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Long noncoding RNA Xist predicts the presence of lymph node metastases in human oesophageal squamous cell carcinoma

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Oesophageal cancer is the eighth most common cancer and the sixth most common cause of cancer death worldwide, developing from epithelial cells in two subtypes: oesophageal adenocarcinoma and oesophageal squamous cell carcinoma (OSCC) [1]. These forms have different risk factors and incidence and, whereas the incidence of OSCC is declining in most parts of the world, oesophageal adenocarcinoma incidence rates have risen sharply in developed countries over the past four decades [2]. Compared with the high incidence of Barrett's associated adenocarcinoma in Europe and the United States, the incidence of OSCC is prevalent in China. Despite the advances in therapy, OSCC is still one of the most lethal malignancies in China, with an overall 5-year survival rate of 20–30% after surgery [3].

Metastasis is a phenomenon of crucial importance in defining prognosis in patients with cancer and is often responsible for cancer-related mortality. Local lymph node metastasis is a typical sign of failure for OSCC clinical treatments, and a link has been established between these metastases and the aberrant expression of specific biomarkers, yet the molecular mechanism of metastatic dissemination remains unclear [4]. Long non-coding RNA (lncRNA) is a class of RNA molecules that with more than 200 bases. They have limited or lack protein-coding capacity, but regulate gene expression at various levels in the form of RNA (epigenetic regulation, transcriptional regulation, post-transcriptional regulation, etc.) [5]. Increasing evidence shows that lncRNAs are involved in certain biological processes of cancer initiation, progression, and metastasis [6].

lncRNA XIST (X-inactive specific transcript) is a product of the XIST gene and the master regulator of X inactivation in mammals. According to recent studies, XIST is up-regulated in several cancers, such as hepatocellular carcinoma [7], gastric cancer [8] and non-small cell lung cancer [9], and overexpression of XIST is associated with growth, invasion, and metastasis of these cancers. It has previously reported that XIST is highly expressed in tumour tissues from OSCC patients, whilst targeting XIST inhibited proliferation, migration and invasion of OSCC cells *in vitro* and suppressed tumour growth *in vivo* [10,11]. In the present study, we hypothesised a link between the relative expression of XIST in tumour versus normal tissue, and the presence of lymph node metastasis in OSCC patients.

This study was approved by the Ethics Committee of the central hospital of Linyi, Shandong, China. Signed informed consent was also obtained. OSCC tumour tissues and normal adjacent tissues were retrospectively selected from 148 OSCC patients who underwent surgery as their first and only treatment between 2005 and 2010 at the central hospital of Linyi. All tissue samples were snap frozen in liquid nitrogen immediately after surgery and stored at −80 °C until RNA extraction. For all samples, clinicopathologic information was available. Corresponding formalin fixed and paraffin embedded tissues were available from 102 of 148 samples. Of 148 patients, 89 had 5-year follow-up information available: median age was 59 years (range, 42–73); 87.6% were males; and median follow-up was 71 (7–104 months).

Total RNA was isolated from frozen tissues using a mirVana RNA isolation kit (Ambion, ThermoFisher, Milton Keynes, UK). The first strand cDNA was synthesised using RevertAid first strand cDNA synthesis kit (Fermentas, ThermoFisher, Milton Keynes, UK), which was then amplified with mirVana qRT-PCR primers sets (Ambion) by using TaqMan gene expression master mix (Applied Biosystems, Foster City, CA, USA) and Applied Biosystems 7300 Real Time PCR system following the manufacturer's instructions. RNU6B served as internal control. All PCR reactions, including no-template controls, were performed in triplicate. The relative expression of XIST was calculated as $2^{-(\Delta Ct-\Delta CNAT)}$. The 75th percentiles of $2^{-\Delta\Delta Ct}$ was used as the cut-off point for patients with high and low levels of XIST.

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Unpaired Student's t and χ^2 test was used to analyse the association between XIST and clinicopathologic parameters. Overall survival was defined as the time from date of diagnosis to the date of death by any cause, assessed using the Kaplan-Meier method. The log-rank test was performed to compare the survival curves of individual groups. Univariate and multivariate analyses were performed for prognostic factors of overall survival using the Cox regression model. The reported results included hazard ratio (HR) and 95% confidence intervals (CI). Results were displayed as mean with SD from at least triplicate experiments for each group. All statistical analyses were performed with SPSS 13.0 software. $p < 0.05$ was considered significant.

The expression of XIST in OSCC tissues was detected by qRT-PCR and the relationship between relative expression of XIST and clinicopathologic information of OSCC patients was analysed. The mean [SD] relative expression of XIST in patients with lymph node metastasis and TNM III was 1.63 [1.22] and 1.64 [1.20], which was significantly increased compared with patients without lymph node metastasis 1.01 [0.83] and TNM I+ II 1.01 [0.86] ($p = 0.004$ and $p = 0.011$, respectively) (Figure 1(a)). No significant association was found between XIST expression and other clinical characteristics such as age, gender, differentiation grade, gross pathological type, tumour position, and T classification (Table 1).

The potential of XIST as a prognosis marker was further investigated in 89 patients who had 5-year follow-up information. This subgroup had similar clinical characteristics as compared with the total 148 patients. Kaplan-Meier survival estimate showed that the patients with high XIST expression ($n = 27$) had a shorter survival compared with the patients with low XIST expression ($n = 61$) ($p = 0.016$; log-rank test)

Table 1. Association between XIST expression and clinicopathologic information.

		XIST expression		
Clinical parameters	Total(n = 148)	Low	High	p-value
Age				0.417
≤60 years	79	55	24	
> 60 years	69	46	23	
Gender				0.278
Female	26	16	10	
Male	122	85	37	
Gross pathology				0.268
Fungating	41	27	14	
Medullary	90	64	26	
Others	17	10	7	
TNM stage				0.019
I+ II	72	56	16	
III	76	45	31	
Lymph node metastasis				0.002
N1	77	66	11	
N0	71	35	36	
Position				0.876
Upper	13	8	5	
Middle	106	76	30	
Lower	29		12	
Differentiation				0.286
well+ moderate	70	42	28	
poor	43	25	18	
T classification				0.095
T1+ T2	22	14	8	
T3	109	76	33	
T4	19	14	5	

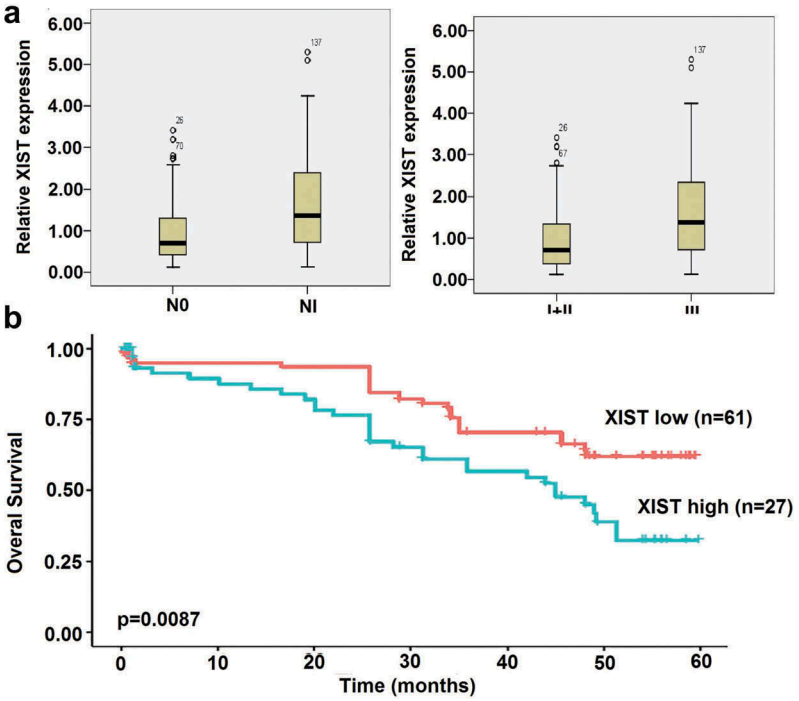

Figure 1. Expression of XIST is associated with lymph node metastasis and survival of OSCC patients. *A*, relative expression of XIST was compared between lymph node metastasis (*N1*) and without lymph node metastasis (*N0*), and between patients in stage I-II and patients in stage III. *B*, 89 OSCC patients were classified into high expression group and low expression group by the 75th percentiles of $2^{\Delta\Delta Ct}$.

(Figure 1(b)). Univariate Cox analysis was performed to determine whether overall survival is associated with other prognostic factors, including differentiation grade and TNM stages. Overall survival was linked with XIST expression (HR/95% CL 2.48, 1.29–4.93, $p = 0.001$), N (2.68, 1.26–6.04, $p = 0.001$), TNM stages (2.32, 0.96–4.17, $p = 0.028$), but not differentiation grade (2.24, 0.89–4.17, $p = 0.089$) and T stage (1.62, 0.59–4.73, $p = 0.284$). Furthermore, multivariate Cox proportional hazard regression analysis was performed using all four variables and showed that high XIST expression was an unfavourable prognostic factor (2.24, 1.13–5.18, $p = 0.017$) along with TNM stage (3.52, 1.18–5.17, $p = 0.042$).

XIST is reported to be upregulated in pancreatic cancer tissues compared to benign tissues and is shown to increase proliferation and migration in pancreatic cancer cell lines [12], suggesting that XIST could be associated with tumour progression and have prometastasis functions. In OSCC, high expression of XIST was associated with poor patient survival [10,11]. Furthermore, targeting XIST significantly inhibited the proliferation, migration and invasion, but induced apoptosis of OSCC cells [11]. We found XIST to be upregulated in OSCC tissues from patients that have lymph node metastasis and high TNM stage. The XIST -based prediction model effectively and accurately classifies post operation OSCC patients into groups with low risk and high risk of developing LNM. This model could be implemented for routine clinical use, and serve as a valuable tool for determining optimal treatment strategies for OSCC patients. This predictive model also may provide new therapeutic targets and facilitate decisions regarding individualised clinical therapies. Previously it has been shown that XIST is up-regulated in several cancers [7–9], and overexpression of XIST is associated with growth, invasion, and metastasis of these cancers. However, in human epithelial ovarian cancer (EOC), upregulation of lncRNA XIST may suppress EOC development through sponging effect to induce hsa-miR-214-3p downregulation [13]. These findings suggest that XIST plays an important role in tumour progression and metastasis formation in multiple cancers. However, the function of XIST appears to depend on the genetic background of the tumour

We initially sought to identify novel markers of lymph node metastasis. Given the increased use and success of XIST for the detection of tumour positive lymph nodes, markers to more accurately assess the risk of nodal involvement beyond imaging studies are very useful. We identified XIST upregulated in OSCC with involved lymph nodes. XIST appear to have potential for further development as markers to assess the risk of lymph node involvement using primary tumour biopsy tissues. Furthermore, we demonstrated that XIST is an independent prognostic marker for OSCC.

This work represents an advance in biomedical science because it shows that quantification of XIST in the OSCC could be useful to estimate the likelihood of the presence of pathologically positive lymph nodes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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