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PRINCIPAL INVESTIGATOR: Gail E. Tomlinson, M.D., Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75235-9105

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Gail E. Tomlinson, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Universitv of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75235-9105

E-Mail: tomlinson@simmons.swmed.edu

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The inheritance of a germ-line mutation of the *BRCA1* or *BRCA2* gene, although associated with a markedly increased incidence of breast cancer, is not solely responsible for the development of breast cancer in predisposed women and multiple other acquired steps appear to be required for the development of breast tumors in predisposed women. It is possible that women with predisposing mutations are at increased risk of cancer because they acquire secondary genetic events at a faster rate than non-predisposed women and that the accumulation of genetic events leads to tumor formation. Increasing evidence suggests that BRCA1 plays a role in DNA repair which adds support to this premise. This study has involved elucidating the pathways by which women with predisposing mutations acquire secondary genetic events. In this study, we have identified a number of women with *BRCA1* mutations in which we have obtained tumors suitable for study. We have studied characteristic histologies of their tumor types. We have developed new assays for quantitating the predisposition to the accumulation of acquired genetic changes.

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Gail E. Tomlinson 3-11-00

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FINAL REPORT

DAMD17 96 - 1 - 6026

Acquired Secondary Events in the Pathogenesis

Of Hereditary Breast Cancer

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INTRODUCTION:

Mutation of the *BRCA1* or *BRCA2* gene accounts for most familial breast cancers. The inheritance of a germ-line mutation of the *BRCA1* or *BRCA2* gene, although associated with a markedly increased incidence of breast cancer, is not solely responsible for the development of breast cancer in predisposed women and multiple other acquired steps appear to be required for the development of breast tumors in predisposed women. It is possible that women with predisposing mutations are at increased risk of cancer because they acquire secondary genetic events at a faster rate than non-predisposed women and that the accumulation of genetic events leads to tumor formation. Increasing evidence suggests that BRCA1 plays a role in DNA repair which adds support to this premise. This study has involved elucidating the pathways by which women with predisposing mutations acquire secondary genetic events. In this study, we have identified a number of women with *BRCA1* mutations in which we have obtained tumors suitable for study. We have studied characteristic histologies of their tumor types. We have developed new assays for quantitating the predisposition to the accumulation of acquired genetic changes.

BODY:

Task 1: Ascertain patients and tumors tissues for proposed study.

All patient materials utilized for the studies proposed derived from the UT Southwestern Familial Breast Cancer Registry directed by the Principal Investigator. For each family or individual enrolled we have calculated the probability of carrying a BRCA mutation by several different algorithms (1-4). Individuals who have a predicted risk of BRCA mutation of 30% or more undergo mutation screening by methods of SSCP and sequence analysis as previously described. In doing so we have ascertained a cohort of mutation carriers sufficient for carrying out the Aims of this study. In the Table below lists mutation carriers determined as a part of our efforts:

Participant Number	Gender	Race	Specific Mutation
BC73-000	F	C	BRCA1-2575-delC
BC92-000	F	C	BRCA1-3549-T
BC92-999	F	C	BRCA1-3549-T

BC98-001	F	C	BRCA1-185delAG
BC98-002	M	C	BRCA1-185delAG
BC110-001	F	C	BRCA1-3450del4
BC113-000	F	C	BRCA1-185delAG
BC129-000	F	C	BRCA1-185delAG
BC129-900	F	C	BRCA1-185delAG
BC130-001	F	C	BRCA1-3600del11
BC130-000	F	C	BRCA1-3600del11
BC131-982	F	C	BRCA1-3888delGA
BC131-000	F	C	BRCA1-3888delGA
BC131-003	F	C	BRCA1-3888delGA
BC132-000	F	C	BRCA1-185insA
BC211-000	F	C	BRCA1-int6spl
BC215-000	F	C	BRCA1-3875del4
BC260-000	F	C	BRCA1-5382insC
BC260-002	F	C	BRCA1-5382insC
BC260-001	F	C	BRCA1-5382insC
BC294-000	F	C	BRCA1-185insA
BC403-004	F	C	BRCA1-185delAG
BC403-001	F	C	BRCA1-185delAG
BC412-000	F	C	BRCA1-185delAG
BC516-000	F	C	BRCA1-185delAG
BC517-000	M	C	BRCA1-185delAG
BC530-000	F	C	BRCA1-185delAG
BC541-000	F	C	BRCA1-2798del4
BC541-100	F	C	BRCA1-2798del4
BC541-901	F	C	BRCA1-2798del4
BC565-000	F	C	BRCA2-6985delCT
BC565-100	F	C	BRCA2-6985delCT
BC581-002	M	B	BRCA1-1623del5
BC581-000	F	B	BRCA1-1623del5
BC581-901	F	B	BRCA1-1623del5
BC593-000	F	C	BRCA1-ex11trunc
BC594-000	F	C	BRCA2-2041insA
BC617-000	F	B	BRCA1-943ins10
BC641-000	F	C	BRCA1-inv4-1G->T
BC694-000	F	C	BRCA2-6503delTT
BC727-000	F	C	BRCA2-4075delGT
BC746-000	F	C	BRCA2-Y1655X
BC750-100	F	C	BRCA2-8525delC
BC751-000	M	C	BRCA2-6051delA

Seven of the above patients have had more than one tumor. Verification of tumor status by pathology report has been obtained on all patients and archival tissue obtained on 30 tumors.

Task 2: Delineate histologic features of malignant and non-malignant tissues.

We have determined the histologic subtype of the tumor types developed in our mutation carriers. Examination of the surrounding normal tissue revealed surprising little distinguishing characteristics. Seventy percent of tumors (N=28) demonstrated intraductal carcinomas, with no distinguishing characteristics other than young age of onset and a higher proportion of estrogen receptor negative status (75%). Fifteen percent of tumors (n = 6) were medullary carcinomas and ten percent were (n=4) were lobular. One was a carcinoma with secretory features and one case was a papillary carcinoma. Five percent of tumors examined had elements of atypia in surrounding normal tissue. Fifteen percent of specimens demonstrated marked hyperplasia of breast epithelium.

Task 3: Allelotyping of familial breast tumors.

We have carried out extensive allelotyping studies to characterize tumors from predisposed individuals. Our most interesting cases have been published separately (5-8). The most consistent pattern of allele loss was seen on chromosome 3p in that over 90% of tumors allelotyped showed a loss of alleles in this region, which was higher than that seen in a meta-analysis LOH patterns in sporadic breast tumor. (Minna lab, unpublished).

Since the initiation of the project, it has become increasingly evident that the *BRCA1* gene plays a major role in maintaining genomic stability. In addition to allelotyping we have developed a new means of measuring the inherent tendency for *BRCA1* mutation carriers to accumulate genetic losses throughout the genome. This has been done in collaboration with Dr. Xifeng Wu in the Department of Epidemiology at the Univ. Texas M.D. Anderson Cancer Center. Because the role of BRCA1 in maintaining genomic instability was unknown at the time that this proposal was formulated, we did not specify the development of such an assay, however the development of this assay has been an extension of the aims of this study as well as a more insightful means of studying acquired genetic changes in inherited breast cancer. The concept behind the development of this assay is that women who carry a mutation of *BRCA1* or *BRCA2* may be more likely to acquire chromosomal breaks leading to loss of heterozygosity and loss of chromosomal material. Women prone to develop breast cancer because of a familial predisposition in *BRCA1* or *BRCA2* who are deficient in DNA repair may acquire genetic changes at a faster rate than women without a predisposing mutation.

We have preliminarily observed a difference in the mean number of chromosomal breaks per normal cells in women with BRCA1 mutations compared to controls. This findings are statistically significant (0.625 breaks per cell in mutation carriers vs 0.47

breaks per cell in controls, $p < 0.05$). A manuscript reporting these findings is in preparation. We are now in the process of correlating these findings with the number of breaks in breast epithelial tissue as well as tumor tissues.

In one very special tumor, we had viable tumor cells available which was developed into a cell line as part of a close collaboration with a DAMD sponsored program to create a repository of breast cancer cell lines. (DAMD17 94-J-0477) This cell line, HCC1937, shows extensive secondary chromosomal changes as outlined in detail in the attached reprint. This cell line, HCC1937, developed and characterized by two collaborative DAMD projects, has been utilized extensively in the breast cancer field to elucidate the function of BRCA1 (9-17) and will undoubtedly continue to be recognized as an important contribution to breast cancer research.

We have allelotyped a subset of our 30 tumors for which we have archival tissues. Examples of two extensively allelotyped tumors is shown in the figure. (page 6)

Task 4:

We have taken several steps in order to determine the timing with regards to tumor development as well as the extent of loss of the wild-type predisposition allele. On breast tumors in which losses on chromosome 3p and 17q are noted, we have seen a loss using 3p14 markers in one of seven informative tumors studied. We have not noted any losses on chromosome 17q in normal breast tissues.

We plan to follow up on our findings outlined above of increased chromosomal breaks in normal tissues of germ-line mutation carriers with a study of cultured breast epithelial cells derived from mutation carriers in which we have measured the sensitivity to radiation. We will perform FISH analysis of chromosome 3p and 17q to determine if these genomic areas demonstrate losses even at low levels.

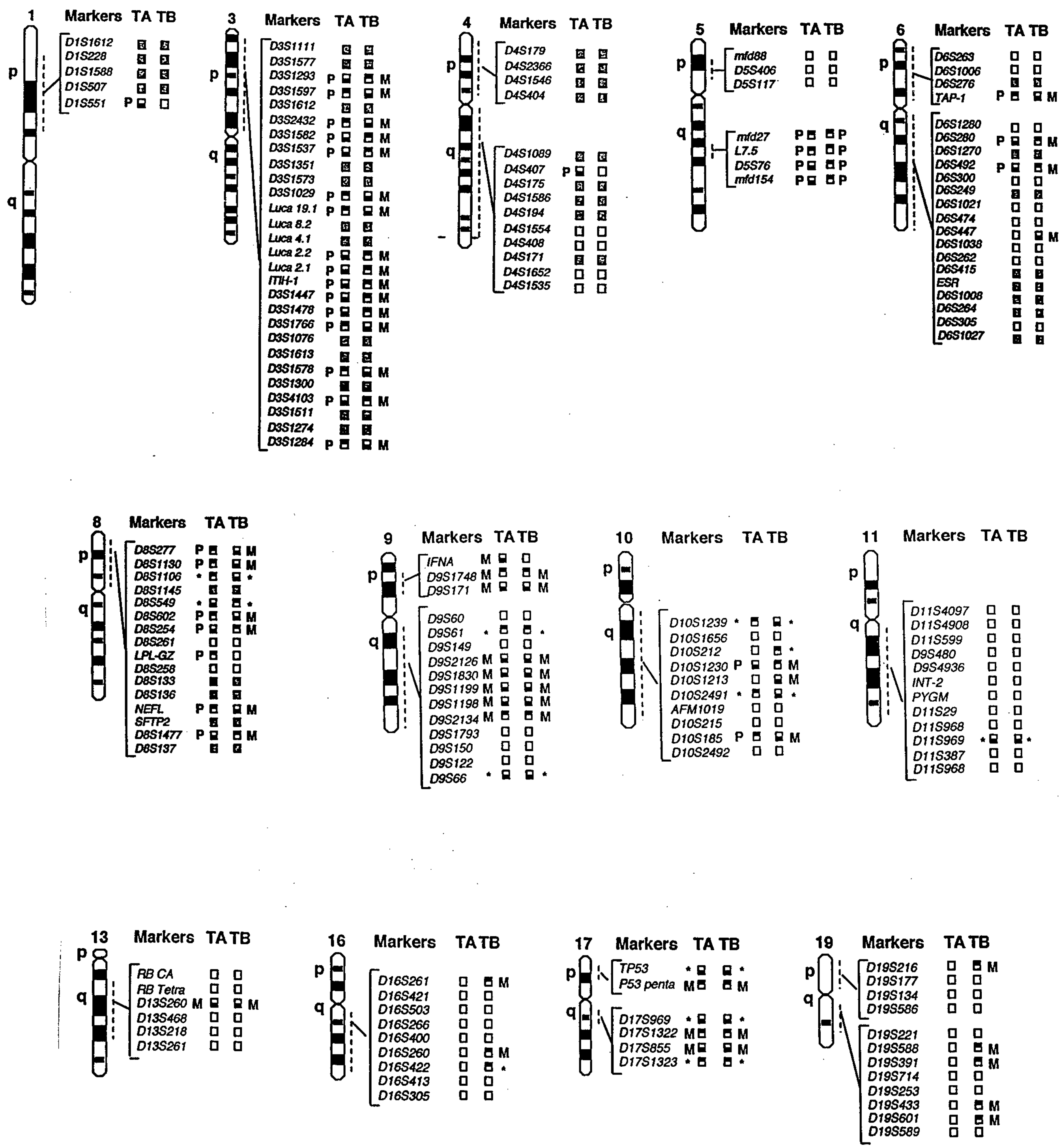
(Task 5: Omitted as a result of initial peer review recommendation.)

CONCLUSION:

We have identified that in familial breast cancer caused by mutation of the *BRCA1* gene that acquired losses occur at high frequency. We found a very high fractional allele loss compared to other breast tumors. In the only report to date of a complete chromosomal karyotype of a BRCA1 associated breast tumor (ref 5, reprint attached) we have also shown a high degree of chromosomal aneuploidy. We have characterized acquired genetic changes in this unique cell line.

We have taken steps to develop new assays for chromosome instability and the tendancy to acquire genetic losses in normal tissues form BRCA mutation carriers.

Figure. Representative genotyping for loss of heterozygosity in tumor tissues. Detailed allelotyping analysis data of two tumors (TA and TB) using 161 microsatellite markers located on 19 chromosomal arms. Clear boxes indicate no loss of alleles. Filled boxes indicate that marker was non-informative. Half-filled boxes indicate loss of heterozygosity observed. (Figure from Wistuba et al, in press.)



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Tomlinson, G., Chen, T., Stastny, V., Virmani, A., Spillman, M., Tonk, V., Blum, J., Schneider, N., Wistuba, I., Shay, J., Minna, J., and Gazdar, A. Characterization of a Breast Cancer Cell Line Derived from a Germ-Line *BRCA1* Mutation Carrier. Cancer Research. 58: 3237-3242, 1998.

Hung J, Mims B, Lozano G, Strong L, Harvey C, Chen, T-Y, Stastny V, Tomlinson G. *TP53* mutation and haplotype analysis of two large African-American kindreds, Human Mutation, 14: 216-221, 1999.

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A fifth manuscript is in preparation describing the patterns of chromosome breaks in BRCA1 mutation carriers.

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[CANCER RESEARCH 58, 3237–3242, August 1, 1998]

Advances in Brief

Characterization of a Breast Cancer Cell Line Derived from a Germ-Line *BRCA1* Mutation Carrier[1]

Gail E. Tomlinson,[2] Tina T-L. Chen, Victor A. Stastny, Arvind K. Virmani, Monique A. Spillman, Vijay Tonk, Joanne L. Blum, Nancy R. Schneider, Ignacio I. Wistuba, Jerry W. Shay, John D. Minna, and Adi F. Gazdar

Departments of Pediatrics [G. E. T.], Pathology [A. K. V., I. I. W., A. F. G.], and Internal Medicine and Pharmacology [J. D. M.] and the Hamon Center for Therapeutic Oncology Research [G. E. T., T. T-L. C., V. A. S., A. K. V., I. I. W., J. D. M., A. F. G.], Southwestern Medical School [M. A. S.], Departments of Cell Biology and Neurosciences [J. W. S.] and Pathology [N. R. S.], University of Texas Southwestern Medical Center, Dallas, Texas 75235; Texas Tech University, Lubbock, Texas 79430 [V. T.]; and Baylor University Medical Center, Dallas, Texas 75246 [J. L. B.]

Abstract

A tumor cell line, HCC1937, was established from a primary breast carcinoma from a 24-year-old patient with a germ-line *BRCA1* mutation. A corresponding B-lymphoblastoid cell line was established from the patient's peripheral blood lymphocytes. *BRCA1* analysis revealed that the tumor cell line is homozygous for the *BRCA1* 5382insC mutation, whereas the patient's lymphocyte DNA is heterozygous for the same mutation, as are at least two other family members' lymphocyte DNA. The tumor cell line is marked by multiple additional genetic changes including a high degree of aneuploidy, an acquired mutation of *TP53* with wild-type allele loss, an acquired homozygous deletion of the *PTEN* gene, and loss of heterozygosity at multiple loci known to be involved in the pathogenesis of breast cancer. Comparison of the primary tumor with the cell line revealed the same *BRCA1* mutation and an identical pattern of allele loss at multiple loci, indicating that the cell line had maintained many of the properties of the original tumor. This breast tumor-derived cell line may provide a useful model system for the study of familial breast cancer pathogenesis and for elucidating BRCA1 function and localization.

Introduction

Mutation of the *BRCA1* gene accounts for most families with an inherited predisposition to breast and ovarian cancer, approximately one-half of families with multiple cases of breast cancer only, and ~8–10% of women with early-onset breast cancer unselected for family history (1–3). These observations suggest that inherited *BRCA1* mutations may account for ~8,000–10,000 new cases of breast cancer in the United States each year. The inheritance of a germ-line mutation of the *BRCA1* gene, although associated with a markedly increased incidence of breast cancer, is not solely responsible for the development of breast cancer in predisposed women. Multiple somatic genetic changes appear to be required in addition for the development of breast tumors in predisposed women (4).

Although the function of the BRCA1 protein is not yet clearly determined, evidence suggests that BRCA1 may play a role in DNA repair, function as a transcription factor, or possibly exist as a secreted granin-like molecule (5–7). If BRCA1 functions in DNA repair, then one would expect an accelerated accumulation of other genetic aberrations in tumors derived from *BRCA1* mutation carriers. Controversy exists as to the cellular localization of BRCA1, either in the nucleus or cytoplasm, or both, according to different stages of the cell cycle and exposures to DNA-damaging agents. Some of the difficulties in determining the cellular localization and potential functions of BRCA1 are due to lack of evidence supporting antibody specificity. However, a major problem also has been the lack of available *BRCA1* null cell lines to facilitate research studies in this area.

Somatic mutation of the *BRCA1* gene is not thought to occur in sporadic breast tumors, although mislocalization of BRCA1 protein has been reported in sporadic breast tumors (8, 9). Although a number of breast cancer cell lines have been established, no breast cancer cell lines have been reported to date that derive from a heterozygous *BRCA1* mutation carrier. The establishment of such a cell line would provide another method to study tumor growth regulation conferred by BRCA1 and could also conceivably serve as a substrate for genetic transfection studies. Reported here is the establishment and characterization of a breast cancer cell line homozygous for a germ-line-inactivating *BRCA1* mutation.

Materials and Methods

Patient Material. The patient was a 24-year-old woman with a nonmetastatic infiltrating ductal carcinoma of the breast. She had had one child previously at the age of 22. Her identical triplet sister had developed breast cancer the previous year at the age of 23. The third identical triplet had a bilateral prophylactic mastectomy at age 24. The patient's mother was reported to have had cancer of the uterine cervix at the age of 22. Both maternal grandparents had died of colon cancer in their sixties. The family is Caucasian and not of known Ashkenazi descent. A pedigree of the family is shown in Fig. 1. After obtaining informed consent for genetic studies, blood and tumor tissue were obtained from the patient and blood from her mother and two sisters. No adjuvant chemotherapy or radiation had been given prior to collection of tumor material.

Tumor Cell Culture Establishment. The patient from whom the breast tumor cell line was derived underwent a mastectomy with gross resection of the primary tumor. A portion of the primary tumor tissue was placed in RPMI 1640 with 5% fetal bovine serum and antibiotics immediately after surgical removal. Tumor tissue was minced and scraped to release tumor cells into the medium. Cells were cultured in T-25 flasks at 37°C with 5% CO_2. Medium was changed weekly, and cultures were observed for cell growth. Cultures were trypsinized and passaged when sufficient colonies of epithelial growth were noted. Estrogen and progesterone receptor studies on the cultured cells as well as the primary tumor were performed by Nichols-Corning Institute using a radioactive binding assay. HER2/neu expression was determined by a quantitative ELISA assay (Calgiochem, Cambridge, MA). Telomerase assay was performed by the telomeric repeat amplification protocol assay (10). For cytogenetic evaluation, cells were cultured on coverslips. Standard methods of harvesting and chromosome banding were used (11). The cell line was designated HCC1937 (for Hamon Cancer Center).

For establishment of a corresponding B-lymphoblastoid cell line, peripheral blood was centrifuged through Histopaque (Sigma Biochemicals, St. Louis, MO), washed in RPMI 1640, and resuspended in initiation medium consisting of RPMI 1640 with 15% fetal bovine serum, 25 mM HEPES, and 1 mM sodium

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[2] To whom requests for reprints should be addressed, at Department of Pediatrics and Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8593. Phone: (214) 648-4907/4903; Fax: (214) 648-4940; E-mail: tomlinson@simmons.swmed.edu.

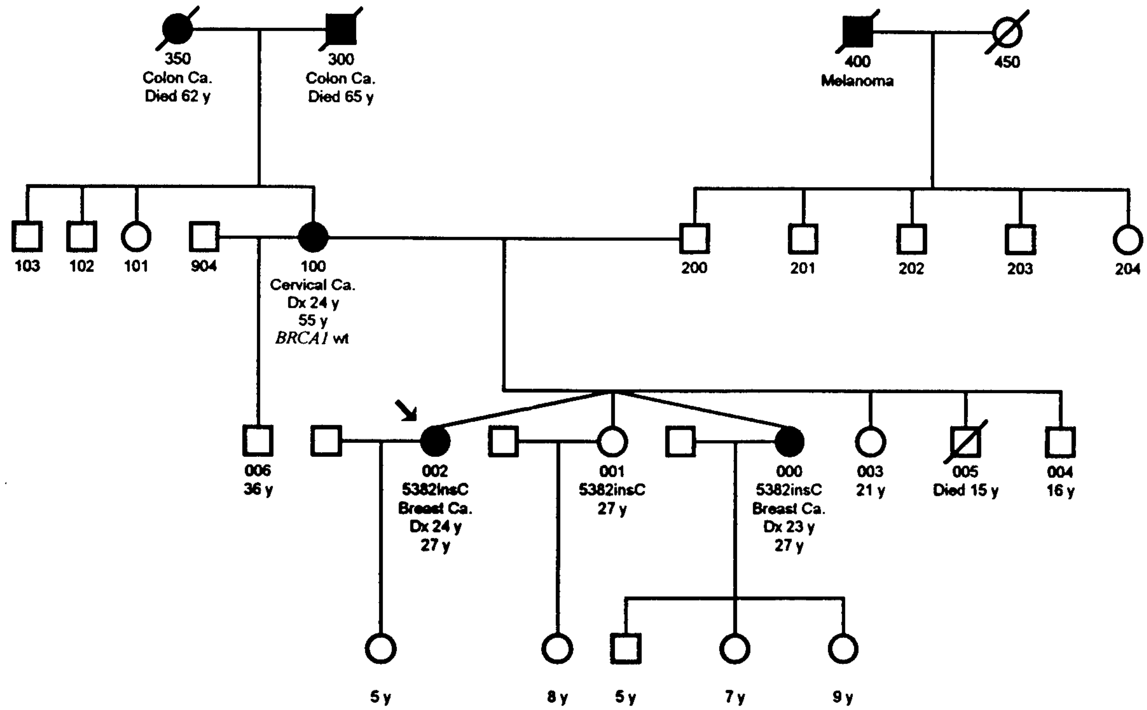


Fig. 1. Pedigree of the family from which the HCC1937 cell line was derived. The patient from which the tumor cell line is derived is indicated by the *arrow*. Germ-line DNA from the patient as well as the affected and one unaffected sister was heterozygous for the *BRCA1* mutation, 5382insC. The patient's mother's DNA demonstrated only wild-type *BRCA1*. DNA from the patient's father was not available for analysis.

pyruvate and 5 ml EBV-conditioned medium from an EBV-producing marmoset cell line (12). Cultures were incubated at 37°C with 5% CO_2. Medium was changed approximately weekly. Cultures were observed daily for approximately 2 weeks, when loose aggregates of nonadherent lymphocytes began to proliferate rapidly. DNA from the tumor cell line HCC1937, the B-lymphoblastoid cell line, and unprocessed peripheral mononuclear blood cells was prepared using standard methods (13).

Allelotyping. Using polymorphic dinucleotide and tetranucleotide microsatellite repeat markers, patterns of allelic losses were studied at loci throughout the genome known to be commonly lost in breast cancer. DNA from the cell line HCC1937 was compared with DNA from the peripheral blood cells as well as the B-lymphoblastoid cell line. Primer sequences were obtained from the Genome Database, and PCR amplification and electrophoresis were performed as described previously (14). For allelotype analysis of the primary tumor, areas were microdissected as described previously (14).

Mutation Analysis. SSCP[3] analysis of genomic DNA was performed by a modification of the technique described by Orita *et al.* (15). Specific genes known to be involved in the pathogenesis of breast cancer were examined as possible secondary acquired changes in the cell line. Coding regions of exons 5–11 of the *TP53* gene, the entire open reading frame of *CDKN2A*, the *PTEN* gene, and the *BRCA1* gene were analyzed (16–21). Primers were designed to amplify fragments 150–200 bp in length. Sequence analysis of DNA fragments demonstrating abnormal mobility on SSCP gels was performed by cloning amplified PCR fragments into pCMV5 vectors and sequencing using Sequenase (United States Biochemical, Cleveland, OH) according to the manufacturer's instructions. ^{35}S-Labeled reactions were electrophoresed on 6% acrylamide gels. A minimum of 8 clones was sequenced for each region of interest. Direct sequence analysis of the entire coding region of the *BRCA2* gene was done by Myriad Genetics (Salt Lake City, UT). Mimatched primer pairs were designed at mutation sites as described in "Results."

Southern blotting was performed to confirm the presence or absence of the *PTEN* coding sequence DNA in the tumor cell line as well as constitutional DNA. Genomic DNA was digested overnight with restriction enzymes *Eco*RI, *Hin*dIII, *Kpn*I, *Bam*HI, and *Mbo*I. Digested DNA was blotted on Hybond (Amersham, Arlington Heights, IL) membranes according to directions provided by the manufacturer. DNA probes were prepared by amplification of the coding region(s) of exons 2–8 of the *PTEN* gene as described previously (22). Hybridization with ^{32}P-labeled probe was carried out using standard techniques (13).

Results

Cell Line Establishment. A breast cancer cell line, designated HCC1937 (Hamon Cancer Center), was established from a grade III infiltrating ductal primary breast tumor from a 24-year-old breast cancer patient with a germ-line *BRCA1* mutation. On histological evaluation of the primary tumor, large vacuoles were observed in many of the cells suggestive of a secretory variant of infiltrating intraductal carcinoma (Refs. 23 and 24, Fig. 2*a*). The cultured tumor cells also contained similar vacuoles and demonstrated a striking resemblance to the primary tumor (Fig. 2*b*). The vacuoles failed to stain with periodic acid-Shiff (with and without diastase treatment), alcian blue, mucicarmine, or oil red O (not shown). These results indicate that the vacuoles lacked glycogen, mucins, or neutral fat. The appearance of these cells was similar to the cytological appearance of cells of secretory carcinoma (25).

The cultured cells grew as an adherent monolayer. During growth phase they had the appearances of small to medium epithelioid cells with finely granular eosinophilic cytoplasm and nuclei demonstrating moderate atypia and occasional mitoses. However, at heavy cell density, a progressively increasing number of the larger vacuolated cells appeared. Approximately 11 months after initiation, it was apparent that a cell line had been established, as evidenced by continuous growth even after recovery from cryopreservation. Immortalization was further demonstrated in that the cells have grown continuously for over 30 months, have undergone multiple passages, and have demonstrated telomerase activity (data not shown).

Progesterone and estrogen receptor radiobinding assays demonstrated no significant levels of progesterone or estrogen binding in

[3] The abbreviations used are: SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity.

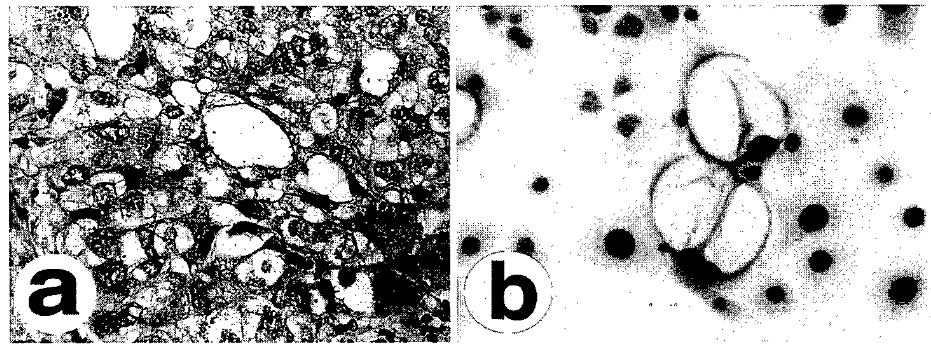


Fig. 2. Morphology of the breast cancer primary tumor and cell line, H&E stain. *a*, the primary breast carcinoma from which HCC1937 was derived. *b*, HCC1937 tumor cell line, cytospin preparation. Giant vacuolated mono- and dinucleated cells are present in both the tumor and cell line. The nonvacuolated cultured cells are medium sized and epithelioid.

either the primary tumor or HCC1937 cultured cells. Only very low levels of HER2/neu expression were observed.

Molecular Analysis. SSCP analysis of *BRCA1* revealed an abnormality in exon 20 in both DNA derived from peripheral blood as well as the cultured cells (Fig. 3). DNA from cells derived from peripheral blood revealed a normal pattern as well as an extra band, whereas SSCP analysis of the tumor cell line revealed an absence of a normal band present in the peripheral blood DNA. The extra abnormal band was also observed in DNA from each of the patient's triplet sisters, but not in the mother. The father's DNA was not available for analysis. Sequence analysis of the PCR product amplified from exon 20 from cell line DNA revealed an inserted C residue at nucleotide 5382. All cloned sequences obtained from HCC1937 DNA contained this mutation. No wild-type sequences were observed. Sequence analysis of microdissected archival tumor tissue also revealed the presence of the 5382insC mutation and lack of normal wild-type *BRCA1* sequence. To provide an alternative rapid method of detecting this mutation without the use of radioactivity, mismatched primers flanking the 53282insC mutation were designed, which resulted in an amplicon of 131 and 132 bp in the wild and mutant type alleles, respectively. The primer sequences are as follows: sense, 5′-CAAAGCGAGCAAGAGAATTCC-3′; and antisense, 5′-GTAATA-AGTCTTACAAAATGAAG-3′. The mismatched base in the sense sequence is underlined. The mismatched primer abolishes a restriction site (CCNNGG) in the wild-type allele, but not the mutant allele, for the enzyme *Bsa*JI (New England Biolabs, Beverly, MA; Fig. 3). The coding sequence of the *BRCA2* gene demonstrated no abnormality.

Single-strand conformation analysis of the *TP53* gene revealed an abnormal band in exon 8. Sequence analysis revealed a substitution of a C for a T nucleotide, resulting in a termination codon at position 306. This change was not present in the germ-line DNA and thus was acquired. The *TP53* mutation was also confirmed by sequencing of DNA from the microdissected primary tumor tissue. Primers were designed for rapid detection of this mutation as follows: sense, 5′-AGGACCTGATTTCCTTACTGC-3′; and antisense, 5′-TGCAC-CCTTGGTCTCCTCCAC-3′. These primers result in an amplicon of 234 bp. The *TP53* gene mutation at codon 306 creates a restriction site (CACNNNGTG) for the restriction enzyme *Dra*III at nucleotides 909–917. The mutant type sequence is cut by *Dra*III, resulting in two fragments of 184 and 50 bp in length (Fig. 4).

Single-strand conformation analysis of the *CDKN2A* gene revealed no abnormality. DNA from HCC1937 repeatedly failed to amplify with primers designed to amplify exons 1–8 of the *PTEN* gene, suggesting the presence of a homozygous deletion, but did amplify exon 9 of this gene. To confirm whether this observation represented a true deletion of the *PTEN* gene, Southern blotting was performed. A Southern blot of DNA from HCC1937, lymphocyte DNA from the patient, as well as DNA from other cell lines, were digested with *Hin*dIII and hybridized with a ^{32}P labeled *PTEN* coding sequence probe (20). An absence of bands corresponding to the *PTEN* coding sequence in HCC1937, with a normal pattern observed in the lymphocyte DNA, was demonstrated (Fig. 5). Similar results were obtained when DNA was digested with *Eco*RI, *Kpn*I, *Bam*HI, *Xba*I, and *Mbo*I. The *PTEN* pseudo-gene, *PTEN2* (22), localized to chromosome 9, was seen in all DNAs and provided an internal control for the *PTEN* homozygous deletion.

Allelotyping Data. Allelotyping results comparing HCC1937 and peripheral blood DNA at 51 informative and 10 uninformative markers are summarized in Table 1. A LOH was observed in the majority of loci examined including chromosomal regions 1p21, 1p36, 3p21, 5q11–5q22, 6q13, 6p21.3, 8p21, 9p21, 10q23–4, 13q12.2–13, 17p13.1, and 17q21, whereas retention of heterozygosity was observed at 3p25, 3q26, 4q33–35, 5p15, 7q31, 8q11.2, 9p12–13, 9q21–33, 11p15.5, 13q14, and 19p12–3. Using comparisons of the mother's DNA, the parental origin of allele loss could be determined at most loci. Both paternal and maternal allele loss was observed. No acquired

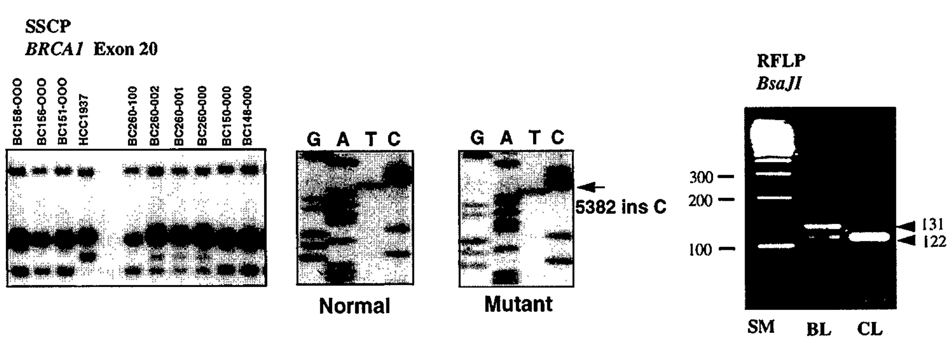
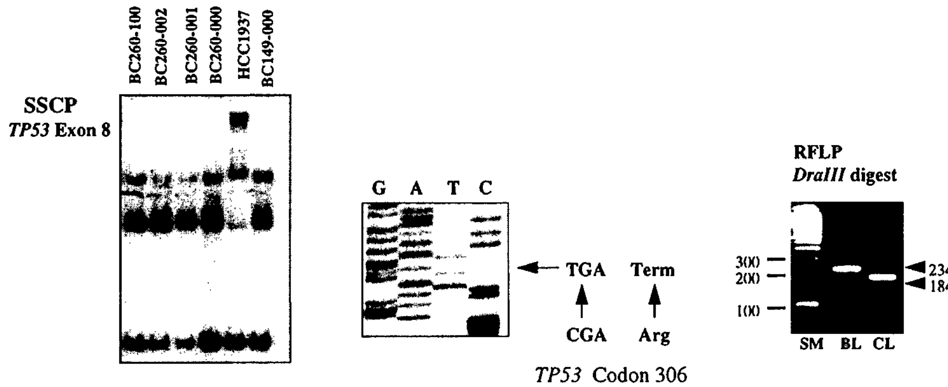


Fig. 3. Molecular analysis of *BRCA1*. Single-strand conformation analysis (*left*) revealed an aberrant band in lymphocyte DNA from the patient (*BC260-002*) and each of her two sisters analyzed (*BC260-001* and *BC260-000*). The tumor cell line demonstrated the mutant band as well as the absence of a wild-type band observed in the constitutional DNA. Sequence analysis (*middle*) revealed an inserted C residue at position 5382. No wild-type sequence at position 5382 was detected in any of the clones analyzed from HCC1937-amplified DNA. Designed restriction fragment length polymorphism analysis using mismatched repair primers as described in "Results" is demonstrated at *right*. Both uncut (131) and cut (122) fragments are detected in the B-lymphoblastoid cell line (*BL*), whereas in the HCC1937 tumor cell line (*CL*), only the cut fragment (122 bp) is observed. *SM*, size marker, 100-bp ladder.

Fig. 4. Molecular analysis of *TP53*. Single-strand conformation analysis of the *TP53* gene revealed an abnormality in exon 8. Sequence analysis demonstrated a point mutation leading to a termination at codon 306. This mutation is also demonstrated by designed restriction fragment length polymorphism method as described in the text. DNA from the lymphoblastoid cell line (*BL*) contained only the wild-type allele, demonstrated by the uncut fragment (234 bp), whereas the cell line HCC1937 (*CL*) demonstrated only the mutant allele, demonstrated by the cut fragment (184 bp).



extraneous bands suggestive of microsatellite instability were noted at any of the loci examined. At selected loci, allelotyping of microdissected archival material was also performed with results identical to the cell line DNA in all loci examined (Table 1). Not all loci examined in the tumor cell line were examined in microdissected archival tissue because of limited archival material.

Cytogenetics. Cytogenetic analysis revealed an extremely complex abnormal karyotype. Of 19 metaphases, no 2 revealed the exact same karyotype. An approximately equal number of metaphases were observed with modal numbers of 51–56 and 92–110 chromosomes, consistent with the evolution of a clone of cells with a near-tetraploid karyotype in addition to a clone of near-diploid cells. Double minute chromosomes were observed rarely in some passages. Numerous marker chromosomes were observed of unknown derivation. The complete composite karyotype of the two modal clones is shown as follows:

51~56,add(X)(q26),−X,add(1)(q32),add(1)(q32),der(1;2)(q10;p10)ins(1;?)(q21;?),+2,der(2)t(2;5)(q31;q13),der(2)del(2)(p11.2)t(2;5)(q31;q13),add(3)(p13),dup(3)(q21q27),der(4;8)(p10;q10)t(1;8)(p22;q24.3),der(4)t(4;4)(p16;q12),i(5)(p10),+7,add(7)(p11.2),der(7)t(7;7)(q11.2;p13),add(8)(p11.2),−10,add(11)(p11.2),der(11)t(11;18)(p11.2;q12.2)del(11)(q23),der(13)t(5;13)(q22;q22),dup(13)(q14q32),−14,add(15)(q24),del(15)(q22q24),+16,add(16)(p11.2)×2,+inv(16)(p13.1q22)×2,der(18)dup(18)(q11.2q21)t(1;18)(q21;q21),add(19)(p13.1),−21,+mar1,+mar2,+6~9mar[cp8 cells]/

93~110<4n>,−X,−X,add(X)(q26)×2,add(1)(q32),der(1;2)(q10;p10)ins(1;?)(q21;?),der(2)t(2;5)(q31;q13),der(2)del(2)(p11.2)t(2;5)(q31;q13),add(3)(p13)×2,−4,−4,der(4;8)(p10;q10)t(1;8)(p22;q24.3)×2,i(5)(p10)×2,−6,−6,add(7)(p11.2)×2,der(7)t(7;7)(q11.2;p13)×2,+8,add(8)(p11.2)×3,−10,−10,+11,+11,add(11)(p11.2)×2,der(11)t(11;18)(p11.2;q12.2),del(11)(q23)×2,−12,−12,dup(13)(q14q32)×2,−14,−14,add(15)(q24)×2,del(15)(q22q24)×2,add(16)(p11.2)×2,inv(16)(p13.1q22)×2,−18,−18,der(18)dup(18)(q11.2q21)t(1;18)(q21;q21),−19,add(19)(p13.1)×2,−21,+mar1×2,+mar2,+mar3×2,+mar4,+mar5,+10~12mar[cp11 cells]

Discussion

In this study, we report the establishment and characterization of breast carcinoma cell line HCC1937, derived from a germ-line *BRCA1* mutation carrier. Histologically, the tumor is characterized as an invasive ductal carcinoma with features of secretory carcinoma. Like many of the mutant *BRCA1*-associated tumors described to date, the tumor and the corresponding cell line lacked estrogen or progesterone receptors (4, 26, 27). Like the majority of disease-associated *BRCA1* mutations, the mutation present in this breast cancer cell line causes a truncated protein product. The inserted C at nucleotide 5382 results in erroneous translation of the protein distal to codon 1755 and termination at codon 1829, whereas wild-type BRCA1 consists of 1863 amino acids. Evidence suggests that the COOH terminus of BRCA1 is essential for function in that patients with a germ-line truncating mutation at codon 1853 are susceptible to early-onset breast cancer, and *in vitro* studies demonstrate that the COOH terminus of BRCA1 is active in transcriptional activation (6, 20). This particular *BRCA1* mutation has been observed in multiple families and is the second most common *BRCA1* mutation reported (28).

Although several series of breast carcinoma cell lines have been reported, no previously established cell line is known to be associated with mutation of *BRCA1*. Yuan *et al.* (29) reported an ovarian cancer cell line that carries a mutation of *BRCA1*, causing a truncation at the

***PTEN*/ 1.13 Kb cDNA Probe (Exon 2 -Exon9)**

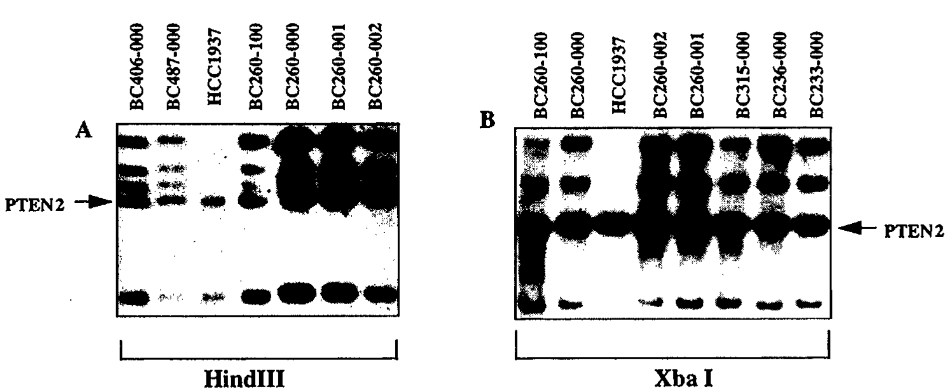


Fig. 5. Southern blot demonstrating absence of the *PTEN* coding sequence in HCC1937. DNA was digested with *Hin*dIII (*left*) or *Xba*I (*right*). The 1.13Kb probe used was prepared from *PTEN* cDNA and contains exons 2–9 (22). Absent bands were observed in the lane containing HCC1937 DNA. Similar results were observed with restriction digests using the enzymes *Eco*RI, *Kpn*I, *Bam*HI, and *Mbo*I (not shown).

Table 1 *Allelotyping of HCC1937 cell line DNA and corresponding primary tumor*

Chromosomal band	Locus[a]	Allelotyping results: Primary tumor	Allelotyping results: Cell line	Parental source of loss
1p36	*D1S1597*		LOH	ND[b]
1p21	*AMY2B*		LOH	Paternal
3p21–31	*D3S1029*		LOH	Paternal
3p14	*D3S1766*	LOH	LOH	Paternal
3p21	*D3S1477*	LOH	LOH	Paternal
3p21	*ITIH*	LOH	LOH	Paternal
3p24.2–p22	*D3S1537*		LOH	Paternal
3p25	*D3S1531*		RH	
3p25	*D3S1537*		RH	
3q26.1–q26.3	*GLUT2*		RH	
4q	*D4S266*		RH	
4q33–35	*mfd22*		RH	
5p15–15.1	*mfd88*		RH	
5p15.1–15.2	*D5S406*		RH	
5p15.3–p15.1	*D5S117*		RH	
5q22–q32	*IL9*		LOH	ND
5q21–q22	*APC*	LOH	LOH	Paternal
5q11.2–q13	*mfd27*		LOH	Paternal
5q33	*mfd154*		LOH	Paternal
5q13–q14	*CRTL*		LOH	Paternal
5cen–5q11.2	*D5S76*		LOH	ND
6p21.3	*TAP1*	LOH	LOH	Paternal
6q13	*D6S280*	LOH	LOH	Paternal
7q31.1–q31.2	*D7S522*		RH	
7q31	*WNT2*		RH	
8q11.2–q12	*D8S285*		RH	
8p21–22	*D8S602*	LOH	LOH	Paternal
8p21–22	*D8S254*	LOH	LOH	Paternal
9p21	*IFNA*	LOH	LOH	Maternal
9p21	*D9S1748*		LOH	ND
9p21	*D9S171*		LOH	Maternal
9p21	*IFNA2*		LOH	ND
9p21	*D9S1747*		LOH	Maternal
9p13	*PAL127*		RH	
9p12	*IF6*		RH	
9q22.3–q31	*9S58*		RH	
9q21.1–q13	*9S146*		RH	
9q31	*9S109*		RH	
9q22	*9S196*		RH	
10q23–q24	*D10S185*	LOH	LOH	Paternal
11p15.5	*TH3.1*		RH	
11p15.5	*IGF2*		RH	
11q	*INT-2*	NI	NI	
11q	*PYGM*	RH	RH	
13q12.3–q13	*D13S267*		LOH	Maternal
13q12.3–q13	*D13S171*		LOH	ND
13q14	*RB*	RH	RH	
17p13.1	*TP53AAAAT*	LOH	LOH	Maternal
17q21	*D17S1322*		LOH	Maternal
19p12	*D19S433*	RH	RH	
19p13.2	*D19S391*	RH	RH	

[a] Markers that were examined that were not informative included *D1S116* (1p31–p21), *D3S1577* (3p12), *D3S1313* (3p14), *KICA* (3p21.3), *RHO1.2* (3q21–q24), *mfd122* (5q31–33.3), *D8S137* (8p11–21), *D6S300* (6q13–14), *D9S126* (9p22), and *D19S253* (19p13.1).
[b] ND, not determinable; RH, retention of heterozygosity; NI, not informative.

COOH-terminal portion of the protein. It is not known whether this *BRCA1* mutation is germ line, although it is quite possible that this line derived from a *BRCA1* mutation carrier because of a separate report of the same germ-line mutation in a breast-ovarian cancer family (30) and because sporadic mutations in ovarian cancer are rare (8, 31).

The cell line HCC1937 demonstrated a considerable degree of aneuploidy as demonstrated by multiple karyotypic abnormalities, a high incidence of LOH at loci involved in breast cancer pathogenesis, and a high DNA index. Of 19 cell lines examined, this tumor demonstrated the highest incidence of LOH.[4] At multiple loci, the corresponding archival tumor tissue was allelotyped as well, with identical findings of allele loss or retention at each locus examined. Marcus *et al.* (32) reported, in a series of hereditary breast cancers using archival tissue, that mutant *BRCA1*-associated tumors demonstrate a considerably higher degree of aneuploidy than either sporadic breast cancers or non-*BRCA1*-related hereditary breast cancers. In addition to a large degree of chromosomal abnormalities, a specific number of other specific molecular changes known to be important in breast cancer pathogenesis were noted to exist in our cell line. The tumor cell line also acquired a *TP53* mutation, not present in the germ line, with loss of the wild-type allele in the tumor. This tumor cell line also demonstrated a homozygous deletion of the *PTEN* gene, the underlying genetic defect in Cowden's syndrome. However, we were unable to detect any mutation, rearrangement, or deletion in the *PTEN* gene in germ-line DNA in this family. In addition, neither the proband nor any of her immediate family members demonstrated signs characteristic of Cowden's syndrome.

The breast cancer risk associated with the *BRCA1* 5382insC mutation is ~55% by age 70 according to one study (33). This risk increases with age, and although the risk at all ages is greater than that of noncarriers at all ages, the observed incidence of breast cancer in the early twenties as observed in this patient and her sibling suggests that other factor(s), either genetic or environmental, may have influenced the development of breast cancer in this family. The question arises as to whether an additional genetic predisposition factor is carried by this family. However, no additional germ-line mutations were found in *BRCA2, PTEN,* or *TP53*. In the rarely observed families in which more than one breast cancer predisposing germ-line mutation occurs in the same individual, the phenotypes are not markedly different with respect to age of onset or number of tumors (34, 35). Perhaps other yet unidentified genetic predisposition genes, genetic modifiers, or environmental factors contributed significantly to early onset of tumor development in this family. The fact that both the patient from whom the cell line derived, as well as her affected sister, had very early-onset breast cancers, and both previously bore children at an early age, suggests that in this family, early child-bearing was not a protective factor. This observation, along with the estrogen and progesterone receptor-negative status, suggests that factors other than hormonal stimulation had stimulated tumor development.

Considerable controversy has existed over the localization of the BRCA1 protein in both normal and malignant tissue. One of the technical challenges in determining the cellular localization of BRCA1 is the specificity of antibodies for the BRCA1 protein. The establishment of a cell line that is null for any COOH-terminal BRCA1 should be useful in sorting out antibody specificity and cellular localization issues. In addition, studies comparing localization of BRCA1 in its mutant form compared with wild-type BRCA1 will be useful in elucidating the role of *BRCA1*. Likewise, transfection studies with wild-type BRCA1 have only been done with breast cancer cells that already contain wild-type BRCA1 (36). It will be of interest to see the effect on cell growth and tumorigenicity of replacing wild-type BRCA1 into the HCC1937 cell line.

Although the tumor from which our cell line derives is distinctive in terms of its histology and very early age of onset, the acquired *TP53* mutation, the estrogen receptor/progesterone receptor negativity, and the marked aneuploidy observed may prove to be characteristic of *BRCA1*-associated tumors. Thus, cell line HCC1937 may serve as a very useful reagent in studying breast cancer pathogenesis in *BRCA1* families.

Acknowledgments

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[4] A. Gazdar, unpublished data.

Note Added in Proof

The cell line HCC1937 has been deposited with the American Type Culture Collection.

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HUMAN MUTATION 14:216–221 (1999)

RAPID COMMUNICATION

TP53 Mutation and Haplotype Analysis of Two Large African American Families

Jaclyn Hung,[1] Betsy Mims,[3] Guillermina Lozano,[3] Louise Strong,[4] Carolyn Harvey,[5] Tina T.-Y. Chen,[1] Victor Stastny,[1] and Gail Tomlinson[1,2]*

[1]*Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas*
[2]*Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas*
[3]*Department of Molecular Genetics, University of Texas M.D. Anderson Cancer Center, Houston, Texas*
[4]*Experimental Pediatrics, University of Texas M.D. Anderson Cancer Center, Houston, Texas*
[5]*Department of Nursing, University of Texas Health Science Center at Tyler, Texas*

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Two large apparently unrelated African American families with a high incidence of breast cancer and other tumors characteristic of Li-Fraumeni breast sarcoma cancer family syndrome were studied. Mutation screening revealed that in both families the affected members carried a germline mutation of the TP53 gene at codon 133 (ATG → ACG, M133T). In order to determine whether an ancestral haplotype was shared by these two families, polymorphic markers within and flanking the *TP53* gene were studied. Haplotype analysis using five markers revealed an identical haplotype shared by the two families. Loss of heterozygosity at the *TP53* locus in the probands' tumor tissues from each family was observed; in each case, the retained allele carried the common haplotype. The frequency of this haplotype in the general African American population is <0.003. This unique haplotype, combined with the rare *TP53* mutation, suggests that these African American families share a common ancestry. This finding suggests that other African Americans may be carriers of this mutation and thus may be at risk of early-onset breast cancer or other cancers characteristic of the Li-Fraumeni breast sarcoma cancer family syndrome. The finding of recurring mutations in African Americans may facilitiate carrier screening and identification in this population. Hum Mutat 14:216–221, 1999.

KEY WORDS: *TP53*; **breast cancer; Li-Fraumeni syndrome; haplotype analysis**

INTRODUCTION

Germline *TP53* (MIM# 191170) mutations in the general population are very rare; however, the presence of such a mutation confers an extremely high risk of developing cancers characteristic of Li-Fraumeni breast-sarcoma cancer family syndrome (LFS; MIM# 151623) in affected individuals [Malkin et al., 1990; Srivastava et al., 1990] Most mutations reported in families with Li-Fraumeni Syndrome have involved point mutations in conserved coding regions of the *TP53* gene [reviewed in Birch et al., 1994] Although some mutations, particularly those in exon 7, have been reported in multiple families, most of the reported mutations have been unique. No haplotye information has been found to determine the origin of these mutations.

METHODS

Two apparently unrelated large African American families were studied. No common surnames or cities or town of residence were shared among the two families. The first family BC54 was ascertained as part of a study of early onset and familial breast cancer. The second family SARC-36, was ascertained from a series of childhood

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*Correspondence to: Gail Tomlinson, Department of Pediatrics, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-8593; Fax: 214-648-4940; E-mail: tomlinson@simmons.swmed.edu

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TABLE 1. Polymorphic Markers at the *TP53* Locus

N	Marker	Locus	Heterozygosity	Reference
1	$(CA)_n$	*TP53* locus	0.90	Jones and Nakamura [1992]
2	$(AAAAT)_n$	Intron 1	0.80	Futreal et al. [1991]
3	16-bp rpt	Intron 3	0.28	Lazar et al. [1993]
4	*Msp*1	Intron 6	0.46	McDaniel et al. [1991]
5	$(ATT)_n$	*TP53* 3′	0.70	Evans et al. [1998]

sarcoma patients. Our proband is a part of a previously described Li-Fraumeni kindred, STS170, with a rare germline *TP53* mutation at codon 133 resulting in a methionine to threonine substitution. [Law et al., 1991]. This M133T mutation was previously shown to segregate with the presence of early-onset cancers in this kindred. The pedigrees of families BC54 and STS170/SARC36 are shown in Figure 1.

Single-strand conformation polymorphism (SSCP) analysis according to the method of Orita et al. [1989], as well as sequence analysis were used to analyze peripheral blood lymphocyte DNA from probands of each family.

Haplotype analysis was performed on peripheral blood lymphocyte DNA as well as microdissected tumor tissue using five polymorphic markers within or flanking the *TP53* locus shown in the Table 1. These markers have been previously described. [Evans et al., 1998; Futreal et al., 1991; Jones et al., 1992; Lazar et al., 1993; McDaniel et al., 1991]. To determine allele frequency of these markers in the African American population, 27 unrelated African Americans were genotyped. Allele frequencies of the intron 3 $(ATT)_n$ marker derived from the 54 chromosomes is shown in Table 2. Determination of allele frequencies of the other polymorphisms was similarly determined in African Americans; however, these were not significantly different from those previously published.

RESULTS

SSCP analysis was performed on peripheral blood and tumor DNA from these two families and identical abnormalities were observed. Sequence analysis revealed that both families carried the same mutation at codon 133 (ATG → ACG, M133T) (Fig. 2).

Allelotype data demonstrated that the proband from the SARC36/STS170 family and two affected individuals from family BC54 were heterozygous for markers #1, #2, and #5 and family BC54 was heterozygous for marker #3. An allele shared between both families was observed for each informative marker. Tumor tissue in both cases demonstrated a loss of heterozygosity with retention of the shared allele. Tumor retention of the common allele using the $(AAAAT)_n$ marker is shown in Figure 3. Allelotyping using the $(ATT)_n$ marker in seven family members of STS170 (SARC36) is shown in Figure 4.

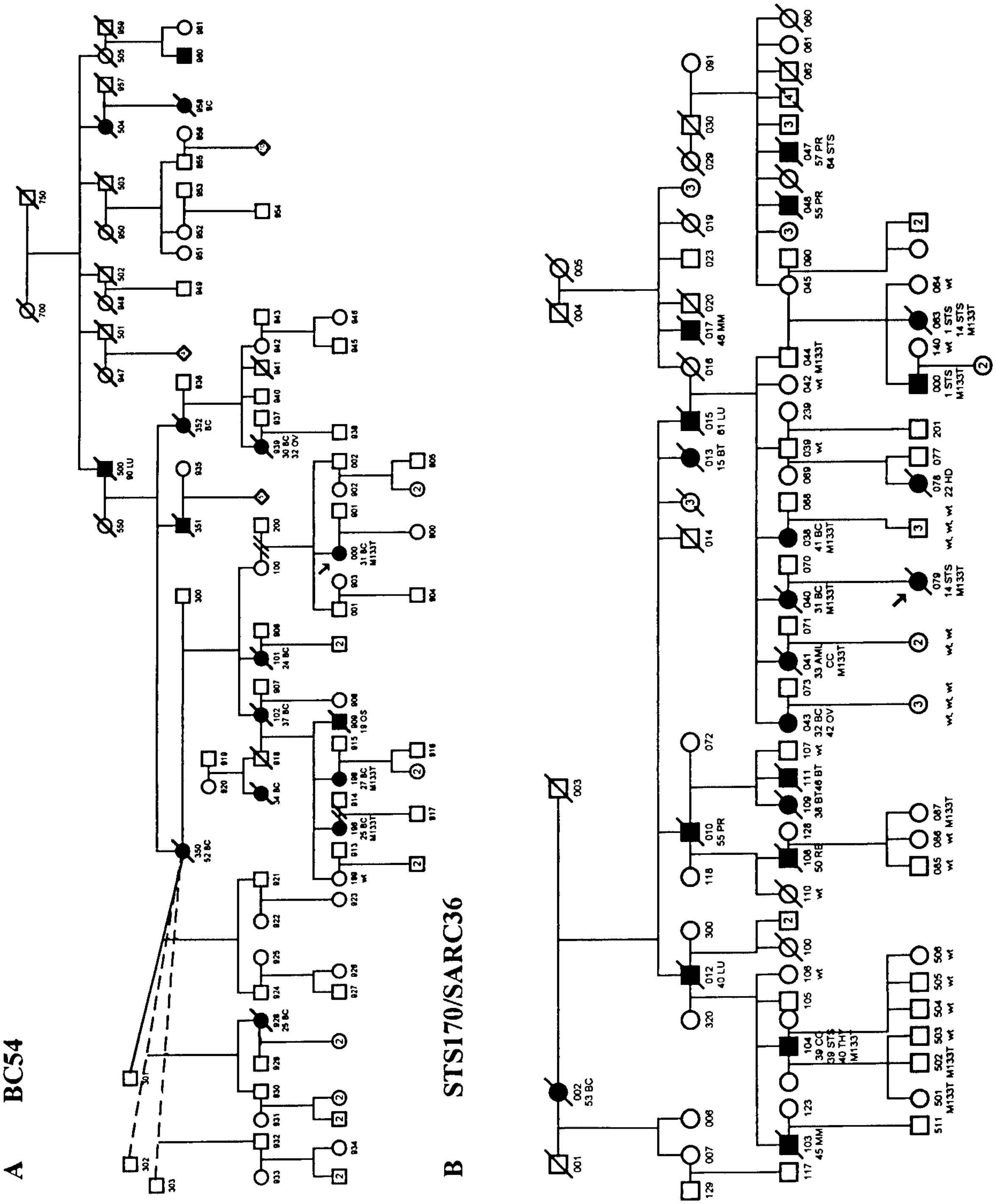
The sizes and frequencies of the alleles shared among affected members of the two families are shown in Table 3. The frequencies of the disease-associated alleles of marker #1, 2, 3, and 5, were 0.24 $(CA)_n$, 0.23 $(AAAAT)_n$, 0.86 (16-base pair [bp] repeat), and 0.07 $(ATT)_n$, respectively, in African Americans. The probability that this haplotype had occurred at random is 0.3% ($0.24 \times 0.23 \times 0.86 \times 0.07 = 0.003$).

A total of 18 members of family STS170 and four members of BC54 were haplotyped. In family STS170, individuals 000, 501, 502, 104, 087, and 063 carry both the M133T mutation as well as the disease-associated $(ATT)_n$ allele. Members 503, 504, 505, 106, 107, 110, 085, and 086 demonstrated neither the mutation or the disease associated haplotype. Four other members demonstrated no mutation and carried the $(ATT)_n$ allele #1; however, complete haplotype analysis at all the markers described in this report was not done on these individuals. In family BC54, individuals 000, 196, and 198 carry both the mutation and the common haplotype, while individual 199 carried neither the mutation nor the associated haplotype.

TABLE 2. Allele Frequencies of *TP53* 3′ $(ATT)_n$ Marker in African Americans

Allele	Size (bp)	Frequency
1[a]	153	0.07
2	150	0.15
3	147	0.05
4	144	0.05
5	141	0.13
6	138	0.15
7	135	0.04
8	132	0.02
9	129	0.05
10	126	0.27

[a]Disease-associated allele in two kindreds described.


A
BC54
B
STS170/SARC36

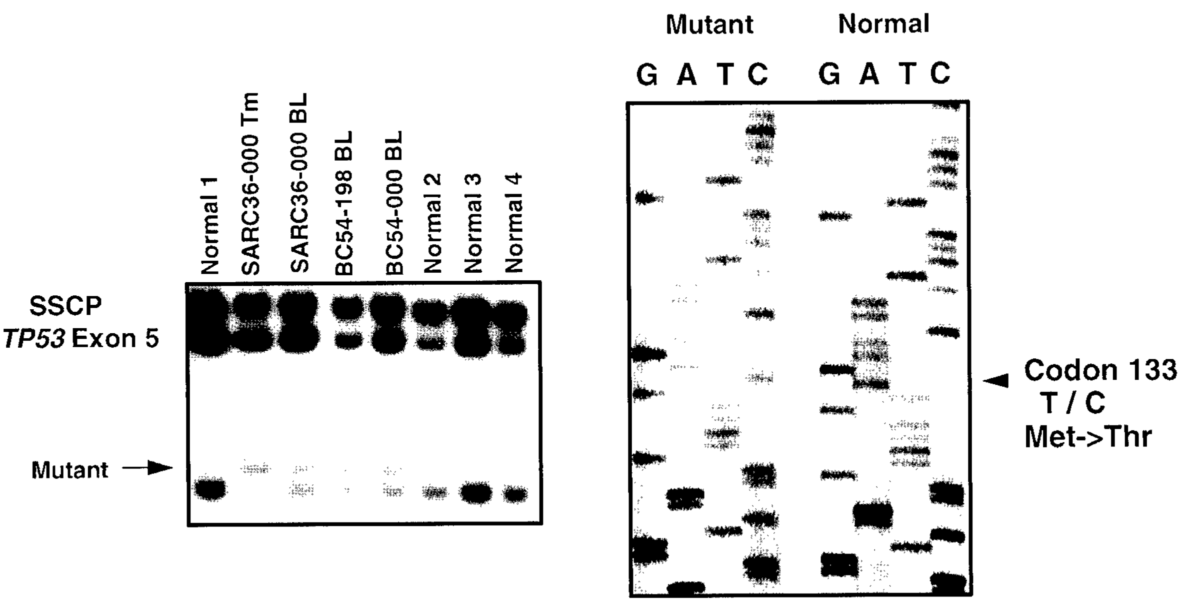


FIGURE 2. Mutation detection by single-strand conformation polymorphism (SSCP) and sequence analysis. At left is SSCP analysis of exon 5 of the*TP53* gene of genomic lymphocyte DNA from affected members of BC54 (BC54-000 BL and BC54-198 BL) and lymphocyte and tumor DNA from SARC36-000 (SARC36-000 BL and SARC36-000 Tm). At right is sequence analysis of lymphocyte DNA demonstrating normal as well the T→ C substitution at codon 133.

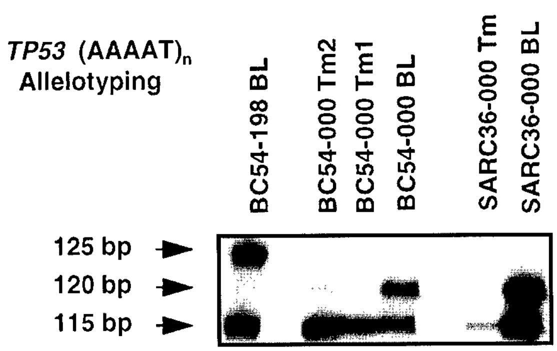


FIGURE 3. Allelotyping of DNA from blood and tumor using the previously described $(AAAAT)_n$ polymorphism. Allele sizes are indicated in base pairs. The 115-bp allele was common in all affected individuals in both families and was retained in a breast tumor from BRC54-000 and a soft tissue sarcoma tumor specimen from SARC36. Both tumors demonstrated loss of other allele. Two different tumor areas from individual BC54-000 were analyzed. Lane marked BC54-000 Tm1 demonstrated complete allele loss, whereas lane marked BC54-000 Tm2 demonstrated small amount of retention of other allele, most likely due to contaminating normal tissue.

FIGURE 1. Pedigrees of families studied. **A:** Pedigree of family BC54, ascertained through a hospital based series of familial and early-onset breast cancer patients. **B:** Pedigree of family STS170/SARC36, ascertained through a proband with a childhood soft tissue sarcoma. Mutation carrier status is indicated by M133T and wild-type status indicated by wt. Numbers directly under symbols refer to unique identifiers of family members. Numbers preceeding diagnoses indicate age of onset. BC, breast cancer; Co, colon cancer; BT, brain tumor; Lu, lung; HD, Hodgkin's disease, STS, soft tissue sarcoma, Pr, prostate; Thy, thyroid, MM, multiple myeloma; Ov, ovarian; OS, osteosarcoma; Re, rectal cancer.

DISCUSSION

Although it is unclear as to how common this mutation is in the general population, a similar haplotype expected to occur at random in 0.3% of the population in these two unrelated African American families together with the identical rare germline *TP53* mutation suggests a founder chromosome affect. Because of the limited number of individuals with this mutation, it is not possible to

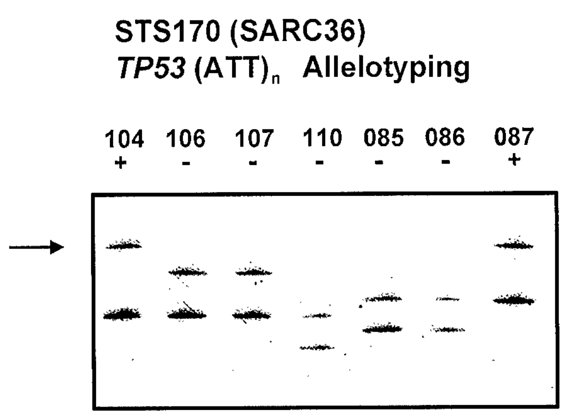


FIGURE 4. Allelotyping of seven members of kindred STS170/SARC36 using the $(ATT)_n$ polymorphism. Lane numbers indicate individual identifiers as shown in pedigree in Figure 1. Mutation status at codon 133 is shown by a + sign, indicating the presence of the M133T mutation or a – sign, indicating no mutation. *Arrow*, presence of allele #1, associated with the presence of the mutation. This was the largest of the alleles observed in any affected or unaffected individuals studied; it has a size of 153 bp.

TABLE 3. Features of Mutation-Associated Haplotype

Marker no.	Marker type	Mutation-associated haplotype size or identification	Population frequency of allele
1	$(CA)_n$	113	0.24
2	$(AAAAT)_n$	115	0.23
3	16-bp rpt	A1	0.86
5	$(ATT)_n$	153	0.07

determine how many generations ago the mutation occurred as is possible with other common germline mutations [Neuhausen et al., 1996], however; on the basis of pedigree analysis, it is evident that this mutation occurred at least five generations ago. The occurrence of this common haplotype also suggests that other African Americans may carry this germline mutation and thus may be at very high risk of breast and other cancers characteristic of the Li-Fraumeni syndrome.

To date, no founder effects have been documented in the *TP53* gene; however, founder effects have been studied in the other breast cancer predisposition genes, *BRCA1* and *BRCA2*. Specific founder effects in the Ashkenazi Jewish population and French Canadians have greatly facilitated carrier identification and genetic counseling [Neuhausen et al., 1996; Simard et al., 1994; Struewing et al., 1995; Tonin et al., 1998]. Other recurring mutations with common haplotypes of *BRCA1* or *BRCA2*, or both, have also been reported in Icelandic, British, Austrian, Dutch, Belgian, Russian, and Hungarian populations [Hakansson et al., 1997; Johannesdottir et al., 1996; Johannsson et al., 1996; Peelen et al., 1997; Ramus et al., 1997]. No definitive recurring mutations in the breast cancer genes *BRCA1* or *BRCA2* with evidence of founder effects have been reported in African Americans; however, two *BRCA1* mutations have been reported as recurring in two distinct African American kindreds each [Gao et al., 1997]. The paucity of haplotype studies of African American breast cancer families may be because the African American population was a previously understudied population. Further studies of *BRCA1* and *BRCA2* or *TP53* in African Americans may reveal additional common recurring mutations in these genes.

Thus, the mutation reported in this article is the first definitive founder mutation in a breast cancer gene in African Americans. It is also the first founder effect to be documented in a Li-Fraumeni family. Other mutations of the *TP53* gene are known to be recurring, particularly those in exon 7; however, it is unclear whether these represent mutations inherited from a common ancestor, or represent distinct mutational events in key residues involved in *TP53* function.

Further studies will be needed to determine the frequency of the M133T mutation among African Americans with early-onset breast cancer or other features of Li-Fraumeni syndrome and in addition whether other recurring breast cancer predisposing mutations occur unique to African Americans. In addition, the identification of multiple kindreds with identical mutations will permit the study of effects of environment, life style and other modifier genes on disease penetrance and spectrum of tumor types associated with identical genetic lesions.

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GENES, CHROMOSOMES & CANCER 28:359–369 (2000)

RESEARCH ARTICLES

Two Identical Triplet Sisters Carrying a Germline *BRCA1* Gene Mutation Acquire Very Similar Breast Cancer Somatic Mutations at Multiple Other Sites Throughout the Genome

Ignacio I. Wistuba,[1,2] Gail E. Tomlinson,[1,3] Carmen Behrens,[1] Arvind Virmani,[1] Joseph Geradts,[4] Joanne L. Blum,[5] John D. Minna,[1,6,7] and Adi F. Gazdar[1,8*]

[1]Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas
[2]Department of Pathology, Pontificia Universidad Catolica de Chile, Santiago, Chile
[3]Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas
[4]Nuffield Department of Pathology and Bacteriology, John Radcliffe Hospital, University of Oxford, Oxford, England
[5]Baylor-Charles A. Sammons Cancer Center, Baylor University Medical Center, Texas Oncology, P.A., Dallas, Texas
[6]Department Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas
[7]Department Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas
[8]Department Pathology, University of Texas Southwestern Medical Center, Dallas, Texas

Monozygotic twins, each of whom has breast cancer, offer a natural study population for gene-environmental interactions as causation of cancer, because they are genetically identical. If heritable factors play a large role in the origin of a neoplasm, disease concordance should be significant in monozygotic twins. Two monozygotic triplet sisters carrying a germline *BRCA1* gene mutation (5382insC) who both developed breast cancer at early ages were studied for loss of heterozygosity (LOH) in their microdissected, paraffin-embedded tumors along with control blood and stromal breast tissue at 19 chromosomal arms using 161 microsatellite markers. Microdissected areas of normal lobular and ductal epithelium and ductal in situ carcinoma were also studied for LOH using a subset of microsatellite markers. The mother's DNA (extracted from peripheral blood lymphocytes) was analyzed to determine the parental allele under LOH in each case. Both tumors demonstrated similar histologic features suggestive of a secretory variant of ductal carcinoma. The tumors from both sisters had similar overall LOH frequency expressed by the fractional allelic loss (FAL) indices (0.56 vs. 0.60) and demonstrated concordance for loss or retention at 82 of 97 informative markers (85% correlation). In addition, detailed mapping analysis of several chromosomal arms revealed that identical breakpoints were detected in both tumors at several chromosome regions. Finally, in both sisters' tumors, when a chromosome exhibited allelic loss, all of the markers exhibited LOH of the same parental allele even when there were intervening regions of retention of heterozygosity. In contrast, 17 archival sporadic breast carcinomas demonstrated a wide range of FAL indexes and highly individual patterns of LOH. Our findings support the hypothesis that inherited factors play a role in the development of the multiple somatic deletions occurring in breast carcinomas. Whether one of these factors is the mutant *BRCA1* allele or some other gene(s) remains to be determined. *Genes Chromosomes Cancer 28:359–369, 2000.* © 2000 Wiley-Liss, Inc.

INTRODUCTION

Family history, suggesting an inherited component in the development of some breast cancers, is one of the strongest known risk factors. It is estimated that 15% to 20% of women with breast cancer have a family history of the disease, and approximately 5% of all breast cancer is attributable to dominant susceptibility alleles (Slattery and Kerber, 1993). Two major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, have been identified (Miki et al., 1994; Wooster et al., 1995; Tavtigian et al., 1996). Breast cancer in families with germline mutations in these genes appears as an autosomal dominant trait, with high penetrance. However, in both sporadic and familial breast cancers, multiple other genetic changes also occur (Couch and Weber, 1998). Breast cancer pathogenesis is characterized by multiple molecular changes, including activation of oncogenes and loss of known and putative tumor suppressor genes (TSGs) (Couch and Weber, 1998). Loss of heterozygosity (LOH) is a frequent marker of TSGs

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*Correspondence to: Dr. Adi F. Gazdar, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390. E-mail: gazdar@simmons.swmed.edu

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because it is a common mechanism of inactivation of one allele (Knudson, 1989). The most common regions of LOH in breast cancer are located at chromosome regions 3p, 6q, 8p, 11p, 13q, 16q, 17p, 17q, and 18q (Callahan et al., 1993; Chen et al., 1994; Yaremko et al., 1995). Recent analyses have demonstrated that allelic losses can be detected early during tumor pathogenesis (Lakhani et al., 1995; Radford et al., 1995; Deng et al., 1996; O'Connell et al., 1998). In fact, some studies suggest that breast tumors occurring in *BRCA1* patients have multiple genetic changes, but no common pattern has emerged (Lakhani et al., 1995; Radford et al., 1995; Deng et al., 1996; O'Connell et al., 1998). Are these secondary, albeit crucial, genetic changes determined only by exogenous (nonhereditary) factors or could their genesis also have an inherited basis? Monozygotic twins offer an experiment of nature for study of the development of these acquired genetic changes in an isogenic situation. If heritable factors play a major role in the origin of these acquired genetic lesions, concordance should be significant in monozygotic twins. Studies of twins comparing maternal and fraternal twin pairs have been used for studies of breast cancer risks related to prenatal and genetic factors (Ahlbom et al., 1997; Swerdlow et al., 1997). In practice, such studies have already been limited by the difficulty of identifying a large number of twins with cancer. For example, there is a relative risk of 35 for monozygotic sisters of women with incidence of breast cancer at ages younger that 35 years, indicating that monozygotic twins of young cases constitute an extraordinarily high-risk group (Swerdlow et al., 1997). However, to the best of our knowledge, acquired genetic abnormalities present in breast carcinomas arising in monozygotic twins have not been studied.

Thus, when we were presented with an unusual opportunity to study breast cancers arising in two of triplet sisters who were genetically identical, both of whom carried a germline *BRCA1* mutation, we were interested in comparing the associated genetic changes in each breast cancer. To do this, we studied the patterns of LOH present in microdissected breast tumors using 161 microsatellite markers spanning 19 chromosomal arms, and we compared them with those present in 17 sporadic breast carcinomas. We were also able to investigate mechanisms associated with allelic loss by determining the parental origin of the alleles under LOH. We have found a remarkable concordance between acquired genetic changes arising in these two breast cancers. We also found specificity of the parental allele that exhibited LOH.

MATERIALS AND METHODS

Patients' Material

Patients A and B were two of three identical triplet sisters carrying a germline *BRCA1* gene mutation (5382insC) who developed invasive breast carcinoma at early ages. The family is Caucasian and not of known Ashkenazi Jewish descent. A pedigree of the family has been published previously (Gazdar et al., 1998; Tomlinson et al., 1998). Patient A was a 24-year-old woman with a nonmetastatic infiltrating ductal carcinoma of the breast. She had had one child previously at the age of 22 years. A breast cancer cell line (HCC1937) homozygous for the *BRCA1* 5382insC mutation was established from this patient's primary tumor, as previously reported (Tomlinson et al., 1998). Patient B, one of her identical sisters, had developed breast cancer the previous year at the age of 23. The third identical sister had bilateral prophylactic mastectomies at age 24. The triplets were raised together, and there was no known shared exposure to carcinogens. The patients' mother was reported to have had cancer of the uterine cervix at the age of 22, but not breast cancer. Both maternal grandparents had died of colon cancer in their 60s. After informed consent for genetic studies was obtained, blood and tumor tissues were obtained from patients A and B and blood from their mother. No adjuvant chemotherapy or radiation had been given prior to collection of tumor material. DNA from cells derived from peripheral blood from each of the triplet sisters demonstrated the same 5382insC *BRCA1* gene mutation, but this mutation was not found in the mother. The father's DNA was not available for analysis but most probably he was a *BRCA1* mutation carrier, although a de novo germline mutation cannot be excluded. In any event, by using maternal DNA, we were able to specify the parental allelic loss in the acquired genetic changes we found. To examine the likelihood that the patterns of LOH in both breast carcinomas from the triplet sisters (patients A and B) occurred by chance we studied a control group of 17 archival invasive breast carcinoma cases. The control group patients ranged in age from 24 to 82 years, although most ($n = 11$) were between the ages of 40 and 60 years. We recognize that an ideal control group would consist of other breast cancers arising in *BRCA1* mutant individuals but a large panel of these archival tumors was not readily available.

Microdissection and DNA Extraction

Archival paraffin-embedded breast tumor tissues were obtained from triplet sisters A and B. Areas of normal ductal and lobular epithelia, ductal in situ carcinoma (DCIS), and invasive carcinoma were identified and precisely microdissected from both cases under microscopic visualization. Areas of invasive carcinoma were also microdissected from the 17 breast carcinoma cases used as control group. Microdissection and DNA extraction were performed from hematoxylin-eosin–stained sections, as previously described (Hung et al., 1995; Wistuba et al., 1998). Normal stromal breast cells or lymphocytes from resected lymph nodes were used as a source of constitutional DNA.

Immunohistochemical Analyses

Immunohistochemical analyses of both sisters' tumors were performed using primary mouse monoclonal antibodies for estrogen receptor (clone 1D5, DAKO, Carpenteria, CA; dilution 1:20), progesterone receptor (clone 1A6, Novocastra, Vector, Burlingame, CA; dilution 1:20), HER2/neu (clone Cb11, cerb-2, Oncor, Gaithersburg, MD; dilution 1:200), p16 (clone G175-505, PharMingen, San Diego, CA; dilution 1:500), *RB* (clone 3C8, QED, San Diego, CA; dilution 1:500), and p53 protein (clone DO7, DAKO; dilution 1:500) expression. Immunostaining was performed using a standard avidin-biotin immunoperoxidase method, as previously described (Geradts et al., 1998).

BRCA1 and *TP53* Gene Mutation Analyses

Microdissected primary breast tumor, DCIS, normal duct, and lobular epithelia from both triplet sisters (patients A and B) were analyzed for the presence of heterozygous or homozygous germline *BRCA1* gene mutation, using a designed restriction fragment length polymorphism method as previously described (Tomlinson et al., 1998). Briefly, mismatched primers flanking the 5382insC mutation were designed, which resulted in an amplicon of 131 and 132 bp in the wild and mutant type alleles, respectively. The mismatched primer abolishes a restriction site (CCNNGG) in the wild-type allele, which is not present in the mutant allele, for the enzyme BsaJI (New England Biolabs, Beverly, MA). Microdissected primary breast tumor, DCIS, normal duct, and lobular epithelia from both triplet sisters (patients A and B) were also examined for exons 5 to 8 *TP53* gene mutations using single-strand conformation polymorphism (SSCP) analysis followed by sequencing, as previously described (Wistuba et al., 1997). The *TP53* gene mutation detected in tumor tissue and the corresponding tumor cell line (HCC1937) obtained from sister A were analyzed in tumor tissue from sister B and stromal cells from both cases, using a designed restriction fragment length polymorphism method as previously described (Tomlinson et al., 1998). Briefly, primers flanking the *TP53* mutation (codon 306, CGA to TGA, Arg to Term) were designed, which resulted in an amplicon of 234 bp. The *TP53* gene mutation at codon 306 creates a restriction site (CACNNNGTG for the restriction enzyme *Dra*III (New England Biolabs, Beverly, MA)) at nucleotides 909–917. The mutant type is cut, resulting in two fragments 184 and 50 bp in length.

Polymorphic DNA Markers and PCR for Loss of Heterozygosity (LOH) Analysis

To evaluate LOH, we used 161 primers flanking dinucleotide and multinucleotide microsatellite repeat polymorphisms located at 19 chromosomal arms (n = 161 markers): 1p (n = 5), 3p (n = 28), 4p (n = 4), 4q (n = 10), 5p (n = 3), 5q (n = 4), 6p (n = 4), 6q (n = 17), 8p (n = 16), 9p (n = 3), 9q (n = 12), 10q (n = 10), 11q (n = 12), 13q (n = 6), 16q (n = 9), 17p (n = 2), 17q (n = 4), 19p (n = 4), and 19q (n = 8). The microsatellite markers utilized in each chromosomal arm are listed in Figure 1 (A and B). Primer sequences can be obtained from the Genome Database, with the exception of the 3p.21.3 polymorphic markers *Luca 2.1*, *Luca 2.2*, *Luca 4.1*, *Luca 8.2*, *Luca 19.1*, and *P1.5*, all located in a 700-kb contig previously identified in our laboratory and previously published (Fondon et al., 1998). For analysis of both triplet sisters' tumors, all 161 polymorphic markers were used. A subset of markers (n = 77) spanning 11 chromosomal arms containing multiple regions frequently deleted in breast cancer were used for analysis of the breast cancer control group. After microdissection and DNA extraction, 5 μl of the proteinase K-digested samples, containing DNA from at least 100 cells, were used for each multiplex PCR reaction as previously described (Wistuba et al., 1998). Nested PCR or two-round PCR (using the same set of primers in two consecutive amplifications) methods were used as previously described (Wistuba et al., 1998). Multiplex PCR was performed during the first amplification, followed by uniplex PCR for individual markers. For each case, constitutional heterozygosity was determined by examination of peripheral blood lymphocytes and normal stromal tissue. LOH was scored by visual detection of complete

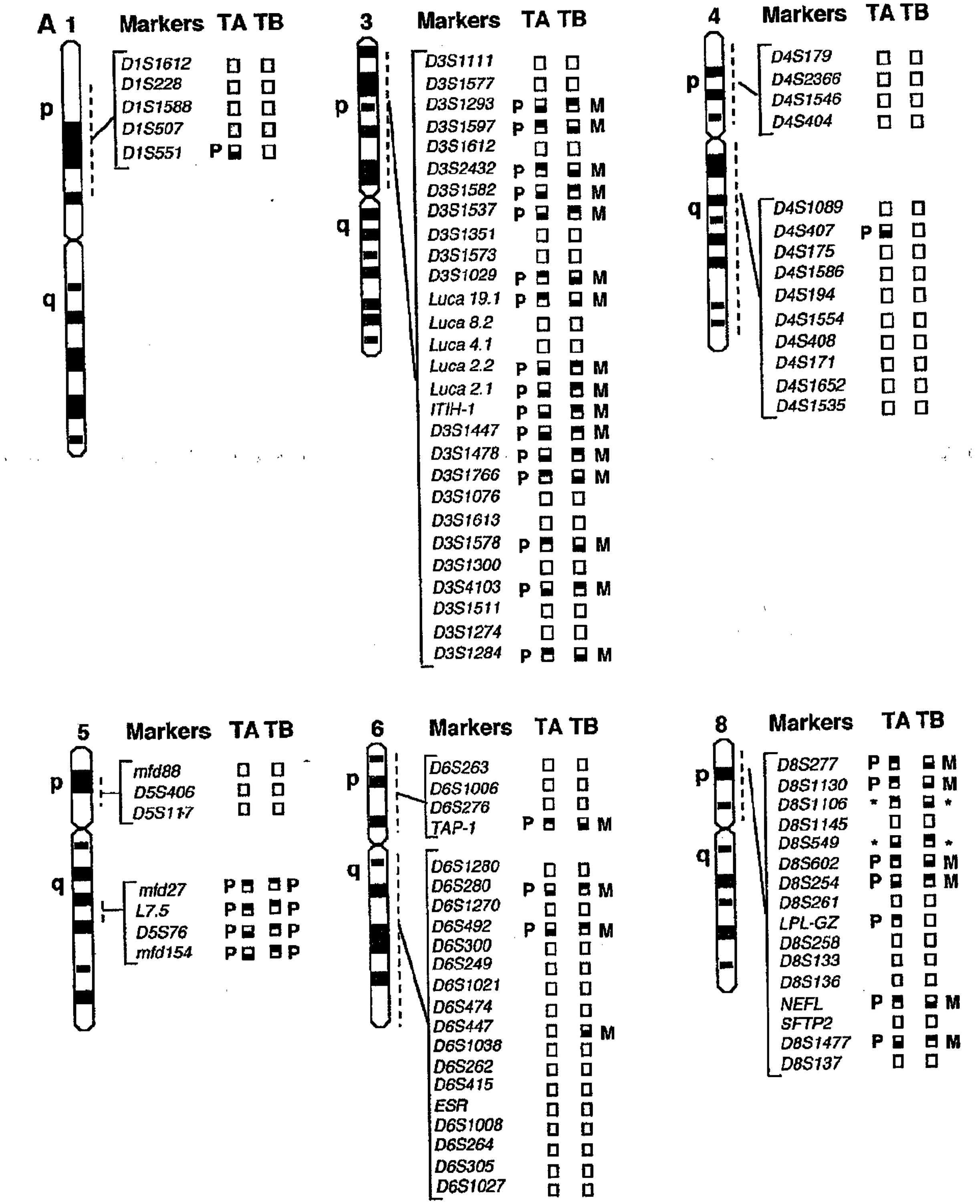


Figure 1. **(A and B)** Detailed allelotyping analysis data of both triplet sisters' breast cancers TA, tumor A, and TB, tumor B, using 161 microsatellite markers located on 19 chromosomal arms. ▄, LOH at the lower allele; ▀, LOH at the upper allele; □, no LOH; □, not informative (homozygous). P, paternal allelic loss; M, maternal allelic loss. Asterisk, parental allelic loss indeterminate (DNA from the mother was available for analysis, but not from the father).

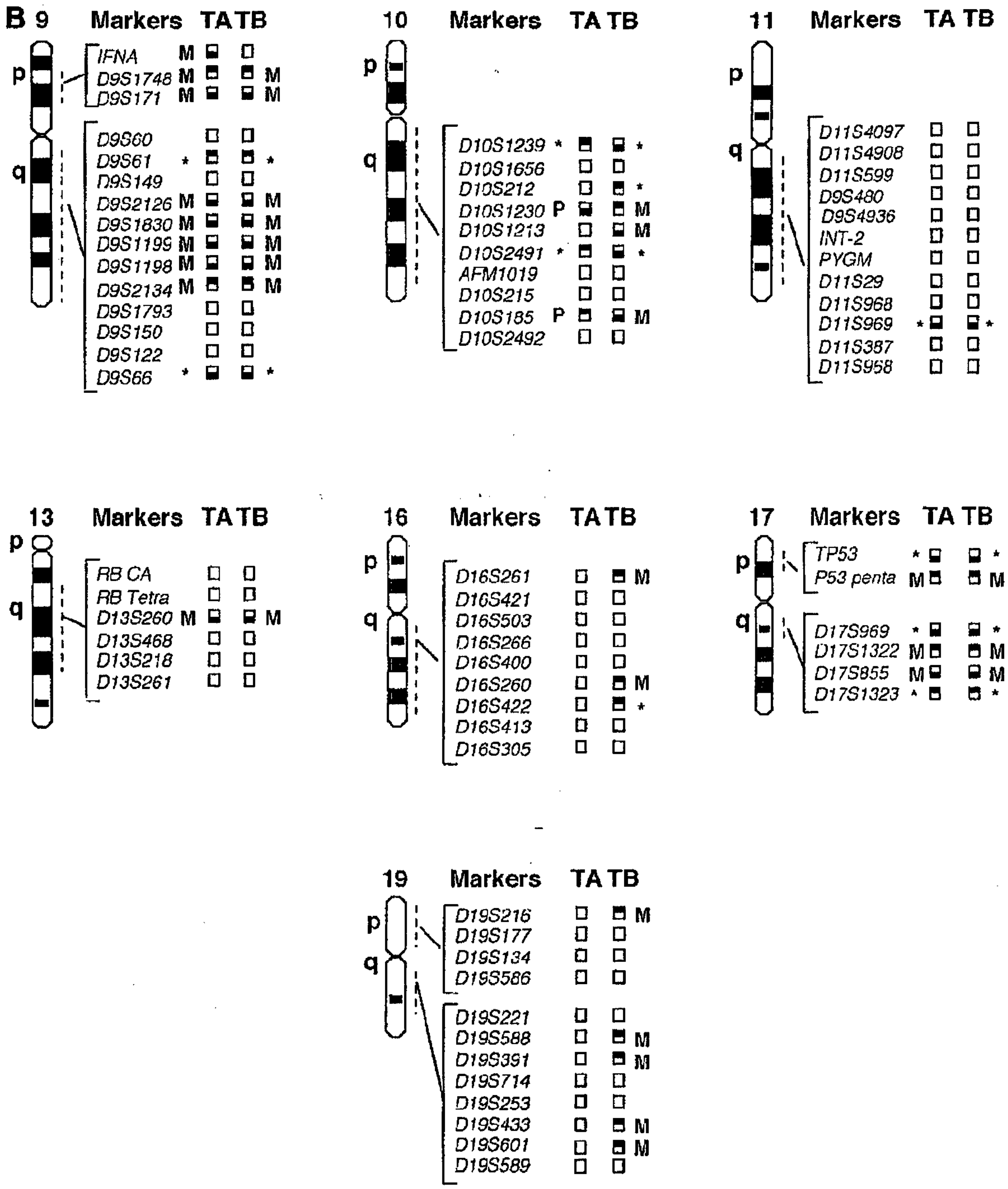


Figure 1. (Continued.)

absence of the one tumor allele in heterozygous (i.e., informative) cases.

Data Analysis

To compare the total frequencies of LOH between microdissected tumor tissues obtained from both triplet sisters (patients A and B) and 17 sporadic invasive breast carcinomas, we utilized the fractional allelic loss (FAL) index. The FAL index was calculated as follows: (total number of loci with LOH)/(total number of informative loci).

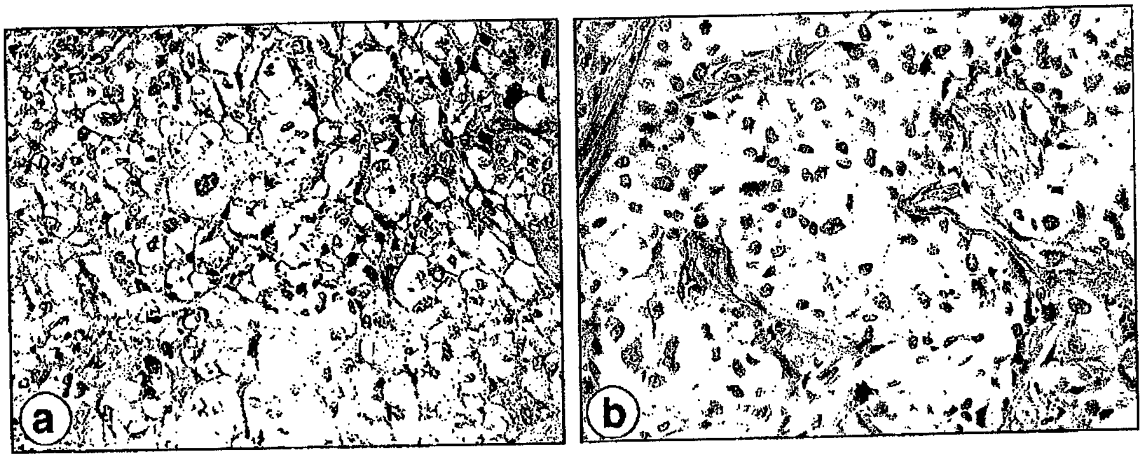


Figure 2. Morphology of both triplet sisters' breast carcinomas (tumors a and b). Both tumors are invasive ductal cell carcinomas with features suggestive of a secretory variant (cell with vacuolated cytoplasm).

RESULTS

Morphologic and Immunohistochemical Features of Breast Carcinomas

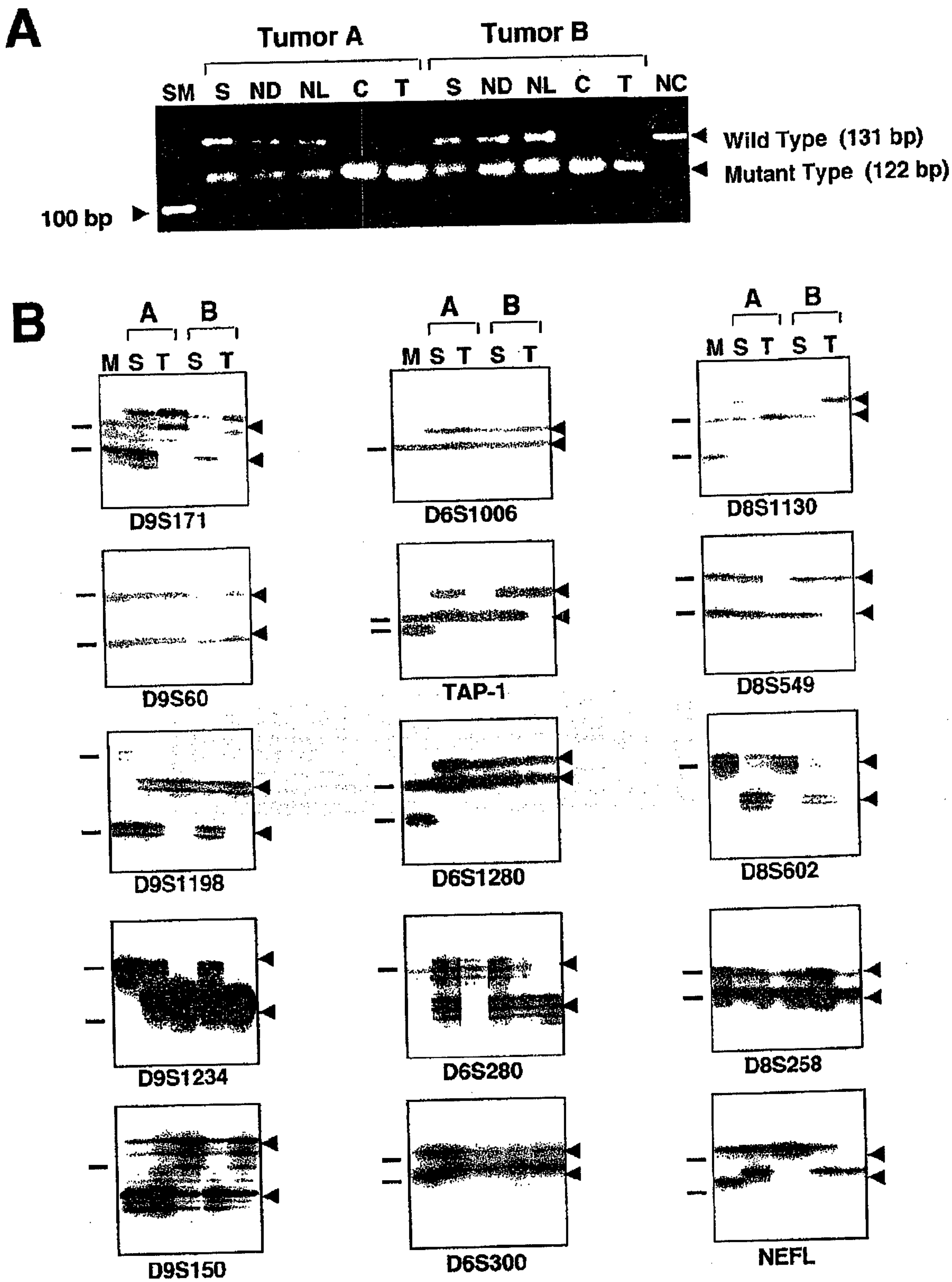
Histologic evaluation of both triplet sisters' tumors (A and B) revealed similar, if not identical, features. Both tumors were poorly differentiated ductal cell carcinomas (grade III) with areas in which large intracytoplasmic vacuoles were observed in many cells, suggestive of a secretory variant of this tumor type (Fig. 2). The vacuoles failed to stain with periodic acid-Schiff (with and without diastase treatment), alcian blue, mucicarmine, or oil red O. These results indicate that the vacuoles lacked glycogen, mucins, or neutral fat. The appearance of these cells was similar to the cytologic appearance of cells of secretory carcinomas. A ductal carcinoma in situ component was detected in both sisters' tumors. In both cases, the pathologic diagnosis was in situ and invasive ductal carcinoma with features of secretory carcinoma.

In addition, both sisters' breast carcinomas retained p16 protein immunohistochemical expression, and immunostaining was negative for *HER2/neu*, progesterone receptor (PR), and estrogen receptor (ER). Tumor A retained *RB* immunohistochemical expression and immunostained negatively for p53 protein. Tumor B lost *RB* immunohistochemical expression and immunostained positively for p53.

BRCA1 and *TP53* Gene Mutations

Microdissected normal ductal and lobular epithelia, DCIS, and invasive breast carcinoma obtained from both sisters' specimens were analyzed for the presence of homozygous or heterozygous *BRCA1* 5382insC mutation, using a designed fragment restriction length polymorphism method as previously described (Tomlinson et al., 1998). Whereas normal epithelial samples adjacent to both sisters' tumors had both wild-type and mutant *BRCA1* alleles, DCIS and tumor samples demonstrated only the mutant allele. Because of the *BRCA1* germline mutation at 17q21 on the paternal alleles, we expected to see LOH of the wild-type maternal allele. Our findings are consistent with the presence of allelic loss at the *BRCA1* gene locus in DCIS and tumor samples but not in normal epithelium in both sisters' specimens (Fig. 3A). A somatic *TP53* gene mutation in exon 8 (C→T, resulting in a termination codon at position 306) and allelic loss at the *TP53* gene locus were detected in the tumor of case A, at both DCIS and

Figure 3. **A:** Agarose gel showing the designed restriction fragment length polymorphism analysis using mismatched primers and BsoJI digestion for the germline *BRCA1* mutation (5382insC) in both twin sisters' normal breast stromal cells (S) and breast cancer specimens (tumors A and B), including invasive carcinoma (T), ductal carcinoma in situ (C), normal lobular (NL), and ductal epithelia (ND). NC, negative control DNA. SM, size marker, 100-bp ladder. Both uncut (wild-type, 131-bp size) and cut (mutant, 122-bp size) fragments are detected in the normal stromal cells and normal epithelial lobular and ductal cells in both cases. In both in situ and invasive carcinomas, only the cut fragment is observed. **B:** Representative autoradiographs showing the allelotyping analysis at chromosomes 9 (left), 6 (center), and 8 (right) of both triplet sisters' breast cancers (tumors A and B) and mother's DNA, illustrating identical breakpoints. M, mother's DNA extracted from normal peripheral lymphocytes; S, stromal normal breast tissue; T, invasive breast carcinoma. Bars represent the main allele bands in the mother's DNA. Arrowheads indicate the main allele bands in the sisters' DNA. In chromosome 9, both sisters' tumors with LOH at the same maternal allele (markers D9S171, D9S1198, and D9S1234). In chromosomes 6 (*TPA-1*, D6S280) and 8 (D8S1130, D8S602, *NEFL*), tumor A demonstrated allelic loss of the paternal allele, whereas tumor B shows LOH at the maternal allele.


A
Tumor A
Tumor B
SM
S
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S
ND
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Wild Type (131 bp)
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100 bp
B
A
B
M S T S T
D9S171
D6S1006
D8S1130
D9S60
TAP-1
D8S549
D9S1198
D6S1280
D8S602
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D6S280
D8S258
D9S150
D6S300
NEFL

Figure 3.

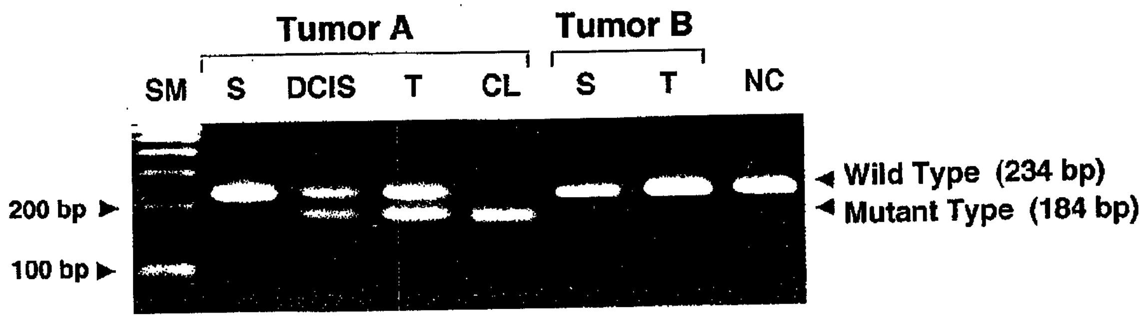

Figure 4. Agarose gel showing the designed restriction fragment length polymorphism analysis using mismatched primers and *DRAIII* digestion for the stop codon *TP53* gene mutation (codon 306, CGA to TGA, Arg to Term) detected in ductal carcinoma in situ (DCIS), invasive breast tumor (T), and corresponding tumor cell lines (HCC1937) from sister A. No mutation was detected in invasive breast tumor tissue obtained form sister B and in normal breast stromal cells (S) from both cases. Both uncut (wild-type, 234-bp size) and cut (mutant, 184-bp size) fragments are detected in the corresponding tumor cell line (HCC1937). Negative control (NC) DNA. SM, size marker, 100-bp ladder.

invasive carcinoma stages (Fig. 4). No mutation at exons 5–8 of the *TP53* gene was detected in the tumor of case B.

Allelotyping Data

Allelic loss analysis of both sisters' breast tumors (labeled tumors A and B in the figures) was performed using 161 polymorphic markers spanning multiple chromosomal regions frequently deleted in breast cancer at 19 chromosomal arms. As expected, identical patterns of allele sizes were detected in the normal samples (peripheral-blood lymphocytes and breast stromal cells or lymphocytes from resected lymph nodes) obtained from both sisters. Ninety-seven of 161 (60%) polymorphic markers were informative (heterozygous) in the sisters' constitutional DNA. Using those 97 informative markers, similar FAL indexes (0.56 vs. 0.60), which are expressions of the overall LOH frequencies, were detected in both sisters' tumors. Of interest is that identical sites of allelic loss ($n = 52$) and retention of heterozygosity ($n = 30$) were detected in 82 of the 97 markers (85% correlation). Discordance of allelic loss was detected in only 15 markers, and the tumor from sister B had more deletions than did the tumor from sister A.

Based on the data that strongly suggest that the allelic loss or retention patterns in the two sisters' tumors were not random, we then asked whether the breakpoints were similar in regions demonstrating allelic loss where we had used several markers. Strikingly, detailed mapping analysis of several chromosomal arms revealed that identical breakpoints were detected in both sisters' tumors. Eight identical breakpoints were detected in both tumors at five chromosomal arms (6p, 6q, 8p, 9q, and 13q), and chromosomal arm 9q demonstrated identity at more than one breakpoint (Figs. 1 and 3B).

We compared the patterns of LOH in the two sisters' tumors to LOH in 17 sporadic breast cancers, using 77 polymorphic markers. Whereas both sisters' tumors had identical FAL (0.63) for this marker subset, a wide range (0.13–0.89) of FAL index was detected in the sporadic breast tumor group (data not shown). Likewise, the patterns of LOH were identical in both sisters' tumors; none of the 17 sporadic breast cancers had a similar pattern of allelic loss (data not shown).

Because of limited material, DCIS foci adjacent to both sisters' tumor specimens were examined for LOH with a subset of polymorphic markers ($n = 113$). Of these, 66 (58%) microsatellite markers were informative (i.e., heterozygous). Identical patterns of LOH were detected in the DCIS samples and their corresponding invasive tumors. As seen in the invasive carcinomas, similar overall frequencies and patterns of LOH were detected in DCIS lesions obtained from both triplet sisters studied. Using the 77 informative markers examined in DCIS lesions, similar FAL indices (0.56 vs. 0.63), an expression of the overall LOH frequency, were detected in both sisters' noninvasive carcinomas. Identical sites of allelic loss ($n = 38$) and retention of heterozygosity ($n = 21$) were detected in 59 of the 66 markers (89% correlation). Discordance of allelic loss was detected in only seven markers, and the DCIS specimens from sister B had more deletions than did the DCIS specimens from sister A.

Parental Source of Allelic Loss

The mother's DNA obtained from peripheral blood lymphocytes was available for allelotyping analysis and was compared with the pattern of alleles present in the normal and tumor samples obtained from each sister. From this analysis, we were able to identify the specific parental allelic loss in 95 comparisons in both tumors (tumor A, 45 comparisons; tumor B, 50 comparisons). Strikingly, each tumor demonstrated LOH of the same parental allele in all microsatellite markers located in individual chromosomes (Fig. 1 A and B). However, the specific parent whose alleles were under LOH in specific chromosomes was not always the same in each sister's tumor. Both tumors demonstrated allelic losses in common on 8 chromosomes. In four chromosomes, alleles of the same parent were under LOH, whereas in four chromosomes the allelic losses were discordant.

Tumor A showed LOH at the paternal allele in 8 of 13 (62%) of the chromosome arms having LOH in which we could establish the parental origin of the allelic loss. In contrast, tumor B demonstrated LOH at the maternal allele in 13 of 14 (93%) of the chromosomal arms demonstrating LOH in which the parental origin of the allelic loss could be established. In the cases in which tumor A had LOH at the maternal allele (chromosomal arms 9p, 9q, 13q, 17p, and 17q), both sisters' tumors demonstrated LOH of the same parental allele. In both sisters, LOH on chromosome 17 involved the maternal allele, which is consistent with a paternal origin or a de novo *BRCA1* mutation. The instances in which tumor B had LOH at the paternal allele were four markers located at chromosome arm 5q.

DISCUSSION

There were two major findings of our study. First, there was a remarkable similarity of morphologic features and patterns of LOH, including several identical breakpoints, detected in both triplet sisters' tumors. Second, in each individual sister's tumor, the same parental allele was under LOH at all the microsatellite markers in an individual chromosome, although there was discordance in the parental loss patterns between the sisters.

On histologic evaluation, both triplet sisters' invasive ductal carcinomas demonstrated features suggestive of a secretory variant of ductal carcinoma. Although secretory carcinoma is an early-age-onset type of breast cancer, occurring more frequently during the first 3 decades of life (Tavassoli, 1992), this histologic type has not been associated with *BRCA1* or *BRCA2* mutations (Marcus et al., 1994; Armes et al., 1998). Secretory breast carcinoma is a very uncommon type of infiltrating breast cancer, representing less than 1% of the breast tumor types (Tavassoli, 1992). Thus, the fact that both sisters' tumors demonstrate histologic features suggestive of this uncommon tumor type is highly unusual.

Although a good concordance between both sisters' tumors was detected for p16, *HER2/neu*, PR, and ER immunostaining, discordance was detected between both tumors in *RB* and p53 immunohistochemical expression. *TP53* mutation analysis of tumor A demonstrated a stop mutation consistent with the lack of p53 immunohistochemical overexpression. Although no *TP53* mutation at exons 5–8 was detected in tumor B, the presence of p53 immunostaining in this tumor suggests that a *TP53* alteration may be present.

Breast cancer arising in monozygotic twins is an experiment of nature that can be used to address several points related to the molecular profile of hereditary breast cancer and the extent of inherited components in breast cancer, although shared exposure to other risk factors cannot be excluded. If heritable factors play a major role in the origin of a neoplasm, disease concordance in these individuals should be significant. Similar overall LOH frequencies and similar patterns of LOH were detected between both triplet sisters' breast carcinomas and their accompanying noninvasive carcinomas, suggesting that similar TSGs have been targeted in both tumors. By contrast, the 17 archival sporadic breast carcinomas demonstrated a wide range of allelic loss frequencies and different patterns of LOH. Our findings suggest that inherited components, potentially related to *BRCA1* gene inactivation, may play an important role in the other somatic changes occurring in the pathogenesis of breast cancer. Several studies have suggested that breast tumors associated with inherited *BRCA1* gene mutations have a very specific tumor phenotype (Armes et al., 1998; Lakhani et al., 1998) and genotype (Tirkkonen et al., 1997; Smith et al., 1999), such as distinct histopathologic features (Armes et al., 1998; Lakhani et al., 1998), a different pattern of chromosomal alterarations studied by comparative genomic hybridization (Tirkkonen et al., 1997), and a high frequency and unusual type of *TP53* mutations (Smith et al., 1999). Our findings add supporting data to this concept. Accumulating data increasingly suggest that *BRCA1* plays a role in multiple cellular functions, including among others cell cycle regulation (Vaughn et al., 1996; Larson et al., 1997; Xu et al., 1999), DNA damage repair (Gowen et al., 1998; Chen et al., 1999), centrosome duplication (Xu et al., 1999), and suppression of estrogen-dependent transcriptional pathways (Fan et al., 1999). Some of those functions are consistent with a caretaker role for the *BRCA1* gene in the pathway to neoplasia and may explain a specific pattern of cumulative mutations if the gene is inactivated.

Detailed mapping analysis of several chromosomal arms revealed that identical breakpoints were detected in both tumors at eight chromosomal regions, involving five chromosomal arms. Although the mechanisms involved in the LOH phenomenon are unknown, it has been suggested that they may occur at high frequency at fragile sites of the genome. Chromosomal fragile sites are specific loci that are especially susceptible to forming gaps, breaks, and rearrangements in metaphase chromosomes when cells are cultured under conditions that inhibit DNA replication. Alleles with fragile sites replicate late in the cell cycle and are more susceptible to the development of breaks (Wang et al., 1999) that might predispose to chromosome rearrangements (Smith et al., 1998). It is tempting to speculate that the identical breakpoints detected in both sisters' breast carcinomas may be related to the presence of genetically programmed fragile sites, common to these triplet sisters. This theory is not applicable if parental allelic loss is discordant.

In each sister's tumor, the same parental allele was under LOH in all the informative microsatellite markers in individual chromosomes. This phenomenon involved both arms of several chromosomes and occurred although several breakpoints were present in some chromosomal arms. According to the theory of Knudson (1989) for the inactivation of TSGs, one normal allele of a TSG is affected usually by mutation that is converted to a homozygous form by a second independent mutation frequently involving deletion. Our findings suggest that the parental allelic loss in a particular chromosome region is not random, but is related to the parental allelic loss at other sites on the chromosome. This, of course, would not be surprising if the whole chromosome were lost by a process such as nondisjunction either with or without duplication of the mutant chromosome, resulting in hemizygosity or homozygosity at all loci in the chromosome. This phenomenon has been demonstrated to happen in chromosome 13 in cases of retinoblastoma (Cavenee et al., 1983). However, in many cases, allelic losses involving the same parental allele at discontinuous regions occurred even with retention of heterozygosity of intervening segments. Although the mechanism is unknown, it is tempting to speculate that in a particular individual, a specific parental chromosome may be protected or susceptible (fragile) to be unstable at several sites and therefore retained or lost. The findings of parental allelic-specific losses associated with known TSGs affected by germline mutations or putative TSGs that underwent a parental imprinting phenomenon support this theory (Feinberg, 1993).

The allelic losses detected in each of the sisters' breast tumors at individual chromosomes appeared not to be random. Whereas the tumor from sister A had a tendency to lose the paternal allele, the tumor from sister B preferentially had LOH at the maternal allele. Although allelic losses of different parental alleles (either paternal or maternal) were detected in the majority of the chromosomes, there were some chromosomes in which both sisters' tumors demonstrated LOH of the same parental allele, especially maternal alleles (at chromosomal arms 9p, 9q, 11q, 13q, 17p, and 17q). A genetic imprinting mechanism of maternal or paternal alleles could explain the preferential LOHs of the same parental alleles at those chromosomes in both tumors. Silencing of one of the parental alleles of a regulatory gene, such as a TSG, by genomic imprinting has been suggested as a pivotal initial step in a cascade of events leading to malignancy (Glassman et al., 1996). However, an alternative mechanism could be the presence of TSGs having a germline mutation in either the paternal or maternal allele that may lead to deletion of the unaffected allele. In theory, any allelic loss should be replicated in the co-twin if related to a heritable mutation, i.e., the same allele should be under LOH in the tumor tissue of both twins. Maternal allelic loss in both sisters' tumors at chromosome arm 17q probably is explained because the germline *BRCA1* mutation is on the paternal allele.

In summary, both sisters' breast carcinomas have somatically acquired genetically similar alterations, and the probability that this occurred by chance is remote. Our findings suggest that inherited factors may regulate the multiple somatic deletions occurring in hereditary breast carcinomas. The allelic loss phenomenon involving one or more microsatellite loci at individual chromosomes is not random and follows a pattern related to the parental allele by which the precise mechanism need to be investigated.

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