

# tRNA<sup>Ini</sup><sub>CAT</sub> inhibits proliferation and promotes apoptosis of laryngeal squamous cell carcinoma cell

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## Primary research

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# Abstract

**Background:** Transfer RNA (tRNA) is a ubiquitous RNA, present in all life forms and considered a housekeeping gene with limited regulatory function. Recent studies have shown that tRNA is dysregulated and involved in the pathogenic process of cancer.

**Methods:** Quantitative reverse transcription-polymerase chain reaction detected the relative expression levels of tRNA<sup>lni</sup><sub>CAT</sub> in paired LSCC tissues and paracancerous tissues and preoperative, postoperative, and healthy human plasma. We analyzed the relationship between its expression level and the clinicopathological features of patients. We also established the receiver operating characteristic curve and predicted its diagnostic value. High expression of tRNA<sup>lni</sup><sub>CAT</sub> was detected in the LSCC cell line. The proliferation, apoptosis, and cycle of cells were analyzed. High expression of tRNA<sup>lni</sup><sub>CAT</sub> was detected in mice with transplanted tumors. The tumor weight and volume in mice were measured. The transplanted tumor was used for pathological analysis and transmission electron microscope observation.

**Results:** tRNA<sup>lni</sup><sub>CAT</sub> was down-regulated in LSCC tissues and plasma. The area under the plasma receiver operating characteristic curve was 0.808. High expression of tRNA<sup>lni</sup><sub>CAT</sub> in the laryngeal carcinoma cell line inhibited cell proliferation and promoted apoptosis. High expression of tRNA<sup>lni</sup><sub>CAT</sub> in transplanted tumor in nude mice inhibited the growth of the transplanted tumor.

**Conclusion:** tRNA<sup>lni</sup><sub>CAT</sub> acts as a tumor inhibitor in LSCC, inhibits cell proliferation, and promotes apoptosis *in vitro* and *in vivo*. tRNA<sup>lni</sup><sub>CAT</sub> may be used as a new biomarker for the early diagnosis of LSCC.

## Background

Laryngeal cancer is a common malignant tumor of the head and neck. It is common among men aged 50–70 years. Its incidence is 1.5–3/100,000, accounting for approximately 1% of all malignant tumors. Laryngeal squamous cell carcinoma (LSCC) is the most common type, accounting for approximately 90% of cases.<sup>1</sup> At present, the incidence of laryngeal cancer is increasing. Treatment is mainly through surgical resection, combined with preoperative or postoperative chemotherapy, radiotherapy, and other comprehensive therapies. However, vocal disorders occur following surgery, affecting the quality of life of patients. Globally, more than 80,000 patients with throat cancer expire each year.<sup>2</sup>

Transfer RNA (tRNA) is a universal RNA present in all forms of life. A mature tRNA is characterized by its secondary structure, which consists of a D ring, a counterpasser ring, a variable ring, a T ring, and an receiving arm, as well as an L tertiary structure maintained by hydrogen bonds.<sup>3</sup> As a basic component of the translation process, tRNA transport amino acids to the ribosome; the genetic information of the nucleotide sequence is transformed into the corresponding polypeptide chain in the manner of codon (mRNA)-anti-crypton (tRNA) interaction;<sup>4,5</sup> this process is essential to maintain the normal life activities

of the body. More than half of RNA modifications occur in tRNA.<sup>6</sup> These modifications affect the structure, stability, and functionality of tRNA, leading to widespread cellular effects; of those, methylation modification is the most common effect.<sup>7</sup>

Early studies reported that tRNA plays only the role of a "porter", and does not possess regulatory function. Nevertheless, as research progresses, tRNA have been found to be involved in various physiological and pathological processes, including cancer, diabetes, and neuronal disease.<sup>8-11</sup> The role of tRNA in LSCC remains unknown and there is no complete tRNA expression map on this disease. This study established tRNA expression profiles from eight pairs of LSCC and paracancerous tissues via a microarray platform. In addition, the relative expression levels of tRNA<sup>Ini</sup><sub>CAT</sub> in tumor and paracancerous tissues, preoperative and postoperative plasma of patients with LSCC and plasma of healthy human were examined. The tRNA<sup>Ini</sup><sub>CAT</sub> was analyzed to determine the relationship between its expression level and clinicopathological features. The work characteristic curves of the subjects were established to predict their diagnostic value. The tRNA<sup>Ini</sup><sub>CAT</sub> was upregulated in laryngeal cancer cell lines. The proliferation, apoptosis, and cycle of cells were analyzed. High expression of tRNA<sup>Ini</sup><sub>CAT</sub> was observed in mice transplanted with tumors. The tumor weight and volume in mice were measured, and the tumors were analyzed by pathology and transmission electron microscopy (TEM). Finally, the usefulness of tRNA<sup>Ini</sup><sub>CAT</sub> as biomarker and potential therapeutic target for LSCC was examined.

## Materials And Methods

### Collection of specimens and clinical data

Patients with LSCC who underwent otorhinolaryngology and head and neck surgery at Li Huili Hospital affiliated to Ningbo University (Ningbo, China) from January 2013 to April 2019 without receiving radiotherapy, chemotherapy, or other antitumor treatments were selected. Tumor tissue and paracancerous tissue samples were obtained from surgery. All LSCC samples were confirmed by at least two senior pathology experts. Healthy human plasma samples were used to obtain plasma samples before and after surgery. Tumor staging was determined according to the American Joint Committee on Cancer tumor-lymph node-metastasis (TNM) staging (2017, 8th edition). This study was approved by the Ethics Committee of Human Research of Ningbo University (No.KY2015PJ012). Written informed consent was provided by all subjects.

### Total RNA preparation and synthesis of cDNA

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and TRIzol LS reagents (Invitrogen) was used to extract the total RNA of tissues/cells and plasma through the SmartSpec Plus spectrophotometer (BioRad, Hercules, CA, USA). The purity and concentration of total RNA were determined. The RNA quality control standards were as follows: A260:A230 ratio > 1.7; A260:A280 ratio 1.8–2.1. According to the rtStar™ tRNA-optimized First-Strand cDNA Synthesis Kit (Arraystar, Rockville, MD, USA) instructions, 2 µg of total RNA was used for demethylation reactions and cDNA synthesis. For assessing the quality of

synthetic cDNA, 1  $\mu$ l of RNA Spike-in quantitative polymerase chain reaction (qPCR) Primer Mix, use  $\mu$ l cDNA, 2.5  $\mu$ l GoTaq qPCR Mixture (Promega, Madison, WI, USA) and 2  $\mu$ l of enzyme-free water were added; only samples with cycle threshold (Ct) values < 30 were used for qPCR detection.

### **qPCR**

The cDNA was diluted with enzyme-free water (1:20). The Applied Biosystems 7900 real-time PCR system (Thermo-Fisher, USA) was used to qPCR. 88 kinds of tRNA primers were bought from Arraystar, Inc, Rockville, MD, USA. U6 was used as a control, and the relative expression level of tRNA was determined by the  $\Delta\Delta$ Ct method. U6 primers were purchased from BGI (Shanghai, China). The U6 sequences were as follows: justice, 5'- GCTTCGGCAGCACATATACTAAAAT-3'; Antimony 5'- CGCTTCACGAATTTGCGTCAT-3'.

### **Cell culture and transfection**

The LSCC cell line AMC-HN-8 was purchased from BeNa Culture Collection, Shanghai, China. The cells were cultured in RPMI 1640 (HyClone, Logan, UT, USA) 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) at 37 °C and 5% CO<sub>2</sub>. Cells were counted using a TC10 automatic cell counter (BioRad). The lentivirus was purchased from GenePharma (Shanghai, China). Stably transfected cells were selected through purine mycin. The tRNA<sup>ini</sup><sub>CAT</sub> of the overexpression lentivirus is termed LV3-tRNA<sup>ini</sup><sub>CAT</sub>.

Sequences: 5'- AGCAGAGTGGCG

CAGCGGAAGCGTGCTGGGCCATAACCCAGAGGTCGATGGATCGAAACCATCCTCTGCTA-3', LV3-NC used as a control, sequence: 5'- TTCTCCGAACGTGTCACGT-3'.

### **Cell inhibition rate test**

AMC-HN-8 cells were digested with trypsin to form a single-cell suspension and inoculated on a six-well plate. A culture medium containing 3 H-TdR was added for 16 h, and the cells were digested and collected. Finally, a Micro Beta 2450 liquid scintillation counter (Perkin Elmer, Waltham, Mass, USA) was used to determine the minutes (cpm), and the inhibition rate was calculated by the average cpm of three wells.

### **Cell cycle and apoptosis analysis**

The cells were starved (serum) to synchronize the cell cycle. Subsequently, the cells were collected, washed with phosphate-buffered saline, and fixed in 70% ethanol at -20 °C for 24 h. Next, staining with DNA dye solution was performed using the Cell Cycle Detection Kit (KeyGEN, Jiangsu, China), and the cells were incubated for 30 min. Lastly, BD FACSCanto II flow cytometry (BD Biosciences, San Jose, CA, USA) Testing was performed. Cells were digested with trypsin (no ethylenediaminetetraacetic acid) into a single-cell suspension, and resuspended in binding buffer. The cells were then stored at room temperature. Using an Annexin V-FITC/PI apoptosis kit (CoWin Biosciences, Beijing, China), the cells were stained for 15 min without light, and the rate of apoptosis was detected using BD FACSCanto II flow cytometry (BD Biosciences).

### **Mouse models of xenotransplantation**

All animal studies were conducted in accordance with the animal protocol approved by the animal protection and use committee of Zhejiang University of Traditional Chinese Medicine (No.2019-007), Hangzhou, Zhejiang, China. BALB/c female nude mice were purchased from Charles River (Beijing, China) and raised in the experimental animal center of Zhejiang University of Traditional Chinese Medicine. The mice were anesthetized with pentobarbital sodium (45 mg/kg); the laryngeal skin was removed and the larynx was inoculated with AMC-HN-8-Luc cell suspension. The mice were randomly divided into three groups (9 or 10 mice per group). Tumor growth was observed using an IVIS Lumina XRMS small animal *in vivo* imager (Perkin Elmer) after 1 week. The mice in each group were injected with 50  $\mu$ l of corresponding medicine solution within the tumor (twice per week for 21 consecutive days). The weight and tumor volume in mice were measured twice per week. The following day, the mice were sacrificed, and the solid tumor was resected and weighed. Conventional hematoxylin-eosin pathological examination and electron microscopy were performed on the tumor tissue.

### **Staining and TEM of hematoxylin-eosin**

The tumor tissue was fixed in 10% formalin, embedded in paraffin, cut into sections (thickness: 8  $\mu$ m) and baked at 45 °C for 5 h. Subsequently, the tissues were dewaxed with xylene for 30 min, treated with different concentrations of ethanol (100%, 90%, 70%); hydrated with distilled water, stained with hematoxylin for 15 min, differentiated in ethanol and ammonia, dehydrated with ethanol, stained with eosin, dehydrated again with ethanol, and washed with xylene. Tissue morphology was observed by optical microscopy. The tumor tissue was fixed with 2.5% glutaraldehyde for 1 h and washed with phosphate-buffered saline for 1 h. The tumor tissue was subsequently fixed in 1% osmium acid for 30 min–1 h, and dehydrated in ethanol and acetone. Finally, the tissue was embedded in epoxy resin and stained with uranyl acetate and lead citrate. The Hitachi H-7650 TEM (Tokyo, Japan) was used to observe the changes in autophagy and apoptosis morphology.

### **Statistical analysis**

The SPSS version 20.0 software (IBM Corp., Armonk, NY, USA) and GraphPad 8.0 (San Diego, CA, USA) were used for data analysis. The relative expression level of tRNA<sup>Ini</sup><sub>CAT</sub> between LSCC tissues and adjacent non-tumor tissues was analyzed by paired sample t test. One-way analysis of variance was used to analyze the level of tRNA<sup>Ini</sup><sub>CAT</sub> expression in the preoperative plasma, postoperative plasma, and plasma of patients with LSCC. The correlation between the expression level and clinicopathological factors was analyzed using the chi-squared test. The diagnostic value was assessed using the Receiver operating characteristic curve (ROC). P-values < 0.05 denoted statistically significant differences.

## **Results**

### **Differences in tRNA expression in LSCC and adjacent tissues**

The cDNA microarray results indicated that the expression levels of 88 tRNA (including intracellular tRNA and mitochondrial tRNA) in LSCC and paracancerous tissues were detected in eight pairs. The results showed that the expression profiles of tRNA in LSCC tissues were different from those observed in

adjacent tissues (Figs. 1 and 2). We found that two tRNA doubled their expression levels in LSCC and paracancerous tissues: tRNA<sup>lni</sup><sub>CAT</sub> (2.24-fold change) and mt-tRNA<sup>Glu</sup><sub>TTC</sub> (- 2.21-fold change). Meanwhile, the first 20 tRNA had higher expression levels in LSCC than in adjacent tissues and were all intracytoplasmic tRNA. In contrast, 18 of the first 20 tRNA with lower expression in LSCC tissues than in adjacent tissues were mitochondrial tRNA (Figs. 3 and 4).

### **Low expression of tRNA<sup>lni</sup><sub>CAT</sub> in LSCC tissues**

To validate the results of the PCR array, tumor and paracancerous tissue samples were collected from a total of 100 patients with LSCC through quantitative reverse-transcription PCR to determine the relative expression levels of tRNA<sup>lni</sup><sub>CAT</sub>. However, as shown in Fig. 5, the expression levels of tRNA<sup>lni</sup><sub>CAT</sub> in tumor tissues were significantly lower than those measured in adjacent paracancerous tissues ( $p = 0.0077$ ). Among those, the expression levels of tRNA<sup>lni</sup><sub>CAT</sub> in 63 LSCC tissues (63%) were lower than those recorded in the corresponding paracancerous tissues (Fig. 6).

### **tRNA<sup>lni</sup><sub>CAT</sub> in plasma of patients with LSCC was downregulated**

By exploring the relative expression levels of tRNA<sup>lni</sup><sub>CAT</sub> in plasma, we obtained consistent results with those reported for tumor tissues. The expression levels of tRNA<sup>lni</sup><sub>CAT</sub> in the preoperative plasma of patients with LSCC were significantly lower than those noted in postoperative plasma ( $p = 0.0032$ ) and healthy human plasma ( $p < 0.0001$ ). Meanwhile, the expression levels in postoperative plasma were significantly lower than those observed in healthy human plasma ( $p = 0.0458$ ) (Fig. 7).

### **tRNA<sup>lni</sup><sub>CAT</sub> expression levels and clinicopathological characteristics of patients with LSCC and potential diagnostic value**

Tissues were divided into low and high expression groups according to the expression levels of tRNA<sup>lni</sup><sub>CAT</sub>. Subsequently, we analyzed the relationship between the expression levels of tRNA<sup>lni</sup><sub>CAT</sub> and clinicopathological factors. The results showed that the expression levels of tRNA<sup>lni</sup><sub>CAT</sub> were not associated with clinicopathological features (Table 1). The area under the ROC curve for tRNA<sup>lni</sup><sub>CAT</sub> in tissues and plasma was 0.575 and 0.808, respectively (Fig. 8).

Table 1

Relationship between the expression levels of tRNA<sup>Ini</sup><sub>CAT</sub> ( $\Delta$ Ct) and clinicopathological features of patients with LSCC

Characteristics	n (%)	Low (%)	High (%)	P-value
All cases	86 (100)	56 (65.1)	30 (34.9)	
Age, years				0.949
≤ 60	34 (39.5)	22 (39.3)	12 (40)	
> 60	52 (60.5)	34 (60.7)	18 (60)	
Primary location				0.851
Supraglottic	24 (27.9)	16 (28.6)	8 (26.7)	
Glottic	62 (72.1)	40 (71.4)	22 (73.3)	
Differentiation				0.732
Well & moderate	67 (77.9)	43 (76.8)	24 (80)	
Poor	19 (22.1)	13 (23.2)	6 (20)	
Lymphatic metastasis				0.571
N0	75 (87.2)	45 (85.7)	27 (90)	
N1–N3	11 (12.8)	8 (14.3)	3 (10)	
Invasion				0.949
T1–T2	52 (60.5)	34 (60.7)	18 (60)	
T3–T4	34 (39.5)	22 (39.3)	12 (40)	
Clinical stage				0.907
I–II	48 (55.8)	31 (55.4)	17 (56.7)	
III–IV	38 (44.2)	25 (44.6)	13 (43.3)	
Smoking history				0.701
Yes	55 (64)	35 (62.5)	20 (66.7)	
No	31 (36)	21 (37.5)	10 (33.3)	
Ct, cycle threshold; LSCC, laryngeal squamous cell carcinoma				

High expression of tRNA<sup>Ini</sup><sub>CAT</sub> inhibits LSCC cell growth

AMC-HN-8 cells were transfected using LV3-tRNA<sup>lni</sup><sub>CAT</sub> to upregulate tRNA<sup>lni</sup><sub>CAT</sub> and negative control (LV3-NC) to assess the role of tRNA<sup>lni</sup><sub>CAT</sub> in tumor progression. Following transfection of LV3-tRNA<sup>lni</sup><sub>CAT</sub> and LV3-NC in AMC-HN-8 cells, green fluorescent protein fluorescence expression was observed (Fig. 9). The levels of tRNA<sup>lni</sup><sub>CAT</sub> in the cells showed a 2.1-fold increase (Fig. 10). A cell inhibition rate analysis was performed to determine the effect of tRNA<sup>lni</sup><sub>CAT</sub> on LSCC cell inhibition. The results showed that overexpression of tRNA<sup>lni</sup><sub>CAT</sub> significantly inhibited cell growth compared with the NC group (Fig. 11).

### **Effects of tRNA<sup>lni</sup><sub>CAT</sub> on cell cycle and apoptosis**

For further exploration of the potential regulatory mechanisms of tRNA<sup>lni</sup><sub>CAT</sub> in cell proliferation, cell cycle and apoptosis were assessed by flow cytometry. The upregulated tRNA<sup>lni</sup><sub>CAT</sub> did not have a significant effect on the cell cycle (Fig. 12). Furthermore, the results of the apoptosis analysis showed that transfection of LV3-tRNA<sup>lni</sup><sub>CAT</sub> significantly promoted apoptosis in AMC-HN-8 cells (Fig. 13). Collectively, these results indicated that tRNA<sup>lni</sup><sub>CAT</sub> promoted apoptosis.

### **tRNA<sup>lni</sup><sub>CAT</sub> inhibits growth of LSCC xenograft**

We used an *in vivo* xenograft mouse model to investigate the role of tRNA<sup>lni</sup><sub>CAT</sub> in inhibiting cancer. The mice were divided into three groups: LV3-tRNA<sup>lni</sup><sub>CAT</sub> (high and low doses) and LV3-NC (Fig. 14). The results indicated that upregulation of tRNA<sup>lni</sup><sub>CAT</sub> significantly inhibited tumor growth (Fig. 15). Among them, the weight of the transplanted tumor in the high dose group was significantly smaller than that in the NC group. During the experiment, there was no significant difference in tumor volume and mouse weight in these groups (Figs. 16 and 17). Pathological findings indicated that the administration of LV3-tRNA<sup>lni</sup><sub>CAT</sub> significantly increased tissue necrosis in nude mice transplanted with tumors, showing a certain role of tRNA<sup>lni</sup><sub>CAT</sub> in promoting apoptosis (Fig. 18). Through TEM, apoptotic cells were shown to have special structural characteristics. The autophagy and apoptosis of the tumor in the LV3-tRNA<sup>lni</sup><sub>CAT</sub> treatment group were significantly increased, the phagocytic structure in the bubble was upgraded, and the nucleus had slightly shrunk (Fig. 19). Therefore, we concluded that tRNA<sup>lni</sup><sub>CAT</sub> promotes LSCC cell apoptosis *in vivo*.

## **Discussion**

The expression spectrum of early LSCC mainly focused on mRNA and miRNA, such as DJ-1,<sup>12</sup> HuR,<sup>13</sup> and miR-34a,<sup>14</sup> which were found to be associated with this disease. In the current study using gene chip technology, the expression profiles of tRNA in LSCC and normal tissues were found to differ. This study provides a complete expression profile of tRNA in LSCC.

tRNA was widely regarded as a housekeeping gene with limited regulatory function. There are only few tRNA with known additional functions,<sup>15</sup> other than their usefulness as adapters for protein synthesis. In recent years, a growing body of evidence suggests that tRNA and its derivatives are dysregulated and

participate in the pathogenic process of cancer.<sup>16</sup> Mutations in tRNA and the involvement of co-proteins produced by tRNA biogenesis and modification were found to be associated with cancer.<sup>17-19</sup> Studies have found that tRNA synthesis was controlled by various oncogenic and tumor suppressor genes. Ras<sup>20</sup> and c-Myc<sup>21</sup> facilitate RNA Pol III transcription; however, Rb<sup>22</sup> and p53<sup>23</sup> inhibit RNA Pol III transcription, leading to severe dysregulation of tRNA levels in multiple types of cancer. In addition, in several types of cancer, tRNA modified enzymes can add some tRNA modification, which changes their codon preferences and leads to an increase in protein expression levels of mRNA with "preference" codons.<sup>24,25</sup> Meanwhile, mutations in mitochondrial tRNA cause mitochondrial dysfunction which is also associated with tumorigenesis.<sup>26</sup>

Contrary to the profiling result, the present study confirmed the relative levels of tRNA<sup>Ini</sup><sub>CAT</sub> expression in 100 pairs of paired tissue samples, and revealed that tRNA<sup>Ini</sup><sub>CAT</sub> expression in LSCC tissues was downregulated (Fig. 5). tRNA<sup>Ini</sup><sub>CAT</sub> was downregulated in LSCC preoperative plasma compared with postoperative plasma; this finding was consistent with the results obtained in tissues (Fig. 7). Moreover, this study demonstrated that tRNA<sup>Ini</sup><sub>CAT</sub> may act as tumor inhibitor in LSCC *in vitro* and *in vivo*. Firstly, we used a LV3-tRNA<sup>Ini</sup><sub>CAT</sub> lentivirus to upregulate tRNA<sup>Ini</sup><sub>CAT</sub> in LSCC cells (Fig. 10). Cell inhibition rate analysis was conducted to determine the effect of tRNA<sup>Ini</sup><sub>CAT</sub> overexpression on cell proliferation. The data showed that overexpression of tRNA<sup>Ini</sup><sub>CAT</sub> inhibited cell growth (Fig. 11). Moreover, cell cycle and apoptosis were evaluated to further investigate the biological functions of tRNA<sup>Ini</sup><sub>CAT</sub> in LSCC cells. The results showed that the upregulation of tRNA<sup>Ini</sup><sub>CAT</sub> did not significantly affect the cell cycle (Fig. 12). However, overexpression of tRNA<sup>Ini</sup><sub>CAT</sub> improved the rate of apoptosis in treated AMC-HN-8 cells (Fig. 13). Lastly, we reached the same conclusion based on experiments in BALB/c mouse xenografts; tRNA<sup>Ini</sup><sub>CAT</sub> inhibited tumor growth and promoted tumor cell apoptosis *in vivo*. Overexpression of tRNA<sup>Ini</sup><sub>CAT</sub> significantly decreased the tumor weight of the xenografts (Fig. 15). By pathological examination and TEM, we found that overexpression of tRNA<sup>Ini</sup><sub>CAT</sub> significantly increased tumor cell apoptosis and autophagy (Figs. 18 and 19).

The findings of this study are consistent with those of previous studies. Numerous studies have confirmed the important role of tRNA in regulating gene expression. Change in bacterial activity tRNA content plays an adaptive role with environmental signal changes.<sup>27</sup> The binding of tRNA to cytochrome C inhibits the effects of cytochrome C and apoptotic proteases, thereby inhibiting apoptosis and enzyme activity.<sup>28</sup> Overexpression of the initial tRNA (tRNA<sub>i</sub><sup>Met</sup>) was observed in normal mammary epithelial cells. Changes in the whole cell tRNA expression levels accelerate the speed of cell proliferation.<sup>29</sup> In carcinoma-associated fibroblasts, tRNA<sub>i</sub><sup>Met</sup> promotes tumor proliferation and angiogenesis.<sup>30</sup> Studies revealed that tRNA<sup>Ile</sup> expression was higher in breast cancer-associated fibroblasts than in normal fibroblasts.<sup>30,31</sup> These abnormally expressed tRNA molecules are expected to be new prognostic markers for such diseases.<sup>32</sup> tRNA<sup>Glu</sup><sub>UUC</sub> and tRNA<sup>Arg</sup><sub>CCG</sub> are highly expressed in breast cancer and enhance

tumor invasion. EXOSC2 and GRIPAP1 are downstream target proteins of tRNA<sup>Glu</sup><sub>UUC</sub> to promote the progression of cancer invasion and metastasis.<sup>33</sup> In human epidermal growth factor receptor 2-positive breast cancer cell lines, free tRNA<sup>Leu</sup> may interact with human epidermal growth factor receptor 3 (ErbB3) and binding protein (EBP1), which in turn activates the ErbB2/ErbB3 signaling pathways, and ultimately promotes cancer cell proliferation.<sup>34</sup> tRNA plays a key role in translation, and its dysregulation may affect cell protein expression. Cancer cells regulate the expression of multiple promoters of cancer progression by regulating the levels of tRNA. This leads to the notion that cancer cells exhibit tRNA regulatory effects in addition to the numerous known regulatory mechanisms that alter the expression of cancer progression promoters. Disorders of tRNA<sup>Ini</sup><sub>CAT</sub> we found in LSCC may cause dysregulation of downstream target protein expression, which in turn changes the ability of tumor proliferation and apoptosis.

## Conclusion

The present study provides a comprehensive tRNA expression spectrum for LSCC. The novel tRNA<sup>Ini</sup><sub>CAT</sub> can inhibit the proliferation of LSCC cells and promote apoptosis *in vitro* and *in vivo*. Studies show that tRNA<sup>Ini</sup><sub>CAT</sub> may be a diagnostic biomarker and a potential therapeutic target in LSCC.

## Abbreviations

LSCC, laryngeal squamous cell carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; tRNA, transfer RNA; TEM, transmission electron microscopy; ROC, Receiver operating characteristic curve.

## Declarations

### Ethics approval and consent to participate

This study was approved by the Ethics Committee of Human Research of Ningbo University, Ningbo, Zhejiang, China. This study was approved by the Animal Ethical Care and Use Committee of Zhejiang University of Traditional Chinese Medicine, Hangzhou, Zhejiang, China.

### Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author upon request.

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### Competing interests

The authors report no conflicts of interest in this work.

### Authors' contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

### Consent for publication

Authors involved in this paper all signed written consent for publishing

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## Figures

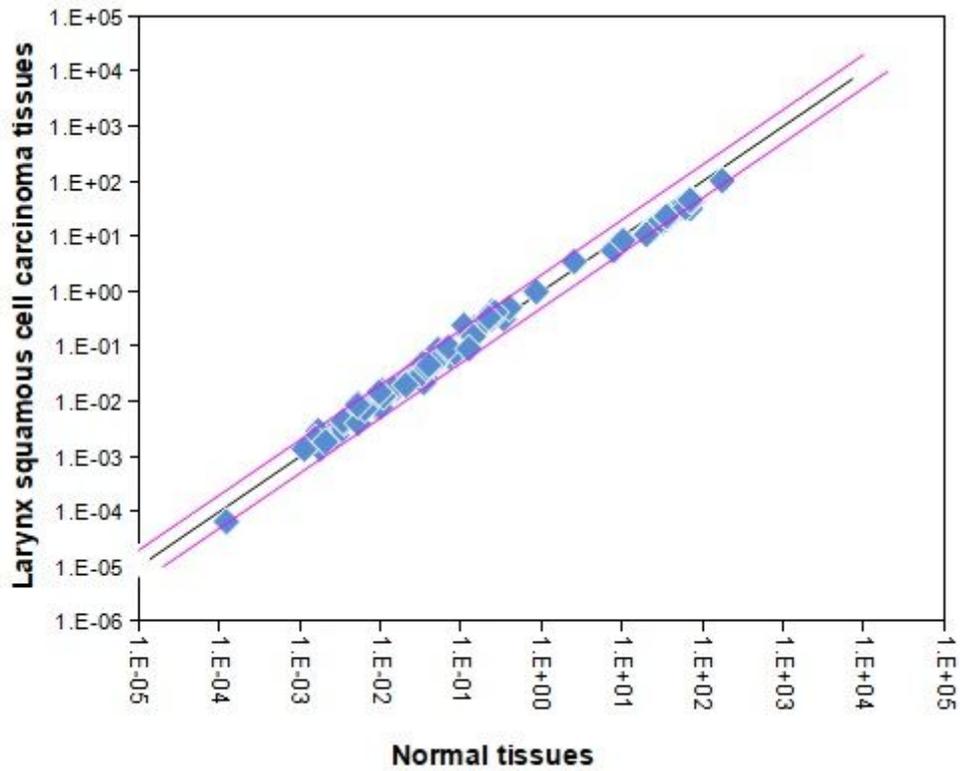
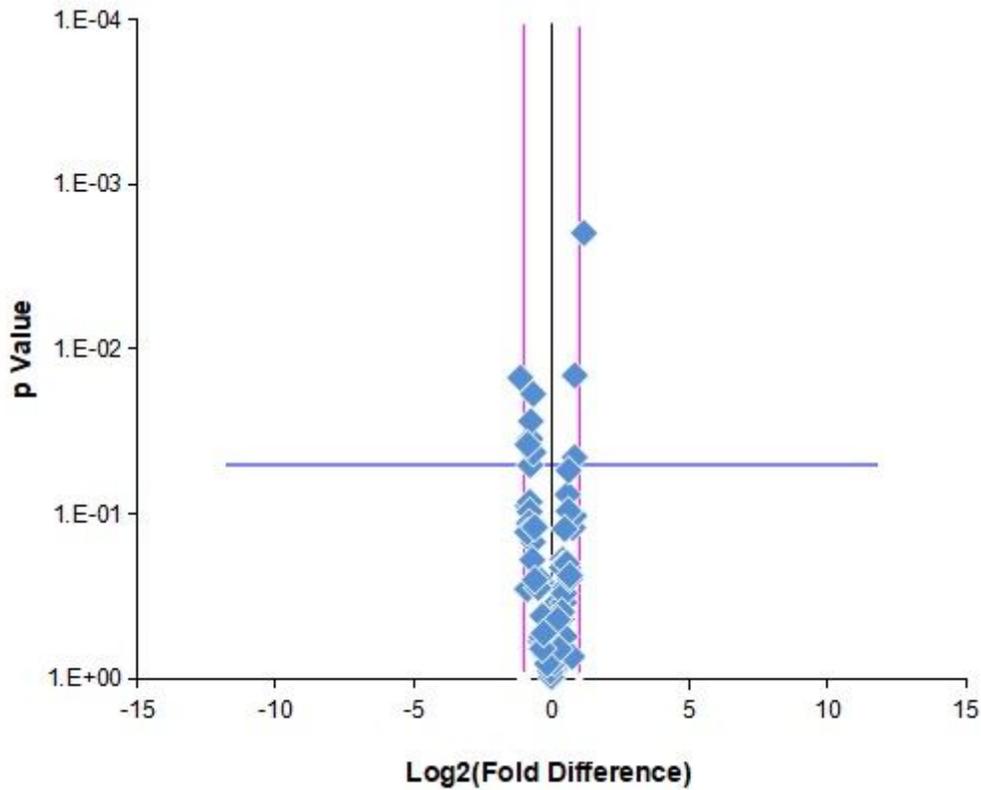


Figure 1

The expression levels of tRNA in LSCC and paracancerous tissues. Black line indicates a multiple change of 1 (no difference). Pink line indicates a multiple change of 2. tRNA, transfer RNA; LSCC, laryngeal squamous cell carcinoma



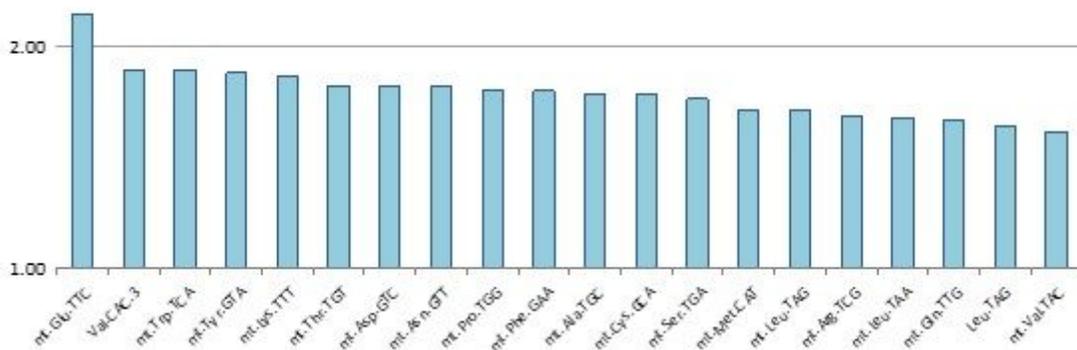
**Figure 2**

The expression levels of tRNA in LSCC and paracancerous tissues. Black line indicates a multiple change of 1. Pink line indicates a multiple change of 2. Blue line indicates the p-value cutoff (0.05). tRNA, transfer RNA; LSCC, laryngeal squamous cell carcinoma



**Figure 3**

Highly expressed tRNA in LSCC tissues (Top 20) tRNA, transfer RNA; LSCC, laryngeal squamous cell carcinoma



**Figure 4**

Lowly expressed tRNA in LSCC tissues (top 20) tRNA, transfer RNA; LSCC, laryngeal squamous cell carcinoma

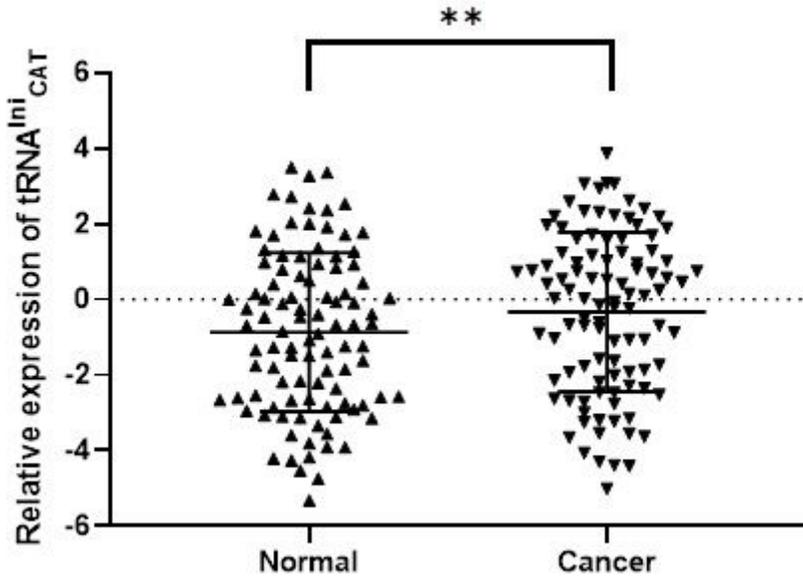


Figure 5

tRNA<sup>Ini</sup><sub>CAT</sub> relative expression levels in LSCC and paracancerous tissues. n=100; \*\*p<0.01. LSCC, laryngeal squamous cell carcinoma

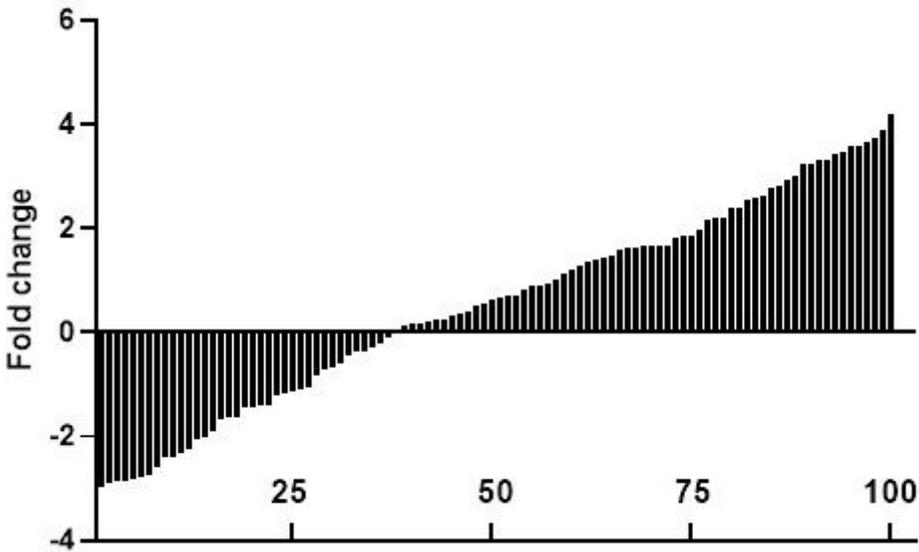


Figure 6

Relative expression levels of tRNA<sup>Ini</sup><sub>CAT</sub> in 100 pairs of LSCC and paracancerous tissues. LSCC, laryngeal squamous cell carcinoma

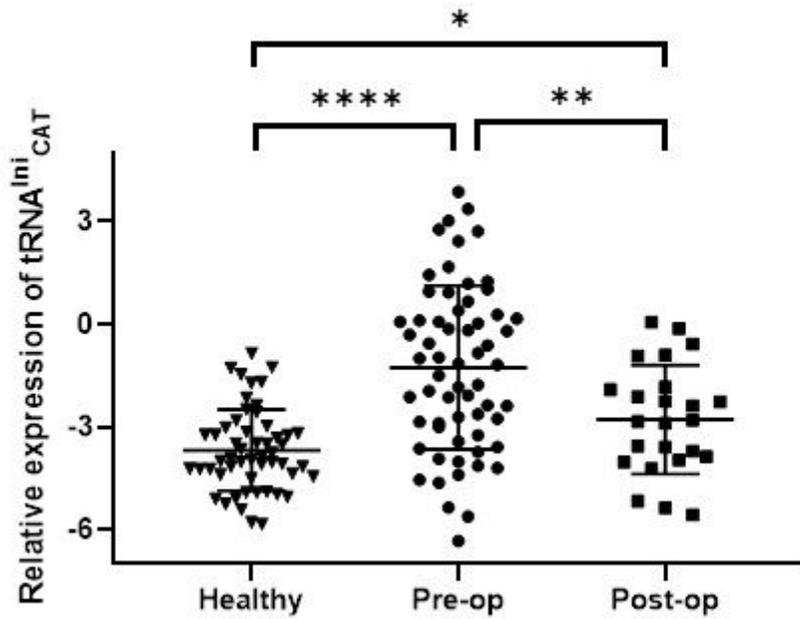


Figure 7

tRNA<sup>Ini</sup>CAT relative expression levels in preoperative plasma (n=62), postoperative plasma (n=24), and healthy human plasma (n=53) of patients with LSCC. \*p<0.05; \*\*p<0.01; \*\*\*\*p<0.0001. LSCC, laryngeal squamous cell carcinoma; Pre-op, preoperative; Post-op, postoperative

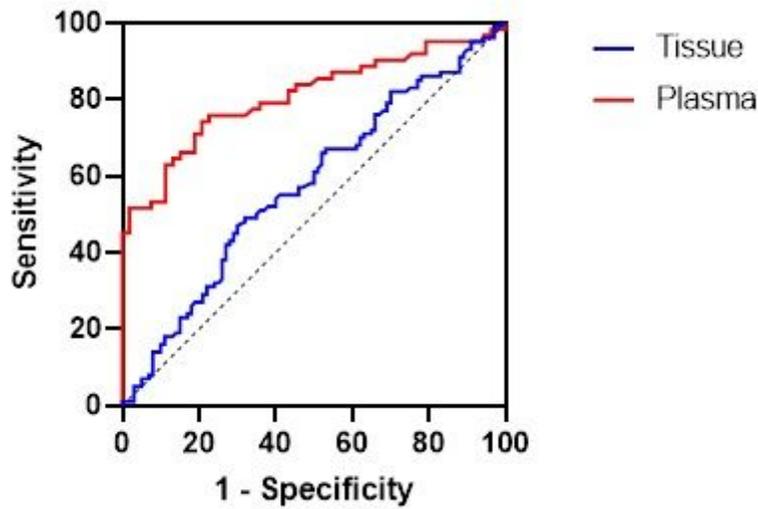
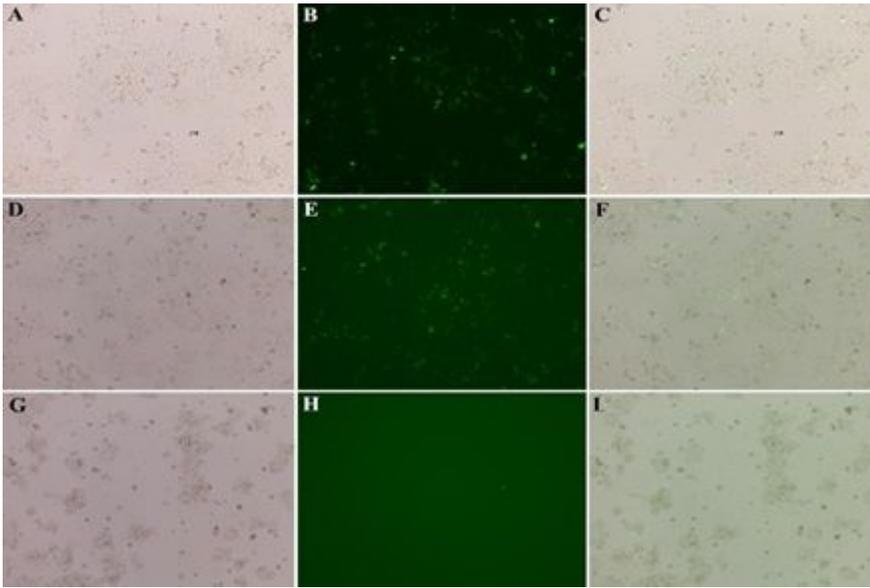


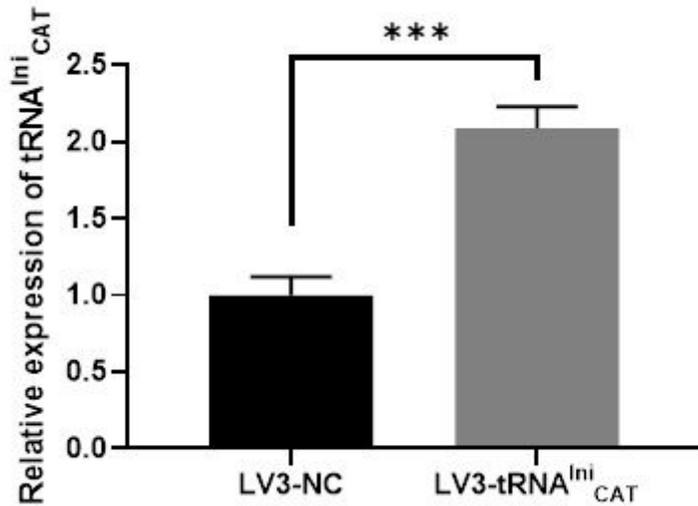
Figure 8

tRNA<sup>Ini</sup>CAT ROC curves in tissues and plasma ROC, receiver operating characteristic



**Figure 9**

Fluorescence microscopy findings showing GFP fluorescence expression in AMC-HN-8 cells after transfection with LV3-tRNA<sup>Ini</sup>CAT and LV3-NC (magnification: ×100). A–C: LV3-tRNA<sup>Ini</sup>CAT groups; D–F: LV3-NC groups; G–I: blank. GFP, green fluorescent protein



**Figure 10**

qRT-PCR analysis of the relative expression levels of tRNA<sup>Ini</sup>CAT in AMC-HN-8 cells. \*\*\*p<0.001. qRT-PCR, quantitative reverse transcription-polymerase chain reaction

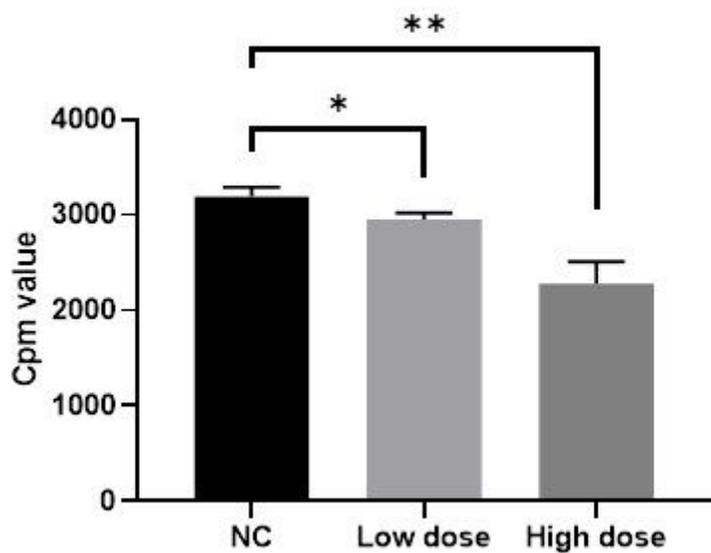


Figure 11

A liquid scintillation counter was used to measure the count per min (cpm) of the three groups. \* $p < 0.05$ ; \*\* $p < 0.01$ . NC, negative control

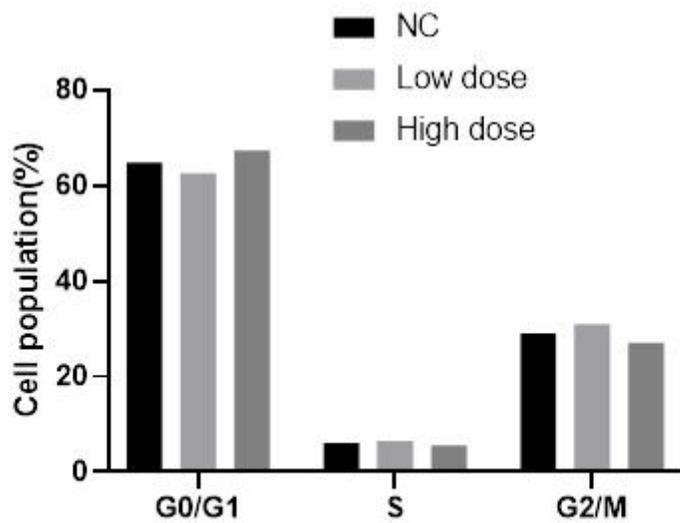
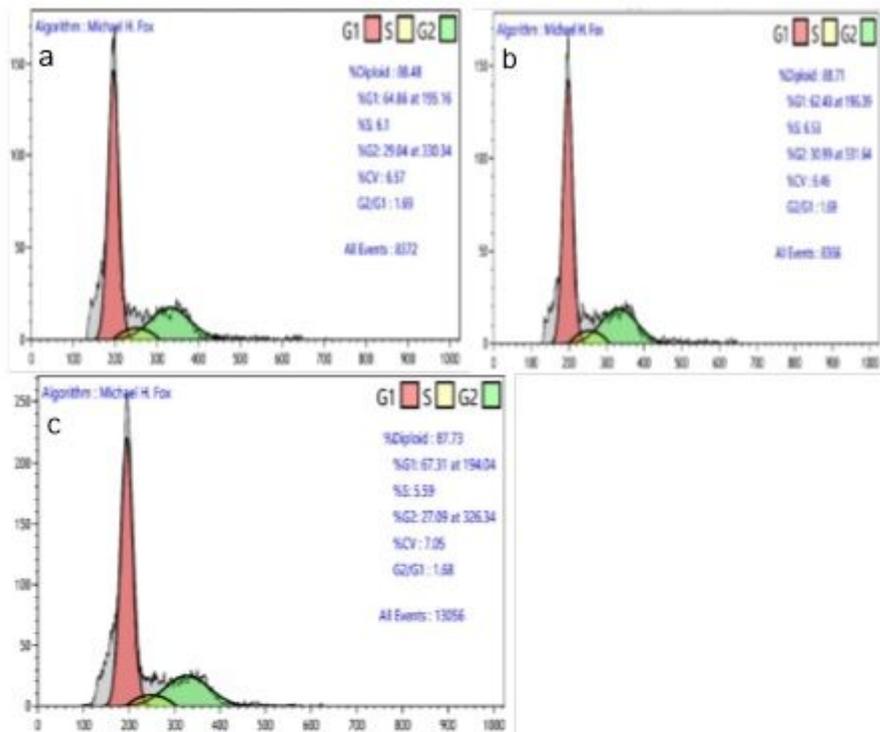
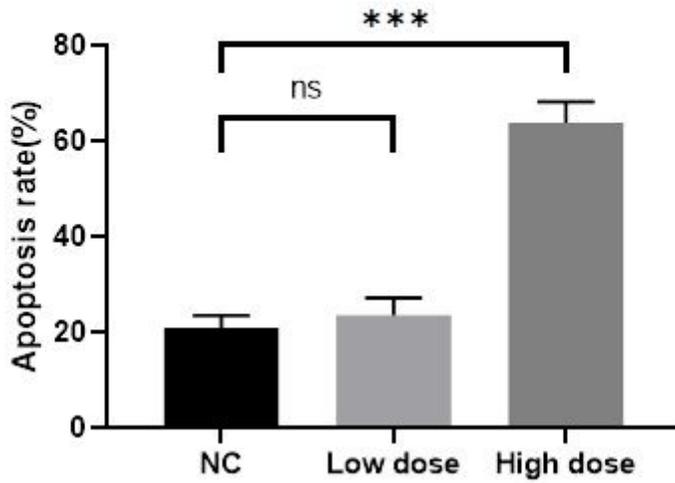
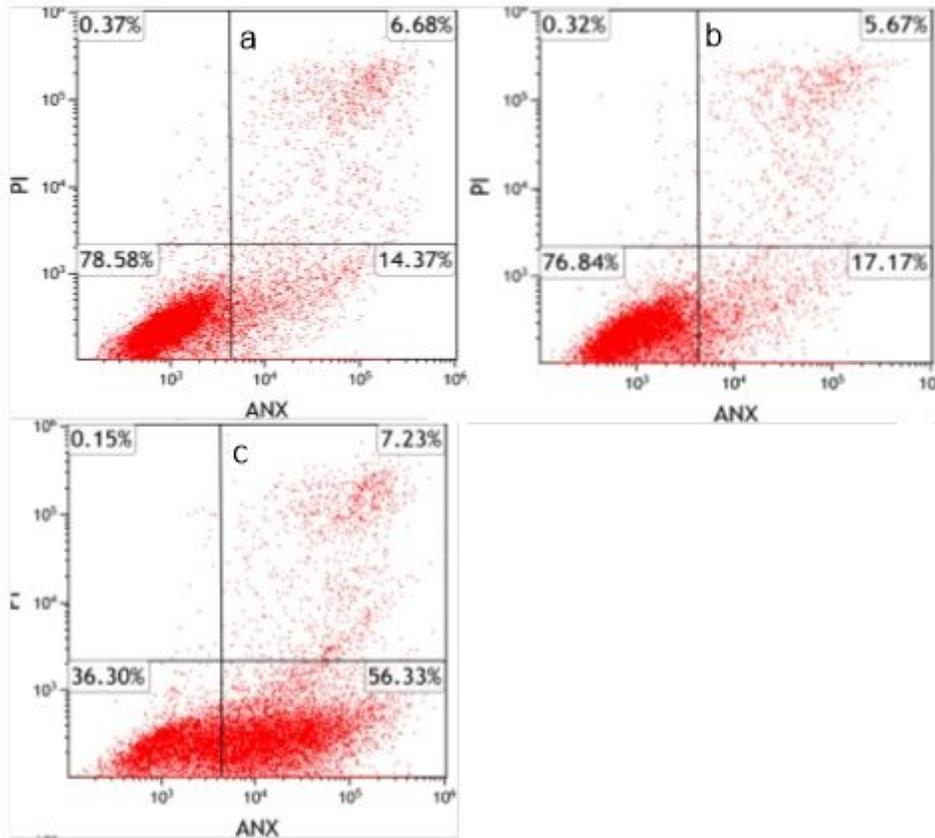


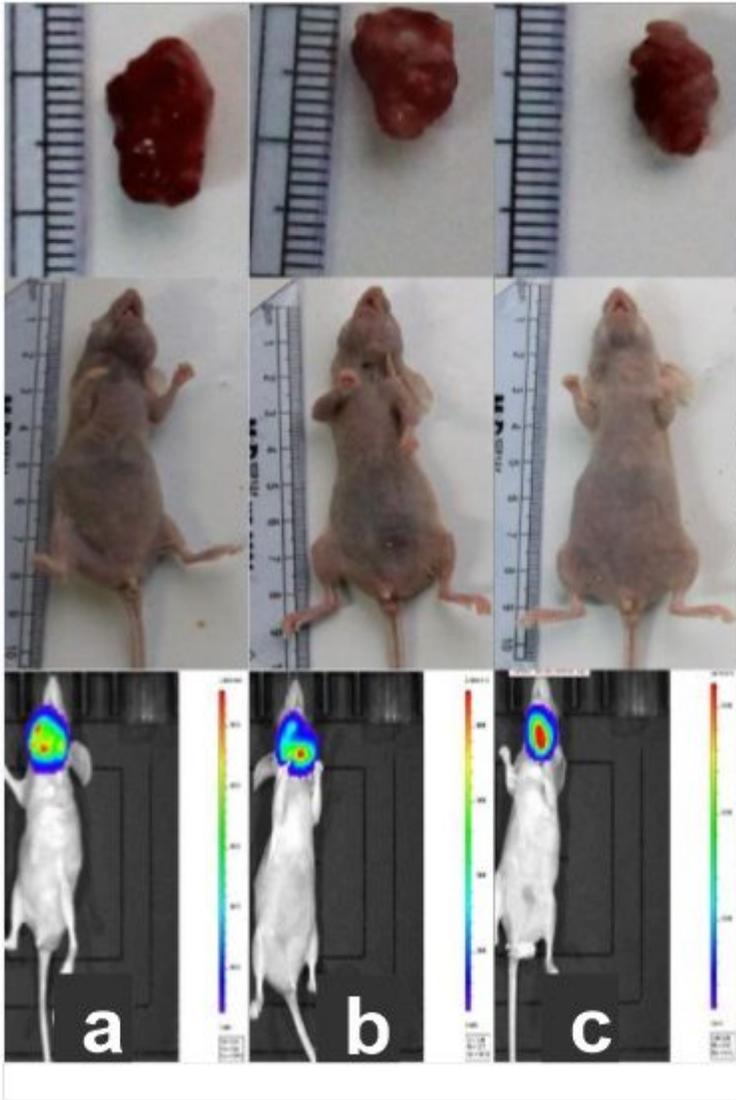
Figure 12

Detection of the effect of high expression tRNA<sup>Ini</sup>CAT on the AMC-HN-8 cell cycle by flow cytometry. (a) NC. (b) Low dose. (c) High dose. NC, negative control



**Figure 13**

Detection of the effect of high expression of tRNA<sup>Asp</sup>CAT on the apoptosis of AMC-HN-8 cells by flow cytometry. (a) NC. (b) Low dose. (c) High dose. \*\*\*p<0.001. NC, negative control



**Figure 14**

Allograft mouse model. (a) NC. (b) Low dose. (c) High dose. NC, negative control

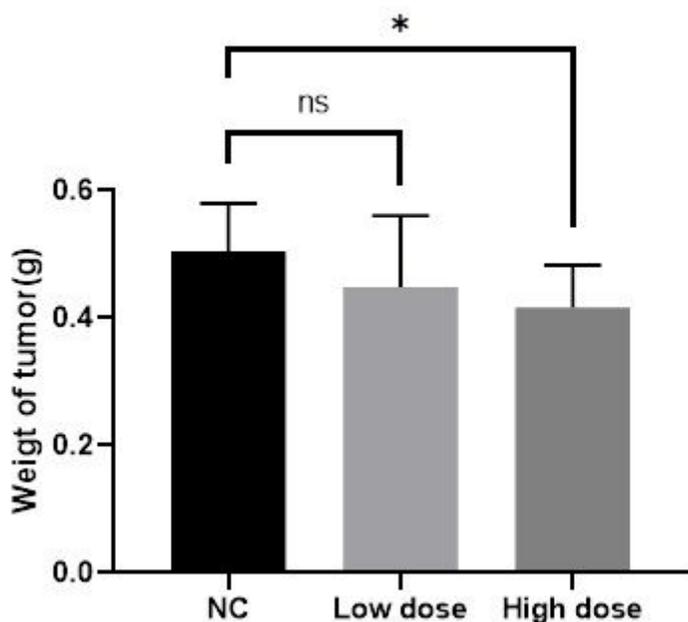


Figure 15

Tumor weight in the high-dose treatment group was significantly higher than that measured in the NC group. \* $p < 0.05$ . NC, negative control

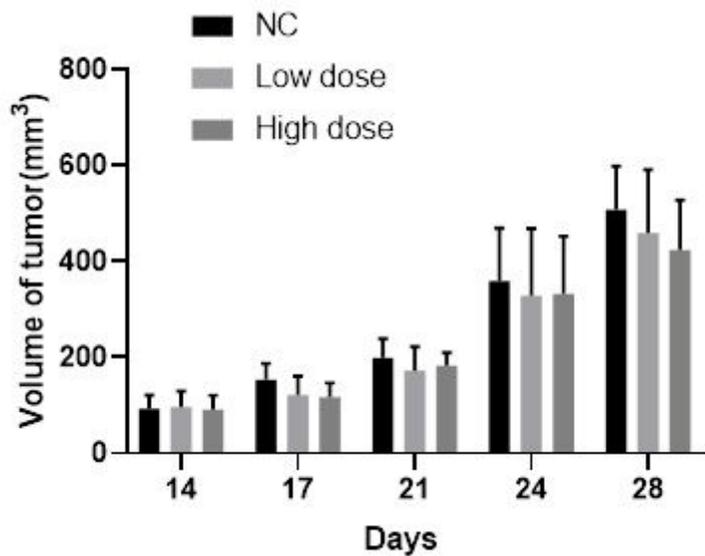
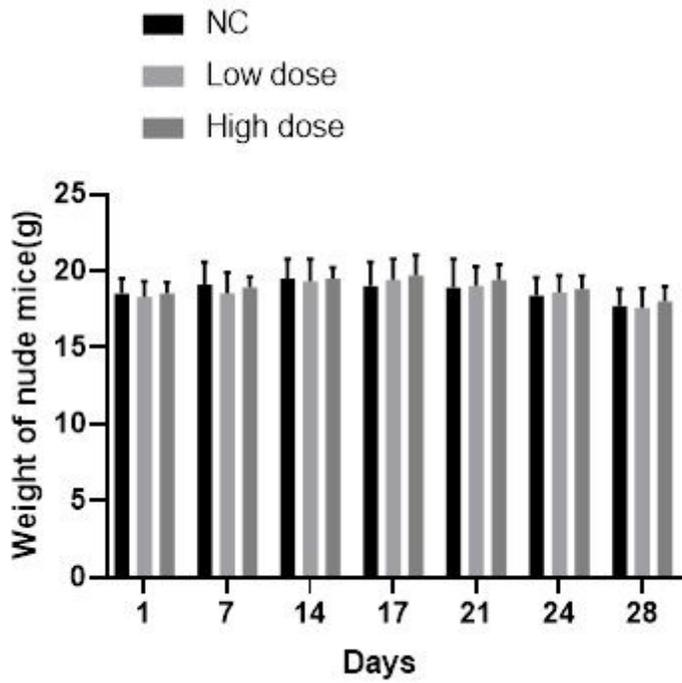


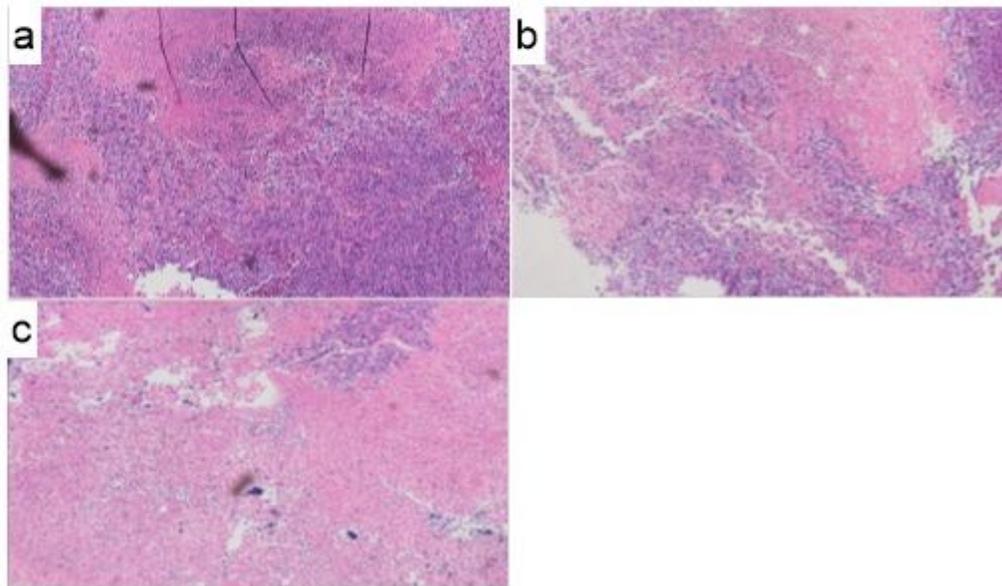
Figure 16

There was no significant difference in tumor volume between each group and the NC group. NC, negative control



**Figure 17**

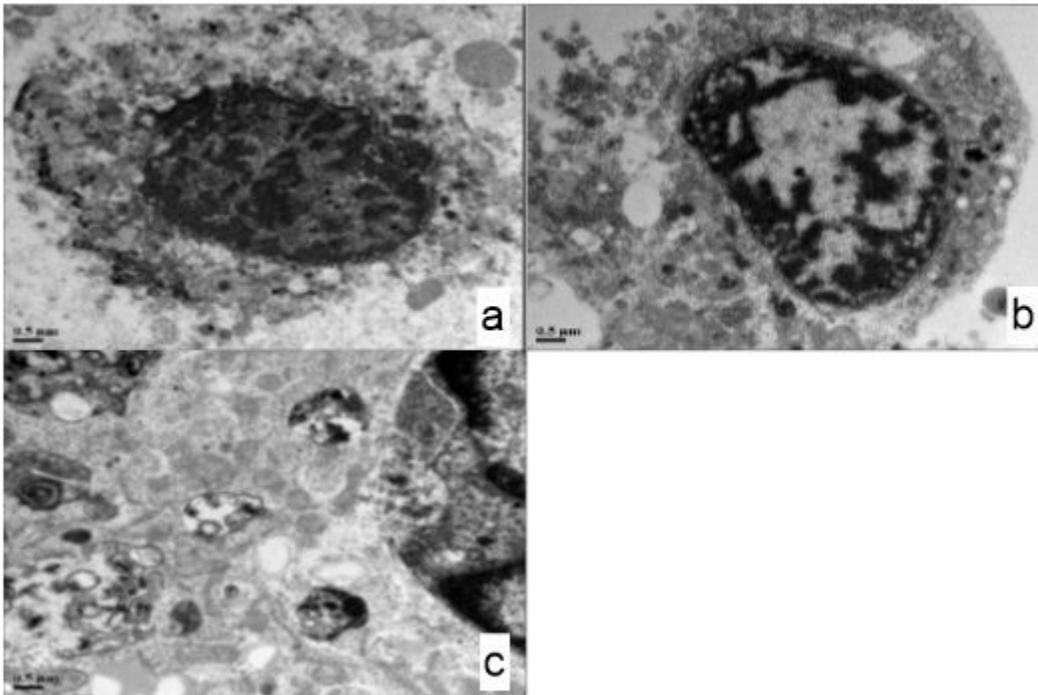
There was no significant difference in the weight of mice between each group and the NC group. NC, negative control



**Figure 18**

Pathological examination showed that tumor necrosis in the high-dose treatment group was markedly decreased compared with that observed in the NC group (magnification  $\times 100$ ). (a) NC. (b) Low dose. (c)

High dose. NC, negative control



**Figure 19**

TEM results showing that LV3-tRNA<sup>Ini</sup>CAT autophagy and apoptosis were observed in the treatment group (b and c) (magnification:  $\times 20,000$ ). (a) NC. (b) Low dose. (c) High dose. TEM, transmission electron microscopy; NC, negative control