Long Non-coding RNA BTG3-7:1 and JUND Co-regulate C21ORF91 to Promote Triple-Negative Breast Cancer Progress

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Background: Triple-negative breast cancer (TNBC) is a type of highly invasive breast cancer with poor prognosis. Recently, massive data reveal that long non-coding RNAs (lncRNAs) play important roles in cancer progress. Recently, although the role of lncRNAs in breast cancer has been well documented, few focused on TNBC. In this study, we aimed to systematically identify functional lncRNAs and to explore its molecular mechanism on TNBC progress.

Methods: The recurrence of lncRNAs and their target genes were validated with TNBC biopsies and cell lines. Total one hundred and thirteen TNBC biopsies, including nineteen patient-matched samples, were collected. The profile of TNBC-related lncRNAs and their target genes were characterized by RNA sequencing (RNA-seq) and bioinformatic analysis. Tumor specific lncRNAs, which also showed biological function correlated with TNBC, were identified as potential candidates; and the target genes, which regulated by the identified lncRNAs, were predicted by the analysis of expression correlation and chromosome colocalization. Cross bioinformatic validation was performed with TNBC independent datasets from the cancer genome atlas (TCGA). The biological functions and molecular mechanism were investigated in TNBC model cell lines by cell colony forming assay, flow cytometry assay, western-blot, RNA Fluorescence in situ Hybridization assay (RNA FISH) and chromatin immunoprecipitation-qPCR (ChIP-qPCR).

Results: Abundant Lnc-BTG3-7:1, which targets gene C21ORF91, was specifically observed in TNBC biopsies and cell lines. Knockdown of Lnc-BTG3-7:1 or C21ORF91 strongly inhibited cell proliferation, promoted cell apoptosis and cell cycle G1-arrested. Meanwhile, investigation of molecular mechanism indicated that Lnc-BTG3-7:1, cooperated with transcription factor JUND, cis-regulated the transcription of C21ORF91 gene, and down-regulation of Lnc-BTG3-7:1/C21ORF91 suppressed GRB2-RAS-RAF-MEK-ERK and GRB2-PI3K-AKT-GSK3β-β-catenin pathways.
INTRODUCTION

Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype and is characterized by poor survival (Shah et al., 2012; Johnson et al., 2020), occurring from younger age and being prone to distant metastasis. Moreover, it is kind of a heterogeneous disease with complex genetic background (Network, 2012), which represents different prognosis and responds to chemotherapies (Masuda et al., 2013; Burstein et al., 2015). However, current treatment outcomes are unsatisfied even under target therapy and immunotherapy, and unfortunately chemotherapy has to remain as the preferred treatment (Pandy et al., 2019). Thus, finding new target and understanding its mechanisms are essential. Recently, many studies report that long non-coding RNAs (LncRNAs) are likely pervasive in cancers and can drive cancers development, drug resistance and progress (Gupta et al., 2010; Martin and Chang, 2012; Bhan et al., 2017; Chi et al., 2019), which can be considered as important cancer biomarkers and play critical roles in cell proliferation, cell cycle, cell apoptosis, cell invasion, and metastasis (Yang et al., 2014; Li et al., 2016; Collina et al., 2019). Recently, although several LncRNAs in breast cancer have been well documented (Mourtada-Maarabouni et al., 2009), only a few of them focus on TNBC, such as MALAT1, LINK-A, HOTAIR, LINP1, LncRNA-RoR, Lnc-RNA BORG, LOC554202, and HULC (Loewer et al., 2010; Augoff et al., 2012; Tripathi et al., 2013; Shi et al., 2014; Tao et al., 2015; Jin et al., 2016; Lin et al., 2016; Zhang et al., 2016; Gooding et al., 2019), and the mechanisms are still unclear. Thus, potential TNBC specific LncRNAs are expected to be explored, and the molecular features of LncRNAs that underlie the development, metastasis, and relapse of TNBC remain to be elucidated.

Therefore, in this study, we aimed to systematically identify specific LncRNA by using RNA sequencing (RNA-seq), and to explore its functions and molecular mechanism on TNBC progress.

MATERIALS AND METHODS

Patients' Tissue Samples

Total one hundred and thirteen TNBC biopsies, including nineteen patient-matched samples, were collected from Breast cancer biobank of Western China Hospital. This experiment was approved by Ethics Committee of Western China Hospital.

Cell Culture

MDA-MB-231, MDA-MB-468, BT-549 cells were used as representative human TNBC and MCF-10A cell was used as human mammary gland epithelial. MDA-MB-231, MDA-MB-468 were cultured by 10% fetal bovine serum in DMEM (Thermo Scientific HyClone, United States). BT-549 was cultured by 10% fetal bovine serum in RPMI 1640 Medium Modified (Thermo Scientific HyClone, United States). MCF-10A was cultured by 5% fetal horse serum (Solarbio, China) in DME/F-12 (Thermo Scientific HyClone, United States) which added with Hydrocortisone (0.5 µg/ml final, Sangon, Shanghai, China), insulin (10 µg/ml final, Novo Nordisk, Bagsvaerd, Denmark), EGF (20 ng/ml final, Sangon, Shanghai, China), cholera toxin (100 ng/ml final, MedChemExpress, New Jersey, United States). All culture mediums were added with 1% penicillin and streptomycin and the four cell lines were cultured at 37.0°C in an atmosphere of 5% CO₂.

RNA Extraction and High-Throughput Sequencing

TRIzol reagent (Invitrogen, Carlsbad, CA, United States) was used to isolated total RNAs from tissues and cells following the manufacturer's protocol. Library preparation and sequencing were performed as described in detail in our previous study (Deng et al., 2019). Briefly, RNA integrity and quality were assessed by 2100 Bioanalyzer RNA Nano Chip (Agilent Technologies, United States) and Nanodrop ND-2000 Spectrophotometer (NanoDrop Technologies, United States). Subsequently, library preparation was performed as described in NEBNext Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, United States) following manufacturer's recommendations. The libraries were then sequenced on the Illuma Hiseq platform (Novogene Bioinformatic Technology Co., Ltd., China), following a 2 × 150-bp paired-end protocol. Sequencing data were available at the NCBI Sequence Read Archive under project number PRJNA553096.

Sequencing Data Processing and LncRNAs Identification

After pre-processing filtering of low-quality reads and adaptor trimming, high-quality sequencing reads were aligned to the human reference genome assembly (GRCh37) using TopHat2 (Kim et al., 2013). Transcript assembly and abundance estimation were performed using Cufflinks (Trapnell et al., 2012) and HTSeq (Anders et al., 2015). The Ensembl, LNCipedia, and non-code databases were chosen as references to retrieve known transcripts. Novel transcripts were subjected to coding potential analysis by Coding-Non-Coding-Index (CNCI) (Sun et al., 2013), Coding Potential Calculator (CPC) (Kong et al., 2007), and Pfam-Scan

Conclusions: In this study, we identified a TNBC specific LncRNA Lnc-BTG3-7:1, which sustained tumor progress. Up-regulation of Lnc-BTG3-7:1 promoted the transcription of oncogene C21ORF91 and activated PI3K-AKT-GSK3β-β-catenin and MAPK pathways. Taken together, our results not only identified a biomarker for diagnosis but also provided a potential therapeutic target against TNBC.

Keywords: Triple-negative breast cancer, Lnc-BTG3-7:1, C21ORF91 gene, GRB2-RAS-RAF-MEK-ERK pathway, GRB2-PI3K-AKT-GSK3β-β-catenin
lncRNAs. To perform differential expression analysis in matching 200 nt but without coding function were defined as candidate (Chojnacki et al., 2017). The transcripts with lengths greater than CTTTTTG, respectively. These shRNAs were annealing and CCTACAGCAAATCCTCGAGGATTTGCTGTAGGAGCTTTG and GCAAAGCT shRNA764 were GGAGCAGTTTGTAAACATTGACTCGAACAGAGTTACGCTCGAGCGTAACTCTGTTAACTGCACC TACAATCAGTACAAAGCAGCTTTTTG and GGTGCAGTTAACAACCCCATTTTTCCTA/GAACGTGTGCATGTGCTAAG forward primer of Lnc-BTG3-7:1 and C21ORF91 Random primer was used for reverse transcription. Then, β Lnc-BTG3-7:1 and C21ORF91 gene using SYBR Green Supermix (Bio-Rad, United States). qPCR was performed synthesized using the ReverseAid First Strand cDNA Synthesis The first strand complementary DNAs of the RNAs were constructed. Protein-protein network (PPI) from STRING database (Szklarczyk et al., 2015) was further integrated into co-expression modules.

**Quantitative Real-Time PCR (qPCR)**
The first strand complementary DNAs of the RNAs were synthesized using the ReverseAid First Strand cDNA Synthesis Kit (Thermo Fisher, United States). qPCR was performed using SYBR Green Supermix (Bio-Rad, United States). Lnc-BTG3-7:1 and C21ORF91 expression in cell lines was normalized to β-actin using the comparative Ct method. Random primer was used for reverse transcription. Then, forward primer of Lnc-BTG3-7:1 and C21ORF91: TGC AACAAACCCCATTTTTCCTA/GAACGTGTGCATGTGCTAAG and reverse primer: AAGAGTTCGGGCTCATCTCACTGAG TACCAGCACCACAAAG, respectively, were used to conduct qPCR.

**Short Hairpin RNA (shRNA) Synthesis and Transfection**
Lnc-BTG3-7:1 and specific shRNAs were synthesized by Sangon Biotech (Shanghai, China). The shRNAs target Lnc-BTG3-7:1 and C21ORF91 gene were shRNA241/shRNA351 and shRNA73/shRNA764, respectively. Sequences of shRNA241 and shRNA351 were: GCTGCTTTTGTAACGCTCGATG TACAATCAGTACAAAGCAGCTTTTTTG and GGTGCAGTTAACAACCCCATTTTTCCTA/GAACGTGTGCATGTGCTAAG and reverse primer: AAGAGTTCGGGCTCATCTCACTGAG TACCAGCACCACAAAG, respectively. These shRNAs were annealing and then inserted into the pLKO.1 vector between the restriction sites Agel and EcoRI. The propagated synthetic construct vectors from *Escherichia coli* were extracted using Plasmid DNA purification kit (Macherey-Nagel, Germany). Lentiviral particles were produced by co-transfecting expression vector sh241_pLKO.1, sh351_pLKO.1, sh73_pLKO.1, and sh764_pLKO.1 with viral particle packaging helper vector into 293T cells. Tires of the four kinds of viral particles were determined by limited serial dilution. The three TNBC cell lines were infected with the four kinds of packaged lentivirus. The efficiency of Lnc-BTG3-7:1 and C21ORF91 single or double knockdown were determined by qPCR.

**Cell Proliferation Assay**
Mock and Lnc-BTG3-7:1/C21ORF91 gene knockdown TNBC cells (MDA-MB-231, MDA-MB-468, and BT-549) were seeded in 35 mm dish with 2 mm grid at 1 × 10^5 cells/dish, respectively. Then, we recorded cell growth curve after culturing for 0, 24, 72, and 96 h to determine short-term cell proliferation capacity.

**Colony Forming Assay**
Mock and Lnc-BTG3-7:1/C21ORF91 gene single or double knockdown TNBC cells were seeded in 12-well plate at 2 × 10^3 cells/well for MDA-MB-231 and MDA-MB-468 cells, and 1 × 10^3 cells/well for BT-549 cells, respectively. Then, cells were cultured for continuous 8 days. After fixation with 4% paraformaldehyde for 30 min, cells were stained with crystal violet solution for 30 min.

**Western-Blot Assay**
Expression of ERK (#4695, CST, United States), p-ERK (#4377, CST, United States), MEK (#4694, CST, United States), p-MEK (#9154, CST, United States), AKT (#9272, CST, United States), AKT-Ser473 (#9271, CST, United States), β-catenin (#9582, CST, United States), c-MYC (#9402, CST, United States) and GSK-3β (#9315, CST, United States) were measured by Western-blot in MDA-MB-231, MDA-MB-468 and BT-549. Each 10 μg of protein was resolved on SDS-PAGE and transferred to PVDF membranes (Millipore, United States). Immunoblotting was performed overnight at 4°C. The membranes were then washed with Tris Buffered saline Tween (TBST) three times and incubated with the corresponding secondary antibodies (1:5000) at room temperature for 1 h. Then, these membranes were washed with TBST for three times. Images were captured by Bio-Rad ChemiDoc MP (Bio-Rad, United States).

**Flow Cytometry Assay**
Mock and Lnc-BTG3-7:1/C21ORF91 gene single or double knockdown TNBC cells (MDA-MB-231, MDA-MB-468, and BT-549) were trypsinized and resuspended to obtain single–cell suspensions, respectively. (1) Cells for cell-cycle analysis were fixed in ice-cold 70% ethanol overnight, and stained with propidium iodide (5 µg/mL) and RNase A (50 units/mL). (2) Cells for apoptosis analysis were stained with fluorescein isothiocyanate-conjugated Annexin V and propidium iodide (Apoptosis Detection kit; 4A Biotech) for 15 min as
recommended by the manufacturer. Samples were tested by CytoFLEX flow cytometry (Beckman, CA, United States) and analyzed by FlowJo V10.0 software (Becton Dickinson, Ashland, OR, United States).

**RNA Fluorescence in situ Hybridization Assay (RNA FISH)**

An oligonucleotide probe that was complementary to Lnc-BTG3-7:1 (designed by Stellaris Designer) and labeled with Cy3 dye at 5’ was purchased from Sangon Biotech (Shanghai, China). A Cy3-labeled sense oligonucleotide was used as negative control. The sequences of the LncRNA probe and negative control (NC) probe were as follows: CCCAGTCAACACTCATACTT, CCACTCCTATACCAATCTCGA, respectively. MDA-MB-231 and BT-549 cells were collected at exponential growth phase and resuspended to obtain single-cell suspensions (5 x 10^4/ml). Then, cells were seeded into multi-chambered coverglass slides and cultured at normal growth conditions overnight. The cells were fixed in 4% formaldehyde for 15 min after washing 5 min for 3 times by phosphate buffer saline (PBS). Then, cells were treated with RNA FISH Kit (Fluorescent *in situ* Hybridization Kit, RiboBio, China) as recommended by the manufacturer, and Lnc-BTG3:7:1 probe and NC probe were cultured at 0.5 μM in 37°C overnight. Then, slides were incubated with JUND antibody (1:400, #5000S, CST, United States) for 2 h and incubated with the corresponding secondary fluorescence antibody (FITC, 1:200, Invitrogen, United States) for 40 min at room temperature after washing by PBS for three times. Last, the slides were sealed with DAPI Fluoromount-G (Yeasen Biotech, China). Images were captured by laser scanning confocal microscope (Nikon, Japan) and analysis with software NIS-Elements BR (Version 5.11.01).

**Chromatin Immunoprecipitation (ChIP)-qPCR**

ChIP assays (Jing et al., 2020) were performed for the MDA-MB-231, MDA-MB-468, and BT-549 cells. Cells (approximately 1 x 10^7 cells) were digested into cell suspension by trypsin and washed with PBS. Then crosslink was performed using 3% formaldehyde for 30 min at room temperature after washing by PBS for three times. Last, the slides were sealed with DAPI Fluoromount-G (Yeasen Biotech, China). Images were captured by laser scanning confocal microscope (Nikon, Japan) and analysis with software NIS-Elements BR (Version 5.11.01).

**Statistical Analysis**

The level of significance was defined as \(P < 0.05\). The difference between mean values was assessed by *t*-test using Prism GraphPad 8.0 (GraphPad Software, United States).

**RESULTS**

**Characterization of LncRNA and Transcriptome of TNBC**

According to the specific structural and non-coding features of LncRNA, 58,163 lncRNAs were identified in at least one sample. Of these, 48,551 (83.47%) were identified as known lncRNAs, and 9,612 novel lncRNAs were detected in TNBC tissues. To investigate the key lncRNAs involved in TNBC progress, the expression profiles of lncRNAs between tumor and normal tissues were compared in order to detect the differentially expressed lncRNAs (DE-lncRNAs). We identified 864 lncRNAs with high occurrence (occurrence > 10) displayed differential expression in TNBC biopsies. Of the dysregulated lncRNAs, 193 lncRNAs had consistent expression patterns in TNBC biopsies. Among them, 62 lncRNAs were highly expressed in tumor tissues, whereas, 131 lncRNAs were down-regulated, compared with normal tissues. Several DE-lncRNAs with high occurrence were further verified in TCGA TNBC cohorts (Figure 1A).

To better understand the role of lncRNAs in TNBC progress, functional analysis of target genes was performed to predict the biological functions of DE-lncRNAs. A total of 671 protein-coding genes were significantly correlated with neighbor lncRNAs (Figure 1B). We then conducted pathway analysis to gain insight into the functions of DE-lncRNA target genes, and found that the target genes of DE-lncRNAs were highly enriched in pathways in cancer (Figure 1C). Moreover, numerous target genes were involved in oncogenic signaling pathways, such as PI3K-AKT signaling pathway (12 target genes), RAS signaling pathway (9 target genes), and MAPK signaling pathway (7 target genes). To find out candidate lncRNAs, we removed DE-lncRNAs whose occurrence less than 10 prior to subsequent analyses (Figure 1C).

**Lnc-BTG3-7:1 and Target Gene C21ORF91 Were Identified in TNBC Specific Pattern**

To predict and identify the potential TNBC specific lncRNAs, DE-lncRNAs were ranked by fold change and occurrence. The top-ranked genes were defined as TNBC specific lncRNAs. Among them, Lnc-BTG3-7:1 was eventually screened out using text mining and highlighted in Figure 2A. The expression levels of Lnc-BTG3-7:1 and its target gene C21ORF91 were compared in order to detect the differentially expressed lncRNAs (DE-lncRNAs). We identified 864 lncRNAs with high occurrence (occurrence > 10) displayed differential expression in TNBC biopsies. Of the dysregulated lncRNAs, 193 lncRNAs had consistent expression patterns in TNBC biopsies. Among them, 62 lncRNAs were highly expressed in tumor tissues, whereas, 131 lncRNAs were down-regulated, compared with normal tissues. Several DE-lncRNAs with high occurrence were further verified in TCGA TNBC cohorts (Figure 1A).

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FIGURE 1 | Characterization and functional analysis differentially expressed LncRNAs (DE-lncRNAs) and differentially expressed target genes (DEGs). (A) Heatmap with expression characteristics of differentially expressed IncRNAs in tumor and normal tissues. The order of DE-lncRNAs was determined by occurrence in comparison group. The top 50 most differentially expressed IncRNAs used for clustering were plotted, of which 25 were up-regulated and 25 down-regulated, respectively. The occurrence of up-regulated and down-regulated patterns were shown as red and blue bars on the left side. Samples were also annotated on top. The DE-lncRNAs verified in TCGA TNBC cohorts were highlighted in red. (B) LncRNA target prediction analysis based on positional relationship and expression correlation (as described in “Materials and Methods”). (C) Pathway enrichment analysis of target genes differentially expressed lncRNAs. The relationships of important biological processes (top 30) were depicted by network. The color was scaled by P-values, and the sizes of the dots represented the percentage of each term.
FIGURE 2 | Identification of functional LncRNA and targeted genes potentially driving TNBC progress. (A) Differential expression of IncRNAs in TNBC biopsies. DE-IncRNAs were ranked by fold change and occurrence. The ten up- and down-expressed Inc-RNAs were labeled. Lnc-BTG3-7:1 was highlighted with a red frame in the up-regulated group. (B) The expression level of Lnc-BTG3-7:1 in TNBC biopsies (n = 113) was higher than normal breast tissue (P < 0.005***), accompanied by the C21ORF91 gene expression increased (P < 0.005***). (C,D) The abundance of Lnc-BTG3-7:1 (C) and C21ORF91 (D) highly increased in TNBC cells compared to MCF10A (P < 0.05). (E) A total of seven TNBC tumor tissue samples and four normal breast tissue samples were tested by qPCR, and a remarkably higher expression of Lnc-BTG3-7:1 in tumor tissues was showed compared to normal tissues (P < 0.005***). (F) Knockdown of lnc-BTG3-7:1 suppressed the C21ORF91 gene expression in MDA-MB-231 (P < 0.005***) and MDA-MB-468 (P < 0.005**), respectively. (G) Gene set enrichment analyses showing the module activity in tumor tissues and adjacent normal tissues. Point size and color represented the normalized enrichment score (NES). (H) Functional annotation based on KEGG functional hierarchy for genes in module M3. Node size represented the number of genes or children terms. Node color indicated the functional classification of pathways. (I) Interaction network suggested a pivotal role of Lnc-BTG3-7:1 and C21ORF91 in TNBC progress via coordinating oncogenic signaling. The genes involved in Wnt/β-catenin, Ras/MAPK, and PI3K/AKT pathways were color-marked. (J) Over-representation analysis of co-expressed genes of C21ORF91. The bar chart showed the gene number in the corresponding pathway. The color of the bar indicated the statistical significance. *P < 0.05 and ***P < 0.001.
Lnc-BTG3-7:1 was verified in patients’ tissue samples by using qPCR, and the same expression trend was observed consistently with RNA-seq (Figure 2E). Furthermore, in order to investigate whether Lnc-BTG3-7:1 targets C21ORF91 gene, we knocked down the Lnc-BTG3-7:1 in MDA-MB-231, MDA-MB-231, and BT-549 cell lines, and the result showed that after knocking down Lnc-BTG3-7:1, the C21ORF91 expression in TNBC cell lines significantly and synchronously decreased (P < 0.05, Figure 2F).

To address the question of how Lnc-BTG3-7:1 function in concert with target gene C21ORF91 to regulate TNBC progress, the possible interaction network was constructed. We then concentrated on module M3, which contained C21ORF91. Gene set enrichment analyses showed module M3 had higher activity in tumor tissues (Figure 2G). Remarkably, not only genes in module M3 participated in signal pathway (Figure 2H), and directly related to Wnt/β-catenin, RAS/MAPK, and PI3K/AKT pathways (Figure 2I), but also functional enrichment analysis revealed that co-expressed genes of C21ORF91 were substantially enriched in PI3K/AKT pathway (Figure 2J). The results indicated that Lnc-BTG3-7:1 and C21ORF91 gene might involve in Wnt/β-catenin, RAS/MAPK and PI3K/AKT pathways.

Lnc-BTG3-7:1 Participates in Regulating TNBC Progress

Lnc-BTG3-7:1 is a TNBC specific lncRNA, and its function still undefined. Thus, to investigate the function of Lnc-BTG3-7:1, we knocked down Lnc-BTG3-7:1 by using shRNA241 and shRNA351, and the knockdown efficiency of shRNA241 was significantly obvious (P < 0.05, Figure 3A). By performing colony forming assay, the long-term proliferation capacity of three TNBC cells was suppressed after knocking down Lnc-BTG3-7:1. The inhibition rates by shRNA241 were 82.1 ± 3.0%, 100 ± 4.5%, and 100 ± 3.9%, and the inhibition rates by shRNA351 were 38.5 ± 8.5%, 100 ± 4.5% and 93.8 ± 7.9% in MDA-MB-231, MDA-MB-468, and BT-549, respectively (P < 0.05**, Figure 3B).

Next, we investigated the effects of Lnc-BTG3-7:1 on cell apoptosis. As shown in Figure 3C, the proportion of Annexin V
(+)/PI (−) and Annexin V (+)/PI (+) apoptotic cells increased significantly after knocking down Lnc-BTG3-7:1 by shRNA241 ($P < 0.05$). Meanwhile, the short-term proliferation capacity of TNBC cells was also significantly suppressed in Lnc-BTG3-7:1 knockdown group. The inhibition rates were $94.8 \pm 23.2\%$, $90.7 \pm 6.1\%$, $94.3 \pm 22.7\%$ in MDA-MB-231, MDA-MB-468 and BT-549, respectively ($P < 0.05^{***}$, Figures 3D–F). Additionally, knockdown of Lnc-BTG3-7:1 by shRNA241 arrested cell cycle in G1 phase in MDA-MB-231 ($55.4 \% \text{ vs. } 63.5\%$, $P < 0.05^{***}$, Figures 3G,I) and BT-549 ($73.2 \% \text{ vs. } 76.6\%$, $P < 0.05^{***}$, Figures 3H,I). Altogether, the results indicated that Lnc-BTG3-7:1 could promote TNBC progress.

**Lnc-BTG3-7:1 Target C21ORF91 Gene to Regulate TNBC Progress**

In previous study, few results showed that C21ORF91 gene was involved in hepatocellular carcinoma cells (HCC) progress, and no report of C21ORF91 gene focus on breast cancer, especially TNBC. Thus, to investigate the functions of C21ORF91 gene in TNBC, we knocked down the expression of C21ORF91 gene using shRNA73 and shRNA764, and the knockdown efficiency of C21ORF91 gene was significantly obvious ($P < 0.05^{**}$, Figure 4A). The result of colony forming assay showed that knockdown of C21ORF91 gene could also inhibit long-term proliferation capacity. The inhibition rates by shRNA73 were 88.9 ± 17.8%, 100 ± 4.5%, 97.7 ± 1.9%, and the inhibition rates by shRNA764 were 78.2 ± 16.3%, 50.6 ± 23.1%, 76.5 ± 16.5% in MDA-MB-231, MDA-MB-468 and BT-549, respectively ($P < 0.05^{***}$, Figure 4B).

Next, we investigated the effects of C21ORF91 gene on cell apoptosis and proliferation. As shown in Figure 4C, the proportion of Annexin V (+)/PI (−) and Annexin V (+)/PI (+) apoptotic cells significantly increased after knocking down C21ORF91 gene by shRNA73 compared to control group ($P < 0.05$). Meanwhile, short-term proliferation capacity of three TNBC cells was also significantly suppressed in C21ORF91 gene knockdown group. The inhibition rates were 78.2 ± 22.6%, 30.8 ± 8.8%, 95.2 ± 23.4% in MDA-MB-231, MDA-MB-468, and BT-549, respectively ($P < 0.05^{**}$, Figures 4D–F). Altogether, above results indicated that C21ORF91 gene was involved in TNBC progress.

To further investigate the co-regulate effects of Lnc-BTG3-7:1 and C21ORF91 gene on anti-TNBC function, Lnc-BTG3-7:1 and C21ORF91 gene were double knocked down by using shRNA241 and shRNA73 in MDA-MB-231, MDA-MB-468, and BT-549. According to the result, double knockdown of Lnc-BTG3-7:1 and C21ORF91 gene inhibited long-term proliferation capacity. The inhibition rates were 99.9 ± 0.14%, 99.8 ± 0.15%, 100 ± 0% in MDA-MB-231, MDA-MB-468 and BT-549, respectively ($P < 0.05^{****}$, Figures 4G,H). Thus, the result showed that Lnc-BTG3-7:1 and C21ORF91 gene co-regulated and involved in TNBC progress.

**Lnc-BTG3-7:1 and JUND Co-regulate the Transcription of C21ORF91 in TNBC**

No previous study has reported the mechanism of how Lnc-BTG3-7:1 regulates C21ORF91. To investigate the underlying mechanism, analysis of lncRNA-protein interaction was used to predict the potential way, and the prediction showed that Lnc-BTG3-7:1 might be synergistic with JUND to co-regulate target genes through cis-acting SE-lncRNA mechanism (Figures 5A,B). Furthermore, in order to confirm the prediction, RNA-FISH and immunofluorescence co-localization analysis were performed to determine the sub-location of Lnc-BTG3-7:1 and co-localization with JUND protein in MDA-MB-231 and BT-549 cells. The location of Lnc-BTG3-7:1, which was mainly in nucleus of TNBC cells and co-located with JUND protein, was observed (Figure 5C). More than that, we further performed ChIP-qPCR (Immunoprecipitation-qPCR), and significant enrichment of Lnc-BTG3-7:1 in JUND specific antibody immunoprecipitation group in MDA-MB-468 ($P < 0.05^{****}$) and BT-549 ($P < 0.05^{****}$) cells were observed compared with the negative control group (Figure 5D), which confirm the combination of Lnc-BTG3-7:1 and JUND. Thus, these results further showed that Lnc-BTG3-7:1 co-regulated with JUND in TNBC.

Altogether, based on the results, we found that Lnc-BTG3-7:1 was synergistic with JUND in the TNBC cells and potentially co-regulated the transcription of C21ORF91.

**Lnc-BTG3-7:1/C21ORF91 Activated AKT-GSK3β and MAPK Pathways in TNBC**

Previous report indicated that GRB2 is one of C21ORF91 potential target proteins (Wang et al., 2008). In line with this conclusion, we also found that C21ORF91 was involved in regulation of GRB2-RAS-RAF-MEK-ERK and GRB2-PI3K-AKT-GSK3β-β-catenin pathways (The data were shown in Figure 2H). To validate the activation of GRB2 signal, we checked the downstream kinase of MAPK and PI3K-AKT-GSK3β pathways. On the one hand, the result indicated that the phosphorylation of AKT at serine 473 a.a. residue was suppressed upon C21ORF91 and Lnc-BTG3-7:1 knockdown in MDA-MB-231, MDA-MB-468 and BT-549 cell lines (Figure 5E). The suppression of p-AKTs473 resulted in hyper phosphorylation of GSK3beta, and subsequentially, down regulation of beta-catenin and c-myc. On the other hand, removal of C21ORF91 or Lnc-BTG3-7:1 inhibited the downstream of canonical MAPK kinases MEK1/2 and ERK1/2 (Figure 5E).

In summary, the potential working model is describe as: (1) JUND along with Lnc-BTG3-7:1 transcriptional upregulates C21ORF91; (2) C21ORF91 translocates from nucleus to cytosol and activates GRB2; (3) GRB2 activates multiple pathways including PI3K and MAPK; (4) upon activation, PI3K signals downstream AKT and GSK3beta which in turn stabilizes b-catenin; (5) similarly, GRB2 activated RAS signals the canonical MAPK cascade, including RAF, MEK, ERK; and (6) these activated kinases promote the proliferation and anti-apoptosis of TNBC (Figure 6).

**DISCUSSION**

Triple-negative breast cancer is a high invasive with poor prognosis subtype breast cancer, occurring from younger age...
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FIGURE 4 | Lnc-BTG3-7:1 and target C21ORF91 gene participates in the process of TNBC progress. (A) The expression levels of C21ORF91 in three TNBC cell lines significantly decreased after C21ORF91 gene knockdown (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.005\)). (B) Knockdown of C21ORF91 significantly reduced colony forming capacity of the three TNBC cell lines (\(P < 0.05\)). (C) Knockdown of C21ORF91 (with shRNA73) induced early apoptosis (Q3: 14.3%) cells and late apoptosis (Q2: 8.74%) cells compared to that of the control group (Q3: 1.35%, Q2: 2.05%) in MDA-MB-468; and early apoptosis (Q3: 0.12%) cells and late apoptosis (Q2: 37.0%) cells compared with the control group (Q3: 0.23%, Q2: 0.55%) in MDA-MB-231 (\(P < 0.05\)), respectively. (D–F) Short-term proliferation capacity of TNBC cell lines were inhibited after knocking down of C21ORF91, especially in MDA-MB-231 and BT-549 (\(P < 0.05\)). (G) The expression levels of C21ORF91 in three TNBC cell lines significantly decreased after double knockdown of Lnc-BTG3-7:1 and C21ORF91 gene (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.005\)). (H) Double knockdown of Lnc-BTG3-7:1 and C21ORF91 gene inhibited long-term proliferation capacity of three TNBC cell lines (\(P < 0.05\)).

and being prone to distant metastasis (Foulkes et al., 2010; Shah et al., 2012). Based on the National Comprehensive Cancer Network guideline (NCCN), there is few clinical success of current therapy, making most TNBC patients have to still rely on chemotherapy (Bianchini et al., 2016; Park et al., 2018), and the median overall survival (mOS) for advanced TNBC patients are only about 9–12 months (Belli et al., 2019). Meanwhile, identifying effective biomarkers for cancer prognosis and drug responsiveness is of great importance in improving the clinical management of cancer, but unfortunately, precise biomarkers of TNBC treatment response and prognosis have not yet been identified (Singh et al., 2010).

Recently, the whole genome and transcriptome sequencing technology have been widely used in discovering latest genome difference (Marotti et al., 2017), which indicate that lncRNAs contribute to a significant portion of the “dark matter” of the human transcriptome (Kapranov et al., 2010) and show a new insight into the regulator heterogeneity of TNBC. Meanwhile, the differential expression of lncRNAs between normal and tumor tissues suggests that it is similar to protein-coding oncogenes (Huardt and Rinn, 2010), and dysfunction of lncRNA strongly associates with cancer progress (Fang et al., 2020). For example, UCA1, LncRNA human ovarian cancer-specific transcript 2 (HOST2) and LncRNA nuclear enriched abundant transcript1 (NEAT1) are associated with lung cancer, ovarian cancer and prostate cancer, respectively (Chakravarty et al., 2014; Gao et al., 2015; Nie et al., 2016). Nowadays, several lncRNAs have been shown to modify critical breast cancer associated molecular
pathways such as GAS5 (Mourtada-Maarabouni et al., 2009). However, so far, there is rare outstanding results concerning TNBC in the field of lncRNAs (Gooding et al., 2019; Zhang et al., 2019), such as HOTAIR, which relates with luminal androgen receptor (LAR) subtype of TNBC (Gupta et al., 2010; Collina et al., 2019).

Also, previous studies have indicated that the C21ORF91 gene encoding cytosolic protein plays a role in biological processes, such as cerebral cortex neuron differentiation, cell differentiation and regulation of dendritic spine development (Danileviciene et al., 2019). Recently, few researches show relationship between the C21ORF91 gene and herpes labialis, in which C21ORF91 gene is identified as direct targets of miR-194 in hepatocellular carcinoma cells (HCC) (Kriesel et al., 2011; Bao et al., 2015), but its function is not clear. The way in which the C21ORF91 gene influences TNBC progress has not been established yet, and how Lnc-BTG3-7:1 regulates C21ORF91 gene is still unknown.

In this study, we first screened out TNBC specific lncRNA Lnc-BTG3-7:1, which sustained tumor progress, and this TNBC
specific lncRNA and its target C21ORF91 gene were involved in Wnt/β-catenin and MAPK pathways, which were associated with cancer cell progress (Liu et al., 2019). According to the bioinformatic analysis, the 1–195th bases of the Lnc-BTG3-7:1 sequence overlapped with the 25–219th bases of the C21ORF91 gene and the coding DNA sequence (CDS) of C21ORF91 started from the 62nd base. Meanwhile, based on analysis of the gene sequence of C21ORF91, we not only found that Lnc-BTG3-7:1 located in the region of super enhance SE_13873/SE_18618, which belonged to cis-acting SE-lncRNA, but also lncRNA-protein interaction prediction showed that JUND and Lnc-BTG3-7:1 had high possibility of interaction. Thus, we predicted that Lnc-BTG3-7:1 might be synergistic with JUND to co-regulate target genes through SE-LncRNA mechanism, which was associated with Wnt/β-catenin and MAPK pathways.

Furthermore, in this study, we observed the functions of the Lnc-BTG3-7:1 and found its regulation mechanism in TNBC cell lines. (1) In functional analysis, single or double knockdown of Lnc-BTG3-7:1 and C21ORF91 gene, strongly induced diminishing of cell proliferation, increasing cell apoptosis and arresting cell cycle in G1. (2) In mechanism analysis, by using IF/FISH and ChIP-qPCR assay, not only Lnc-BTG3-7:1 was co-located with JUND in nucleus, but also Lnc-BTG3-7:1 was enriched in JUND specific antibody immunoprecipitation groups in TNBC cell lines. Taken together, our result demonstrated that Lnc-BTG3-7:1 co-regulate the transcription of C21ORF91 gene with nuclear synergistic transcription factor JUND. Meanwhile, Western-blot test showed that Lnc-BTG3-7:1 was involved in GRB2-PI3K-AKT-GSK3β-β-catenin and GRB2-RAS-RAF-MEK-ERK pathways in TNBC.

Thus, from the above, our findings suggested that Lnc-BTG3-7:1 and the target C21ORF91 gene were TNBC specific factors and participated in TNBC progress.

**CONCLUSION**

In our study, a TNBC specific lncRNA Lnc-BTG3-7:1 was screened out and verified, which was involved in TNBC progress and could activate PI3K-AKT-GSK3β and MAPK pathways.
by regulating transcription of the target C21ORF91 gene. In conclusion, our results not only identified Lnc-BTG3-7:1 as a biomarker for diagnosis, but also provided a potential therapeutic target against TNBC.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA553096.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

**AUTHOR CONTRIBUTIONS**

LY and Jj: conceptualization. ZD and HX: formal analysis. ZD, HX, LT, ZX, and Yj: methodology. ZD, LY, JJ, and ZH: project administration. LY: writing – original draft. ZD, HX, and LY: writing – review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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