Decreased Expression of BRCA2 Accelerates Sporadic Breast Cancer Progression

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Abstract Human tumor suppressor BRCA2 has been known to function in the repair of DNA double strand break. Germ line mutation in BRCA genes predisposes individuals to familial breast and ovarian cancer. The aim of present study was to characterize the implication of BRCA2 expression in cases of non-hereditary (sporadic) breast cancer. Female breast cancer patients aged between 20 and 75 years who underwent surgery were randomly selected. Patients with Stage IV disease at time of primary diagnosis or previous history of any other malignancy other than breast carcinoma or undergoing neoadjuvant chemotherapy were excluded from the study. Total 48 patients full filled these criteria. Patients were treated with either modified radical mastectomy or breast conservation therapy. Concentration of BRCA2 was measured by Sandwiched method of ELISA. Immunohistochemistry was used to determine the hormonal receptor status. Stage of the disease was not found to have any significant correlation on the BRCA2 expression. In patients with higher grade of tumors the level of BRCA2 expression was found to be low. Decreased expression of BRCA2 was found to be triple negative, had aggressive features and associated with higher chances of axillary metastasis. Genetic instability caused by decreased expression of BRCA2 could trigger mutations in

sporadic breast cancer cases and mutations in turn leads to uncontrolled proliferation and invasive growth.

Keywords BRCA2 decreased expression · Double Strand Break (DSB) · Homology-directed Recombination (HR) · Triple Negative Breast Cancer (TNBC)

Abbreviations

DNA	De-oxy ribonucleic acid
BRCA	Breast cancer susceptibility protein
ATM/ATR	Ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related protein
TP53	tumor protein 53
CHK2	checkpoint kinase-2
Rad51	Rad51 recombinase
ER	Estrogen receptor
PR	Progesterone receptor
HER2	Human epidermal growth factor receptor protein 2
USG	Ultrasonography
MRI	Magnetic resonance imaging
ELISA	Enzyme linked immunosorbent assay

Introduction

The treatment responses and outcome of breast cancer patients with the same stage of disease can be markedly different. The lymph node status and histological grade fail to classify breast tumors accurately according to their clinical behavior [1–3].

The expression of specific genes contributes to the pathogenesis and aggressiveness of breast cancers. Some breast cancers have a high degree of genomic instability. DNA (de-oxy ribonucleic acid) double-strand break (DSB) repair occurs following error-free homology-directed recombination using

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a network of proteins that are also implicated in breast cancer syndromes, including ATM/ATR (Ataxia telangiectasia mutated/ataxia telangiectasia and Rad3-related protein), TP53 (tumor protein 53), CHK2 (checkpoint kinase-2), NBS1, BRCA1 (breast cancer susceptibility type 1), and BRCA2 (breast cancer susceptibility type 2) [4]. This suggests that defects in DNA double-strand break repair are associated with a genetic predisposition to breast cancer.

BRCA1 and BRCA2 co-localize with Rad51 (Rad51 recombinase) to form complexes [5, 6]. Rad51 proteins are required for recombination during mitosis and meiosis, as well as for Homologous recombination (HR) repair of DSBs [7]. Rad51 coats single-stranded DNA to form a nucleoprotein filament that invades and pairs with a homologous region in duplex DNA, and then activates strand exchange to generate a crossover between the juxtaposed DNA [8, 9].

Venkitaraman A R et al. showed that BRCA1 does not directly regulate Rad51, since interactions between BRCA1 and Rad51 are indirect and stoichiometrically negligible [10]. BRCA1 and BRCA2 have different roles in the repair of DSBs by HR.

BRCA2 has a more direct role in repair of DSBs by HR. BRCA2-deficient cells exhibit increased sensitivity to ionizing radiation, indicative of a defect in DSB repair, whereas the cell cycle checkpoint and apoptotic RING domain check point kinase 2 (CHK2) responses to DNA damage remain intact. In addition, BRCA2-deficient cells accumulate chromosomal breaks and aberrant mitotic exchanges during culture. Rad51-deficient cells show similar phenotypes, providing genetic evidence that interactions of BRCA2 with Rad51 are fundamental for the maintenance of cell division and chromosome structure [11].

One inherited defective copy of the gene is sufficient for cancer predisposition, but the loss of the wild-type allele is required for tumorigenesis [12]. More than 1,000 mutations have been identified in BRCA1&2, and most of them result in the truncation of these proteins.

We have characterized expression of the familial breast and ovarian cancer gene, *BRCA2*, in cases of non-hereditary (sporadic) breast cancer. BRCA2 levels were found to be markedly decreased in the patients with breast cancer and more so in invasive cancer. This study suggests that BRCA2 may normally serve as a negative regulator of mammary epithelial cell growth whose function is compromised in breast cancer either by direct mutation or alterations in gene expression.

Materials and Methods

Selection of Patients Female breast cancer patients who underwent surgery from January, 2014 – April, 2015 at the Department of General Surgery were considered for inclusion

in this study. Patients were randomly selected between age limits 20–75 years. Patients with Stage IV disease at time of primary diagnosis or previous history of any other malignancy other than Breast carcinoma or undergoing neoadjuvant chemotherapy were excluded from the study. Total 48 patients full filled these criteria.

Ethical Permission Before commencement of project, human ethical committee clearance was obtained for carrying out the project work. Written informed consent form in three languages (English, Hindi & Bengali) was obtained from each of the patients prior to their recruitment in the study.

Treatment The first line treatment was surgery with curative intent. Patients were treated with either modified radical mastectomy (MRM) or breast conservation therapy (BCS). After completion of surgery, radiotherapy and appropriate adjuvant chemotherapy or hormone therapy was administered as per international guidelines.

Collection of Samples and Clinical Data

Blood samples were collected in clotting vials from all patients and serum was separated by centrifuging the samples at 1000–1200 r.p.m for 5–7 min. Post surgery tissue blocks (both mastectomy and axillary dissection specimen) and clinico pathological characteristics of tumor (tumor size, grade and lymph node metastasis) were collected.

Assay Procedure

A] Enzyme linked immunosorbent assay (ELISA) Concentration of BRCA2 was measured by Sandwiched method of ELISA. Assay was performed according to MyBioSource Kit (Cat No. MBS262713; Lot No. 36253669). The kit detects normal human BRCA2 protein level which ranges from 0.5 to 2.1 ng/ml of serum sample. Briefly serum samples and known concentration of BRCA2 standards were added to the corresponding wells. ELISA plate was sealed and incubated at 37 °C for 90 min. Plate was washed three times. Biotinylated human BRCA2 antibody was added to each well. Plate was incubated again at same temperature for 60 min. After washing the wells, enzyme conjugate was added and incubated for 30 min. Color reagent was added and the mixture was incubated in the dark. After development of color gradient for known standard, the reaction was stopped by adding stop solution. Optical density was read by ELISA plate reader (Robonik Readwell Touch) at 450 nm within 15 min.

B] ImmunoHistochemistry 2–7 µm section of tissues were sliced by microtome and layered on poly-L-lysine coated glass slides. Slides were put in the incubator at 65 °C for

30–40 min, dipped in xyline-I and then xyline II. Slides were dipped in different concentration of alcohol for dehydration. All slides were then immersed in hot citrate buffer for antigen retrieval, cooled and washed with Tris buffer. Slides were allowed for peroxide blocking for 5–8 min and then for protein blocking for 8–10 min. Slides were wiped clearly. Primary antibody for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor protein 2 (HER2/neu) were applied for 45 min and washed. With post primary block, slides were incubated for 30 min. Novolink polymer was added for 30 min. All slides were washed with Tris Buffer. DAB (diaminobenzidine) chromogen was applied for 3–5 min. After rinsing properly, haematoxyline was applied for 5 min. Dehydration was done again as mentioned before. Finally all the slides were mounted before examining. [Novolink Min Polymer Detection System-Leica, Ref RE7290-K, LOT 6030030. Anti-ER: Rabbit monoclonal, Anti-PR: Rabbit monoclonal antibody, Anti-Her2/Neu: Mouse monoclonal antibody; Cell Marque, USA. All Antibodies are ready to use form (diluted in TBS, pH: 7.3–7.7 with carrier protein and preservatives.)].

Follow Up Follow-up was performed by history taking, physical examination and haematological (liver function tests, complete blood count) and radiological tests (chest radiography, high resolution abdominal and breast ultrasonography (USG), mammography and magnetic resonance imaging (MRI). Investigations were done meticulously for detection of local or distant recurrence.

Statistics The collected data was statistically analyzed and the significance levels were ascertained. Statistical Analysis was performed with SPSS. Chi-square test was used to compare the variables. Significance level was set at $p < 0.05$.

Results and Analysis

The mean age (mean $\pm$ s.d.) of all patients was 48.15 ± 11.60 years with range 29.00–71.00 years and the median age was 49.5 years.

BRCA2 levels of different clinico-pathological groups was shown in the Table 1. Expression of BRCA2 at < 0.5 ng/ml was considered as decreased expression according to kit reference. In higher age group (≥ 40 years), 10 (76.9 %) patients had decreased BRCA2 (< 0.2 ng/ml) level. Decreased level (< 0.2 ng/ml) of BRCA2 was found more in postmenopausal (53.8 %) patients than premenopausal patients (46.2 %), ($p = 0.6282$). Although the association of age and menopausal status with BRCA expression was not statistically significant, the

findings suggest that BRCA2 mutation increases with age and hence the overall expression of BRCA2 is less.

According to clinical stage, 6 (7.7 %) patients who were in stage II, 10 (76.9 %) patients who were in stage III and 2 (15.4 %) patients in stage IV had low BRCA2 level which was statistically significant ($p = 0.0112$). In BRCA2 level between 0.2 and 0.49 ng/ml, 2 (7.1 %) patients were in stage I, 3 (10.7 %) patients were in stage II, 19 (67.9 %) patients were in stage III and 4 (14.3 %) patients were in stage IV. In BRCA2 level 0.5–2.1 ng/ml, 2 (28.6 %) patients were in stage I, 3 (42.9 %) patients were in stage II, 1 (14.3 %) patients were in stage III and 1 (14.3 %) patients were in stage IV. Hence stage of the disease did not have any impact on the BRCA2 expression.

Nine (69.2 %) patients had large (≥ 5 cm) tumors and 4 (30.8 %) patients with middle sized (2–4.99 cm) tumors had decreased level of BRCA2 and this association was not statistically significant ($p = 0.1226$). Hence tumor size did not relate with BRCA2 expression.

The histological grades were measured by Modified Bloom-Richardson Grading Scheme. In grade II out of 10 patients 2 (15.4 %) patients and in grade III out of 26 patients 11 (84.6 %) patients had decreased level of BRCA2 and this was statistically significant ($p = 0.0030$). In BRCA2 level between 0.2 and 0.49 ng/ml, 5 (17.9 %) patients were in grade II and 23 (82.1 %) patients were in grade III. Whereas in patients with normal BRCA2 levels (0.2–0.49 ng/ml) 2 (28.6 %) patients were in grade I, 3 (42.9 %) patients were in grade II and 2 (28.6 %) patients were in grade III. Hence, in patients with higher grade of tumors the level of BRCA2 expression was found to be low. Figure 1 was representing that the mean level of BRCA2 for grade-III tumor was low compared to that of grade-I and II tumor.

Nine (69.2 %) patients with metastasis in 4–9 lymph nodes and 4 (30.8 %) patients with more than 9 lymph nodes metastasis were found with decreased level of BRCA2 and this was statistically significant ($p = 0.0055$). Patients with BRCA2 level between 0.2 and 0.49 ng/ml, 16 (57.1 %) patients had metastasis in 4–9 lymph nodes and 16 (57.1 %) patients had > 9 lymph nodes metastasis. Patients with normal level of BRCA2 expression had no lymph node metastasis in 3 (42.9 %) patients, 1–3 lymph node metastasis in 2 (28.6 %) patients and 4–9 lymph node metastases in 2 (28.6 %) patients. These findings suggested that decreased expression of BRCA2 was associated with higher chances of axillary metastasis and hence upstaging the disease. Figure 2 had shown that BRCA2 level was found to be normal range in nodal metastasis 0 and it decreased most in case of lymph node metastasis more than 9.

According to histological type, 12 (92.3 %) patients with infiltrating ductal carcinoma (IDC) and 1 (7.7 %) patients in

Table 1 Clinicopathological details according to BRCA2 level

		<0.2 ng/ml n (%)	0.2–0.49 ng/ml n (%)	0.5–2.1 ng/ml n (%)	p- value
Age	<40	3(23.1)	7(25.0)	2(28.6)	0.9640
	≥40	10(76.9)	21(75.0)	5(71.4)	
Menopausal Status	Post	7(53.8)	19(67.9)	5(71.4)	0.6282
	Pre	6(46.2)	9(32.1)	2(28.6)	
Clinical Stage	I	0(0.0)	2(7.1)	2(28.6)	0.0112*
	II	6(7.7)	3(10.7)	3(42.9)	
	III	10(76.9)	19(67.9)	1(14.3)	
	IV	2(15.4)	4(14.3)	1(14.3)	
Tumor Size (cm)	<2	0(0.0)	1(3.6)	0(0.0)	0.1226
	2–4.99	4(30.8)	10(35.7)	6(85.7)	
	≥5	9(69.2)	17(60.7)	1(14.3)	
Lymph Node Metastasis	0	0(0.0)	1(3.6)	3(42.9)	0.0055*
	1–3	0(0.0)	5(17.9)	2(28.6)	
	4–9	9(69.2)	16(57.1)	2(28.6)	
	>9	4(30.8)	6(21.4)	0(0.0)	
Grade	I	0(0.0)	0(0.0)	2(28.6)	0.0030*
	II	2(15.4)	5(17.9)	3(42.9)	
	III	11(84.6)	23(82.1)	2(28.6)	
Histological Type	DCIS	1(7.7)	0(0.0)	1(14.3)	0.3977
	IDC	12(92.3)	27(96.4)	6(85.7)	
	LC	0(0.0)	1(3.6)	0(0.0)	
Subtypes	Luminal A	2(15.4)	4(14.3)	3(42.9)	0.0010*
	Luminal B	0(0.0)	0(0.0)	2(28.6)	
	HER-2/neu positive	0(0.0)	8(28.6)	0(0.0)	
	Triple Negative	11(84.6)	16(57.1)	2(28.6)	

*Statistically significant, *DCIS* Ductal carcinoma insitu; *IDC* Invasive Ductal Carcinoma; *LC* Lobular Carcinoma

ductal carcinoma insitu (DCIS) had low level of BRCA2 and this association was not statistically significant ($p=0.3977$). So, histological type did not show any correlation with BRCA2 expression.

Decreased level of BRCA2 was more common in triple negative tumors (11; 84.6 %) than luminal-A tumors (2; 15.4 %) which was statistically significant ($p=0.0010$). 4 (14.3 %) tumors were luminal A, 8 (28.6 %) tumors were HER-2/neu positive and 16 (57.1 %) tumors were triple negative with BRCA2 level 0.2–0.49 ng/ml. 3 (42.9 %) tumors were luminal A, 2 (28.6 %) tumors were luminal

B and 2 (28.6 %) tumors were triple negative with BRCA2 level 0.50–2.1 ng/ml. So to state, the patients with aggressive features had decreased expression of BRCA2 and were found to be triple negative. Figure 3 had shown BRCA2 levels among four molecular subgroups. All subgroups had decreased BRCA2 level. Compared to other subgroups, triple negative type of tumors exhibited most decreased level.

Discussion

We characterized expression of BRCA2 in cases of non-hereditary (sporadic) breast cancer and found that BRCA2 serve as a negative regulator. Its function is compromised in breast cancer either by direct mutation or alterations in gene expression. Hence in cases where BRCA2 is under expressed, it may be considered that it is mutated. With our limited resources, using ELISA and IHC we could demonstrate that patients with very aggressive breast carcinoma (triple negative hormonal receptor status, large number of axillary node metastasis, and higher grade of tumor) had decreased expression of BRCA2.

Fig. 1 Mean level of BRCA2 with respect to different grades; the value was low in grade-III tumor compare to grade-I and grade-II tumor

Fig. 2 Mean level of BRCA2 according to increasing lymph node metastasis; the level was decreased most in tumors with >9 lymph node metastasis compared to others tumor

Marilyn E et al. analyzed the effect of antisense inhibition of BRCA on the proliferative rate of mammary epithelial cells. Experimental inhibition of BRCA expression with antisense oligonucleotides produced accelerated growth of normal and malignant mammary cells, but had no effect on non-mammary epithelial cells [13]. In our study also we found that patients with breast cancer had decreased levels of BRCA2 expression and had more aggressive tumors, pointing towards alteration in gene expression or mutation.

The roles played by BRCA1 and BRCA2 in the repair of DSBs by HR differ. Evidence suggested a more direct role of BRCA2 [14]. In addition, BRCA2-deficient cells accumulate chromosomal breaks and aberrant mitotic exchanges during culture [15]. In present study also, we found that in patients with higher grade of tumors the level of BRCA2 expression was low. Decreased expression of BRCA2 was also found to be associated with higher chances of axillary metastasis and hence upstaging the disease.

Scully R, et al. showed that mutant BRCA1-associated tumors display aggressive features, including early age of onset, high tumor grade, ER and PR negativity, and a high proliferation rate [16, 17]. Typical mutant BRCA2-associated tumors exhibit ER/PR pleomorphism and molecular features that are similar to those found in sporadic breast cancers. The BRCA1

Fig. 3 Mean BRCA2 levels of four molecular subtypes. Decreased level was observed in triple negative subtype compared to other subtypes

phenotype is defined by markers of basal-like or triple negative breast cancer based on gene expression profiles.

Conversely, in our study, we found similar level of aggressive features in patients having decreased expression of BRCA2. Decreased level of BRCA2 was more common in triple negative tumors (84.6 %) than luminal-A tumors (15.4 %) which was statistically significant. A number of other studies showed that the triple negative subtype has the worst overall and disease-free survival compared to the other subtypes [18, 19].

This study also revealed that decreased expression of BRCA2 was associated with higher histological grades and metastatic deposits in larger number of axillary lymph nodes. Decreased expression of BRCA2 was also associated with increasing incidence of infiltrating duct carcinoma on post operative histopathological examination.

Further studies suggested that BRCA2 mediates G2/M-phase control by interacting with a novel protein, BRCA2-associated factor 35 (BRAF35), which binds to branched DNA structures. Nuclear staining revealed a close association of BRAF35/BRCA2 complex with condensed chromatin, coincident with histone H3 phosphorylation. Antibody microinjection experiments suggested a role of BRCA2/BRAF35 complex in modulation of metaphase progression [20].

Since BRCA2 has a major role in DNA repair, its suppression is thought to induce unrepaired DNA lesions, which cause cell cycle arrest by activating checkpoint signaling, including mitotic progression [25]. Dysfunction of p53 as a result of mutation leads to uncontrolled cell cycle checkpoints, inducing uncontrolled proliferation and invasive growth [21].

In this study, our limitation was that we could not clearly demonstrate the exact nature of mutations in BRCA2 in patients in whom BRCA2 was under expressed. Gene profiling would have clearly demonstrated the mutations in the BRCA2 in patients where we found decreased expression of BRCA2. We were limited also to find out why a BRCA-related predisposition to cancer is apparently site-specific, affecting the breast and ovary only.

Conclusion

Genetic instability caused by loss of BRCA2 due to decreased expression could trigger mutations in sporadic breast cancer cases. Mutation leads to uncontrolled proliferation and invasive growth. There is an indirect role of BRCA2 in cell cycle regulation. More work will be required to determine whether BRCA2 regulates cell cycle control, as distinct from its role in DNA repair.

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Conflict of Interest All authors have declared no conflict of interest.

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