LncRNA XIST promotes liver cancer progression by acting as a molecular sponge of miR-200b-3p to regulate ZEB1/2 expression

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Abstract

Objective: To evaluate the predictive value of long non-coding RNA (lncRNA) X-inactive specific transcript (XIST) for survival, and determine the involvement of miRNA(miR)-200b-3p and zinc finger E-box-binding homeobox (ZEB) 1/2 in the pro-tumor effect of lncRNA XIST in liver cancer.

Methods: We evaluated lncRNA XIST expression in liver cancer tissues and cell lines by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and analyzed the correlation between its expression and overall survival of liver cancer patients by Kaplan–Meier analysis. Its effects on cell proliferation, migration, and invasion were analyzed by Cell-Counting Kit-8 and Transwell assays. The association between lncRNA XIST and miR-200b-3p, and the effects of lncRNA XIST on ZEB1/2 expression were explored using luciferase reporter assays, real-time PCR, and western blotting.

Results: The lncRNA XIST was significantly upregulated in liver cancer, and increased lncRNA XIST expression was associated with a poor prognosis. The lncRNA XIST promoted liver cancer cell proliferation, migration, and invasion in vitro, and acted as a molecular sponge for miR-200b-3p, and also regulated the expression of ZEB1/2 via miR-200b-3p.

Conclusion: The lncRNA XIST is an oncogenic lncRNA that promotes liver cancer metastasis, and its pro-metastatic phenotype can be partially attributed to the lncRNA XIST/miR-200b-3p/ZEB1/2 signaling axis.
Keywords
Long non-coding RNA, X-inactive specific transcript, miR-200b-3p, zinc finger E-box-binding homeobox 1/2, liver cancer, metastasis

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Introduction
Liver cancer is one of the most common and aggressive malignancies worldwide, with an extremely poor prognosis, especially in China.\textsuperscript{1,2} Despite extensive progress in recent decades, the overall survival rate of liver cancer patients is still unsatisfactory because of the high incidence of recurrence and metastasis.\textsuperscript{3,4} Metastasis is a complex, multi-step process, in which epithelial–mesenchymal transition (EMT) endows cells with enhanced invasive potential, and may thus play a crucial role in the metastatic dissemination of tumor cells.\textsuperscript{5} EMT is currently believed to be responsible for liver cancer metastasis, therapeutic resistance, and recurrence.\textsuperscript{6} Elucidation of the molecular mechanisms underlying the control of EMT progression thus provides a new focus for the development of liver cancer treatments.

Long non-coding RNAs (lncRNAs) are commonly defined as non-coding RNAs with a length of more than 200 nucleotides,\textsuperscript{7} and have been shown to play crucial regulatory roles in gene expression.\textsuperscript{8} Emerging evidence has also shown that numerous lncRNAs are dysregulated in liver cancer and participate in regulating liver cancer growth, apoptosis, migration, and invasion, as either oncogenes or tumor suppressor genes.\textsuperscript{9} For example, lncRNA HULC is highly upregulated as an oncogene in liver cancer, and promotes tumorigenesis and metastasis by regulating EMT,\textsuperscript{10} while maternally expressed gene 3 inhibits liver cancer tumorigenesis and metastasis by regulating miR-29a.\textsuperscript{11} In addition, lncRNA expression signatures are correlated with liver cancer metastasis and prognosis.\textsuperscript{12} The lncRNA X-inactive specific transcript (XIST) has been reported to be dysregulated in various cancers, including gastric, colorectal, and lung cancer. One study reported that lncRNA XIST expression was increased in esophageal squamous cell carcinoma, and that this increase was related to a poor prognosis\textsuperscript{13}, while lncRNA XIST was also shown to promote esophageal carcinoma metastasis by regulating miR-101/enhancer of zeste homolog.\textsuperscript{13} In addition, lncRNA XIST promoted cell invasion by regulating the miR-497/metastasis-associated in colon cancer-1 axis in gastric cancer, and was associated with a poor prognosis.\textsuperscript{14} Although these studies indicated that lncRNA XIST plays a crucial role in tumorigenesis; its role in liver cancer metastasis has received less attention.

In the current study, we examined lncRNA XIST expression levels in liver cancer cells and tissues, and analyzed the correlation between its expression and prognosis in patients with liver cancer. We also investigated the biological functions and underlying molecular mechanism of lncRNA XIST in liver cancer metastasis by determining the effects of lncRNA knockdown on liver cancer cell proliferation, migration, and invasion. We further explored the role of lncRNA XIST as a molecular sponge for miR-200b-3p, and investigated the regulatory relationship...
between lncRNA XIST and zinc finger E-box-binding homeobox (ZEB) 1/2. The results of this study regarding the lncRNA XIST/miR-200b-3p/ZEB1/2 signaling axis indicate a possible candidate target for liver cancer treatments.

Materials and methods

Cell culture and human tissue specimens

Fresh frozen human liver cancer tissues and paired normal adjacent liver tissues were collected from patients undergoing surgery at Zhejiang Provincial People’s Hospital from August 2008 to October 2010. No patients received radiochemotherapy or immunotherapy before surgery. Liver cancer tissues were taken from non-necrotic sites in the center of the lesion, and adjacent tissues were taken from sites more than 3 cm from the edge of the tumor. Tissue samples were stored in liquid nitrogen within 30 minutes after excision. This study was approved by the Ethics Committee of Zhejiang Provincial People’s Hospital (approval number: 201900158) and all patients or their caregivers provided consent (written or oral) for participation in the study.

The human liver cancer cell lines HepG2 (HB-8065), SNU-398 (CRL-2233), and SNU-449 (CRL-2234), and transformed human liver epithelial-2 (THLE-2), MHCC97-H, and MHCC97-L cells were obtained from American Type Culture Collection (Manassas, VA, USA) or Shanghai Biological Sciences Institute (Shanghai, China). All cell lines were cultured in Dulbecco’s Modified Eagle Medium or RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics at 37°C with 5% CO₂.

RNA and microRNA (miRNA) extraction and quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction, reverse transcription, and RT-qPCR were performed as described previously. For miRNA detection, mature miR-200b-3p was reverse transcribed with specific RT primers, quantified using a TaqMan probe, and normalized to U6 RNA using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA).

RNA immunoprecipitation

SNU-449 cells were co-transfected with pcDNA3.1-MS2-XIST, pcDNA3.1-MS2, pcDNA3.1-MS2-XIST-Mut, and pMS2-GFP (System Biosciences, Shanghai, China) following the manufacturer’s protocol and used for RNA immunoprecipitation experiments using a Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions.

Cell transfection

HepG2 and SNU-449 cells were transfected with small interfering RNA (siRNA) against XIST (siRNA-XIST), negative control siRNA, miR-200b-3p mimics, or miRNA inhibitor (RiboBio, Guangzhou, China), according to the manufacturer’s instructions.

Lentiviral vectors

Wild-type (WT) and mutant (MT) XIST sequences were amplified and cloned into GV369-GFP (System Biosciences) to obtain cells stably expressing lncRNA-XIST-WT and lncRNA-ATB-MT. We then constructed XIST-WT and XIST-MT lentiviral vectors and co-transfected SNU-449 cells with GV369-GFP-XIST-WT or GV369-GFP-XIST-MT and the packaging
plasmids using Lipofectamine 3000 reagent (Invitrogen, Waltham, MA, USA). The resulting stably overexpressing SNU-449 cell line was identified and confirmed by qPCR.

Cell proliferation assay
The effect of lncRNA XIST on cell proliferation was determined by Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan), as described previously.15

Cell migration and invasion assays
Cell migration and invasion assays were performed as described previously.15

Luciferase reporter assays
XIST-WT and XIST-MT sequences were amplified from HEK-293 cell genomic DNA by PCR and cloned into the luciferase reporter sequence in the pmiR-CheckREPORT vector. SNU-449 cells were co-transfected with the pmiR XIST-WT or pmiR-XIST-MT reporter vectors together with either miR-200b-3p mimic or negative control in 96-well plates using Lipofectamine 3000 (Invitrogen). Relative luciferase activity was normalized to Renilla luciferase activity 48 hours after transfection.

Western blotting
Total protein was extracted from cells or tissues using standard protocols.15 Protein expression levels were examined using antibodies against ZEB1, ZEB2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, UK) and normalized to GAPDH. Western blot analysis was performed as described previously.15

Statistical analysis
Survival curves were generated using the Kaplan–Meier method and assessed using the log-rank test. Statistical analyses were performed using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). The results are presented as mean ± standard error. P < 0.05 was considered statistically significant.

Results
Expression of lncRNA XIST in liver cancer cell lines and tissues and association with prognosis in liver cancer patients
Fresh frozen liver cancer tissues and paired normal adjacent liver tissues were collected from 42 patients undergoing surgery (Table 1). Expression levels of lncRNA XIST were significantly upregulated in liver cancer tissues compared with adjacent normal tissues (Figure 1a). We also measured lncRNA XIST expression in liver cancer cell lines. LncRNA XIST expression was significantly elevated in liver cancer cell lines compared with transformed human liver epithelial-2 (THLE-2) cells (Figure 1b).

We divided the 42 liver cancer patients into two groups according to the median lncRNA XIST expression level and examined the correlation between lncRNA XIST expression and clinicopathological characteristics. High lncRNA XIST expression was significantly correlated with tumor size (P = 0.049) and microvascular invasion (P = 0.005) (Table 1). However, there were no significant associations between lncRNA XIST expression and any other clinicopathological characteristics.

We further determined if lncRNA XIST expression was associated with survival among liver cancer patients. High lncRNA XIST expression in liver cancer tissues was significantly associated with poorer survival.
recurrence-free survival ($P = 0.039$) (Figure 1c) and overall survival ($P = 0.028$) (Figure 1d), determined by Kaplan–Meier survival estimates. Taken together, these results suggested that high lncRNA XIST expression might be critically involved in liver cancer progression.

**Effect of lncRNA XIST knockdown on liver cancer cell proliferation, migration, and invasion**

We examined the biological functions of lncRNA XIST in liver cancer cells to clarify the possible association between high lncRNA XIST expression and liver cancer progression. We transfected SNU-449 and HepG2 cells with siRNA-XIST and confirmed its downregulation by quantitative reverse transcription PCR. The resulting decreased expression of lncRNA XIST significantly inhibited SNU-449 and HepG2 cell proliferation compared with the negative control groups, as shown by CCK-8 assay (all $P < 0.05$) (Figure 2a and 2b). Moreover, the migration capacities of SNU-449 and HepG2 cells were reduced following transfection with siRNA-XIST compared with the corresponding negative control cells (Figure 2c and 2d). Invasion of HepG2 and SNU-449 cells was also weakened when endogenous lncRNA XIST was silenced with siRNA-XIST (Figure 2e and 2f). These results demonstrated that

**Table 1.** Relationship between lncRNA XIST expression and clinicopathological parameters in 42 patients with liver cancer.

| Parameter n (%) | IncRNA XIST expression | | | | |
|----------------|------------------------|-----------------|-----------------|-----------------|-----------------|
|                | All cases, age (years) | High (≥2 fold) | Low (<2 fold) | | |
|                | All cases | 42 (100) | 21 | 21 | | |
|                | ≤50 | 19 (45.2) | 9 | 10 | 0.756 |
|                | >50 | 23 (54.8) | 12 | 11 | | |
|                | Sex | | | | |
|                | Male | 26 (61.9) | 14 | 12 | 0.525 |
|                | Female | 16 (38.1) | 7 | 9 | | |
|                | Hepatitis B virus | | | | |
|                | Positive | 31 (73.8) | 16 | 15 | | |
|                | Negative | 11 (26.2) | 5 | 6 | 0.725 |
|                | Alpha-fetoprotein$^b$ | | | | |
|                | >400 | 18 (42.9) | 12 | 6 | 0.061 |
|                | ≤400 | 24 (57.1) | 9 | 15 | | |
|                | Tumor size | | | | |
|                | ≤5 cm | 28 (66.7) | 11 | 17 | 0.049 |
|                | >5 cm | 14 (33.3) | 10 | 4 | | |
|                | Histological grade | | | | |
|                | Moderate | 25 (59.5) | 11 | 14 | | |
|                | Poor | 17 (40.5) | 10 | 7 | 0.345 |
|                | Microvascular invasion | | | | |
|                | Positive | 17 (40.5) | 13 | 4 | 0.005 |
|                | Negative | 25 (59.5) | 8 | 17 | | |

$^a$Pearson’s $\chi^2$ tests, $^b$measured in $\mu$g/L. Results considered statistically significant at $P < 0.05$.

lncRNA, long non-coding RNA; XIST, X-inactive specific transcript.
lncRNA XIST promoted liver cancer cell proliferation, migration, and invasion in vitro.

Role of lncRNA XIST as a molecular sponge for miR-200b-3p

Some lncRNAs have previously been reported to function as competing endogenous RNAs (ceRNAs) for miRNAs, and as molecular sponges to competitively suppress miRNAs.\(^\text{16,17}\) We searched for miRNAs that might regulate lncRNA XIST expression using the bioinformatics tool MicroInspector (http://bioinfo.uni-plovdiv.bg/microinspector/) and starBase database (http://starbase.sysu.edu.cn/). We identified miR-200b-3p,
which has been reported to inhibit EMT and tumor metastasis by targeting ZEB1/2,18,19 and lncRNA XIST contains three predicted binding sites for miR-200b-3p (Figure 3a), and we therefore selected miR-200b-3p as a candidate.

We determined the occurrence of direct binding between miR-200b-3p and lncRNA XIST by RNA immunoprecipitation assay, to pull down endogenous miRNAs associated with lncRNA XIST (Figure 3b). The lncRNA XIST immunoprecipitate in SNU-449 cells was significantly enriched with miR-200b-3p compared with immunoprecipitates of IgG, empty vector (MS2), and lncRNA XIST with mutant miR-200b-3p binding sites, according to RT-qPCR (P = 0.008) (Figure 3c). We also examined the expression level of miR-200b-3p in lncRNA XIST-expressing SNU-449 cells, and showed similar expression levels compared with negative control cells (Figure 3d). These data suggest that lncRNA XIST functions as a molecular sponge to adsorb miR-200b-3p, rather than inducing the degradation of miR-200b-3p.

We further confirmed the specific association between lncRNA XIST and miR-200b-3p by constructing XIST-WT and XIST-MT luciferase reporter vectors. Overexpression of miR-200b-3p reduced the luciferase activity of the XIST-WT but not the XIST-MT reporter or empty vector (Figure 3e), indicating that miR-200b-3p was a direct target of lncRNA XIST.

Figure 2. Knockdown of long non-coding RNA (lncRNA) X-inactive specific transcript (XIST) suppressed liver cancer cell proliferation, migration, and invasion. (a, b) HepG2 and SNU-449 cells were transfected with small interfering RNA (siRNA) against XIST (siRNA-XIST) or negative control siRNA at a final concentration of 50 nM. Cell proliferation was analyzed by Cell Counting Kit-8 (CCK-8) assay at different time points post-transfection. (c, d) Migration abilities of HepG2 and SNU-449 cells were evaluated by Transwell assays. (e, f) Invasion abilities of HepG2 and SNU-449 cells were evaluated by Transwell assays. All values presented as mean ± standard error based on at least three independent experiments. *P < 0.05, **P < 0.01.
To further determine if lncRNA XIST was regulated by miR-200b-3p, we transfected miR-200b-3p mimic or negative control into SNU-449 cells, and found that overexpression of miR-200b-3p had no significant effect on lncRNA XIST expression (Figure 3f). Taken together, these results suggest that lncRNA XIST might act as a molecular sponge to competitively inhibit miR-200b-3p.

Effects of lncRNA XIST on expression of miR-200b-3p target ZEB1/2

Previous reports have indicated that miR-200b-3p can act as a tumor suppressor to inhibit EMT progression by targeting ZEB1/2. We therefore determined if lncRNA XIST affected the expression of the miR-200b-3p targets ZEB1/2. We examined lncRNA XIST expression in SNU-449 cells transduced with XIST-WT, XIST-MT, and negative control vectors. LncRNA XIST expression was significantly increased in the XIST-WT and XIST-MT groups compared with the negative control group (P = 0.0006) (Figure 4a). In addition, protein expression levels of the miR-200b-3p targets ZEB1/2 were upregulated in SNU-449 cells stably overexpressing XIST-WT but not XIST-MT (Figure 4b). However, ZEB1 and ZEB2 mRNA levels were similar in the XIST-WT and negative control groups (Figure 4c). These results suggested that lncRNA XIST regulated the expression of ZEB1/2.
To further confirm if lncRNA XIST regulated the expression of ZEB1/2 via miR-200b-3p, we transfected SNU-449 cells with siRNA-XIST or miR-200b-3p inhibitor to decrease endogenous lncRNA XIST and miR-200b-3p expression, respectively (Figure 4d). ZEB1 and ZEB2 protein levels, but not mRNA levels, were decreased by knockdown of lncRNA XIST, and this decrease was abolished by inhibition of miR-200b-3p (Figure 4e and 4f). These data demonstrated that the regulation of ZEB1/2 by lncRNA XIST was dependent on the specific binding of miR-200b-3p.

**Discussion**

In this study, we demonstrated that lncRNA XIST was upregulated in liver cancer tissues and cells compared with adjacent normal liver tissues and cells, consistent with its previously reported upregulation in liver, colorectal, and gastric cancers. Increased lncRNA XIST expression was associated with tumor size and microvascular invasion, and high lncRNA XIST expression in liver cancer tissues indicated worse recurrence-free survival and poor overall survival among liver cancer patients. We also comprehensively
investigated the biological functions of lncRNA XIST in vitro, and showed that lncRNA XIST knockdown significantly suppressed liver cancer cell proliferation, migration, and invasion, while enhanced expression promoted liver cancer cell migration and invasion. These results indicate that lncRNA XIST acts as an oncogene to promote liver cancer progression.

Some lncRNAs have recently been reported to act as ceRNAs by competitively binding common miRNAs. The current results demonstrated that lncRNA XIST acts as a molecular sponge for miR-200b-3p in liver cancer. Similar results indicating that lncRNA XIST can promote the progression of several kinds of tumors have also been reported. For example, lncRNA XIST acted as a molecular sponge for miR-133a and promoted pancreatic cancer proliferation by targeting epidermal growth factor receptor. Li et al. reported that lncRNA XIST promoted EMT by regulating the miR-367/ZEB2 axis in non-small-cell lung cancer, and Kong et al. showed that lncRNA XIST acted as a miR-194-5p molecular sponge to regulate mitogen-activated protein kinase 1 in liver cancer cells. These results suggested that lncRNA XIST can act as a ceRNA to bind miRNAs, thus affecting the expression of miRNA targets that may play a critical role in cancer progression. However, lncRNA XIST has also been found to be downregulated in liver cancer, and Zhuang et al. reported that lncRNA XIST inhibited liver cancer cell metastasis by targeting miR-92b. The lncRNA XIST can thus function as either an oncogene or tumor suppressor in liver cancer, and its role may depend on the tumor type or cellular context.

Members of the miR-200 family, including miR-200a, miR-200b, miR-200c, and miR-429, have been reported to inhibit EMT and tumor invasion by targeting ZEB1 and ZEB2, which play vital roles in EMT progression and can bind to and repress the activity of the E-cadherin promoter. The current results demonstrated that lncRNA XIST indirectly regulated ZEB1/2 by sponging miR-200b-3p, and ZEB1 and ZEB2 were directly targeted by miR-200b-3p, consistent with previous reports. The miRNA-mediated inhibition of protein production might occur at the post-transcriptional level. In the present study, we demonstrated that miR-200b-3p bound to ZEB1/2 and dramatically decreased protein but not mRNA levels of ZEB1/2, thus supporting a mechanism involving post-transcriptional regulation of ZEB1/2. Taken together, the results of our study revealed that lncRNA XIST acted as a ceRNA for miR-200b-3p to regulate ZEB1/2 expression. In the current study, the number of liver cancer tissue specimens was limited. Therefore, additional samples are needed to confirm our data in the future.

In conclusion, our research indicates that lncRNA XIST acts as a prognostic factor in liver cancer, and promotes liver cancer progression by acting as an miR-200b-3p sponge. These findings indicate that lncRNA XIST could be a potential and important biomarker or therapeutic target for liver cancer.

Availability of data and materials
The datasets are available from the corresponding author.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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