LncRNA \textit{DANCR} upregulates PI3K/AKT signaling through activating serine phosphorylation of RXRA

Jianming Tang¹, Guangsheng Zhong², Haibo Zhang¹, Bo Yu³, Fangqiang Wei⁴, Liming Luo¹, Yao Kang⁵, Jianhui Wu⁶, Jiaxiang Jiang⁷, Yucheng Li¹, Shuqiang Wu¹, Yongshi Jia¹, Xiaodong Liang¹ and Aihong Bi¹

Abstract
Conventional therapies and novel molecular targeted therapies against breast cancer have gained great advances over the past two decades. However, poor prognosis and low survival rate are far from expectation for improvement, particularly in patients with triple negative breast cancer (TNBC). Here, we found that lncRNA \textit{DANCR} was significantly overregulated in TNBC tissues and cell lines compared with normal breast tissues or other type of breast cancer. Knockdown of \textit{DANCR} suppressed TNBC proliferation both in vitro and in vivo. Further study of underlying mechanisms demonstrated that \textit{DANCR} bound with RXRA and increased its serine 49/78 phosphorylation via GSK3β, resulting in activating PIK3CA transcription, and subsequently enhanced PI3K/AKT signaling and TNBC tumorigenesis. Taken together, Our findings identified \textit{DANCR} as an pro-oncogene and uncovered a new working pattern of lncRNA to mediate TNBC tumorigenesis, which may be a potential therapeutic target for improving treatment of TNBC.

Introduction
Breast cancer is the most prevalent malignant tumor in women, and the efficacy of currently available therapies seems far from satisfactory, which severely threatens the health of females from all over the world¹². Generally, breast cancer can be classified into four subtypes, including luminal type A, luminal type B, human epidermal growth factor receptor-2 (HER2) positive type, and triple-negative breast cancer (TNBC) type³. TNBC demonstrates a lack of ER, PR, and HER2 expression by immunohistochemical results, and acts as a highly invasive subtype comprising about 20% of all breast cancer patients⁴⁵. Furthermore, TNBC can exhibit high invasiveness, metastasis, high recurrence risk, and mortality rates, resulting in poor prognosis⁶. The lack of molecular targeted therapies and the poor survival of TNBC patients have fostered great endeavors to discover precise molecular targets for clinical treatment strategies. A growing body of evidence suggests that long non-coding RNAs (LncRNAs) are involved in TNBC progression through regulating tumor-related gene expression⁷⁻⁹. However, the precise molecular mechanisms by which lncRNA mediates TNBC progression remain unclear.

Differentiation antagonizing non-protein coding RNA (DANCR), an lncRNA encoded on human chromosome 4q12, has been identified as an oncogene in multiple malignant tumors, including colon cancer¹⁰, esophageal cancer¹¹, hepatocellular carcinoma¹², osteosarcoma¹³, TNBC¹⁴. DANCR was previously demonstrated to contribute to suppression of cell differentiation due to acting as a negative regulator¹⁵⁻¹⁶. Recent studies demonstrated
that DANC\(\text{R}\) directly interacts with miR-758-3p\(^{17}\) and miR-577\(^{18}\) in non-small cell lung cancer and colorectal cancer, respectively. DANC\(\text{R}\) represses the expression of TIMP2/3 through physical binding with EZH2 in prostate cancer\(^{19}\). DANC\(\text{R}\) is also shown as a direct target of MYC in cancer\(^{20}\). Furthermore, DANC\(\text{R}\) expression is correlated with survival and/or prognosis of patients with hepatocellular carcinoma\(^{12}\), gastric cancer\(^{21}\), and colorectal cancer\(^{10}\). However, due to the molecular and phenotypic heterogeneity within and between different tumor types, mechanism explorations are required to elucidate the precise biological behaviour of DANC\(\text{R}\) in tumors, especially TNBC.

Here, we showed that DANC\(\text{R}\) is more highly expressed in TNBC compared with that in normal breast tissues, which exhibits poor prognosis. DANC\(\text{R}\) promoted proliferation and tumorigenesis in TNBC through activating ser49/78 phosphorylation of RXRA, and thus promoting PIK3CA expression. We also showed that signaling axis DANC\(\text{R}\)-RXRA-P13K/AKT plays important roles in TNBC proliferation and tumorigenesis in vitro and vivo, respectively. In conclusion, the present study elucidated the function of DANC\(\text{R}\) in TNBC and might provide a novel of signaling pathway in the treatment of TNBC.

**Materials and methods**

**Clinical samples**

Between August 2013 and August 2015, clinical specimens containing breast cancer tissues including 60 triple-negative (TNBC) type, 15 HER2 type, 15 Luminal A type, and 15 Luminal B type, and 10 normal breast tissues were obtained from department of Breast Surgery in Meizhou People’s Hospital. All these patients had not received chemotherapy and radiotherapy before the operation and all clinical samples were confirmed by pathology. This study protocol approval from the Research Ethics Committee of the Meizhou People’s Hospital, and written informed consent from each participant were obtained.

**Cell lines**

BT549, MCF7, T47D, MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells were purchased from Cell Bank of the Chinese Scientific Academy (Shanghai, China), and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, Life Technologies, CA) with 10% fetal bovine serum (Hyclone, Life Technologies, CA). MCF10A was also from Cell Bank of the Chinese Scientific Academy (Shanghai, China), and was cultured in DMEM/F12 (Hyclone, Life Technologies, CA) supplemented with 5% horse serum (Invitrogen, Carlsbad, CA), 20 ng/ml hEGF (Sigma-Aldrich), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), and 100 U/ml penicillin-streptomycin (Sigma-Aldrich). BT549, MCF7, T47D, MDA-MB-231, MDA-MB-453, MDA-MB-468, and MCF10A were recently authenticated through using STR DNA fingerprinting from Shanghai Biowing Applied Biotechnology Co., Ltd. Moreover, by using LookOut Mycoplasma PCR Detection kit (Sigma-Aldrich), we detected the mycoplasma infection.

**RNA extraction and quantitative RT-PCR**

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and extracted according to the manufacturers’ protocol (Sigma-Aldrich). Reverse transcription (RT) was performed using the M-MLV Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative RT–PCR was performed by means of Power qPCR Premix (SYBR Green) (Shanghai Generay Biotech Co., Ltd). GAPDH was used as a control. Primers are contained in Supplementary Table S1.

**Western blot analysis**

Western blot was performed as we previously described\(^{22}\). The specific antibodies for WB as follows: anti-RXRA (21218-1-AP, 1:1000, Proteintech), Phospho-RXRA (PA5-64630, 1:1000, Invitrogen), Akt (#4685S, 1:1000, Cell Signaling Technology), Abcam), Akt (#4685S, 1:1000, Cell Signaling Technology), PIK3CA (MA5-17149, 1:1000, Thermo Fisher), GSK3β (ab65740, 1:1000, Abcam), Akt (#4685S, 1:1000, Cell Signaling Technology), PIK3CA (MA5-17149, 1:1000, Thermo Fisher), GAPDH (#5174S, 1:1000, Cell Signaling Technology).

**ChIP-qPCR**

ChIP assay was performed using EZ-Magna ChiP™ A/G Chromatin Immunoprecipitation Kit (Millipore-17-408) referring to the manufacturer’s protocol.

Input genomic DNA and the purified immunoprecipitated DNA were used for qRT-PCR. Primers are contained in Supplementary Table 1.

**Luciferase promoter assay**

pG3-P3-PIK3CA promoter wild or mutant type of the RXRA binding was performed co-transfection with or without RXRA using Lipofectamine RNAiMAX Reagent (Thero Fisher) referring to the manufacturer’s protocol. pRL Renilla luciferase control reporter vector (Promega) was presented as a control. A dual-luciferase assay was analyzed 48 h after co-transfection using the Promega E1960 Dual-Luciferase® Reporter System in accordance with the manufacturer recommendation.

**RNA immunoprecipitation (RIP) and RNA pull-down assays**

The EZ-Magna RIP Kit (17-701, Millipore) was used for RIP assay with 10 μg anti-RXRA (21218-1-AP, Proteintech) referring to the manufacturer recommendation. The purified immunoprecipitated RNA and input genomic RNA were detected by quantitative RT-PCR. Biotin-labeled RNA was transcribed with Biotin RNA Labeling
Mix (Roche 11685597910) and T7 RNA polymerase (Roche 10881775001), mixed with DNase I recombinant (Roche 04716728001), and purified with RNeasy Mini Kit (Qiagen 74904). Cell nuclear proteins were extracted using the Pierce 78833 NE-PER (R) Nuclear and Cytoplasmic Extraction Reagents. Cell nuclear extract was mixed with Biotin-labeled RNA. Washed streptavidin agarose beads (Sigma-Aldrich) were added to each reaction. The binding protein was analyzed by Western blot assay.

Cell proliferation and soft agar colony formation

A WST-1 Assay Kit (Roche) was used for cell proliferation assay. Cells were seeded in a 48-well plate and then incubated at 37 °C. Cell numbers were assessed with the WST-1 Assay Kit. For soft agar colony formation, cells were split into the suspension of a single-cell, and then seeded in a media containing 0.4% top layer agar and 0.8% bottom layer agar in a 6-well plate. Cell culture media was changed every 4 days after seeding. Colonies were fixed with 4% paraformaldehyde and stained with 2% crystal violet solution after 2–3 weeks, respectively. The visible colony numbers scored and data were analyzed.

Construction of vectors

The cDNA encoding DANC, PIK3CA,GSK3β, RXRA were amplified from MCF10A cells and sequenced, and then subcloned into the pcDNA3 vector (Invitrogen), subsequently named pCDNA3-DANC, pCDNA3-PIK3CA, pCDNA3-GSK3β, pCDNA3-RXRA. pLVX-DANC, pLVX-PIK3CA, pLVX-GSK3β, and pLVX-RXRA was generated from pCDNA3-DANC, pCDNA3-PIK3CA, pCDNA3-GSK3β, pCDNA3- RXRA, respectively. PIK3CA promoter was PCR-amplified from MCF10A cells and sequenced, and then subcloned into pGL3 vector (Promega). A Quik Change Site-Directed Mutagenesis Kit (Stratagene) was used for point mutations. shRNAs for DANC were designed (shDANC-1 target sequence: 5′-GGAGCTAG AGCAGTGACAAT G-3′; shDANC-2 target sequence 5′-GGTCACCAGAC TTGCT ACACC-3′), and RXRA (shRXRA target sequence: 5′-GGCAAGCACTATGG AGTGCA-3′, respectively.

Tumorigenesis studies

BALB/c-nude mice female at an age of 4–5 weeks (SLAC, Shanghai, China) were randomly divided into 4 per group, and then MDA-MB-231 cells (3 × 10⁶) were implanted subcutaneously into mammary fat pads of each mice. All animal experiments procedures were approved by Zhejiang Provincial People’s Hospital the Guidance of Institutional Animal Care and Use Committee (IACUC). The IVIS Lumina imaging station (Caliper Life Sciences) was used for bioluminescence imaging. Different investigators independently performed mice allocation, surgery and the outcome assessing.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism version 5.0. The significance of the data from patient specimens was determined by Pearson’s correlation coefficient. The significance of data from the in vitro and vivo between experimental groups was determined by the Student’s t test or Mann–Whitney U-test *P < 0.05 was considered statistically significant.

Results

Expression of DANC in clinical TNBC specimens

We examined DANC expression using clinical samples consisting of TNBC tissues and normal breast tissues. DANC was found to be significantly upregulated in TNBC tumor tissues compared with that in normal breast tissues (Fig. 1a). To support our finding, we downloaded the TCGA RNA-seq dataset and microarray dataset of invasive breast cancer specimens, respectively. In these datasets, There was a clear trend that the TNBC tissues exhibits higher DANC expression compared to the paired peritumoral tissues (Fig. 1e and Supplementary Figure 1A). Interestingly, higher level of DANC was demonstrated to be associated with bigger tumor size (Fig. 1b). We then detected DANC expression in breast cancer samples of various subtypes. DANC showed significantly higher expression level in TNBC than that in the other subtypes of breast cancer (Fig. 1c). Furthermore, patients with high DANC expression (n = 30) suffered poorer overall survival (OS) as compared to low expression group (n = 30) (Fig. 1d). Similarly, TCGA microarray dataset also demonstrated the result (Supplementary Figure 1B). Gene amplifications in DANC were observed in mRNA overexpression (TCGA database from cBioPortal) (Supplementary Figure 1C). Taken together, this data demonstrated that DANC is amplified and over-expressed in TNBC tumors.

DANC inhibition suppressed cell proliferation and tumor growth in TNBC

To further demonstrate the involvement of DANC in TNBC, we tested DANC expression in six breast cancer cell lines (BT549, MCF7, T47D, MDA-MB-231, MDA-MB-453, and MDA-MB-468) and a normal mammary epithelial cell line (MCF10A). DANC expression was markedly upregulated in breast cancer cell lines as compared to that in MCF10A cell lines (Fig. 2a). Importantly, among all the six cancer cell lines, DANC exhibited highest expression levels in two TNBC cell lines (MDA-MB-231 and MDA-MB-468).

We then tested the effect of DANC inhibition on TNBC cell growth. Here, we knocked down the DANCR
expression through treating MDA-MB-231 and MDA-MB-468 cells with the short hairpin RNA-mediated DANCR silencing (shRNA) or non-silencing control (shC), and found that depletion of endogenous DANCR significantly suppressed cell proliferation in both TNBC cell lines compared with the controls (Fig. 2b, c). Moreover, DANCR knockdown also impaired soft agar colony formation in MDA-MB-231 and MDA-MB-468 cells (Fig. 2d, e).

To further elucidate the TNBC tumorigenesis with DANCR, analysis of the orthotopic breast cancer model was performed. MDA-MB-231 cell lines transduced with shDANCR-1, shDANCR-2 or shControl (shC) were separately injected into the mice mammary gland fat pads. Remarkably, knockdown of DANCR significantly decreased TNBC tumor growth compared with controls (Fig. 2f, g). These data suggested that DANCR contributes to TNBC cell proliferation and tumor growth.

**DANCR interacts with RXRA in TNBC cells**

Recent studies have demonstrated that IncRNAs mainly function as sponges to bind functional proteins and then influence their downstream genes expression. Thus, we hypothesized that DANCR-regulated tumorigenesis depends on its binding proteins. DANCR was examined for transcription factor binding sites using a JASPAR database of transcription factor binding profiles, which identified two RXRA-binding sites as the most potential candidates. It is predicted that RXRA may bind with DANCR at both 211 to 225 and 269 to 283 sites (Fig. 3a). To test this, we performed RIP quantitative PCR with an antibody against RXRA from nuclear extracts of both MDA-MB-231 and MDA-MB-468 cells. We demonstrated that DANCR specifically bound to endogenous RXRA protein (Fig. 3b).

We next constructed DANCR plasmids containing a wild type and three mutant types including DANCR-
RXRA binding site1 (Mut1), site2 (Mut2), or both sites (Mut1/2). As we predicted, re-expression of shRNA-resistant DNAcr cDNA encoding the wild type restored the binding of DNAcr with RXRA, whereas re-expression of a DNAcr shRNA-resistant plasmid containing the DNAcr-RXRA binding site1, site 2, or two sites mutants did not rescue it, suggesting that these two sites are critical for DNAcr-RXRA binding (Fig. 3c, d).

To further validate the binding between DNAcr and RXRA, we performed RNA pulldown (Fig. 3e), and deletion-mapping methods (Fig. 3f, g) to demonstrate whether RXRA would bind within specific regions of DNAcr. These data identified a 300nt region at the 5′ end of DNAcr required for the binding with RXRA (Fig. 3g). Taken together, the RIP, RNA pulldown, and deletion-mapping data validate a specific binding between RXRA and DNAcr.
DANCR regulates RXRA phosphorylation

To explore the role of RXRA in DANCR-mediated TNBC tumor growth, we first assessed RXRA protein and expression in DANCR depletion TNBC cells. We found that DANCR knockdown did not affect the protein level of RXRA in MDA-MB-231 and MDA-MB-468 cells (Fig. 4a). Moreover, DANCR depletion had no effect on RXRA mRNA level (Fig. 4b). However, Western blot analysis showed that lower phosphorylation level of RXRA was found in the DANCR-knockdown group as compared to that of the controls (Fig. 4c). Furthermore, re-expression of shRNA-resistant DANCR wild type and RXRA-binding mutant types. RIP-qPCR assay of effects of re-expression of shRNA-resistant DANCR wild type or mutant types on RXRA binding. e Biotinylated DANCR was incubated with nuclear extracts (MDA-MB-231 and MDA-MB-468 cells), targeted with streptavidin beads, and binding proteins were resolved in a gel. Western blotting assay of the specific binding of RXRA and DANCR. f, g RNAs corresponding to fragments in different regions of DANCR were treated as in (e), and binding RXRA was detected by western blotting assay. Error bars ± SD. *P < 0.05. **P < 0.01. ***P < 0.001. Data are representative from two independent experiments.

DANCR facilitates PIK3CA transcription in a RXRA-mediated manner

Since PI3K signaling is critical for TNBC proliferation and Akt is a dominant downstream effector of PI3K signaling pathway, we explored the effects of DANCR depletion on expression of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) in MDA-MB-231 and MDA-MB-468 cells. As shown in Fig. 5a, b, knockdown of DANCR significantly inhibited PIK3CA protein and mRNA expression levels in both TNBC cells. However, knockdown of RXRA significantly rescued DANCR depletion-inhibited PIK3CA expression (Fig. 5c). These data suggest that DANCR may mediate RXRA to regulate PIK3CA expression.

To validate that RXRA regulates PIK3CA expression in TNBC cells, we predicted RXRA-binding sites to the promoter of PIK3CA using the JASPAR database of...
transcription factor binding profiles. One RXRA binding site was detected in the promoter of PIK3CA at the −1148 to −1134 site (Fig. 5d). RXRA Knockdown increased PIK3CA mRNA levels by qRT-PCR assays (Supplementary Figure 3). ChIP-qPCR assays using the antibody against RXRA demonstrated that RXRA bind to PIK3CA promoter (Fig. 5e). Promoter luciferase assays further validated that RXRA overexpression markedly decreased PIK3CA promoter transcriptional activity compared with the control, whereas the transcriptional activity was definitely restored after the mutation of RXRA binding site in PIK3CA promoter (Fig. 5f). Taken together, these data indicate that RXRA acts as a transcription repressors to inhibit PIK3CA expression.

To validate that DANCR/RXRA/PIK3CA signaling pathway regulates TNBC tumor growth, we downregulated RXRA and overexpressed PIK3CA in MDA-MB-231 and MDA-MB-468 cells with a RXRA shRNA or a PIK3CA vector (Fig. 5g and Supplementary Figure 2A). Knockdown of RXRA rescued DANCR depletion-inhibited PIK3CA and Akt phosphorylation (Fig. 5g and Supplementary Figure 2A), cell proliferation (Fig. 5h and Supplementary Figure 2B), colony formation (Fig. 5i and Supplementary Figure 2C). Moreover, overexpression of PIK3CA restored Akt phosphorylation (Fig. 5g and Supplementary Figure 2A), cell proliferation (Fig. 5h and Supplementary Figure 2B), colony formation (Fig. 5i and Supplementary Figure 2C) inhibited by DANCR knockdown. Furthermore, DANCR expression was found to be correlated with PIK3CA expression (Supplementary Figure 2D). These data further demonstrate that DANCR mediates RXRA to upregulate PIK3CA expression, resulting in enhancing PI3K/AKT signaling pathway and promoting TNBC tumor growth.

**DANCR-mediated RXRA phosphorylation depends on GSK3β**

Since the glycogen synthase kinase 3 beta (GSK3β) had been reported to promote RXRA phosphorylation in colorectal cancer cells, we detected whether DANCR-regulated RXRA phosphorylation depends on GSK3β. As shown in Fig. 6a, compared with the control group, overexpression of DANCR increased RXRA binding with GSK3β, RXRA phosphorylation, Akt phosphorylation and PIK3CA expression in both TNBC cells (Fig. 6a). Knockdown of DANCR decreased RXRA binding with GSK3β, RXRA phosphorylation, Akt phosphorylation and PIK3CA expression in both TNBC cells (Fig. 6b). Furthermore, overexpression of GSK3β increased RXRA phosphorylation, Akt phosphorylation and PIK3CA expression (Fig. 6c) inhibited by DANCR knockdown. Consistent with previous study, overexpression of RXRA increased its binding with GSK3β and decreased Akt phosphorylation and PIK3CA expression in MDA-MB-231 and MDA-MB-468 cells (Fig. 6d). Overexpression of RXRA significantly restored GSK3β association and Akt phosphorylation and PIK3CA expression inhibited by DANCR knockdown (Fig. 6d). These data support that DANCR depends on GSK3β to mediate RXRA phosphorylation in both TNBC cells.
Ser49/Ser78 sites of RXRA protein are critical for DANCR-mediated TNBC tumor growth

Since the roles of RXRA phosphorylation mediated by DANCR in TNBC is not well understood, we focused on the role of RXRA phosphorylation in DANCR-regulated TNBC cell proliferation. To detect whether RXRA phosphorylation is necessary to activate PI3K/AKT signaling pathway, we generated the GSK3β kinase deficient mutant plasmid, GSK3β-K58A. As shown Fig. 7a, overexpression of GSK3β cDNA encoding the wild type restored DANCR knockdown-inhibited RXRA phosphorylation, Akt phosphorylation and PIK3CA expression, whereas transfection of GSK3β kinase deficient mutant type did not rescue it. Next, we predicted potential serine phosphorylation sites of the RXRA protein using the Phosphor Motif Finder Program. Two serine phosphorylation candidate sites at Ser49 and Ser78 were identified. Transfection of RXRA wild type in MDA-MB-231 cells restored its binding with GSK3β inhibited by DANCR knockdown, and further decreased DANCR depletion-inhibited Akt phosphorylation, PIK3CA expression, cell proliferation, and soft agar colony formation (Fig. 7b–e). However, transfection of RXRA^S49A/S78A mutant did not rescue it compared with the wild type group (Fig. 7b–e). Taken together, these data demonstrates that serine phosphorylation of RXRA plays a crucial role in activating the PI3K/AKT signaling pathway.

Fig. 5 DANCR facilitates PIK3CA transcription in a RXRA-mediated manner. a, b Effects of DANCR knockdown on PIK3CA protein (a) and mRNA (b) expression in MDA-MB-231 and MDA-MB-468 cells. c Knockdown of RXRA restores DANCR depletion-inhibited PIK3CA expression in MDA-MB-231 and MDA-MB-468 cells. d Schematic diagram of the putative RXRA binding site in PIK3CA promoter. e ChIP-qPCR assay of the binding of RXRA with the PIK3CA promoter in MDA-MB-231 cells. f Luciferase assay of RXRA mediation on the PIK3CA promoter activity in MDA-MB-231 cells. g Knockdown of RXRA and overexpression of PIK3CA in DANCR depletion cells in MDA-MB-231 cells. h, i Knockdown of RXRA and overexpression of PIK3CA rescue DANCR knockdown-inhibited cell proliferation (h) and soft agar colony formation (i) in MDA-MB-231 cells. Error bars ± SD. *P < 0.05. **P < 0.01. ***P < 0.001. Data are representative from two independent experiments.
Discussion

In this study, we demonstrated a new mechanism by which DANCR-upregulated PI3K/AKT signaling pathway through activating serine phosphorylation of RXRA protein is important for TNBC cell proliferation and tumor growth (Fig. 8). By means of facilitating connection between RXRA and GSK3β, DANCR promotes the phosphorylation of RXRA, leading to suppress RXRA induced inhibition of PIK3CA transcription, thereby activating PI3K/AKT downstream signaling and ultimately promoting TNBC tumorigenesis.

Our data demonstrate that DANCR promotes TNBC tumorigenesis. DANCR was previously identified to play an important role in maintaining the undifferentiated cell state\(^{15,16}\). Previous work demonstrated that DANCR interacts with EZH2 to form a complex to promote stem cell characteristics\(^{16,28}\). It has been well documented that DANCR was overexpressed and amplified in breast cancer, and knockdown of DANCR significantly inhibited cell proliferation and invasion in breast cancer through facilitating binding of EZH2 to the promoters of ABCG2 and CD44 genes\(^{14}\). Here, we report that DANCR is upregulated in clinical TNBC samples, and higher DANCR level is positively correlated with poorer prognosis of TNBC patients. Knockdown of DANCR significantly inhibited TNBC cell proliferation, colony formation in vitro, and tumor growth in vivo. These results strongly support that DANCR is critical for TNBC tumorigenesis.

Our results also demonstrate that DANCR mediates TNBC through RXRA. RXRA had been reported to play...
critical roles in breast cancer cell progression. Phosphorylation of the RXRA at serine 260 decreases its coactivator recruitment ability, leading to inhibition of RXRA transcriptional activity and enhanced cancer cell proliferation. DANCR has also been identified as a molecular sponge mediating miR-758-3p and miR-577 in non-small cell lung cancer and colorectal cancer, respectively. Furthermore, some studies showed that DANCR regulate breast cancer through binding and phosphorylating EZH2. In this study, our data showed that DANCR directly bound with RXRA and mediated its phosphorylation. Knockdown of DANCR enhanced RXRA phosphorylation. Re-expression of shRNA resistant DANCR wild type impaired DANCR knockdown-promoted RXRA phosphorylation, whereas re-expression of shRNA resistant mutant type of the DANCR-RXRA binding did not affect it. Knockdown of RXRA rescued DANCR depletion-inhibited cell proliferation and soft agar colony formation. Taken together, our data demonstrate that DANCR regulate TNBC cell proliferation through RXRA phosphorylation.

Our results further suggest that DANCR mediates TNBC through RXRA- downregulating PI3K/AKT signaling pathway. Activating PI3K/AKT signaling was reported to be associated with poor OS in patients with breast cancer. PIK3CA activation was also demonstrated to be critical for enhancing PI3K/AKT signaling. RXRA was reported to mediate PI3K/AKT signaling in response to stem cell differentiation and provoke tumor suppression. Inhibition of the N-terminally truncated RXRA association with the PI3K p85α subunit also resulted in suppression of PI3K/AKT signaling activation. Here, we found that RXRA binds with the promoter of PIK3CA and downregulates PIK3CA transcription. We also observed that knockdown of DANCR inhibited RXRA protein phosphorylation and PIK3CA expression level.
Interestingly, knockdown of RXRA rescued DANCN depletion-inhibited PIK3CA expression, cell proliferation, soft agar colony formation, and the promoter transcriptional activity of PIK3CA. Furthermore, we found DANCN markedly promotes RXRA association with GSK3β. In consistent with the previous study31,32, our data demonstrate that DANCN-induced RXRA phosphorylation suppresses RXRA-inhibited PIK3CA transcription in TNBC cells and ultimately activates the downstream PI3K/AKT signaling, whereas overexpression of GSK3β mutant type with deficient kinase activity or RXRA with mutated binding site with GSK3β dramatically blocked it. These data suggest that DANCN promoted TNBC tumorigenesis depends on its binding and phosphorylating RXRA, which leads to PI3K/AKT signaling pathway activation.

In summary, our results identified DANCN as an oncogene promotes TNBC tumorigenesis through a distinctive mechanism by which DANCN facilitated RXRA phosphorylation depending on GSK3β, thereby inhibiting the function of RXRA as a transcription repressors, and ultimately enhanced downstream PI3K/AKT signaling. Our findings have shed light into a novel roles of DANCN in TNBC tumorigenesis which have significant implications on better understanding the function of DANCN in human cancers. We hope that the newly established roles of DANCN and RXRA in tumorigenesis may provide a strong rationale for targeting them to improve the treatment of TNBC patients.

Acknowledgements

This study was supported in part by grants from the Zhejiang Provincial Nature Science Foundation of China (Grant number: LY18H160035 to Xiaodong Liang).

Author details

1 Department of Radiation Oncology, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China. 2 Department of Thyroid and Breast Surgery, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China. 3 Department of Oncology, Taixing People’s Hospital, Taixing, Jiangsu 225300, P.R. China. 4 Department of Hepatobiliary and Pancreatic Surgery, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China. 5 Department of Orthopaedic Surgery, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China. 6 Department of Otolaryngology, Meizhou People’s Hospital, Meizhou, Guangdong 514000, P.R. China. 7 Department of Cardiology, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, Zhejiang 310014, P.R. China

Conflict of interest

The authors declare that they have no conflict of interest.

Publisher’s note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information accompanies this paper at (https://doi.org/10.1038/s41419-018-1220-7).

Received: 12 October 2018 Revised: 1 November 2018 Accepted: 14 November 2018

Published online: 05 December 2018

References

1. Desantis, C., Ma, J., Byram, L. & Jemal, A. Breast cancer statistics, 2013. CA Cancer J. Clin. 64, 52–62 (2014).
2. Donepudi, M. S., Kondapalli, K., Amos, S. J. & Venkateshan, P. Breast cancer statistics and markers. J. Cancer Res. Ther. 10, 506 (2014).
3. Wiegelt, B., Eberle, C., Cowell, C. F., Ng, C. K. & Reis-Filho, J. S. Metastatic breast carcinoma more than a special type. Nat. Rev. Cancer 14, 147–148 (2014).
4. Denkert, C., Liedtke, C., Tutt, A. & van, M. G. Molecular alterations in triple-negative breast cancer: the road to new treatment strategies. Lancet 389, 2430 (2016).
5. Bianchini, G., Ballo, J. M., Mayer, I. A., Sanders, M. E. & Gianni, L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. Nat. Rev. Clin. Oncol. 13, 674 (2016).
6. Lehmann, B. D. et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J. Clin. Investig. 121, 2750 (2011).
7. Li, W. et al. The FOXN3-NEAT1-SIN3A complex promotes progression of hormonally responsive breast cancer. J. Clin. Investig. 127, 3421–3440 (2017).
8. Zhou, W. et al. The IncRNA H19 mediates breast cancer cell dedifferentiation by differentially sponging miR-200b/c and let-7b. Sci. Signal. 10, 483 (2017).
9. Lin, A. et al. The LINK-A IncRNA interacts with PtdIns(3,4,5)P3 to hyperactivate AKT and confer resistance to AKT inhibitors. Nat. Cell Biol. 19, 238–251 (2017).
10. Liu, Y., Zhang, M., Liang, L., Li, J. & Chen, Y. X. Over-expression of IncRNA DANCN is associated with advanced tumor progression and poor prognosis in patients with colorectal cancer. Int. J. Clin. Exp. Pathol. 8, 11480 (2015).
11. Shi, H. et al. Long non-coding RNA DANCN promotes cell proliferation, migration, invasion and resistance to apoptosis in esophageal cancer. J. Hepatocell. Dis. 10, 2573 (2013).
12. Yuan, S. X. et al. Long non-coding RNA DANCN increases stemness features of hepatocellular carcinoma via de-regression of CTNNB1. Hepatology 63, 499–511 (2016).
13. Jiang, N. et al. IncRNA DANCN promotes tumor progression and cancer stemness features in osteosarcoma by upregulating AXL via miR-33a-5p inhibition. Cancer Lett. 405, 46–55 (2017).
14. Sha, S., Yuan, D., Liu, Y., Han, B. & Zhong, N. Targeting long non-coding RNA DANCR inhibits triple negative breast cancer progression. Biol. Open 6, 1310–1316 (2017).
15. Kretz, M. et al. Suppression of progenitor differentiation requires the long noncoding RNA ANCR. Genes Dev. 26, 338 (2012).
16. Zhu, L. & Xu, P. C. Downregulated lncRNA-ANCR promotes osteoblast differentiation by targeting EZH2 and regulating Runx2 expression. Biochem. Biophys. Res. Commun. 432, 612–617 (2013).
17. Wang, S. & Jiang, M. The long non-coding RNA-DANCR exerts oncogenic functions in non-small cell lung cancer via miR-758-3p. Biomed. Pharmacother. 103, 94–100 (2018).
18. Wang, Y. et al. Long noncoding RNA DANCR promotes colorectal cancer proliferation and metastasis via miR-577 sponging. Exp. Mol. Med. 50, 57 (2018).
19. Jia, J. et al. Long noncoding RNA DANCR promotes invasion of prostate cancer through epigenetically silencing expression of TIMP2/3. Oncotarget 7, 37868–37881 (2016).
20. Lu, Y. et al. MYC targeted long noncoding RNA DANCR promotes cancer in part by reducing p21 levels. Cancer Res. 78, 64–74 (2018).
21. Hao, Y. P., Qiu, J. H., Zhang, D. B. & Yu, C. G. Long non-coding RNA DANCR, a prognostic indicator, promotes cell growth and tumorigenicity in gastric cancer. Tumour Biol. 39, 1010428317699798 (2017).
22. Tang, J., Zhong, G., Wu, J., Chen, H. & Jia, Y. SDF2 recruits KLF4 to regulate nasopharyngeal carcinoma proliferation via P38/ATK signaling. Oncogenesis 7, 61 (2018).
23. Xu, M. D. et al. A positive feedback loop of lncRNA-PVT1 and FOXM1 facilitates gastric cancer growth and invasion. Clin. Cancer Res. 23, 2071 (2016).
24. Wilusz, J. E., Sunwoo, H. & Spector, D. L. Long noncoding RNAs: functional surprises from the RNA world. Genes Dev. 23, 1494–1504 (2009).
25. Z. L. et al. The degradation of EZH2 mediated by lncRNA ANCR attenuated the invasion and metastasis of breast cancer. Cell Death Differ 24, 59 (2016).
26. Hatem, R. et al. Targeting mTOR pathway inhibits tumor growth in different molecular subtypes of triple-negative breast cancers. Oncotarget 7, 48206–48219 (2016).
27. Liang, J. et al. miR-27a-3p targeting RXRα promotes colorectal cancer progression by activating Wnt/β-catenin pathway. Oncotarget 8, 82991–83008 (2017).
28. Jia, Q., Jiang, W. & Ni, L. Down-regulated non-coding RNA (lncRNA-ANCR) promotes osteogenic differentiation of periodontal ligament stem cells. Arch. Oral. Biol. 60, 234–241 (2015).
29. Peng, X., Yun, D. & Christov, K. Breast cancer progression in MCF10A series of cell lines is associated with alterations in retinoic acid and retinoid X receptors and with differential response to retinoids. Int. J. Oncol. 25, 961–971 (2004).
30. Sun, S. Y. & Lotan, R. Retinoids and their receptors in cancer development and chemoprevention. Crit. Rev. Oncol. Hematol. 41, 41–55 (2002).
31. Matsushima-Nishiwaki, R. et al. Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma. Cancer Res. 61, 7675 (2001).
32. Macoritto, M. et al. Phosphorylation of the human retinoid X receptor alpha at serine 260 impairs coactivator(s) recruitment and induces hormone resistance to multiple ligands. J. Biol. Chem. 283, 4943–4956 (2008).
33. Qiu, Z., Li, Y., Zeng, B., Guan, X. & Li, H. Downregulated CDKN1C/p57kip2 drives tumorigenesis and associates with poor overall survival in breast cancer. Biochem. Biophys. Res. Commun. 497, 187 (2018).
34. Zhang, R. et al. RXRα provokes tumor suppression through p53/p21/p16 and P38/ATK signaling pathways during stem cell differentiation and in cancer cells. Cell Death Dis. 9, 532 (2018).
35. Chen, L. et al. Sulindac-derived RXRα modulators inhibit cancer cell growth by binding to a novel site. Chem. Biol. 21, 596–607 (2014).