Recently, many researches have shown that HOTAIR interacts with PRC2 to recruit PcG protein to their target genes in cancer [41]. In the recent research, Li et al. [42] found that HOTAIR was overexpressed and regulated PTEN methylation in laryngeal cancer. However, they did not present a comprehensive investigation on lncRNAs in laryngeal cancer.

MALAT1 is a nuclear lncRNA of more than 8000nt that is expressed from chromosome 11q13. Research report indicated that MALAT1 regulated gene expression and also posttranscriptionally modified primary transcripts [37]. MALAT1 was highly expressed in lung, pancreas and multiple types of cancers [43]. Lai et al. [44] reported that patients with high level of MALAT1 were associated with tumor recurrence after liver transplantation. These associations imply that targeting MALAT1 may show important clinical implication by selectively affecting disseminated cancer cells or residual cancer cells after surgery. Moreover, functional domain study also showed that 3' end of MALAT1 played a crucial role in cell proliferation, migration and invasion [45]. Our research results indicated that MALAT1 was highly expressed in laryngeal tumors,

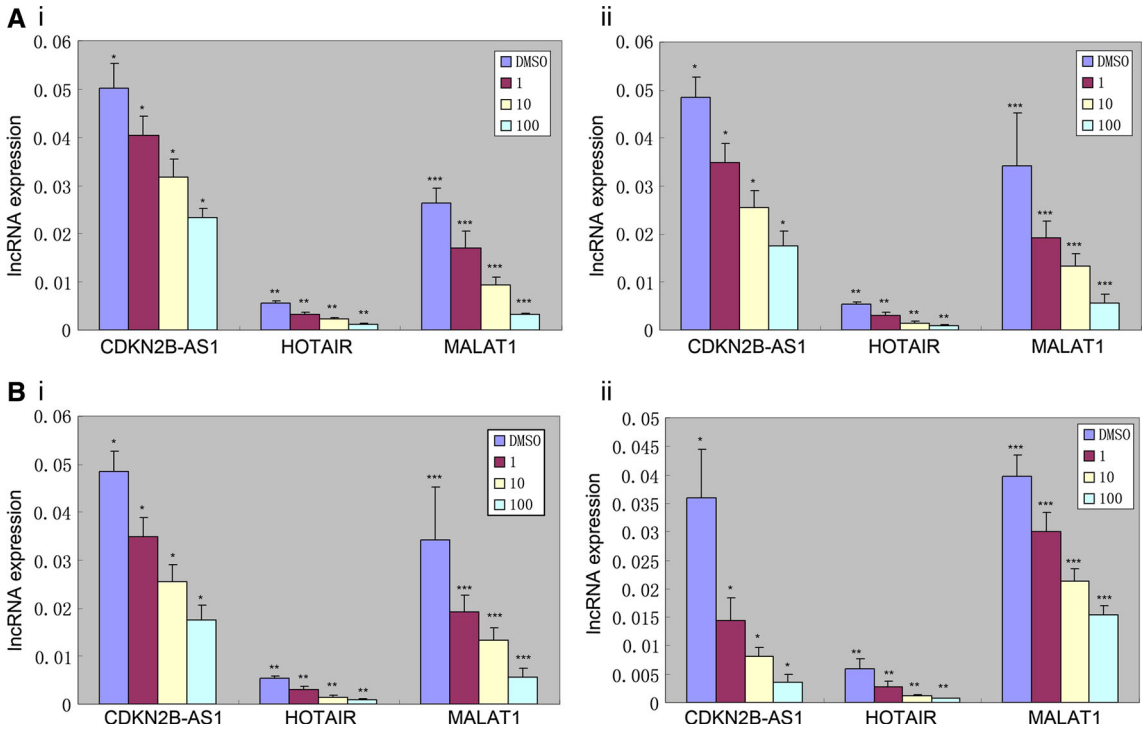

Fig. 4 The expressions of CDKN2B-AS1, HOTAIR and MALAT1 were influenced by increasing the concentration of chemotherapy agents. **a** The chemotherapy assay was performed in laryngeal cancer Hep-2 cells. (**i**) Added 1, 10, 100 μM cisplatin and 0.1 % control DMSO into Hep-2 cells, with the volume of 100 μl. Compared the expressions of the lncRNAs of each concentration group by real-time PCR after incubating for 3 h. The lncRNA expression among each concentration group had statistical difference. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$). (**ii**) Added 1, 10, 100 μM paclitaxel and 0.1 % control DMSO into Hep-2 cells, with the volume of 100 μl. Compared the expression of lncRNAs of each concentration, which got statistical significance. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$). **b** The chemotherapy assay was performed in laryngeal cancer AMC-HN8 cells. (**i**) Treated AMC-HN8 cells with 1, 10, 100 μM cisplatin and 0.1 % control DMSO. The expressions of the lncRNAs of each concentration group were evaluated, which presented significant difference. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$). (**ii**) Treated AMC-HN8 cells with 1, 10, 100 μM paclitaxel. Same volume of 0.1 % DMSO as control. The expressions of lncRNAs of each concentration group were accessed. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$)

which was coherent with published reports and also suggest a possible role in tumor biology.

In this research, we also discovered two significantly lowly expressed lncRNAs, RRP1B and SRA1. RRP1B was firstly identified as a metastasis susceptibility gene in breast cancer. Lee et al. [46] found that RRP1B suppressed metastasis progression, and Crawford et al. [47] also reported that RRP1B might be a novel susceptibility gene for breast cancer progression and metastasis. Our finding also indicated that RRP1B was differentially lowly expressed in laryngeal tumor, which might be a novel significant gene for the cancer treatment. SRA is a unique modulator of steroid receptor transcriptional activity. It appeared highly expressed in liver, skeletal muscle, adrenal gland and pituitary gland, whereas brain and other typical steroid-responsive tissues such as prostate, breast, uterus and ovary contained low levels [48]. According to previous research, the overexpression of SRA core sequence during breast tumorigenesis in patients more likely to survive under Tamoxifen treatment, which suggested SRA1 an unusual bifaceted gene products with suspected relevance to breast cancer [49]. It was the first time that we discovered SRA1 was significantly low expressed in laryngeal tumor; however, considering its bifaceted characteristics, we need more further research to investigate its function and we are making efforts to it.

From previous research, we have discovered the comprehensive microRNA profile in LSCC [28], and the interaction between microRNAs and lncRNAs in laryngeal cancer attracted our attention. Through searching in one of the most comprehensive lncRNA database, we found the possible microRNAs, which interacted with the selected lncRNAs, which provided a deeper aspect for the investigation of laryngeal cancer. Together with our previous research, we assure to find out a microRNA–lncRNA pathway, which plays an important role in laryngeal cancer pathogenesis in future.

In order to find out the influence of chemotherapeutics on the selected high-expressed lncRNAs, chemotherapy assay was performed. Cisplatin and paclitaxel were the

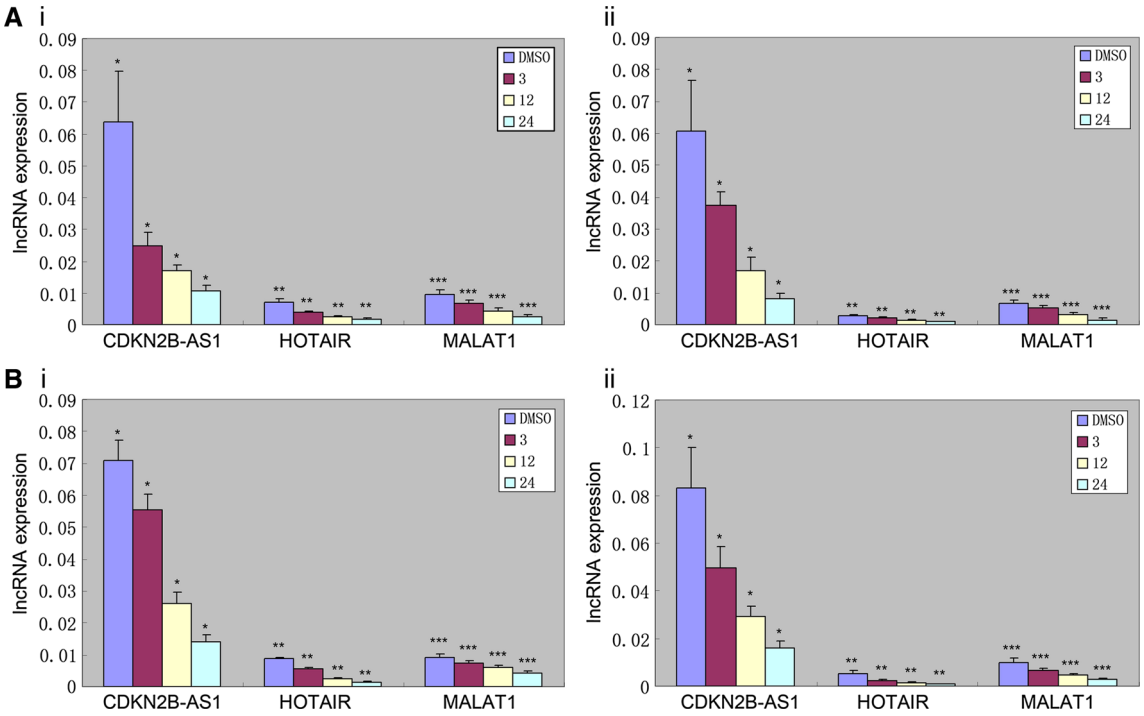

Fig. 5 The expressions of CDKN2B-AS1, HOTAIR and MALAT1 were influenced by lengthening the treating period of chemotherapy agents. **a** The chemotherapy assay was performed in laryngeal cancer Hep-2 cells. (**i**) Added 1 μM cisplatin and 0.1 % control DMSO into Hep-2 cells, with the volume of 100 μl. Incubated for 3, 12 and 24 h, respectively. Compared the expressions of the lncRNAs of each treating period group by real-time PCR. The lncRNA expression among each period group had statistical difference. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$). (**ii**) Added 1 μM paclitaxel and 0.1 % control DMSO into Hep-2 cells. After incubating for different periods, compared the expression of lncRNAs of each period group, which got statistical significance. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$). **b** The chemotherapy assay was performed in laryngeal cancer AMC-HN8 cells. (**i**) Treated AMC-HN8 cells with 1 μM cisplatin and 0.1 % control DMSO. Incubated cells for 3, 12 and 24 h, respectively. The expressions of the lncRNAs of each period group were evaluated, which presented significant difference. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$). (**ii**) Treated AMC-HN8 cells with 1 μM paclitaxel. Same volume of 0.1 % DMSO as control. The expressions of lncRNAs of each treating period group were accessed. Results were mean ± SD ($n = 3$; * $p < 0.05$; ** $p < 0.05$; *** $p < 0.05$)

most common chemotherapeutics in LSCC, and their effects were reached consensus. We treated laryngeal Hep-2 cell line and AMC-HN8 cell line with cisplatin and paclitaxel, respectively, and the influences of the concentration and the treating period on the lncRNAs expression were evaluated. The results revealed that both in Hep-2 and AMC-HN8 cells, with the increasing of the concentration, the relatively expressions of CDKN2B-AS1, HOTAIR and MALAT1 were significantly declined. Moreover, when the treating duration prolonged, the CDKN2B-AS1, HOTAIR and MALAT1 expressions were also dramatically descended. From this result, we obtained one more confirmation of previous experiments' results, and it also implied that these lncRNAs were the targets of the chemotherapeutics, which might have effects on the pathways. Since RRP1B and SRA1 were newly discovered in laryngeal cancer and their expression levels were quite low, chemotherapy assay was not accurate enough to investigate the influence. We intend to utilize further genetic approaches to clarify this later. So far, the investigations of chemotherapeutics like cisplatin and paclitaxel with regard to lncRNAs are a brand new topic, and few published reports about it, which gives us both challenges and hope. With our findings, it helps to promote the development of new chemotherapeutics with a better understanding of both the cancer pathogenesis and pharmacological mechanisms.

In conclusion, for the first time, we reported a comprehensive analysis of lncRNAs in LSCC, and we identified five differentially expressed lncRNAs, including CDKN2B-AS1, HOTAIR, MALAT1, RRP1B and SRA1. Furthermore, we demonstrated a prediction of microRNA–lncRNA interactions through searching comprehensive database and obtain the possible microRNAs, which have interaction effects with confirmed lncRNAs. Additionally, chemotherapy assay indicated that cisplatin and paclitaxel had significant target function on the identified lncRNAs, which presented novel molecular targets to cure patients with LSCC and also led an orientation for the new drug development.

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Conflict of interest None.

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