Overexpression of long non-coding RNA n346372 in bladder cancer tissues is associated with a poor prognosis

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Abstract. Accumulating evidence has confirmed that dysregulated long non-coding RNAs (lncRNAs) participate in the initiation and progression of a number of solid tumors and have potential applications for early diagnosis, targeted therapy, and the prognosis of patients with bladder cancer. In the present study, via high-throughput sequencing technology and bioinformatics analysis, a total of 169 lncRNAs with significantly differential expression between bladder cancer tissues and paired adjacent normal tissues (n=10) were initially identified by screening. Reverse-transcription-quantitative polymerase chain reaction was carried out to validate the expression levels of lncRNA-n346372 in 60 pairs of tissue samples from bladder cancer patients. The results indicated that lncRNA-n346372 was upregulated in bladder cancer tissues compared with the matched adjacent normal tissues ($P<0.05$). In addition, the results of fluorescence *in situ* hybridization analysis of bladder cancer cells and tissues demonstrated that lncRNA-n346372 is located in the cytoplasm, and the expression of lncRNA-n346372 in bladder cancer tissues was significantly increased compared with the paired normal tissues. Following a χ^2 test with common clinical variables among the patients, the expression level of lncRNA-n346372 was demonstrated to be positively associated with advanced tumor stage and poor histological differentiation of bladder cancer. Kaplan-Meier survival analysis revealed that patients with high expression of n346372 were more likely to have a poor prognosis compared with patients with low n346372 expression. Finally, univariate and multivariate analyses indicated that the relative level of n346372, apart from tumor stage and histological grade, may serve as an independent prognostic factor of bladder cancer. To the best of the authors' knowledge, this is the first study to verify the dysregulated expression of lncRNA-n346372 in bladder cancer; an association of this lncRNA with overall survival of bladder cancer patients was also uncovered in the present study, suggesting that lncRNA-n346372 may contribute to the initiation and/or progression of bladder cancer with potential applications in the clinic.

Introduction

Bladder cancer is a common genitourinary disease worldwide, particularly in the male population, with ~74,000 newly diagnosed cases in the United States in 2014 (1,2). Despite advances in surgical techniques, including the widespread application of minimally invasive surgery as well as an improved understanding of multimodal treatments involving chemotherapy, radiotherapy, and immunotherapy, the 5-year cancer-specific survival for patients with advanced bladder cancer remains ~50% without any improvement in the past two decades (3-6). Furthermore, the decision making in clinical practice depends on the results of cystoscopy examination, imaging and histopathological examination, which cannot provide sufficient information regarding patients' prognosis (7). Therefore, it is necessary to investigate novel molecular biomarkers that may aid increased accuracy of prognostic evaluation.

Long non-coding RNAs (lncRNAs), which can be located in the nucleus or cytoplasm, are RNA molecules of >200 nucleotides with limited protein-coding potential (8,9). With the help of microarray and next-generation sequencing, accumulating evidence has confirmed that a number of dysregulated lncRNAs in bladder cancer tissues and cell lines may serve critical roles in tumor formation, progression, and/or metastasis, e.g., urothelial cancer associated 1 (UCA1), maternally expressed gene 3 (MEG3), H19 (10-13), and these lncRNAs when detected in cancerous tissues and body fluids may serve as biomarkers for early diagnosis, as therapeutic targets, and/or prognostic markers of bladder cancer. For example, associated studies have identified that the upregulated lncRNA-UCA1 in bladder cancer may

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Abbreviations: FISH, fluorescence *in situ* hybridization; lncRNA, long non-coding RNA; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

Key words: long non-coding RNA, bladder cancer, prognosis

contribute to tumor proliferation, patient mortality and tumor invasiveness, and its detection in the urine sediment may improve the sensitivity and specificity of bladder cancer diagnoses (14,15).

In addition, certain upregulated lncRNAs in bladder cancer, including lncRNA-n336928, cervical carcinoma expressed proliferating cell nuclear antigen-regulatory lncRNA and promoter of cyclin-dependent kinase inhibitor 1 antisense DNA damage-activated RNA, have not only been reported to have the ability to promote tumorigenesis, but have also been implicated in poor prognosis and may serve as independent prognostic factors (7,16,17). Therefore, lncRNAs as novel biomarkers could be useful in clinical procedures involving the diagnosis and prognosis of bladder cancer.

In the present study, based on previous high-throughput sequencing applied to 10 pairs of tissue samples, the aim was to screen RNAs for significantly differentially expressed lncRNAs that could be novel molecular biomarkers for the prognosis of bladder cancer. It is noteworthy that differential expression patterns of lncRNA-n346372 were identified in bladder cancer tissues and corresponding normal tissues; furthermore, the association of this lncRNA with clinical variables and prognosis of patients with bladder cancer were investigated.

Patients and methods

Patients, tissue specimens and cell lines. A total of 60 patients with bladder cancer and paired normal tissues adjacent to the tumor were included in the present study; all patients provided written informed consent. Following a radical cystectomy, all the resected specimens were snap-frozen in liquid nitrogen immediately and then stored at -80˚C (in a freezer) until analysis. Initially, 10 pairs out of the total number of samples, including five pairs of non-muscle-invasive bladder cancer (NMIBC; Ta three cases; T1 two cases) and five pairs of muscle-invasive bladder cancer (MIBC; T2a three cases; T2b two cases) were analyzed by high-throughput transcriptome sequencing. The present study's protocol was approved by the Ethics Committee of Changhai Hospital of the Second Military Medical University (Shanghai, China).

T24 bladder cancer cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS; both Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 1% ampicillin (100 units/ml) and streptomycin (100 units/ml; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidifed atmosphere of 95% air and 5% CO_2. T24 cells were plated at a density of $5x10^4$ per well in 6-well plates.

Clinical data collection. Clinical parameters in the present study, including age, sex, smoking history, tumor size, tumor number, tumor stage and histological grade, are summarized in Table I. The 2002 Tumor Node Metastasis criteria were adopted to evaluate the tumor tissue stage and the 2004 World Health Organization classification was employed to evaluate the histological grade (18); the pathological diagnosis of each specimen was made independently by two pathologists. In particular, there were 15 cases diagnosed with NMIBC and 45 cases of MIBC, and out of all the samples 17 cases were classified as low-grade bladder cancer and 43 cases as high-grade bladder cancer. Follow-up information came from outpatient visits and regular telephone interviews.

High-throughput transcriptome sequencing. Total RNA was extracted from 10 pairs of samples using the TRIzol reagent (Thermo Fisher Scientific, Inc.), followed by quantification and a purity check on a NanoDrop-2000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and an integrity check based on formaldehyde denaturing 2% agarose gel electrophoresis (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following DNA digestion with DNase I (Bio-Rad Laboratories, Inc.), enrichment of total RNA was conducted to isolate mRNA or removal of ribosomal RNAs using magnetic beads with oligo (dT) (New England BioLabs, Inc., Ipswich, MA, USA). Mixed with Fragmentation Buffer (Ambion; Thermo Fisher Scientific, Inc.), the enriched mRNAs were broken down into short fragments, which could serve as reverse-transcription templates for the subsequent cDNA synthesis. These short fragments were purified and resolved in Elution buffer (Ambion; Thermo Fisher Scientific, Inc.) for end repair and single-nucleotide A (adenine) addition. After being attached to adapters and following agarose gel electrophoresis analysis, they were selected as suitable templates for polymerase chain reaction (PCR) amplification using OneTaq® DNA polymerase (New England BioLabs, Inc.). The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 30 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. Subsequently, the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) and the ABI StepOnePlus Real-Time PCR System (Agilent Technologies, Inc.) were used for quantification and quality evaluation of the established sample library and were designated as the quality control steps. Finally, the library was sequenced on a HiSeq™ 2000 instrument (Illumina, Inc., San Diego, CA, USA). The National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/genome/gdv/browser/?context=genome&acc=GCF_000001405.38) was used to search sequence assignment of lncRNA-n346372.

Reverse transcription-quantitative PCR (RT-qPCR). Following extraction from bladder cancer tissues and corresponding normal tissues with the TRIzol reagent (Thermo Fisher Scientific, Inc.), total RNA was reverse-transcribed to cDNA using the PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan) at 37˚C for 15 min and 95˚C for 5 sec, followed by storage at 4˚C. RT-qPCR was conducted using SYBR® Premix Ex Taq™ (Takara Bio, Inc.) with an Applied Biosystems Step One Plus (Agilent Technologies, Inc.). The thermocycling parameters were as follows: 95˚C for 30 sec, followed by 30 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. A total of 20 μl PCR reaction mixture was prepared for incubation in a 96-well optical plate and melting curve analysis was carried out to assess the specificity of the PCR products.

Table I. Correlation analysis between ln346372 expression level and clinical-pathological data of all enrolled patients in the present study.

		Ln346372 expression			
Variable	Group	Low, n (%)	High, n (%)	Total	P-value
Age, (years)	≤60	4 (26.7)	11 (73.3)	15	0.133
	>60	22 (48.9)	23 (51.1)	45	
Sex	Male	21 (39.6)	32 (60.4)	53	0.377
	Female	4 (57.1)	3 (42.9)	7	
Smoking history	No	20 (50.0)	20 (50.0)	40	0.064
	Yes	5 (25.0)	15 (75.0)	20	
Tumor size (cm)	≤3	6 (50.0)	6 (50.0)	12	0.608
	>3	19 (39.6)	29 (60.4)	48	
Tumor number	Unifocal	4 (50.0)	4 (50.0)	8	0.513
	Multifocal	21 (40.4)	31 (59.6)	52	
Tumor stage	≤T1	11 (73.3)	4 (26.7)	15	0.004
	T2-T4	14 (31.1)	31 (68.9)	45	
Tumor grade	Low	14 (82.4)	3 (17.6)	17	<0.001
	High	11 (25.6)	32 (74.4)	43	

ln346372, long non-coding RNA 346372.

The sequences of primers were as follows: n346372 Forward primer, 5'-ATGTGATGGTGAGTAGGAG-3', and reverse primer, 5'-GAAGCAGTGTCATTTAGAGCA-3'; β-actin forward primer, 5'-GAGCTACGAGCTGCCTGACG-3', and reverse primer, 5'-CCTAGAAGCATTTGCGGTGG-3'. The relative expression level of lncRNA-n346372 was calculated by the $2^{-\Delta\Delta Cq}$ method (19) with β-actin as an endogenous gene for normalization.

Fluorescence in situ hybridization (FISH). A Ribo™ lncRNA FISH kit (cat. no. C10910) purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China) was employed for RNA FISH to identify the location and expression of lncRNA-n346372 in T24 bladder cancer cells and patients tissue samples, according to the manufacturer's protocol. In brief, cells were cultured (and 4-mm-thick tissue slices were prepared), and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells and tissues were permeabilized with Triton-100 for 5 min at 4˚C (Beyotime Institute of Biotechnology, Shanghai, China), and Cy3-labelled n346372 and DAPI-labelled 18S-RNA probes (Guangzhou RiboBio Co., Ltd.) were detected. In the dark, DNA was stained with DAPI for 10 min at room temperature, followed by washing in PBS three times every 5 min. Slides were mounted and examined by confocal fluorescence microscopy (x200; Olympus Corporation, Tokyo, Japan).

Statistical analysis. The data were presented as the mean ± standard deviation of at least three independent experiments. All statistical analyses were conducted using the SPSS software, version 22.0 (IBM Corp., Armonk, NY, USA). Continuous variables were analyzed by the Student's t test and categorical variables were subjected to the χ^2 test. The overall survival rates were calculated by the Kaplan-Meier method with the log-rank test to assess the level of significance between two the survival curves. Univariate and multivariate Cox regression models were employed to investigate independent predictive factors of overall survival. Heat maps were generated with MeV 4.8 software (mev.tm4.org). P<0.05 was considered to indicate a statistically significant difference.

Results

Numerous lncRNAs are significantly upregulated in bladder cancer tissues compared with matched adjacent normal tissues. According to the results of the high-throughput sequencing applied to 10 pairs of bladder cancer tissues and matched adjacent non-cancerous tissues, with P<0.05, a fold change value >2, and false discovery rate <0.05 as the screening strategy, a total of 169 lncRNAs were identified in accordance with the above criteria. These 169 lncRNAs were demonstrated to exhibit significantly differential expression between bladder cancer tissues and matched adjacent normal tissues, including 32 upregulated lncRNAs exhibited in a heat map (P<0.05; Fig. 1) and 137 downregulated lncRNAs. Notably, in the upregulated group, eight lncRNAs with the fold change >3.0 were identified; in descending order they were n346372, n337945, n341831, n344768, n377154, n387149, n385823 and n373941.

Relative expression level of n346372 increases in bladder cancer tissues compared with the matched adjacent normal tissues. The following verification was performed on 60 pairs of tissue samples and the results of the RT-qPCR experiment demonstrated that the relative expression level

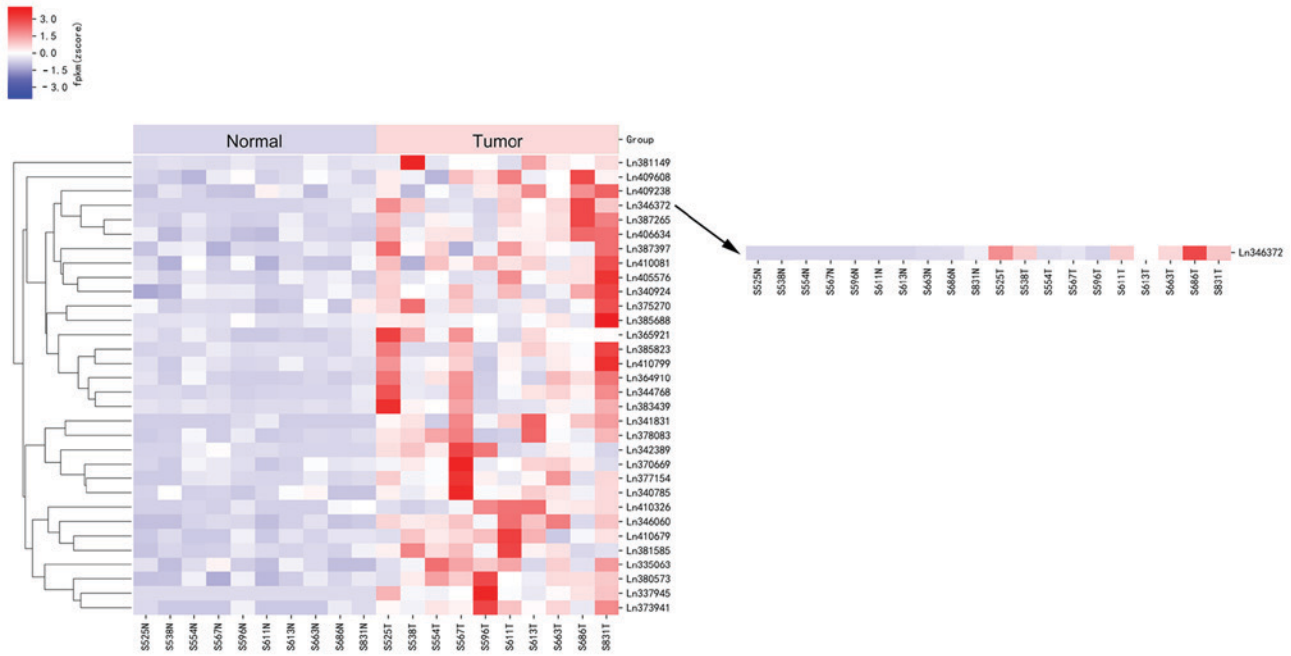

Figure 1. Hierarchical clustering analysis of 32 upregulated lncRNAs with a fold change >3.0 according to high-throughput sequencing. A total of 10 pairs of bladder T tissues and paired adjacent N tissues samples are indicated below the heat map. The heat map reflecting lncRNA-n346372 expression has been copied and presented separately adjacent to the main heat map. lnc, long non-coding; T, tumor; N, normal.

of n346372 was significantly increased in bladder cancer tissues compared with matched adjacent normal tissues; this result was consistent with the sequencing data (P<0.001; Fig. 2). Furthermore, following the RNA FISH assay of T24 bladder cancer cells and 10 pairs of tissue samples, it was demonstrated that upregulated n346372 was located in the cytoplasm (Fig. 3), and the fluorescent signal of n346372 in bladder cancer tissues exceeded that in matched adjacent normal tissues (Fig. 4), providing further evidence of the differential expression trend between bladder cancer tissues and corresponding normal tissues.

Overexpression of n346372 in bladder cancer tissues is associated with a poor prognosis and can serve as an independent prognostic factor of overall survival. Initially, the median level of tumor n346372 in all enrolled patients was selected as a cut-off level and on this basis, the study population was divided into two groups: Low-expression (n=25) and high-expression (n=35). First, it was demonstrated that ~68.9% of patients with the diagnosis of MIBC exhibited high n346372 expression in the tumor, whereas only 26.7% of patients with NMIBC exhibited such a high level in the tumor. Similarly, 74.4% of patients with poorly differentiated tumor tissue overexpressed n346372, while only 17.6% of patients with a low histological grade of the tumor overexpressed n346372. Following analysis of the relative level of n346372 and certain common clinical variables, the overexpression of n346372 in bladder cancer was identified to be positively associated with advanced tumor stage and higher histological grade (P<0.05; Table I). Furthermore, following examination of the overall survival rate with the Kaplan-Meier method used in terms of the relative level of n346372, the results demonstrated that the overall survival in the low-expression group was significantly improved

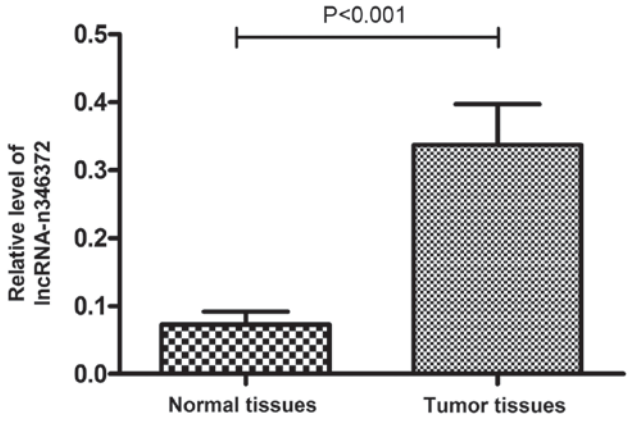

Figure 2. Dysregulation of lncRNA-n346372 expression. The relative levels of lncRNA-n346372 in bladder cancer tissues were significantly increased compared with the paired adjacent normal tissues. lnc, long non-coding.

compared with the high-expression group (P<0.05; Fig. 5). Univariate and multivariate Cox regression analyses were performed to determine the prognostic significance of n346372 expression levels, and the univariate analysis indicated that the n346372 expression, tumor stage, and tumor grade were significantly associated with the overall survival of bladder cancer patients (P<0.01; Table II), whereas the multivariate analysis demonstrated that apart from tumor stage and histological grade, the n346372 expression level was also an independent prognostic factor of bladder cancer (P<0.05; Table II).

Discussion

Accumulating evidence has confirmed that despite initially being regarded as spurious transcriptional noise, lncRNAs

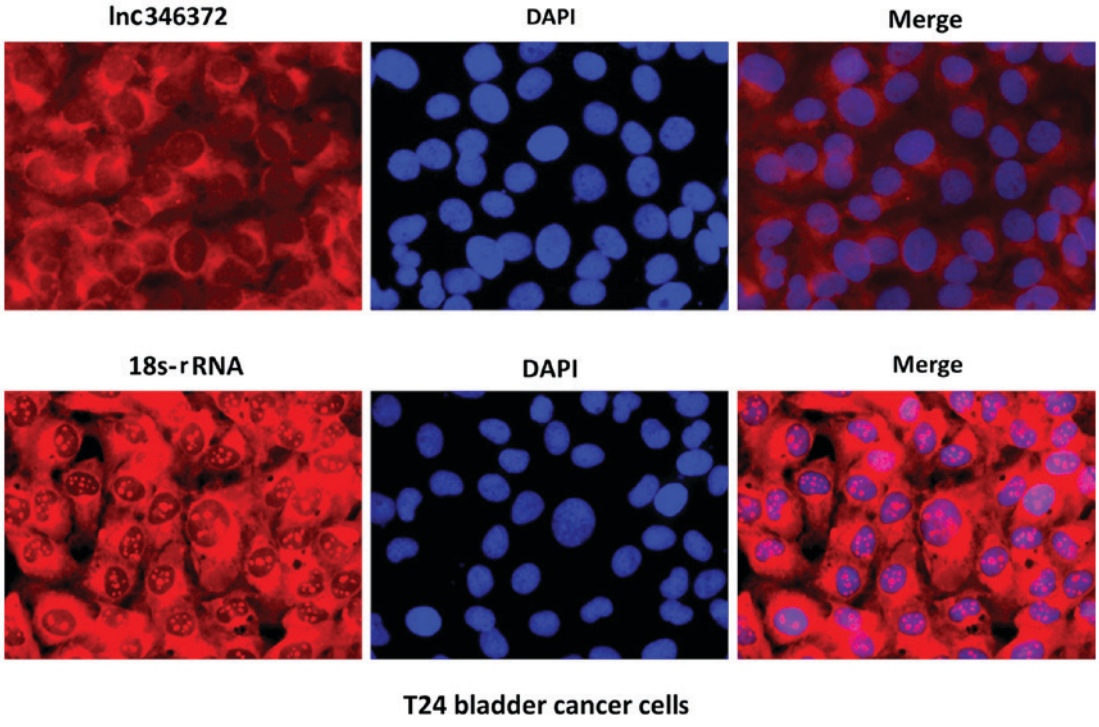

Figure 3. Fluorescence *in situ* hybridization analysis of subcellular location of lncRNA-n346372 and control 18S rRNA in T24 bladder cancer cells. Nuclei were stained blue (DAPI) and lncRNA-n346372 and control 18S rRNA was stained red. lncRNA-n346372 is mainly located in the cytoplasm. Magnification, x400. rRNA, ribosomal; lnc, long non-coding.

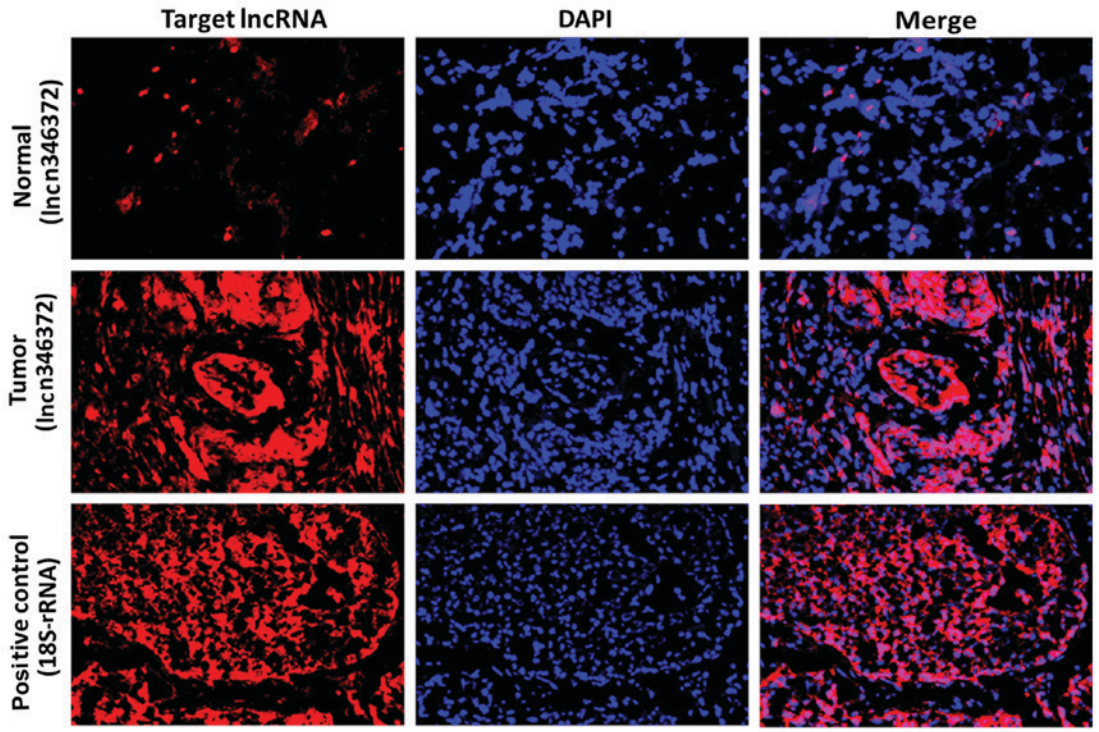

Figure 4. The expression levels of the analyzed lncRNAs according to RNA fluorescence *in situ* hybridization in bladder cancer tissues and paired adjacent normal tissues. Nuclei were stained blue (DAPI) and lncRNA-n346372 and control 18S ribosomal RNA was stained red. LncRNA-n346372 expression in bladder cancer tissues was upregulated compared with the paired adjacent normal tissues. Magnification, x200. Lnc, long non-coding; rRNA, ribosomal RNA.

exert strong regulatory action on diverse biological processes, particularly cellular development and metabolism. LncRNAs can also be expressed abnormally in a variety of solid tumors as well as serving as ideal molecular biomarkers for the diagnosis and prediction of the prognosis of a number of different types of cancer (20-25). Additionally, a handful of dysregulated lncRNAs identified in previous studies serve important roles in bladder cancer pathogenesis and may

Table II. Univariate and multivariate Cox regression analysis for prognostic factors predicting overall survival of patients with bladder cancer.

	Univariate analysis			Multivariate analysis		
Covariant	HR	95% CI	P-value	HR	95% CI	P-value
n346372 expression	5.563	1.992-15.536	0.001	3.729	1.127-12.346	<0.001
Age (year)	0.998	0.369-2.696	0.997			
Sex	1.682	0.570-4.961	0.346			
Smoking history	0.938	0.369-2.384	0.893			
Tumor size (cm)	1.658	0.388-7.091	0.495			
Tumor number	1.507	0.511-4.443	0.457			
Tumor stage	16.420	2.148-125.526	0.007	8.338	1.066-65.201	0.043
Tumor grade	13.816	1.854-102.945	0.010	8.187	1.043-64.245	0.045

HR, hazard ratio; CI, confidence interval.

increase diagnostic efficacy and prognostic accuracy (26-31). For example, protein sprouty homolog 4-intronic transcript 1, HOX antisense intergenic RNA and metastasis-associated lung adenocarcinoma transcript 1 have been demonstrated to be substantially upregulated in bladder cancer, and further investigation indicates that these upregulated lncRNAs in bladder cancer are closely associated with an advanced pathological stage, recurrence of the Ta/T1 stage and poor survival, respectively. Furthermore, a small interfering RNA-mediated knockdown of these genes significantly inhibits the biological functions of bladder cancer cells, including their proliferation, invasiveness and migration capability (32-33). In contrast, several lncRNAs downregulated in bladder cancer have also been reported, for example, MEG3, a gene encoding a lncRNA, which is expressed in normal tissues while its expression is lacking in a number of cell lines as well as in bladder cancer; additionally, the downregulation of lncRNA-MEG3 can activate autophagy and increase cell proliferation and is negatively associated with bladder cancer formation (10,12,34).

In the present study, significant overexpression of lncRNA-n346372 was first identified in bladder cancer tissues compared with paired adjacent normal tissues; the gene locus of this lncRNA is located on chromosome 22 (chr22: 16189344-16192955) with a mature transcript of 228 bp in length. The relative level of n346372 was validated by RT-qPCR in 60 pairs of bladder cancer tissues in addition to the results of FISH in tissue samples, suggesting that the overexpression of n346372 in bladder cancer may be involved in certain pathways contributing to tumorigenesis and/or cancer progression. Nonetheless, sequence assignment based on the NCBI database revealed that this region contains no protein-coding genes and the specific regulatory function and a possible target gene(s) remain unclear; therefore, future studies at the cellular and molecular levels need to be conducted to investigate the detailed molecular mechanism of action of n346372.

Notably, following the analysis of the expression level of n346372 and clinical variables, the results demonstrated that 68.9% of the patients with an advanced tumor stage (T2-T4) are more likely to exhibit upregulation of n346372 in the tumor, and the two parameters exhibit a positive association, indicating that n346372 may promote the process of muscle invasion in bladder cancer. Additionally, 74.4% of patients with a high histological grade tended to highly express this lncRNA in the tumor and a similar association between upregulated n346372 and poor differentiation was identified, suggesting that the overexpression of n346372 may contribute to tumor progression and poor prognosis of patients with bladder cancer. To prove this hypothesis, a comparison of overall survival between the two groups was conducted and the results revealed that patients in the high-expression group are more likely to have a poor prognosis. Finally, multivariate analysis indicated that n346372 expression correlates with the prognosis of bladder cancer as well as the tumor stage and histological grade described in a series of guidelines. These data demonstrated that n346372 may serve as an independent prognostic factor of bladder cancer. There are certain limitations to the present study. Independent verification of the results of the present study

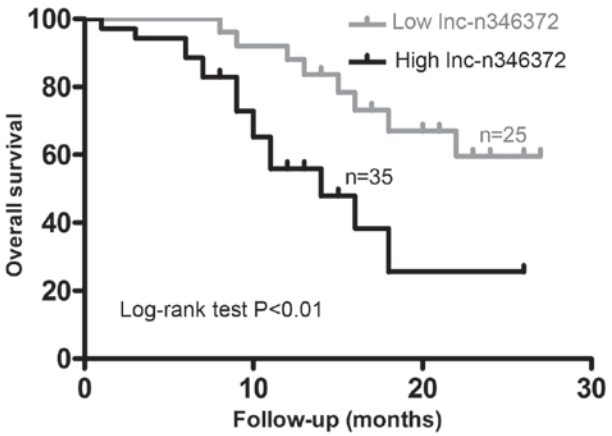

Figure 5. The comparison of overall survival rates between groups with high lnc-n346372 expression (n=35) and low lnc-n346372 expression (n=25) by the Kaplan-Meier method. Patients in the high-expression group exhibited much shorter survival compared with those in the low-expression group (P<0.01; log-rank test). Lnc, long non-coding.

is not guaranteed because of the small sample size used. A larger number of samples need to be subsequently analyzed for validation. In addition, it is necessary to perform independent studies to confirm the prognostic value of n346372 in bladder cancer.

In conclusion, to the best of the authors' knowledge this is the first study describing differential expression of n346372 in bladder cancer tissues compared with paired adjacent normal tissues. A positive association of n346372 overexpression with advanced tumor stage and poor differentiation was also noted. Furthermore, bladder cancer patients with upregulated n346372 are more likely to have an unfavorable prognosis in contrast with patients who exhibit downregulation and consequently, n346372 was demonstrated in the present study to hold promise as an independent prognostic factor of bladder cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AL, ZZ and CX designed the study. AL performed the experiments. ZZ drafted this manuscript. CX critically revised the manuscript. WX coordinated the experiments, interpreted the data and prepared the manuscript. SQ performed the statistical analysis. MH collected and analyzed the clinical samples. SZ designed the RNA-FISH and high-throughput transcriptome sequencing experiments.

Ethics approval and consent to participate

The present study's protocol was approved by the Ethics Committee of Changhai Hospital of the Second Military Medical University.

Patient consent for publication

All of the patients provided written informed consent.

Competing interests

The authors declare they have no competing interests.

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