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Annexin A2 mediated posttranscriptional destabilization of BRCA1 mRNA in sporadic breast cancer

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ABSTRACT

BRCA1 is a nuclear phosphoprotein involved in genome integrity by regulating cell cycle checkpoints, DNA repair and apoptosis. BRCA1 down regulation occurs in sporadic breast cancer (BC). Posttranscriptional regulation of gene expression has evolved as a means of fine-tuning of protein levels. There are several posttranscriptional regulatory motifs, including CU-rich, U-rich and AU-rich elements, which are usually located in the 5' and 3' UTR's. Post-transcriptional regulation of BRCA1 is poorly characterized and underappreciated. To elucidate the molecular mechanism of AnxA2 mediated posttranscriptional regulation of BRCA1 mRNA in sporadic breast cancer. In order to investigate BRCA1 mRNA levels in MDAMB-231 & MCF-7 cells, we conducted DRB chase experiment. The BRCA1 mRNA destabilization is significantly increased upon AnxA2 induction in MCF-7 cells. Further we knocked down AnxA2 in MDAMB-231 cells we found, BRCA1 mRNA stabilization which proves AnxA2 destabilizes BRCA1 mRNA. In RIP-CHIP experiment we found BRCA1 immunoprecipitated with AnxA2. This supports our finding that AnxA2 has role in the regulation of BRCA1 at mRNA level. Luciferase reporter assay showed decrease in luciferase activity with BRCA1 3'UTR. On treating with AnxA2 binding oligo from BRCA1 3'UTR showed decrease in cell viability.

KEY WORDS: ANXA2, BRCA1, RNA BINDING PROTEIN, RENILLA LUCIFERASE

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INTRODUCTION

Breast Cancer (BC) is one of the leading causes of death among women and has 10% life time risk of developing malignancies in many countries in the western world (Newman 1988). Hereditary BC Syndrome accounts for 5-10% of all BC cases, while the other 90-95% of BC is "Sporadic. According to Population Based Cancer Registry (PBCR); In India, Breast cancer accounts for 25 - 35% of all female cancers in metro cities like; Mumbai, Delhi, Bengaluru, Kolkata, Chennai, Ahmadabad, Bhopal, etc. and it is second common cancer in rural areas. This implies, practically, one fourth of all female cancer cases are breast cancers, (Adnan 2018). Several studies have been reported the evidences, suggesting involvement of BRCA1 in the etiology of sporadic breast and ovarian cancer through reduced expression. Decreased levels of BRCA1 mRNA are frequently observed in breast tumors, (George 1998, Adnan, 2018, Nicolas 2018, Harahap 2018, Huszno 2019).

Lower or undetectable levels of expression of the BRCA1 protein have been observed in sporadic BC (McCoy 2003). Different mechanisms have been shown to be responsible for the reduced expression of BRCA1; epigenetic silencing of the BRCA1 gene at the transcriptional level by means of promoter methylation is another mechanism involved (Das 2004, Choic 2009, Robertson 2002, Baylin 2006). The BRCA1 gene was identified and cloned in 1994. BRCA1 known for its multiple vital functions such as tumor suppressor activity, including roles in cell cycle progression, DNA damage repair processes DNA damage responsive cell cycle check point regulation of a set of specific transcriptional pathways and apoptosis (Rice 1998). Since, BRCA1 gene is rarely mutated in sporadic breast cancer. It has been suggested that, dysregulation of BRCA1 expression causes reduced mRNA or protein levels in BC (Thompson 2011).

Post-transcriptional regulation of gene expression has evolved as a mean of fine tuning protein levels and generating rapid temporal or spatial changes in protein expression following an environmental stimulus (Saunus 2008). Regulation of the dynamics of mRNA molecule is a wide spread phenomenon and there are many such examples of genes regulated in this way including tumor suppressor genes (Rosen 2003, Maity 1997). The mechanisms often involve RNA-binding proteins and non-coding RNAs (transacting factors) which recognizes sequence motif in their target transcripts and 'tag' them for recognition by macromolecular complexes involved in RNA metabolism such as mRNA transport granules, RNA induced silencing complexes (RISC), the exosome, GW bodies and stress granules(Thanin 2018, Cok 2001, Fu 1997, anderson 2006). There are many examples of post-transcriptional regulatory motifs, including CU-rich, U-rich and AU-rich elements which are usually located regions of regulated transcripts (Moore 2005, Kedeersha 2002, Engels 2006). It can be difficult to predict the regulatory functions of individual motif based on primary sequence alone, as they often associate with multiple transacting factors with different and sometimes opposing activities.

BRCA1 is post-transcriptionally regulated by mRNA binding proteins, the identity of which is unknown. HuR is an RNA binding protein already proven to play role in the event of post-transcriptional regulation of BRCA1 by Jodi M Saunus et al. HuR is ubiquitously expressed RNA binding protein that regulates the stability and translation of transcripts that function in multiple cellular pathways, such as p21WAFI, COX, TP24, Cyclin A and B and P27. As like HuR other mRNA binding proteins like Annexin A2 (AnxA2) expression increases in basal breast cancer where BRCA1 expression is very less or null. AnxA2 is a multifunctional calcium dependent phospholipid binding protein. AnxA2 shows functional diversity like it regulates membrane traffic and cytoskeleton organization, extracellular activities and targeted gene disruption (Lopez 2005). AnxA2 is highly expressed in the surface of human tumor cells and promotes cell migration and invasion by activating plasminogen and cleaving extracellular matrix[25]. AnxA2 stimulates cell proliferation, angiogenesis and invasion (Wein 2003, Wiklund 2002, Maji 2016, Volker 2002). This study demonstrates the hypothesis of AnxA2 mediated post-transcriptional regulation of tumor suppressor BRCA1 gene contributes to the development and progression of sporadic breast cancer.

MATERIALS & METHODS

Cell Culture

Human breast cancer cell lines, MDA-MB-231 and MCF-7 were obtained from National Center for cell Sciences (NCCS), Pune and grown in respective media as prescribed by the supplier.

Real Time RT-PCR

Total RNA was isolated using TRIZOL (Invitrogen). cDNA was synthesized using RevertAid First strand cDNA synthesis kit by Thermo Fischer scientific with following primers; For BRCA1 (F: 5'-GGTTCTGATGACTCACATGATGGG-3'&R: 5'-TCTGTGGCTCAGTAACAAATGCTC-3'),AnxA2(5'TAACTTTGATGCTGAGCGGG-3'&R:5'-TAATTTCCTGCAGCTCCTGG-3') and for GAPDH (5'-GAGCGAGATCCCTCCAAA-3' & R: 5'-ACTGTGGTCATGAGTCCTTC-3'. BRCA1, AnxA2 & GAPDH were amplified by DyNAmo Color Flash SYBR Green q-PCR kit (Thermo Fischer scientific) on Cepheid Smart cycler. PCR set up for

BRCA1 was 2 minutes for 95^0 C for one cycle, followed by 30 second 94^{0}C, 55^{0}C & 72^{0}C for 35 cycles. PCR reaction set up for AnxA2 & GAPDH used was 2 minutes at 95^{0}C and 40 cycles of 30 s at 95^{0}C, 30 s at 54^{0}C, and 30 s at 68^{0}C. The comparative ΔCt method was used to determine relative BRCA1, AnxA2 mRNA expression.

RNA stability:

RNA stability was analyzed by transcription-chase experiment. The cells were stimulated to over express BRCA1 & AnxA2 by selected agonists respectively and then treated with DRB to inhibit ongoing transcription, after which total RNA was isolated at selected time points and cDNA was synthesized and amplified as described earlier.

RNA Immunoprecipitation: Formaldehyde was added to cosslink protein-RNAs in MDAMB-231 Cells grown in specific medium, at RT after 15 minutes stopped by addition of 2M glycine and incubated for 5 minutes. Cells were collected in 500µl of RIPA lysis buffer containing RNase inhibitor. The lysate was centrifuged at 12000g. Annexin A2 specific primary antibody added and incubated at 4^{o}C overnight on shaker. 50 µl of Protein A/G beads added to the lysate and incubated at 4^{o}C for 4 hours. The lysate was washed twice with RIPA lysis buffer. The crosslinking reversed by 6µl of 5M NaCl and 20 µg of proteinase k added to the lysate and incubated 1 hour at 42^{o}C and 1 hour at 65^{o}C. RNA was extracted by following TRIZOL RNA extraction protocol. RNA was reverse transcribed and amplified for BRCA1 by specific primers as described earlier. PCR products were resolved on ethidium bromide–stained 1.5% agarose gel.

Luciferase reporter assays

Luciferase reporter used was BRCA1 3'UTR luciferase reporter construct, made by ligating the full-length BRCA1 3'UTR downstream of the luciferase of pLuc. MDAMB-231 cells in 12 well plates were transiently transfected with equimolar amounts of luciferase reporter constructs. After 24 h of luciferase expression, Firefly luciferase reporter activity was determined relative to Renilla using a Dual Luciferase Reporter Assay kit (Promega) and Promega Luminometer.

MTT assay:

The MTT (3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay conducted to check cell proliferation by transfecting the MDAMB-231 cells by 50 oligo nucleotide specific binding to BRCA1 3'UTR analyzed by NCBI BLAST software and read by Epoch analyzer.

RESULTS & DISCUSSION

A detailed understanding of the mechanisms regulating BRCA1 expression is very much essential to identify the regulatory abnormalities in breast cancer (Diaz 2004, Blasi 1999). Reduction in BRCA1 expression in sporadic breast cancer is well documented (Tarui 2002). In our earlier data, we have demonstrated that the expressions of BRCA1 and AnxA2 protein are reciprocally regulated, which indicate the possibility of AnxA2 involvement in BRCA1 posttranscriptional regulation. The posttranscriptional regulation of BRCA1 in the sporadic breast cancer is underappreciated and poorly studied. This study suggests BRCA1 mRNA stability is regulated by AnxA2 in sporadic breast cancer (Wein 2003, Wiklund 2002, Huszno 2019).

BRCA1 & AnxA2 mRNA expression in different cells

Levels of BRCA1 & AnxA2 mRNA were analyzed in BT-549, MCF-7 & MDAMB-231 cells. We found abundant AnxA2 mRNA in MDAMB-231 & BT-549 cells than MCF-7. On the other hand MCF-7 cells showed abundant BRCA1 mRNA as compared to BT-549 & MDAMB-231 (Figure 1).

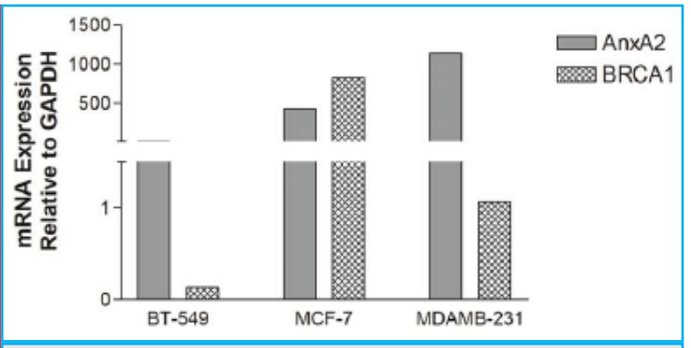

FIGURE 1. BRCA1 & AnxA2 mRNA expression in BT-549, MCF-7 & MDAMB-231 cells

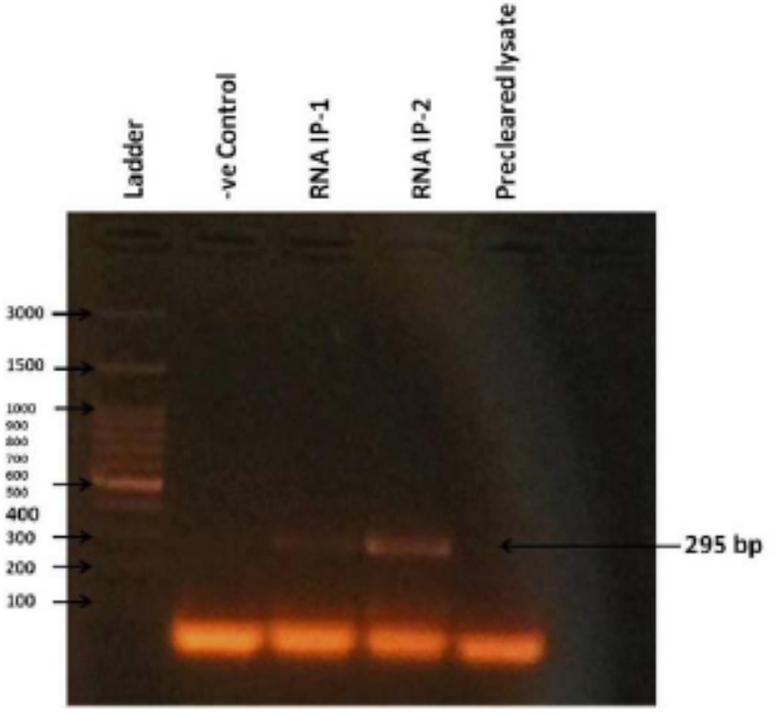

FIGURE 2. RNA Immunoprecipitation assay - anti AnxA2 antibody and BRCA1 specific primers. AnxA2 coimmunoprecipitates with BRCA1 mRNA in MDAMB-231 breast cancer cells. BRCA1 PCR products were undetectable without anti AnxA2 antibody. Lane -1 without AnxA2 antibody, Lane-2 & 3 with AnxA2 antibody and Lane - 4 Pre-cleared cell lysate.

Endogenous AnxA2 and BRCA1 mRNA are binding partners

To determine the association between endogenous AnxA2 and BRCA1 mRNA transcripts, we performed an IP-RT-PCR assay using an anti AnxA2 antibody and BRCA1 specific primers. We are able to show that AnxA2 coimmunoprecipitates with BRCA1 mRNA in MDAMB-231 breast cancer cells (figure 2). Conversely, BRCA1 PCR products were undetectable without anti AnxA2 antibody. The results provide clear evidence that AnxA2 associates with BRCA1 mRNA TNBC cells.

AnxA2 Regulates BRCA1 Post-transcriptionally

To address AnxA2 associated with BRCA1 mRNA, we investigated the effect of AnxA2 over expression on the regulation of BRCA1 mRNA stability. MCF-7 cells were induced to over express AnxA2 and studied the effect on BRCA1 mRNA stability. We observed decreased BRCA1 mRNA stability with time dependent manner, which shows AnxA2 overexpression degrades BRCA1 mRNA. To validate, AnxA2 associated in the regulation of BRCA1 mRNA. We knocked down AnxA2 by specific siRNA in MDAMB-231 cells and analyzed for BRCA1 mRNA stability. We found increase in BRCA1 mRNA stability in time dependent manner (figure 3). This shows AnxA2 is involved in regulation of BRCA1 mRNA. To study whether post-transcriptional regulation of BRCA1 occurs in sporadic breast cancer, we investigated BRCA1 expression and mRNA stability by DRB chase experiment. BRCA1 mRNA was normalized to control mRNA. We observed decrease in BRCA1 mRNA stability when we over expressed AnxA2 in TNBC cells. Interestingly, when we knocked down AnxA2 by AnxA2 specific siRNA we found increase in BRCA1 mRNA stability (figure 3). This data suggests that AnxA2 may be involved in the post transcriptional processes as well as in the destabilization of BRCA1 mRNA (Tali 2017, Harahap 2018, Zhang 2018).

To investigate, AnxA2 is involved in the regulation of BRCA1 mRNA through the binding of BRCA1 3'UTR region. We designed pLuc vector having multiple cloning site downstream to a luciferase reporter gene in the coding region. The cloning of BRCA1 3'UTR construct

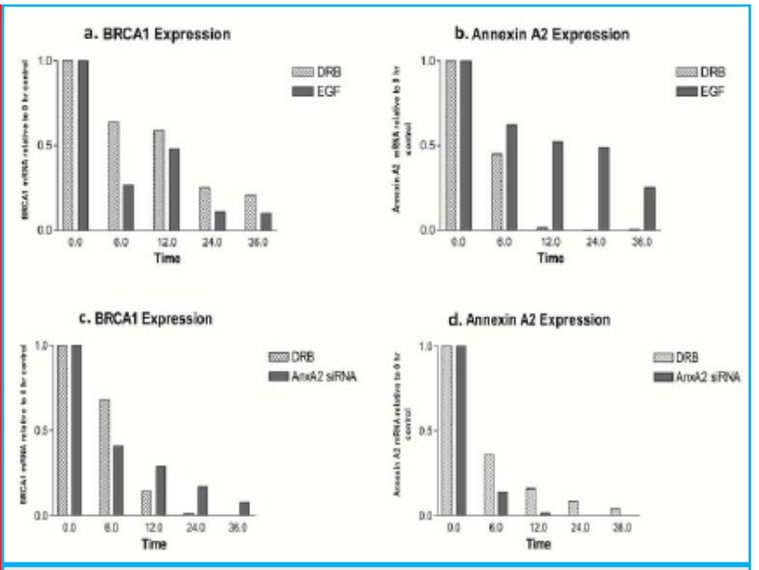

FIGURE 3. DRB Chase experiment –a) BRCA1 mRNA stability decreased on EGF induced MCF-7 cells. b) AnxA2 mRNA stability increased on EGF induction in MCF-7 cells. c) BRCA1 mRNA stability increased on AnxA2 knock down. d) AnxA2 knock down caused decrease in AnxA2 mRNA stability.

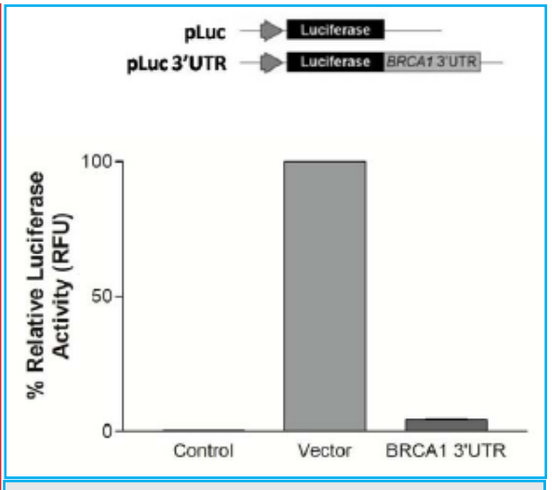

FIGURE 4. Luciferase Reporter Assay - BRCA1 3'UTR construct allows the luciferase reporter expression that mimic putative AnxA2 target in the 3' UTR of BRCA1 gene. As a control, firefly luciferas construct having targeted sequence. In each well, the transfection mixture included a Renilla luciferase reporter vector, allowing readout for normalizing the transfection efficiency at 24 hours after transfection in MDAMB-231 cells. The lysate was collected and analyzed firefly luciferase activity, which was normalized to Renilla luciferase activity.

allows the luciferase reporter expression that mimic putative AnxA2 target in the 3' UTR of BRCA1 gene. As a control, firefly luciferase construct having targeted sequence was tested. In each well, the transfection mixture included a Renilla luciferase reporter vector, allowing readout for normalizing the transfection efficiency at 24 hours after transfection in MDAMB-231 cells. The lysate was collected and analyzed firefly luciferase activity, which was normalized to Renilla luciferase activity. We found reduced luciferase expression containing BRCA1 3' UTR as compared to the control and pLuc vector control (figure 4). We identified that AnxA2 interacts with BRCA1 3'UTR, leading to decreased luciferase activity (Maji 2016).

Effect of AnxA2 putative binding oligo on cell viability

To study the similarity or association of AnxA2 protein sequence with BRCA1 3'UTR, with the help of Vienna RNA web service for secondary structure prediction and BLAST of NCBI software we selected 50 nucleotide oligo having affinity of AnxA2 to bind BRCA1 3'UTR. We transfected MDAMB-231 cells with 50 oligo nucleotides and analyzed cell viability by MTT experiment. We found decrease in cell viability of oligo treated cells than without treatment (Figure 5). Finally, putative 50 oligo nucleotide transfected MDAMB-231 cells showed increased apoptosis upon performing cell viability assay. This suggests that, putative oligo nucleotide spares BRCA1 3'UTR from AnxA2 to accelerate tumor suppres-

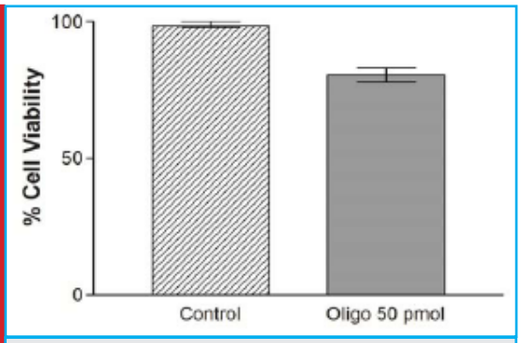

FIGURE 5. MDAMB-231 cells with 50 oligo nucleotides putative binding sequence and analyzed the cell viability by MTT.

sor activity in turn promoting the cells to undergo apoptosis (Shetty 2012).

In summary, we demonstrate that AnxA2 is involved in BRCA1 gene expression at the posttranscriptional level as well as destabilization on BRCA1 in sporadic breast cancer.

CONCLUSION

In the present study, we demonstrate that AnxA2 has a role in posttranscriptional regulation of BRCA1 expression.

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