MYC Is Amplified in *BRCA1*-Associated Breast Cancers

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ABSTRACT

Purpose: **Germ-line mutations in the *BRCA1* tumor suppressor gene predispose to early onset breast cancers with a distinct phenotype characterized by high tumor grade, aneuploidy, high proliferation rate, and estrogen receptor-negativity. The molecular mechanisms and cooperative oncogenes contributing to multistep tumor progression in cells lacking BRCA1 are not well defined. To examine whether *C-MYC* (*MYC*), a transforming oncogene associated with genetic instability, contributes to multistep tumor progression in *BRCA1*-associated breast cancer, we have analyzed tumors from women with hereditary *BRCA1*-mutated and sporadic breast cancers.**

Experimental Design: **We performed fluorescence *in situ* hybridization using a *MYC:CEP8* assay on formalin-fixed paraffin-embedded tumor tissues from 40 women with known deleterious germ-line *BRCA1* mutations and 62 sporadic cases, including 20 cases with hypermethylation of the *BRCA1* gene promoter.**

Results: **We observed a *MYC:CEP8* amplification ratio ≥2 in 21 of 40 (53%) *BRCA1*-mutated tumors compared with 14 of 62 (23%) sporadic tumors ($P = 0.003$). Of the 14 sporadic cases with *MYC* amplification, 8 (57%) were *BRCA1*-methylated. In total, *MYC* amplification was found in a significantly higher proportion of tumors with BRCA1 dysfunction (29 of 60, 48% *versus* 6 of 42, 14%; $P = 0.0003$). In a multivariable regression model controlling for age, tumor size, and estrogen receptor status, *BRCA1*-mutated tumors demonstrated significantly greater mean *MYC:CEP8* ratio than sporadic tumors ($P = 0.02$).**

Conclusions: **Our data indicate that *MYC* oncogene amplification contributes to tumor progression in *BRCA1*-associated breast cancers. Thus, we conclude that the aggressive histopathological features of *BRCA1*-associated tumors are in part due to dysregulated MYC activity.**

INTRODUCTION

BRCA1 (MIM 113705) is a classical tumor suppressor gene, the loss of the wild-type allele of which is required for breast and ovarian tumorigenesis in germ-line mutation carriers (1, 2). *BRCA1* encodes a multifunctional protein, which together with other proteins contributes to homologous recombination, DNA damage response, and transcriptional regulation, and serves to maintain genomic stability (3). Breast cancers arising in *BRCA1* mutation carriers are usually high grade, aneuploid, highly proliferative, and estrogen receptor (ER)-negative (ER−; Ref. 2). Moreover, the *BRCA1*-mutated tumors have unique gene expression profiles (4, 5). Interestingly, hypermethylation of the *BRCA1* promoter may be an important mechanism for functionally inactivating *BRCA1* in nonhereditary forms of breast cancer (6), as 7–31% of sporadic breast tumors are reported to be *BRCA1*-methylated. It appears that *BRCA1*-methylated sporadic tumors may display pathological features and gene-expression profiles similar to *BRCA1*-mutated hereditary breast cancers (4, 6, 7). However, the cooperating oncogenes and tumor suppressor genes contributing to multistep carcinogenesis in BRCA1-deficient cells remain largely unknown. In a previous study, we showed that *HER-2/neu* oncogene amplification is not a feature of *BRCA1*-associated breast cancers despite the clinicopathological similarities among *HER-2/neu*-amplification-positive tumors and *BRCA1*-associated tumors (8).

MYC[9] oncogene encodes a proliferative nuclear DNA-binding protein, the deregulated expression of which has been shown to play an important role in the induction and progression of lymphomas, lung cancer, and breast cancer (9, 10). *MYC* amplification has been reported as a poor prognostic biomarker

Received 6/26/03; revised 10/17/03; accepted 10/28/03.

Grant support: O. I. O. is supported by the United States Army Department of Defense Grant DAMD17-99-1-9123, the Falk Medical Research Trust, the Breast Cancer Research Foundation, and CA 14955 to the University of Chicago Cancer Research Center. O. I. O. is a Doris Duke Distinguished Clinical Scientist. B. L. W. is supported by grants from the Breast Cancer Research Foundation, the NIH (CA57601), and is an Investigator of the Abramson Family Cancer Research Institute at the University of Pennsylvania Cancer Center.

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[9] *MYC* is the officially accepted name for *C-MYC* (Human Genome Organization Gene Nomenclature Committee http://www.gene.ucl.ac.uk/nomenclature/).

Table 1 Clinical and pathological features of *BRCA1*-mutated hereditary and sporadic breast cancers

Pair-wise comparisons indicated that *BRCA1*-mutated tumors differ from both sporadic tumor groups with respect to age and tumor size; *BRCA1*-mutated and sporadic *BRCA1*-methylated tumors were more likely than sporadic unmethylated tumors to be estrogen receptor (ER)-negative.

		Sporadic tumors (n = 62)		
Feature	*BRCA1*-mutated tumors (n = 40)	*BRCA1*-methylated (n = 20)	*BRCA1*-unmethylated (n = 42)	P^a
Age at diagnosis (years, mean ± SD)	n = 40	n = 19[b]	n = 38	<0.0001
	43 ± 11	56 ± 15	60 ± 16	
Tumor size (cm, mean ± SD)	n = 29	n = 18	n = 39	0.0007
	2.0 ± 1.5	4.5 ± 3.8	3.5 ± 1.9	
Tumor type	n = 35	n = 18	n = 42	0.29
Infiltrating ductal carcinoma	30 (85.7)[c]	16 (88.9)	38 (90.4)	
Intraductal carcinoma	3 (8.5)	0 (0.0)	1 (2.4)	
Medullary carcinoma	2 (5.7)	0 (0.0)	1 (2.4)	
Infiltrating lobular carcinoma	0 (0.0)	2 (11.1)	2 (4.8)	
ER status	n = 31	n = 20	n = 38	0.01
Negative (−)	22 (71.0)	12 (60.0)	13 (34.2)	
Positive (+)	9 (29.0)	8 (40.0)	25 (65.8)	
Nodes involved	n = 27	n = 16	n = 33	0.47
0	9 (33.3)	5 (31.2)	14 (42.5)	
1–3	11 (40.1)	3 (18.8)	11 (33.3)	
4–9	4 (14.8)	6 (37.5)	4 (12.1)	
≥10	3 (11.1)	2 (12.5)	4 (12.1)	
Tumor grade	n = 27	n = 11	n = 28	0.22
1	3 (11.1)	1 (10.0)	4 (14.3)	
2	5 (18.5)	6 (50.0)	10 (35.7)	
3	19 (70.4)	4 (40.0)	14 (50.0)	

[a] Three-group comparison.
[b] Total n per characteristic differs in some cases due to unknown values.
[c] Numbers in parentheses, percentages.

in ~25% (1–48%) of breast tumors and is associated with tumor aggressiveness, including genetic instability, high tumor grade, and ER-negativity (10, 11).

In the present study, using breast tumors from *BRCA1* mutation carriers and sporadic tumors with known *BRCA1* promoter methylation status, we tested the hypothesis that *MYC* oncogene is preferentially amplified in *BRCA1*-associated cancers. To our knowledge, this is the first study to evaluate *MYC* amplification in BRCA1-deficient breast cancer.

MATERIALS AND METHODS

Patient Materials. The study was conducted under research protocols approved by the University of Chicago, University of Pennsylvania, and Creighton University Institutional Review Boards. *BRCA1* mutation carriers were identified through the high-risk clinics at the corresponding institutions where genetic testing is provided as a clinical service. The details of patient enrollment have been reported previously (8). All of the *BRCA1* mutation carriers for whom tumor blocks were available were included. Sporadic breast tumors were identified from cases operated on at the University of Chicago Hospitals as described previously (8). The 102 breast tumors analyzed in the present study were composed of 40 specimens from *BRCA1* germ-line mutation carriers and 62 sporadic cases, used as control for our hybridization assays. Twenty-two of the 40 (55%) specimens from *BRCA1*-mutation carriers were included in our previous study and were negative for HER-2/*neu* amplification/overexpression (8, 12). Specimens from *BRCA1*-mutation carriers were predominantly from young patients (mean age 43), with infiltrating ductal carcinomas (89%) of high grade (70%) that were ER− (71%). The sporadic cancers were skewed toward cases with tumors large enough to contribute to a tissue bank. Pathological features such as tumor grade and ER status were generally distributed in a manner consistent with that in the general population of women with breast cancer. Sporadic tumors were from older individuals and were more frequently ER-positive relative to the *BRCA1*-mutated cases. All 62 of the sporadic cases were evaluated for methylation of the *BRCA1* promoter region. Twenty of the 62 sporadic tumors were *BRCA1*-methylated and displayed tumor characteristics that were intermediate between hereditary and sporadic unmethylated tumors (Table 1).

Fluorescence *in Situ* Hybridization (FISH). Probes and corresponding hybridization mixtures (Vysis/Abbott, Inc., Downers Grove, IL) included *MYC*, labeled with *SpectrumOrange*, and chromosome enumeration probe *CEP8*, labeled with *SpectrumGreen*. The *MYC* probe contains DNA sequences specific for the *MYC* human gene locus and hybridizes to 8q24.12-q24.13 of human chromosome 8. The *CEP8* probe contains α-satellite DNA that hybridizes to the centromeric region of chromosome 8 (8p11.1-q11.1). The *CEP8* probe was used in dual hybridizations with the *MYC* probe as an internal control for chromosome 8 aneusomy. FISH for detection of *MYC* gene amplification in 4–6 μm thick formalin-fixed, paraffin-embedded breast tumor tissue specimens was performed according to protocols described previously (8). In each tumor sample an average of 86 (30–200) well-defined malignant nuclei and in each normal sample an average of 40 (20–70)

nonmalignant nuclei were scored (8). Both the absolute number of *MYC* signals and the ratio of *MYC* signals to chromosome 8 centromere signals were recorded. Tumors with a ratio of *MYC:CEP8* signals of ≥2.0 were considered *MYC*-amplified. The chromosome 8 copy number alteration was estimated by scoring the reduction of *CEP8* signals to one copy (monosomy) and gain of *CEP8* signals to three or more copies (polysomy; Ref. 8). The *CEP9* probe, labeled with *SpectrumOrange*, and *CEP17* probe, labeled with *SpectrumAqua*, contains α-satellite DNA that hybridizes to the centromeric regions of chromosome 9 (9p11-q13) and chromosome 17 (17p11.1-q11.1), respectively. Tumor ploidy was identified by scoring and comparing the mean numbers of *CEP8*, *CEP9*, and *CEP17* signals per cell in a triple-color FISH experiment conducted on a representative group of 12 tumors (7 *BRCA1*-mutated and 5 sporadic).

***BRCA1* Methylation Analysis.** Methylation analysis was performed on DNA isolated from patient tumor slides using a methylation-specific PCR-based approach (13). Methylation-specific primers were designed to the promoter region in exon 1A in the 5′ untranslated region of the *BRCA1* gene (14) taking care to design primers to regions that differ between the *BRCA1* gene and its pseudogene (15). A nested PCR protocol was used. Primers that do not discriminate between methylated and unmethylated *BRCA1* sequence (5′-GTATTTTGAGAGGTTGTTGTTTAG-3′ and 5′-CTAAAAAACCCCACAACCTATC-3′, annealed at 62°C for 15 cycles) were first used followed by primers specific to the methylated and unmethylated sequence (methylated sequence-specific primers: 5′-TCGTGGTAACGGAAAAGCGC-3′ and 5′-AACGAACTCACGCCGCGCAA-3′ annealed at 66°C for 35 cycles; and unmethylated sequence-specific primers: 5′-TTGAGAGGTTGTTGTTTAGTGG-3′ and 5′-AACAAACTCACACCACACAA-3′ annealed at 54°C for 40 cycles). The methylated primers resulted in a 68-bp product, whereas the unmethylated primers resulted in a 100-bp product. As a control for the methylated-specific primers, Sss1 methylase-treated DNA was used that generates DNA completely methylated at all of the CpG sites.

Statistical Analysis. Demographic and disease characteristics expressed on a continuous scale were summarized and compared between *BRCA1*-mutated and sporadic case groups using the F test, followed by pair-wise comparisons where warranted using *t* tests. For discrete characteristics, frequency distributions were compared using Fisher's exact test. Paired comparisons of *MYC* signals, *CEP8* signals, and the *MYC:CEP8* ratio between tumor and normal cells from the same individual were conducted using the paired *t* test.

The mean number of *MYC* signals was plotted against the mean number of *CEP8* signals per tumor for *BRCA1*-associated and sporadic tumors. For each tumor, the ratio of mean *MYC* signals to mean *CEP8* signals was computed. Distributions of these ratios (and the *MYC* and *CEP8* signals) were compared between groups using the F test, followed by pair-wise *t* tests where indicated. To obtain more symmetric distributions suitable for these tests, logarithms of values were used. For these tests and those described above, results of the parametric procedures and their nonparametric counterparts (*e.g.*, Kruskal-Wallis, Wilcoxon rank-sum, and Wilcoxon signed-rank tests) were similar. *MYC:CEP8* ratios were also classified into amplification status categories (*e.g.*, no amplification, amplification) and proportions with amplification compared between groups using Fisher's exact test. Analogous tests were computed for amplification status cross-classified with tumor aneusomy status (monosomic, disomic, or polysomic).

Linear model methods were used to evaluate the relationship between tumor group and *MYC* amplification taking into account differences in patient/tumor characteristics between *BRCA1*-mutated and sporadic tumors. Specifically, a linear regression model was fit, with the logarithm of the *MYC:CEP8* ratio as the response variable and covariates for tumor group and other patient/tumor characteristics that differed between tumor groups as the predictors.

RESULTS

Control values for gene copy numbers for tumor cells were established from evaluation of the adjacent nontumor breast epithelium from 18 *BRCA1*-mutation carriers and from 10 patients with sporadic tumors. Because mean signal copy numbers per cell and copy number ratios were similar between the two groups (data not shown), the control values were evaluated from a pool of all 28 of the nontumoral specimens. The mean number of *MYC* copies per nontumoral cell was 1.87 ± 0.08 (mean ± SD) with no case from either group having more than four *MYC* signals per nucleus. Similarly, the mean number of *CEP8* copies per cell was 1.88 ± 0.06, resulting in a mean ratio *MYC:CEP8* = 1.00 ± 0.05. The mean proportion of cells having only one copy of *CEP8* was 12.5% (± 5.1%). The mean proportion of cells with two *CEP8* signals per nucleus was 87.2% (± 5.4%), and the mean proportion of cells with more than two *CEP8* signals per nucleus was 0.4% (± 0.11%).

Among both *BRCA1*-mutated and sporadic cases, tumor cells had on average significantly greater number of *MYC* copies per cell than the adjacent normal cells ($P < 0.01$ in both cases). In *BRCA1*-mutated tumors, the mean number of absolute *MYC* signals per cell (5.57 ± 2.62) was greater than in the sporadic group (4.10 ± 3.58; $P = 0.02$). *CEP8* copies per cell did not differ between groups, suggesting that *MYC* was targeted for amplification in the *BRCA1*-mutated tumors (Table 2). The mean *MYC:CEP8* ratio in *BRCA1*-mutated tumors (2.37 ± 1.17) was significantly greater than the ratio among sporadic tumors (1.80 ± 1.29; $P = 0.002$). Fig. 1 shows the mean number of *MYC* copies per cell plotted against the mean number of *CEP8* copies per cell, depicted separately for tumors from *BRCA1*-mutated (Fig. 1*A*) and sporadic (Fig. 1*B*) groups. Fourteen (23%) of the sporadic tumors had a *MYC:CEP8* ratio of two or higher, a proportion of tumors comparable with the ~25% of breast tumors that have been reported with *MYC* amplification in the literature (10). However, the proportion of *MYC*-amplified tumors was significantly higher in the *BRCA1*-mutated group (21 of 40, 53%; $P = 0.003$). (A representative photomicrograph of a *MYC*-amplified *BRCA1*-mutated tumor is shown on Fig. 2*A*).

Next we asked whether *MYC* amplification in the sporadic cases was associated with *BRCA1*-methylation. Twenty of the 62 sporadic tumors were *BRCA1*-methylated, and 8 of these showed *MYC* amplification (Fig. 1*B*). We observed significant heterogeneity among these tumors because *BRCA1* methylation was incomplete in the majority of cases (methylated and un-

Table 2 Mean numbers of *MYC* and *CEP8* copies per cell and mean ratio in tumor tissues from *BRCA1*-mutated and sporadic cases

Parameter	*BRCA1*-mutated tumors ($n = 40$)	Sporadic tumors ($n = 62$)		*P* (three-group comparison)	*P* (pair-wise test)		
		BRCA1-methylated ($n = 20$)	*BRCA1*-unmethylated ($n = 42$)		*BRCA1 vs.* methylated	*BRCA1 vs.* unmethylated	Methylated *vs.* unmethylated
MYC	5.57	5.32	3.53	0.0005	0.21	<0.0001	0.07
CEP8	2.46	2.23	2.29	0.43	–	–	–
MYC:CEP8	2.37	2.36	1.53	0.0005	0.48	<0.0001	0.06

methylated DNA species were observed; data not shown). The mean absolute *MYC* copy number per cell was 5.32 ± 5.09 in *BRCA1*-methylated tumors, compared with 3.53 ± 2.45 in unmethylated tumors ($P = 0.07$); mean *MYC:CEP8* signal ratios were 2.36 ± 1.91 in *BRCA1*-methylated tumors and 1.53 ± 0.74 in unmethylated tumors ($P = 0.06$; Table 2). Pairwise comparisons between the *BRCA1*-mutated and sporadic groups (*i.e.*, *BRCA1*-methylated, unmethylated), however, revealed that *BRCA1*-mutated tumors had significantly higher *MYC* copy numbers per cell ($P < 0.0001$) and *MYC:CEP8* signal ratios ($P < 0.0001$) than sporadic unmethylated cancers (Table 2).

Because the three groups differed with respect to age, tumor size, and ER status (Table 1), we additionally examined whether the *MYC:CEP8* signal ratio remained greater in *BRCA1*-mutated tumors after these differences were taken into account. A multiple regression model was fit, with log (*MYC:CEP8* signal ratio) as the response variable, and the tumor group (*BRCA1*-mutated *versus* all sporadic), age, tumor size, and ER status as the predictors. Results indicated a significant difference in mean *MYC:CEP8* ratio values between the *BRCA1*-mutated and sporadic groups after adjustment for age and tumor variables ($P = 0.02$; Table 3). This finding was similar to that of the univariate comparison of *BRCA1*-mutated tumors to all of the sporadic cases ($P = 0.002$). Another regression analysis comparing *BRCA1*-mutated, *BRCA1*-methylated, and unmethylated cases was also conducted, with similar findings, in that *BRCA1*-mutated and *BRCA1*-methylated tumors had significantly greater *MYC:CEP8* ratio than sporadic unmethylated tumors after adjustment for age, tumor size, and ER ($P = 0.005$ and $P = 0.05$, respectively; data not shown). In both of these analyses, age, tumor size, and ER status were not significant predictors of *MYC* amplification. It is interesting that *BRCA1* methylation was observed in 57% (8 of 14) of *MYC*-amplified sporadic tumors (Fig. 1*B*), but in only 25% (12 of 48) of those without *MYC* amplification ($P = 0.05$), strengthening the association between *MYC* amplification and loss of BRCA1 function. (A representative photomicrograph of a *MYC*-amplified *BRCA1*-methylated tumor is shown in Fig. 2*B*). When *BRCA1*-mutated and *BRCA1*-methylated tumors were grouped (BRCA1-deficient tumors) and compared with sporadic unmethylated tumors, a higher proportion of BRCA1-deficient tumors had *MYC* amplification (29 of 60, 48% *versus* 6 of 42, 14%; $P = 0.0003$).

It was of interest to know whether the type of *BRCA1* mutation can predict *MYC* amplification, because in our study not all of the *BRCA1*-mutated tumors displayed *MYC* amplification. Table 4 summarizes the *BRCA1* mutation status and *MYC* amplification status of tumors from *BRCA1* mutation carriers. We did not observe any clear genotype/phenotype correlation as the mutations were scattered all over the gene. *MYC* amplification was observed not only in tumors with mutations in the regions preceding or directly associated with MYC binding sites of BRCA1 (16), but also in cases with mutations beyond the MYC binding sites (Table 4).

Lastly, we assessed *CEP8* signals to determine aneusomy for chromosome 8 in tumor cell. The pattern of chromosome 8 aneusomy was similar between *BRCA1*-mutated and *BRCA1*-methylated tumors (data not shown); hence, we compared BRCA1-deficient and sporadic unmethylated groups. Computation of the mean number of *CEP8* signals per cell revealed that the majority of tumors from both the BRCA1-deficient and sporadic unmethylated groups had definite gains of chromosome 8 as has been reported in breast cancer literature (Ref. 17; Table 5; mean *CEP8* copies per cell ≥2; Fig. 1, *A* and *B*). However, as described above, the mean absolute *MYC* signals per cell was greater in the BRCA1-deficient cases (5.49 ± 3.59) than in the sporadic unmethylated group (3.53 ± 2.45; $P = 0.0002$), as was the mean *MYC:CEP8* ratio (2.37 *versus* 1.53; $P < 0.0001$). In all, 35 of 102 (34%) tumors analyzed in our study were *MYC*-amplified (Table 5), and the majority of tumors with *MYC* amplification displayed chromosome 8 polysomy (Fig. 1, *A* and *B*). To demonstrate that the gain of chromosome 8 was not random or due to tissue preparation artifact, we evaluated aneusomy for chromosome 8 relative to chromosome 9 and chromosome 17 in triple color *CEP8/CEP9/CEP17* FISH on a representative group of 12 tumors (7 *BRCA1*-mutated and 5 sporadic). The comparisons among *CEP17*, *CEP8*, and *CEP9* in *BRCA1*-mutated tumors indicate significant reduction of *CEP17* to one copy as compared with either *CEP8* (mean paired difference = −0.77) or *CEP9* copy number (mean difference is −1.01). There was a higher mean copy number of *CEP8* per cell and a larger proportion of cells with ≥3 copies/cell than was observed with *CEP9* (data not shown). It does appear that *CEP8* tended to differ by *MYC* amplification status, whereas the *CEP9* and *CEP17* did not (data not shown). Thus, consistent with our previous publication (8) we observed increased copy number of chromosome 8 (correlated with *MYC* amplification), reduction of chromosome 17 to one copy, and more or less stable copy number of chromosome 9.

DISCUSSION

In the present study, we showed that *MYC* amplification is a frequent event in breast tumors from *BRCA1* germ-line mutation carriers and in sporadic tumors with *BRCA1* promoter hypermethylation. This observation is in contrast to our previous

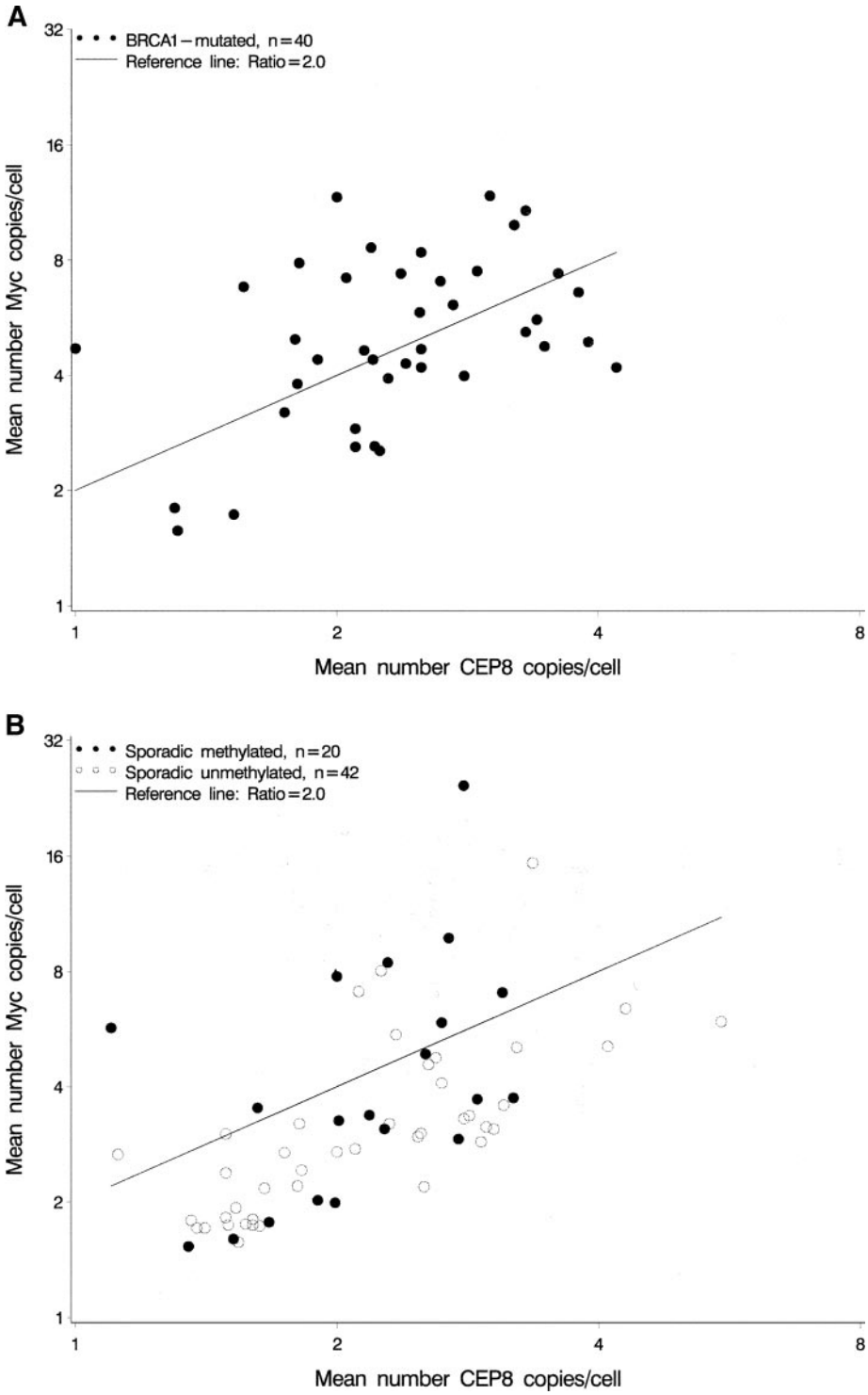

Fig. 1 Distribution of the mean number of *MYC* copies relative to chromosome 8 centromere signals in breast cancer tissues: *BRCA1*-mutated (*A*), sporadic methylated (*B*, ●) and unmethylated (*B*, ○). Ratio ≥2, the cutoff point for *MYC* amplification, is indicated by a *solid line*. *A* and *B*, *MYC*:*CEP8* ratios were greater for *BRCA1*-mutated tumors as compared with all sporadic cases (mean 2.37 *versus* 1.80; $P = 0.002$). *MYC* amplification was present in 53% of *BRCA1*-mutated tumors compared with 23% of sporadic tumors ($P = 0.003$). *B*, 20 of the 62 sporadic tumors were *BRCA1*-methylated, and 8 (40%) of these showed *MYC* amplification. The mean *MYC*:*CEP8* ratio was greater for BRCA1-deficient tumors (*A* and *B*, ●) compared with sporadic unmethylated tumors (*B*, ○; mean 2.37 *versus* 1.53; $P < 0.0001$). *MYC* amplification was present in 48% of BRCA1-deficient tumors compared with 14% of sporadic tumors with presumably normal function of BRCA1 ($P = 0.0003$).

finding that no *BRCA1*-associated tumors had high levels of *HER-2/neu* amplification (8). Thus, our data support an association of *MYC* amplification with breast tumors from *BRCA1* mutation carriers. In addition, the similarity observed between tumors from germ-line *BRCA1*-mutation carriers and some methylated sporadic tumors suggest that *MYC* is a cooperative oncogene in tumor cells lacking BRCA1. To our knowledge, this is the first study to address the contribution of *MYC* to hereditary *BRCA1*-mutated and *BRCA1*-methylated sporadic breast cancers.

MYC amplification in sporadic breast cancers has been intensely studied using different methods. However, the results

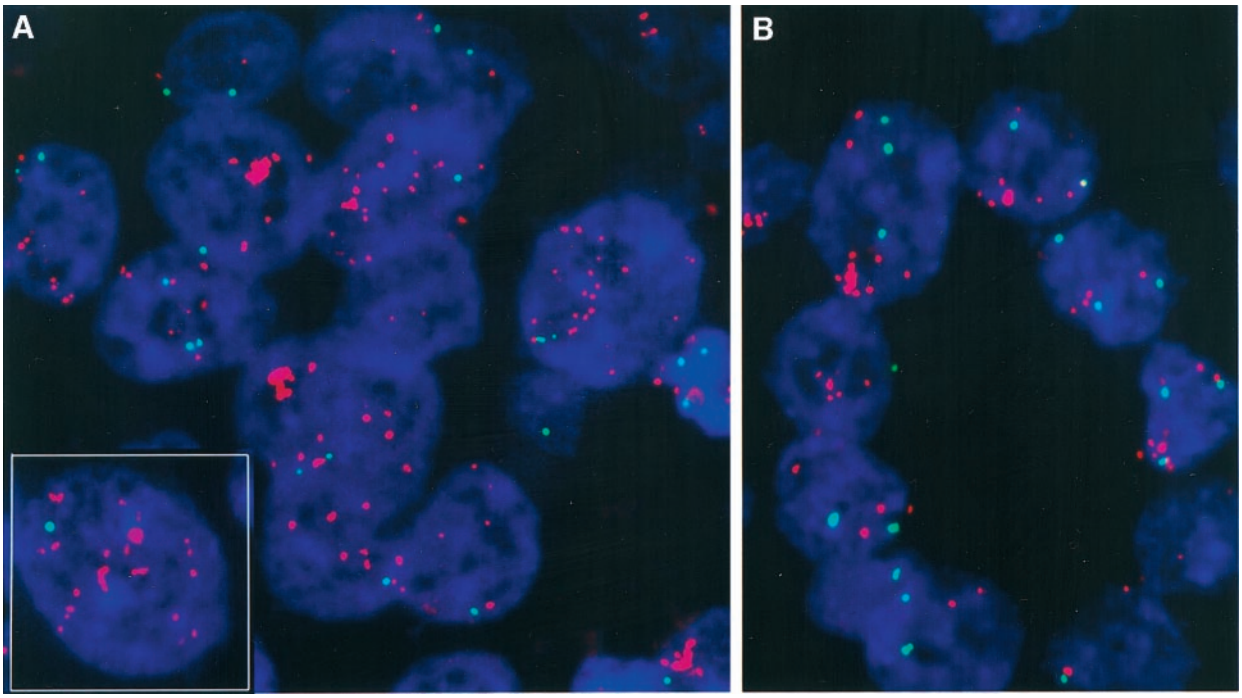

Fig. 2 Representative photomicrographs of breast tumor tissue sections from BRCA1-deficient cancers after fluorescence *in situ* hybridization. The *MYC* gene is identified by red fluorescent signals, and the chromosome 8 centromere (*CEP8*) is identified by green fluorescent signals. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue*). Original magnification, ×1250. *A*, highly *MYC*-amplified cancer from *BRCA1* germ-line mutation carrier (ratio = 6.0). A mean of 12 (3–40) *MYC* signals per nuclei were observed. The tumor was heterogeneous, monosomic/polysomic for chromosome 8. The patient was 32 years old. *Inset*, nucleus from the neighboring field of the same tissue section. *B*, *MYC*-amplified *BRCA1*-methylated sporadic tumor (ratio = 3.9). The mean copy number of *MYC* per cell was 8 (4–14); 75% of nuclei revealed two to three *CEP8* signals. The patient was 40 years old.

have been controversial, partly due to low sensitivity of some of the analytic methods used (10, 18). Studies, in which *MYC* amplification was detected by FISH method, listed in Table 5, show that 12–45% of breast cancer cases have *MYC* amplification. The proportion of *MYC*-amplified tumors found in our study is comparable with the majority of these reports. Similar to previous studies, we did not find any correlation between *MYC* amplification and age or tumor size. We also found no association between *MYC* amplification and ER-negativity, which is in agreement with some reports (17, 19, 20) but in contradiction to others (21). For the first time, however, we found, that *MYC* oncogene amplification can be associated with BRCA1 inactivation status.

An association among *BRCA1* methylation, loss of BRCA1 transcripts, and reduced or undetectable BRCA1 protein expression has been described in about 20–30% of sporadic tumors (6, 22). We observed *MYC* amplification in 40% of *BRCA1*-methylated sporadic tumors. Interestingly, despite the methylation heterogeneity, the mean *MYC* and *CEP8* copy numbers per cell, copy number ratios, the proportion of *MYC*-amplified tumors, and the pathological features of *BRCA1*-methylated tumors were comparable with the parameters of *BRCA1*-mutated hereditary tumors and appear to be intermediate between *BRCA1*-mutated hereditary and sporadic unmethylated tumors. Thus, these data suggest that loss of BRCA1 in some sporadic breast cancers through epigenetic mechanism(s) such as promoter methylation contribute to the development of those tumors (6), and appears to precede and, hence, promote *MYC* amplification, as we observed in the hereditary *BRCA1*-mutated tumors.

Our data are consistent with previous studies in familial breast cancers. DNA microarray-based analyses have suggested

Table 3 Multivariable regression model for *MYC:CEP8* ratio

The analysis indicates that *BRCA1*-mutated tumors have significantly greater *MYC:CEP8* ratio than sporadic tumors after adjustment for age, tumor size, and estrogen receptor (ER). None of these factors were significant predictors of *MYC:CEP8* ratio.

Covariate[a]	$\hat{\beta}$	SE ($\hat{\beta}$)	*P* for test: $\beta = 0$
BRCA1-mutated (*vs.* all sporadic)	0.333	0.142	0.02
Age at diagnosis	−0.0006	0.004	0.88
Tumor size >2.0 cm (*vs.* ≤2.0 cm)	0.114	0.126	0.37
Tumor size unknown (*vs.* ≤2.0 cm)	0.056	0.171	0.74
ER negative (*vs.* ER positive)	0.140	0.114	0.22

[a] Tumor group, tumor size, and ER are represented as (0.1) indicator variables in the model, and coefficients represent change in log *MYC:CEP8* ratio relative to reference category shown in parentheses. Analysis is based on 88 of 102 cases with complete data for age and ER. Because tumor size was unavailable for 15 cases, a category "unknown" was created and used as a covariate in order to retain these cases in the model. Results of the same analysis omitting these 15 cases were similar. The estimated intercept for the model is 0.353.

Table 4 Association between *BRCA1* mutation and *MYC* amplification in 40 hereditary breast cancers

BRCA1 mutation designation[a]	Exon	Amino acid change	Mutation effect	N of cases (n = 40)	Amplified (n = 22)	Nonamplified (n = 18)
185delAG	2	Stop 39	F[b]	6	4	2
188del11	2	Stop 39	F	1	0	1
230delAA	3	Stop 39	F	1	1	0
C64Y	5	Cys to Tyr	M	2	1	1
C64G	5	Cys to Gly	M	1	0	1
E143X	7	Glu to stop	N	1	0	1
IVS8+2T-A	**8**	**–**	**S**	**1**	**1**	**0**
633delC	**8**	**Stop 233**	**F**	**2**	**1**	**1**
300Cys-Gly (T-G)	*11A*	*Cys to Gly*	*?*	*1*	*1*	*0*
1205delGA	*11A*	*Stop 364*	*F*	*1*	*0*	*1*
1374 delG	*11A*	*Stop 419*	*F*	*1*	*0*	*1*
1406insA	***11A***	***Stop 434***	***F***	***1***	***1***	***0***
2080delA	11B	Stop 700	F	1	0	1
2187delA	11B	Stop 699	F	1	0	1
Q780X	11B	Gln to stop	N	2	2	0
2576delC	11C	Stop 845	F	1	0	1
E908X	11C	Glu to stop	N	2	1	1
Y978X	11C	Tyr to stop	N	1	0	1
R1443X	*13*	*Arg to stop*	*N*	*2*	*1*	*1*
P1637L	*16*	*Pro to Leu*	*UV*	*1*	*1*	*0*
5272-2delA	*19*	*Stop 1719*	*F*	*1*	*0*	*1*
5296del4	*19*	*Stop 1728*	*F*	*3*	*3*	*0*
69222del 80582[c]	*19*	*Del exons 19 to 22*	*F*	*1*	*1*	*0*
5382insC	*20*	*Stop 1829*	*F*	*1*	*0*	*1*
5385insC	*20*	*Stop 1829*	*F*	*2*	*1*	*1*
R1835X	*24*	*Arg to stop*	*N*	*2*	*2*	*0*

[a] Mutations that directly affect MYC-binding sites (175–303 and 433–511aa) are marked in bold. Mutations that directly affect Nmi-binding sites (298–683 and 1301–1863aa) are marked in *italic*.
[b] F, frameshift; M, missense; N, nonsense; S, splice; UV, unclassified variant.
[c] Genomic destination of mutation is shown.

that breast cancers arising in the setting of germ-line *BRCA1* mutations have unique gene expression profiles, and sporadic tumors with methylated *BRCA1* may be misclassified with the *BRCA1*-mutation-positive group (4, 5). We reviewed the set of genes published by Hedenfalk *et al.* (Ref. 4; 7 *BRCA1* tumors) and by van 't Veer *et al.* (Ref. 5; 18 *BRCA1* tumors), and found that *MYC* on 8q was overexpressed in *BRCA1* mutation carriers (data not shown). By conventional comparative genomic hybridization, 8q23–24 amplicon has been described in hereditary as well as in sporadic breast cancers, and *MYC* has been suggested

Table 5 Frequency of *MYC* amplification in fluorescence *in situ* hybridization studies of breast cancer reported in the literature and in current study

Cases analyzed	*MYC* amplified	Correlations and comments	Reference
261	38 (14.6%)	DNA aneuploidy, PR(−),[a] high tumor grade, node positive, high S-phase, tumor aggressiveness. No association with ER(−), age, tumor size, metastases.	Rummukainen *et al.*, 2001b (20)
177	25 (14%)	DNA aneuploidy, PR(−), high tumor grade, node positive, high S-phase. No association with ER(−), age, tumor size, metastases.	Rummukainen *et al.*, 2001a (19)
100	21 (21%)	S phase, DNA index and ER(−). No correlation with tumor size. Only node negative IDC analyzed.	Persons *et al.*, 1997 (21)
74	9 (12%)	No clinicopathologic parameters mentioned. Amplification detected in tissue microarray.	Schraml *et al.*, 1999 (31)
53	24 (45%)	Hypertetraploidy/hypertetrasomy. All cells aneusomic for chromosome 8 exhibited extensive *MYC* amplification.	Janocko *et al.*, 2001 (17)
26	22 (86%)	DNA aneuploidy, chromosome 8 polysomy, high tumor grade, high tumor stage.	Visscher *et al.*, 1997 (32)
23	7 (30.4%)	High tumor grade. No correlation with chromosome 8 polysomy. Only DCIS analyzed.	Fiche *et al.*, 2000 (33)
20	5 (25%)	DNA aneuploidy. No other clinicopathologic parameters mentioned. Amplification detected in fine needle aspirates.	Heselmeyer-Haddad *et al.*, 2002 (34)
102	35 (34%)	*BRCA1* status (mutation and methylation). No correlation with age, tumor size, ER(−). 60 BRCA1-deficient (40 hereditary *BRCA1*-mutated and 20 sporadic *BRCA1* methylated) and 42 sporadic tumors with presumably normal BRCA1 were analyzed.	Grushko *et al.* (current study)

[a] PR, progesterone receptor; ER, estrogen receptor.

as a target of this amplification (11, 23, 24). Moreover, mice carrying conditional *BRCA1* mutation display gain of chromosome 15 (orthologous to human chromosome 8q24) by CGH and overexpression of MYC protein by Western blot analysis (25, 26). However, the observation of preferential *MYC* amplification in our study does not itself rule out the possible importance of other genes in the 8q24 region, which may be coamplified with *MYC* (27).

The observed similarities between *BRCA1*-mutated and *BRCA1*-methylated sporadic tumors support a tumor progression model in which early loss of BRCA1 function causes defects in chromosome structure, cell division, and viability, so that a BRCA1-deficient cell must acquire additional alterations that overcome these problems and presumably force tumor evolution down a limited set of pathways (3). Our data suggest that MYC function might be critical or important in these pathways. BRCA1 protein contains several functional domains that interact directly or indirectly with a variety of molecules, and it likely serves as an important central component in multiple biological pathways (22). BRCA1 contains at least two nuclear localization sequences, which are required for translocation into the nucleus. The presence of a transactivation domain and the association of BRCA1 with the RNA polymerase II holoenzyme suggest that BRCA1 might be involved in gene transcription. Consistent with this notion, Wang *et al.* (16) demonstrated that BRCA1 physically binds to MYC and represses its transcriptional and transforming activity. Furthermore, they showed that BRCA1 reverses the phenotype of rat embryonic fibroblasts transformed by *myc-ras* activation. Another group found that in addition to direct binding to C-MYC, BRCA1 specifically binds to Nmi (N-MYC-interacting protein; Ref. 28) and that later is functioning as an adaptor molecule to recruit MYC to a complex with BRCA1. The authors showed that through disruption of Nmi-BRCA1-MYC tri-complex constructs with BRCA1 mutations within Nmi binding sites are unable to indirectly suppress the oncogenic potential of MYC. These data indicate that BRCA1 is a component of a transcription factor complex and may in part function as a tumor suppressor by regulating MYC activity (28). Thus, our observation that MYC activation through gene amplification occurs in a high proportion of human *BRCA1*-mutated hereditary and *BRCA1*-methylated sporadic cases provides additional support for a role for MYC in *BRCA1*-associated tumor progression. Future work will evaluate the mechanisms of *MYC* amplification in BRCA1-deficient cells.

In our study not all of the *BRCA1*-mutated tumors displayed *MYC* amplification, suggesting a possible association between the type of *BRCA1* mutation and *MYC* amplification. However, no such association was found. It was shown previously that BRCA1 contains two regions that independently interact with MYC and require amino acid residues 175–303 and 343–433. The two regions span exons 8, 9, and 10 and the NH_2-terminal portion of exon 11 (16). *MYC* amplification was observed not only in tumors with *BRCA1* mutations located upstream or within MYC binding sites, but also in cases with mutations located downstream of binding sites. Apparently, the regions downstream of MYC binding sites may indirectly affect BRCA1-MYC interaction, or the BRCA1 truncated protein formed might be unstable and incapable of strong interaction with MYC. For example, mutations of BRCA1 within Nmi binding sites (298–683 and 1301–1863 amino acids) may disrupt Nmi-BRCA1-MYC tricomplex thereby suppressing the oncogenic potential of MYC (Ref. 28; Table 4). In addition, because DNA methylation is a quantitative process, the heterogeneity observed in the *BRCA1*-methylated cases is possibly related to the degree of methylation of the promoter region (the quantity and density of CpG islands involved), causing different levels of concentration or complete absence of the protein product.

The *BRCA1* mutant tumors appear to have a profile that is most consistent with the basal-like subtype suggested by Perou *et al.* (29) and Sorlie *et al.* (30) based on the following observations. First, both (meaning sporadic basal-like tumors and *BRCA1* mutant tumors) tend to be high grade, ER/progesterone-receptor negative and *HER-2/neu*-negative, and both show *MYC* amplification. In fact, *MYC* emerged as one of the most relevant genes that defined the basal-like group and was expressed 2–4 fold above background in the majority of cases.[10] Moreover, we have shown previously that *BRCA1*-mutated tumors express specific basal cytokeratins in a manner suggestive of an ER–, basal-like epithelial cell of origin (12) and are never associated with high levels of *HER-2/neu* amplification (8). Therefore, it is reasonable to suggest that *BRCA1*-mutated tumors are mostly basal-like (ER−, HER2−), and that *MYC* amplification additionally defines a subset of these tumors. Additional studies of a larger cohort of *BRCA1*-associated tumors are ongoing to dissect the role of cooperative oncogenes and tumor suppressor genes in the progression of these breast cancers.

ACKNOWLEDGMENTS

We thank Dr. Katrin Carlson for critical reading of the manuscript.

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