TNRC9 Downregulates BRCA1 Expression and Promotes Breast Cancer Aggressiveness

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Abstract

Although the linkage between germline mutations of BRCA1 and hereditary breast/ovarian cancers is well established, recent evidence suggests that altered expression of wild-type BRCA1 might contribute to the sporadic forms of breast cancer. The breast cancer gene trinucleotide-repeat-containing 9 (TNRC9; TOX3) has been associated with disease susceptibility but its function is undetermined. Here, we report that TNRC9 is often amplified and overexpressed in breast cancer, particularly in advanced breast cancer. Gene amplification was associated with reduced disease-free and metastasis-free survival rates. Ectopic expression of TNRC9 increased breast cancer cell proliferation, migration, and survival after exposure to apoptotic stimuli. These phenotypes were associated with tumor progression in a mouse model of breast cancer. Gene expression profiling, protein analysis, and in silico assays of large datasets of breast and ovarian cancer samples suggested that TNRC9 and BRCA1 expression were inversely correlated. Notably, we found that TNRC9 bound to both the BRCA1 promoter and the cAMP-responsive element-binding protein (CREB) complex, a regulator of BRCA1 transcription. In support of this connection, expression of TNRC9 downregulated expression of BRCA1 by altering the methylation status of its promoter. Our studies unveil a function for TNRC9 in breast cancer that highlights a new paradigm in BRCA1 regulation. Cancer Res; 73(9); 2840–9. ©2013 AACR.

Introduction

Breast malignancies are a leading cause of female cancer-related deaths, with an estimated worldwide mortality of more than 458,000 deaths in 2008 (1). Progress in reducing the societal impact of this disease has been limited because of its biologic and pathologic diversity resulting in differences in prognosis and responsiveness to therapy (2). The heterogeneous nature of breast cancer has been attributed to the variability in expression of genes that control cell growth and malignant potential (3, 4). Although the majority of breast cancers are sporadic, 5% to 10% of cases are caused by inherited mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 (5–7).

A genome-wide association study (GWAS) revealed 5 low penetrance common susceptibility alleles that are associated with an increased risk for breast cancer (8). Although the association of one of these genes, trinucleotide repeat-containing 9 (TNRC9/TOX3), has been further validated by similar GWAS studies in populations of diverse ethnicity, the mechanism by which it confers breast cancer risk is currently unclear (8–11). This prompted us to investigate what role TNRC9 plays in breast cancer. TNRC9 is a nuclear protein containing a nuclear localization signal and a high mobility group-box domain that can modify chromatin structure. TNRC9 regulates Ca²⁺-dependent transcription in neurons through interaction with cAMP-responsive element-binding protein (CREB; ref. 12). It was also reported that exogenous delivery of TNRC9 induced a complex with CREB that protected neuronal cells from cell death through activation of BCL-2 (13).

Recent evidence suggests that downregulation of the expression of wild-type BRCA1 or alterations in BRCA1-related pathway(s) might contribute to nonhereditary forms of breast cancer (14, 15). However, the mechanism underlying BRCA1 downregulation is not well understood. It was also reported that transcriptional elements that include CREB might influence the constitutive expression of BRCA1 (16). These data suggest that transcriptional regulation of BRCA1 is dependent upon a dynamic equilibrium between transcriptional...
coactivators and corepressors that govern histone acetylation and DNA accessibility at the BRCA1 promoter (7). Yet putative coactivators/corepressors modulating the binding of CREB to the proximal promoter site of BRCA1 remain unknown.

The potential role of TNRC9 in chromatin structure and function along with the data suggesting that the transcriptional regulation of BRCA1 might be dependent upon epigenetic mechanisms (7) led us to hypothesize that constitutive expression of TNRC9 could be relevant to breast cancer biology through the modulation of BRCA1 activity. An effective strategy to identify potential drivers of oncogenesis is the comparison of germline with somatic variation in gene copy number (17, 18); therefore, we started our investigation by assessing genomic areas that could differentiate advanced versus localized breast cancers with a particular focus on TNRC9. Because we observed a high prevalence of the TNRC9 gene amplification in the germline and tumor DNA in the patients with advanced breast cancer, we extended our work with transcriptional and functional analyses to identify a potential mechanism by which TNRC9 may affect breast cancer risk and progression.

Materials and Methods

Study population and specimens

Under protocols approved by the Weill Cornell Medical College (WCMC)-Qatar Institutional Review Boards (Doha, Qatar) and the Hamad Medical Corporation Ethics committee (Doha, Qatar), and the Tunisian National Ethical Committee, all subjects signed informed consent documents for participation in the study. Human blood genomic DNA samples of 315 patients with sporadic breast cancer and 228 healthy controls and 32 human breast cancer tumor samples were obtained from WCMC-Qatar Genetic Medicine Database and the Arab Breast Cancer Consortium Bio-Repository.

Controls and patients, included in the present study, were selected from the same Arab population (Tunisians) and including only unrelated subjects. All patients included in this study had primary breast cancer, with unilateral breast tumors, and with no family history of the disease. The diagnosis of cancer was confirmed by histopathologic analyses. The patients had a mean age of 47.8 ± 10.7 years. The median follow-up was 65 months (range, 1–276 months). At the time of analysis, 94 patients relapsed (local or distant recurrence) and 61 patients experienced metastasis. Among them, 56 (17.8%) patients died from breast cancer.

Controls were healthy women having a mean age of 53.2 ± 13.6 years. They were blood donors with no evidence of any personal or family history of cancer (or other chronic illness). Samples from healthy controls were age matched to the cases.

Antibodies, reagents, and constructs

Anti–TNRC9, anti–Ki-67, anti–BRCA1, anti–DNMT1, and anti–GAPDH, and the secondary horseradish peroxidase-conjugated antibodies were purchased from Abcam. Anti–TNRC9 (only used in Supplementary Fig. 3), anti–hemagglutinin (HA), and anti–myc were products of Sigma-Aldrich. Other detailed information is listed in Supplementary Materials and Methods. The full-length TNRC9, CREB, and mutant TNRC9 were cloned into pCDEF3 vector (Invitrogen). The short hairpin RNAs (shRNA) targeting TNRC9 were constructed using pSuper RNAi system (Oligogene). The BRCA1 promoter was cloned into PGL4 basic luciferase reporter plasmid (Promega).

Cell culture and transfection

MDA-MB-231, ZR-75-1, MCF7, and HEK293 cells were obtained from American Type Culture Collection. Cells were maintained in Dulbecco’s Modified Eagle’s Medium/F12 supplemented with 10% FBS (Thermo Scientific Hyclone). Fugene HD (Promega) was used for the transfection of MDA-MB-231 and ZR-75-1, and Lipofectamine 2000 (Invitrogen) was used for the transfection of MCF7 and HEK293.

Nucleic acid preparation

Genomic DNA from blood samples and cells were extracted with QIAamp DNA Blood Maxi Kit (Qiagen), or GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Genomic DNA and RNA from slices of archived formalin-fixed paraffin block were extracted with RecoverAll Total Nucleic Acid Isolation Kit (Ambion).

Copy number amplification and DNA methylation analyses

All the quantitative real-time PCR (qRT-PCR) were conducted on 7500 real-time PCR system (Applied Biosystems) using GoTaq qPCR Master Mix (Promega). The genomics region of FUT2 and PTPN4 were used as references to assess the copy number variation of TNRC9 genomic region. When \( \min (C_{\text{FUT2}}, C_{\text{PTPN4}}) - \max (C_{\text{TNRC9 exon1}}, C_{\text{TNRC9 exon2}}) > 1 \), it was concluded that the sample had a copy number amplification (CNA) of TNRC9 gene. Bisulfite conversion of 2 μg of DNA was conducted in a thermocycler using the Qiagen EpiTect Bisulfite Kit according to manufacturer’s instructions. The bisulfite-converted DNA was analyzed by quantitative PCR (qPCR) using primers for methylated BRCA1 promoter region. The bisulfite-converted DNA was analyzed by qPCR using primers for methylated and unmethylated BRCA1 promoter region. The description of the methylation qPCR and the bisulfite sequencing analyses are provided in the Supplementary Materials and Methods.

Protein level evaluation of archived sample and xenograft mouse model

Immunostaining procedure of the formalin-fixed and paraffin-embedded sections with TNRC9 and BRCA1 antibody is described in the Supplementary Materials and Methods. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Brown University (Providence, RI). NU/NU nude mice are purchased from Charles River Laboratories. Detailed procedures are provided in Supplementary Materials and Methods.

MTT Assay

The quantity of viable cells was evaluated by MTT assay. The cells were incubated with growth medium with 1 mg/mL MTT for 3 hours at 37°C. The medium was then replaced with 100 μL dimethyl sulfoxide. The plate was covered with tinfoil and
agitated on orbital shaker for 15 minutes. Absorbance was recorded at 570 nm with a filter reference at 620 nm using EnVision Multilabel Plate Reader (PerkinElmer).

**Boyden chamber migration assay**

The quantitative cell migration (QCM) 24-well colorimetric cell migration assay (Chemicon) was used for Boyden chamber migration assay according to the manufacturer’s protocol. Briefly, cells (2 x 10^5) in 500 µL serum-free medium were brought into the top chamber and then inserted into the bottom chamber with 500 µL of medium with FBS. Cells were allowed to migrate for 24 hours at 37°C before fixing and staining. Non-migrated cells were removed by cotton swab. Migrated cells were stained and counted under microscope and photographed. Three independent experiments were carried out under the same condition. Two fields (x 10 magnification) of each chamber were imaged, and stained cells in 6 representative fields were counted manually.

**Colony formation assay**

Colony formation ability was assessed using a soft agar assay (19). In brief, 5,000 cells in 1.5 mL 0.35% agarose containing growth media were overlaid with 1.5 mL 0.5% agarose containing growth media, and the cells were incubated for 4 weeks. The whole-well images were photographed by digital camera and 3 fields (x 10 magnification) of each well were imaged, and colonies in 9 representative fields were counted manually.

**Gene expression profiling**

RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent). The amplification, labeling, and fragmentation of RNA and hybridization with GeneChip Human Genome U133 Plus 2.0 Array were conducted using GeneChip 3’ IVT Express Kit according to the manufacturer’s protocol (Affymetrix). The microarrays were scanned and images were assessed for quality by Affymetrix scanner. Cel file data from each microarray was exported, normalized using Robust Multi-array, and quality by Affymetrix scanner. Cel microarrays were scanned and images were assessed for quality by Affymetrix scanner. Cel file data from each microarray was exported, normalized using Robust Multi-array, and quality by Affymetrix scanner. Cel file data from each microarray was exported, normalized using Robust Multi-array, and quality by Affymetrix scanner. Cel file data from each microarray was exported, normalized using Robust Multi-array, and quality by Affymetrix scanner.

**Chromatin immunoprecipitation assay**

Cells were grown in 10 cm dishes and treated with or without 10 nmol/L E2 or 500 ng/mL trichostatin A (TSA) for 24 hours before harvesting. Chromatin immunoprecipitation (ChIP) assay was conducted using ChIP Kit (Abcam). The ChIP products were analyzed by qRT-PCR. Primer sequences are listed in Supplementary Materials and Methods.

**Luciferase reporter assay**

MCF7 cells were seeded in 24-well plates at a concentration of 10^5 cells per well and were transfected with pGL4-promBRCA1 (100 ng) and different amount of HA-TNRC9 as indicated in the following day. When necessary, empty pCDEF3 plasmid was supplemented to ensure that the total amount of DNA was kept constant. pRL-TK Renilla plasmid (10 ng) was cotransfected to normalize transfection efficiency. Twenty-four hours after transfection, cells were lysed, and luciferase activity was analyzed using the Dual Luciferase Reporter Assay System (Promega) on EnVision Multilabel Plate Reader.

**Statistical analyses**

We used SPSS 13 software to conduct the statistical analysis of our data. Two-tailed Student t tests were used for simple significance testing, and Pearson χ² tests were used for correlation analysis. Two-sided P < 0.05 was considered to be significant. Unless otherwise stated, the error bars represent SEM of the biologic triplicates.

**Results**

**TNRC9 gene is amplified and associated with poor prognosis in advanced breast cancer**

As shown in Table 1, qRT-PCR of TNRC9 gene in blood genomic DNA of 315 patients with sporadic breast cancer and

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NOTE: The odds ratios (OR) and their 95% confidence interval (CI) were calculated using the unconditional logistic regression analysis. The P value was estimated on the basis of the χ² test.
aTumor stage.
228 healthy controls revealed that TNRC9 amplification is more frequent in patients compared with controls (OR = 2.053; P = 0.0001). TNRC9 gene amplification was found to be particularly frequent in patients with advanced breast cancer (T3–4 stages) compared with those with local breast cancer (T1–2 stages; OR = 5.050; P = 0.00007). We further investigated the relationship between the presence of TNRC9 gene CNA and disease progression. No significant difference was seen in the overall survival rates between patients with TNRC9 gene amplification (CNA+) and without (CNA−). However, patients with advanced breast cancer carrying an amplified TNRC9 gene had reduced relapse-free and metastasis-free survival rates (Fig. 1A and B). It has been shown that the germline gene amplification is very often associated with significant modification of gene dosage in cancer cells (22). In agreement with this observation, TNRC9 mRNA was consistently found overexpressed in tumors with high levels of TNRC9 gene amplification (8 CNA+ vs. 16 CNA−; Fig. 1C). Immunohistochemical staining of breast cancer specimens with anti-TNRC9 antibody confirmed that TNRC9 expression was significantly elevated in high-grade compared with low-grade breast cancer (Fisher Exact test, P = 0.033). High levels of TNRC9 expression were found in more than 63.2% (12 of 19) of high-grade breast tumors, whereas only 23.1% (3 of 13) of low-grade tumors were faintly positive for TNRC9 (Fig. 1D).

Taken together, the correlation between TNRC9 gene amplification and protein expression with advanced stages of breast cancer and poor prognosis suggests that TNRC9 expression could serve as marker for breast cancer aggressiveness.

TNRC9 promotes cancer cell proliferation, migration, and survival both in vitro and in vivo

To elucidate the role played by TNRC9 in cancer biology, we transfected MDA-MB-231, an endogenous TNRC9 low-expressing breast cancer cell line, with HA-tagged TNRC9 expression plasmid. The MTT cell proliferation assay showed that ectopic expression of TNRC9 significantly promoted growth of MDA-MB-231 cells (Supplementary Fig. S1B; P = 0.01). In addition, a wound-healing assay showed that ectopic expression of TNRC9 promoted the migration of MDA-MB-231 cells (Supplementary Fig. S1C).

To further confirm the role played by TNRC9 in growth and migration of breast cancer cells, we repressed TNRC9 expression using 2 short hairpin (sh) TNRC9 constructs, sh1 and sh2 in MCF7 and ZR-75-1 breast cancer cells that constitutively express high levels. Western blot analysis confirmed that both of the shRNA constructs interfered the expression of TNRC9. The silencing effect was more effective with sh2 (Supplementary Fig. S2). Therefore, we used sh2 for the subsequent experiments. The gene silencing of TNRC9 significantly inhibited cell growth in both MCF7 and ZR-75-1 cells (Fig. 2A; P = 0.014 and P = 0.022, respectively). The wound-healing assay indicated that the depletion of TNRC9 impaired the cell migration ability (Fig. 2B). A Boyden chamber cell migration

Figure 1. TNRC9 amplification is associated with advanced breast cancer and poor prognosis. A and B, Kaplan–Meier estimates of disease-free (A) and metastasis-free (B) survival curves of patients with breast cancer with advanced breast cancer stratified by CNA+ and CNA−. Survival curves were plotted according to Kaplan and Meier (35). The differences between groups were calculated by the log-rank test (36). *" represents each censored sample. C, TNRC9 mRNA expression in 25 breast cancer tumor specimens grouped by TNRC9 CNA+ and CNA−. Relative TNRC9 expression was calculated by 2−ΔΔCt. Data are shown as median and 5 to 95 percentiles (whiskers). *, P = 0.03. D, immunohistochemical staining of TNRC9 in high- and low-grade breast cancer tumors (magnification, ×40).
assay further confirmed that TNRC9 played a role in promoting the cell migration (Fig. 2C; $P = 0.004$). To evaluate the ability of TNRC9 to induce morphologic transformation of cancer cells, we examined its effect on anchorage-independent colony formation. As shown in Fig. 2D, control cells generated more colonies in soft agar than TNRC9-silenced cells by day 28 after inoculation ($P = 0.014$ for MCF7 and $P = 0.022$ for ZR-75-1). Furthermore, control cells tended to form larger colonies than TNRC9-silenced cells (Fig. 2E).

To examine the role of TNRC9 in tumor growth in vivo, we tested TNRC9 shRNA-targeted ZR-75-1 cells and control ZR-75-1 cells behavior in nude mice. Mice injected with TNRC9-silenced cells did not develop tumors before day 10, whereas those injected with control cells developed tumors by day 4 (Fig. 3A). The average tumor size was consistently smaller in mice injected with the TNRC9-silenced cells compared with controls during the time frame of the experiment (Fig. 3A; $P = 0.007$). All mice were sacrificed on day 28 after injection. Mice injected with control cells had formed major tumor burden, whereas only 3 mice injected with TNRC9-silenced cells had developed barely palpable tumors (Fig. 3B). The proliferation status of control and TNRC9-silenced cells was confirmed by immunostaining with anti-Ki-67 antibody (Fig. 3C). Moreover, the tumors from the control group appeared denser than the TNRC9-silenced group (Supplementary Fig. S3).

**Abrogation of TNRC9 sensitizes cancer cells to apoptosis signal**

To explore the role of TNRC9 on cell survival to apoptotic stimuli, we assessed the response of TNRC9 knockdown MCF7...
and ZR-75-1 cells and corresponding control cells to UV light exposure, serum deprivation, and cisplatin treatment. In all conditions, MTT assay displayed that the TNRC9-abrogated cancer cells had a lower survival rate (Supplementary Fig. S4A–S4C). This result suggests that TNRC9 protects breast cancer cells from cell death due to apoptotic stimuli.

**TNRC9 and BRCA1 expression are inversely correlated**

To identify putative TNRC9 targets involved in breast cancer biology, we analyzed the transcriptome of MCF7 breast cancer cells transfected with control vector and TNRC9 shRNA vector. Gene expression profiling of TNRC9-abogated breast cancer cells (K1-K3: Supplementary Fig. S6A) displayed patterns different from those of control cells (C1-C3: Supplementary Fig. S6A). Compared with control, 103 transcripts were underexpressed and 124 overexpressed in TNRC9-depleted cells (P<0.05; Supplementary Table S1 and Supplementary Fig. S6B). Figure 4A represents profiles of a subset of genes, differentially expressed between TNRC9-abrogated and MCF7 control cells, which are involved in cell apoptosis, motility; response to stimuli, and DNA repair including BRCA1 gene (Supplementary Fig. S6C). As shown in Supplementary Fig. S6, upregulation of BRCA1 in TNRC9-abrogated cells was confirmed by Western blot analysis. Quantification of TNRC9 and BRCA1 mRNAs in breast cancer specimens along with the immunohistochemical analyses indicated that TNRC9 and BRCA1 expressions are often inversely correlated (Fig. 4B).

To confirm the inverse correlation between TNRC9 and BRCA1 expression, we analyzed NCBI GEO datasets from 266 patients with breast cancer via in silico assays for the expression of TNRC9 and BRCA1. A self-organizing heatmap displaying the TNRC9 and BRCA1 genes showed that they have opposite expression in 127 tumors of the 266 breast cancer specimens (47.7%; Fig. 4C). The matrix in Fig. 4C, III suggests an overall negative correlation between TNRC9 and BRCA1 (n = 127, Pearson P = -0.4) suggesting that a negative regulation may occur between the 2 genes in a significant number of breast cancer tumors. Similar observations were also found in 105 of 200 patients with ovarian cancer, which is also dependent, at least in hereditary cases, upon alteration of the BRCA1 and BRCA2 pathways (Supplementary Fig. S7).

**TNRC9 binds to CREB and BRCA1 promoter and downregulates BRCA1 promoter activity**

The enhanced frequency of TNRC9 gene amplification in advanced breast cancers, its effects on breast cancer cell behavior, and the inverse correlation between TNRC9 and BRCA1 expression stimulated our search for targets of TNRC9. Because it is known that the CREB complex binds to the promoters of BRCA1 and BRCA2 and regulate their expression (23, 24), and because it has been recently shown that exogenous
TNRC9 transfection into mouse cells induces its binding to the CREB complex (15), we examined the interaction between endogenous TNRC9 and the CREB complex in human cells. First, we transfected human HEK293 cells, on which TNRC9 is endogenously expressed, with the CREB gene (Myc-CREB; Myc fragment is used as a tag). Then, we used anti-TNRC9 antibodies to immunoprecipitate cell lysates and anti-Myc antibody to reveal immunocomplexes. As shown in Fig. 5A, a specific band corresponding to the molecular weight of CREB protein was revealed, indicating the interaction between the endogenous TNRC9 and CREB in human cells.

We next investigated whether TNRC9 binds to the promoter of BRCA1 using ChIP assays. qPCR analyses with the ChIP products showed that TNRC9 binds to the promoter of BRCA1, but not BRCA2 (Fig. 5B). The inverse correlation between TNRC9 and BRCA1 expression suggested a potential suppression of BRCA1 expression by TNRC9. To assess the effect of TNRC9 on BRCA1 promoter, we made 2 plasmid constructs: a chimeric construct encompassing a 602 bp promoter region of BRCA1 (−148−145) and the luciferase reporter gene; a mutated TNRC9 construct without DNA-binding domain (HA-TNRC9mut). Then, we cotransfected the MCF7 cells with different plasmid construct combinations. As indicated in Fig. 5C and Supplementary Fig. S8, luciferase assay showed that overexpression of TNRC9 substantially inhibited the activity of BRCA1 promoter in a dose-dependent manner, but mutated TNRC9 did not ($P > 0.05$).

It is well known that both estrogen 17β-estradiol (E2) and histone deacetylase (HDAC) inhibitor TSA treatment can induce the expression of BRCA1 (25, 26). Thus, estrogen treatment and HDAC inhibition are inferred to abolish the binding ability of TNRC9 to the BRCA1 promoter. To test our assumption, we assessed the effects of E2 and TSA treatment on the TNRC9–BRCA1 interaction in ZR-75-1 cells. ChIP-qPCR analyses showed that estrogen and TSA treatment inhibited the binding of TNRC9 to BRCA1 promoter (Fig. 5D).

**TNRC9 induces BRCA1 promoter methylation**

It has been suggested that methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA level in...
We compared the methylation status of the promoter of \textit{BRCA1} in TNRC9 knockdown cells and in ZR-75-1 control cells. As shown in Fig. 5E, the proportion of unmethylated promoter is higher in TNRC9-abrogated cells \((P = 0.01)\), indicating that TNRC9 induces the methylation of \textit{BRCA1} promoter, which results in decreased levels of \textit{BRCA1}. The bisulfite sequencing analysis of a 639-bp region of \textit{BRCA1} promoter indicates that...
the methylation of 6 CpGs of this region could be regulated by TNRC9 (Supplementary Fig. S9). To confirm the contribution of TNRC9 to BRCA1 promoter methylation in vivo, we measured the percentage of methylated BRCA1 promoter in 23 formalin-fixed paraffin-embedded sections of breast cancer tissues. The results indicated that methylation of BRCA1 promoter was positively associated with increased TNRC9 expression (Fig. 5F; \( r^2 = 0.63 \) and \( P < 0.0001 \)). Because it has been shown that the DNA methyltransferase-1 (DNMT1) binds to BRCA1 promoter inducing its methylation (30) and large-scale 2-hybrid assay revealed that TNRC9 interacted with DNMT1 in mice (31), we examined the role of TNRC9 in DNMT1 activity. The gene silencing of TNRC9 in ZR-75-1 breast cancer cells decreased significantly DNMT1 binding to BRCA1 promoter (Fig. 5G). A similar effect was observed when ZR-75-1 control cells were treated with estrogen or TSA (Fig. 5H). Analysis of immunoprecipitated ZR-75-1 cell lysates with anti-TNRC9 or anti-DNMT1 antibodies did not reveal TNRC9–DNMT1 complexes (data not shown).

Discussion

Genetic variants in TNRC9 gene are highly associated with breast cancer. We have now shown the clinical implications of TNRC9 in breast cancer and uncovered a molecular basis for a TNRC9 role in tumor progression and invasion. Initially, we showed that the TNRC9 gene is amplified and overexpressed in breast cancer, particularly in advanced stages. Our previous investigations suggest that Arab women may experience an earlier age of onset and a preponderance of aggressive breast cancer phenotype (32) and highlighted the importance of BRCA1 gene in the etiology of breast cancer in Arab population (33). Our current findings, showing a significant association between TNRC9 gene amplification and reduction in disease-free and metastasis-free survival rates, suggest that TNRC9 could be involved in the onset of aggressive forms of breast cancer, found more frequently in Arab populations. A key component of the cancer paradigm is tumor progression through cancer cell migration, invasion, and metastasis. Although extensively investigated, the biochemical and molecular events underpinning and controlling these processes in most cancers have remained poorly defined. In the present study, we identified biologic functions of TNRC9 that had not been previously deciphered. Our results show that increased expression of TNRC9 is sufficient to promote the proliferation, aggressive behavior, and survival of breast cancer cells both in vitro and in vivo conditions, whereas opposite effects were observed when TNRC9 is knocked down. Interestingly, analysis of the literature revealed that TNRC9 has been implicated in the development of breast cancer metastases to the bone (34). Therefore, it is reasonable to conceive that TNRC9 confers risk for enhanced survival, and increased aggressiveness of cancer cells and, consequently, enhanced expression of TNRC9 may represent a marker for invasive potential. The effects of TNRC9 on breast cancer cell behavior along with our striking finding, showing the inverse correlation of TNRC9 and BRCA1 expression in several breast cancer cell lines and in a large number of breast and ovarian tumor specimens, prompted us to search for targets of TNRC9. First, we showed the binding of TNRC9–CREB complex, a BRCA1 and BRCA2 transcriptional regulator, in human cells. We next showed that TNRC9 binds specifically to BRCA1 promoter and downregulates BRCA1 expression by altering the methylation status of its promoter. BRCA1 expression was found to be reduced in a significant proportion of human breast tumors (8, 9), up to one-third of which can be explained by promoter hypermethylation (27–29). Understanding the underlying mechanisms of BRCA1 gene methylation is critical for generating effective strategies for reestablishing BRCA1 expression and thus restoring its tumor suppressor function.

Taken together, our results suggest that TNRC9-mediated suppression of BRCA1 expression might result in the disruption of normal DNA damage response resulting in the accumulation of DNA damage. This will likely cause genomic instability and finally lead to tumorigenesis. However, the current studies do not rule out the involvement of other essential mechanisms by which TNRC9 influences breast cancer risk and progression. High-throughput molecular and functional analyses will be in use to identify genes and pathways involved in TNRC9 regulation.

In conclusion, this study provides novel evidence that TNRC9 is a risk factor for breast cancer development and progression that exerts its role through pleotropic effects; TNRC9 not only plays a role in tumorigenesis, but also may increase the cell survival of cancer cells, thus qualifying as a potential prognostic biomarker and as a target for therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The contents are solely the responsibility of the authors and do not necessarily represent the official views of the Qatar National Research Fund.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Shan, S. Douszua, S. Bakhru, E. Al-Azawi, M.L. Asciento, J. Malek, E. Mathiowitz, E. Wang, F.M. Marincola, L. Chouchane
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Kizhakayil, I. Agha, L. Chouchane
Study supervision: I. Al-Bozom, S. Gehani, E. Mathiowitz, L. Chouchane

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References


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