ORIGINAL ARTICLE

LncRNA TCONS_l2_00002973 correlates with less advanced tumor stage and favorable survival, and also inhibits cancer cells proliferation while enhancing apoptosis in triple-negative breast cancer

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Summary

Purpose: This study aimed to investigate the correlation of long non-coding RNA (lncRNA) TCONS_l2_00002973 expression with clinicopathological features as well as overall survival (OS) in triple-negative breast cancer (TNBC) patients, and further explore the regulatory effect of lncRNA TCONS_l2_00002973 on cells' proliferation and apoptosis in TNBC cells.

Methods: 96 TNBC patients undergoing surgery were consecutively enrolled in this prospective cohort study. LncRNA TCONS_l2_00002973 expression in tumor tissue sample and adjacent non-tumor sample was detected by qPCR. Normal control (NC) shRNA (NC(-) group), lncRNA TCONS_l2_00002973 shRNA (Lnc(-) group), NC overexpression (NC(+) group) and lncRNA TCONS_l2_00002973 overexpression plasmids (Lnc(+) group) were transfected into MDA-MB-231 breast cancer cells. Cell proliferation ability, cell apoptosis rate and apoptosis-related protein expressions were detected using CCK-8 assay, annexin V (AV)/propidium iodide (PI) assay and Western blot assay respectively.

Results: LncRNA TCONS_l2_00002973 expression was lower in tumor tissue compared to paired adjacent tissue (p<0.001), and its low expression was associated with increased T stage (p=0.002), raised N stage (p=0.003) and advanced TNM stage (p=0.005) in TNBC patients. Poor OS was found in lncRNA TCONS_l2_00002973 low expression group compared to lncRNA TCONS_l2_00002973 high expression group (p=0.006). Further in vitro experiments disclosed that lncRNA TCONS_l2_00002973 expression was reduced in various breast cancer cell lines compared to normal breast cell line, and lncRNA TCONS_l2_00002973 repressed cell proliferation and enhanced cells apoptosis in MDA-MB-231 cells.

Conclusions: LncRNA TCONS_l2_00002973 correlates with less advanced tumor stage and favorable survival, and it also inhibits cancer cells proliferation while enhances apoptosis in TNBC.

Key words: cell apoptosis, cell proliferation, lncRNA TCONS_l2_00002973, overall survival, triple-negative breast cancer

Introduction

Breast cancer is the most common cancer in females and the leading cause of cancer-related deaths among females worldwide [1-3]. An estimated 1,700,000 new cases and 520,000 deaths occurred in 2012 around the world, while 268,600 new cases and 69,500 deaths occurred in 2015 in females in China [4-6]. Among all types of breast cancers, triple-negative breast cancer (TNBC) is the

most aggressive subtype characterized by dismal prognosis as well as rapid recurrence, which is characterized by lack of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expressions [7]. Partly due to its molecular characteristics, TNBC is difficult to benefit from common therapies for breast cancer, such as endocrine therapy and HER-2 targeted therapy, and it's easy to develop resistance to chemotherapy and radiotherapy [8-14]. Thus, there is an urgent need to explore novel targets to monitor disease progression and prognosis in TNBC patients.

Long non-coding RNAs (lncRNAs), a class of endogenous RNAs with more than 200 nucleotides, is known to participate in multiple biological processes, and their aberrant expressions are associated with several cancer biological activities such as cell proliferation, apoptosis and metastasis [15-18]. LncRNA TCONS_l2_00002973, located on chromosome 10 from 37261767 to 37284630, was initially discovered to be downregulated in TNBC tissues compared with paired normal adjacent tissues by analysis of Agilent human lncRNA microarray chips, and in consideration of its downregulation in TNBC, we suspected that it may correlate with progression and prognosis of TNBC, whereas the role of lncRNA TCONS_l2_00002973 in TNBC is still largely unknown [19]. Thus, we conducted this study to investigate the correlation of lncRNA TCONS_l2_00002973 expression with clinicopathological features as well as overall survival (OS) in TNBC patients, and further explore the regulatory effect of lncRNA TCONS_l2_00002973 on proliferation and apoptosis in TNBC cells.

Methods

Patients and data collection

96 TNBC patients operated between 2013/1/1 and 2014/12/31 at the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, were consecutively enrolled in this prospective cohort study. Baseline key tumor features including pathological grade and TNM stage were documented, and patients were regularly followed up for 36 months, the last follow-up date being 2018/1/1. The protocol of this study was approved by the Ethics Committee of the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, and each patient signed informed consent before inclusion.

Detection of lncRNA TCONS_l2_00002973 expression in TNBC tissue and adjacent normal tissue sample

TNBC tissue sample and paired adjacent normal samples were obtained during the surgery and stored in liquid nitrogen for measurement of lncRNA TCONS_

l2_00002973 expression. LncRNA TCONS_l2_00002973 expression in TNBC tissue and adjacent normal tissue was determined by quantitative polymerase chain reaction (qPCR) assay.

Cells culture and detection of lncRNA TCONS_l2_00002973 expression

Breast cancer cell lines including BT474, MCF7, MDA-MB-453 and MDA-MB-231 were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). In addition, normal breast epithelial cell line MCF10A was also purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). BT474 cells were cultured in 90% RPMI-1640 sodium (GIBCO, USA) with 10% fetal bovine serum (FBS) (GIBCO, USA), MCF7 cells were cultured in 90% MEM sodium (GIBCO, USA) with 10% FBS (GIBCO, USA), MDA-MB-453 and MDA-MB-231 cells were cultured in 90% L-15 sodium (GIBCO, USA) with 10% FBS (GIBCO, USA) and MCF10A cells were cultured in MEGM kit (Sigma, USA). All cells were cultured in an incubator with 5% CO_2 at 37°C. LncRNA TCONS_l2_00002973 expression in each cell line was determined using qPCR assay.

Transfection

ShRNA plasmids pGPU6 (NTCC, China) and overexpression plasmids pEX-2 (NTCC, China) were used in the transfection of TNBC cells (MDA-MB-231). After construction, NC shRNA, lncRNA TCONS_l2_00002973 shRNA, NC overexpression and lncRNA TCONS_l2_00002973 overexpression plasmids were transfected into MDA-MB-231 cells and divided into NC(-) group, Lnc(-) group, NC(+) group and Lnc(+) group. MDA-MB-231 cells were cultured in 90% L-15 sodium (GIBCO, USA) with 10% FBS (GIBCO, USA).

Detection of cell proliferation and apoptosis

After transfection, lncRNA TCONS_l2_00002973 expression in each group was detected at 24 h using qPCR assay to validate the transfection results. Cell proliferation ability was detected at 0, 24, 48 and 72 h using Counting Kit-8 (CCK-8) (Abcam, USA) following the instructions of the manufacturer. Cell apoptosis rate was detected at 72 h using Annexin V (AV)/propidium iodide (PI) assay according to the instructions of the manufacturer, while the apoptotic marker C-Caspase 3 and anti-apoptotic marker P-P38 protein expressions were detected at 72 h using Western blot assay.

qPCR assay

qPCR was used to determine the expression of lncRNA TCONS_l2_00002973 in tissues and cells. Firstly, total RNA was extracted from tissues or cells using TRIzol solution (Invitrogen, USA); secondly, RNA was reversely transcribed using PrimeScript RT reagent (Takara, Japan); thirdly, qPCR was carried out using the One Step SYBR PrimeScript RT-PCR Kit (Takara, Japan). GAPDH was used as internal reference in qPCR assay, and lncRNA TCONS_l2_0000297 expression was calculated using $2^{-\Delta\Delta Ct}$ formula. The primer of lncRNA TCONS_l2_00002973 was: Forward 5' GCCATGCTCAG-

GATCTAGGAAG 3', Reverse 5' AGATGCCAAAGAACAC-CAGACT 3'; the primer of GAPDH was: Forward 5' GAGTCCACTGGCGTCTTCAC 3', Reverse 5' ATCTTGAG-GCTGTTGTCATACTTCT 3'

Western blot assay

One mL RIPA buffer (Sigma, USA) was added to cells on ice, and subsequently the cells suspension was centrifuged (16000 rpm, freezing condition). The collected supernatant and the total protein concentration were determined by bicinchoninic acid (BCA) kit (Pierce Biotechnology, Rockford, IL, USA). Thermal denaturation was performed, and 20 μg protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Thermo, USA), and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore,

Bedford, USA). Five% skim milk was used to block the membranes, which were subsequently incubated with the corresponding primary antibody overnight at 4°C, and further incubated for 90 min with the appropriate HRP-conjugated secondary antibody at room temperature. Finally, the bands were exposed to X-ray film and visualized with RapidStep™ ECL Reagent (Millipore, Bedford, USA). Antibodies used in Western blot assay are listed in Table 1.

Statistics

Statistical analyses were performed using SPSS 19.0 software (IBM, USA) and GraphPad Prism 5.01 software (GraphPad Int, USA). Overall survival (OS) was calculated from the date of surgery to the date of death. Data were mainly expressed as mean±standard deviation (SD),

Table 1. Antibodies used in Western blot assay

Antibody	Company (Country)	Dilution
Primary antibody		
Cleaved Caspase-3 Rabbit mAb	CST (USA)	1:1000
Caspase-3 Antibody	CST (USA)	1:1000
p38 MAPK Antibody	CST (USA)	1:1000
Phospho-p38 MAPK Antibody	CST (USA)	1:1000
GAPDH Rabbit mAb	CST (USA)	1:1000
Secondary antibody		
Anti-rabbit IgG, HRP-linked Antibody	CST (USA)	1:2000

CST: Cell Signalling Technology

Table 2. Correlation of characteristics of TNBC patients with lncRNA TCONS_l2_00002973

| Parameters	lncRNA TCONS_l2_00002973 low expression (N=48) n (%)	lncRNA TCONS_l2_00002973 high expression (N=48) n (%)	p value
Age (years)	54.27±14.16	56.08±10.73	0.481
Pathological grade			0.069
Grade 1	6 (21.5)	14 (29.2)	
Grade 2	38 (79.2)	33 (68.8)	
Grade 3	4 (8.3)	1 (2.1)	
T stage			0.002
T1	9 (18.8)	17 (35.4)	
T2	24 (50.0)	29 (60.4)	
T3	15 (31.3)	2 (4.2)	
N stage			0.003
N0	7 (14.6)	18 (37.5)	
N1	16 (33.3)	20 (41.7)	
N2	17 (35.4)	4 (8.3)	
N3	8 (16.7)	6 (12.5)	
TNM stage			0.005
Stage I	1 (2.1)	4 (8.3)	
Stage II	22 (45.8)	34 (70.8)	
Stage III	25 (52.1)	10 (20.8)	

Data were presented as mean±standard deviation or percents. Comparison was determined by t-test or chi-square test. P<0.05 was considered as significant. TNBC: Triple-negative breast cancer; lncRNA: long non-coding RNA

standard error of the mean (SEM) or percents. Comparison between two individual groups was detected by *t*-test or x^2 test. Comparison between two paired groups was detected by Wilcoxon signed rank sum test, and OS was calculated with the Kaplan-Meier method and compared with log-rank test. P<0.05 was considered as significant.

Results

Comparison of lncRNA TCONS_l2_00002973 expression between tumor tissue and paired adjacent normal tissue

As displayed in Figure 1, lncRNA TCONS_l2_00002973 expression was remarkably decreased
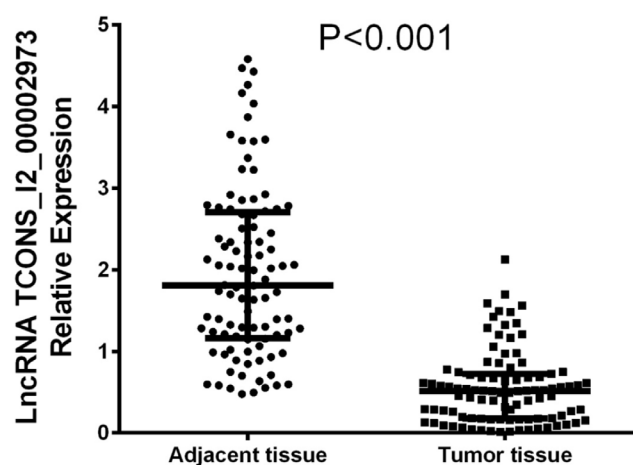
Figure 1. LncRNA TCONS_l2_00002973 expression in tumor tissue and adjacent normal tissue. LncRNA TCONS_l2_00002973 was upregulated in tumor tissue compared to adjacent tissue. Comparison between the two groups was determined by paired t-test.
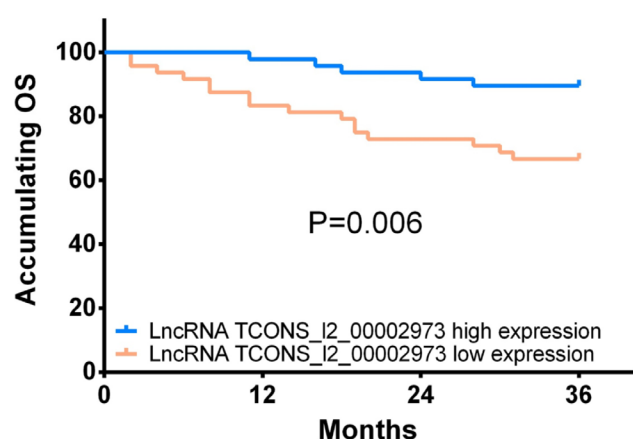
Figure 2. Accumulating OS in lncRNA TCONS_l2_00002973 high expression group and lncRNA TCONS_l2_00002973 low expression group. Patients with lncRNA TCONS_l2_00002973 low expression presented worse overall survival (OS) compared to patients with lncRNA TCONS_l2_00002973 high expression. Kaplan-Meier method was used to display OS and log-rank test to compare OS between two groups.

in tumor tissue compared to paired normal adjacent tissue in TNBC patients (p<0.001).

Correlation of lncRNA TCONS_l2_00002973 expression with tumor features of TNBC

All TNBC patients were divided into lncRNA TCONS_l2_00002973 high expression group (N=48) and lncRNA TCONS_l2_00002973 low expression group (N=48) according to its median value for the following analysis. LncRNA TCONS_l2_00002973 low expression was associated with increased T stage (p=0.002), raised N stage (p=0.003) and advanced TNM stage (p=0.005), and its low expression numerically correlated with higher pathological grade (p=0.069) in TNBC patients. However, no association of lncRNA TCONS_l2_00002973 expression with age was observed (p=0.481) (Table 2).

Comparison of OS between lncRNA TCONS_l2_00002973 high expression group and lncRNA TCONS_l2_00002973 low expression group

Kaplan-Meier curve and log-rank test were used for the comparison of OS between lncRNA TCONS_l2_00002973 high and low expression patients (Figure 2), and poor OS was found in lncRNA TCONS_l2_00002973 low expression group compared to lncRNA TCONS_l2_00002973 high expression group (p=0.006).
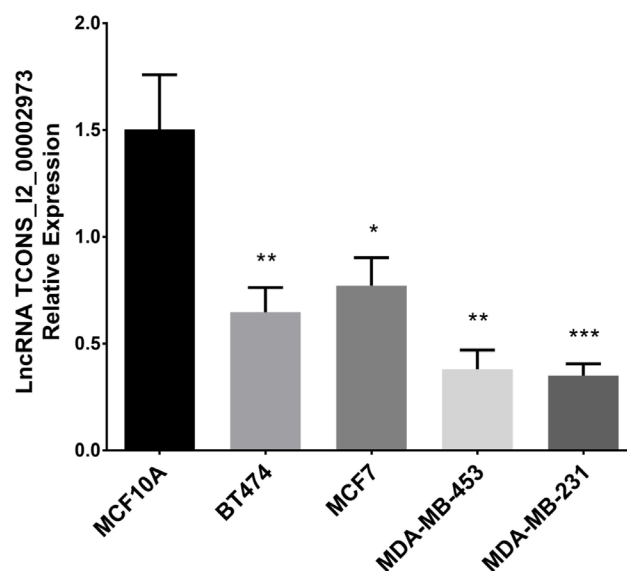

Figure 3. LncRNA TCONS_l2_00002973 relative expression in various cancer cell lines and normal breast cell line. Compared with normal breast cancer cell line MCF10A, lncRNA TCONS_l2_00002973 expression in breast cancer cell lines including BT474, MCF7, MDA-MB-231 and MDA-MB-453 was downregulated. Comparison between two groups was determined by t-test. * P<0.05, ** P < 0.01, *** P<0.001.

Comparison of lncRNA TCONS_l2_00002973 expression between various breast cancer cells and normal breast cell line

To explore the underlying mechanism of lncRNA TCONS_l2_00002973 in breast cancer, we further performed cell experiments. Compared to normal breast cell line MCF10A cells, lncRNA TCONS_l2_00002973 expression was reduced in breast cancer cell lines including BT474 cells ($p<0.01$), MCF7 cells ($p<0.05$), MDA-MB-453 cells ($p<0.01$) and MDA-MB-231 cells ($p<0.001$) (Figure 3). Considering that the numerically lowest lncRNA TCONS_l2_00002973 expression was observed in TNBC cell line (MDA-MB-231 cells), it was chosen for the subsequent experiments.
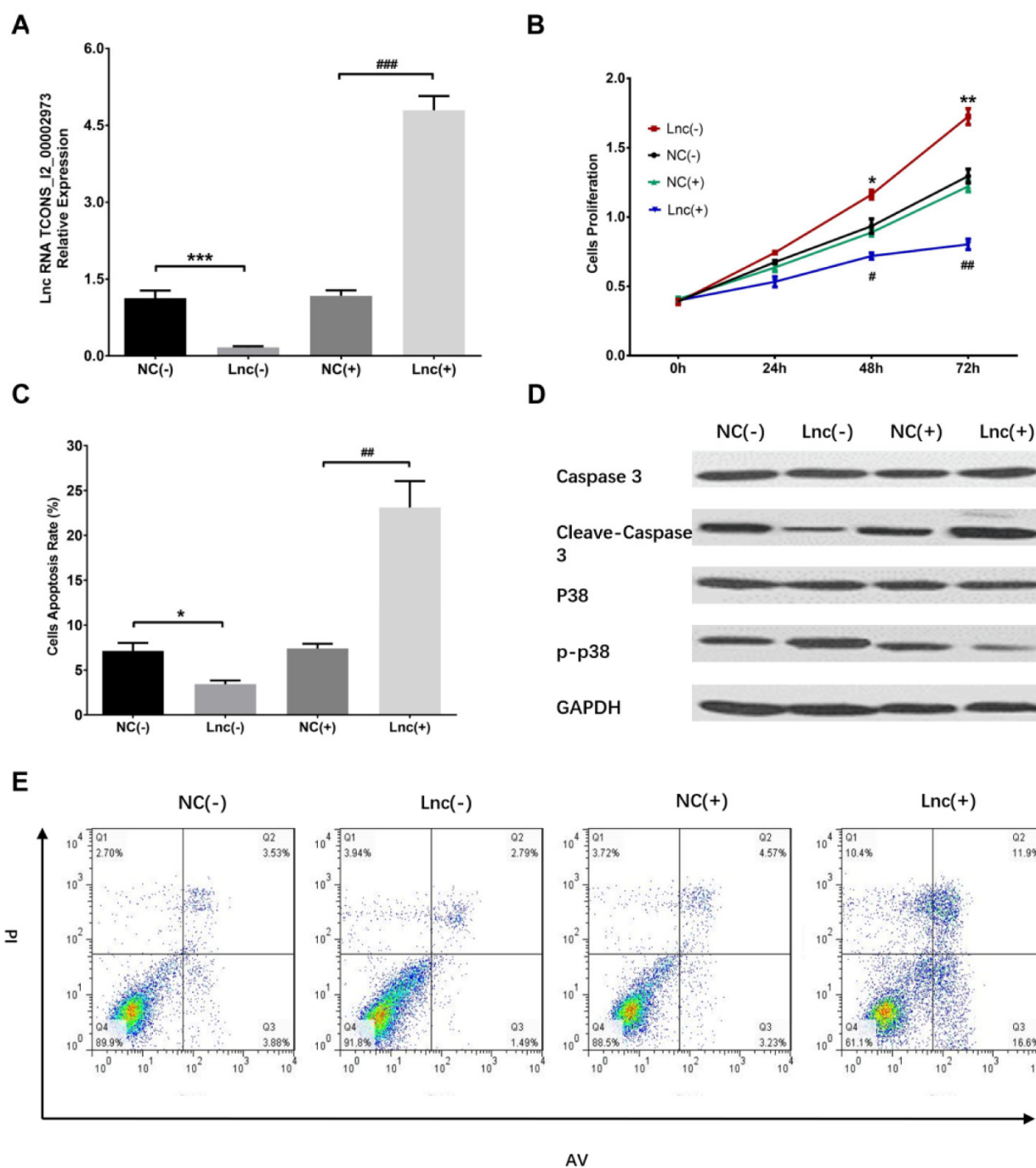
Figure 4. Cells proliferation and apoptosis after lncRNA TCONS_l2_00002973 transfection. LncRNA TCONS_l2_00002973 expression was reduced in Lnc (-) group compared with NC (-) group but raised in Lnc (+) group compared to NC (+) group **(A)**. CCK-8 assay disclosed that lncRNA TCONS_l2_00002973 inhibited cell proliferation **(B)**, meanwhile, AV/PI assay revealed that lncRNA TCONS_l2_00002973 enhanced apoptosis in MDA-MB-231 cells **(C-E)**. Comparison between two groups was determined by t-test. *: NC(-) vs Lnc(-), * $P<0.05$, ** $P<0.01$, *** $P<0.001$. #:NC(+) vs Lnc(+), #, $P<0.05$, ## $P<0.01$, ### $P<0.001$.

LncRNA TCONS_l2_00002973 inhibited cells proliferation and promoted cells apoptosis in MDA-MB-231 cells

At 24 h after transfection, lncRNA TCONS_l2_00002973 expression in Lnc (-) group was greatly decreased compared to NC (-) group (p<0.001), while it was dramatically increased in Lnc (+) group compared to NC (+) group (p<0.001) (Figure 4A). CCK-8 assay was performed for cell proliferation, which revealed that cell proliferation rate was elevated in Lnc (-) group compared to NC (-) group at 48 h (p<0.05) and 72 h (p<0.01), whereas it was reduced in Lnc (+) group at 48 h (p<0.05) and 72 h (p<0.01) compared to NC (+) group (Figure 4B). As to cell apoptosis rate, it was decreased in Lnc (-) group compared to NC (-) group (p<0.05), while it was increased in Lnc (+) group compared to NC (+) group (p<0.01) (Figure 4C, 4E). Based on Western blot assay, Cleave-Caspase 3 expression was lower in Lnc (-) group compared to NC (-) group but higher in Lnc (+) group compared to NC (+) group, while p-p38 expression was elevated in Lnc (-) group compared to NC (-) group but reduced in Lnc (+) group compared to NC (+) group (Figure 4D). These results suggested that lncRNA TCONS_l2_00002973 repressed proliferation and enhanced apoptosis in MDA-MB-231 cells.

Discussion

In this study, we found that: (1) lncRNA TCONS_l2_00002973 was lowly expressed in tumor tissue compared to paired normal adjacent tissue, and its low expression correlated with worse TNM stage as well as shorter OS in TNBC patients; (2) lncRNA TCONS_l2_00002973 repressed proliferation and induced apoptosis in TNBC cells.

LncRNAs are RNA molecules with no ability to encode proteins, while they present diverse regulatory functions on chromatin status, transcription, RNA processes and others during the post-genome period, and have become a focus related to the gene function and cellular processes in several cancers including breast cancer [20-24]. For instance, an interesting experiment discloses that lncRNA nuclear factor - κB (NF-κB) inhibits metastasis by blocking inhibitor of NF-κB (IκB) phosphorylation in breast cancer [21]. Another study reveals that depletion of lncRNA neighbor of BRCA1 gene 2 (NBR2) weakens the activation of energy-stress-induced AMP-activated protein kinase (AMPK), causes aberrant cell cycling, interferes with apoptosis/autophagy response and enhances oncogenesis in different cancers, particularly in breast cancer [22]. Hence, these previous studies indicate that lncR-

NAs play critical roles in pathologic processes of breast cancer.

In clinical trials, several lncRNAs expressions (such as lncRNA Rhabdomyosar-coma 2-associated transcript (RMST) expression and lncRNA growth arrest-specific 5 (GAS5)) have been reported to be dysregulated in breast cancer, including TNBC [25-28]. For the correlation of lncRNAs with clinicopathological characteristics in breast cancer patients, a previous study displays that low lncRNA NKILA expression correlates with large tumor size and advanced TNM stage in breast cancer patients, and another research discloses that downregulated lncRNA BC040587 expression correlates with poor tumor differentiation in breast cancer patients [28,29]. However, few previous studies investigate the association of lncRNA TCONS_l2_00002973 with clinicopathological characteristics in patients with breast cancer, and there is just one report disclosing that lncRNA TCONS_l2_00002973 expression is downregulated in TNBC according to the analysis of Agilent human lncRNA microarray chips, thus we suspected that lncRNA TCONS_l2_00002973 played a favorable role in TNBC [19]. In the present study, we explored the association of lncRNA TCONS_l2_00002973 with clinicopathological characteristics in TNBC and found that lncRNA TCONS_l2_00002973 was downregulated in tumor tissue compared to paired normal adjacent tissue, and its expression was negatively associated with T stage, N stage and TNM stage in TNBC patients. The possible reasons were that downregulated lncRNA TCONS_l2_00002973 might facilitate cells proliferation, migration or invasion to induce faster tumor growth or more tumor metastasis, thereby resulting in worse disease conditions in TNBC patients such as T stage, N stage and TNM stage, and our subsequent *in vitro* experiments also disclosed that lncRNA TCONS_l2_00002973 induced TNBC cell apoptosis while promoted proliferation. The detailed mechanism of lncRNA TCONS_l2_00002973 in TNBC is rarely known, thus further investigation is needed.

The predictive value of lncRNA TCONS_l2_00002973 on the treatment outcomes of TNBC patients is also seldom reported [20]. In this study, we performed Kaplan-Meier analysis and log-rank test to evaluate the correlation of lncRNA TCONS_l2_00002973 with OS in TNBC patients and we found that its low expression was associated with poor OS in TNBC patients. These might be resulted from: (1) lncRNA TCONS_l2_00002973 might interact with multiple genes or pathways to affect cellular activities and further decrease tumor growth and tumor metastasis, thereby contributing to prolonged OS in TNBC patients, thus down-

regulated lncRNA TCONS_l2_00002973 might be related to severer tumor progression and lead to unfavorable survival in TNBC patients; (2) downregulated lncRNA TCONS_l2_00002973 might enhance the resistance to therapy in TNBC, thereby reducing treatment efficacy and causing unfavorable OS in TNBC patients. These hypotheses needed to be validated in future studies.

Limited experiments disclose that some lncR-NAs, such as lncRNA GAS5 and lncRNA RMST, could repress cell proliferation or promote cell apoptosis in TNBC cells, whereas the underlying mechanism of lncRNA TCONS_l2_00002973 in breast cancer is rarely known [25,26]. In order to explore its role in TNBC, we further performed *in vitro* experiments, including qPCR assay, CCK8 assay, AV/PI assay and Western blot assay. What we found was that lncRNA TCONS_l2_00002973 expression was downregulated in various breast cancer cell lines compared to normal breast cell line, and moreover, it inhibited cell proliferation and enhanced cell apoptosis in MDA-MB-231 cells, suggesting that lncRNA TCONS_l2_00002973 might function as antioncogene by reducing cell proliferation and promoting cell apoptosis in TNBC.

In conclusion, lncRNA TCONS_l2_00002973 correlates with less advanced tumor stage and favorable survival and it also inhibits cancer cell proliferation while enhances apoptosis in TNBC.

Conflict of interests

The authors declare no conflict of interests.

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