Expression of long non-coding ribonucleic acid metastasis-associated lung adenocarcinoma transcript-1 is correlated with progress and apoptosis of laryngeal squamous cell carcinoma

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Abstract

Introduction

Long non-coding ribonucleic acids are pervasively transcribed in the genome and potentially involved in the progress of malignant tumours. The role of long non-coding ribonucleic acid metastasis-associated lung adenocarcinoma transcript-1 in human laryngeal squamous cell cancer is not well understood.

Methods

The expression level of metastasis-associated lung adenocarcinoma transcript-1 in 72 laryngeal squamous cell cancer and the corresponding adjacent non-neoplastic tissues was examined using real-time polymerase chain reaction. The correlation between the expression of metastasis-associated lung adenocarcinoma transcript-1 and clinicopathologic parameters was determined. A methylthiazol tetrazolium assay was used to detect the proliferation of laryngeal squamous cell cancer cells after metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acid transfection in vitro. The growth of mice xenografts was observed after an intra-tumoral injection of metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acid lentivirus. Moreover, the apoptosis cells were increased in metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acid lentivirus-treated laryngeal squamous cell cancer xenografts than the control.

Results

The level of metastasis-associated lung adenocarcinoma transcript-1 in laryngeal squamous cell cancer was significantly higher than that in the corresponding adjacent non-neoplastic tissues. Patients with a poor histological grade or an advanced clinical stage have a higher expression of metastasis-associated lung adenocarcinoma transcript-1. Cell proliferation was significantly inhibited after metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acid transfection in vitro. The growth of xenografts was significantly suppressed by a repeated injection of metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acid lentivirus. Moreover, the apoptosis cells were increased in metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acid lentivirus-treated laryngeal squamous cell cancer xenografts than the control.

Conclusion

Taken together, these data suggest an important role for metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acid in vitro. The growth of mice xenografts was observed after an intra-tumoral injection of metastasis-associated lung adenocarcinoma transcript-1 short interfering ribonucleic acids. Furthermore, apoptosis in the xenografts was determined by terminal uridine nick-end labelling stain and transmission electron microscopy.

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common malignancy of the upper respiratory tract. Current treatments including surgical intervention, radiation therapy and chemotherapy have a moderate effect on early-stage cases, but are less effective for more advanced cases. A recent meta-analysis has reported an overall five-year survival rate of LSCC of 64.2%1. Therefore, determination of the molecular mechanisms underlying LSCC carcinogenesis or progression is important for the development of effective therapeutic strategies. In addition to the classical long protein-coding messenger ribonucleic acids (mRNAs), there are a wide variety of non-coding RNA (ncRNA) transcripts in mammalian genomes. As a type of ncRNA, microRNAs (miRNA) have been identified to play critical roles for ncRNAs in cancer. miRNAs are associated with diagnosis, staging, progression, prognosis and treatment of malignant tumours2. Our previous study has shown that miR-21 plays an important role in the proliferation and apoptosis of LSCC. However, in addition to the relatively well-known miRNAs, growing knowledge of long ncRNA (lncRNA) has been gained in the mammalian transcript. Similar to miRNA, lncRNA can also promote cellular pathways that lead to the development and progression of cancer4-5. Recent studies have shown that metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1) is significantly overexpressed in tumours such as hepatocellular carcinoma and lung cancer4-7. However, the biological roles of MALAT-1 in LSCC are still poorly understood. Therefore, in this study, we first detected the expression of MALAT-1 in LSCC by real-time polymerase chain reaction (PCR) and found a significant up-regulation of MALAT-1 in LSCC cancer tissue. Moreover, we found that MALAT-1 knockdown can suppress proliferation and induce apoptosis of LSCC.
Our findings suggest that MALAT-1 plays an oncogenic role and may serve as a therapeutic target in LSCC.

**Materials and methods**

**Patients and samples**

Patients were enrolled between June 2005 and October 2006. Seventy-two patients with laryngeal cancer who had undergone partial or total laryngectomy at the Department of Otorhinolaryngology, the Second Affiliated Hospital of Harbin Medical University, under an approved protocol of Harbin Medical University, were included in the study. The patients had not received any therapy before admission.

After surgery, the matched specimens of LSCC and the corresponding adjacent non-neoplastic tissues obtained from patients were preserved in liquid nitrogen within 5 min of excision, and then transported frozen to the laboratory and stored at –80°C. All tissues obtained from the neck dissection were marked according to their regions and the lymph nodes were serially sectioned, stained with haematoxylin–eosin and microscopically observed.

**Real-time reverse transcription-PCR**

Total RNA was extracted from cancerous/non-cancerous specimens or cell lines, and the expression level of MALAT-1 was determined by quantitative PCR (qPCR) as described previously. The primers for MALAT-1 detection were 5′-GAAATGCTGCTTGAACGTTAAGCTTTGAT-3′ (sense) and 5′-GACTTCAATTCAATACCCGCTGAGT-3′ (antisense). The relative MALAT-1 expression was calculated using 2^−ΔΔCt with the cycle threshold (CT) values normalized using 18S rRNA as internal control.

**Lentivirus vectors for MALAT-1 short interfering RNA (siRNA)**

siRNA of the human MALAT-1 lentivirus gene transfer vector harbouring the green fluorescent protein (GFP) sequence was constructed by GenePharma (Shanghai, China). The sequence of the siRNA was as follows: 5′-GATCCATAATCGGTTCAGG-3′ (sense) and 5′-GAAACGATTATGGAATCTT-3′ (antisense). The recombinant lentivirus of MALAT-1 siRNA and the control lentivirus (GFP-lentivirus) were prepared and titred to 10^8 TU/mL (transfection unit).

**Cell culture and virus transduction**

The human epithelial-2 (Hep-2) cells of human LSCC were kindly provided by the Laboratory of Cell Pathology, Harbin Medical University. Cells were cultured in DMEM medium containing 10% foetal bovine serum (Gibco) and incubated in a humidified (37°C, 5% CO₂) incubator. Hep-2 cells were plated in 24-well plates (2 × 10⁴ cells/well) overnight. The lentiviruses were diluted in 0.2 mL (10^⁷ TU/mL) of complete medium containing polybrene (8 μg/mL) and added to the cells for 1 h of incubation at 37°C, followed by incubation in 0.3 mL of freshly prepared polybrene-DMEM for another 24 h, which was replaced with fresh DMEM medium, and the cells were cultured for the next 48 h.

**Methylthiazol tetrazolium (MTT) assay**

After MALAT-1 siRNA transfection of Hep-2 cells for varying time periods (20, 44, 68 and 92 h), 20 μL of sterile MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, tetrazole) dye (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added and incubation was continued for another 4 h at 37°C. Then, 150 μL of dimethyl sulfoxide was added to each well and thoroughly mixed for 10 min. Spectrometric absorbance at 492 nm was measured on an enzyme immunoassay analyzer (model 680; Bio-Rad Laboratories, Hercules, CA, USA). The cell growth rate was calculated using the following formula:

\[
\text{Cell growth rate (\%)} = \left(\frac{\text{mean absorbance in six wells of the treatment group}}{\text{mean absorbance in six wells of the control group}}\right) \times 100
\]

**Animal experiments**

Fourteen BALB/c mice (provided by The Central Animal Facility, Harbin Medical University) were 5–6 weeks old and about 20 g in weight. They were bred under aseptic conditions and maintained at a constant humidity and temperature (25–28°C) according to standard guidelines under a protocol approved by Harbin Medical University. All mice were subcutaneously injected into the dorsal scapula region with a 100 μL suspension (1 × 10^⁶) of Hep-2 cells. The size of the tumour was measured twice a week with callipers, and the volume of the tumour was determined using the simplified formula of a rotational ellipsoid (length × width^2 × 0.5). Once tumours reached approximately 0.5–0.6 cm³ in size, the mice received an injection into the tumour once a week for three weeks. The seven mice in the experimental group were treated with 100 μL of the MALAT-1 siRNA lentivirus; the remaining seven mice, in the control group, received an injection of 100 μL of the GFP-lentivirus. Tumours were harvested one week after the end of the treatment.

**TUNEL stain**

Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method in situ apoptosis detection kit (Roche), according to the manufacturer’s instructions. After deparaffinization and dehydration and inactivation of intrinsic peroxidase activity, 20 paraffin sections of each tumour specimen were incubated with 2 μL proteinase K at 37°C for 15 min. The sections were then treated with terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP. After stopping the reaction with tris-borate-ethylene diaminetetraacetic acid (TB-EDTA) buffer (30 mmol/L sodium chloride and 30 mmol/L sodium citrate), the samples were investigated by microscopy. Controls for the TUNEL procedure were treated.
Expression of MALAT-1 level


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Knockdown of MALAT-1 suppresses tumour growth in vivo

All 14 mice developed detectable tumours after they were subcutaneously injected with Hep-2 cells. The growth of the LSCC xenografts was significantly inhibited in mice treated with the MALAT-1 siRNA lentivirus compared with those treated with the GFP-lentivirus (Figure 4). The average tumour weight (1.275 ± 0.253 g) in the MALAT-1 siRNA-treated LSCC xenografts was statistically lower (p < 0.001) than the tumours in the control group (2.397 ± 0.383 g).

Knockdown of MALAT-1 induces apoptosis of Hep-2 cells

The extent of DNA fragmentation was determined using TUNEL stain, which indicated many apoptotic cells in MALAT-1 siRNA Lentivirus-treated Hep-2 xenografts (Figure 5a). No TUNEL-positive staining was detected in the control xenograft sections (Figure 5b).

The tumour cells in the MALAT-1 siRNA-treated group showed a typical apoptotic morphology under transmission electron microscopy (Figure 6b), characterised by homogeneous condensation of chromatin to one side or the periphery of the nuclei. The inner matrix of some mitochondria showed increased electron density as is typically observed with apoptotic cells. Furthermore, autophagy was also found in MALAT-1 siRNA-treated tumours (Figure 6c). These ultrastructural changes were unique in tumour cells of the MALAT-1 siRNA-treated group but were absent in tumour cells of the control group (Figure 6a) which showed intact membranes and intact morphology of organelles.

Discussion

Accumulating evidence has confirmed that the mammalian genome encodes thousands of IncRNAs that are pervasively transcribed. Although IncRNAs have frequently been disregarded as transcriptional ‘noise’, there is substantial evidence to suggest that they are functional. One of the primary functions of IncRNAs appears to be as epigenetic regulators of protein-coding gene expression. Dysregulated expression of IncRNAs in cancer correlates to disease progression and the important function of IncRNAs in tumours has become a new field for cancer research. MALAT-1 was originally identified as a transcript showing significant expression correlated with metastasis of non-small-cell lung tumours, and subsequently, research showed that it is overexpressed in many human carcinomas such as breast, colon, endometrial stromal sarcoma, bladder and hepatocellular carcinoma. In this study, we examined the expression pattern of MALAT-1 in LSCC tissues and investigated its clinical implications. qPCR analysis showed that the MALAT-1 was overexpressed in primary LSCC compared with adjacent non-cancerous tissues. Furthermore, the patients with poor differentiation, lymph node metastasis or advanced clinical stages were detected with high MALAT-1 expression. These data suggest that the increased level of MALAT-1 may play an...
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influencing the expression of motility-related genes such as CCT4 and HMMR. Moreover, Ying and colleagues found that up-regulated MALAT-1 contributes towards bladder cancer cell migration by inducing epithelial-to-mesenchymal transition. In agreement with these findings, our data showed a significant decrease in the proliferative ability of Hep-2 cells after MALAT-1 down-regulation. Furthermore, to investigate the function of MALAT-1 in LSCC in vivo, the LSCC xenografts of mice were treated by the MALAT-1 siRNA lentivirus. The average tumour weight was significantly lower in the mice compared with the control, thus suggesting that MALAT-1 knockdown could effectively suppress the progression of LSCC in vivo.

Apoptosis plays a crucial role in cancer development. Lai and colleagues have demonstrated that MALAT-1 siRNA can suppress invasion and induce apoptosis of hepatocellular carcinoma cells. Consistent with these findings, our results suggested that MALAT-1 siRNA-treated LSCC xenografts showed increased apoptosis cells compared with the control. This indicated that overexpression of MALAT-1 may increase the malignant phenotype of LSCC cells such as apoptosis resistance.

In summary, we have identified that MALAT-1 is overexpressed in LSCC tumour tissues and is associated with metastasis and progress. Moreover, MALAT-1 is not only involved in the regulation of cell proliferation but is also an important regulator in cell apoptosis of LSCC. MALAT-1 may play an oncogenic role and should be investigated further as a potential therapeutic target in LSCC.

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Figure 4: MALAT-1 siRNA suppresses Hep-2 tumour growth in vivo. Tumours from mice injected with Hep-2 cells were dissected one week after the treatment. (a) Tumours in the MALAT-1 siRNA lentivirus treated group. (b) Tumours in the control group. (c) Difference in tumour weight between the MALAT-1 siRNA lentivirus-treated group and the control group (n = 7). *, p < 0.01.

Figure 5: TUNEL staining of Hep-2 xenografts. In vivo TUNEL staining of the MALAT-1-RNAi-lentivirus-treated Hep-2 xenograft tumour sections also showed a number of apoptotic cells (a), whereas no obvious apoptotic cell was found in tumours of the control group (b).

important regulative role in the carcinogenesis and malignant progression of LSCC and may be useful for therapeutic intervention. A previous study has shown that inhibition of MALAT-1 suppressed the proliferation and invasion of cervical cancer cells through the modulation of caspase-3, -8, Bax, Bcl-2 and Bcl-xL genes. Similarly, a recent study has reported that MALAT-1 enhances cell motility of lung adenocarcinoma cells by

