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GRANT NUMBER DAMD17-94-J-4077

TITLE: A Comprehensive Repository of Normal and Tumor Human Breast Tissue and Cells

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REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, 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
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE
A Comprehensive Repository of Normal and Tumor Human Breast Tissue and Cells

5. FUNDING NUMBERS
DAMD17-94-J-4077

6. AUTHOR(S)
Jerry W. Shay, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas
Dallas, Texas 75235-9016

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, 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 words)*

We have successfully established a breast tissue repository which contains over 250 indivudual breast specimens. We have cryopreserved and cultured cells from human breast tumor tissues and have established 21 new human breast tumor cell lines. The repository also includes breast epithelial and stromal cell strains derived from non cancerous breast tissue as well as peripheral blood mononuclear cells.

14. SUBJECT TERMS
Breast Cancer , tumor cell lines and strains from non malignant tissue

15. NUMBER OF PAGES
49

16. PRICE CODE

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

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JWS Roy 7/3/98

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A Comprehensive Repository of Normal and Tumor Human Breast Tissues and Cells
Jerry W. Shay, Ph.D., Program Director
(Total grant period: July 1, 1994 - July 31, 1998)
(Progress report period: July 1, 1997 - June 30, 1998)

Introduction

Statement of background/purpose:

Progress in understanding the development of human breast cancer has been made by studying tumor tissue obtained from patients during surgery and by establishing breast tumor cell lines. However, in such cases, the researcher may be analyzing one or more late events in the progression of the disease. The development of breast cancer is likely to be a multi-step, progressive process with several heritable alterations accumulated during the evolution to malignancy. Since one of the major objectives of breast cancer research is to provide a means for early intervention, it is important to define the specific molecular alterations at each stage in this process. The study of such alterations is greatly aided by having not only tumor cells but also corresponding non-malignant breast cells of stromal and epithelial origin. Because non-malignant breast cells may have undergone genetic alterations, a self-replicating source of constitutional DNA is also of great importance. The purpose of our project is to establish a unique repository of materials for the biologic and genetic study of breast cancer. The repository will contain cryopreserved and cultured cells from tumor tissues, non-malignant epithelial and stromal cells as well as peripheral blood mononuclear cells. Patient demographic and clinical data, and family history will be collected and entered onto a relational database.

Statement of the principal objectives of the program are to:

1) obtain and cryopreserve from breast cancer patients, peripheral blood mononuclear cells, tumor tissue, and non-malignant adjacent breast tissue; include samples from women with familial breast cancer and with non-invasive breast cancers; collect patient demographic, family, clinical and pathological data;
2) prepare and cryopreserve breast tissue organoids from which both epithelial and stromal cells can be cultured;
3) characterize breast epithelial and stromal cells
4) establish and characterize breast tumor cell lines from patients with breast carcinoma;
5) establish and cryopreserve Epstein Barr Virus-transformed B-lymphoblastoid cells as a source of constitutional DNA;
6) maintain computerized records of all data, materials accessioned, and cell characterization;
7) make samples available and publicize information about the repository and to make its resources readily available to the scientific community with minimal restrictions.
8) maintenance of cell repository and backup
9) obtain future stable monetary support for repository

Body

Task 1. **Obtain and Cryopreserve Normal and Tumor Surgical Specimens**

In July 1994 we initiated a repository for multiple areas of breast cancer research. It was an ambitious project, and during the first year of the parent grant we obtained and cryopreserved 84 tissue samples. Of these samples we established and cryopreserved a total of five human breast tumor cells lines. During the second year of the parent grant we obtained and cryopreserved 55 additional tissue samples. Efforts were undertaken to obtain early and premalignant breast tissue samples and during the second year of the parent grant we obtained one ductal carcinoma *in situ*, three lobular carcinoma *in situ*, 20 fibroadenomas, and 17 other benign conditions.

Prior to the initiation of our breast tumor and cell repository 85 breast cancer specimens were accessioned. These consisted of 62 primary breast cancers and 23 metastatic lesions. When available, primary tumor tissue, adjacent non-malignant tissue, and cryopreserved peripheral blood mononuclear cells were obtained. In summary, during the four years of the parent breast tumor and cell repository grant, we have obtained and cryopreserved approximately 165 samples so that at the present time we have accessioned a total of 250 individual breast specimens. Thus most of our effort during the early years has been to accession samples and establish the cell lines. During the third and fourth year we have also initiated and now completed most of the characterization of our primary tumors and tumor derived cells lines and most manuscripts are in press or published while a few are still being prepared. One of our tumor derived cell lines obtained during the third year has a BRCA-1 (inherited breast cancer susceptibility locus) mutation. This line has been characterized (see appendix, reference 11) and additional interesting specimens will be obtained as they become available. All patient demographic, family, clinical and pathological data are maintain on the computerized database (see appendix, reference 12).

The samples have been characterized for DNA ploidy, karyotype, progesterone/estrogen receptors, Ber-EP4 (breast specific antigen), BRST-1 (breast specfic antigen), BRST-2 (breast specific antigen), cytokeratins, Her2-neu (breast amplified oncogene), p53 mutations and telomerase activity (references 2,4,6,7,9,10). Detailed experimental methods are described in the published manuscripts.

Task 2. **Culture and Cryopreserve Organoids from "Normal" Breast Tissue Samples and Separate Epithelial from Stromal Cells**

We were successful in culturing and cryopreserving breast epithelial and stromal cell cultures. During the four year project, a total of 23 human breast epithelial and 25 stromal cell strains have been cryopreserved. In addition, we have 50 additional organoid cultures frozen which have not been established into epithelial and stromal strains. Due to limited manpower, we have elected to only characterize those epithelial and stromal cells in which tumor cell lines are established. Since it requires at least 4-6 months of culture to be confident that a primary tumor is successfully established, we generally

make breast tissue organoids and in some instances primary cultures and then cryopreserve them until such time as the tumor cell data are obtained. We now have matched tumor derived cell lines and normal epithelial and stromal cells from five of our accessioned specimens (see appendix). We have finalized the characterization of these strains, scaled them up and cryopreserved early passages for future distribution from the repository.

Task 3. **Characterize Breast Epithelial and Stromal Cells**

One of the epithelial cell cultures obtained from a patient with Li-Fraumeni syndrome spontaneously immortalized in cell culture (see reference 2). The 5 stromal and epithelial samples with matching tumor derived lines have been characterized for DNA ploidy, karyotype, progesterone/estrogen receptors, Ber-EP4 (breast specific antigen), BRST-1 (breast specfic antigen), BRST-2 (breast specific antigens), cytokeratins, Her2-neu (breast amplified oncogene), p53 mutations, telomere length, and telomerase activity.

Task 4. **Establish and Characterize Breast Tumor Cell Lines from Primary Breast Carcinoma**

We recognized at the onset that establishing breast tumor cell lines would be the rate limiting component to the success of the repository. At the end of the first year of the parent grant we had clearly established one additional breast tumor cell line (for a total of 5 new breast tumor cell lines). During the second through fourth years we made a special effort to initiate and obtain additional human breast tumor cell lines. We were successful in establishing 16 additional lines for a total of 21 lines that are currently in the repository (see appendix). These new human breast tumor cells lines were almost all derived from primary invasive ductal breast carcinomas and have been characterized for DNA ploidy, karyotype, progesterone/estrogen receptors, Ber-EP4, BRST-1, BRST-2, cytokeratins, Her2-neu, p53 mutations and telomerase activity. In addition, a manuscript describing the FRA3b and FHIT characterization of the cells was published (reference 9). Two additional manuscripts are in press (reference 11 and 12). The tumor derived breast cell lines have been provided to the American Type Culture Collection for distribution to the scientific community.

Task 5. **Establish and Cryopreserve EBV-transformed B-lymphoblastoid Cell Lines**

We have cryopreserved peripheral blood mononuclear cells from patients from whom we obtained permission, but decided that we would transform only those samples with EBV when we had preliminary evidence that the tumor lines were successfully established and cryopreserved. Of the 21 breast tumor cell lines that we have established, we have 16 EBV-transformed peripheral blood mononuclear cultures established as lines for a source of constitutional DNA. In addition, two of these EBV-transformed B-lymphoblastoid cell lines have accompanying normal breast and stromal cell strains as well as a tumor derived cell line. This is a unique combination of materials from these two individuals and will be a valuable asset for breast cancer research.

Task 6. **Maintain a Computerized Database**

All entries are currently made and will continue to be made on a MacIntosh computer in the co-investigator's laboratory (Dr. Gazdar). Patient demographic information, and relevant clinical and family data are collected and entered onto a computerized relational database written in the Fourth Dimension software program with access by password. A database has been appropriately modified by Mr. David Wheeless, Computer Specialist, at the University of Texas Southwestern Medical Center. Only Drs. Shay, Gazdar, and personnel with a need to know have access to patient identification. Informed consents and other hard copies of patient data are stored in locked, limited access cabinets. Responsibility for computer entries are given to a single person (with the confirmation of correct entry given to a second person). Backup of the data base is made weekly onto a tape drive (automatic via network).

Task 7. **Making Samples Available to Breast Cancer and Other Researchers**

Our homepage announcing the availability of our tissue/cell repository is now on line (http://www.swmed.edu/bcrep). During the first four years approximately 100 individuals have obtained tissues and cells from our repository. We have contacted existing breast tissue banks to coordinate data base interconnections. We have contacted Dr. Steve Ethier at the University of Michigan Cancer Center who has just established a web site for their breast tumor repository (http://www.cancer.med.umich.edu/). In addition, Dr. Martha Stampfer (Lawrence Berkeley Laboratory, California) has also established a home page on human mammary epithelial cells (http://www.lbl.gov/~mrgs) and we have contacted her. We have now establish our own web site and hope to link our site with the Michigan and California site as well as the Cell Line Data Base and the Breast Cancer Information Core. In addition, many of our reagents (especially the tumor derived cell lines) developed have been submitted to the American Type Culture Collection for broad distribution to the scientific community (The ATCC will also be linked to our homepage). Those reagents such as primary biopsies, which are limited in quantity, will be maintained in our repository for distribution.

Task 8. **Maintenance of Cell Repository and Backup**

At present, all samples are split and maintained in both Dr. Gazdar's and Dr. Shay's laboratories. All samples are coded, divided and maintained in both liquid nitrogen and -150°C freezers (with automatic alarms). The freezers are located in separate buildings. Only designated personnel are able to access the repository. During the fourth year of this project many of the reagent will be provided to the American Type Culture Collection as a permanent source for the distribution of the tumor derived cell lines.

Task 9. **Future Stable Monetary Support for Repository**

We have now completed the requested four years of the grant. We were not successful in obtaining long term support for the repository, thus we have requested and obtained permission to carry over unexpended funds for an additional year. We are

planning to only maintain and distribute from the repository during this extension year. However, for during this extension year, expansion of the repository will only be in areas that are exceptionally interesting or important (e.g. additional LiFraumeni Syndrome and BRCA-1 mutations). Thus, our plan during this extension year is to continue to maintain and send out materials from the repository. We are considering applying for funds to sustain the repository from several foundations. The present rules do not permit us to request maintenance support from the USAMRC.

Conclusions

All subtasks have been completed with the exception of obtaining future stable support for the repository. We believe we have had a very successful effort since the initiation of the repository. Initially we had to recruit and train new research assistants and establish lines of communication for successfully obtaining and distributing samples. We were somewhat disappointed in the first year that we had not clearly established more tumor cells lines, but during the second through the fourth years we had had considerably more success. One of our biggest successes was the development of an improved telomerase activity assay which we used to characterize almost all the 250 human breast tumors, 55 adjacent noncancerous breast tissue specimens, and other noncancerous lesions including 20 fibroadenomas and 17 fibrocystic disease specimens (see references 1,2,3,4,7,8,10). In addition, we successfully established a breast epithelial cell line from a patient with Li-Fraumeni syndrome (one of the first spontaneously immortalized human breast epithelial lines reported, reference 2) and have another breast cell line with a BRCA-1 mutation (reference 11 and appendix). Finally, and perhaps most importantly for future breast cancer research, we have successfully established 21 new human breast tumor cell lines and from 16 of these we have corresponding non-malignant blood lymphocytes. In addition, we also have non cancerous human breast epithelial and stromal cell strains from 5 of these patients. Overall, we have tumor derived cell lines, lymphocytes, epithelial and stromal cells from two individuals. These new reagents should facilitate progress in breast cancer research in the future.

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[CANCER RESEARCH 58, 0000–0000, August 1, 1998]

Advances in Brief

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

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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 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

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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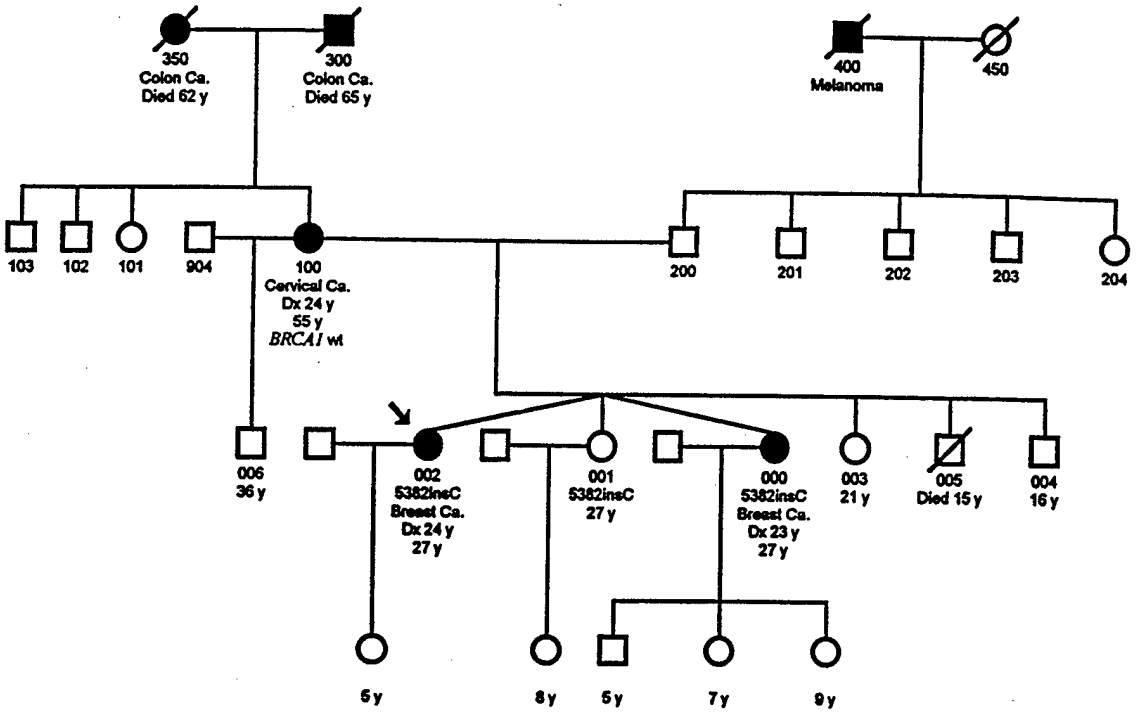

Fig. 1. Pedigree of the family from which the HCC1937 cell line was derived. The patient from which the tumor cell line is derived is indicated by the *arrow*. Germ-line DNA from the patient as well as the affected and one unaffected sister was heterozygous for the *BRCA1* mutation, 5382insC. The patient's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

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).

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

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

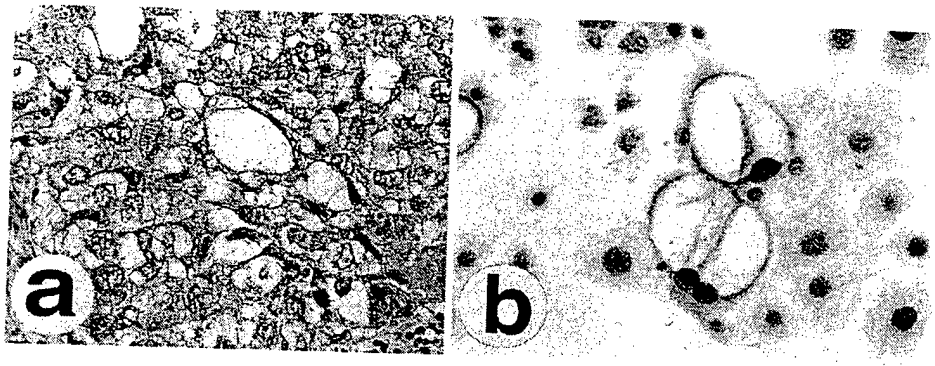

Fig. 2. Morphology of the breast cancer primary tumor and cell line. H&E stain. *a*, the primary breast carcinoma from which HCC1937 was derived. *b*, HCC1937 tumor cell line, cytospin preparation. Giant vacuolated mono- and dinucleated cells are present in both the tumor and cell line. The nonvacuolated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

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 5323insC 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′-CTTCATTTTGTAAGACTTATTAC-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′-TGCACCCTTGGTCTCCTCCAC-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, and *Mbo*I. The *PTEN* pseudo-gene, *PTEN2* (20), 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 extraneous bands suggestive of microsatellite instability were noted at

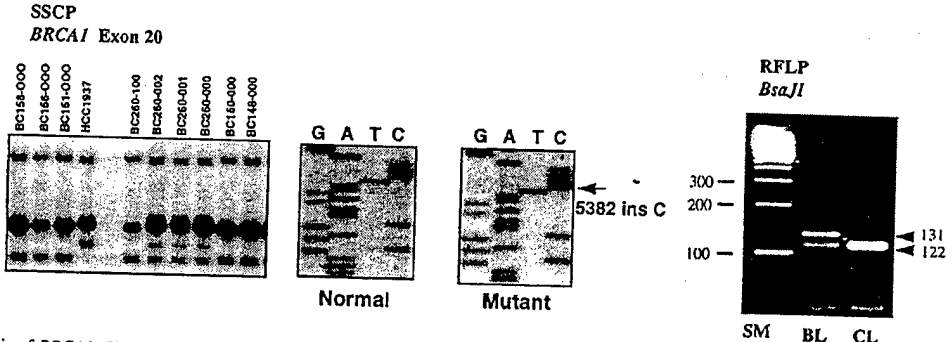

Fig. 3. Molecular analysis of *BRCA1*. Single-strand conformation analysis (*left*) revealed an aberrant band in lymphocyte DNA from the patient (*BC260-002*) and each of her two sisters analyzed (*BC260-001* and *BC260-000*). The tumor cell line demonstrated the mutant band as well as the absence of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

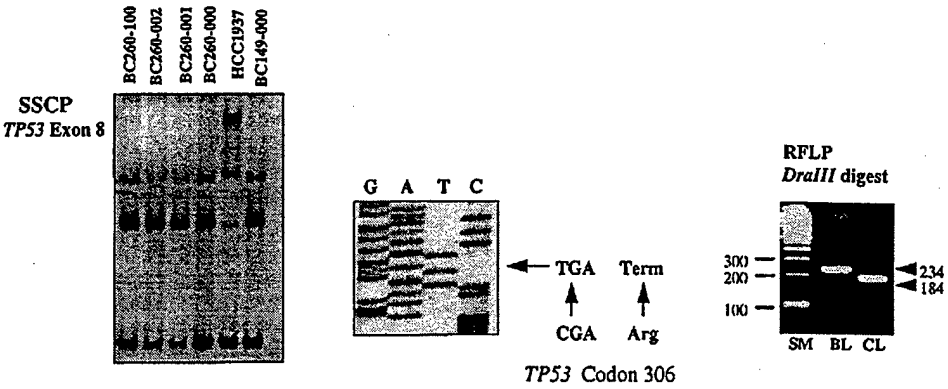

Fig. 4. Molecular analysis of *TP53*. Single-strand conformation analysis of the *TP53* gene revealed an abnormality in exon 8. Sequence analysis demonstrated a point mutation leading to a termination at codon 306. This mutation is also demonstrated by designed restriction fragment length polymorphism 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 (122 bp).

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 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,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 COOH-terminal portion of the protein. It is not known whether this *BRCA1* mutation is germ line, although it is quite possible that this

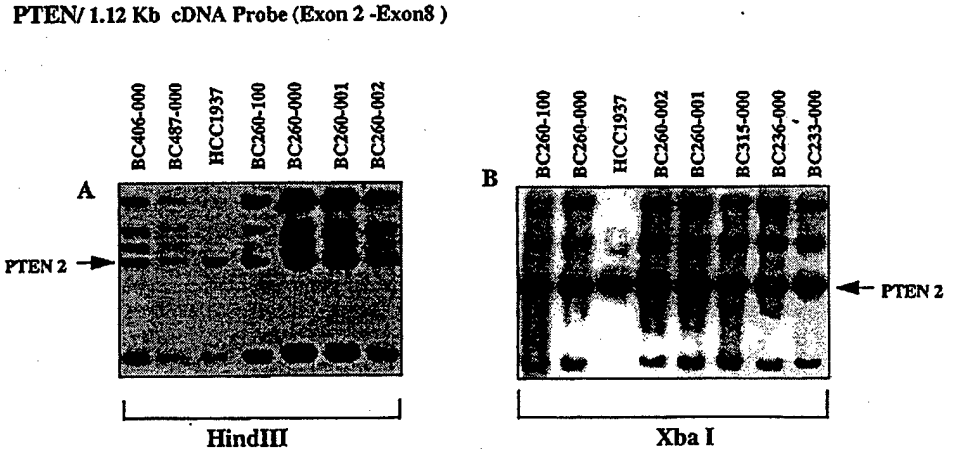

Fig. 5. Southern blot demonstrating absence of the *PTEN* coding sequence in HCC1937. DNA was digested with *Hin*dIII (*left*) or *Xba*I (*right*). The Y737 probe used was prepared from *PTEN* cDNA and contains exons 2–8 (22). Absent bands were observed in the lane containing HCC1937 DNA. Similar results 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] RH, retention of heterozygosity; ND, not determinable.

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 of whether an additional genetic predisposition factor is carried by this family arises. 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] Gazdar, unpublished data.

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In Press: Int. Journal of Cancer 1998

CHARACTERIZATION OF PAIRED TUMOR AND NON-TUMOR CELL LINES ESTABLISHED FROM PATIENTS WITH BREAST CANCER

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Paired breast tumor cell lines

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Funding Agency: U.S. Army

Grant Number: DAMD 17-94-J-4077

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ABSTRACT

Our study aim was to develop a panel of tumor cell lines along with paired non-malignant cell lines or strains collected from breast cancers, predominantly primary tumors. From a total of 189 breast tumor samples consisting of 177 primary tumors and 12 metastatic tissues, we established 21 human breast tumor cell lines that included 18 cell lines derived from primary tumors and 3 derived from metastatic lesions. Cell lines included those from patients with germline *BRCA1* and *FHIT* gene mutations and others with possible genetic predisposition. For nineteen tumor cell lines, we also established one or more corresponding non-malignant cell strains or B lymphoblastoid (BL) lines, that included 16 B lymphoblastoid lines and 7 breast epithelial (2) or stromal (5) cell strains. The present report describes clinical, pathological, and molecular information regarding the normal and tumor tissue sources along with relevant personal information and familial medical history. Analysis of the breast tumor cell lines indicated that most of the cell lines had following features: They were derived from large tumors with or without axillary node metastases; were aneuploid and exhibited a moderate to poorly differentiated phenotype; ER- and PR-negative, and over expressed p53 and HER2/neu proteins. Of 13 patients with primary breast cancers receiving curative intent mastectomies, seven were dead after a mean period of 10 months. Our panel of paired tumor and non-malignant cell lines will provide important new reagents for breast cancer research.

INTRODUCTION

In vitro permanent cell lines derived from primary or metastatic cancers provide important experimental systems for studying the biology and genetic changes associated with tumor initiation and progression. Cell lines provide an unlimited, self replicating source of cells which can be widely distributed to facilitate comparative studies. The usefulness of the modest number of breast cancer cell lines being utilized today has been somewhat restricted by the absence of readily available sources of constitutional DNA. Further, most of these breast tumor derived cell lines have been established from metastatic tumors (Leibovitz, 1994), raising questions as to their relationship to primary tumors.

Since the establishment of the first human breast carcinoma cell line in 1958 (Lasfargues, 1958), many attempts have been made to establish additional permanent breast tumor cell lines. Human breast tumor cell lines, however, are difficult to establish in culture (Smith, 1984; Smith *et al.*, 1987). Moreover, breast tumor cells are frequently contaminated with normal epithelial, stromal or mesothelial cells that demonstrate initial *in vitro* growth, making it difficult to determine the source of the proliferating cultured cells (McCallum and Lowther, 1996). Although about 50 to 70 human breast cancer lines have been described in the literature, the number of breast tumor cell lines that have been adequately characterized and are widely used is only about 20 (Leibovitz, 1994). To date, only a few breast tumor cell lines are available that also include paired non-malignant cell lines or strains (Band *et al.*, 1990), and none have been established from patients with germline mutations in predisposing genes such as BRCA1.

The majority of breast carcinoma cell lines have been initiated from tumor metastases, in particular malignant pleural effusions (Band *et al.*, 1990; Mahacek *et al.*, 1993), while relatively few have been established from primary tumors (Ethier *et al.*, 1993; Petersen *et al.*, 1990). Almost all of the primary tumor cell lines were derived from patients who also had nodal metastases (Leibovitz, 1994). Additional problems with the use of currently available breast cancer

cell lines include slow growth rates *in vitro* (McCallum and Lowther, 1996), and lack of hormonal responses. MCF-7 is the most widely studied breast carcinoma cell line because of its steroid receptor status and estrogen sensitivity (Levenson, 1997), while other cell lines which have low steroid receptor expression (such as PMC42) are not widely used (Leibovitz, 1994). In this report, we describe the initiation and characterization of a relatively large panel of paired tumor-non malignant human cell lines and strains derived from patients with primary and metastatic tumors. Our panel should compensate for many of the shortcomings associated with existing breast tumor cell lines.

MATERIALS AND METHODS

Initiation and culture of breast cancer cell lines

Tumor tissues were obtained from breast cancer patients following surgical resection, and processed for culture as described earlier (Leibovitz, 1994; Oie *et al.*, 1996). Briefly, solid tumor tissues were placed in a petri dish containing RPMI-1640 (Gibco-BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum, dissected into small pieces with a surgical blade, and scraped to release tumor cells into the surrounding media. The tumor cells along with finely dissected tumor fragments were transferred to a T75 flask, and cultured in 12-15 ml of RPMI-1640 or ACL4 medium (Gazdar and Oie, 1986) supplemented with 5% fetal bovine serum. The media were changed weekly and the flasks were monitored periodically for epithelial cell growth. When sufficient colonies of epithelial growth were noted, the cultures were trypsinized (for adherent epithelial cells) and passaged, or aliquots of non-adherent epithelial cells were passaged.

Because our aim was to establish cell lines from primary tumors, we obtained only occasional samples from metastases. A total of 189 tumor samples were utilized for culture consisting of 177 primary tumors and 12 metastatic tissues. In some cases, adjacent non-malignant breast tissues were used to initiate non-malignant cell strains. The tumor samples were collected over a five year period (1991-1996), and included patients from diverse age, ethnic and racial groups representing various stages of disease progression as determined by the TNM staging (Table I). Appropriate Institutional Review Board permission and patient informed consent were obtained for these studies.

Initiation of non-malignant cell strains

Non-malignant mammary tissues from the same patients were processed, and epithelial and stromal cell strains were established as described earlier (Hammond *et al.*, 1984; Shay *et al.*, 1995; Stampfer and Yaswen, 1993). Briefly, following surgical excision, the tissues were

minced and digested overnight (8-16 hrs) at 37°C with collagenase (400-800 units/ml) and hyaluronidase (200 units/ml). The resulting fragments, which included large organoids of ductal cells were filtered to separate smaller organoids, and cryopreserved in MCDB170 (Gibco-BRL) medium containing 10% DMSO (Shay *et al.*, 1995). To initiate epithelial and stromal cell strains, aliquots of the processed organoids were placed in either growth factor-supplemented MCDB170 media for epithelial cells, or DMEM (Gibco-BRL) supplemented with 10% iron-fortified serum (HyClone Laboratories, Inc., Logan, UT) for stromal cell growth (Hammond *et al.*, 1984).

Epstein-Barr virus (EBV) lymphocyte transformation

B lymphoblastoid cell lines were initiated essentially as described earlier (Louie and King, 1991). Briefly, samples of blood were obtained from patients, and were processed to separate mononuclear cells by centrifugation through Histopaque (Sigma Biochemicals, St. Louis, MO), washed twice in RPMI-1640 and resuspended in initiation medium (RPMI-1640 with 15% fetal bovine serum, 25 mM Hepes, 1mM sodium pyruvate). The cell suspension (~ 1.0 - 5.0 X 10^6 lymphocytes) was transferred to a T25 culture flask containing 5 µg/ml phytohemagglutinin-p (Sigma) and 5 ml EBV-conditioned initiation medium from a EBV producing marmoset cell line, and incubated at 37 °C with 5% CO_2. The media were changed weekly until aggregates of non-adherent lymphocytes having cytoplasmic processes (uropods) began to proliferate rapidly.

DNA fingerprinting of breast tumor cell lines

DNA fingerprinting was performed using the AmpliType™ PM PCR Amplification and Typing kit (Perkin Elmer, Branchburg, NJ). Six genetic loci, HLA, low density lipoprotein receptor (LDLR), glycophorin A (GYPA), hemoglobin G gammaglobin (HBGG), D7S8, and group specific component (GC) were used for fingerprint analysis of paired cell lines.

Cytogenetic analysis

Cytogenetic analysis was performed as described previously (Virmani *et al.*, 1998). Metaphase spreads were prepared as described earlier from seven cell lines initiated from primary tumors, and analyzed by G banding using standard cytogenetic techniques .

Flow cytometry (FACS)

FACS analysis to determine expression profiles of surface antigens was performed as described earlier (Latza *et al.*, 1990). Log phase cultures were harvested, and washed with phosphate buffered saline and bovine serum albumin. Cell aggregates were disrupted gently using a pipette, and the resulting single cell suspension was adjusted to 1 X 10^7 cells/ml. 1 X 10^6 cells were incubated with an appropriate primary antibody for 30 min on ice. Following incubation, cells were washed with saline-albumin and were incubated with the FITC-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO) in the dark on ice. Cells were washed once with saline-albumin, fixed in 1% paraformaldehyde and analyzed using a FACScan Flow Cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

Ploidy analysis

For ploidy analysis, cells were collected from log phase growth cultures, fixed briefly in formaldehyde, pelleted and suspended in agar (1.5%), and then embedded in paraffin. The paraffin-embedded samples were used to prepare 5 μm sections, and the sections were Feulgen-stained using Thionin Schiff's reagent. DNA ploidy was determined by image analysis using a Roche Pathology work station (Roche Image Analysis, Elon College, NC). A minimum of 200 tumor cells were analyzed for ploidy using stromal cells as an internal control.

Immunostaining

Immunostaining analyses were performed as described earlier (Yu, 1992) using standard avidin biotin techniques after a microwave antigen retrieval step. Paraffin-embedded culture

samples were prepared, and sectioned as described previously. The sections were used for evaluation of immunostaining markers. The panel of antibodies used for immunostaining analyses included: estrogen receptor (DAKO Corp., Carpenteria, CA; dilution 1:160); progesterone receptor (Zymed, San Francisco, CA; dilution 1:200); p53 (DO-7 clone that reacts with wild and mutant forms of the intracellular p53 protein; DAKO, 1:80), and HER2/neu (c-erb-B2; DAKO, 1:14,000). A minimum of 15 fields or 30,000 μm^2 of nuclear area was quantified. The samples were scored as follows (Table III): negative (-); low (+) if expression was restricted to 0-30% cells, moderate (++) for 30-70% positive cells, and strongly positive (+++) if >70% of cells stained positive.

RT-PCR

RT-PCR analysis was performed as described earlier (Sakaguchi *et al.*, 1998). RNA extracted from log-phase tumor lines was used to prepare cDNA. Expression of two genes was determined: epithelial glycoprotein 2 (EGP2), a cell surface glycoprotein present in most epithelial cells and tumors, and cytokeratin-19 (K19), a primitive keratin expressed by all epithelial cells . Using gene-specific oligonucleotide primers, cDNA was subjected to 30 cycles of PCR, and the amplified products (a 515 bp fragment for EGP2 and a 460 bp fragment for cytokeratin 19) were resolved by polyacrylamide gel electrophoresis (Sakaguchi *et al.*, 1998).

Estrogen/progesterone receptor assays

Enzyme immunoassays for estrogen/progesterone receptor assays were performed by the Corning Nichols Institute (San Juan Capistrano, CA). Cytosolic fractions were prepared after homogenization in a buffer containing molybdate to preserve the receptors followed by ultracentrifugation. Receptors in the cytosol were captured by antibody-coated beads and detected by a second, horseradish peroxidase conjugated antibody. The receptor concentration was determined from a standard curve.

HER2/neu expression assays

HER2/neu expression was determined by a quantitative ELISA assay (Calbiochem, Cambridge, MA) using the manufacturer's suggested protocol. The relative expression levels of the HER2/neu protein in tumor cell lines were expressed as a multiple of the mean level expressed by two human epithelial cell strains.

RESULTS

Establishment of cell lines and strains

Human breast tumor specimens from 189 patients with breast cancer were accessioned during a five year period. Using these specimens, 21 tumor cell lines were established. The overall success rate was 21/189 attempts or approximately 11%, a percentage higher than previously reported (Meltzer *et al.*, 1991). A pair of tumor cell lines, HCC1007 and HCC1008, was established from the primary tumor and axillary node metastasis, respectively, of the same patient.

As shown in Table I, the initiation period for establishment of tumor cell lines (i.e. the time before sufficient tumor cell growth occurred for initial cell passage) ranged from 4 months to 44 months, with a median time of 12 months, a period considerably longer than that reported by others (4-5 months) (Meltzer *et al.*, 1991). During this long latent period, stromal fibroblasts tended to grow faster than the epithelial cells, requiring selective enrichment of the epithelial tumor cells. Although the majority (15) of cell lines were initiated in RPMI1640 (Gibco-BRL) medium supplemented with 5% fetal bovine serum, ACL4 medium (Gibco-BRL) supplemented with 5% serum was used for initiating 6 cell lines. After establishment, the two media were generally interchangeable, and most cell lines grew equally well in either medium, with approximate doubling periods of 24-72 hours.

We established paired, non-malignant B lymphoblastoid cell lines corresponding to 16 of the 21 breast tumor cell lines, and in some instances, breast tissue derived epithelial and stromal cell strains. Our current collection includes a total of 16 paired B lymphoblastoid lines, 2 epithelial and 5 stromal strains. One or more corresponding non-malignant cell strains/lines were available for 19 of the 21 tumor cell lines. DNA fingerprinting and other molecular analyses confirmed that in all cases the paired lines were derived from the same individuals.

Patient clinical, demographic and tumor information

The panel of cell lines were established from breast cancer patients ranging in age from 24 to 82 years, although most (12) were between the ages 40 through 60 (Table I). These patients represented diverse ethnic and racial backgrounds: 10 white, six African-American, two Hispanic, one Oriental and one East Indian. Most patients (n = 18) had primary invasive carcinomas, of which 17 were ductal (one with metaplasia), while one primary tumor had a diagnosis of acantholytic squamous carcinoma. Three tumor cell lines were derived from metastases (one from a lymph node with metasastic ductal carcinoma, and two from malignant pleural effusions (one from a metastatic lobular carcinoma, and one from adenocarcinoma, not otherwise specified). Analysis of the TNM staging indicated that these patients were suffering from varying degrees of tumor extent, ranging from stage I (2 patients) through stage IV (3 patients). Fourteen of the 20 patients had grade 3 tumors while the other 6 had grade 2 tumors. Five of the patients received prior chemotherapy (corresponding to cell lines HCC1187, HCC1395, HCC1419, HCC1428, and HCC1569), and two received prior radiation therapy (HCC1954 and HCCHCC2185).As shown in Table I, of 13 patients with primary breast cancers receiving curative intent mastectomies, 7 were dead after a mean period of about 10 months, ranging from 5 to 22 months.

Of 15 primary tumors successfully cultured whose nodal status was determined, 10 (66%) had lymph node metastases, while 5 (33%) did not. As shown in Table I, the majority of the cell lines were derived from large tumors that were positive for lymph node metastases. As mentioned earlier, cell lines HCC1007 and HCC1008 were established from the primary tumor and axillary node metastasis, respectively, of the same patient.

Genetic predisposition

Of the patients from whom the 21 cell lines were established, a detailed family history was obtained from 10. Of these, five had some feature suggestive of a genetic predisposition as indicated by the prevalence of familial breast cancer, corresponding to cell lines HCC38, HCC1500, HCC1395, HCC1428 and HCC1937. Two of these cell lines were derived from

patients having germline mutations in known (*BRCA1*, HCC1937; see below) or potential (*FHIT;* HCC1569) predisposing genes. One cell line (HCC2218) was derived from a patient who suffered from an early onset, at age 38, of breast cancer. The patient pedigrees for HCC38, HCC1500, HCC1569 and HCC2218 (see below) are shown in Figure 1. A brief summary of the four patients follows:

HCC38 was initiated from a 50 yr old woman with a primary breast tumor as well as a second primary leiomyosarcoma (Fig 1A). In addition, her mother had died of breast cancer. Because the history of both breast and sarcoma was somewhat suggestive of Li-Fraumeni Syndrome, her lymphocyte DNA was examined for possible mutation of the *TP53* gene however no mutation was found in exons 4 through 8 of the *TP53* gene.

HCC1500 (Fig 1B) was initiated from a 32 yr old woman with a significant family history of early-onset colon cancer as well as a sister with breast cancer. This cell line was associated with a homozygous deletion at chromosome 3p21, an area known to show frequent LOH in breast cancers (Sekido *et al.*, 1998).

One cell line, HCC1569, which was found to have a mutation of the *FHIT* gene which proved to be heritable in that the patient's daughter also carried the same alteration. The tumor arose in an older patient (age 70) without a family history of breast cancer (Fig 1C). Further details of the mutation have been published elsewhere (Ahmadian *et al.*, 1997). It is unclear whether the germline alteration which occurred in this breast tumor (which otherwise appeared to be sporadic) was a causative factor in the development of her cancer.

Cell line HCC2218 derived from a woman age 38 at diagnosis (Fig. 1D). The early age of onset has been associated with a 12% incidence of carrying a *BRCA1* alteration, although to date we have not documented a germline mutation in this family.

As reported separately in greater detail, HCC1937 was derived from a 24 yr old woman with a germline *BRCA1* mutation, insertion C at nucleotide 5382 (Tomlinson *et al.*, 1998). This mutation was present in two other family members.

Morphology and growth characteristics

Actively growing, sub-confluent tumor cell lines were examined for their morphological and growth properties. The cell lines were also evaluated for substrate attachment and differentiation status. Examples of cell culture appearances are presented in Fig. 2. The majority (n = 16) of the cell lines (Table II) exhibited partial (n = 8) or complete (n = 8) substrate attachment (Figs. 2a-c). However, five lines lacked substrate adherence (Fig. 2d). Four of the partially or non-adherent cultures formed duct-like and hollow morula-like structures that were strikingly similar to the *in vivo* ductal morphology during breast development (Figs. 2c-d). Domes represent hemispherical elevations of the monolayer resulting from vectorial fluid transport. We regard these cultures as demonstrating partial differentiation. Although tumor cell lines HCC1007 and HCC1008 were derived from primary and metastatic tumors respectively from the same patient and exhibited a similar partially adherent growth morphology, the formation of duct and gland-like structures was less pronounced in HCC1007 compared to HCC1008. HCC1007 cells, however, were more extensively vacuolated than those observed in HCC1008. The other adherent cultures failed to form duct or morula-like structures, and lacked dome formation. They were regarded as poorly differentiated. Adherent cells were, in general, large and exhibited characteristic epitheliod 'cobblestone' morphology and were occasionally multi-nucleated or vacuolated. At confluence, adherent cell line HCC1937 exhibited a considerable subpopulation of giant vacuolated cells.

Non-malignant cell strains

Non-malignant cell strains were initiated as described in the Methods, and evaluated for their growth patterns and morphological features in culture. Immortalized B lymphoblastoid lines grew as rapidly dividing non-adherent cell aggregates demonstrating radially oriented cytoplasmic processes ("uropods"). They expressed CD45 cell surface antigen, and lacked expression of the epithelial cell specific markers CK19 and EGP2. Epithelial cell strains derived from non-cancerous breast tissue grown in defined medium and exhibit a cobblestone cell morphology, and grew as monolayers with 16-24 hr population doublings. They expressed CK19 and EGP2, but, unlike

their malignant cell counterparts, they had a finite lifespan (average of 50 population doublings, range from 40 to 90), and lacked telomerase enzyme activity (data not shown). When breast epithelial cell strains become senescent, they enlarged, flattened, become multi-nucleated and vacuolated, and remain attached to the culture dish for several months before detaching. Mammary stromal cell strains growing in serum-containing medium exhibited fusiform fibroblast morphology, grew in monolayers with a 20-24 hr population doubling period. They lacked expression of CD45, CK19 and EGP2, and had a finite lifespan (average of 50 population doublings, range from 30 to 60), and lacked telomerase enzyme activity.

Characterization of the tumor cell lines

The tumor cell lines were analyzed using the molecular markers EGP2 and cytokeratin 19 to confirm their epithelial origin. The expression of EGP2 protein was analyzed by flow cytometry and EGP2 and K19 RNA transcripts by RT-PCR for as described in the Methods. All of the 21 cell lines were positive for EGP2 expression as analyzed by FACS (Table II). These results were confirmed by RT-PCR for all 15 of the cell lines tested, with complete concordance between the data from the two methods. In addition, the tumor cell lines were also tested for K19 expression by RT-PCR. As shown in Table II, all 21 cell lines were positive for K19 transcripts.

To determine if the paired tumor cell lines corresponded to their non-malignant cell lines or strains derived from non-malignant breast tissues or B lymphocytes, DNA fingerprinting analysis was performed using the AmpliType™ kit as described in the Methods. DNA fingerprinting analysis of the 16 tumor cell lines having corresponding 16 non-malignant lymphocyte cultures demonstrated 100% correlation for the six genetic loci tested in all of the pairs.

Ploidy and cytogenetic analysis

The ploidy index of the 21 breast tumor cell lines ranged from diploid (DNA index of 1.0) to polyploid (DNA index of 2.0 or greater) (Table II). The majority of the cell lines were characterized by an abnormal ploidy index, including aneuploidy (10/21), tetraploidy (4/21) and

multiploidy (8/21) (Non-aneuploid index values greater than 2.0, indicated by >2 in Table II). Cytogenetic analysis was performed for 7 (HCC38, HCC202, HCC1008, HCC1187, HCC1395, HCC1569, and HCC1937) of the 21 cell lines. In all cases, extensive chromosomal rearrangements were observed on chromosome 3. HCC38, the first cell line to be initiated, and HCC1937 had undergone extensive rearrangements on chromosomes 1, 3, 9, 11, 13 and 17. The modal chromosome number among the 7 cell lines ranged from 46 through 111. Three of the cell lines, HCC1008, HCC1395 and HCC1937 also exhibited the presence of double minute chromosomes, ranging from 1-12. Double minute chromosomes were more numerous (4-12) in HCC1937.

Histological and biochemical analyses

Cells of the actively proliferating tumor cell line cultures were prepared for histological analysis as described in the Methods. Histological sections were analyzed for the expression of estrogen receptor (ER) and progesterone receptor (PR); the tumor suppressor gene, p53; and the oncogene HER2/neu. The expression of the HER2/neu protein was also determined using an ELISA assay as described in the Methods. Table III contains a summary of the data from these studies.

The expression of hormone receptors was measured using biochemical assays that determined the levels of total cytosolic protein, and ranged from low (<15 fmol/mg) to 984 fmol/mg for PR in HCC1500. Twelve tumor cell lines were ER- and PR-negative, while 4 were ER or PR positive, and only one cell line (HCC1500) exhibited expression of both ER and PR. Immunostaining analysis of the hormone receptor status indicated a high concordance (11/14 or 79% for ER; 13/14 or 93% for PR). The functional relevance of the expression of these receptors in these is not yet determined. The expression of p53, however, was up-regulated (indicated by ++ or +++ in Table III) in nine of 20 (45%) cell lines while the overexpression of HER2/neu (as determined by immunostaining) also was frequent (8/20 or 40%; indicated by ++ or +++ in Table III). Immunostains also confirmed (as expected) that ER, PR and p53 proteins were localized in

nucleus, while the transmembrane receptor tyrosine kinase, HER2/neu was surface-localized at the cell membrane.

The expression profiles of HER2/neu from the ELISA assays were consistent with the immunostaining data which also showed that HER2/neu was up-regulated in 40% of the cell lines (Table III). Relative to its expression in control non-malignant breast epithelial strains, HER2/neu expression was highly up-regulated (10-30 fold) in 9 cell lines (HCC202, HCC1007, HCC1008, HCC1187, HCC1419, HCC1569, HCC1954, HCC2185, and HCC2218). In other tumor cell lines (8/21), HER2/neu expression was up-regulated only moderately, ranging from 2-fold in HCC70 to 3-4 fold in others such as HCC2157. A comparison of the data from the two methods suggested that ELISA may be more sensitive, and that HER2/neu overexpression in excess of 4-fold (by ELISA) was detectable by immunostaining. Thus, while a direct correlation of the expression profiles between the two methods was not possible, all eight of cell lines that showed HER2/neu overexpression by immunostaining, also showed several-fold overexpression levels by ELISA, indicating concordance between the two methods. Seven of these 8 cell lines showed HER2/neu expression levels 10-30 fold higher than the levels in non-malignant epithelial cell strains (Table III). HER2/neu expression levels were similar in the paired tumor cell lines HCC1007 and HCC1008 derived from the same individual.

DISCUSSION

The present study describes the establishment and characterization of 21 new breast cancer cell lines, most of which (18/21) were derived from primary breast cancers, including 5 from node negative tumors. The success rate for primary breast cancers was 18 (10%) of 177 attempts, while our success rate for metastatic tumors was three (25%) of 12 attempts. The success rate for all tumors was 21/189 or 11%. A notable feature of the current study was the establishment of paired normal and tumor cell lines from 19 individual patients. While non-malignant cell lines and strains are extremely important for comparative studies on genetic predisposition and allelotyping, to date, only a few paired breast cell lines are available (Band *et al.*, 1990). In the present study, we

have established the largest collections of paired cell lines, and characterized them using DNA fingerprinting and other molecular analyses. The epithelial origin of the tumor cell lines was confirmed by expression of the epithelial cell specific markers EGP2 and CK19.

Until recently, the success rate of establishing cell lines from breast carcinomas has not progressed greatly since the initiation of the first cell line (Lasfargues, 1958), and only a modest number of cell lines have since been established (Meltzer *et al.*, 1991). Caillieau et al. reported a culture success rate of approximately 10% from metastatic tumors, while culture attempts from approximately 300 primary breast carcinomas were completely unsuccessful (Cailleau *et al.*, 1978). In another study, only one of 136 (0.7%) primary tumors was successfully cultured (Amadori, 1993). More recently, McCallum and Lowther (McCallum and Lowther, 1996) established long term cultures from 10 (7.4%) of 135 primary tumors. However, the axillary node status of the patients whose tumors were successfully cultured was not stated. These cultures have exceedingly long doubling times (16-60 days), greatly limiting their usefulness as research tools and casting doubt as to whether they truly represent immortalized cell lines.

Human breast tumor-derived cells require exogenous growth factors in culture (Band and Sager, 1989; Ethier *et al.*, 1993). Because normal breast cells differentiate and cease to grow in serum-containing media (Band and Sager, 1989), special media formulations have been used to establish short or long term cell lines from primary and metastatic tumors (Band *et al.*, 1990). However, the success rates of these studies have been low. We utilized such a formulation for the growth of epithelial and stromal strains from the non-malignant areas of the mastectomy specimens. For establishment of tumor lines, our methods of tumor handling, disaggregation and culture conditions used in our studies were not unusual. Thus, one major factor in the relatively high success rate in the present study may have been extreme patience with prevention of overgrowth by fibroblasts. Although the majority (66%) of the tumor cell lines described herein were derived from large tumors that were positive for lymph node metastasis (TNM stage IIB or higher), some cell lines (24%) of primary tumor origin were derived from node-negative patients.

Analysis of the established tumor cell lines and the primary tumors from which they were derived suggests a profile of the subset of primary tumors that are most likely to develop into continuous cell lines. These features include: a) large tumor size with or without axillary lymph node metastases; b) hyperploidy or aneuploidy; c) relatively poor degree of differentiatiation; d) steroid receptor negative; e) HER2/neu over expression and f) positive immunostaining detection of p53 protein expression. In addition, over half (7/13) of the patients receiving curative intent mastectomies were dead within about 10 months. Some of these properties were also noted in a previous study establishing breast tumor cell lines (Meltzer *et al.*, 1991). These results also are consistent with the observation that a possible relationship may exist between the loss of hormone receptors and over expression of HER2/neu (Ito *et al.*, 1995). In addition, the successfully cultured tumors (and the resultant cultures) have a higher than expected frequencies of allelic losses at the p53 gene and at one or more regions on chromosome 3p (data not shown).

Breast cancer cell lines have been utilized for a number of biological and biochemical studies including expression of receptors for growth factors and steroid receptors. Compared to non-malignant breast epithelial cell strains, all of the breast carcinoma cell lines expressed increased amounts of HER2/neu, an important prognostic marker and a member of the receptor tyrosine kinase growth factor receptor superfamily whose expression is up-regulated several-fold in many breast carcinomas. The expression values of this protein ranged from two to thirty fold greater than the values measured in non-malignant epithelial cell strains. In eleven cell lines, this increase was modest (two to four fold) while in ten cell lines it was considerable (seven to thirty fold).

Of the patients whose tumors were successfully cultured, 10 were investigated for a genetic predisposition. Evidence suggestive or conclusive for a genetic predisposition was discovered in five of these 10 subjects (50%), a relatively high incidence. They included (as described herein and elsewhere) patients with germline mutations of the *BRCA1* gene (cell line HCC1937) at 17q21 (insertion C at nucleotide 5382) (Tomlinson *et al.*, 1998), and the *FHIT* gene (HCC1569) at 3p14.2 (G > T at nucleotide 651) (Ahmadian *et al.*, 1997).

Cell lines containing homozygous deletions are useful for the identification of the putative recessive oncogenes in the deleted regions. As detailed elsewhere (Sekido *et al.*, 1998), four homozygous deletions at three chromosomal 3p regions were identified in the tumor cell lines. These regions are 3p12 (cell line HCC38) (P. Rabbitts et al., in preparation), *FHIT* gene at 3p14.2 (cell lines HCC1428 and HCC1806) (Ahmadian *et al.*,) and 3p21.3 (cell line HCC1500) (Sekido *et al.*, 1998). Because the putative recessive oncogenes at two of these regions (3p12, 3p21) have not been identified, the lines provide important new reagents for gene localization, cloning and characterization.

Paired tumor-non-malignant cell lines provide useful reagents for detailed allelotyping. Availability of paired normal and tumor-derived cells from the individual patients should provide important tools for comparative studies on gene expression patterns, cell cycle control mechanisms, efficacy of therapeutic drugs, and diagnostic and prognostic markers. Further information regarding the breast tumor-derived cell lines described here is available on the world wide web (Breast Tissue Repository at the Hamon Center for Therapeutic Oncology, The University of Texas Southwestern Medical Center at Dallas; **http://www.swmed.edu/bcrep**). The breast tumor-derived cell lines and the B lymphoblastoid cell lines have been depositied with the American Type Culture Collection (ATCC; Rockville, MD) for broad distribution to the scientific community. In summary, the human breast tumor-derived cell lines and their paired non-malignant cell strains described in this paper represent the largest collections of its kind, and will provide valuable research tools for investigating breast cancer biology.

ACKNOWLEDGEMENTS

Supported in part by the U.S. Army Grant DAMD 17-94-J-4077.

We thank Dr. David Euhus and Dr. George Peters for their help in making the normal and tumor tissues available for this study. We also thank Ms. Yu-Yan Liu and Ms. Bilue Thomas for tissue collection and technical assistance.

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FIGURE LEGENDS

Fig.1 Pedigrees of four of the five patients with known genetic predisposition (see Results for more information). A, Pedigree of a woman with a primary breast tumor (corresponding to cell line HCC38) with prior history of sarcoma, whose mother had died of breast cancer. B, Pedigree of a young woman with a significant family history of cancer. Cell line HCC1500 was initiated from this patient. C, Pedigree of an older patient, corresponding to cell line HCC1569, with a germline mutation of *FHIT* gene. D, Pedigree of the 38 yr old patient from whom cell line HCC2218 was initiated.

Fig. 2 Morphological appearances of breast carcinoma cell lines. For adherent or partially adherent cell lines (Figs. 1A-C), cells were cultured on acid cleaned glass slides, fixed in alcohol and stained with hematoxylin and eosin. For purely non-adherent cultures (D), cytospin preparations were handled as described for adherent cultures. A, cell line HCC1395. Adherent epitheloid cells and partially adherent or non-adherent subpopulations (darkly staining cell masses) are present. B, cell line HCC1428. Large epitheloid cells with occasional vacuole formation. C, cell line HCC712. Adherent subpopulation demonstrating prominent dome formation. Domes represent hemispherical elevations of the monolayer resulting from vectorial fluid transport. D, cell line HCC1500. Non-adherent culture showing morula-like formation on the right (consisting of a hollow spherical structure lined by a single layer of cells) and duct-like structure on left (consisting of irregular tubular structure which may be hollow or solid, with occasional branching).

TABLE I. Clinical and pathological features of breast tumors used for initiation of cell lines

Cell line	Patient Age/Race	Current Status (mos)[2]	Tumor Source	Tumor Type	TNM Stage	Tumor Size[3]	Grade	LN metastasis	Prior therapy	Culture Date	Growth period (months)	Paired line / strain
HCC38	50/W	NA	Pr.Breast	Ductal ca	IIB	3	3	3 / 28		4/27/92	32	BL
HCC70	49/B	Dead (5)	Pr.Breast	Ductal ca	IIIA	6	3	4 / 17		6/3/92	44	NA
HCC202	82/W	NA	Pr.Breast	Ductal ca	IIIA	8	3	4 / 19		9/5/92	41	NA
HCC712	41/A	Dead (22)	Pr.Breast	Ductal ca	IIB	5	2	44 / 46		12/20/92	13	BL
[1]HCC1007	67/B	Dead (10)	Pr. Breast	Ductal ca	IIA	2	3	12 / 12		6/7/94	9	BL
[1]HCC1008	67/B	Dead (10)	LN	Met ductal ca	IIA	2	3	12 / 12		6/7/94	12.5	BL
HCC1143	52/W	NA	Pr.Breast	Ductal ca	IIA	5	3	0 / 15		8/30/94	29	BL
HCC1187	41/W	Dead (12)	Pr.Breast	Ductal ca	IIA	2.5	3	NA	C	9/13/94	4.5	BL
HCC1395	43/W	Alive (24)	Pr.Breast	Ductal ca	I	1.8	3	0 / 34	C	12/14/94	14	BL, St
HCC1419	42/H	Dead (6)	Pr.Breast	Ductal ca	IIIA	3	2	5 / 5	C	12/28/94	9	St
HCC1428	49/W	Dead (6)	Pl.Effusion	Met adenoca	IV	NA	3	NR	C	1/4/95	15	BL
HCC1500	32/B	Alive (11)	Pr.Breast	Ductal ca	IIB	4	2	4 / 24		2/8/95	14	St, Ep
HCC1569	70/B	Dead (12)	Pr.Breast	Metaplastic ca	IV	16	3	4 / 18	C	3/8/95	19	St
HCC1599	44/W	NA	Pr.Breast	Ductal ca	IIIA	12	3	NA		3/28/95	10	BL
HCC1739	51/W	Alive (24)	Pr.Breast	Ductal ca	I	1.5	3	0 / 33		6/14/95	15.5	BL, St, Ep
HCC1806	60/B	Dead (7)	Pr.Breast	Acantholytic Sq ca	IIB	9.5	2	0 / 18		7/31/95	10	BL
HCC1937	24/W	Alive (29)	Pr.Breast	Ductal ca	IIB	3.9	3	NA		10/13/95	11.5	BL
HCC1954	61/EI	Alive (26)	Pr.Breast	Ductal ca	IIA	3.1	3	0 / 27	R	10/30/95	4	BL
HCC2157	48/B	Alive (24)	Pr.Breast	Ductal ca	IIIA	6.5	2	1 / 9		3/4/96	8	BL
HCC2185	49/WH	NA	Pl.Effusion	Met lobular ca	IV	9	2	NR	C, R	3/18/96	7.5	BL
HCC2218	38/W	NA	Pr.Breast	Ductal ca	IIIA	6.5	3	42 / 43		4/10/96	6	BL

NA, not available; NR, not relevant; B, Black; W, White; A, Asian; EI, East Indian; H, Hispanic; Pr, primary; LN, lymph node ; Pl, pleural; CA, carcinoma; CIS, carcinoma-in-situ; Sq, squamous; T, tumor; BL, blood lymphocytes; St, stromal; Ep, epithelial; Met, Metastatic; C, Chemotherapy; R, Radiotherapy

[1] HCC1007 and HCC1008 were initiated from the primary carcinoma and the axillary lymph node metastasis, respectively, of the same patient; [2]Time period in months between surgery (Date sample received) and Death/Last date of contact; [3]Tumor size in cms

TABLE II. Characterization of breast tumor cell lines

Cell line	ATCC No.	Substrate Adherence	Morphology / Differentiation Status	Ploidy Index[1]	EGP2 FACS[2]	EGP2 RT-PCR	K19 RT-PCR
HCC38	CRL2314	Partial	PD	1.9	58	NA	+
HCC70	CRL2315	Adherent	PD	2.0	120	+	+
HCC202	CRL2316	Partial	PD	2.0	85	+	+
HCC712	CRL2317	Partial	Domes and Duct-like	1.2	184	+	+
HCC1007	CRL2318	Partial	Duct and morula-like	1.8	181	+	+
HCC1008	CRL2320	Partial	Duct and morula-like	Multiple	50	+	+
HCC1143	CRL2321	Partial	PD	Multiple	169	NA	+
HCC1187	CRL2322	Non-adherent	PD	Multiple	54	+	+
HCC1395	CRL2324	Adherent	Vacuolated, PD	Multiple	30	+	+
HCC1419	CRL2326	Adherent	PD	1.9	119	+	+
HCC1428	CRL2327	Adherent	PD	2.0	154	+	+
HCC1500	CRL2329	Adherent	PD	0.9	30	NA	+
HCC1569	CRL2330	Adherent	PD	2.3	127	NA	+
HCC1599	CRL2331	Non-adherent	PD	Multiple	165	+	+
HCC1739	CRL2333	Partial	PD	2.7	290	+	+
HCC1806	CRL2335	Adherent	PD	1.4	151	+	+
HCC1937	CRL2336	Partial	Vacuolated, PD	Multiple	451	+	+
HCC1954	CRL2338	Partial	PD	NA	324	+	+
HCC2157	CRL2340	Non-adherent	Duct and morula-like	1.7	79	+	+
HCC2185	CRL2342	Non-adherent	PD	1.4	41	NA	+
HCC2218	CRL2343	Non-adherent	PD	2.0	228	NA	+

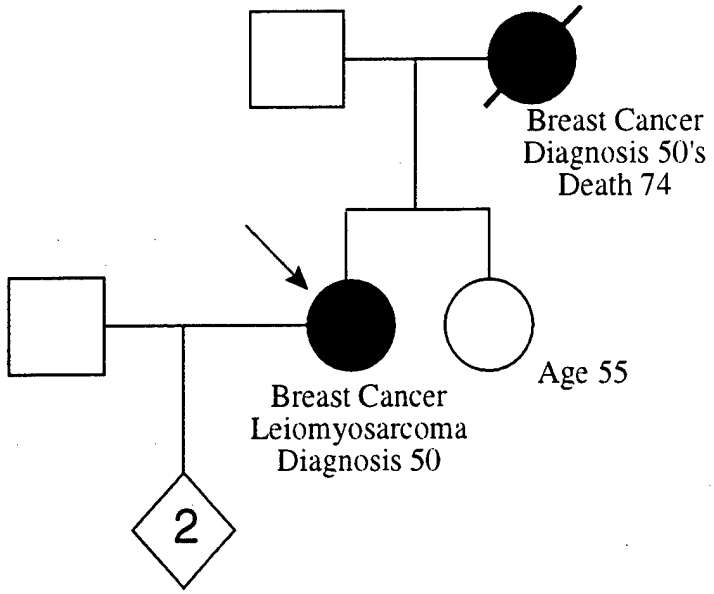
Abbreviations: NA, Not Available; PD, poorly differentiated
[1]Multiple indicates multiploid cell population with several ploidy indices
[2]FACS data is expressed in arbitrary mean channel fluorescence units

TABLE III. Characterization of breast carcinoma cell lines

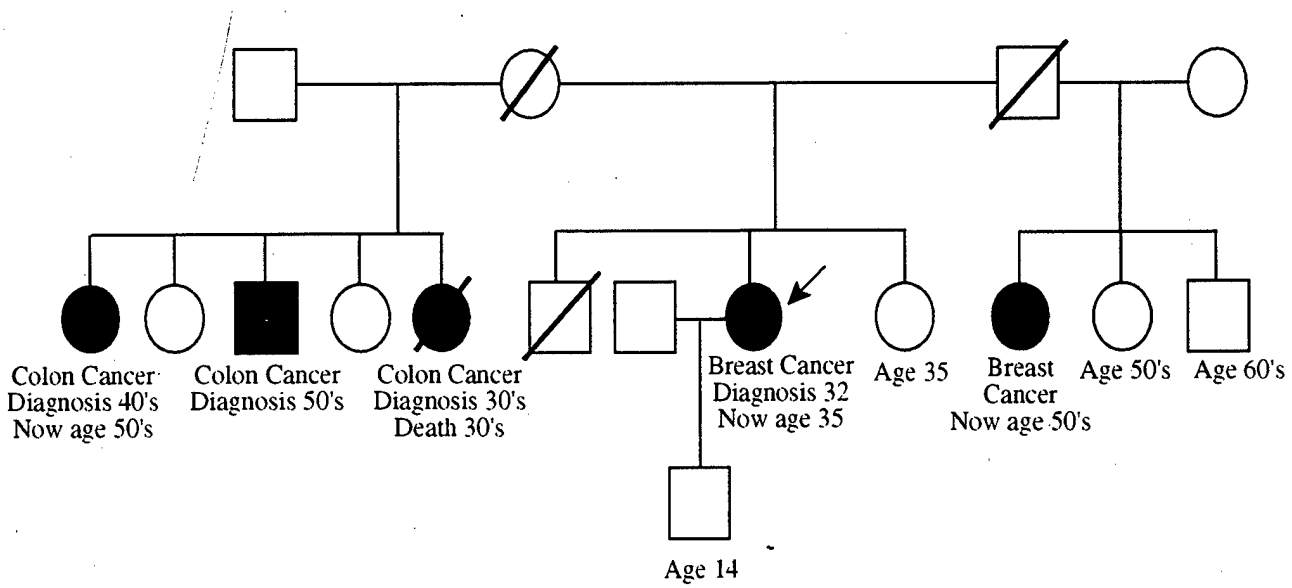
Cell Line	Estrogen Receptor (IS)	Estrogen Receptor (cytosol) (fmol/mg)	Progesterone Receptor (IS)	Progesterone Receptor (cytosol) (fmol/mg)	p53 (IS)	HER2/neu (IS)	Relative HER2/neu Expression[1]
HCC38	-	< 15	-	< 15	+	-	6.5
HCC70	+	< 15	-	< 15	+++	-	2
HCC202	-	< 15	-	< 15	-	++	30
HCC712	+	NA	+	NA	+	-	4
HCC1007	-	< 15	-	< 15	+++	+++	11
HCC1008	-	< 15	-	< 15	+++	+++	12
HCC1143	-	NA	-	NA	+++	-	4
HCC1187	-	< 15	-	< 15	+++	-	12
HCC1395	-	57	-	< 15	++	-	3
HCC1419	-	< 15	-	< 15	-	+++	29
HCC1428	+	< 15	+	133	-	-	2.5
HCC1500	++	55	+++	984	+	-	3
HCC1569	-	< 15	-	< 15	-	+++	30
HCC1599	-	< 15	-	43	-	-	4
HCC1739	-	NA	-	NA	++	-	1.5
HCC1806	-	NA	-	NA	-	-	4
HCC1937	-	< 15	-	< 15	-	-	4
HCC1954	NA	< 15	NA	< 15	NA	NA	28.5
HCC2157	-	NA	+	NA	++	++	4
HCC2185	-	NA	-	NA	+++	+++	10
HCC2218	-	< 15	-	< 15	+	+++	28

IS, immunostaining; -, negative; +, low; ++, moderate; +++, high; NA, not available; [1]Compared to the expression levels in non-malignant human mammary epithelial cell strains using ELISA (mean value equals 0.5 fmole/μg)

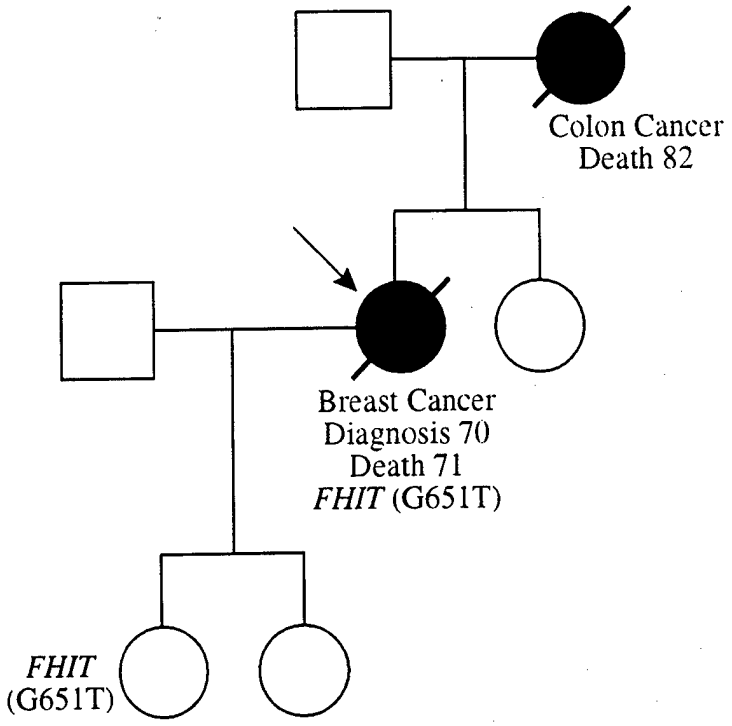
A. HCC38



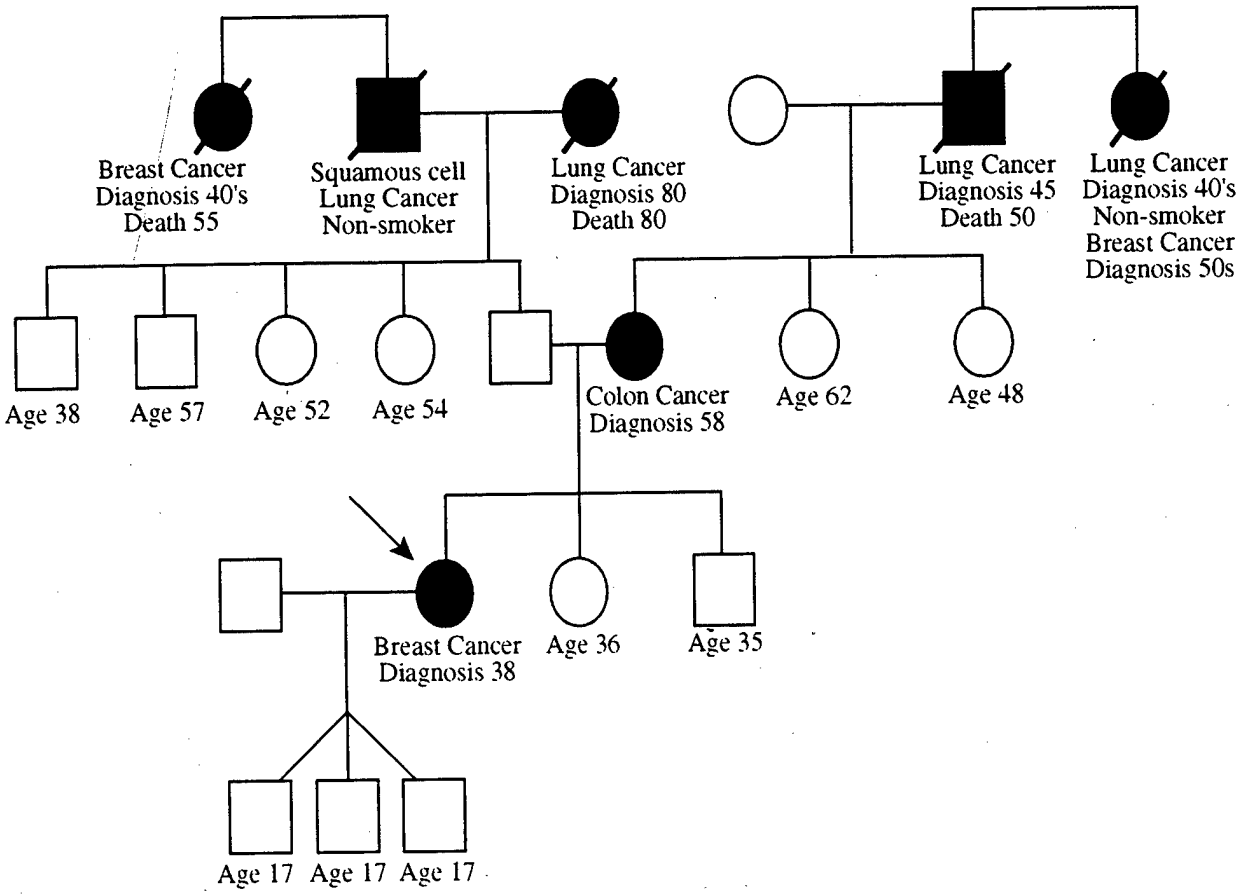
B. HCC1500

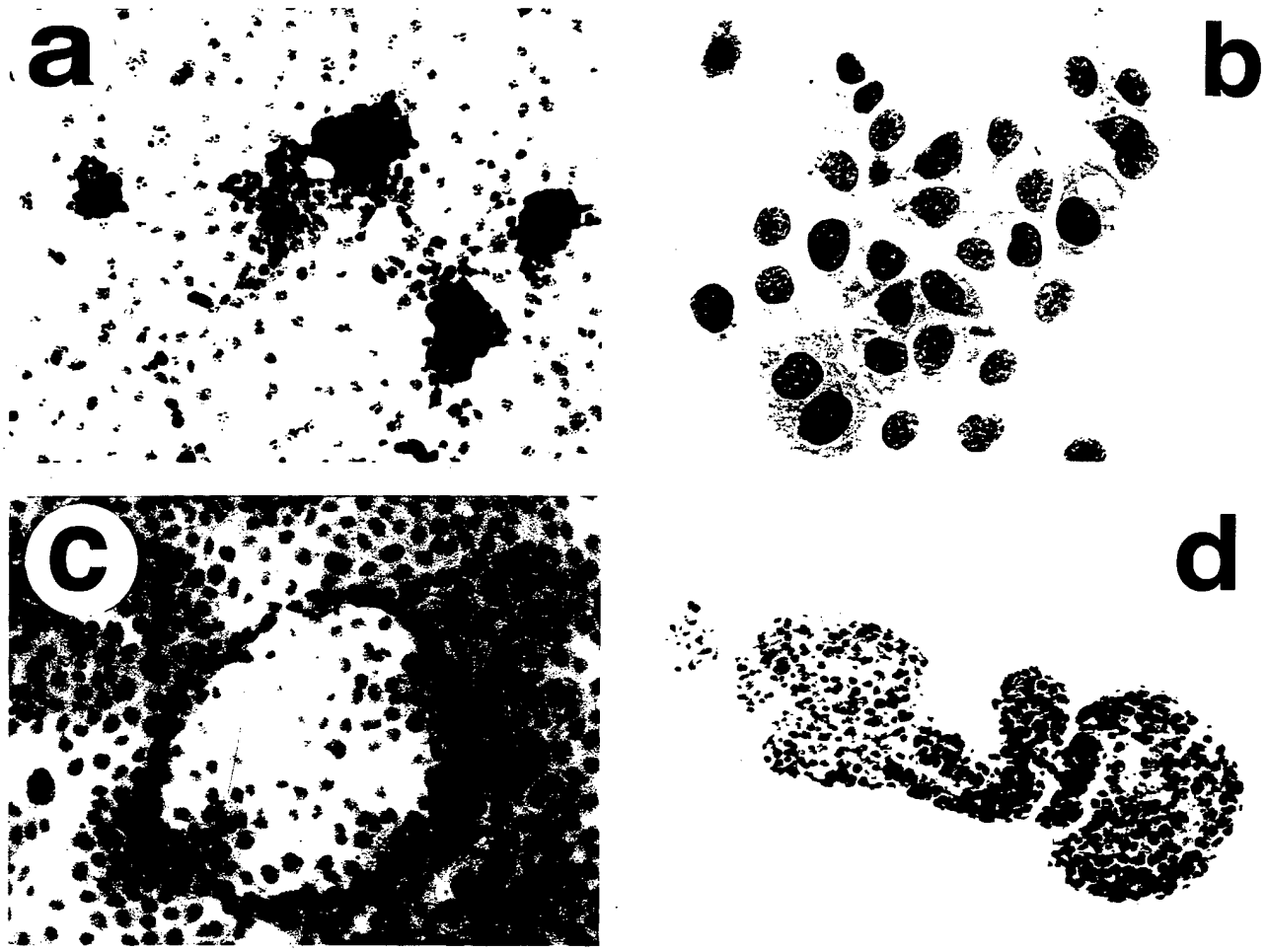


C. HCC1569



D. HCC2218




a
b
c
d