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GRANT NUMBER DAMD17-96-1-6206

TITLE: Acquired Secondary Events in the Pathogenesis of Hereditary Breast Cancer

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REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (1 Sep 97 - 31 Aug 98)

4. TITLE AND SUBTITLE
Acquired Secondary Events in the Pathogenesis of Hereditary Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6206

6. AUTHOR(S)
Gail E. Tomlinson, M.D., Ph.D.

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

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Mutation of the *BRCA1* gene accounts for most families with an inherited predisposition to breast and ovarian cancer and many families with multiple cases of breast cancer only. The inheritance of a germline 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 and multiple other acquired steps appear to be required for the development of breast tumors in predisposed women. In this study we have identified over 50 women with *BRCA1* mutations for which tumor tissue is available for study. We present here acquired mutations in tumors from individuals who carry a predisposing mutation in *BRCA1*. We compare acquired mutations in a BRCA1 associated tumor and a unique tumor cell line derived from this tumor. We compare and contrast acquired genetic abnormalities in tumors from two otherwise genetically identical siblings.

14. SUBJECT TERMS			15. NUMBER OF PAGES
Breast Cancer	Molecular Pathology		30
BRCA1; TP53	Microdissection		16. PRICE CODE
Genetic Predisposition			

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRA(
Unclassified	Unclassified	Unclassified	Unlimited

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Acquired Secondary Events in the

Pathogenesis of Hereditary Breast Cancer

DAMD17-96-1-6206

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

TABLE OF CONTENTS

INTRODUCTION: Mutation of the *BRCA1* gene accounts for most families with an inherited predisposition to breast and ovarian cancer, approximately half of families with multiple cases of breast cancer only and approximately 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 approximately 8-10,000 new cases of breast cancer in the U.S.A. each year. The inheritance of a germline 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 and multiple other acquired steps appear to be required for the development of breast tumors in predisposed women. Some of these observed acquired changes have been described. (4)

Although the function of the BRCA1 protein is not yet clearly determined, evidence suggests that *BRCA1* may play a role in DNA repair, may function as a transcription factor or may possibly exist as a secreted granin-like molecule. (5-7) If *BRCA1* indeed functions in DNA repair, then one would expect an accelerated accumulation of other genetic aberrations in tumors derived from *BRCA1* mutation carriers. In this project we have identified and begun to characterize *BRCA1* associated tumors derived from *BRCA1* carriers. Genetic changes include activation oncogenes and the loss of function of tumor suppressor genes. In this study we focus on the latter, loss of tumor suppressor genes. Loss of heterozygosity (LOH) is a marker of possible inactivation of such tumor suppressor genes.

No models currently exist for the study of breast cancers associated with *BRCA1* mutations. Somatic mutation of the *BRCA1* is thought not to occur in sporadic breast tumors. (8) Although a modest number of breast cancer cell lines have been established, no breast cancer cell lines have been reported to date which derive from a *BRCA1* predisposed individual. The establishment of such a cell line could not only provide the means of study of the secondary changes which develop during tumor development, tumor

growth characteristics conferred by *BRCA1,* and could conceivably serve as a substrate for genetic transfection studies. The work described here has resulted in the characterization of a breast cancer cell line mutant for *BRCA1* including an extensive LOH profile. This work is described in detail in the accompanying manuscript (Tomlinson et al. Cancer Research 58: 3237-3242). In addition, the work we have compared and contrasted acquired genetic changes in breast tumors from two genetically identical individuals harboring a *BRCA1* mutation. In doing so, we have not only reported the changes which occur in the *BRCA1* associated tumors, but have described a previously unreported phenomena of parental-specific loss of heterozygosity in breast tumors. (manuscript in preparation).

BODY:

TASK 1

Patient Selection: Patients have been selected from Registry families for *BRCA1* and *TP53* mutation screening. Families are screened for germline mutation of *BRCA1* if breast and ovarian cancers are observed in first degree relatives or in the same individual or if three or more members of the family have had breast cancer. We have also screened our Registry members who have developed very early onset breast cancer (less than 30 years of age) for both *TP53* and *BRCA1.*

Mutation analysis: SSCP analysis for exons 5 through 9 of the *TP53* gene and exons 2 and 20 of the *BRCA1* gene was performed as a modification of the technique as described by Orita et al (9). The protein truncaton assay was performed on exon 11 according to the method described by Specific genes known to be involved in the pathogenesis of breast cancer were examined as possible secondary acquired changes.

Sequence analysis has been facilitated by the acquisition by our Center of an automated sequencer (ABI) by which we are able to sequence bands which show altered mobility by SSCP screening.

During this year we identified two new families with a *TP53*. One of these families is a Hispanic family which demonstrates a unique truncation mutation of exon 4 which will be reported separately (manuscript in preparation). Two of the affected family members in this highly-cancer prone kindred developed a total of seven different primary cancers which were treated in our institution and these tumor blocks have been identified and are available for study.

We also determined mutations of *BRCA1* in a total of twenty eight families including 55 women affected by breast cancer. We thus have met the accrual goal for Task 1.

TASK 2: We have identified tumor blocks from 30 of the mutation carriers mentioned above. Many of these tumor blocks have been processed for microdissection. The review of slides and notations is in progress.

TASK 3: The comparison of familial and sporadic breast tumors is in process. We have established all PCR conditions necessary to perform this work on parafiin embedded specimens. Chromosomal loci to be studied will include those frequently lost in breast cancer, 3p, 6q, 8p, 11p, 13q, 17p, 17q and 18q, as well as selected loci which show loss frequent loss. A total of 161 markers at a total to 20 different chromosomal loci. Using polymorphic dinucleotide and tetranucleotide microsatellite repeat markers, patterns of allelic losses were studied at loci known to be commonly lost in breast cancer. Primer sequences were obtained from the Genome Database. PCR amplification and electrophoresis was performed as described previously. (10) The amplification of some of these markers examined for LOH is shown in Figure 1 on page 6. A list of markers used along with the patterns of loss of heterozygosity in two tumors from the same family carrying a 5323insC is shown in the Figure 2 on page 7.

TASK 4: We have identified several tumor specimens of *BRCA1* and *TP53* individuals which also demonstrate premalignant lesions. These tumors will first be studied to determine LOH patterns in the tumor tissue and then a comparison will be made to corresponding premalignant lesions.

TASK 5: This task has been eliminated as recommended by the study section reviewers.

CONCLUSION: We have made substantial progress this year by completing the ascertainment of necessary *BRCA1* who are mutations carriers who are candidates for characterization of the acquired changes in their tumors. We are in the process of analyzing these tumors. We have completed the characterization of a unique tumor and corresponding cell line, (Tomlinson et al, Cancer Research 58: 3237-42), have compared and contrasted acquired genetic changes in genetically equivalent individuals (Wistuba et al, in preparation) and have described in detail two separate interesting *TP53* mutations. (Hung et al, submitted; Rutherford et al, in preparation). Complete citations of work published or prepared for publication as related to this grant are listed below:

Publications derived from this grant:

Tomlinson GE, Chen TL, Stastny VA, Virmani A, Spillman MA, Tonk VJ, Blum, JL, Schneider NJ, Wistuba I, Minna JD and Gazdar AF. 1998. Characterization of a breast cancer cell line.derived from a germ-line *BRCA1* carrier. Cancer Research, 58: 3237-42.

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, submitted.

Wistuba I, **Tomlinson G,** Behrens C, Geradts J, Blum J, Minna JD, Gazdar AF. Parental allele specific deleions in breast cancer arising in two identical triplet sisters carrying a germline BRCA1 gene mutation, in preparation.

Rutherford C, Chen TL, **Tomlinson GE.** A novel exon 4 truncating germline mutation of the *TP53* gene, in preparation.

Figure 1. Photomicrograph of gels demonstrated patterns of LOH at multiple different loci in two sisters with BRCA1- associated familial breast cancer.

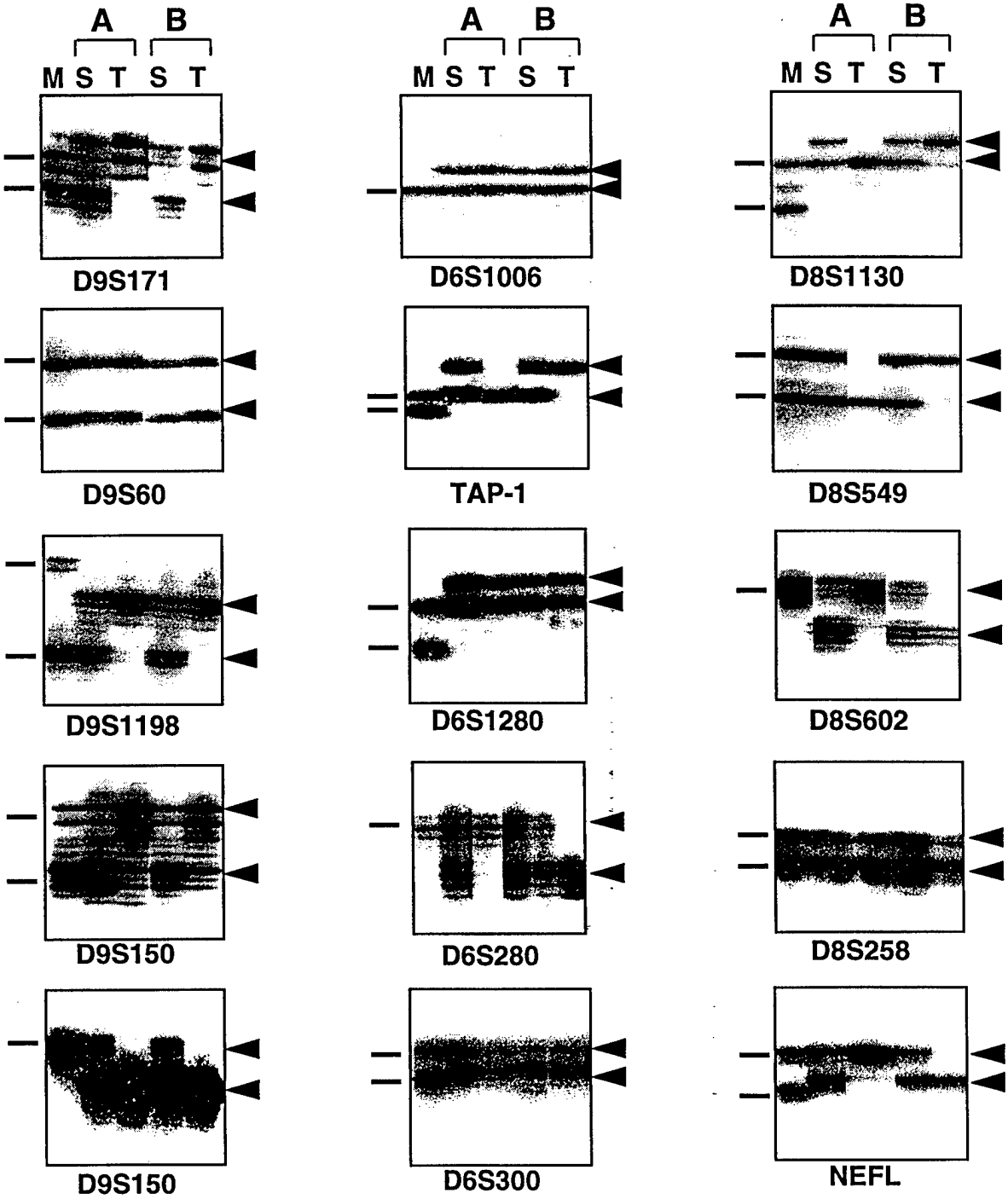
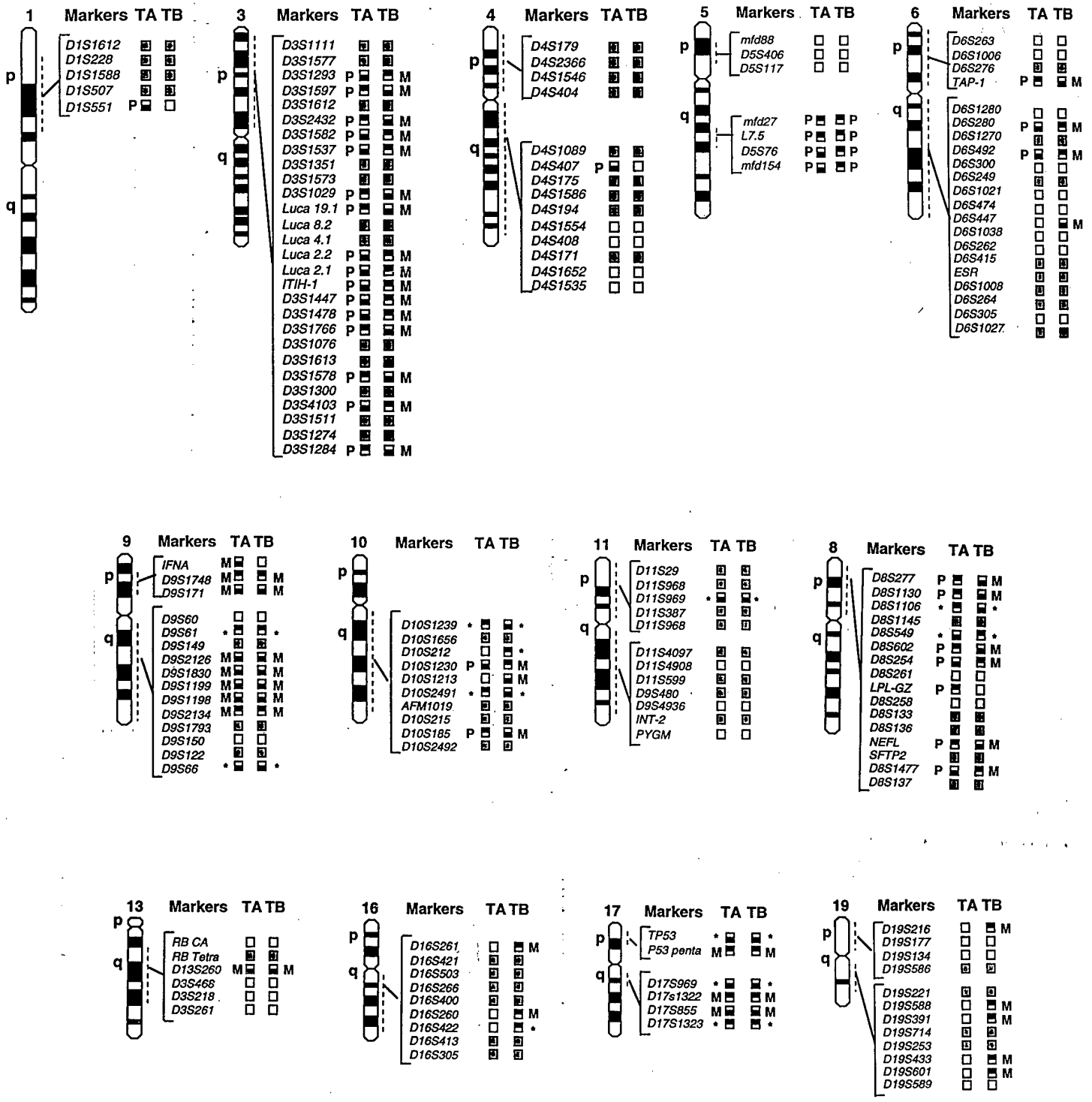


Figure 2. Comparitive allele loss in two familial breast cancers with an underlying germline BRCA1 mutation



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M., Frye, C., Weaver-Feldhaus, J., Ding, W., Gholami, Z., Sodervist, P., Terry, L., Jhanwar, S., Berchuck, A., Iglehart, J., Marks, J., Ballingler, D., Barrett, J., Skolnick, M., Kamb, A. and Wiseman, R. BRCA1 mutations in primary breast and ovarian carcinomas. Science, *266:* 120-122, 1994.

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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

Received 5/21/98; accepted 6/16/98.

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[1] Supported by Grants NCI CA70472-03 (to G. E. T.), DAMD 17-96-1-6206 (to G. E. T.), and DAMD 17-94-J-0477 (to J. W. S. and A. F. G.) and the Susan G. Komen Foundation (to G. E. T., J. L. B., J. W. S., and J. D. M.) and Clay Weed Memorial Fund (to G. E. T.).

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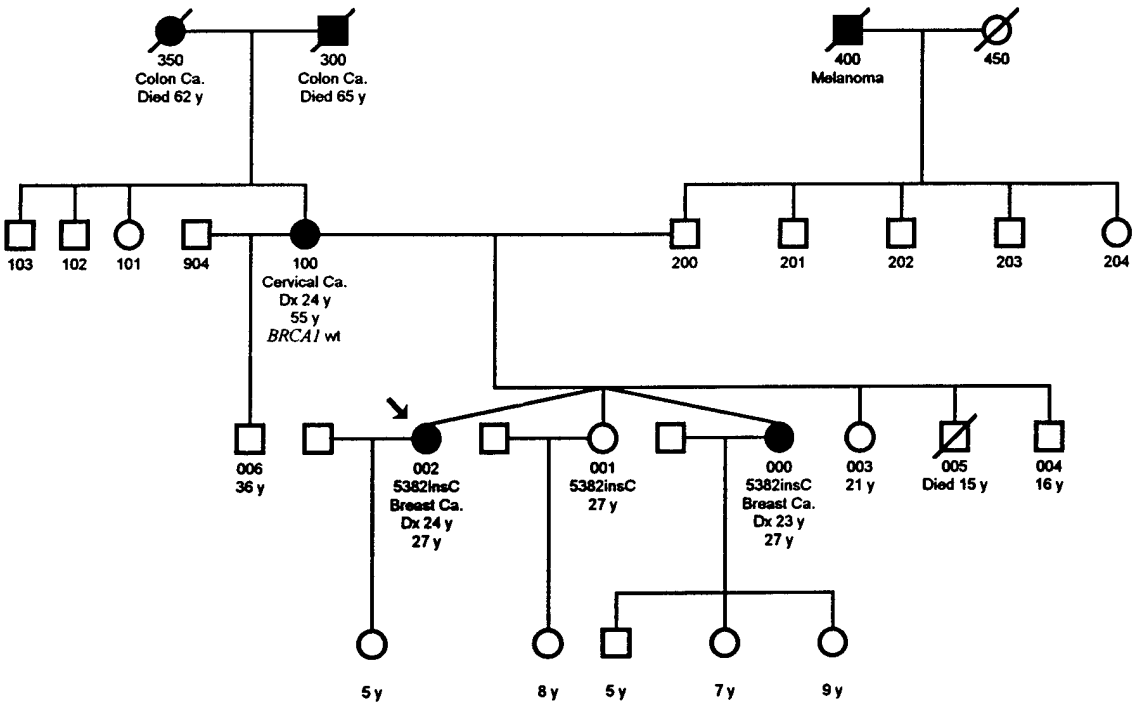

Fig. 1. Pedigree of the family from which the HCC1937 cell line was derived. The patient from which the tumor cell line is derived is indicated by the *arrow*. Germ-line DNA from the patient as well as the affected and one unaffected sister was heterozygous for the *BRCA1* mutation, 5382insC. The patient's 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.

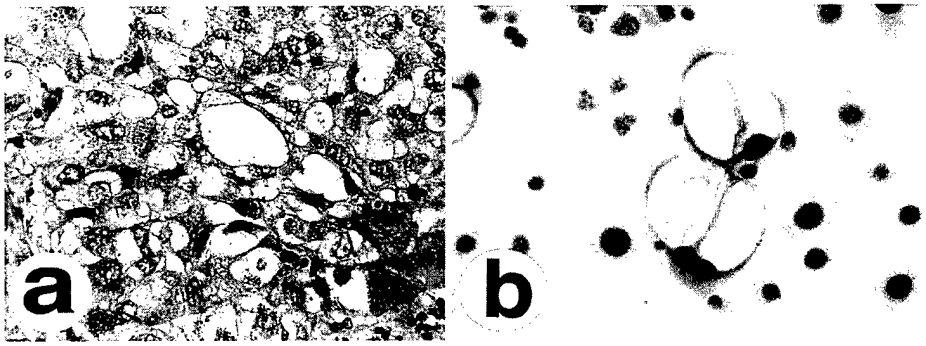

Fig. 2. Morphology of the breast cancer primary tumor and cell line, H&E stain. *a*, the primary breast carcinoma from which HCC1937 was derived. *b*, HCC1937 tumor cell line, cytospin preparation. Giant vacuolated mono- and dinucleated cells are present in both the tumor and cell line. The nonvacuolated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

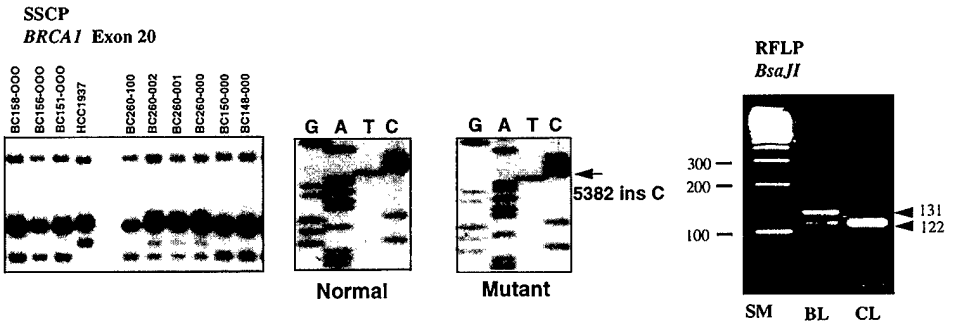

Fig. 3. Molecular analysis of *BRCA1*. Single-strand conformation analysis (*left*) revealed an aberrant band in lymphocyte DNA from the patient (*BC260-002*) and each of her two sisters analyzed (*BC260-001* and *BC260-000*). The tumor cell line demonstrated the mutant band as well as the absence of 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.

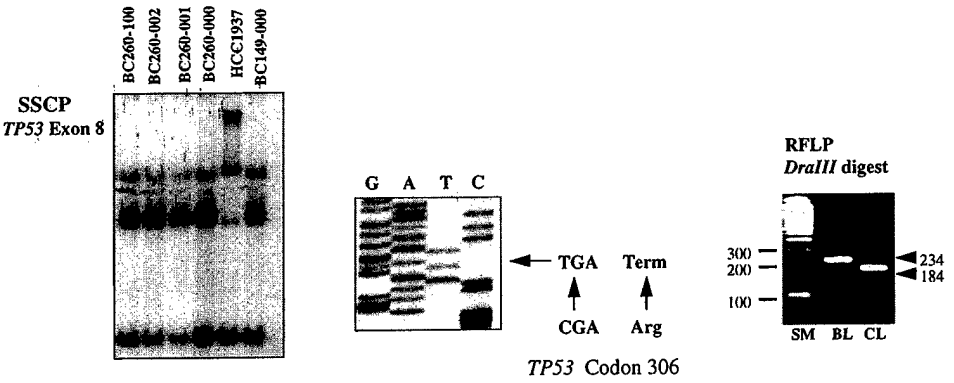

Fig. 4. Molecular analysis of *TP53*. Single-strand conformation analysis of the *TP53* gene revealed an abnormality in exon 8. Sequence analysis demonstrated a point mutation leading to a termination at codon 306. This mutation is also demonstrated by designed restriction fragment length polymorphism 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

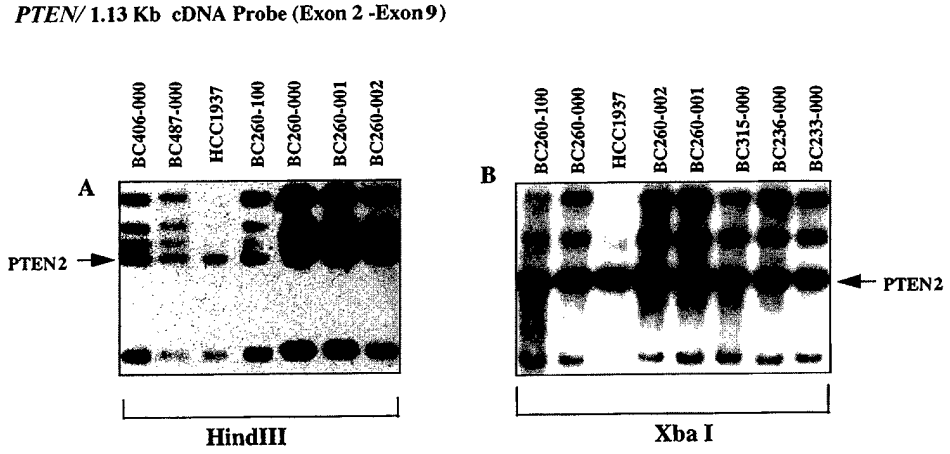

Fig. 5. Southern blot demonstrating absence of the *PTEN* coding sequence in HCC1937. DNA was digested with *Hin*dIII (*left*) or *Xba*I (*right*). The 1.13Kb probe used was prepared from *PTEN* cDNA and contains exons 2–9 (22). Absent bands were observed in the lane containing HCC1937 DNA. Similar results 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	*1F6*		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

We thank Eva Forgacs for help with the *PTEN* analysis, Gabriella Ethington and Kristin Shelby for help in obtaining the family history and blood samples for analysis, Scott Armstrong for help in procuring the tumor sample, and Jeffrey Doolittle and Scott Donovan for excellent technical assistance with the cytogenetic analysis.

[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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TP53 MUTATION AND HAPLOTYPE ANALYSIS OF TWO LARGE AFRICAN AMERICAN FAMILIES

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Supported by NIH PO1-CA34936 (LCS) and DAMD 17-96-1-6206 (GET)

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Abbreviated title: *TP53* in African Americans

Abstract

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, Met -> Thr). In order to determine if an ancestral haplotype was shared by these two families, polymorphic markers within and flanking the *TP53* gene were studied. Haplotype analysis using four 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 and in each case the retained allele carried the common haplotype. The frequency of this haplotype in the general African-American population is less than 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.

Introduction

Germline *TP53* mutations in the general population are very rare, however the presence of such a mutation infers an extremely high risk of developing cancers characteristic of Li-Fraumeni breast-sarcoma cancer family syndrome 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 yet exists 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 SARC36, was ascertained from a series of childhood 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 second family BC 54 was ascertained as part of a study of early onset and familial breast cancer. The pedigrees of these families 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. Four of the markers have been previously described (Futreal et al. 1991; Jones and Nakamura 1992; Lazar et al. 1993; McDaniel et al. 1991). Primer sets for fifth marker, a previously undescribed intron 3 $(ATT)_n$ polymorphism are as follows: F: TCTGGTCCCCTGTGTTCC; R: CCTGGGCGATAGAACAAG. To determine allele frequency of this intron 3 $(ATT)_n$ 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 than 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, Met -> Thr). (Figure 2)

Allelotype data revealed 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. (Figure 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 (28 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 x 0.23 x 0.86 x 0.07 = 0.003).

Discussion

Although it is unclear as to how common this mutation is in the general population, a common 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. Due to the limited number of individuals with this mutation, it is not possible to determine how many generations ago the mutation occurred as is possible with other common germline mutations (Neuhausen et al. 1996b), however from pedigree analysis it is evident that this mutation occurred at least six 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. 1996a; Simard J 1994; Struewing et al. 1995; Tonin et al. 1998) Other recurring mutations with common haplotypes of *BRCA1* and/or *BRCA2* 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* have been yet reported in African Americans. This may be due to the African American population being a previously under-studied population. Further studies of *BRCA1* and *BRCA2* in African Americans may reveal common recurring mutations in these genes.

The mutation we report here thus 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 as to 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 enable to study the effects of environment, life style and other modifier genes on disease penetrance and spectrum of tumor types associated with identical genetic lesions.

Figure Legends

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 was previously published. (Law et al. 1991) 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. Numbers directly under symbols refer to unique identifiers of family members. Numbers preceeding diagnoses indicate age of onset.

Figure 2. Mutation detection by 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 a previously described $(AAAAT)_n$ polymorphism. The lower 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.

Table 1

Polymorphic Markers at the *TP53* locus

Number	Marker	Locus	Heterozygosity	Reference
1	$(CA)_n$	*TP53* locus	.90	(Jones and Nakamura 1992)
2	$(AAAAT)_n$	Intron 1	.80	(Futreal et al. 1991)
3	28 bp rpt	Intron 3	.28	(Lazar et al. 1993)
4	Msp1	Intron 6	.46	(McDaniel et al. 1991)
5	$(ATT)_n$	Intron 3	.70	This report

Table 2

Allele frequencies of TP53 intron 3 $(ATT)_n$ marker in African Americans

Allele	Size (base pairs)	Frequency
1	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

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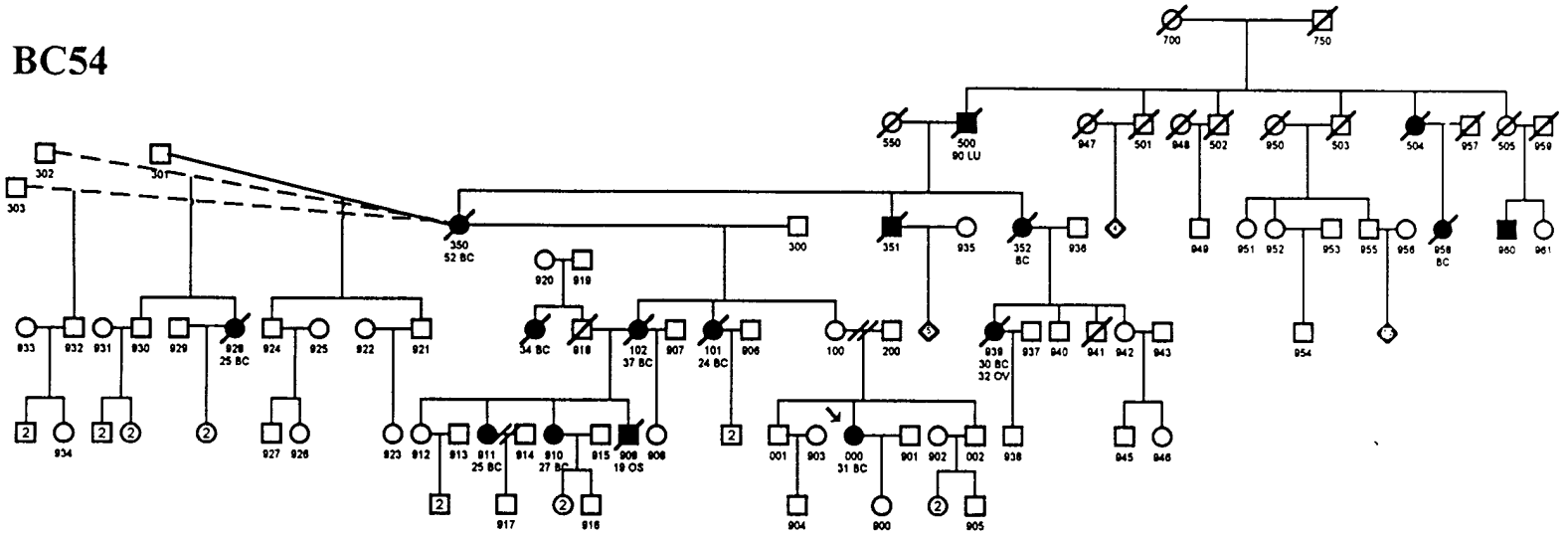
A BC54



B STS170/SARC36

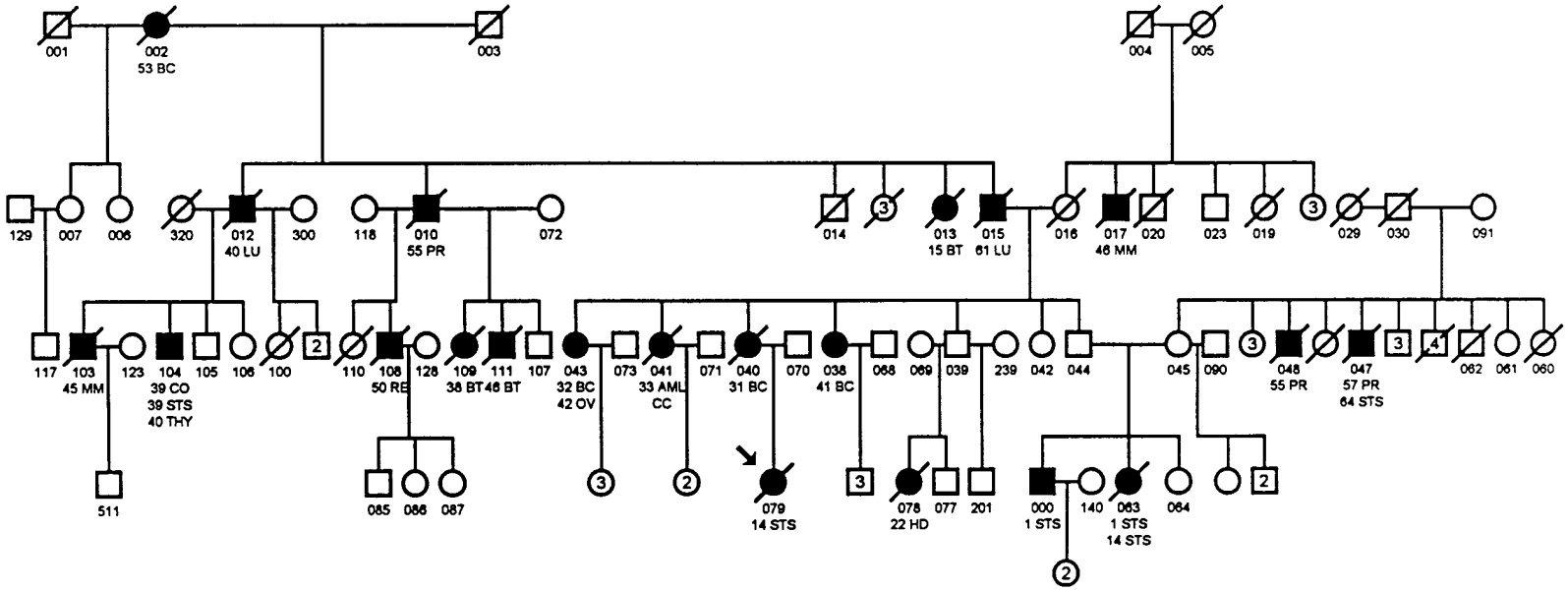
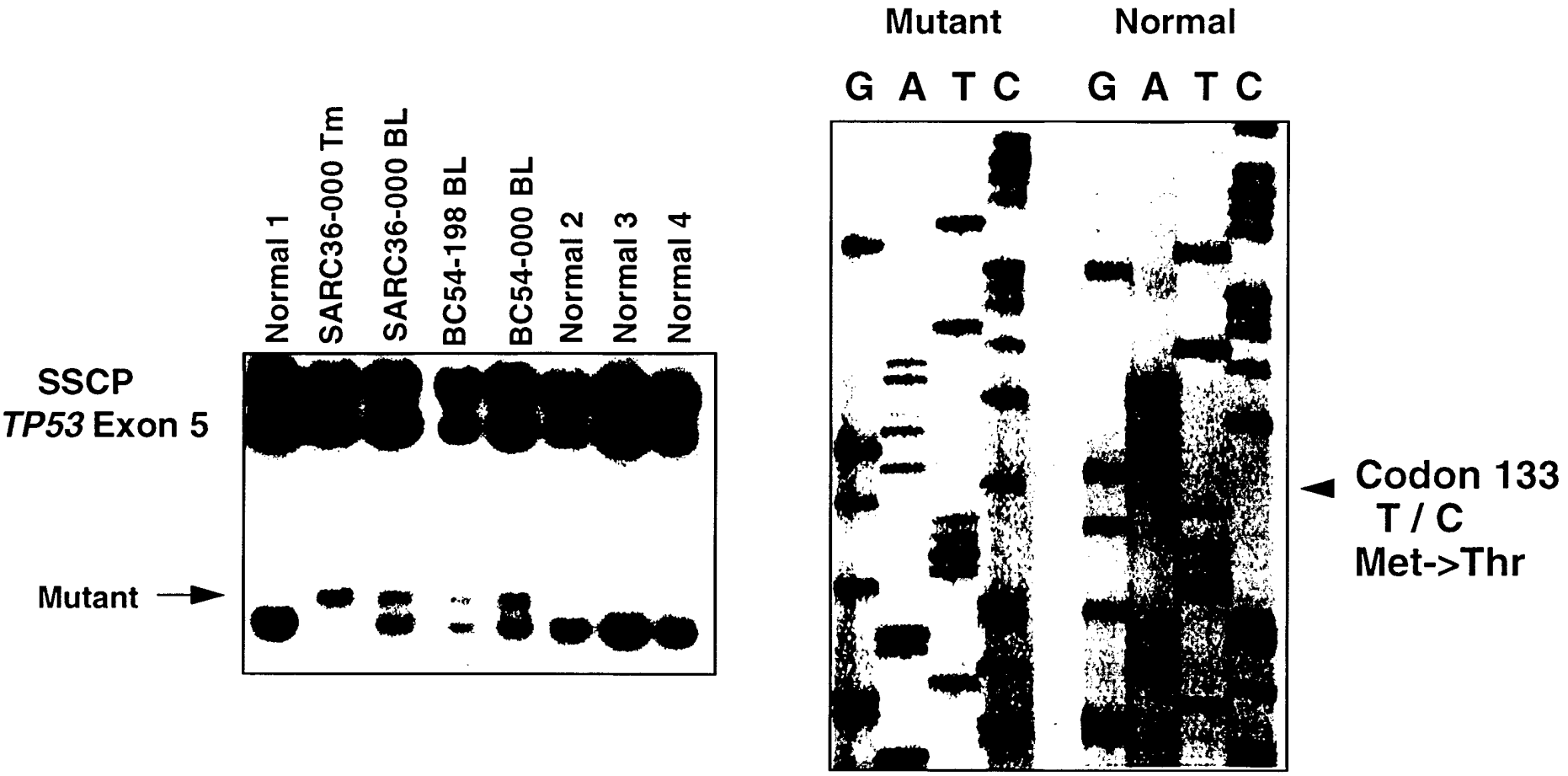


Figure 1


Normal 1
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SARC36-000 BL
BC54-198 BL
BC54-000 BL
Normal 2
Normal 3
Normal 4
SSCP
TP53 Exon 5
Mutant
Mutant
Normal
G A T C
G A T C
Codon 133
T / C
Met->Thr

Figure 2

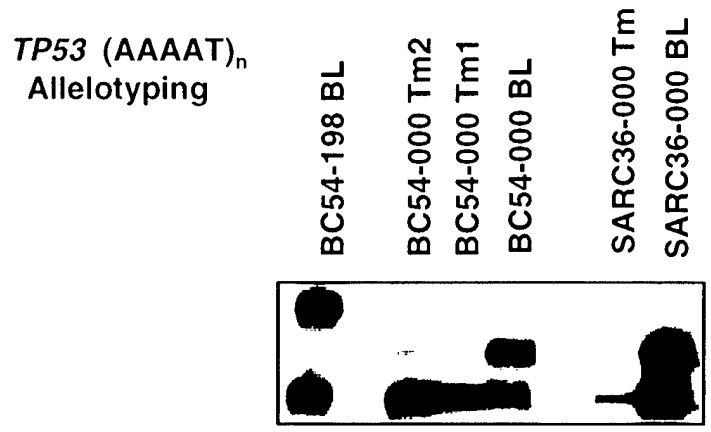
TP53 (AAAAT)$_n$
Allelotyping
BC54-198 BL
BC54-000 Tm2
BC54-000 Tm1
BC54-000 BL
SARC36-000 Tm
SARC36-000 BL

Figure 3