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Dysfunctional telomeres in human *BRCA2* mutated breast tumors and cell lines

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ABSTRACT

In the present study the possible involvement of telomeres in chromosomal instability of breast tumors and cell lines from *BRCA2* mutation carriers was examined. Breast tumors from *BRCA2* mutation carriers showed significantly higher frequency of chromosome end-to-end fusions (CEFs) than tumors from non-carriers despite normal telomere DNA content. Frequent CEFs were also found in four different *BRCA2* heterozygous breast epithelial cell lines, occasionally with telomere signal at the fusion point, indicating telomere capping defects. Extrachromosomal telomeric repeat (ECTR) DNA was frequently found scattered around metaphase chromosomes and interstitial telomere sequences (ITSs) were also common. Telomere sister chromatid exchanges (T-SCEs), characteristic of cells using alternative lengthening of telomeres (ALT), were frequently detected in all heterozygous *BRCA2* cell lines as well as the two ALT positive cell lines tested. Even though T-SCE frequency was similar in *BRCA2* heterozygous and ALT positive cell lines they differed in single telomere signal loss and ITSs. Chromatid type alterations were more prominent in the *BRCA2* heterozygous cell lines that may have propensity for telomere based chromosome healing. Telomere dysfunction-induced foci (TIFs) formation, identified by co-localization of telomeres and γ-H2AX, supported telomere associated DNA damage response in *BRCA2* heterozygous cell lines. TIFs were found in interphase nuclei, at chromosome ends, ITSs and ECTR DNA. In conclusion, our results suggest that BRCA2 has an important role in telomere stabilization by repressing CEFs through telomere capping and the prevention of telomere loss by replication stabilization.

1. Introduction

Breast carcinoma, the most common cancer in women in the Western world, frequently shows complex karyotypes with multiple chromosomal changes [1]. Genomic alterations are more common in familial breast tumors with *BRCA1* or *BRCA2* mutations than in sporadic tumors and show a distinct genomic profile [2–6]. As BRCA1 and BRCA2 are known to be involved in homologous recombination (HR) repair of DNA double strand breaks (DSBs), mutations may result in gross chromosomal rearrangements in tumors [7]. Human breast tumors with *BRCA2* germline mutation and murine cells deficient in the BRCA2 homolog have been shown to have multiple chromosomal abnormalities [8,9]. The abnormalities include broken chromosomes and chromatids, markers of defective mitotic recombination, and tri- and quadriradial chromosomes [10]. Spectral karyotyping reveals complex chromosomal rearrangements such as translocations or deletions, as well as chromosome end-to-end fusions (CEFs) that include multiple, non-homologous chromosomes [11]. Similar structural aberrations occur in Brca1-deficient mouse cells [12]. These abnormalities establish that *BRCA* gene products are essential for preserving chromosome structure, suggesting that they behave as caretakers of genome stability and play key roles in networks involved in DNA damage response (DDR) [10].

Telomere repeats $(TTAGGG)_n$ on chromosome ends also play a critical role in maintaining chromosomal stability [13]. When the telomere length declines below a certain threshold due to continuing cell divisions, a growth arrest state named replicative senescence is triggered [14]. Most cancer cells escape senescence by expression of telomerase, a reverse transcriptase that carries its own RNA template [15]. However, some cancers can maintain telomere lengths by one or more mechanisms that do not involve telomerase, such as the so called alternative lengthening of telomeres (ALT) which is characterized by increased rates of telomeric recombination [16]. Telomeric sister chromatid exchanges (T-SCEs) are abundant in cells using ALT and can be examined with chromosome orientation fluorescence *in situ* hybridization (CO-FISH) [17,18].

Studies on aging telomerase-deficient *p53* mutant mice showed development of epithelial cancers, including breast carcinomas, and formation of complex non-reciprocal chromosome translocations by breakage–fusion–bridge (BFB) cycle [19]. However, other tumors harbour dysfunctional telomeres without telomere erosion due to incomplete telomere end-capping [20]. Dysfunctional

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telomeres may lead to the initiation of chromosomal instability, caused by either telomere erosion or capping defects [21]. The shelterin protein complex that shapes and safeguards human telomeres contains six proteins; TRF1, TRF2 and POT1, that directly recognize the TTAGGG repeats and TIN2, TPP1 and Rap1, that are interconnected [22]. The shelterin complex enables cells to distinguish telomeres from sites of damage. Many other proteins are involved in telomere capping and maintenance which also have an important role in DDR and show increased CEFs in deactivated mode [23,24]. Uncapped telomeres have been found to be associated with DDR factors, such as γ-H2AX. They are recognized as telomere dysfunction-induced foci (TIFs) and are markers for telomere dysfunction [25].

Recently, BRCA2 has been shown to contribute to telomere maintenance by facilitating telomere replication [26]. Therefore, genomic instability is believed to be caused in part by telomere dysfunction in BRCA2 deficient cells and tumors. In the present study we further analysed telomere dysfunction in *BRCA2* deficient breast epithelial cell lines and tumors. We found high frequency of CEFs in metaphase cells from breast tumors with a *BRCA2 999del5* germline mutation and in *BRCA2* heterozygous breast epithelial cell lines. Using telomere CO-FISH analysis on *BRCA2* heterozygous breast epithelial cell lines we estimated the T-SCE frequency to be similar to that of the ALT positive cell lines and CEFs occasionally had telomere signal at fusion points. These findings suggest that BRCA2 might have more roles in telomere maintenance than just facilitating telomere replication.

2. Materials and methods

2.1. Chromosomal analysis of breast tumor tissue

Tumor tissue obtained directly from surgery was finely minced in a drop of culture medium and processed for chromosomal analysis. Chromosomes were harvested after 3–11 days of short-term culture as previously described [1]. Colcemid (0.016 μg/ml) was added for the final 4–6 h depending on accumulation of mitoses. Hypotonic treatment was done with 0.4% KCl for 25 min at room temperature followed by fixation with freshly made methanol–acetic acid (3:1). Cell suspension was spread on ice cold slides. Chromosomes were G-banded with Wright's stain. The number of cells analysed per tumor ranged from 17 to 86, with a mean of 45.7. Tumor karyotypes were previously described for cases 2, 11, 16, 20, 32 [1] and 23 [27]. Dicentric chromosomes were defined as having CEF. Frequency of CEF was calculated per metaphase and per chromosome to exclude the aneuploidy effect. Breast tumor samples were obtained from the Department of Pathology, Landspitali, The National University Hospital of Iceland with permission from the Icelandic Data Protection Committee (2006050307) and the Bioethics Committee (VSNb2006050001/03-16).

2.2. Allele specific quantitative PCR

Allele-specific quantitative PCR (qPCR) reactions were carried out as previously described [28]. Relative proportions of the wild-type and *999del5 BRCA2* alleles in breast tumors and epithelial cell lines were quantitatively determined using a single forward primer for both alleles and distinguishable reverse primers for the wild-type (wt) and mutated (mut) allele. A single *BRCA2* specific TaqMan minor groove binding (MGB) probe was used, with 5′-end FAM labelling and a nonfluorescent quencher at the 3′-end. The qPCR assay primers and TaqMan-MGB probe were: forward primer: 5′-CATGATGAAAGTCGTAAGAAA-3′, reverse primer (mut): 5′-CATGACTTGCAGCTTCTCTTTGTG-3′, reverse primer (wt): 5′-CATGACTTGCAGCTTCTCTTTGAT-3′, TaqMan-MGB probe: 5′-TTTATCGCTTCTGTGACA-3′. The *BRCA2* wild type allele loss was measured by differences in fluorescence intensity of FAM performed in duplicate and the Ct values averaged.

2.3. Telomere slot-blotting

Average telomere length for each breast tumor sample was measured on slot blot [29] using the Telo-TAGGG Telomere Length Assay Kit for probes and telomere length controls (Roche Applied Science). Telomeric probe hybridization signals were normalized against centromeric probe hybridization signals to correct for DNA loading in each sample. To calculate the values for telomere length normalized telomeric probe hybridization signals were then plotted against telomere length controls, provided by the kit, with a known average telomere length of 10.2 kb and 3.9 kb. Measured average telomere length less than 80% of the 10.2 kb control telomere content was considered as telomere erosion. Average telomere length was analysed in tumor samples from patients carrying the *BRCA2* 999del5 mutation [30] and from sporadic tumors without mutations in *BRCA1*, *BRCA2* and *TP53*.

2.4. TERT immunohistochemistry

Immunohistochemical staining for the catalytic subunit of the TERT protein was performed as previously described [27]. Briefly, polyclonal rabbit anti-human antibody (anti-telomerase, human Ab-2, catalog no. 58005; Calbiochem) was used in dilution 1:400 and detected with EnVision System (catalog no. K4010; Dako) using peroxidase as the final enzymatic reaction. The TERT staining was scored: <10% positive nuclei, 10–50% positive nuclei, and >50% positive nuclei.

2.5. Cell lines

Three human E6/E7 transformed mammary epithelial cell lines derived from normal and tumor breast tissue of *BRCA2 999del5* mutation carriers were used; BRCA2-999del5-1N, BRCA2-999del5-2N and BRCA2-999del5-2T [31]. Another mammary epithelial cell line, HME348, derived from *BRCA2 6872del4* mutation carrier and immortalised with ectopic expression of telomerase (TERT) was also used [32]. MCF10a mammary epithelial cell line derived from normal tissue and Saos-2 and U2-OS ALT positive osteosarcoma cell lines, kindly provided by Dr. Sarantis Gagos, Biomedical Research Foundation of the Academy of Athens, Greece, were used as controls. Saos-2 and U2-OS cell lines were grown in DMEM basal medium supplemented with 10% fetal bovine serum, MCF10a and *BRCA2 999del5* cell lines were grown in DMEM-Ham's F12 basal medium (GIBCO) with supplements as previously described [31] and the HME348 cell line was grown in MEGM medium (Lonza).

2.6. Telomere fluorescence in situ hybridization

For the detection of CEFs, ready-to-use PNA probe for telomeric sequences was used from the Telomere PNA FISH kit/Cy3 (K5326 from Dako) in mixture with FITC-conjugated PNA centromeric probe for the detection of all centromeres (Dako). Hybridization was done according to the manufacturer's instructions.

Dual colour CO-FISH analysis was done following the established basic protocol [33] with several modifications. Briefly, subconfluent cell monolayers were cultured in fresh medium containing 5′-bromo-2′-deoxyuridin (BrdU; Sigma) at a final concentration of 1×10^{-5} M for up to 24 h, depending on the length of the cell cycle. The *BRCA2 999del5* cell lines along with the $SaOS_2$ cell line all had cell cycle of 22–24 h, whereas HM348, MCF10a and U2OS cell lines had cell cycle of 17–19 h. Alphoid satellite PNA probe was used in combination with telomere PNA probe to verify the length of one cell cycle by CO-FISH (supplementary Fig. 1). CO-FISH pre-treatment was briefly as follows. Slides were treated with RNase A (0.5 μg/ml) for 10 min at 37 °C, stained with Hoechst 33258 (0.5 μg/ml; Sigma) for 15 min at room temperature, mounted with 2× SSC buffer, and exposed to 365 nm UV black-light (Oshram, L 18 W/73) for 60 min at room temperature. The BrdU substituted DNA was digested with Exonuclease III (3 U/μl; New England Biolabs) in 1× NEB buffer provided with the enzyme for 10 min at 37 °C. CO-FISH was performed using the Cy3-conjugated telomere lagging strand probe $(CCCTAA)_n$ following manufacturer's instruction for the DAKO Telomere FISH kit/Cy3 with the addition of the same concentration of FAM-conjugated PNA telomere leading strand $(TTAGGG)_3$ probe (Panagene). The efficiency of CO-FISH method was confirmed on normal blood lymphocytes that were found to have stable telomere signal distribution of one telomere lagging strand red signal and other telomere leading strand green signal on each sister chromatid. A T-SCE was detected when both sister chromatids had telomere signals of the same colour, either of a single colour (red or green) or a combination of both colours. Single telomere signals with a combination of both colours were also shown to have a T-SCE. Chromosome ends with telomere signal on one of the two sister chromatids were defined as having single telomere signal and chromosome ends with no signals as telomere signal free.

2.7. Immunofluorescence detection of γ-H2AX at telomeres

Mitotic cells were collected by adding colcemid to the cell cultures for 2–3 h. Cells were detached by trypsinization and allowed to swell in 0.075 M KCl hypotonic buffer for 10–20 min at 37 °C. About 200 μl of the mitotic cell suspension was added to the cytospin funnel clamped to a regular glass slide and centrifuged at 1800 rpm for 8 min. The slides were immersed in prechilled 90% EtOH (−20 °C) for 30 min, air dried and further fixed in 4% formaldehyde in PBS for 10 min following PBS ×1 rinsing and dehydration in ethanol series. Telomere FISH was performed as previously described using FAM-conjugated PNA telomere $(TTAGGG)_3$ probe. Following post-hybridization washes blocking solution (2% fetal bovine serum and 1% bovine serum albumin in PBS ×1) was added to the slides for 10 min. The γ-H2AX antibody (BL178, catalog number A300-081A; Bethyl Laboratories) was used in dilution 1:1000 with detection of the secondary antibody (anti-rabbit Alexa 546, Molecular Probes) in dilution 1:1000. Slides