Seminar/Conference attended
Poster presentation:

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**Alterations of FANCC and BRCA2 in primary breast cancer of Indian patients**, 81st Annual Meeting of the Society of Biological Chemists (India) and Symposium on Chemistry and Biology: Two Weapons Against Diseases, November 8th -11th, 2012, Kolkata, India.


**Frequent alterations of homologous recombination repair pathway genes in breast carcinoma of Indian patients**, 34th Annual Convention of Indian Association for Cancer Research, February 19th-21st, 2015, Jaipur, India.

Platform presentation:


**Molecular analysis of Homologous Recombination Repair pathway genes in primary breast cancer of Indian patients**, One day Seminar on ‘Biological Chemistry’ of Society of Biological Chemists (Kolkata Chapter), April 26, 2014.
FANCC (Fanconi anaemia complementation group C)

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Identity

Other names: FAC, FACC, FA3
HGNC (Hugo): FANCC
Location: 9q22.32
Local order: Next to PTCH and XPAC !!

DNA/RNA

Description
14 exons; spans 80 kb.

Transcription
mRNA of 2.3, 3.2, and 4.6 kb (alternative splicing in 5', variable 3' untranslated region, exon 13 skipping).

Protein

Description
558 amino acids; 63 kDa.

Expression
Wide, in particular in the bones; high expression in proliferating cells, low in differentiated cells.

Localisation
Cytoplasmic (mostly) and nuclear.

Function
- FANCA and FANCG form a complex in the cytoplasm, through a N-term FANCA (involving the nuclear localization signal) - FANCG interaction; FANCC join the complex; phosphorylation of FANCA would induce its translocation into the nucleus. This FA complex translocates into the nucleus, where FANCE and FANCF are present; FANCE and FANCF join the complex. The FA complex subsequently interacts with FANCD2 by monoubiquitination of FANCD2 during S phase or following DNA damage. Activated (ubiquinated ) FANCD2, downstream in the FA pathway, will then interact with other proteins involved in DNA repair, possibly BRCA1; after DNA repair, FANCD2 return to the non-ubiquinated form.
- FANCC may have multifunctional roles, in addition to its involvement in the FA pathway. FANCC binds to cdc2 (mitotic cyclin-dependent kinase), STAT1, GRP94 (a chaperon protein), NADPH, and a number of other proteins; involved in DNA repair and in suppressing interferon gamma induced cellular apoptosis.

There are 15 FA genes that make up the FA pathway. Among these FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCM and FANCL form the core complex.

During G1 phase of cell cycle these proteins are localized in the cytoplasm. During S phase or during DNA damage FANCA and FANCG at first form a complex in the cytoplasm followed by its interaction with FANCC.

Then the complex translocates to the nucleus. In the nucleus other FA proteins like FANCE, F, B, M and L interact with the complex. They cooperatively bind to
form the core complex. FANCL has E3 ubiquitin ligase activity.

The core complex then activates FANCD2 and FANCI by monoubiquitination.

The activated FANCD2-FANCI complex then interact with other FA genes like FANCP/SLX4, FANCD1/BRCA2, FANCI/BRIP1 and FANCN/PALB2 for efficient DNA repair.

FANCC helps in accumulation of FANCE and it has role in foci formation of MRE11/RAD50/NBS1 complex in response to intrastrand crosslink inducers.

FANCC binds to cdc2 (mitotic cyclin-dependent kinase), it is necessary for DNA damage-induced G2/M checkpoint in vitro and in vivo.

In response to oxidative DNA damage, FANCC prevents premature senescence in hematopoietic stem cells.

It interacts with cytochrome p-450 reductase and NADPH during increased production of reactive oxygen species (ROS).

In hematopoietic stem cells it regulates apoptosis, self renewal capacity and cell cycle control. It inhibits activity of dsRNA dependent protein kinase mediated death signaling pathway by interacting with Hsp70.

FANCC and p53 cooperatively work in apoptosis. It has role in suppressing interferon gamma induced cellular apoptosis.

In normal oral epithelium, a gradual increase of FANCC protein expression from basal to parabasal layer to spinous layer suggesting its role in cellular proliferation and differentiation.

FANCC is important for proper functioning of monocytes/macrophages. It suppresses TNFα production in mononuclear phagocytes by suppressing TLR8 activity.

FANCC interacts with STAT1, GRP94 (a chaperon protein). It has role in telomere attrition and telomere recombination.

**Homology**

No known homology.

**Mutations**

**Germinal**

Most mutations are found in exon1, intron 4, and exon 14.

**Implicated in**

**Fanconi anaemia (FA)**

**Note**

FACC is implicated in the FA complementation group C; it represents about 15% of FA cases.

**Disease**

Fanconi anaemia is a chromosome instability syndrome/cancer prone disease (at risk of leukaemia).

**Prognosis**

- Fanconi anaemia's prognosis is poor; mean survival is 16 years: patients die of bone marrow failure (infections, haemorrhages), leukaemia, or androgen therapy related liver tumours.
- It has recently been shown that significant phenotypic differences were found between the various complementation groups. FA group C patients had less somatic abnormalities. However, there is a certain clinical heterogeneity.

**Cytogenetics**

Spontaneous,chromatid/chromosome breaks; increased rate of breaks compared to control, when induced by breaking agent.

**Hybrid/Mutated gene**

Mutations in exon 4, 13 leading to deletion of exon 9 were reported in Brazilian Fanconi Anemia patients.

**Oncogenesis**

Fanconi anemia patients are prone to develop head and neck, esophageal, gastrointestinal, vulvar and anal cancers.

Frequent deletion and promoter methylation are observed in FANCC gene in oral cancer, breast cancer, acute leukemia and pancreatic cancer.

**Diabetes and obesity**

**Note**

FANCC prevents diabetes and obesity.

**To be noted**

Apart from its function in DNA damage repair, FANCC plays important role in apoptosis, cell cycle, differentiation and innate immunity.

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Frequent alterations of homologous recombination repair pathway in primary and chemotolerant breast carcinomas: clinical importance

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Aim: To understand the importance of homologous recombination repair pathway in development of breast carcinoma (BC), alterations of some key regulatory genes like BRCA1, BRCA2, FANCC and FANCD2 were analyzed in pretherapeutic/neoadjuvant chemotherapy (NACT)-treated BC samples. Materials & methods: Alterations (deletion/methylation/expression) of the genes were analyzed in 118 pretherapeutic and 41 NACT-treated BC samples. Results: High deletion/methylation (29–68%) and 64–78% overall alterations of the genes were found in the samples. Concordance was evident between alteration and protein expression of the genes. Estrogen/progesterone receptor-negative tumors showed significantly high alterations even in NACT-treated samples having low CD44 and proliferating cell nuclear antigen expression. Pretherapeutic patients with alterations showed poor prognosis. Conclusion: Alterations of homologous recombination repair pathway genes are needed for the development of BC.

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Breast carcinoma (BC) is the most common cancer among women worldwide [1]. BC accounts for 25% of cancer burden in eastern Indian population [2]. Genetic predisposition, exposure to ionization radiation, human papillomavirus infection, hormonal exposures and obesity are some of the etiological factors of BC [3-6]. The BC in younger women (age ≤40 years) is more aggressive and shows lower survival rates compared with the BC in older women [7]. Therapy, clinical response and prognosis of BC vary in different subtypes with estrogen receptor (ER)/progesterone receptor (PR)-negative BC showing worst prognosis [8]. BC with high expression of proliferation marker proliferating cell nuclear antigen (PCNA) and stem cell marker CD44 shows poor response to therapy [9,10]. The recent trend in therapy of BC is the administration of neoadjuvant chemotherapy (NACT) to shrink the locally advanced tumors [11]. This treatment is followed by surgery and adjuvant therapies. The major chemotherapeutic drugs used for BC are doxorubicin/epirubicin with alkylating agents like cyclophosphamide [12,13]. In locally advanced tumors, only 4–31% showed pathological complete response even after NACT [14-16]. CD44 expression is higher in triple-negative BC [17]. But subtype-specific expression of CD44 and PCNA is not clear and the actual mechanism of chemotherapeutic tolerance of BC is not well understood.

Sensitivity of the tumors to the chemotherapeutic drugs is inversely correlated with DNA damage response (DDR) competency [18-21]. The DDR pathways play a potential role in preventing

Keywords

• breast carcinoma • HRR pathway • neoadjuvant chemotherapy

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cancerogenesis. Among all DDR pathways homologous recombination repair (HRR) pathway is important due to presence of several BC susceptible genes [22]. In this pathway, formation of fanconi anemia (FA) core complex is an important phenomenon, which activates FANCD2 and subsequently recruits BRCA1 and BRCA2 to the DNA damage site for error-free repair by homologous recombination. Hence, some of the key regulatory genes in this pathway are BRCA1, BRCA2, FANCC and FANCD2. Alteration in any of these genes can impair the whole pathway. Different studies showed variable frequencies of deletion (20–70%) and promoter methylation (10–60%) in BRCA1/BRCA2 genes in pretherapeutic BC [23,24]. In addition, 40–65% deletion and promoter methylation in FANCC gene as well as 10–20% reduced protein expression of FANCD2 were seen in BC [25,26]. High BRCA1 deletion is reported in ER(-) compared with ER(+) in pretherapeutic BC [27]. But, in NACT-treated BC, the alterations of HRR pathway genes are not well documented. The analysis of alterations of the key regulatory genes of HRR pathway in different subtypes of BC in both pretherapeutic and NACT-treated samples may put some light in understanding the chemotherapeutic tolerance and prognosis of the disease.

Thus, our study has been focused on the following aspects in primary BC and NACT-treated BC of different subtypes:

- Analysis of alterations (deletion/methylation) of BRCA1, BRCA2, FANCC and FANCD2 genes;
- Expression analysis of BRCA1, BRCA2, FANCC and FANCD2 proteins by immunohistochemistry;
- Expression analysis of CD44 and PCNA proteins; and
- Clinicopathological correlation of alterations of these genes with progression of BC.

Our data showed high alterations of the HRR pathway genes in ER/PR(-) than ER/PR(+) samples of both pretherapeutic and NACT-treated BC. The alterations of the genes showed worst prognosis in pretherapeutic BC patients. However, the chemotolerance and better prognosis in NACT-treated BC might be due to low expression of CD44 and PCNA in spite of high alterations of the HRR pathway genes.

Materials & methods

- **Collection of clinical specimens**

  Freshly operated 118 pretherapeutic and 41 NACT-treated primary BC samples, adjacent normal tissues along with 5 ml blood of the corresponding patients were collected from 159 unrelated patients from the hospital section of Chittaranjan National Cancer Institute, Kolkata. NACT-treated samples were operated after 2–6 months of NACT treatment. Informed consent from the patients and approval from the Research Ethics Committee of the Institute were obtained for sample collection. All tumors were staged according to the International Union against Cancer tumor node metastasis classification. Detailed clinicopathological information like stage/grade/nodal status/age of onset/molecular subtypes are listed in **Tables 1 & 2**.

- **Microdissection & DNA extraction**

  The contaminant normal cells in the BC lesions were removed by microdissection from cryosections (5 μm) using surgical knives under a dissecting microscope (Leica MZ16, Germany) [28]. The representative sections from various regions of the samples were stained with hematoxylin and eosin for pathological diagnosis as well as for marking the tumor-rich regions. Microdissected samples containing >60% tumor cells were taken for DNA isolation. High-molecular-weight DNA from the microdissected tumors and their corresponding normal tissue/peripheral blood lymphocyte (PBL) were isolated according to standard protocol. The quality and quantity of DNA were checked by measuring optical density at 260–280 nm [29].

- **Deletion analysis**

  Deletion analyses of BRCA1, BRCA2, FANCC and FANCD2 were done by microsatellite-based deletion mapping [28]. A standard PCR was carried out by using a (γP32)-ATP-labeled forward primer. PCR products were electrophoresed on 7% denaturing polyacrylamide sequencing gel and autoradiographed [28]. The scoring of loss of heterozygosity, hemizygous deletion, microsatellite size alterations of one allele, microsatellite size alterations of two alleles, biallelic alterations like LMA (loss of heterozygosity + microsatellite size alteration), homozygous deletion was done on an autoradiogram [28]. The detailed information about the microsatellite markers is given in **Supplementary Table 1A & B**.
Promoter methylation analysis
Promoter methylation status of BRCA1, BRCA2, FANCC and FANCD2 was analyzed in CpG-rich islands in the promoter region of the respective genes by PCR-based methylation-sensitive restriction analysis [30]. Approximately, 100 ng of DNA samples were individually digested overnight with HpaII enzyme (CCGG) (Promega, WI, USA), its methylation-insensitive isoschizomer MspI (Sibenzyme, Russia) and with another methylation-sensitive enzyme HhaI (GCGC) (Sibenzyme, Russia) separately. The 445 bp fragment of β-3A-adaptin gene (K1) and 229 bp fragment of RARβ2 (K2) were used as digestion and integrity controls, respectively [31]. The primer sequences are listed in Supplementary Table 1C.

Immunohistochemical analysis
The molecular subtyping for all 159 samples were done by analyzing the ER, PR and HER2 expression by immunohistochemistry [32]. Scoring was done as per the recommended American Society of Clinical Oncology guidelines. The protein expressions of BRCA1, BRCA2, FANCC, FANCD2, CD44 and PCNA were also analyzed in 42 pretherapeutic and 17 NACT-treated samples. The primary antibodies used were ERα (C-311, sc-787), PR (H-190, sc-7208), HER2/neu (F-11, sc-7301), BRCA1 (IMG-80317), BRCA2 (H-299, sc-28235), FANCC (C-14, sc-18110), FANCD2 (H-300, sc-28194), PCNA (F-2, sc-25280) and CD44 (DF1485, sc-7291) along with appropriate horseradish peroxidase-conjugated secondary antibodies (sc-2005, sc-2010, sc-2768 and sc-2777) of Santa Cruz Biotechnology (CA, USA). Diaminobenzidine (sc-24982, Santa Cruz Biotechnology) was used for color development, and hematoxylin was used as counterstain. The staining intensity (1 = weak, 2 = moderate, 3 = strong) and the percentage of positive cells (<1 = 0, 1–20 = 1, 20–50 = 2, 50–80 = 3 and >80 = 4) were detected by two independent observers. By combining the two scores, final evaluation of expression was done (0–2 = low, 3–5 = intermediate, 6–7 = high) [33]. The staining patterns of the proteins in tumors were compared with the staining patterns of the ducts of normal samples.

Treatment of 5-Aza-2′-deoxycytidine on BC cell lines
To determine the effect of promoter methylation on expression of HRR genes, MCF-7 and MDA-MB-231 cell lines were grown in presence of 10 and 20 μM 5-Aza-2′-deoxycytidine (5-aza-dC) for 5 days [34]. Total RNA was isolated from the 5-aza-dC treated/untreated MCF-7 and MDA-MB-231 samples using TRIzol reagent according to manufacturer’s protocol (Invitrogen, CA, USA). The real time-PCR quantification of the above genes was performed using SYBR Green PCR assay (Applied Biosystems, CA, USA) with β2-microglobulin gene as control. The primer sequences are listed in Supplementary Table 1D.

Statistical analysis
Fisher’s exact test was used for analysis of contingency table to find the differences among proportions. Survival analysis was done using
Kaplan–Meier method, and log-rank test was used to compare the survival patterns of the different groups. A Cox hazard model was used to estimate the effect on survival after adjusting the confounding factors. Disease-free survival (DFS) was measured from the date of surgery for pretherapeutic patients and date of commencement of NACT for NACT-treated patients to the date of most recent follow-up, recurrence, metastasis or death (up to 5 years). The detailed follow-up records were available for 159 BC patients. All the statistical analyses were performed using statistical programs Epi Info 6.04, SPSS 10.0 (SPSS, IL, USA). A t-test was performed to compare the CD44 and PCNA expressions between pretherapeutic and NACT-treated samples. p < 0.05 was considered to be statistically significant.

### Results

#### Subtype status of the BC samples

In immunohistochemical analysis of the BC samples, ER and PR expressions were seen in the nuclei, and HER2 was mainly present in membranes. In pretherapeutic BC samples, the frequencies of luminal A, luminal B, HER2 enriched and triple-negative subtypes were 20, 26, 32 and 22%, respectively; whereas, in NACT-treated samples, their frequencies were 32, 29, 10 and 29%, respectively (Tables 1 & 2). In pretherapeutic BC samples, luminal A type showed significant association with early tumor stage (p = 0.035) and lymph node-negative status (p = 0.001); whereas, triple-negative BC showed borderline significance with increased grade (p = 0.09).

#### Deletion analysis of the candidate HRR pathway genes

The deletion analysis of BRCA1, BRCA2, FANCC and FANCD2 loci showed frequent deletion of one allele with infrequent alterations of both allele deletion, microsatellite size alterations of one allele, microsatellite size alterations of two alleles and LMA (Figure 1A–F). The deletion patterns of the HRR genes in pretherapeutic BC samples were seen in the following order: BRCA1 (68%; 73/108) > BRCA2 (60%; 58/97) > FANCC (36%; 42/118) > FANCD2 (29%; 34/118) (Figure 1G). Similarly, the deletion frequencies of NACT-treated BC samples were in the following order: BRCA2 (62%; 21/34) > BRCA1 (60%; 24/40) > FANCC (34%; 14/41) > FANCD2 (32%; 13/41) (Figure 1H). No significant differences were found in deletion frequencies based upon the ER/PR status and age of onset in both pretherapeutic and NACT-treated BC samples (Figure 1G & H). Infrequent MA (1–3%) and LMA (1–2%) in BRCA1 and BRCA2 loci and infrequent homozygous deletion in FANCC (2.5%) and FANCD2 (5%) loci were observed in only pretherapeutic samples (Supplementary Table 2).

No significant association was seen among deletion of the HRR pathway genes in pretherapeutic BC and NACT-treated BC samples (data not shown). However, 86% (101/118) of pretherapeutic and 85% (35/41) of NACT-treated samples harbored deletion in at least one of the genes (Supplementary Table 2). This shows deletion of HRR genes plays an important role in development of BC. Similarly, deletion in more than or equal to two genes was found in 61% (72/118)
pretherapeutic and 68% (28/41) NACT-treated BC. Moreover, deletion in all the genes was showed by 10% (12/118) pretherapeutic BC. (Supplementary Table 2).

- **Promoter methylation analysis of HRR pathway genes**
  The differential methylation pattern in promoter regions of the HRR genes was seen in the BC samples (Figure 2A–D). In pretherapeutic BC samples, the methylation frequencies were seen in the following order: FANCD2 (60%; 71/118) > BRCA2 (53%; 63/118) > FANCC (45%; 53/118) > BRCA1 (37%; 44/118) (Figure 2C). Similar pattern of methylation was seen in NACT-treated BC samples – that is, FANCD2 (56%; 23/41) > BRCA2 (54%; 22/41) > FANCC (46%; 19/41) > BRCA1 (29%; 12/41) (Figure 2D). A significant increase in promoter methylation of BRCA2 was seen in both pretherapeutic and NACT-treated samples in ER/PR(-) BC compared with ER/PR(+) BC (p = 0.048, 0.005, respectively) (Figure 2C & D). Also, the same increase was observed in early age of onset BC than the late age of onset BC (p = 0.006).
Figure 2. Methylation analysis of HRR genes. (A & B) Promoter methylation status of HRR genes analyzed by MSRA showing methylation in different samples (#1089, #593, #5822 and #812). (A) Schematic representation of promoter regions of HRR genes revealing distribution of HhaI (GGGC: arrowhead) and HpaII (CCGG: star) restriction sites: (→): location of methylation primers; (white boxes): transcription start site. (B) Methylation status of HRR genes in BC. (C & D) Pattern of promoter methylation of HRR genes in ER/PR(+/−) BC and early/late-onset BC. (C) Pretherapeutic BC (D) NACT-treated BC.

*Significant difference in methylation of the genes between ER/PR (+/−) BC.

**Significant difference in methylation of the genes between early/late-onset BC. p < 0.05 was considered to be statistically significant.
Figure 2. Methylation analysis of h RR genes (cont.).
BC: Breast carcinoma; ER: Estrogen receptor; Hha: HhaI-digested DNA; Hpa: HpaII-digested DNA; HRR: Homologous recombination repair; K1 and K2: Controls for DNA digestion and integrity check, respectively; MSP: MSP1-digested DNA; MSRA: Methylation-sensitive restriction analysis; NACT: Neoadjuvant chemotherapy; PR: Progesterone receptor; U: Undigested DNA.

For NACT-treated samples (Figure 2D). Similarly significant increase in methylation of FANCD2 was seen in the ER/PR(-) NACT-treated samples than the respective ER/PR(+) samples (p = 0.028) (Figure 2D).

No significant association was seen among methylation frequencies of the HRR genes in both pretherapeutic and NACT-treated BC samples (data not shown). About 94% (111/118) of pretherapeutic and 83% (34/41) of NACT-treated samples harbored methylation in at least one of the genes (Supplementary Table 2). Methylation in more than or equal to two genes was found in 70% (82/118) pretherapeutic and 61% (25/41) NACT-treated BC (Supplementary Table 2). In addition, promoter methylation in all the genes was observed in 4% (5/118) of pretherapeutic and 10% (4/41) of NACT-treated BC samples (Supplementary Table 2).

Interestingly, all the HRR pathway genes showed methylation in MCF-7 and MDA-MB-231 cell lines (Supplementary Figure 1A & B). Hence, promoter methylation of HRR genes is one of the inactivation mechanisms in breast tumorigenesis.

- Validation of promoter methylation of HRR genes in BC cell lines

In BC cell lines, gradual increase in expression of HRR pathway genes was seen with increasing concentration of 5-aza-dC. At 20 μM 5-aza-dC treatment in MCF7 cells, increased expression of the genes was in the following order BRCA2 (75-fold) > FANCC (4.7-fold) > FANCD2 (4.2-fold) > BRCA1 (2.8-fold) (Supplementary Figure 1C). In MDA-MB-231 cells, increased expression of the HRR genes was in the following order BRCA2 (3.7-fold) > BRCA1 (2.4-fold) > FANCC (twofold) > FANCD2 (1.8-fold) with 20 μM 5-aza-dC treatment (Supplementary Figure 1D).

- Overall alterations of the HRR pathway genes

Overall alterations (deletion/methylation) of the HRR pathway genes in pretherapeutic BC were in the following order: BRCA1 (78%: 92/118) > BRCA2 (72%: 85/118) > FANCD2 (70%: 83/118) > FANCC (64%: 76/118) (Figure 3A). Almost similar pattern of overall alterations was seen in NACT-treated BC samples – that is, BRCA1 (76%: 31/41) > BRCA2 and FANCD2 (71%: 29/41) > FANCC (68%: 28/41) (Figure 3B). In pretherapeutic BC, significantly higher alterations in ER/PR(-) BC were found in BRCA1 (p = 0.012), BRCA2 (p = 0.044) and FANCD2 (p = 0.044) compared with ER/PR(+) BC (Figure 3A). In NACT-treated BC, significantly high alterations of BRCA1 (p = 0.028), BRCA2 (p = 0.006), FANCC (p = 0.04) and FANCD2 (p = 0.04) were observed in ER/PR(-) BC compared with ER/PR(+) BC (Figure 3B). In addition, the BRCA1 alteration was significantly high (p = 0.01) in early age of onset than the late age of NACT-treated BC (Figure 3B).

In chi-square analysis of pretherapeutic BC samples, significant co-alterations were seen among BRCA1 with BRCA2 and FANCD2 (Supplementary Table 3A) and similar trend was observed in ER/PR(-) BC (Supplementary Table 3B). Significant co-alterations were seen between BRCA1 and FANCD2 in ER/PR(+) BC. However, in early age of onset BC, significant co-alterations were seen between BRCA1 and FANCD2 and between BRCA2 and FANCC. Whereas in late age of onset BC, significant co-alterations between BRCA1 and BRCA2 were observed (Supplementary Table 3C). In NACT-treated BC, significant co-alterations were seen between BRCA1 and BRCA2 and between FANCC and FANCD2 (Supplementary Table 3A). Similar trend of association of BRCA1 and BRCA2 was found in ER/PR(-) BC and in late age of onset BC. The association of FANCC and FANCD2 was seen in ER/PR(+), early age of onset and late age of onset BC (Supplementary Table 3A–C).

Alteration in at least one of the genes was evident in 97% (115/118) of pretherapeutic and 93% (38/41) of NACT-treated samples (Figure 3C & D). However, alteration in more than or equal to two genes was found in 86% (102/118) pretherapeutic and 88% (36/41) NACT-treated BC. The frequencies of alteration in more than or equal to two genes were significantly higher in ER/PR(-) than in ER/PR(+) BC for both pretherapeutic and NACT-treated BC (p = 0.048 and 0.047, respectively) (Figure 3C & D). Moreover, 35% (41/118) pretherapeutic and 41% (17/41) NACT-treated BC
samples showed alteration in all the genes where alteration was significantly higher in ER/PR(-) BC compared with ER/PR(+) BC for both pretherapeutic and NACT-treated BC \( (p = 0.001 \text{ and } 0.02, \text{ respectively}) \) \( (\text{Figure 3C & D}) \). Biallelic alterations (both deletion and methylation) of \textit{BRCA1}, \textit{BRCA2}, \textit{FANCC} and \textit{FANCD2} were 22\% \((26/118)\), 32\% \((38/118)\), 16\% \((19/118)\) and 18\% \((21/118)\) in pretherapeutic BC and 12\% \((5/41)\), 34\% \((14/41)\), 12\% \((5/41)\) and 20\% \((8/41)\) in NACT-treated BC samples, respectively \( (\text{Supplementary Table 3D}) \). Except \textit{BRCA2} \( (p = 0.016) \) in pretherapeutic BC, no significant association between deletion and methylation of HRR genes was found in both pretherapeutic and NACT-treated BC \( (\text{Supplementary Table 3D}) \). Biallelic co-alterations among \textit{BRCA1}, \textit{BRCA2}, \textit{FANCC} and \textit{FANCD2} were 3–11\% in pretherapeutic and 2–12\% in NACT-treated BC samples.

- **Immunohistochemical analysis in BC samples**

  HRR proteins expression

  Protein expression of HRR pathway genes was analyzed to study its concordance with molecular
alteration (Figure 4A–D). In normal breast tissue, predominant nuclear expression of BRCA1, BRCA2, FANCC and FANCD2 was observed in myoepithelial and luminal epithelial cells (Figure 4A–D). Reduced or absence of expression of BRCA1, BRCA2, FANCC and FANCD2 proteins was seen in 55% (23/42), 55% (23/42), 38% (16/42) and 52% (22/42) of the pretherapeutic samples, respectively. In NACT-treated BC, reduced expression of BRCA1, BRCA2, FANCC and FANCD2 proteins was seen in 47% (8/17), 35% (6/17), 59% (10/17) and 47% (8/17) samples, respectively. Expression of BRCA1, BRCA2, FANCC and FANCD2 proteins showed concordance with their molecular alterations in the pretherapeutic (p < 0.001 for BRCA1, BRCA2 and FANCC and 0.004 for FANCD2) as well as in NACT-treated BC samples (p = 0.036, 0.035, 0.02217/fon-2016-0289

Figure 4. Representative immunohistochemical patterns of HRR proteins in breast carcinoma: negative stain, high expression in luminal and myoepithelial cells of adjacent normal, moderate and low expression in tumors. (A) BRCA1 expression in breast samples (#1010, #5000, #1743, #2443); (B) BRCA2 expression in breast samples (#3103, #5000, #3964, #6632); (C) FANCC expression in breast samples (#228, #5000, #3947, #3032); (D) FANCD2 expression in breast samples (#3370, #5000, #1084, #5751). The arrow indicates nuclear expression.

Scale bar = 50 μm, magnification = 40×. From the 40× images the insets were made by zooming it four-times.

BC: Breast carcinoma; D+/−: Deletion positive/negative; HRR: Homologous recombination repair; M+/−: Methylation positive/negative; N: Normal; T: Tumor.
CD44 & PCNA expression
The immunohistochemical analysis of CD44 and PCNA was done to see the prevalence of stem cells and proliferation index in the BC samples respectively. In normal breast ducts, membrane expression of CD44 was present in 6–9% of myoepithelial cells; whereas, the nuclear expression of PCNA was present in 8–14% myoepithelial cells (Figure 5A). Luminal epithelium cells showed very low expression of these proteins. CD44 mainly expressed in the membranes of pretherapeutic (3–33%) and NACT-treated (1–12%) BC cells, though 10–15% cells also showed nuclear expression. PCNA expression was mostly found in the nucleus of pretherapeutic (11–83%) and NACT-treated (3–59%) BC cells, but 15–27% cytoplasmic expression was also observed (data not shown). The average percentage of expression or the frequencies of CD44 and PCNA was significantly higher (p = 0.003 and 0.004, respectively) in pretherapeutic samples than NACT-treated samples irrespective of any subtypes (Figure 5B & C). Thus, the NACT-tolerant cells have low frequency of CD44+ cells and low proliferative index.

Clinicopathological association & survival analysis
High (50–74%) overall alteration of these genes was found in stage I and II tumor samples and became comparable in the later stages (stages III and IV) (68–81%) in both pretherapeutic and NACT-treated samples. A similar trend was also evident in tumor grades. Significantly high alterations were found in BRCA1 and FANCC in lymph node invasive tumors of pretherapeutic patients (p = 0.014 and 0.043, respectively) than in lymph node noninvasive tumors (data not shown).

The pretherapeutic BC patients with alterations in BRCA1, BRCA2, FANCC and FANCD2 showed significantly poor DFS (p = 0.003–0.013) and worst prognosis was seen among the ER/PR(-) BC patients having alteration in any of the HRR pathway genes (Figure 6A–D). Most importantly, in pretherapeutic BC patients, multiple gene alteration of HRR pathway (more than or equal to two, more than or equal to three and all four) was significantly associated with poor DFS (p < 0.001–0.035).

But the prognostic difference between ER/PR+/-tumors decreases with the increase in number of HRR genes altered (Figure 6E–G). NACT-treated patients failed to show any such association with their survival (Supplementary Figure 2A–G).

The multivariate Cox model showed a significant association (p = 0.014–0.049) with alterations in BRCA1, BRCA2, FANCC and FANCD2 genes, positive lymph node status and high grade (III) with the poor DFS of pretherapeutic BC patients (Supplementary Table 5A). NACT-treated patients did not show any such correlations (Supplementary Table 5B).

Discussion
The present study was undertaken to understand the role of HRR pathway in development of BC by analyzing the molecular alterations of four key regulatory genes, BRCA1, BRCA2, FANCC and FANCD2, in pretherapeutic and NACT-treated samples, and intends to predict its role in chemotherapeutic tolerance of the disease. Alterations of these genes were correlated with different clinicopathological parameters, such as ER/PR/HER2 expression status, pathological stage, grade, nodes at pathology, age of onset and prognosis of the disease.

In pretherapeutic BC samples, the frequencies of ER/PR(-) samples were more prevalent (54%) than ER/PR(+) samples (46%). The different molecular subtypes of BC vary between 20 and 32%. Similar trends of ER/PR positivity and the molecular subtypes were reported in different studies including Indian patients [35–37]. However, different patterns of molecular subtypes were also reported in other studies [38,39]. This might be due to differences in ethnicity, sample distribution among different stages, grades and age of onset. In contrary to our pretherapeutic samples, NACT-treated samples showed higher frequencies of ER/PR(+) (59%) samples compared with ER/PR(-) (41%) samples. The frequencies of different molecular subtypes of BC included in the study were 10–32% in NACT-treated samples. To the best of our knowledge, frequencies of different subtypes in NACT-treated samples are not well documented.

The deletion frequencies in pretherapeutic BC were comparatively high in BRCA1 and BRCA2 (68 and 60%, respectively) than in FANCC and FANCD2 (36 and 29%, respectively), irrespective of their ER/PR status and age. Similar pattern of deletion of BRCA1, BRCA2 and FANCC was reported in our previous studies and also in

[35–37].

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Figure 5. Expression patterns of CD44 and PCNA in breast carcinoma samples. (A) Representative immunohistochemical patterns of CD44 and PCNA proteins in BC: negative stain, expression in luminal and myoepithelial cells of adjacent normal, pattern of expression in pretherapeutic BC and pattern of expression in NACT-treated BC. The first panel represents CD44 expression in breast samples (#1010, #5000, #1904 and #1656) and the second panel represents PCNA expression in breast samples (#228, #5000, #1089 and #1656). The arrow indicates membrane expression in CD44 and nuclear expression in PCNA. Scale bar = 50 μm, magnification = 40X. From the 40X images the insets were made by zooming it four-times. (B) Frequencies of cells expressing CD44 in different subtypes of pretherapeutic and NACT-treated BC; (C) frequencies of cells expressing PCNA in different subtypes of pretherapeutic and NACT-treated BC. *Statistically significant difference (p < 0.05).

BC: Breast carcinoma; T: Tumor; N: Normal; NACT: Neoadjuvant chemotherapy.

Other studies on BC [23,25,40]. High frequencies of deletion of BRCA1 and BRCA2 (44–87%) were also reported in ovarian cancer [41]. Comparable frequencies of deletion (32–42%) were reported in FANCC in head and neck squamous cell carcinoma (HNSCC) samples [42]. To the best of
our knowledge, deletion of FANCD2 is not well documented in any cancer. Deletion in at least one of the genes was high (83–87%) in pretherapeutic BC irrespective of ER/PR status and age. But, co-deletion of the HRR genes were prevalent in ER/PR(-) BC than in ER/PR(+) BC.
for pretherapeutic BC hinting toward the differences in pathogenesis of the molecular subtypes of BC. For NACT-treated BC, similar pattern of deletion was seen in the HRR genes.

The methylation frequencies in pretherapeutic BC were highest in FANCD2 (60%) and lowest in BRCA1 (37%). Variable frequencies of methylation of BRCA1 (10–20%) and BRCA2 (10–60%) were reported in BC and ovarian cancer. Similar pattern of methylation of FANCC was reported in our previous study on BC and HNSCC. To our knowledge, FANCD2 methylation is not well reported in BC. Methylation in at least one of the genes was seen in 93–95% pretherapeutic samples irrespective of ER/PR status and age. Similarly methylation in multiple genes was comparable in different groups. Like pretherapeutic BC, comparatively low frequency of methylation of BRCA1 (29%) was seen in NACT-treated BC than the other genes (46–56%). However, the methylation frequencies were prevalent in ER/PR(-) than in ER/PR(+) samples in NACT-treated BC. Similarly, prevalent methylation of BRCA1, BRCA2 and FANCC was seen in early age of onset than in late age. The co-methylation of the genes was also prevalent in ER/PR(-) BC and in early age of onset BC. Thus, our data indicate that promoter methylation is one of the inactivation mechanisms of these genes in BC. This has been validated by hypomethylation experiment in BC cell lines, MCF-7 and MDA-MB-231.

The overall alteration (deletion/methylation) of HRR genes was high (64–78%) in pretherapeutic BC with significantly high alteration of BRCA1, BRCA2 and FANCD2 in ER/PR(-) BC than ER/PR(+) BC. Approximately, 97% (115/118) of pretherapeutic samples showed alterations in at least one of the genes irrespective of ER/PR status and age, indicating the importance of HRR pathway in the development of BC. Differential association of alterations of these genes was seen in this tumor. But, co-alterations of the genes were significantly high in ER/PR(-) BC than ER/PR(+) BC indicating its importance in prognosis of the disease. Like pretherapeutic BC, similar trend of overall alteration was observed in NACT-treated BC. Bi-allelic alterations (both deletion and methylation) of HRR genes were harbored by 12–34% BC samples suggesting deletion and methylation act cooperatively for inactivation of the genes.

In normal breast duct, expression of the HRR proteins was evident in both myoepithelial and luminal epithelial cells. The reduced expressions of these proteins in both pretherapeutic and NACT-treated BC showed concordance with their molecular alterations (deletion and/or methylation). Reduced mRNA and protein expression of BRCA1 was reported in BC. Reduced mRNA and protein expression of FANCC was found in our previous study on BC. In BC, reduced expressions of BRCA2 and FANCD2 proteins were also found.

In pretherapeutic samples, 97% of the samples with altered HRR pathway showed high expression of CD44/PCNA. Interestingly, in NACT-treated samples, in spite of high alteration in HRR pathway, CD44/PCNA expressions were significantly low compared with pretherapeutic samples. There might be two possible reasons behind this. First, the BC cells might show reduced expression of CD44/PCNA followed by NACT to tolerate the initial drug toxicity. This might be due to the low proliferation rate and modulation of CD44 signaling pathways in the drug-tolerant cells. Second, after NACT, high CD44/PCNA expressing cells might have been killed and only low CD44/PCNA expressing cells remained persistent. After NACT, low CD44 expression was associated with better disease outcome. Like our data, in response to NACT, BC showed reduced expression of PCNA. Similar results were also seen in our previous study on HNSCC samples. We did not find any association of CD44/PCNA expression with alterations of HRR pathway in both pretherapeutic and NACT-treated BC. Hence, the alteration in HRR pathway is an important factor in development of the disease.

BRCA1 and FANCC alterations showed significant association with nodes at pathology in pretherapeutic BC. Significant association of BRCA1 alterations with early age of onset in NACT-treated BC was also observed. However, alterations of these genes showed worst patient outcome in ER/PR(-) pretherapeutic samples, suggesting prognostic importance. In addition, in multivariate analysis, alterations of BRCA1, BRCA2, FANCC and FANCD2 were important risk factors in pretherapeutic BC patients with positive lymph node invasion and higher histological grades. In NACT-treated BC, no significant association of patients’ survival with the alteration in these genes was found irrespective of ER/PR status. It seems that low prevalence
of BC stem cells and proliferative index might restrict the disease of NACT-treated patients in spite of frequent alterations of HRR genes.

**Conclusion**

Alteration of HRR pathway might be one of the early events in development of BC with preferential alterations in the ER/PR(-) tumors. The alteration of this pathway has prognostic implication in pretherapeutic samples but not in chemotolerant NACT-treated samples. However, detailed analysis in this regard is warranted in understanding the molecular pathogenesis of both pretherapeutic and NACT-treated BC samples.

**Supplementary data**

To view the supplementary data that accompany this paper please visit the journal website at: http://www.futuremedicine.com/doi/full/10.2217/fon-2016-0289

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**References**

Frequent alterations of HRR pathway in primary & chemotolerant breast carcinomas

RESEARCH ARTICLE


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