RNA-Sequencing-Based lncRNA Biomarker Profiling on Triple Negative Breast Cancer

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Abstract: Basal-like triple-negative breast cancer (TNBC) is highly heterogeneous and lack of effective molecular targets for therapy. In this study, we developed the lncRNA signatures of TNBC as molecular biomarkers. RNA-sequencing in 12 paired breast cancer and adjacent tissues identified up-regulated and down-regulated lncRNAs of Basal subtype in contrast to Luminal A, Luminal B and HER2 subtypes. Additionally, Kaplan-Meier analysis revealed that high expression of lncRNA (ZEB1-AS1 and TMEM254-AS1) had a poor relapse-free survival rate (RFS), while high expression of lncRNA (LINC01087, LINC01122 and LINC00856) had a positive correlation with RFS. Furthermore, qRT-PCR analysis showed that the mRNA expressions of the ZEB1-AS1 and TMEM254-AS1 lncRNA were up-regulated in TNBC tissues, while the mRNA expression of lncRNA, including LINC01087, LINC01122 and LINC00856 were down-regulated in TNBC tissues. Taken together, our results elucidated that 5 novel lncRNAs, including ZEB1-AS1, TMEM254-AS1, LINC01087, LINC01122 and LINC00856 contributed to the progression of invasive TNBC. These lncRNAs could be molecular biomarkers for the development of TNBC treatment.

Keywords: TNBC, long noncoding RNA (lncRNA), RNA-sequencing, ZEB1-AS1, TMEM254-AS1, LINC01087, LINC01122, LINC00856.

INTRODUCTION

Targeted therapy of breast cancer (BCa) has achieved great advance in the past decades [1], however, BCa with the potential invasiveness and metastasis remains a major challenge for treatment. Triple negative breast cancer (TNBC) is defined that it does not express the estrogen receptor (ER), the progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) proteins. TNBC is generally more aggressive and easily metastasized due to tumor heterogeneous than other types of breast cancer [2]. The incidence and mortality of TNBC are increasing recently. Though standard-of-care chemotherapy is applied, a part of the patients still has not obviously responded to treatment. It has been a long-lasting challenge that cancer heterogeneity of TNBC and the lack of better molecular targets [3]. Therefore, it is necessary to exploit novel and effective biomarkers for TNBC patients on personalized treatment.

Long noncoding RNAs (lncRNA) are a kind of non-coding RNA, and the lengths of RNA are more than 200 nucleotides. LncRNA may involve in cancer progression. Most of lncRNA cannot translate into proteins due to lacking significant open reading frames. However, some research also has been reported that lncRNA is able to encode small peptides. Huang *et al*. suggested that the conserved 53-aa small peptide was

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ISSN: 1929-2260 / E-ISSN: 1929-2279/21

encoded by lncRNA HOXB-AS3, that was a suppressor of colon cancer and could regulate aerobic glycolysis by modulating PKM splicing [4]. In breast cancer, lncRNA is found to promote epithelial-mesenchymal transition and metastasis through competing with miRNAs at binding sites of target genes [5]. In addition, lncRNA DSCAM-AS1 was a potentially ER positive modulated lncRNA in acquired endocrine therapy-resistant patients of BCa [6], that provided novel insight into anti-oestrogen therapy. The high expression of lncRNA HOTAIR was found to accelerate the aggressiveness of BCa tumor through regulating the expression of targets [7]. However, it still lacks an effective approach to develop key molecular targets for TNBC. Thus, it would have substantial value to screen effective targets for TNBC patients.

In the present study, we aimed to exploit and identify availably prognostic lncRNA from RNA sequencing. We developed the biomarkers of lncRNA that were driven by TNBC drawing from patient tissues RNA-seq cohort. Our findings could open an avenue in both comprehending the molecular basis of TNBC progression and developing novel treatment strategies overcoming this disease.

MATERIALS AND METHODS

Ethics Statement

All the use of surgical samples of BCa tissues was approved by the Institutional Review Board of the Second Clinical Medicine College of Jinan University (LL-KY-2019435).

Breast Cancer Subtypes and Survival Analysis

The BCa tissues and tumor adjacent tissues were obtained from patients diagnosed between 2019 and 2021 at Shenzhen People's Hospital (including 3 Luminal A, 3 Luminal B, 3 Her2 and 3 TNBC subtypes). The differential analysis of genes was obtained by edger analysis between the 3 basal (TNBC) samples of patients and 9 other subtype sample of BCa data [8]. The significant difference was *P*-value<0.05 and the threshold $\log_2$[fold change] ≥2. Kaplan-Meier survival curve for the outcome of BCa patients were performed using Kaplan-Meier database (www.kmplot.com).

Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was isolated from BCa tissues using RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was performed using TransScript One-Step RT-PCR SuperMix (Transgen Biotech), and 1 μg of RNA was used to produce cDNA according to the manufacturer's instructions. The BIO-RAD CFX96 Real-Time System (BIO-RAD) was utilized for qRT-PCR reactions. QRT-PCR primers were designed by NCBI (https://www.ncbi.nlm.nih.gov/) and Integrated DNA Technologies (https://sg.idtdna.com/pages). All sequences of primers were found in Supplementary Table **S1**. QRT-PCRs were performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific). The mRNA relative expression was analysed by the comparative Ct values. Three replicates were performed in each assay.

RNA-Sequencing

Total RNA was extracted from human BCa tissues and tumor-adjacent tissues. The concentration of RNA was qualitied by Qubit system, and the purity of RNA was detected by Nanodrop system (OD260/280), and the integrity of RNA was performed by Agilent 2100 system. The library was established by NEB library. RNA sequencing was performed at Beijing Novogene, using the Illumina Novaseq 6000 platform.

Statistical Analysis

All statistical analyses were performed according to SPSS 21.0 software. Relapse-free survival curves were generated by the Kaplan–Meier plotter and analysed using the long-rank test. QRT-PCR results were showed as the mean ± standard deviation (SD). All data were analysed for a normal distribution and homogeneity of variance. Means were compared using independent-samples T-tests or One-way analysis of variance (ANOVA). A **P*<0.05, ** *P*<0.01, ****P*<0.001.

RESULTS

Identification of Differential Expression of Genes in Different Subtypes of BCa

We evaluated the differential expression of genes by RNA-seq. BCa samples were classified as 4 subtypes based on clinical histopathologic diagnosis. These subtypes contained the following: (1) Luminal A subtype featured by positive estrogen receptor and progestogen receptor and negative Her2 receptor; (2) Luminal B subtype featured by positive estrogen receptor and progestogen receptor and Her2 receptor; (3) Her2 subtype featured by positive human epithelial factor receptor; (2) TNBC (Basal-like) featured by negative estrogen receptor and progestogen receptor

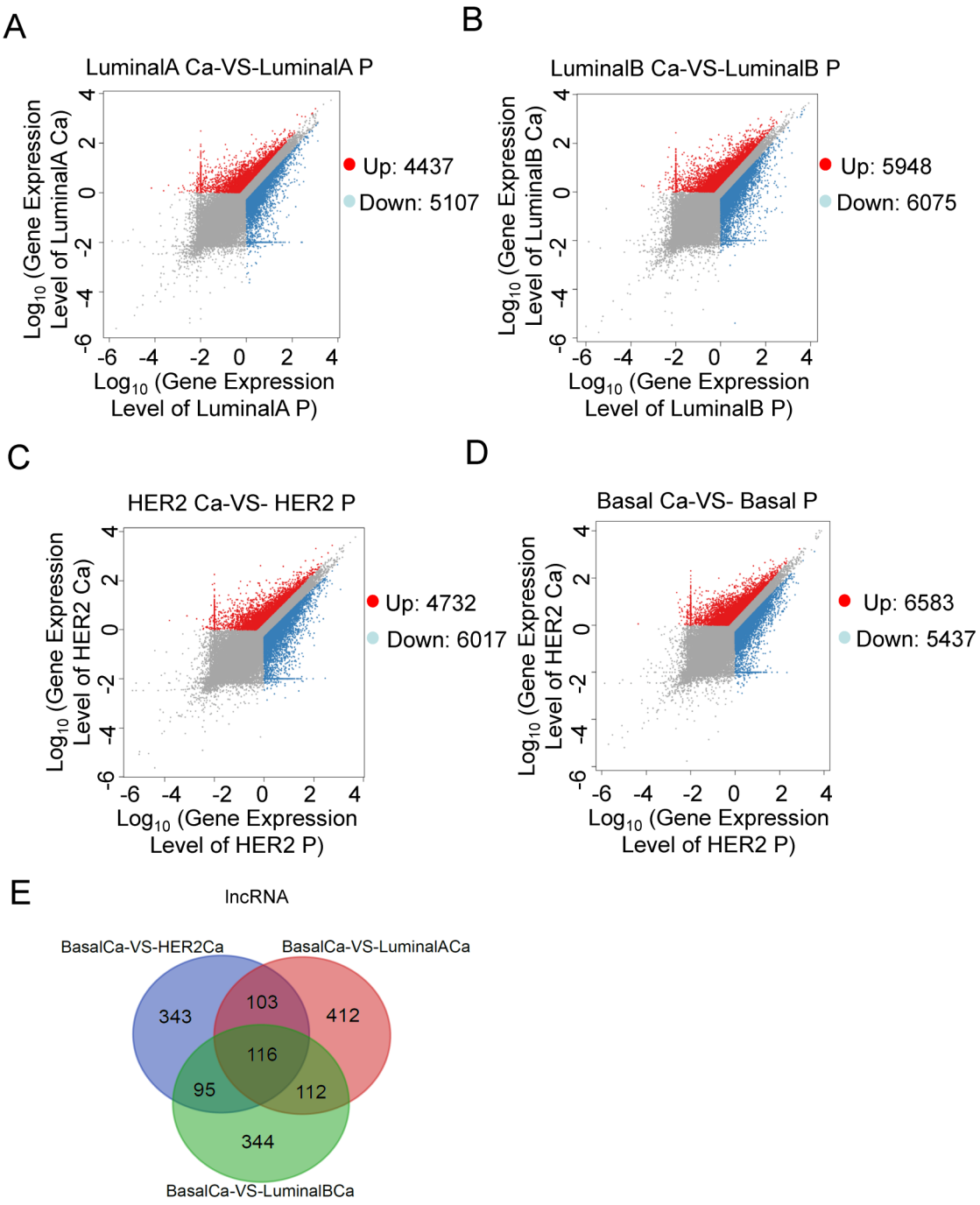

Figure 1: Differential expression analysis of four subtypes of BCs by RNA-sequencing data. A Up-regulated and down-regulated genes in Luminal A subtype (Luminal A Ca) compared with adjacent tissue (Luminal A P) (n=3). **B** Up-regulated and down-regulated genes in Luminal B subtype (Luminal B Ca) compared with adjacent tissue (Luminal B P) (n=3). **C** Up-regulated and down-regulated genes in HER2 subtype (HER2 Ca) compared with adjacent tissue (HER2 P) (n=3). **D** Up-regulated and down-regulated genes in Basal subtype (Basal Ca) compared with adjacent tissue (Basal P) (n=3). **E** Venn diagram analysis of the significant discrepancy of lncRNA in Basal Ca versus Luminal A Ca and Basal Ca versus Luminal B and Basal Ca versus HER2 Ca after filtering the respective lncRNA of adjacent tissue.

and Her2 receptor. The NOI-seq algorithm [9] identified 9544 differentially expressed genes, 5107 down-regulated and 4437 up-regulated in Luminal A tissues compared with adjacent tissues (Figure **1A**). It identified 12023 differentially expressed genes, 6075 down-regulated and 5948 up-regulated in Luminal B tissues compared with adjacent tissues (Figure **1B**). It identified 10749 differentially expressed genes, 6017 down-regulated and 4732 up-regulated in Her2 tissues compared with adjacent tissues (Figure **1C**). In addition, it identified 12020 differentially expressed genes, 5437 down-regulated and 6583 up-regulated in Basal-like cancer tissues compared with adjacent tissues (Figure **1D**).

There is mounting evidence showed that lncRNA are differentially expressed in cancers and involve in regulating cellular metabolism, invasion, metastasis, and drug resistance [10]. To elucidate cancer-related lncRNA in Basal subtype, after filtering out the gene expression of adjacent tissues in different BCa subtypes, we found that 743 expressed genes had significantly differences in Basal subtype compare with Luminal A subtype. The 667 differentially expressed genes were found to change in Basal subtype compare with Luminal B subtype. Besides, it also identified that 657 expressed genes had observably differences in Basal subtype compare with Her2 subtype (Figure **1E**). Given that a deficiency of known driver lncRNA for TNBC, these basal-peculiar lncRNA might provide molecular targets for TNBC.

Candidate lncRNA Screening and Differential Expression Analysis

To extract lncRNA which involved in the regulation of Basal subtype of BCa, we chose differentially expressed lncRNA which are significantly changes [Log_2 (Fold change) >7.0, p-value<0.05] in Basal subtype compared with adjacent tissues. These results showed that a total of 41 lncRNA met this standard (Figure **2A**). From these, 5 lncRNA (ITGB2-AS1, P2RY8-OT2, ATP2B4-AS1, ZNF518B-OT1, ARHGAP18-AS1) were up-regulated, while 8 lncRNA (TEX14-OT1, LINC01122, TMEM161B-AS1, DSCR8, ECE1-AS1, LINC00856, RTN4-AS1, LINC01122) were down-regulated in Basal subtype compared with Lunimal A subtype (Figure **2A**, Table **1**); One lncRNA (TMEM254-AS1) were up-regulated, while 8 lncRNA (MSC-AS1, CKMT2-AS1, CACNA2D4-AS1, WDPCP-OT3) were down-regulated in Basal subtype compared with Luminal B subtype (Figure **2A**, Table **2**); 2 lncRNA (SPATA6-AS1, FAM174B-AS1) were up-regulated, while 13 lncRNA (PIK3CD-AS2, ZNF804A-OT1, OCIAD1-AS1, ANO10-AS1, BHLHE40-AS1, CSNK1A1L-AS1, MS4A6A-OT1, GPR18-OT3, MCPH1-AS1, TRNT1-OT1, CLIC5-AS2, IL7-OT1, ANKUB1-OT1) were down-regulated in Basal subtype compared with Her2 positive BCa (Figure **2A**, Table **3**). In addition, we also found that the expression of 4 lncRNA (LINC01169, C1orf162-OT3, MYH13-OT1, ZEB1-AS1) were increased and the expression of 4 lncRNA (COL4A6-OT3, LINC01087, SPRY4-AS1, HLA-DPA1-OT2) were decreased in Basal subtype in contrast to Luminal A& Luminal B& Her2 positive BCa (Figure **2A**, Tables **4, 5**).

Increasing evidence suggested that lncRNA contributed to target regulation and stability by promoting their targets expression or interacting with microRNA [5]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway approach identified that the differently signal pathways induced by above-mentioned differentially expressed lncRNA. Importantly, the functional enrichment analysis revealed that “Fat digestion and absorption”, “Mineral absorption”, “Glycerolipid metabolism”, “Glycerophospholipid metabolism”, “Viral protein interaction with cytokine and cytokine receptor”, “Pancreatic secretion”, “Aldosterone synthesis and secretion”, “Thiamine metabolism”, “Nitrogen metabolism” were activated as the top nine pathways (Figure **2B**). These differentially expressed targets were found by lncRNA-mRNA co-expression and co-location pairs, such as CEL, PNLIP, FABP2, SLC26A9, MT1HL1, CHAT, ETNPPL, IL20, CXCL13, MC2R, KCNK9, ALPG, and CA3 genes.

Analysis of Survival-Associated lncRNA

To explore a role of these identified differentially lncRNA in BCa clinical prognosis, the survival of invasive TNBC patients was analysed using Kaplan-Meier plotter (www.kmplot.com). Restrict analysis to subtypes included in ER negative, PR negative, HER2 negative, and intrinsic basal subtype of TNBC. The results showed that high expression of lncRNA (ZEB1-AS1 and TMEM254-AS1) had poor relapse-free survival rate (RFS, n=88, ZEB1-AS1:HR=2.11, P=0.012; TMEM254-AS1:HR=2.58, P=0.0016) compared with low expression (Figure **3A**,Bs), while high expression of lncRNA (LINC01087, LINC01122 and LINC00856) had positive correlation with RFS (n=88, LINC01087:HR=0.48, P=0.014; LINC01122: HR=0.48, P=0.014; LINC00856:HR=0.53, P=0.03) (Figure **3C-E**). Others had no significant differences (data not shown). Therefore, the results indicated that these biomarkers might serve as usable prognostic indicators for clinical diagnosis of TNBC.

Real-Time qPCR Identification of Survival-Associated lncRNA

To further identify the authenticity of screening lncRNA by RNA-sequence, qRT-PCR analysis showed that the mRNA expressions of the lncRNA, including ZEB1-AS1, TMEM254-AS1 were up-regulated in TNBC tissues compared to adjacent tissues (Figure **4**). On the contrary, the mRNA expressions of lncRNA, including LINC01087, LINC01122 and LINC00856 were down-regulated in TNBC tissues compared to adjacent tissues. Thus, the qRT-PCR results validated the analysis obtained by RNA-sequence data (Figure **4**).

A

Table 1. All differentially expressed LncRNA between Basal and Luminal A

Gene_ID	Gene name	log_2(fold change)	*P*-value	Up/Down
ENSG00000121101	TEX14-OT1	-20.8078917646438	5.4E-08	Down
ENSG00000233723	LINC01122-204	-26.1344519480268	2.33E-10	Down
ENSG00000227039	ITGB2-AS1-202	11.3810088517827	0.0002	Up
ENSG00000247828	TMEM161B-AS1-207	-20.7055247395713	6.06E-08	Down
ENSG00000198054	DSCR8-203	-20.8170518734616	6E-08	Down
ENSG00000117298	ECE1-AS1	-7.53559148833483	0.04	Down
ENSG00000182162	P2RY8-OT2	9.34749646789377	0.002	Up
ENSG00000058668	ATP2B4-AS1	8.35078206373302	0.015	Up
ENSG00000230417	LINC00856-207	-20.8733933477574	4.73E-08	Down
ENSG00000115310	RTN4-AS1	-21.0565641381349	4.27E-08	Down
ENSG00000233723	LINC01122-201	-26.1344519480268	2.33E-10	Down
ENSG00000178163	ZNF518B-OT1	22.1083636813478	1.01E-08	Up
ENSG00000146376	ARHGAP18-AS1	24.2799938738262	1.11E-09	Up

Table 2. All differentially expressed LncRNA between Basal and LuminalB

Gene_ID	Gene name	log_2(fold change)	*P*-value	Up/Down
ENSG00000235531	MSC-AS1-207	-23.9187405636798	2.49E-09	Down
ENSG00000230091	TMEM254-AS1-202	24.9420952623797	9.83E-10	Up
ENSG00000247572	CKMT2-AS1-201	-25.0487599445589	9.92E-10	Down
ENSG00000151062	CACNA2D4-AS1	-9.46851642179227	0.0234	Down
ENSG00000143951	WDPCP-OT3	-26.0461305972269	4.08E-10	Down

Table 3. All differentially expressed LncRNA between Basal and HER2

Gene_ID	Gene name	log_2(fold change)	*P*-value	Up/Down
ENSG00000231789	PIK3CD-AS2-201	-21.3367059527513	7.16E-08	Down
ENSG00000170396	ZNF804A-OT1	-7.67094165277261	0.031	Down
ENSG00000248256	OCIAD1-AS1-201	-8.85184022471439	0.041	Down
ENSG00000160746	ANO10-AS1	-25.4945428788784	1.06E-09	Down
ENSG00000235831	BHLHE40-AS1-202	-10.0167381091245	0.002	Down
ENSG00000180138	CSNK1A1L-AS1	-21.4976470307641	3.64E-08	Down
ENSG00000110077	MS4A6A-OT1	-10.8075270059552	0.007	Down
ENSG00000125245	GPR18-OT3	-24.1973311647625	3.5E-09	Down
ENSG00000249898	MCPH1-AS1-203	-20.8687635700174	7.94E-08	Down
ENSG00000072756	TRNT1-OT1	-7.15151468484095	0.014	Down
ENSG00000112782	CLIC5-AS2	-11.3502609746371	0.004	Down
ENSG00000104432	IL7-OT1	-11.5748489769009	0.004	Down
ENSG00000206199	ANKUB1-OT1	-9.54602529298167	0.022	Down
ENSG00000132122	SPATA6-AS1	22.2757907234644	1.54E-08	Up
ENSG00000185442	FAM174B-AS1	21.8581606085617	2.44E-08	Up

Table 4. All differentially expressed LncRNA between Basal and Luminal A/LuminalB

Gene_ID	Gene name	log_2(fold change)	*P*-value	Up/Down
ENSG00000197565	COL4A6-OT3	-8.80848274866248	0.035	Down
ENSG00000224559	LINC01087-201	-9.44945635288289	0.002	Down
ENSG00000259471	LINC01169-201	22.6810754259996	5.18E-09	Up
ENSG00000143110	C1orf162-OT3	22.2173016660523	8.88E-09	Up
ENSG00000006788	MYH13-OT1	23.9679626196533	1.49E-09	Up

Table 5. All differentially expressed LncRNA between Basal and HER2/Luminal A/LuminalB

Gene_ID	Gene name	log_2(fold change)	*P*-value	Up/Down
ENSG00000148516	ZEB1-AS1	22.8255726317403	9.45E-09	Up
ENSG00000231185	SPRY4-AS1-202	-24.4484774565741	2.57E-09	Down
ENSG00000231389	HLA-DPA1-OT2	-28.2388156823631	6.88E-11	Down

B

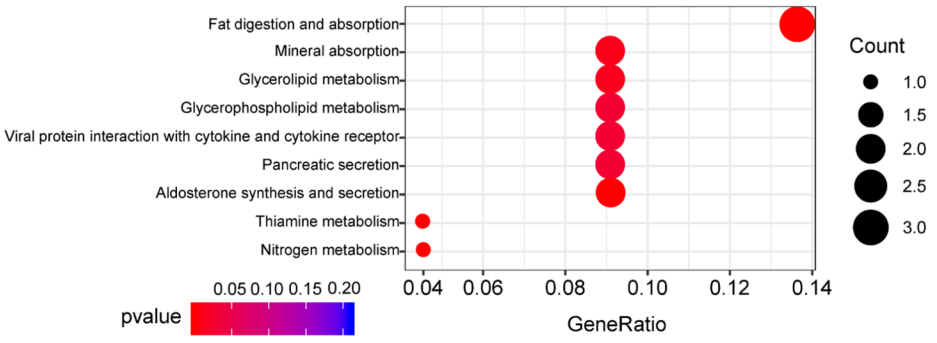

Figure 2: Screening of cancer-associated lncRNA in Basal subtype. A Differential lncRNA analysis was listed for overexpressed and deficient lncRNA in Basal versus Luminal A (Table **1**), Basal versus Luminal B (Table **2**), Basal versus HER2 (Table **3**), Basal versus Luminal A versus Luminal B (Table **4**), Basal versus Luminal A versus Luminal B versus HER2 (Table **5**). **B** Enrichment differential pathways were shown by KEGG analysis.

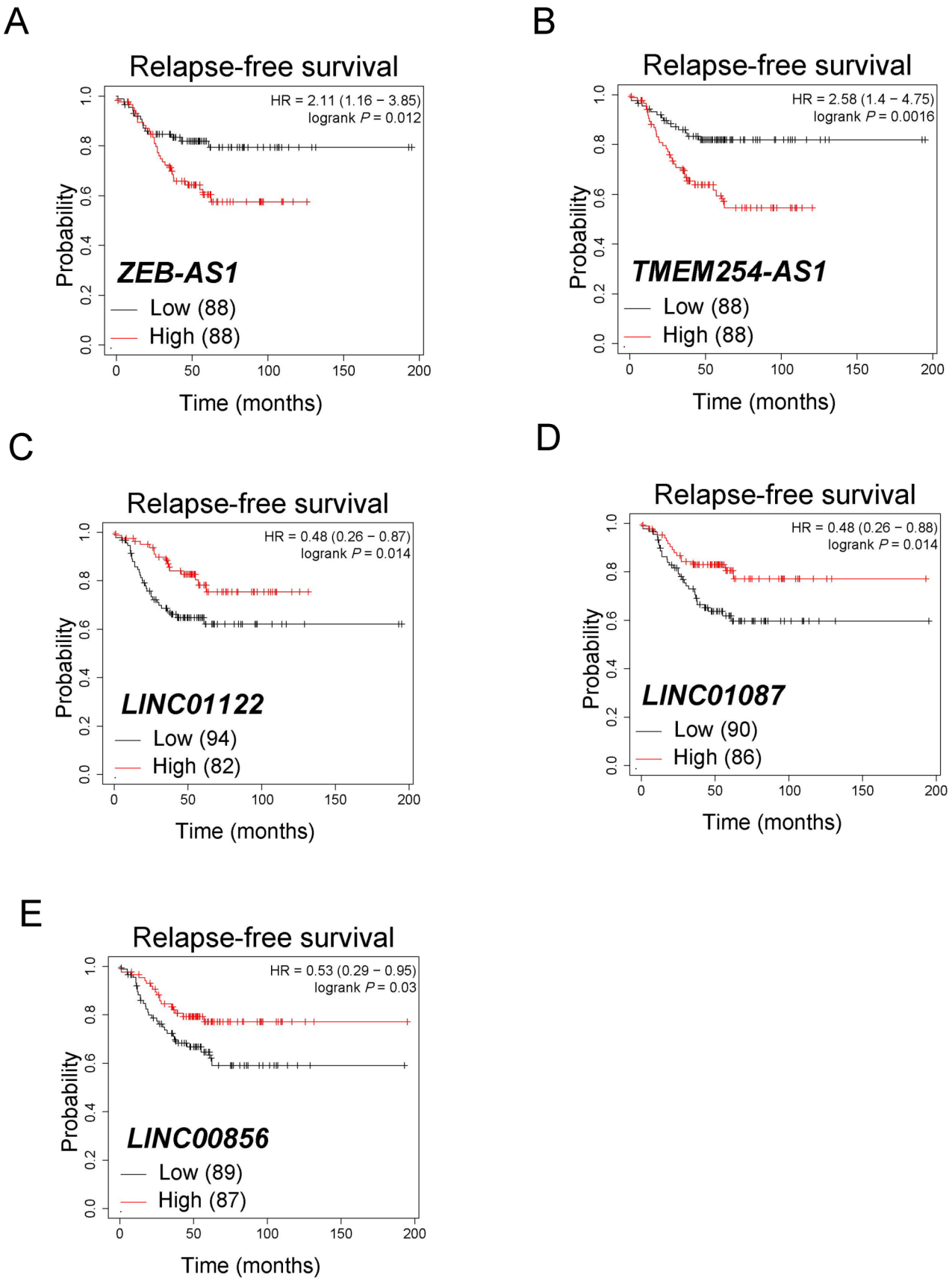

Figure 3: The correlation between the RNA expression levels of identified lncRNA and relapse-free survival in Basal subtype BCa from Kaplan-Meier plotter dataset. Kaplan-Meier analysis of relapse-free survival of BCa patients from 'high' and 'low' mRNA expression levels of (A) *ZEB-AS1*, (B) TMEM254-AS1, (C) LINC01087, (D) LINC01122 and (E) LINC00856.

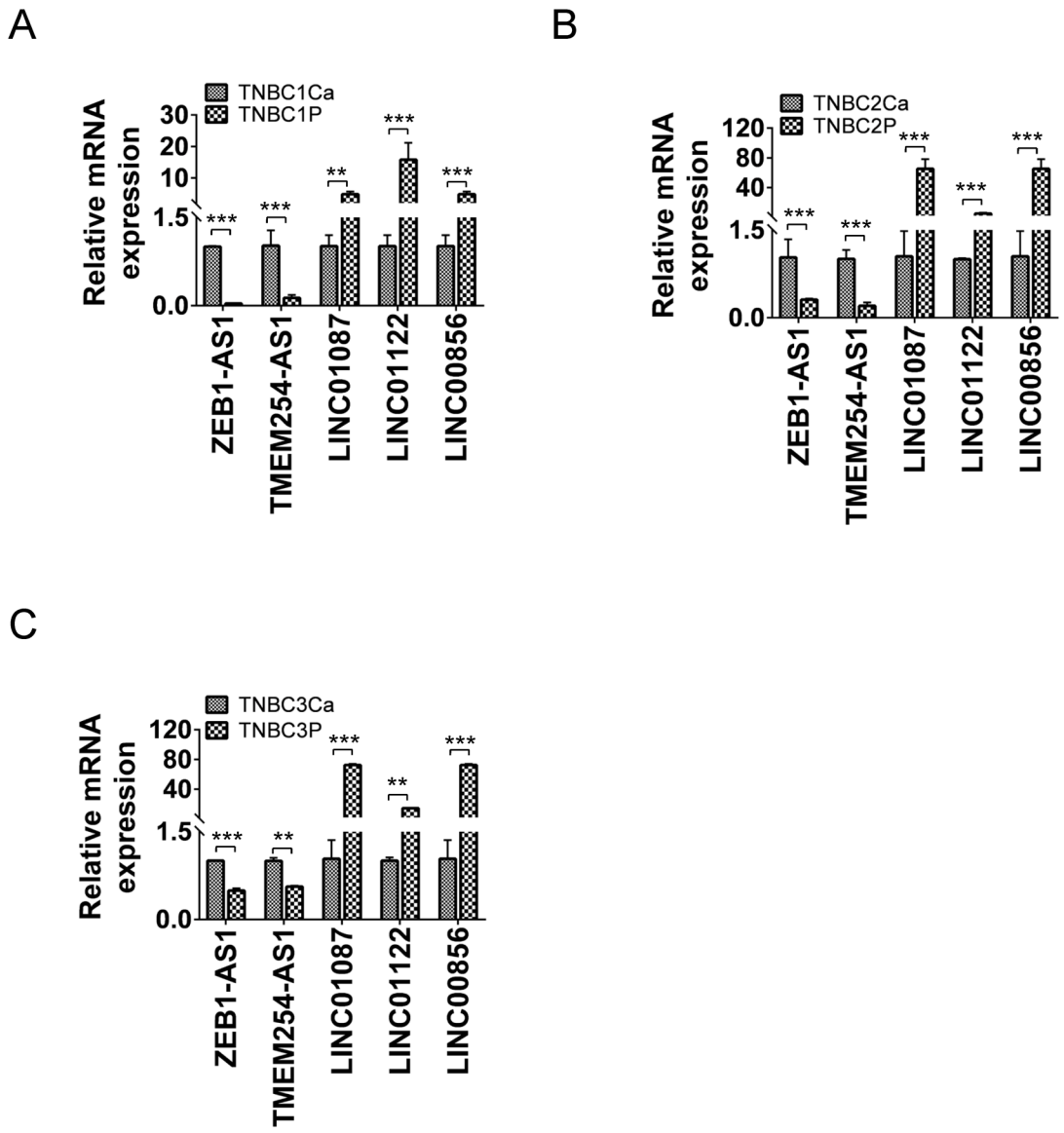

Figure 4: QRT-PCR analysis was used to identify the expression of lnRNA. Each experiment was performed in triplicate and data was represented as mean ± S.D. One-Way ANOVA and Dunnett's multiple comparison test were used to analyse the data (*P < 0.05, **P < 0.01, ***P < 0.001).

DISCUSSION

TNBC is a heterogeneous tumor, that has high invasiveness, high mitotic rates and chemotherapy resistance [11]. Our data provided 5 potential biomarkers for TNBC. Previous studies showed that ZEB1-AS1 could promote tumor progression of hepatocellular carcinoma by up-regulating its target ZEB1 [12]. Besides, the high expression of ZEB1-AS1 had correlated with poor outcome of malignancy including glioma and prostate cancer [13, 14]. It was found that TMEM254-AS1 contributed to be upregulated in persistence cells of neoadjuvant chemotherapy for TNBC by single-cell lncRNA transcriptome, that might serve as novel therapeutic target against TNBC [15]. Previous studies suggested that LINC01087 had high expression levels in BCa. LINC01087 was found to be significantly upregulated in Luminal subtype of BCa in contrast to TNBC subtype [16]. However, its molecular mechanism remains unknown on BCa. In addition, the correlation and regulatory function between LINC01122 or LINC00856 and cancers has not been reported. Due to the limitation of tissue sample size for RNA-sequencing, further identification needs to be analysed through expanding sample size.

In conclusion, we identified that 5 novel lncRNAs, including ZEB1-AS1, TMEM254-AS1, LINC01087, LINC01122 and LINC00856 contributed to the progression of invasive TNBC. The overexpression of ZEB1-AS1 or TMEM254-AS1 was related to poor

prognosis of TNBC patients, while downregulation of LINC01087, LINC01122 and LINC00856 was associated with poor outcome of TNBC patients. In brief, this study identifies novel lncRNAs in TNBC progression and could be potential therapeutic targets against TNBC.

Data Availability Statements

All data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS

We thank the Cancer Genome Atlas and Kaplan-Meier Plotter for providing their data platform. This work was supported by grants from the China Postdoctoral Science Foundation Grant 2019M660232 (Lin Gao), the National Natural Science Foundation of China 82002929 (Lin Gao), Basic and Applied Basic Research Foundation of Guangdong Province [2020A1515111072 (Lin Gao), 2019B151512003 (Chang Zou), 2020B1515120032 (Chang Zou)], the Science and Technology Foundation of Shenzhen JCYJ20190806154610953 (Lin Gao), JCYJ20180305164128430 (Chang Zou), the International Cooperation Foundation of Shenzhen GJHZ20180928171602104 (Chang Zou), the Shenzhen Economic and Information Committee "Innovation Chain and Industry Chain" integration special support plan project 20180225112449943 (Chang Zou), the Shenzhen Public Service Platform on Tumor Precision Medicine and Molecular Diagnosis (Chang Zou), the Ministry of Science and Technology of the People's Republic of China (No. 2018ZX09201018), the National Natural Science Foundation of China(No. 81803183), the Shenzhen Key Medical Discipline Construction Fund (SZXK053).

CONFLICT OF INTERESTS

The authors declared no competing interests.

AUTHOR CONTRIBUTIONS

Lin Gao designed the project and wrote the manuscript. Jinquan Xia, Malin Hong and Jingyi Huang were responsible to analysed for RNA-seq data. Pan Zhao performed the experiments. Yong Dai, Wenbin Zhou and Qinhe Yang provided BCa samples. Li Fu and Jigang Wang supported clinical and pathological information. Chang Zou designed and supervised this project and revised the manuscript.

SUPPLEMENTAL TABLE

The supplemental Table can be downloaded from the journal website along with the article.

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Received on 05-04-2021 Accepted on 20-05-2021 Published on 25-05-2021

https://doi.org/10.30683/1929-2279.2021.10.01