Distinct Molecular Pathogeneses of Early-Onset Breast Cancers in *BRCA1* and *BRCA2* Mutation Carriers: A Population-based Study[1]

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ABSTRACT

Breast cancers arising in women with and without a germline mutation in the *BRCA1* or *BRCA2* gene display different histological features, which suggests unique mechanisms of molecular pathogenesis: We used a molecular pathological analysis to define the genetic abnormalities relevant to these specific pathogeneses. Tumor material was studied from 40 women with breast cancer diagnosed before 40 years of age, sampled from a population-based study and stratified by *BRCA1* and *BRCA2* germline mutation status. Cases were not selected for family history or ethnic origin, and none were known to be genetically related. Thus, germline mutation itself is likely to impact on the molecular pathogenesis of these tumors, with no substantial influence due to modifying genetic or environmental factors. Breast cancers occurring in *BRCA1* mutation carriers had significantly higher levels of p53 expression, including the preinvasive (carcinoma *in situ*) stage of disease, compared with cancers occurring in *BRCA2* mutation carriers or women with no detectable germline mutation. These cancers also had a higher proliferation rate as measured by Ki-67 antibody. Expression of the prognostic factors c-erbB-2, cyclin D1, and estrogen receptor was significantly less common in *BRCA1* mutation carriers. Lower levels of cyclin D1 were also found in cancers from *BRCA2* mutation carriers compared with non-mutation carriers. Direct p53 mutation analysis revealed mutations in 18% of all of the early-onset breast cancers within the study and included rare insertion and deletional mutations in cancers from *BRCA1* mutation carriers. Our data indicate that a *BRCA1* breast cancer phenotype may be recognized by an exceptionally high proliferation rate and early and frequent p53 overexpression but infrequent selection for overexpression of several other prognostic factor proteins known to be involved in breast oncogenesis. In contrast, breast cancers arising in *BRCA2* mutation carriers have a more heterogeneous phenotypic profile.

INTRODUCTION

There is increasing evidence that specific histological phenotypes can be recognized in breast cancers occurring in women with germline mutations in the breast cancer susceptibility genes, *BRCA1* and *BRCA2* (1–7). Typically, breast cancers occurring in *BRCA1* mutation-carrying heterozygotes are high-grade and have a high proliferation rate, with medullary or atypical medullary cancers being overrepresented. In contrast, lobular cancers and extensive intraduct cancers are more frequent in women with germline *BRCA2* mutations. Certain histological subtypes of invasive breast cancers have been associated with altered somatic expression of tumor suppressor genes

and oncogenes, such as the reduced E-cadherin expression and increased cyclin D1 expression reported in infiltrating lobular cancers (8, 9). Thus, the distinct histological phenotypes identified in breast cancers occurring in *BRCA1* and *BRCA2* mutation carriers may be a consequence of both the germline mutation and subsequent specific somatic mutations occurring in breast tissue.

Recent studies on breast cancers diagnosed in all age groups have indicated that those in *BRCA1* mutation carriers are typically ER-,[3] PR-, and c-erbB-2-negative (10–13). In addition, high rates of mutation in the tumor suppressor gene *p53* have been identified in breast cancers from *BRCA1* and *BRCA2* mutation carriers (14–16). However, one study of Jewish women found no difference in p53 protein expression in BRCA1 and BRCA2-associated breast cancers diagnosed before age 42 years when compared with breast cancers in noncarriers (13). A further study demonstrated that p53 over-expression in cancers occurring in *BRCA1* mutation carriers was predominantly associated with germline mutations leading to protein truncation within the ring finger domain of the *BRCA1* gene (17). The majority of these molecular data have been accrued from families with multiple individuals affected by breast cancer or from Jewish or Icelandic women, who are known to have a high prevalence (because of founder effects within their populations) of a germline mutation at specific sites within these two genes. Thus it is possible that the pathogenesis of breast cancers occurring in these defined groups is influenced not only by the specific site of the germline *BRCA1* or *BRCA2* mutation but also by other modifying genetic or environmental factors specific to these groups of women. Conversely, it is not known whether the somatic molecular alterations described in the cancers of these selected population subgroups resemble those seen in the general population.

The histological and biological characteristics of breast cancers occurring in premenopausal women are said to differ from those diagnosed in older women. Compared with later-onset breast cancers, early-onset breast cancers are commonly high-grade with a high proliferation rate, and medullary or atypical medullary cancers are more common (18–20). Early-onset breast cancers also commonly lack ER expression and show more frequent p53 overexpression (20, 21). All of the above characteristics have also been reported in cancers arising in *BRCA1* mutation carriers. Hence, there is uncertainty as to the extent to which breast cancers occurring in women with germline *BRCA1* mutations can be distinguished from early-onset cancers arising in women without germline mutations in this gene. In addition, little is known about the molecular pathogenesis of early-onset breast cancer in general during its development and progression to invasive cancer. In this study, we investigate the molecular and immunophe-

[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; ABCFS, Australian Breast Cancer Family Study; TE buffer, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA; PTT, protein truncation test; EGFR, epidermal growth factor receptor; LSAB, labeled streptavidin-biotin; LOH, loss of heterozygosity; DCIS, ductal carcinoma *in situ*; SSCP, single strand confirmation polymorphism.

Table 1 *Antibodies used for immunohistochemistry profile, blocking, and detection kits*

Antibody	Class	Dilution	Source	Antigen retrieval	Blocking	Detection kit
p53 (DO7)	MM[a]	1:200	Novocastra Laboratories (Newcastle-Upon-Tyne, United Kingdom)	PC		LSAB+
p21	MM	1:8000	Santa Cruz Biotechnology (Santa Cruz, CA)	PC	PBA	LSAB+
Cyclin D1	RP	1:400	Upstate Biotechnology (Lake Placid, NY)	PC		LSAB+
bcl-2	MM	1:50	DAKO Corporation (Carpinteria, CA)	PC		LSAB2
Ki-67	RP	1:100	DAKO Corporation	PC		LSAB2
PCNA	MM	1:400	DAKO Corporation	None		LSAB2
c-erbB-2	RP	1:400	DAKO Corporation	MW		LSAB2
EGFR	MM	1:100	Biogenex (San Ramon, CA)	E	PBA	LSAB+
ER	MM	1:50	DAKO Corporation	PC		LSAB2
PR	MM	1:50	DAKO Corporation	PC		LSAB2
pS2	RP	1:100	Novocastra Laboratories	None	PBA	LSAB2
Cathepsin D	RP	1:600	DAKO Corporation	PC		LSAB2
E-cadherin	MM	1:3000	Zymed Laboratories (San Francisco, CA)	PC		LSAB+
β-Catenin	GP	1:2000	Santa Cruz Biotechnology	PC	PBA	LSAB+

[a] MM, mouse monoclonal; RP, rabbit polyclonal; GP, goat polyclonal; PC, pressure cooker; MW, microwave; E, enzyme digestion with pepsin; PBA, protein blocking agent; LSAB, labeled streptavidin-biotin.

notypic events that occur during the preinvasive and invasive stages of early-onset breast cancers occurring in a population-based sample, either with or without a *BRCA1* or *BRCA2* germline mutation.

MATERIALS AND METHODS

Case Selection. We analyzed tissue from 40 women with breast cancer diagnosed before the age of 40 years, selected from the population-based ABCFS (22, 23). Participants in the ABCFS were recruited from incident, histologically confirmed, first primary cases of invasive breast cancer as recorded on the Victorian and New South Wales State Cancer Registries. Verification of every cancer reported in a relative was sought through cancer registries, pathology reports, hospital records, treating clinicians, and death certificates. All of the women gave informed consent to participate, and the study was carried out with the approval of all of the relevant institutional ethics committees. A total of 467 breast cancer cases, diagnosed between 1992 and 1995, were studied, and blood samples were available from 388 (83%) of these. Mutation detection for *BRCA1* and *BRCA2* identified 10 cases with a germline *BRCA1* mutation and 9 cases with a germline *BRCA2* mutation (1). In addition, 21 cases were randomly selected, who did not carry a mutation in either *BRCA1* or *BRCA2*, and were designated as "control" cancers for comparisons with mutation carriers.

BRCA1 and BRCA2 Mutation Detection. *BRCA1* and *BRCA2* mutation detection was performed on DNA extracted from peripheral blood-derived buffy coat using a Progenome II DNA extraction kit (Progen, Ipswich, Australia) and stored in TE buffer. The PTT (24) was used to detect truncating mutations in exon 11 of *BRCA1,* using primers described by Hogervorst *et al.* (25) and Friedman *et al.* (26). Exon-flanking primers based on the *BRCA2* genomic sequence (27) were used for the PTT of *BRCA 2* exons 10 and 27, and primers to three overlapping exon fragments were used for exon 11. Mutations detected by PTT were confirmed by cycle sequencing (Perkin-Elmer). If no mutation was found by these methods, sequencing of the remaining exons of *BRCA1* and *BRCA2* was performed except in 4 cases for whom no further peripheral blood was available. The PCR primers used for cycle sequencing were based on those described by Simard *et al.* (28) for *BRCA1*, and Wooster *et al.* (27) for *BRCA2*.

Immunohistochemical Studies. Immunohistochemical studies were performed on sections obtained from paraffin blocks of tissues fixed in 10% neutral buffered formalin. Three-μm sections cut from paraffin wax blocks were placed onto silane-coated slides and dried at 60°C for 30 min. The sections were dewaxed in histolene and rehydrated through graded alcohols. Antigen retrieval was used for the antibodies indicated in Table 1. The antigen retrieval methods used were 2 min at pressure in a pressure cooker or 20 min on low setting in a microwave, both in 10 mM sodium citrate (pH 6.0), or 5 min enzyme digestion with pepsin at 37°C. The DAKO Autostainer was used for the immunohistochemistry staining. All of the wash steps used 50 mM Tris-HCl (pH 7.6) and 0.05% Tween 20. The sections were treated with 3% hydrogen peroxide for 10 min. Some of the antibodies had a blocking step for 5 min using Protein Blocking Agent (Immunon, Pittsburgh, PA) as indicated in Table 1. The sections were incubated in primary antibody at the dilutions listed

in Table 1 for 30 min at room temperature with the exception of EGFR, which was incubated at 37°C for 60 min. Sections were incubated with biotinylated secondary antibody, followed by peroxidase-conjugate Streptavidin using DAKO LSAB2 or LSAB+ kits as indicated in Table 1. LSAB2 incubation times were 10 min for each step; LSAB+ incubation times were 15 min for each step. Staining was visualized using 3-amino-9-ethyl-carbazole, washed in water, and counterstained with hematoxylin. Crystal Mount (Biomedia, Foster City, CA) was applied to sections and dried on a 60°C hotplate. The sections were then coverslipped with DPX mountant.

For all of the antibodies except Ki-67, the intensity of staining and proportion of positive cells were determined for the normal breast epithelium, DCIS, and invasive carcinoma components from each case, modified from the method described by Allred *et al.* (29) The proportion score represented the estimated fraction of positive staining cells (0, ≤10%; 1, 11–25%; 2, 26–50%; 3, 51–75%; 4, 76–90%; 5, ≥91%). The intensity score represented the estimated average staining intensity of positive cells (0, none; 1, weak; 2, moderate; 3, strong). The results were then analyzed by two different methods. The first was a simple estimate of whether the cellular component was positive or negative for each antibody, positivity being defined as greater than 10% of cells positive. The second method took into account both the intensity and the proportion of positive cells to give a semiquantitative estimate of the expression levels of antigen in the tissue. Samples with intensity scores of 0 or 1 were designated as negative-to-weak expression. For intensity scores of 2 and 3, a combined score was derived by adding the intensity and proportion scores. Combined scores of 2 and 3 were designated as negative-to-weak expression, 4–6 as moderate expression, and 7 or 8 as strong expression. For each antibody, only the cellular compartment that was expected to express the antigen of interest was scored (*i.e.,* either nuclear, cytoplasmic, or membranous staining). The expression level was not calculated for Ki-67 antibody staining because this antibody was used simply as an indication of the number of cells in the cell cycle. Instead the proportion score was calculated at both 10% and 50% positive cell cutoff points.

Microdissection and DNA Extraction. Microdissection of target tissue was performed on dewaxed, methyl green-stained slides using a 21-gauge needle. Microdissected material (approximately 500 cells per μl of buffer) was placed directly into a 0.5-ml tube containing Proteinase K buffer (10 mM Tris-KCl (pH 8.0), 10 mM MgCl, 0.5% Tween 20, and 0.4 mg/ml Proteinase K). Tubes were then incubated for 72 h at 55°C with Proteinase K (0.3 μl of 20 mg/ml Proteinase K per 15 μl of buffer) added after 24 and 48 h. After 72 h, Proteinase K was inactivated by boiling for 15 min.

PCR-LOH Analysis. Table 2 summarizes the primers used for PCR amplification of DNA fragments spanning microsatellite sites at different chromosome loci. The PCR amplification reaction contained 2 μl of microdissected DNA sample, 10 mM Tris, 50 mM KCl (pH 8.3), 1.5–4.0 mM MgCl, 1 μM of each primer, 0.1 mM dTTP, 0.1 mM dCTP, 0.1 mM dGTP, 4.0 μM dATP, reaction buffer (1X; Perkin-Elmer), 1 unit of AmpliTaq Gold (Perkin-Elmer) and 0.5 μCi [^{33}P]dATP (2000 Ci/mmol). DNA was amplified in a 9600 Thermocycler (Perkin-Elmer) as follows: 94°C for 5 min, 35 cycles of 55°C for 1 min, 72°C for 1 min, and 94°C for 1 min with a final 72°C hold for 10 min. The amplified products were separated in a 6% denaturing polyacryl-

Table 2 *Microsatellite positions and primer conditions for LOH analysis*

Name	Position	Sequence	Size (bp)[a]	Type	Reference or footnote
D17S855	17q21	[b]F5′GGATGGCCTTTTAGAAAGTGG R5′ACACAGACTTGTCCTACTGCC	143-155	DN	(46, 47)
D17S858	17q21	F5′GCACTCTGACTAGAATCTGGGG R5′TCCAAGTGGGAATGAGTGC	131-127	DN	(46)
D17S1322	17q21	F5′CTAGCCTGGGCAACAAACGA R5′GCAGGAAGCAGGAATGGAAC	130 min	TN	c
D17S1323	17q21	F5′TAGGAGATGGATTATTGGTG R5′AAGCAACTTTGCAATGAGTG	155 min	DN	c
D13S260	13q12.3	F5′AGATATTGTCTCCGTTCCATGA R5′CCCAGATATAAGGACCTGGCTA	NA	DN	(47, 48)
D13S171	13q12.3	F5′CCTACCATTGACACTCTCAG R5′TAGGGCCATCCATTCT	227-241	DN	(47, 48)
TP53.PCR15	17p13.1	F5′ACTGCCACTCCTTGCCCCATTC R5′AGGGATACTATTCAGCCCGAGGTG	103-135	DN	(49)
TP53	17p13.1	F5′GAATCCGGGAGGAGGTTG R5′AACAGCTCCTTTAATGGCAG	NA	PN	(50)

[a] bp, base pair(s).
[b] F, forward primer; R, reverse primer; min, minimum bp size documented; NA, details not published; DN, dinucleotide repeat; PN, pentanucleotide repeat.
[c] D. Goldgar, 1994. Personal communication in OMIM (Online Mendelian Inheritance in Man). MIM Numbers GDB 375331 and 375323: Johns Hopkins University, Baltimore, MD. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/

amide gel and visualized by autoradiography. LOH was considered to be present if the intensity of alleles varied by more than 50%.

p53 **Mutation Detection.** Mutations in exons 5–10 of the *p53* gene were detected by cycle sequencing, SSCP, or sub-cloning. Mutations that were detected at least twice from different PCR reactions by the above methods were designated *p53* mutation-positive.

Microdissected DNA (1–2 μl) was amplified with primers and MgCl$_2$ (at concentrations described in Table 3), in a total reaction volume of 25 μl containing 2 mM dNTPs, 10 mM Tris (pH 8.3), 50 mM KCl, and 0.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer). Touch-down PCRs were performed in a 9600 Thermocycler (Perkin-Elmer) with the initial annealing temperature 8°C higher than the final temperature. DNA was denatured for 10 min at 94°C initially, followed by cycles of 94°C for 1 min, variable annealing temperatures for 1 min, 72°C for 1 min, followed by a final 72°C hold for 10 min. The annealing temperature was decreased every 2 cycles by 2°C, touching down at the final annealing temperature (see Table 3). An additional 30 cycles were performed, and amplification was confirmed by agarose gel electrophoresis.

For cycle sequencing, PCR products were purified using a Sephaglas Bandprep kit (Pharmacia Biotech) and eluted in TE buffer. Purified products (6 μl) were sequenced using AmpliCycle Sequencing Kits (Perkin-Elmer) incorporating [^{33}P]dATP (Amrad Pharmacia Biotech) and the same primers used for the amplification reaction. Fragments were analyzed using standard 6% polyacrylamide/urea sequencing gels, run for 1–2 h at 60 W, then dried, and exposed to X-ray film (Biomax-MR, Kodak) overnight.

PCR products were ligated into pGEM-T Easy vectors (Promega) according to the manufacturer's instructions and transformed into JM109 High Efficiency Competent Cells (Promega). Recombinant plasmid DNA was cycle-sequenced as described above.

SSCP analysis was performed using 1 μl of microdissected DNA, amplified in a total volume of 25 μl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2 mM MgCl; 200 μM (each) dGTP, dTTP, and dCTP; 20 μM dATP; 1 μCi [α-^{32}P]dATP; 400 nM appropriate primers (see Table 3), and 0.5 unit of AmpliTaq Gold (Perkin-Elmer). PCR was performed using the touch-down protocol described above.

Five μl of PCR product was diluted in SDS/EDTA to give a final concentration of 0.1% SDS-10 mM EDTA. Equal volumes of diluted product were mixed with loading dye comprising; 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.02% xylene cyanole FF. Samples were denatured for 5 min at 95°C and immediately placed on ice, and 5 μl was loaded onto a 6% polyacrylamide gel containing 90 mM Tris-borate, 2 mM EDTA, and 10% glycerol and run at 60 W for 6 h at 4°C. Gels were dried and exposed to overnight autoradiography. Shifted bands were cut out of the gel, DNA was eluted in 20 μl of H$_2$0 at 60°C and 5 μl was used to amplify the appropriate exon as described above. PCR products were cleaned and sequenced as described.

Statistical Methods. Statistical comparisons between groups were assessed by Fisher's exact test. Nominal *P*s are given without adjustment for multiple comparisons. Because of the small numbers of cases in each category, raw data are provided together with the nominal *P*s.

RESULTS

Tumor material from 40 women selected from the ABCFS were analyzed: 19 cases with a germline *BRCA1* or *BRCA2* mutation (10 *BRCA1* and 9 *BRCA2*) and 21 cases for whom there were no detectable germline mutations in these genes. The 19 cases with germline mutations comprised all but one of the 20 mutations detected in the ABCFS (1). One woman with a germline protein-truncating mutation in both *BRCA1* and *BRCA2* was excluded from the statistical analysis because the tumor could not be classified into either the *BRCA1* or *BRCA2* category. Ten different *BRCA1* protein-truncating mutations and 7 different *BRCA2* protein-truncating mutations were represented

Table 3 *PCR primers and reaction conditions for p53 mutation analysis*

Exon	Primer pair (5′-3′)[a]	Primer concentration (μM)	MgCl concentration (μM)	Annealing temperature (°C)
5	F; TTCCTCTTCCTGCAGTACTC R; GCCCCAGCTGCTCACCATCG	0.8	2	58
6	F; GCTGCTCAGATAGCGATGG R; AGACCCCAGTTGCAAACCAG	0.4	2	55
7	F; GTGTTATCTCCTAGGTTGGC R; AAGTGGCTCCTGACCTGGAG	0.125	2	55
8	F; AGTGGTAATCTACTGGGACG R; AGGCATAACTGCACCCTTGG	0.4	2.5	55
9	F; CAAGGGTGCAGTTATGCC R; AACTTTCCACTTGATAAGAGGTC	0.8	2	58
10	F; GTACTGTGAATATACTTACTTCTCC R; GGGCTGAGGTCACTCACC	0.8	2	58

[a] F, forward primer; R, reverse primer.

Table 4 *Proportion of cancers showing greater than 10% of cells positive by immunohistochemistry*

Antibody[a]	Germline mutation status			Nominal *P* values[b]		
	BRCA1 mutation carriers	*BRCA2* mutation carriers	Control cancers	*A1 vs. A2*	*A1 vs. C*	*A2 vs. C*
p53	7/10	1/9	9/20	**0.02**	0.3	0.1
p21	7/10	6/9	6/20	1.0	0.06	0.1
Cyclin D1	3/9	5/9	19/19	0.6	**<0.001**	**0.01**
bcl-2	3/10	5/9	7/18	0.4	0.7	0.5
Ki-67[c]	4/7	0/9	1/19	**0.02**	**0.01**	1.0
PCNA	8/8	8/9	10/13	1.0	0.3	0.3
c-erbB-2	4/10	7/9	18/20	0.2	**0.01**	0.5
EGFR	1/10	0/9	2/20	1.0	1.0	1.0
ER	1/10	6/9	11/20	**0.02**	**0.02**	0.7
PR	1/10	4/9	6/20	0.1	0.4	0.7
pS2	1/10	3/9	7/20	0.3	0.2	1.0
Cathepsin D	2/8	7/9	8/17	0.06	0.4	0.2
E-cadherin	7/8	7/7	10/14	1.0	0.6	0.3
β-Catenin	7/8	4/8	8/11	0.3	0.6	0.4

[a] Not all of the cases could be stained for each antibody because of the unavailability of tissue.
[b] *P* shown in bold type are nominally significant at the 0.05 level.
[c] Cutoff for Ki-67 staining was 50% of cells positive by immunohistochemistry.

(1). None of the 40 women were known to be genetically related. There was no difference in the proportion with a family history of breast cancer between carriers of a germline BRCA1 or BRCA2 mutation and noncarriers ($P > 0.1$; Ref. 1). The mean ages (SD) of diagnosis of breast cancer for the *BRCA1* and *BRCA2* mutation carriers and the control cancers were 32.3 (5.3), 34.9 (3.7) and 36.5 (2.6) years, respectively. Immunohistochemical and molecular information could not be obtained on the invasive component of one control cancer because it was microinvasive and the invasive component was insufficient for study. A detailed histological analysis of these breast cancers has been previously reported (1).

LOH at the *BRCA1* and *BRCA2* Loci. LOH at one or more intragenic *BRCA1* loci was common in cancers arising in *BRCA1* mutation carriers, being present in seven of eight informative cancers. Two cases were homozygous for all of the markers studied, and in one case, LOH could not be consistently demonstrated. A focus of DCIS was also available for analysis in three of the informative cases. LOH was again documented in this preinvasive stage of disease

Similarly, LOH was commonly seen in *BRCA2* germline mutation carriers, with the loss of one or more microsatellite markers approximately 0.5 cM and 2.1 cM from the *BRCA2* locus demonstrated in all of the seven informative cancers. LOH was analyzed in the DCIS component of five of these. Loss of the same allele was demonstrated in four cases, and in three of these, multiple sites of DCIS were dissected, all showing similar loss. LOH was not demonstrated in the DCIS accompanying an invasive carcinoma in one case, despite the DCIS having an homogeneous cellular morphology with no apparent contamination by other cell types.

Immunohistochemistry. The immunophenotypes of invasive breast cancers in *BRCA1*, *BRCA2*, and non-mutation carriers are shown and contrasted in Table 4. Compared with the other two groups, p53 positivity was more common in tumors arising in *BRCA1* mutation carriers. In addition, when both the intensity and extent of staining was scored, all of the positive breast cancers in the *BRCA1* mutation carriers showed strong p53 expression (Fig. 1*A*). In contrast, none of the cancers in the other two groups showed strong expression ($P < 0.002$). Positive p53 expression was not related to the site of germline mutation in *BRCA1* mutation carriers (data not shown). Strong p53 expression was also seen in the DCIS component of all of the cancers from the *BRCA1* mutation carriers with positive p53 staining in the invasive component (Fig. 1*B*). However, strong p53 expression was not present in the morphologically normal breast epithelium. Overall, 44% of all of the invasive breast cancers studied were p53-positive at the 10% cutoff level.

Positive cyclin D1 staining was rare in cancers arising in *BRCA1* or *BRCA2* mutation carriers compared with control cancers ($P < 0.01$, Table 4). The normal breast epithelium showed variable staining. The accompanying DCIS component was cyclin D1-negative in all but one of these cancers in *BRCA1* mutation carriers. Infrequent cyclin D1 staining in cancers arising in *BRCA1* mutation carriers was further accentuated when the degree of antigen expression was assessed as strong or moderate *versus* weak or negative. Only 11% (1 of 9) of
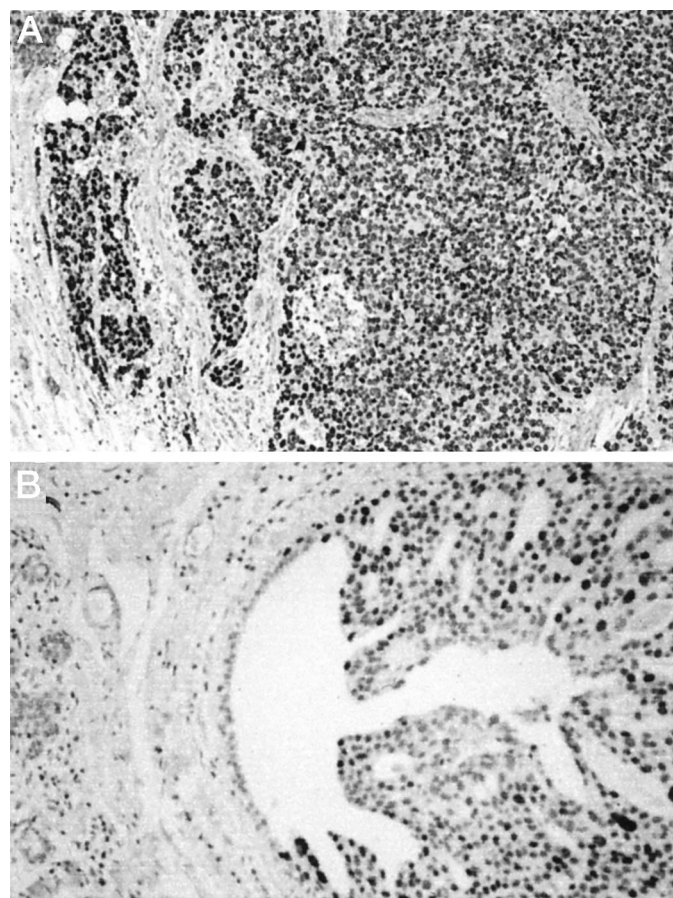

Fig. 1. p53 immunohistochemical staining in cancers arising in *BRCA1* germline mutation carriers. *A*, invasive carcinoma showing strong nuclear staining for p53 in the majority of tumor cells present. *B*, ductal carcinoma *in situ* showing strong nuclear staining for p53. The normal breast epithelium is negative.

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Table 5 *Cancers positive for p53 mutations by germline mutation status, histologic grade, heterozygosity at the p53 locus, and p53 immunophenotype*

Case	Germline mutation status	Histologic grade	Mutated exon	Codon	Mutation type	Amino acid change	LOH	Immunophenotype
1	*BRCA1*	3	5	130	Insertion 6 bp	Ins. pro;ala	LOH	Positive
2	*BRCA1*	3	10	332	Deletion C	Stop 345	No LOH	Positive
3	*BRCA1*	3	7	245	Missense G>A	gly>ser	LOH	Positive
4	*BRCA2*	3	5	152	Missense G>A	pro>pro[a]	Homozygous	Positive
5	Control	3	7	245	Missense G>A	gly>asp	No LOH	Positive
6	Control	3	7	254	Missense T>C	ile>thr	No LOH	Positive
7	Control	2	9	325	Missense G>A	gly>glu	ND[b]	Negative

[a] Silent mutation.
[b] ND, not done.

cancers in *BRCA1* mutation carriers showed strong or moderate staining, compared with 63% of control cancers ($P = 0.02$).

Fifty-seven per cent of the cancers in *BRCA1* mutation carriers, compared with none of the cancers in *BRCA2* mutation carriers and only 5% of the control cancers, showed exceptionally high Ki-67 staining (defined as >50% of cells positive; Table 4). Ki-67 staining was also exceptionally high in the DCIS component of disease in *BRCA1* mutation carriers (data not shown).

c-erbB-2 positivity was less frequent (40%) in *BRCA1* mutation carriers compared with 78 and 90% of cancers in *BRCA2* mutation carriers and control cancers, respectively (Table 4). In addition, none of the cancers in *BRCA1* mutation carriers showed strong expression of c-erbB-2, whereas 44% (4 of 9) of cancers in *BRCA2* mutation carriers and 45% (9 of 20) of control cancers showed strong membranous expression (*BRCA1 versus BRCA2*, $P = 0.03$; *BRCA1 versus* control cancers, $P = 0.01$).

ER-positive cancers were rare in the cancers from *BRCA1* mutation carriers (10%, Table 4). The DCIS component was also negative in all of the ER-negative *BRCA1* mutation carriers. These nine ER-negative cases were also negative for PR and pS2. In contrast, 67% of invasive cancers in *BRCA2* mutation carriers and 55% of control cancers were ER-positive. PR and pS2 staining were variable in these cancers. Overall, 44% of all of the breast cancers studied were ER-positive. The ER status of the normal epithelial component was variable in all of the cancers examined. There was no difference in Cathepsin D positivity when cancers from BRCA1 mutation carriers were compared with cancers from BRCA2 mutation carriers and control cancers. Nonetheless, when the antigen expression level was assessed, only 13% (one of eight) of cancers in BRCA1 mutation carriers showed strong or moderate staining, compared with 67% (six of nine) of cancers in BRCA2 mutation carriers ($P = 0.05$). No obvious differences between the three categories of breast cancer were noted in the immunophenotype of the other antibodies studied.

Mutation Detection and LOH at the *p53* Locus. p53 mutation was detected in 7 of the 39 invasive cancers (18%). The mutations were scattered over four of the six exons examined (Table 5). Three of the mutations were detected in cancers from germline *BRCA1* mutation carriers, and three from control cancers. No functional mutations were detected in the *BRCA2* mutation carriers, although one silent mutation (pro>pro) was detected. Five mutations were missense; all of them were transitions with 4 being G>A transitions. Five of the six cases with functional mutations showed positive immunohistochemical staining for p53, whereas one—with a mutation in exon 9—was negative by immunohistochemistry. Conversely, no p53 mutations were identified in 12 other carcinomas in which p53 antigen was detected by immunohistochemistry. DCIS was available for study in two cases with p53 mutations in exon 7 of the invasive component (two control cancers). Identical mutations were documented in the DCIS component of each case.

Cancers from 37% (13 of the 35 tested) of cases were shown to have LOH at one or more intragenic *p53* microsatellite locus (4 in cancers from *BRCA1* mutation carriers, 5 from *BRCA2* mutation

carriers, and 4 from control cancers). However, only 6 of the 13 cases with LOH showed p53 antigen expression by immunohistochemistry. Only two of the six cases with functional p53 mutations detected by molecular methods showed LOH at the *p53* locus (Table 5). No LOH was detected in three of the p53 mutation-positive cases, and one case could not be assessed because of insufficient material.

DISCUSSION

This paper has demonstrated, for the first time on a population basis, that breast cancers occurring in young *BRCA1* or *BRCA2* mutation carriers show distinct molecular pathogeneses, and that these pathways are different from cancers occurring in mutation-negative women. Previous studies of multiple-case *BRCA1* or *BRCA2*-associated breast cancer kindreds and studies performed on populations with strong founder mutations have identified reduced expression of ER, PR, and c-erbB-2 in cancers from *BRCA1* mutation carriers (10–13). In addition, somatic mutations in p53, or p53 protein overexpression, have been documented in cancers occurring in *BRCA1* and *BRCA2* mutation carriers (14–17). Although the number of cases with *BRCA1* or *BRCA2* mutations was small in our sample and, therefore, some of the nominally significant findings reported herein may not be reproducible in subsequent population-based studies, our study confirms the above findings drawn from selected groups at high-risk of *BRCA1* or *BRCA2* germline mutations. In addition, the present study also serves to identify molecular and immunophenotypic changes that are independent of germline mutation site or presence of a family history. In this way, we hope to emphasize the molecular pathogenic consequences of the germline mutations themselves and, as far as possible, minimize the impact of modifying genetic or environmental factors.

Elevated p53 expression seems to be common in cancers from *BRCA1* mutation carriers. However, data from the present series and Crook *et al.* (15) indicate that the type and site of mutation documented in cancers arising in *BRCA1* mutation carriers are unusual compared with breast cancers in general. We were able to show that p53 overexpression consistently occurred at the preinvasive (DCIS) stage of the disease, but not in morphologically normal breast epithelium. Hence, these data suggest p53 overexpression is an important and early event in the molecular pathogenesis of cancers arising in *BRCA1* mutation carriers.

We found few cancers in germline *BRCA2* mutation carriers to have p53 overexpression or mutation. This is in contrast to two previous studies using cancer-dense, predominantly European-based families and Icelandic women (15, 16). This discrepancy is interesting in light of the two distinct histological phenotypes reported for cancers arising in *BRCA2* mutation carriers. High-grade carcinomas, without an excess of lobular carcinoma, have been described in Icelandic and European-based patients (2, 30), whereas we (1) and Marcus *et al.* (6) have described lower-grade cancers in *BRCA2* mutation carriers, which frequently show a lobular pattern of growth. Thus it is possible that p53 mutations in cancers arising in *BRCA2* germline mutation carriers confer a high-grade phenotype to these tumors. Unfortunately,

there is no comprehensive follow-up data available on cancer patients with germline *BRCA2* mutations, and, therefore, the effect of high-grade histology and p53 mutation cannot be assessed.

Cyclin D1 is a G_1 cyclin, active in the regulation of G_1-S cell cycle progression (31, 32). Cyclin D1 is overexpressed in several cancer types and acts as an oncogene in some breast cancers (9, 33). We found an unusually high rate of cyclin D1 overexpression over our entire sample of early-onset breast cancers (73%) compared with previous studies on breast cancers occurring at all ages (30–50%; 34, 35). One possible explanation for this high rate may be high circulating estrogen levels within these patients with early-onset breast cancers because estrogen increases cyclin D1 protein synthesis *in vivo* (36). Similar variance according to hormonal status is well described for other proteins affected by estrogen stimulation, notably the ER. However, cyclin D1 expression was not solely a consequence of physiological estrogen stimulation because cyclin D1 expression in the normal breast epithelium in individual cases was not identical to the staining properties of the invasive cancer.

Despite the overall high rate of cyclin D1-positive tumors in our sample, the invasive and accompanying *in situ* cancers arising in *BRCA1* mutation carriers were rarely positive for cyclin D1 compared with the control cancers. However, there was variable staining in the normal breast epithelium. These findings suggest that cyclin D1 overexpression is rarely involved in the oncogenesis of breast cancers arising in germline *BRCA1* mutation carriers. One previous study has examined cyclin D1 expression in breast cancers arising in *BRCA1* or *BRCA2* mutation carriers (13). In this series no difference was seen between cyclin D1 expression in Jewish women harboring one of three founding mutations in the *BRCA1* and *BRCA2* genes compared with women from the same population without these mutations. However, we have found that the frequency of cyclin D1 expression in *BRCA2* mutation carriers is intermediate between *BRCA1* mutation carriers and control cancers, and, therefore, it is likely that combining *BRCA1* and *BRCA2* mutation carriers into one group, as in the above study, would mask any differences between *BRCA1* mutation carriers and control cancers.

In our study, cancers arising in *BRCA1* mutation carriers were commonly ER-negative, which contrasted with the majority of cancers in *BRCA2* mutation carriers and control cancers, which were ER-positive. These data, which suggested reduced or absent ER pathway function in *BRCA1*-associated cancers, were confirmed by the absence of expression of the estrogen/ER-inducible proteins pS2 and PR in these cancers. A similar lack of ER positivity in *BRCA1*-associated cancers has been previously reported in studies involving Jewish patients and patients from cancer-dense families (10–13). Few studies have specifically addressed ER status in confirmed *BRCA2* mutation carriers. However, high rates of ER positivity have been demonstrated in two studies in Jewish and Icelandic women with specific sites of germline mutation in *BRCA2* (11, 37).

The infrequent c-erbB-2 staining in cancers of *BRCA1* mutation carriers within our sample is interesting in light of the high histological grade of these cancers inasmuch as c-erbB-2 overexpression is an adverse prognostic indicator that has been previously associated with high-grade cancers (38). This report supports the previously reported lack of c-erbB-2 expression in cancer-dense families (10). Taken together, these data suggest that neither hormone receptor nor c-erbB-2 stimulation seem to be important in the pathogenesis of cancers arising in women with *BRCA1* germline mutations.

Data defining the molecular and immunophenotypic profiles of breast cancers arising in *BRCA1* and *BRCA2* mutation carriers can be used in conjunction with other experimental approaches to elucidate the mechanisms of carcinogenesis induced by aberrant function of these genes. Both *BRCA1* and *BRCA2* are thought to be involved in the maintenance of genomic integrity because both proteins are known to associate with RAD51 (39, 40). There is also mounting evidence that *BRCA1* is involved in regulation of cellular proliferation: Over-expression of wild-type *BRCA1* leads to growth retardation of breast and ovarian cancer cells *in vitro* and tumor inhibition in nude mice (41), and decreased expression of *BRCA1* leads to increased cellular proliferation (42). In addition, recent data suggest that *BRCA1* transactivates the expression of the cyclin-dependent kinase inhibitor, p21 and inhibits cell cycle progression to the S phase (43). *BRCA1* is also known to bind p53 and stimulate p53-dependent transcriptional pathways, including the p53-dependent induction of p21 (44, 45). Importantly, we and others have previously shown that breast cancers arising in *BRCA1* mutation carriers have an exceptionally high proliferation rate measured by their cellular mitotic count and, as described in this study, a high Ki-67 index (1–5). Thus, it is reasonable to postulate that once early inactivation of the normal *BRCA1* allele and p53 stabilization has occurred in the breast tissue of germline *BRCA1* mutation carriers, there is little selection pressure for the recruitment of additional oncogenic mechanisms of cell growth. In contrast, the molecular pathogenesis of cancers arising in *BRCA2* mutation carriers would indicate that additional somatic mutations are advantageous for tumor growth, perhaps implying a less direct effect of BRCA2 on control of cellular proliferation.

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Distinct Molecular Pathogeneses of Early-Onset Breast Cancers in _BRCA1_ and _BRCA2_ Mutation Carriers: A Population-based Study

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