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Human *BRCA1*-Associated Breast Cancer: No Increase in Numerical Chromosomal Instability Compared to Sporadic Tumors

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Key Words
BRCA1 · Chromosomal instability · CIN · Fluorescence in situ hybridization · Hereditary breast cancer

Abstract
BRCA1 is a major gatekeeper of genomic stability. Acting in multiple central processes like double-strand break repair, centrosome replication, and checkpoint control, BRCA1 participates in maintaining genomic integrity and protects the cell against genomic instability. Chromosomal instability (CIN) as part of genomic instability is an inherent characteristic of most solid tumors and is also involved in breast cancer development. In this study, we determined the extent of CIN in 32 breast cancer tumors of women with a *BRCA1* germline mutation compared to 62 unselected breast cancers. We applied fluorescence in situ hybridization (FISH) with centromere-specific probes for the chromosomes 1, 7, 8, 10, 17, and X and locus-specific probes for 3q27 *(BCL6)*, 5p15.2 (D5S23), 5q31 *(EGR1)*, 10q23.3 *(PTEN)*, and 14q32 (*IGH@*) on formalin-fixed paraffin-embedded tissue microarray sections. Our hypothesis of an increased level of CIN in *BRCA1*-associated breast cancer could not be confirmed by this approach. Surprisingly, we detected no significant difference in the extent of CIN in *BRCA1*-mutated versus sporadic tumors. The only exception was the CIN value for chromosome 1. Here, the extent of CIN was slightly higher in the group of sporadic tumors.

In the move towards personalized medicine, it is becoming increasingly important to know the *BRCA* mutation status at the time of breast cancer diagnosis. Targeted therapies for mutation carriers with cross-linking platinum-based regimens or inhibitors of poly(ADP-ribose) polymerase (PARP) are currently entering the clinical stage [Audeh et al., 2010; Byrski et al., 2010; Tutt et al., 2010]. Nowadays, the strategy to identify mutation carriers includes molecular genetic testing after evaluation of the personal and the family history for breast and ovarian cancer [McIntosh et al., 2004]. Morphologic characteristics of the tumor, like e.g. triple negativity, have turned out to be helpful as additional selection criteria [Lakhani et al., 2002; Gadzicki et al., 2009].

There is an urgent need for the identification of further markers that enable a differentiation between *BRCA*-related and *BRCA*-non-related tumor tissue.

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Genomic instability is seen as such a characteristic of tumor development. Through the accelerated rate of genetic diversity, cancer cell clones with a favorable genotype, acquiring functional capabilities that allow them to survive, proliferate and disseminate, can arise and grow [Hanahan and Weinberg, 2011]. Instability of the genome is inherent to the great majority of human cancer cells during tumor initiation and progression. As demonstrated for colorectal cancer by Lengauer et al. [1997], there are 2 major mutually exclusive forms of instability: microsatellite instability (MIN), caused by alterations of mismatch repair genes, and chromosomal instability (CIN). Recently, Geigl et al. [2008] defined CIN as the rate of gain or loss of whole chromosomes or fractions of chromosomes. Despite the impact of CIN for tumorigenesis, the mechanisms responsible for the induction of CIN are not yet completely understood.

BRCA1 is a key protein to maintain genomic stability. Indeed, in vitro knock-out models clearly demonstrate that inactivation of BRCA1 results in increased genomic instability, in supernumerary centrosomes that lead to unequal chromosome segregation and thus probably to aneuploidy [Xu et al., 1999; Ko et al., 2006]. Loss of BRCA1 or its CHEK2-mediated phosphorylation leads to spindle formation defects and CIN [Stolz et al., 2010].

BRCA1 is involved in multiple pathways to maintain genomic integrity. CIN can be subdivided into numerical versus structural CIN, with the latter involving gene amplification, deletions, etc., often resulting from breakdowns of the replication fork that cause double strand breaks in DNA and defects in the DNA damage response. Numerical CIN can be due to an impaired chromosome segregation as a result of centrosomal defects or defects in the spindle checkpoint.

A fundamental task of BRCA1 is its role in DNA damage response and repair and chromatin remodeling. BRCA1 is co-localized at the site of DNA damage with other proteins like RAD51 and BRCA2 to accomplish homologous recombination [Scully and Livingston, 2000; Venkitaraman, 2003]. Impaired repair of chromatid breaks can lead to chromosomal end-to-end fusions and therefore to structurally aberrant chromosomes [Moynahan et al., 2001; Derheimer and Kastan, 2010]. BRCA1 also acts in cell cycle checkpoint control and centrosome duplication keeping numerical chromosomal balance. BRCA1 participates in the checkpoint control after ATM/ATR-mediated cell cycle arrest and in centrosome maintenance [Cortez et al., 1999; Lee et al., 2000]. By interacting with BARD1 (BRCA1-associated RING domain 1), a heterodimeric complex is formed that exhibits E3 ubiquitin ligase activity and tags proteins, e.g. cell cycle regulators or centrosomal proteins, for proteasomal degradation [Gudmundsdottir and Ashworth, 2006]. Newer data assume that numerous cell cycle regulators, among them BRCA1, lead to chromosome mis-segregation through pathways not completely understood [Thompson et al., 2010]. An erroneous microtubule attachment of the sister kinetochores promoting unequal chromosome segregation fidelity during mitosis is possible.

Considering these functional properties of BRCA1 in double-strand break repair and centrosome regulation, we hypothesized that BRCA1-deficient tumors should have a higher degree of CIN than sporadic breast cancer and that this could serve as a marker for *BRCA1*-associated breast cancer. We therefore aimed to determine the level of CIN in breast cancer of patients with a *BRCA1* germ-line mutation by fluorescence in situ hybridization (FISH) analysis and compare it with the level of CIN in sporadic breast cancer from a hospital-based series.

Materials and Methods

Patient Samples

Formalin-fixed paraffin-embedded tissue microarray sections of breast cancer from 32 *BRCA1* mutation carriers and from 62 unselected, consecutively ascertained breast cancers were obtained from the tumor bank of the Institute of Pathology, Hannover Medical School, which is the reference pathology of the German Consortium for Hereditary Breast and Ovarian Cancer. This study was approved by the local ethics committee (approval number 4121). *BRCA1*-related tumors were distributed in 2 tissue microarrays blocks, and 1 tissue microarray block comprised the sporadic cancers.

Standard pathological review included the analysis of grading, estrogen receptor (ER), progesterone receptor (PR), and Ki-67 expression as well as human epidermal growth receptor (HER2/neu) (detailed information for all samples in online supplementary table 1; for all online supplementary material, see www.karger.com/doi/10.1159/000332005). For the expression of ER and PR, a modified Allred score led to a 4-point scoring system. HER2/neu was evaluated according to a slightly modified 3-point category (0–3+) DAKO system proposed for the evaluation of the HercepTest.

Probe Selection

Centromere-specific probes for chromosomes 1, 7, 8, 10, 17, and X as well as locus-specific probes were selected (Abbott Molecular, Des Plaines, Ill., USA). CIN as defined by Geigl et al. [2008] should be detectable with every of the selected probes, because no clonal evolution or selection pressure occurs to the cells from the last mitosis to the time point of measurement. According to Rennstam et al. [2003] and Tsuji et al. [2010], chromosomes 7 and 10 are rarely aberrant in breast cancer and were therefore chosen as internal controls. Recently, distinct patterns of genomic

Table 1. Literature for *BRCA*-specific chromosomal aberrations

	1	3	5	7	8	10	14	17	X
Gain									
Waddell et al. [2009]					8q				Xq
Melchor et al. [2008]	1q	3q			8q			17q	
Joosse et al. [2009]		3q22–25, 3q26–29		7q22–36		10p14–12, 10p15–14			
Melchor et al. [2005]	1q	3q25–26.3			8q21–23, 8q24			17q22–25	
Jönsson et al. [2007]		3q27.2–27.3, 3q29		7q21.11–21.12					
van Beers et al. [2005]		3q13–27				10pter–q21			
Wessels et al. [2002]		3q		7p	8q	10p		17q	
Probes used in our study	cen 1	3q		cen 7	cen 8	cen 10		cen 17	cen X
Loss									
Waddell et al. [2009]			5q						
Melchor et al. [2008]		3p	5q		8p	10q	14q		
Joosse et al. [2009]			5p13–12, 5q11–23, 5q31–35	7p22–15			14q22–23		
Melchor et al. [2005]			5q11–23		8p21–23				
Johannsdottir et al. [2006]			5q14.2, 5q33.1, 5q35.3						
Brozek et al. [2009]								17q11.2, 17q23.2, 17q25.3	
Tirkkonen et al. [1997]			5q						Xq21–qter
van Beers et al. [2005]			5cen–q23						
Wessels et al. [2002]		3p	5q						
Probes used in our study	cen 1		5p, 5q	cen 7	cen 8	cen 10, 10q	14q	cen 17	cen X

alterations have been observed using array-based comparative genomic hybridization. Gains of 1q, 8q, and 17q were among the most frequent copy number changes. These data are consistent with earlier studies by Fehm et al. [2002]. Here, aneusomy for at least one of the chromosomes 1, 8, and 17 was found in 92% of 74 primary breast cancer samples. In 88% of the tumors, alterations of chromosome 1 were detected, and this was thus the most frequently affected chromosome, followed by 69% for chromosome 8 and 60% for chromosome 17. Using FISH with centromere-specific probes, Farabegoli et al. [2001] also identified alterations of chromosomes 1, 8, and 17 and suggested those of chromosomes 1 and 17 to be early changes. To extend our study to include the investigation of CIN affecting chromosome arms, locus-specific probes for 3q27 *(BCL6)*, 5p15.2 (D5S23), 5q31 *(EGR1)*, 10q23.3 *(PTEN)*, and 14q32 *(IGH@)* were additionally applied. These regions were previously reported to be frequently gained or lost in breast cancer from patients with germ-line *BRCA1* and *BRCA2* mutations (table 1) [Tirkkonen et al., 1997; Wessels et al., 2002; Melchor et al., 2005; van Beers et al., 2005; Johannsdottir et al., 2006; Jönsson et al., 2007; Melchor et al., 2008; Brozek et al., 2009; Joosse et al., 2009; Waddell et al., 2009]. We reasoned that the chance to detect CIN would be higher if we analyzed chromosomes or chromosome arms that are frequently affected in breast cancer, particularly in breast cancer from women with germ-line *BRCA1* mutations.

Fluorescence in situ Hybridization

FISH experiments were performed as described previously by Wilkens et al. [2005] on 5-μm sections. The tissue microarrays were co-hybridized with the FISH probes for centromere 7 and centromere 8, 5p15.2 and 5q31, centromere 10 and 10q.23.3 *(PTEN)*. All the other FISH probes were hybridized separately. Only integer, non-overlapping nuclei with at least 1 signal per color from an area with sufficient hybridization were counted. Signals from an average of 50–100 interphase nuclei were counted for each tumor and FISH probe by 2 independent scientists.

Control Experiments

FISH analyses were performed on tissue sections of reduction mammoplasties to determine the normal range of the CIN value due to cutting artifacts present on 5-μm sections of normal mammary cells. The chromosomes 7, 8, and X were each investi-

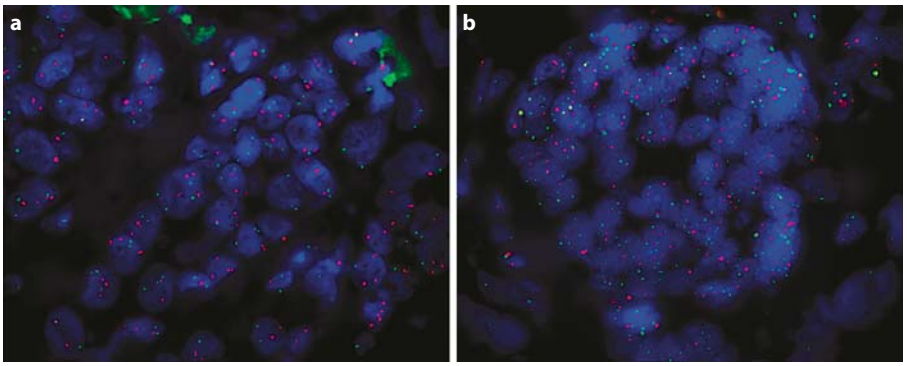

Fig. 1. FISH analysis of two different formalin-fixed paraffin-embedded breast cancer tissues with **a** low CIN values (CIN index 16 for chromosome 7 and 28 for chromosome 8) and **b** high CIN values (CIN index 77 for chromosome 7 and 81 for chromosome 8). FISH probes were specific for the centromeres of chromosome 7 (red) and chromosome 8 (green).

Table 2. Mean and median values of CIN [%] for each chromosome

	Mean			Median	
	BRCA1	sporadic	p value	*BRCA1*	sporadic
Chr. 1	42.42	52.10	0.017	46.0	54.0
3q	36.70	42.18	n.s.	34.0	40.0
5p	32.63	32.59	n.s.	25.0	28.0
5q	19.14	25.23	n.s.	16.0	21.0
Chr. 7	46.41	45.34	n.s.	47.0	46.5
Chr. 8	48.16	48.07	n.s.	50.5	49.0
Chr. 10	32.57	30.46	n.s.	28.0	28.0
10q	30.55	27.00	n.s.	28.0	27.0
14q	43.42	44.88	n.s.	43.0	47.0
Chr. 17	34.00	40.16	n.s.	31.0	38.0
Chr. X	46.68	43.08	n.s.	50.0	46.0

Chr. = Chromosome; n.s. = not significant.

gated in 300–550 cells from 4 different normal mammary tissues.

In all these experiments, the modal chromosome count was 2, as expected for normal cells. The average CIN index was 14.31% with a range from 8.4–24%. According to Ward et al. [1993], the cut-off level was calculated as the mean +3 SD. This results in a cut-off level of 28.27%. Subsequently, for every single probe, tumors with a greater extent of CIN were considered as chromosomally unstable for this probe.

CIN Index and Statistical Analysis

CIN values (CIN index) were determined as percentages of cells with a non-modal chromosome count [Nakamura et al., 2003]. The modal count is the number of chromosomes which occur most frequently within the cells of a distinct tumor. Non-tumor cells have a modal count of 2 and tetraploid cells a count of 4. Based on the modal count, the CIN index rises with the increase of highly variant distributions of chromosome numbers. The more different chromosome counts are present in a tumor, the higher the CIN index will be. Student's t test and Fisher's exact test were applied for testing significance. Statistical analysis was performed using Graphpad prism 5.02 (GraphPad Software Inc., La Jolla, Calif., USA). A p value <0.05 is considered to be statistically significant.

Results

The copy numbers of chromosomes 1, 7, 8, 10, 17, and X and of chromosomal regions at 3q, 5p, 5q, 10q, 14q were determined in 94 primary breast cancers, among them 32 breast cancers from women with a germ-line *BRCA1* mutation and 62 cancers from unselected hospital cases as a comparative group. According to the criteria of Lengauer et al. [1997] and Nakamura et al. [2003], the CIN value was defined as the percentage of cells with a non-modal chromosome count. The CIN values were calculated for each chromosome and each chromosome region for each tumor. Figure 1 shows examples of a breast cancer with high and another with low CIN. The CIN values for each of the investigated chromosomes and chromosome arms for each *BRCA1*-associated and each sporadic breast cancer is given in online supplementary tables 2 and 3. Table 2 and figure 2 summarize the results for all distinct chromosomes and chromosomal regions investigated. There are no significant differences between *BRCA1*-associated and sporadic breast cancers. The only exception is seen for chromosome 1 with a mean CIN value of 42.4 for *BRCA1*-associated versus 52.1 for sporadic breast cancer (Student's t test, $p = 0.017$). The classification of the cases into chromosomally stable and chromosomally unstable based on the evaluated cut-off value showed that significantly more sporadic than *BRCA1*-associated breast cancers had an elevated CIN for chromosome 1 (Fisher's exact test, $p = 0.0024$). Table 3 and figure 3 summarize the results of the classification of sporadic and *BRCA1*-associated breast

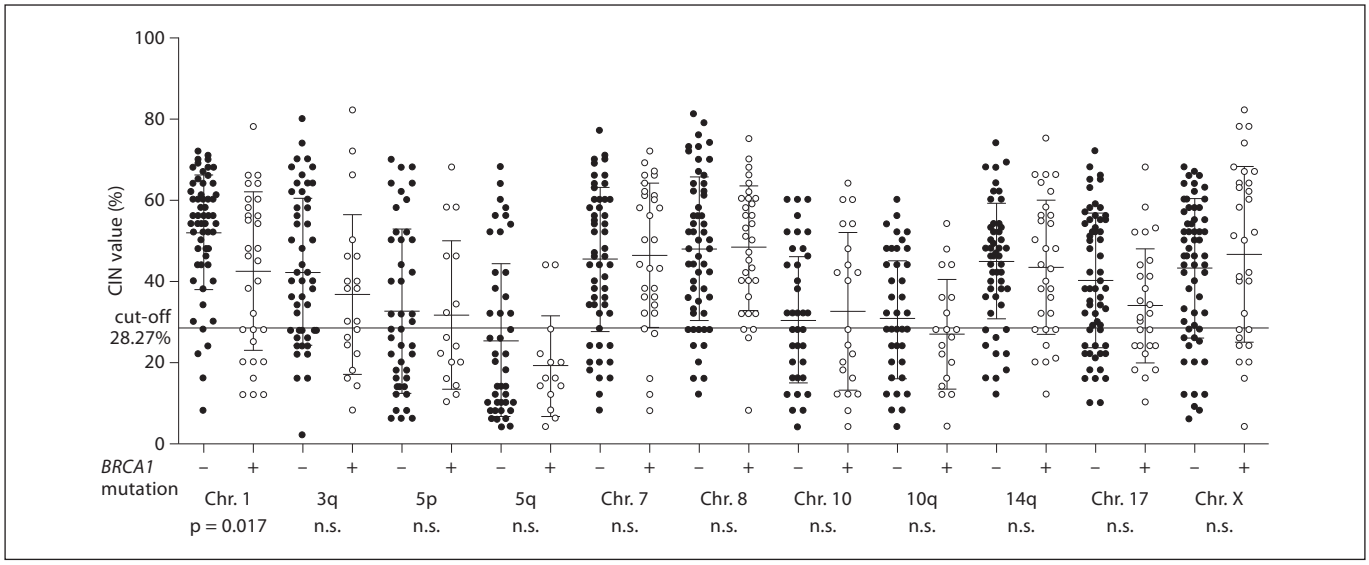

Fig. 2. CIN data of all investigated chromosomes. The *BRCA1* status is shown at the bottom, the group of sporadic tumors is marked with (–) and the group of *BRCA1*-mutated tumors with (+). Student's t test was performed to compare the mean values. The mean values of chromosome 1 differ significantly (p = 0.017), all other mean values are not significantly (n.s.) different. The cut-off value was calculated as the mean +3 SD from the investigation of 1,400 nuclei from normal breast tissue by FISH with 3 different probes.

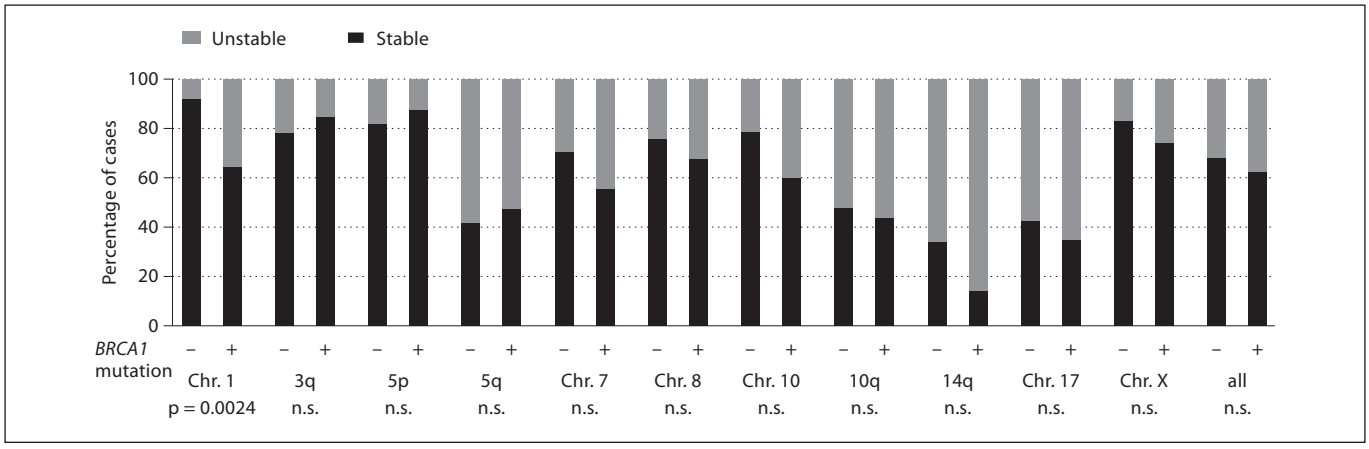

Fig. 3. Distribution of chromosomally stable and unstable cases. The *BRCA1* mutation status is shown at the bottom, the group of sporadic tumors is marked with (–) and the group of *BRCA1*-mutated tumors with (+). The cut-off was calculated as mean +3 SD from the investigation of normal breast tissue by FISH and was determined to be 28.27%. Chromosomally unstable cases were defined as cases with a CIN index greater than the cut-off value. The evaluation of significance was done by applying Fisher's exact test (n.s., not significant).

cancers into chromosomally stable and unstable. A detailed analysis of the distribution of chromosomally unstable cases is shown in online supplementary table 4. Both groups of samples mostly harbor 4–6 unstable chromosomes or loci (*BRCA1*: 53.13%; sporadic tumors: 38.70%). Furthermore, tumors in which almost all investigated chromosomes/loci (10–11) showed instability were clearly overrepresented in the comparative group of sporadic tumors (*BRCA1*: 3.13%; sporadic tumors: 17.70%).

Thus, no increased numerical CIN was observed in primary breast cancer of women with a *BRCA1* germ-line mutation.

Table 3. Results of the calculation of Fisher's exact test after the classification of the cases into chromosomally stable and unstable based on the evaluated cut-off value of 28.27% (number of tumors given)

	Sporadic		*BRCA1*		p value
	stable	unstable	stable	unstable	
Chr. 1	5	57	11	20	0.0024
3q	21	36	8	12	n.s.
5p	23	21	9	7	n.s.
5q	29	15	12	2	n.s.
Chr. 7	12	44	5	27	n.s.
Chr. 8	11	49	4	28	n.s.
Chr. 10	28	20	11	10	n.s.
10q	27	20	13	7	n.s.
14q	10	49	8	23	n.s.
Chr. 17	18	43	12	15	n.s.
Chr. X	15	47	10	21	n.s.

Chr. = Chromosome; n.s. = not significant.

Discussion

We investigated the degree of numerical CIN in human primary breast cancer of *BRCA1* mutation carriers compared to sporadic breast cancer. Based on the knowledge of the role of BRCA1 in double-strand break repair, regulation of centrosome duplication, and genomic maintenance, we hypothesized a higher degree of CIN in BRCA1-deficient tumors [Deng, 2002; Ko et al., 2006; Sankaran et al., 2007; Kais and Parvin, 2008; Shimomura et al., 2008].

According to Lengauer et al. [1998], CIN is a matter of rate, and the existence of a genetic alteration (state) provides no information about the rate of occurrence. Because it is impossible in human tumors to apply time-based measurements, which would be the best approach to determine how frequently the karyotypes of sister or daughter cells are not identical to each other or to their mother cells, we decided to measure the degree of cell-to-cell variability using FISH [Geigl et al., 2008]. FISH as a single cell approach offers the possibility to screen hundreds of cells with the selected probe set. We are aware that the FISH approach did not directly measure an increased rate of aberrations but evaluated the status of chromosomal imbalances at a given time point only. However, the chromosomal heterogeneity present in the cells may serve as a good indicator for CIN. Therefore, in our opinion, FISH using a broad set of probes is the best practical way to mirror the numerical chromosomal situation and thus obtain an indication of chromosomal stability or instability in primary tumors.

Regarding its role as 'caretaker' of the genome, we hypothesized that BRCA1 deficiency could lead to numerical CIN. This instability will not be directed at specific chromosomes, and it should be possible to measure it with randomly chosen chromosomes. Many studies investigated the regions of copy number aberrations that are specific for *BRCA1*-mutated tumors [Wessels et al., 2002; Melchor et al., 2005; van Beers et al., 2005; Jönsson et al., 2007; Joosse et al., 2009]. Our approach was to take probes for chromosomes or regions of chromosomes that were previously reported as gained or lost, e.g. 3q, 5q, 14q, but also chromosomes that did not show a clear tendency towards gains or losses in BRCA1-deficient tumors, e.g. centromere 7 or centromere X. None of the selected chromosomes or regions showed an elevated extent of instability in the group of BRCA1-deficient breast tumors. Applying centromere-specific probes for 6 chromosomes and locus-specific probes for 5 different chromosome regions, we were surprised not to find any evidence of an increased CIN in *BRCA1*-associated breast cancer.

Moynahan et al. [2001] reported about chromosome aberrations, including chromatid breaks and exchanges, chromosome breaks, deletions and translocations in *Brca1*-deficient murine embryonic stem cells but, interestingly, aneuploidy was not evident.

We cannot rule out with certainty that there are BRCA1-deficient tumors among the breast cancers classified as sporadic, in particular among the 7 triple-negative tumors, since this is the prevalent subtype of *BRCA1*-associated tumors [Young et al., 2009]. Inactivation through promoter hypermethylation or germ-line mutation can not be excluded. However, the latter seems unlikely assuming a carrier frequency for a *BRCA1* mutation of 0.1% [Turnbull and Rahman, 2008]. Regarding the mean CIN values of the triple-negative tumors, there are no obvious significant differences (data not shown).

Investigating the degree of CIN via FISH for the centromeres of the chromosomes 1, 11, and 17 in *BRCA1*-associated hereditary breast cancers compared to normal breast tissue, Miyoshi et al. [2002] detected higher CIN values in tumors with positive p53 immunostaining harboring a somatic *TP53* mutation. Despite the very small number of tumors per group (4 in the p53-negative and 3 in the p53-positive group), the result is reasonable since the loss of cell cycle checkpoint control seems to allow

proliferation of cells with an abnormal chromosome count. We could not confirm these results with our study. Although we saw a tendency towards a positive correlation between a positive p53 immunostaining and a higher CIN level within the sporadic tumors, this observation could not been confirmed for the *BRCA1*-mutated tumors (data not shown).

Furthermore, *BRCA1*-related tumors are mostly p53 positive [Lakhani et al., 2002]; thus an increased CIN level would have been expected for *BRCA1*-related tumors, which is, however, not the case in our study.

Notably, in our study an even higher degree of CIN for chromosome 1 was seen in sporadic breast cancers. By dividing the tumors into chromosomally stable and chromosomally unstable, again a higher percentage of chromosomally unstable cases was found in the group of sporadic than in the group of *BRCA1*-associated breast cancer. Aberrations of chromosome 1, often resulting in a gain of 1q, are among the most frequent changes in solid tumors and also in breast cancer [Wilkens et al., 2004; Steinemann et al., 2006; Takehisa et al., 2007; Mitelman et al., 2011].

There is a clear discrepancy between the increased genomic instability seen in in vitro and animal models compared with our results in primary breast cancer of *BRCA1* mutation carriers. Centrosomal aberrations and CIN in BRCA1-deficient breast cancer are described as an early event in tumor development [Lingle et al., 2002; Guo et al., 2007]. Pihan et al. [2003] identified centrosomal aberrations and CIN in sporadic pre-invasive carcinomas of the breast, prostate, and uterine cervix. In contrast, late-stage primary breast cancers were investigated in this study. Therefore, we cannot rule out that *BRCA1*-related tumors are initially more unstable and karyotypically heterogeneous and undergo the phenomenon known as 'karyotypic convergence' during tumor progression [Heim et al., 1988]. As a consequence, late-stage tumors show a homogeneous karyotype with a high extent of aneuploidy and stable chromosomal aberrations. Karyotypic heterogeneity disappears during clonal evolution towards the selection of the most favorable chromosomal composition [Chiba et al., 2000]. The last step is the acquisition of a mutation that suppresses CIN, making CIN a target of selective pressure. For example, overexpression of the *Bub1b* spindle checkpoint kinase was reported to have this effect in *Trp53*$^{-/-}$ murine embryonic fibroblasts [Oikawa et al., 2005]. A high rate of CIN may prove to be its Achilles' heel and lead to a high amount of apoptosis and a lower rate of proliferation [Lengauer et al., 1998; Ganem et al., 2009]. Thus, cells are forced to acquire a relatively stable chromosomal composition during tumor development.

Furthermore, it could be speculated that there are no obvious differences between the sporadic and *BRCA1*-related tumors, because centrosomal alterations leading to the degree of CIN are the common end path of all cumulative events that occur within tumor development and progression, independent of the initial origin.

For the first time, a representative number of *BRCA1*-related human primary breast cancers were investigated to unravel the role of CIN in BRCA1 deficiency. We show that there is no difference in numerical CIN between *BRCA1*-associated and sporadic late-stage primary breast cancer. Therefore, the level of CIN is not appropriate as a marker for *BRCA1*-associated breast cancer.

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