AD_____

Award Number DAMD17-94-J-4077

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

PRINCIPAL INVESTIGATOR: Jerry W. Shay, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75235-9016

REPORT DATE: July 1999

TYPE OF REPORT: Final

PREPARED FOR: 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 1999	Final (1 Jul 94 - 30 Jun 99)

4. TITLE AND SUBTITLE
A Comprehensive Repository of Normal and Tumor Human Breast Tissues 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)
The University of Texas Southwestern Medical Center at Dallas
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 individual breast specimens. In addition, we have cryopreserved and cultured cells from human breast tumor tissues and have established 21 new human breast tumor cell lines (including one with a BRCA-1 mutation). The repository also includes breast epithelial and stromal cell strains derived from non cancerous breast tissue as well as peripheral blood mononuclear cells. The new breast tumor cell lines have been deposited at the ATCC repository for unrestricted distribution. The rest of the respository is widely available for distribution at our world wide web homepage: http://www.swmed.edu/home_pages/bcrep/ with links to other breast tissue banks.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	47
	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

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18 298-102

USAPPC V1.00

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

JWS ✓ Where copyrighted material is quoted, permission has been obtained to use such material.

JWS ✓ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

JWS ✓ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

JWS NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

JWS ✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JWS ✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JWS ✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

JWS ✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Jeremy W. Sherman 9 July 1999

PI Signature Date

Table of Contents

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 - June 30, 1999)
(Final report period: July 1, 1998 - June 30, 1999)

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 was to establish a repository of materials for the biologic and genetic study of breast cancer and to provide these to other scientists. The repository contains 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 have been entered onto a database.

Statement of the principal objectives of the program were 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 was to obtain 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. In addition, since normal epithelial cells are a limited non renewable resource, we are presently introducing telomerase into the strains with matched tumor cell lines so they will have an extended proliferation capacity. This may permit us to more widely distribute these in the future.

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. 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 8). Two additional manuscripts have been published characterizing some aspects of these new breast cancer resources (reference 11 and 12). The tumor derived breast cell lines have been provided to the American Type Culture Collection for unrestricted 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 have linked 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 is also 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, most but not all samples are 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. Last year many of the reagents were 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**

Last year we completed the requested four years of the grant. We were not successful in obtaining long term support for the expansion of the repository, thus we requested and obtained permission to carry over unexpended funds for an additional year. In addition to maintaining and distributing from the repository during this extension year, we did make some additional progress in characterizing the reagents we had

obtained. We were able to get some help from the Susan B. Komen Foundation to sustain the repository for the present time and for the forseeable future.

Key Research Accomplishments

- accessioned 250 individual breast tumor and 55 noncancerous specimens
- cryopreserved 23 human breast epithelial and 25 stromal cell strains
- established, characterized and cryopreserved 21 human breast tumor cell lines and provided these to the ATCC for unrestricted distribution
- established 16 EBV-transformed peripheral blood mononuclear lines as a source of constitutional DNA and provided these to the ATCC for unrestricted distribution
- established and continue to maintain a web site announcing the availability of our tissue/cell repository http://www.swmed.edu/bcrep with links to other data bases

Reportable Outcomes

Manuscripts acknowledging support from the parent grant DAMD17-94-J-4077:

1. Kim, N-W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay J. W., Specific association of human telomerase activity with immortal cells and cancer, *Science*, 266:2011-2015, 1994.
2. Shay, J. W., G. Tomlinson, M. A. Piatyszek, and L. S. Gollahon. Spontaneous in vitro immortalization of breast epithelial cells from a Li-Fraumeni patient. *Mol. Cell. Biol.*, 15:425-432, 1995.
3. Piatyszek, M. A., Kim N. W., Weinrich S. L., Hiyama, K., Hiyama, E., Wright W. E., Shay, J. W. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci.*, 17:1-15, 1995.
4. Hiyama, E., Gollahon, L., Kataoka, T., Kutoi, K., Yokoyama, T., Gazdar, A.F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors. *J Natl. Cancer Inst.*, 88:116-122, 1996.
5. Holt, S. E., Gollahon, L. S., Willingham, T., Barbosa, M. S., and Shay, J. W. p53 levels in human mammary epithelial cells expressing wild-type and mutant human papillomavirus type 16 (HPV-16) E6 proteins: relationship to reactivation of telomerase and immortalization. *Int. J. Oncol.*, 8:262-270, 1996.
6. Gollahon, L. and Shay, J. W. Immortalization of human mammary epithelial cells transfected with mutant p53 (273his). *Oncogene*, 12:715-726, 1996.
7. Hiyama, E., Gollahon, L., Kataoka, T., Kutoi, K., Yokoyama, T., Gazdar, A.F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors (correspondence). *J Natl. Cancer Inst.*, 88:839-840 1996.
8. Ahmadian, M., Wistuba, I. I., Fong, K. M., Behrens, C., Kodagoda, D. R., Saboorian, M. H., Shay, J. W., Tomlinson, G. E., Blum, J., Minna, J. D., Gazdar, A. F. Analysis of the *FHIT* gene and *FRA3B* region in sporadic breast cancer, preneoplastic lesions and familial breast cancer probands *Can Res.* 57:3664-3669, 1997. (see appendix)
9. Pearson, A. S., Gollahon, L. S., O'Neal, N. C., Saboorian, H., Shay, J. W., and Fahey, T. J. Detection of telomerase activity in breast masses by fine needle aspiration

Annals of Surgical Oncology, 5:186-193, 1998.
10. Yashima, K., Milchgrub, S., Gollahon, L., Maitra, A., Saboorian, H., Shay, J. W., Gazdar, A. F. Telomerase enzyme activity and RNA expression during the multistage pathogenesis of breast carcinoma. *Clin. Can. Res* 4:229-234, 1998. (see appendix)
11. Tomlinson, G. E., Chen, T, T-L., Stastny, V. A., Virmani, A. K., Spillman, M. A., Tonk, V., Blum, J. L., Schneider, N. R., Wistuba, I. I., Shay, J. W., Minna, J. D., Gazdar, A. F. Characterization of a breast cancer cell line derived from a germline BRCA1 mutation carrier *Cancer Res.* 58: 3237-3242. 1998. (see appendix)
12. Gazdar, A. F., Kurvari, V., Virmani, A. K., Gollahon, L. S., Sakaguchi, M, Westerfield, M., Kodagoda, D., Stasny, V., Cunningham, T., Wistuba, I., Tomlinson, G., Tonk, V., Ashfaq, R., Minna, J. D., and Shay, J. W. Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. *Intern. J. Can.*, 78:766-774, 1998 (see appendix)
13. Wistuba, I.I., Behrens, C, Milchgrub, S., Syed, S., Ahamadian, M., Virmani, A.K, Kurvari, V., Cunningham, T.H., Ashfaq, R., Minna, J.D., and Gazdar, A.F. Comparison of features of human breast cancer cell lines and their corresponding tumors. *Clin. Cancer Res.* 4:2931-2938, 1998. (see appendix)

Conclusions

All subtasks have been completed. 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). 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.

References

1. Kim, N-W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay J. W., Specific association of human telomerase activity with immortal cells and cancer, *Science*, 266:2011-2015, 1994.
2. Shay, J. W., G. Tomlinson, M. A. Piatyszek, and L. S. Gollahon. Spontaneous in vitro immortalization of breast epithelial cells from a Li-Fraumeni patient. *Mol. Cell. Biol.*, 15:425-432, 1995.
3. Piatyszek, M. A., Kim N. W., Weinrich S. L., Hiyama, K., Hiyama, E., Wright W. E., Shay, J. W. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci.*, 17:1-15, 1995.
4. Hiyama, E., Gollahon, L., Kataoka, T., Kutoi, K., Yokoyama, T., Gazdar, A.F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors. *J Natl. Cancer Inst.*, 88:116-122, 1996.
5. Holt, S. E., Gollahon, L. S., Willingham, T., Barbosa, M. S., and Shay, J. W. p53 levels in human mammary epithelial cells expressing wild-type and mutant human papillomavirus type 16 (HPV-16) E6 proteins: relationship to reactivation of telomerase and immortalization. *Int. J. Oncol.*, 8:262-270, 1996.
6. Gollahon, L. and Shay, J. W. Immortalization of human mammary epithelial cells transfected with mutant p53 (273his). *Oncogene*, 12:715-726, 1996.
7. Hiyama, E., Gollahon, L., Kataoka, T., Kutoi, K., Yokoyama, T., Gazdar, A.F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors (correspondence). *J Natl. Cancer Inst.*, 88:839-840 1996.
8. Ahmadian, M., Wistuba, I. I., Fong, K. M., Behrens, C., Kodagoda, D. R., Saboorian, M. H., Shay, J. W., Tomlinson, G. E., Blum, J., Minna, J. D., Gazdar, A. F. Analysis of the *FHIT* gene and *FRA3B* region in sporadic breast cancer, preneoplastic lesions and familial breast cancer probands *Can Res.* 57:3664-3669, 1997.
9. Pearson, A. S., Gollahon, L. S., O'Neal, N. C., Saboorian, H., Shay, J. W., and Fahey, T. J. Detection of telomerase activity in breast masses by fine needle aspiration *Annal of Surgery*, 5:186-193, 1998.
10. Yashima, K., Milchgrub, S., Gollahon, L., Maitra, A., Saboorian, H., Shay, J. W., Gazdar, A. F. Telomerase expression during multistage pathogenesis of breast carcinoma. *Clin. Can. Res* 4:229-234, 1998.
11. Tomlinson, G. E., Chen, T, T-L., Stastny, V. A., Virmani, A. K., Spillman, M. A., Tonk, V., Blum, J. L., Schneider, N. R., Wistuba, I. I., Shay, J. W., Minna, J. D., Gazdar, A. F. Characterization of a breast cancer cell line derived from a germline BRCA1 mutation carrier *Cancer Res.* 58: 3237-3242. 1998.
12. Gazdar, A. F., Kurvari, V., Virmani, A. K., Gollahon, L. S., Sakaguchi, M, Westerfield, M., Kodagoda, D., Stasny, V., Cunningham, T., Wistuba, I., Tomlinson, G., Tonk, V., Ashfaq, R., Minna, J. D., and Shay, J. W. Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. *Intern. J. Can.*, 78:766-774, 1998.

List of Personnel Receiving Pay from the Research Effort (1994-1999)

1. Principal and co-investigator
Jerry Shay, Ph.D.
Adi Gazdar, M.D.

2. Research Fellows
Lauren Gollahon, Ph.D.
Brittney Herbert, Ph.D.
Venkatesh Kurvari, Ph.D.
James Norton, Ph.D.
Harold Werbin, Ph.D.

3. Research Technicians
Mari Johnson
Martha Liao
Jeffrey Rohde
Richard Squires
Jean-Fancois Train
Max Westerfield

Appendix

Copies of the following 5 key manuscripts are attached:

1. Ahmadian, M., Wistuba, I. I., Fong, K. M., Behrens, C., Kodagoda, D. R., Saboorian, M. H., Shay, J. W., Tomlinson, G. E., Blum, J., Minna, J. D., Gazdar, A. F. Analysis of the *FHIT* gene and *FRA3B* region in sporadic breast cancer, preneoplastic lesions and familial breast cancer probands *Can Res.* 57:3664-3669, 1997.
2. Yashima, K., Milchgrub, S., Gollahon, L., Maitra, A., Saboorian, H., Shay, J. W., Gazdar, A. F. Telomerase enzyme activity and RNA expression during the multistage pathogenesis of breast carcinoma. *Clin. Can. Res* 4:229-234, 1998.
3. Tomlinson, G. E., Chen, T, T-L., Stastny, V. A., Virmani, A. K., Spillman, M. A., Tonk, V., Blum, J. L., Schneider, N. R., Wistuba, I. I., Shay, J. W., Minna, J. D., Gazdar, A. F. Characterization of a breast cancer cell line derived from a germline BRCA1 mutation carrier *Cancer Res.* 58: 3237-3242. 1998.
4. Gazdar, A. F., Kurvari, V., Virmani, A. K., Gollahon, L. S., Sakaguchi, M, Westerfield, M., Kodagoda, D., Stasny, V., Cunningham, T., Wistuba, I., Tomlinson, G., Tonk, V., Ashfaq, R., Minna, J. D., and Shay, J. W. Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. *Intern. J. Can.*, 78:766-774, 1998.
5. Wistuba, I.I., Behrens, C, Milchgrub, S., Syed, S., Ahamadian, M., Virmani, A.K, Kurvari, V., Cunningham, T.H., Ashfaq, R., Minna, J.D., and Gazdar, A.F. Comparison of features of human breast cancer cell lines and their corresponding tumors. *Clin. Cancer Res.* 4:2931-2938, 1998.

[CANCER RESEARCH 57, 3664–3668, September 1, 1997]

Advances in Brief

Analysis of the *FHIT* Gene and FRA3B Region in Sporadic Breast Cancer, Preneoplastic Lesions, and Familial Breast Cancer Probands[1]

Mohsen Ahmadian, Ignacio I. Wistuba, Kwun M. Fong, Carmen Behrens, Dulmini R. Kodagoda, M. Hossein Saboorian, Jerry Shay, Gail E. Tomlinson, Joanne Blum, John D. Minna, and Adi F. Gazdar[2]

Hamon Center for Therapeutic Oncology Research [M. A., I. I. W., K. M. F., C. B., D. R. K., G. E. T., J. D. M., A. F. G.] and Departments of Internal Medicine [J. D. M.], Pediatrics [G. E. T.], Pharmacology [J. D. M.], Cell Biology [J. S.], and Pathology [M. H. S., A. F. G.], University of Texas Southwestern Medical Center, Dallas, Texas 75235-8593; Department of Pathology, Pontificia Universidad Catolica de Chile, Santiago, Chile [I. I. W.]; and Texas Oncology, P. A., Dallas, Texas 75246 [J. B.]

Abstract

The *FHIT* gene, which spans the *FRA3B* fragile site at chromosome 3p14.2, is a candidate tumor suppressor gene in breast and other cancers. We investigated *FHIT* and *FRA3B* for loss of heterozygosity (LOH); homozygous deletions; abnormal transcripts; and acquired/germ-line point mutations in breast cancer cell lines ($n = 32$), breast epithelial and stromal cell cultures ($n = 18$), microdissected invasive ($n = 16$) and ductal *in situ* carcinomas ($n = 6$), and their accompanying normal and abnormal epithelial foci ($n = 14$). LOH at 3p14.2, especially at *FHIT* intragenic marker *D3S1300*, was found in 6 of 16 microdissected invasive tumors and 3 of 6 ductal *in situ* carcinomas. In accompanying preneoplastic foci, LOH occurred in two of eight intraductal hyperplasias but not in histologically normal ductal epithelium ($n = 6$). Three of 32 (9%) breast cancer cell lines demonstrated homozygous deletions of *FHIT* exon 4 (two cases) and exon 5 (one case), which correlated with exon 4-deleted transcripts and loss of the cDNA transcript containing the coding exons 5–9, respectively. Normal mammary cultures and 31 of 32 tumor cell lines (97%) expressed wild-type coding transcripts as well as a minor exon 8-deleted message. Single-strand conformation polymorphism analysis of the coding exons in the 32 tumor and 18 normal breast cell lines and their sequencing revealed four silent polymorphisms and a germ-line histidine triad point mutation (651 G→T) in a tumor arising in a 70-year-old woman. This mutation was also present in one of her two thus far unaffected daughters. Analysis of additional DNAs from 280 probands of high-risk breast cancer families for other *FHIT* exon 8 mutations detected an intronic point mutation 13 bases upstream of exon 8. Thus, we have demonstrated relatively early abnormalities of the *FHIT/FRA3B* region in breast cancer and discovered two rare *FHIT* germ-line mutations. The expression of a transcript containing the coding exons in nearly all cell lines, including those with germ-line mutations, suggests the possibility that another gene in the *FRA3B* region may be involved in the pathogenesis of breast cancer.

Introduction

Multiple genetic abnormalities characterize invasive breast cancers (1–4), including LOH[3] at chromosomal sites that harbor known or putative TSGs. In breast cancer, LOH frequently occurs at several 3p regions and includes 3p14.2 (location of *FHIT* and *FRA3B* fragile site) and 3p21 (5–7). The candidate TSG *FHIT*, which spans *FRA3B*, appears to be the target of hemi- and homozygous deletions in various human cancers and may be associated with aberrant cDNA transcripts (7, 8). Whereas other investigators have identified *FHIT* abnormalities in breast cancer (9, 10), to investigate the involvement of *FHIT/FRA3B* during the development of breast cancer, we analyzed the *FHIT/FRA3B* region in microdissected breast cancers, normal breast epithelium, and breast cancer preneoplasia (intraductal hyperplasia and DCIS), as well as normal and breast tumor cell lines. In addition, to investigate the possible role of *FHIT* in familial breast cancer, we searched for *FHIT* germ-line mutations in DNA from probands of families determined to be at high risk of having genetic predisposition to breast cancer.

Materials and Methods

Archival Samples and Microdissection. Formalin-fixed sections of paraffin-embedded nonmalignant and tumor (prefix T) samples from 19 breast cancer patients undergoing mastectomy or local excision from the Parkland Memorial Hospital (Dallas, TX) surgical pathology archives were selected (15 infiltrating ductal carcinomas, 1 infiltrating lobular carcinoma, and 3 DCIS without an invasive component). The LOH studies identified seven tumors with 3p loss (see "Results"), from which six specimens of histologically normal ductal epithelium, eight specimens of intraductal hyperplasia, and three DCIS were also microdissected and studied. Microdissection under direct microscopic visualization and DNA extraction from archival paraffin-embedded sections were performed as described previously from noncoverslipped, H&E-stained slides (11).

Detection of LOH and MAs. Four highly polymorphic 3p12–21 microsatellite markers (listed in Fig. 1) within and flanking *FHIT* were used for LOH studies. Primer sequences can be obtained from the Genome Database. Because of the small amount of material available from the microdissected tissue, we used a nested PCR strategy as described previously (12). LOH was identified in informative samples by complete loss of an allele in most cases, whereas, in a few cases, a faint band was seen (*e.g.*, Fig 1*B*, *case 9*). MAs were evident by a shift and/or gain of one electrophoretic band.

Cell Lines. Sixteen breast cancer-derived cell lines (14 from primary tumors and 2 from metastases; prefix HCC), nine normal breast epithelial (prefix HME) and nine breast stromal lines (prefix HMS) were initiated by our group.[4] In addition, 16 breast cancer-derived cell lines (3 from primary tumors and 13 from metastases; prefix HTB) were obtained from the American Type Culture Collection (Rockville, MD).

Identification of Homozygous Deletions. Intronic DNA adjacent to *FHIT* exons 3–9 was sequenced following amplification of individual intron/exon boundaries with primers described previously (13). These intronic sequences are available in the Genome Database (see ref. 13 for accession numbers). New primers were designed such that the product size would be less than 200 bp and suitable for use in multiplex PCR with formalin-fixed, paraffin-embedded samples. The primer sequences are available from the authors upon request. In brief, multiplex PCRs were performed with seven different primer sets ampli-

Received 4/11/97; accepted 7/17/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by United States Army Research Grant DAMD17–94-J-4077 and by grants from the G. Harold and Leila Y. Mathers Charitable Foundation and the Susan G. Komen Foundation. M. A. was supported by National Service Award 1-T32-CA66817–01A1 from the National Cancer Institute, K. F. was supported by an American Society of Clinical Oncology Young Investigator Award, and G. T. was supported by NIH Grant CA-70472.

[2] To whom requests for reprints should be addressed, at Hamon Center for Therapeutic Oncology, University of Texas Southwestern Medical Center, Building NB, Room 106, 5323 Harry Boulevard, Dallas, TX 75235-8593. Phone: (214) 648-4900; Fax: (214) 648-4940; E-mail: gazdar@simmons.swmed.edu.

[3] The abbreviations used are: LOH, loss of heterozygosity; wt, wild type; MA, microsatellite alteration; BL, B lymphoblastoid; TSG, tumor suppressor gene; DCIS, ductal carcinoma(s) *in situ*; RT, reverse transcription; SSCP, single-strand conformation polymorphism; nt, nucleotide.

[4] Manuscript in preparation.

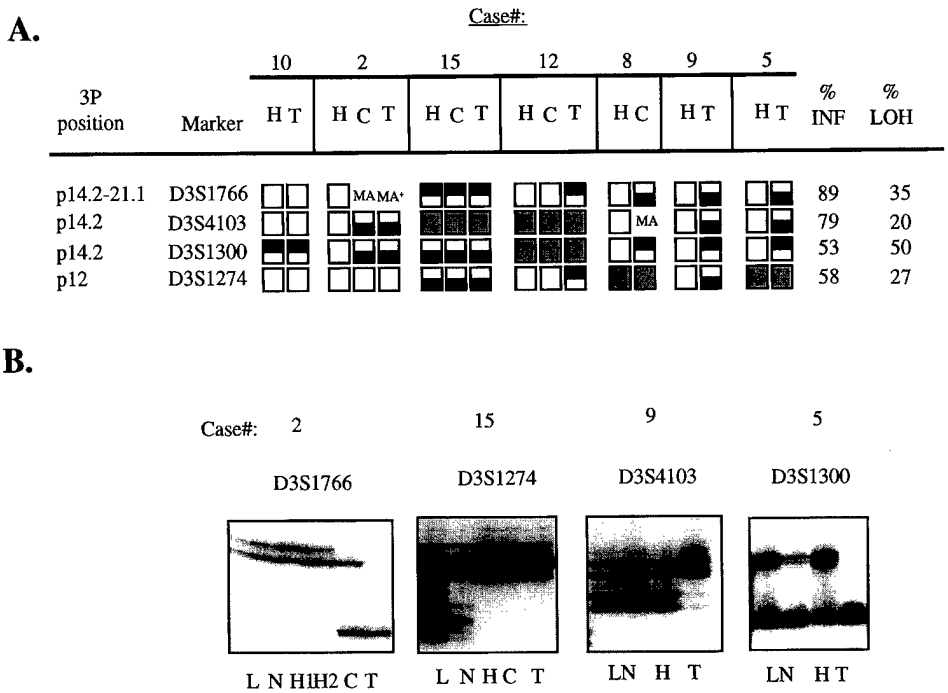

Fig. 1. LOH of the 3p12–3p21 region in breast cancers and accompanying preneoplastic lesions. *A*, schematic representation of the location of chromosome 3p markers (*top to bottom*, telomeric to centromeric) and allelotyping results for microdissected samples with 3p LOH (designated at the top of the figure). The informativeness (*INF*) and frequency of LOH are shown on the right for 16 invasive breast cancer and 6 DCIS lesions. Seven cases demonstrating LOH and their accompanying preneoplastic lesions are shown. ▣ and ▣, LOH of the upper allele or the lower allele, respectively; ▩, uninformative cases; □, retention of heterozygosity. *H*, intraductal hyperplasia; *C*, DCIS; *T*, invasive carcinoma; MA^+, MA and LOH. *B*, representative autoradiographs demonstrating LOH and/or MA in microdissected cases 2, 15, 9, and 5. *L*, lymphocytes; *N*, normal ductal epithelium; *H*, intraductal hyperplasia; *C*, DCIS; *T*, invasive tumor. Two separate intraductal hyperplasias were studied from case 2 (*H1* and *H2*). *Case 2*, D3S1766: MA in DCIS and MA and LOH in invasive tumor. *Case 15*, D3S1274: LOH in intraductal hyperplasia, DCIS, and invasive tumor. *Case 9*, D3S4103: LOH in invasive tumor. *Case 5*, D3S1300: LOH in invasive tumor.

fying exons 3, 4, 5, and 8 (four sets) or exons 6, 7, and 9 (three sets) in 1× Perkin-Elmer buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 0.5 μM of each primer, 0.05 μl of 3000 mCi/mmol of [^{32}P]dCTP (Amersham Corp.), and 3.5 units of AmpliTaq Gold (Perkin-Elmer Corp.) with either 100 ng of genomic DNA from cell lines or about 100 microdissected cells using touch down PCR. PCR products were separated on a 5% nondenaturing polyacrylamide gel and visualized by autoradiography.

RT-PCR, SSCP, and Sequencing Analysis. Total RNA from cell lines was isolated by RNAStat (Tel-test "B," Inc., Friendswood, TX) according to the manufacturer's protocol. RT-PCR and RT-PCR/SSCP analysis using overlapping inner sets of primers have been described in detail (14). Genomic SSCP analysis of exon 8 in breast cancer cell lines and paraffin-embedded tissue, using forward primer SSX (5′-GAGAGCATCACTGTCAAG-3′) and reverse primer 698R (5′-GCTGTCATTCCTGTGAAAGTCTCC-3′), was performed with touch down PCR as used for allelotyping. Sequencing was performed with an Applied Biosystems, Inc. 373 sequencer or Thermosequenase Kit from Amersham according to the manufacturer's protocol. Sequencing products were separated on 6% denaturing polyacrylamide gel (National Diagnostics, Atlanta, GA) and visualized by autoradiography.

PCR-RFLP Analysis. Primer *Afl*III (5′-GTACATTTTCAGCACGTA-CAC-3′) with a T-to-A (underlined) change in *FHIT* nt 645 (GenBank accession no. U46922) introduced an artificial *Afl*III site in the wt sequence when combined with primer 710R (5′-CTCCTCATAGATGCTGTCATTCC-3′) to amplify exon 8. PCR products were then digested with *Afl*III and were separated on 3% Metaphor gels (FMC Bioproducts, Rockland, ME) and visualized with ethidium bromide staining.

Breast Family Repository and Patient Information. Tumor cell line HCC1569 was derived from the invasive breast cancer of a 70-year-old African-American woman. Although her mother died of colon cancer at age 82, her family history was not suggestive of familial breast cancer. Normal DNA from peripheral blood lymphocytes of 280 breast cancer probands were obtained from the University of Texas Southwestern Familial Breast Cancer Registry. These individuals were believed to be at high risk of carrying a genetic predisposition to breast cancer either because of a positive family history or very early age of cancer onset. Informed consent was obtained from each individual, which permitted the study of possible cancer predisposition genes. We are currently analyzing the status of known predisposition genes such as *BRCA1, BRCA2,* and *p53* in the Southwestern Familial Breast Cancer Registry. Thus, cases with possible germ-line mutations in known predisposition genes were not excluded.

Results and Discussion

Allele loss at several 3p regions occurs frequently in breast cancer cell lines and tumors and may appear early in preneoplastic breast lesions (5, 15). A recently described candidate TSG, *FHIT,* localizes to 3p14.2, which also contains the *FRA3B* fragile site (7). *FHIT* allele loss has been detected in 25% of primary breast tumors and was associated with transcript abnormalities in ~30% of the cases studied (9). To further delineate the role of the *FHIT* gene in the development of breast cancer, we studied acquired and germ-line abnormalities associated with breast cancers and their accompanying preneoplastic lesions.

3p14 Allele Loss Is a Frequent Event In Breast Carcinomas. Nineteen microdissected breast carcinomas were analyzed for LOH using two 3p14.2 microsatellite markers within *FHIT* and two markers in flanking regions. Overall, LOH at one or more of these markers was found in 6 of 16 (37%) primary breast cancers and 3 of 6 (50%) cases of DCIS (Fig. 1*A*). Two of three DCIS cases with LOH were associated with an invasive component. The highest frequency of LOH (50%) occurred at *D3S1300* (*FHIT* intron 5). Others have also identified a similar frequency of LOH at the *D3S1300* locus (44%) in DCIS (16).

From six primary invasive cancers and one DCIS without invasion that showed *FHIT* LOH, we microdissected six samples of normal ductal epithelium and eight intraductal hyperplasias and tested them for LOH. None of the samples from normal epithelium but two of eight (25%) intraductal hyperplasias demonstrated 3p14.2 LOH (Fig. 1*A*). In these cases, the same allele was always lost in hyperplastic and DCIS lesions as in the corresponding tumor samples (Fig. 1*A*). MAs affected two of the markers in 2 of 19 (11%) breast carcinomas (Fig. 1*A*). In case 2, the same MA at D3S1766 was also noted in the DCIS accompanying the invasive carcinoma (Fig. 1*B*). The invasive component also demonstrated LOH at D3S1766, whereas the DCIS did not. We conclude from these studies that LOH in *FHIT/FRA3B* occurs often in primary breast carcinomas and occasionally in accompanying hyperplastic lesions. Furthermore, these findings are consistent with the invasive tumor, DCIS, and intraductal hyperplasia in each patient

being clonally related and indicate that 3p14.2 LOH can be an early event in the multistep model of progressive genetic events leading to carcinogenesis.

Screening for *FHIT* Intragenic Homozygous Deletions and Aberrant Transcripts. We devised a multiplex-PCR technique to search for homozygously deleted *FHIT* exons. Primers were designed so that we could amplify individual exons (exons 3–9) in two different multiplex reactions so that each exon was a different size fragment and suitable for analysis of microdissected samples (Fig. 2*A*). Using this technique, we found intragenic homozygous deletions affecting *FHIT* exons in 3 of 32 (9%) breast cancer cell lines but not in 18 normal mammary epithelial/stromal cell lines. HTB130 (MDA-MB-436) was confirmed to be deleted for exon 5 as reported previously by Negrini *et al.* (9). Two new breast cancer cell lines (HCC1428 and HCC1806) were also identified to be deleted homozygously for exon 4 (Fig. 2*A*). DNA from corresponding BL cell lines of HCC1428 and HCC1806 were intact for exons 3–9 and heterozygous for flanking markers, indicating that these exon 4 homozygous deletions were acquired somatically. We were unable to identify intragenic homozygous deletions in 19 microdissected primary breast tumors using this strategy. The absence of such deletions in these samples may have resulted from a small amount of contamination by nontumor cells, which would have resulted in a false positive result. Alternatively, the homozygous deletion could have arisen as a result of culturing of the breast cancer cell lines.

Nonnested RT-PCR of *FHIT* exons 5–9 (nts 378–788) from cDNA synthesized from total RNA demonstrated that, with the exception of a single tumor cell line (HTB130), all epithelial, stromal, and tumor breast cultures expressed a transcript of the wt size (411 bp; Fig. 2*B*). Most breast carcinoma cell lines (84%) as well as all 18 normal breast epithelium and stromal lines also demonstrated a faint 342-bp band, which was identified as an exon 8-deleted transcript by sequencing (Fig. 2*B*). Furthermore, 4 of 32 (12%) breast cancer cell lines also expressed a faint 380-bp transcript that was deleted for exon 7 in addition to the wt transcript. We confirmed the published finding that HTB130 (9), which has a homozygous deletion of exon 5, does not express wt *FHIT*-coding exons (exons 5–9; Fig. 2*B*), which would argue that *FHIT* is the target of the 3p14.2 homozygous deletion. Lack of wt exon 5–9 transcript in this cell line was confirmed by two independent RT-PCR strategies: nested PCR of exons 3–10 (nts 203–904) and nonnested PCR of exons 3–8 (nts 203–698). Moreover, aberrant bands were observed by nonnested exon 3–8 RT-PCR in HTB130 and were reminiscent of those observed by Negrini *et al.* (Ref. 9; data not shown). In contrast, the exon 4 deletions in cell lines HCC1428 and HCC1806 did not affect the expression of the wt *FHIT*-coding exons (exons 5–9). Additional testing of HCC1428 and HCC1806 with the nested RT-PCR strategy amplifying *FHIT* exons 3–10 revealed expression of exon 4-deleted transcripts as expected (Fig. 2*C*). Moreover, nonnested RT-PCR amplification of *FHIT* exons 3–8 (nts 203–698) confirmed the presence of a 402-bp transcript, consistent with exon 4 deletion, in HCC1428 and HCC1806 (data not shown). Thus, the two cell lines with exon 4 homozygous deletions had intact wt transcripts for the coding exons 5–9, indicating that exon 4 deletions may not necessarily abrogate *FHIT* function unless the expression of the *FHIT* protein is affected by the exon 4-deleted transcripts, a possibility that has been suggested by others (13). Of interest, the region between *FHIT* exons 4 and 5 contains a viral integration site (17, 18).

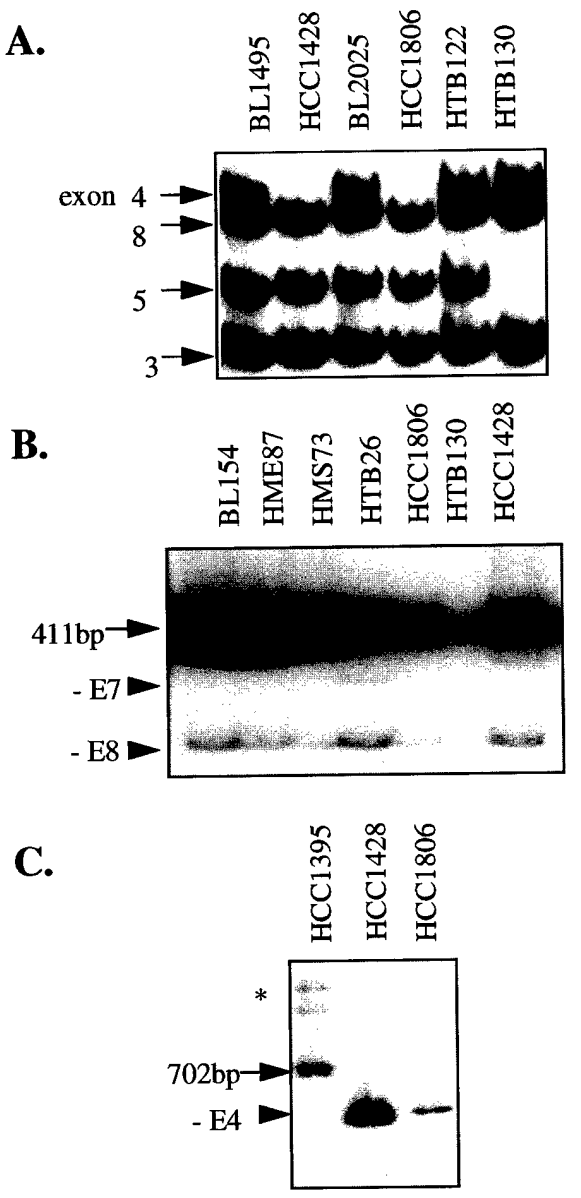

Fig. 2. Molecular analyses of *FHIT* exonic homozygous deletions and aberrant cDNA transcripts. *A*, representative autoradiographs demonstrating multiplex PCR for detection of homozygous deletions in exons 3, 4, 5, and 8. *Arrows* denote the position of each amplified exon. HCC1428 and HCC1806 exhibited homozygous deletions of exon 4, which were retained in the corresponding BL lines (BL1495 and BL2025, respectively). HTB130 contained a homozygous deletion of exon 5, as reported previously (9). *B*, Nonnested RT-PCR of *FHIT* nts 378–788 (exons 5–9) fractionated by nondenaturing PAGE for a panel of cell lines showing the wt 411-bp product (*arrow*) as well as the less abundant 342-bp alternative transcript lacking exon 8 (*−E8, arrowhead*) in the majority of normal or breast cancer cell lines tested. The position of exon 7-deleted transcripts (*−E7, arrowhead*) is shown in HTB26. The HTB130 cell line, containing a homozygous deletion of exon 5, predictably lacked a wt transcript. Two cell lines having homozygous deletions of exon 4 (HCC1428 and HCC1806) and a BL line from a breast cancer family proband having a germ-line mutation in intron 7 (BL154; see text) express the usual pattern of wt and exon 8-deleted transcripts. *C*, nested RT-PCR (first-step amplification of *FHIT* nts 203-1038 followed by second-step amplification of nts 203–904) of breast cancer cell lines fractionated by nondenaturing PAGE. *Arrow*, position of the normal-sized 702-bp transcript, which on lighter exposures composed a doublet due to alternate splicing of nts 812–822; *, the two resultant larger heteroduplex forms; *arrowhead*, the transcripts that were sequenced from the HCC1428 and HCC1806. In these cell lines, exon 4-deleted variant (*−E4*) was detected (data not shown).

In the 29 remaining cancer cell lines without homozygous deletions, nested RT-PCR showed the wt 702-bp transcript and/or aberrant bands: 6 breast cancer lines (20%) only expressed wt-sized bands, 15 (52%) contained wt and aberrant bands, and 4 (14%) expressed aberrant nested transcripts only. Four (14%) cell lines did not amplify any nested transcripts but each had expressed nonnested wt exons 5–9. Sequencing of representative aberrant bands revealed that most of the aberrant bands consisted of deletions of various exons including, exons 4, 3–9, 3–7, 4–6, 5–7, and 8, as described previously (data not

shown; Ref. 14). However, some bands appeared to be artifactual by sequencing, and there was a background of faint bands with this very sensitive nested RT-PCR method. Hence, caution must be used with the extremely sensitive nested RT-PCR methodology, particularly as "aberrant" bands may be found in normal cell lines, *e.g.,* 12 of 18 (67%) normal epithelial and stromal cell lines also demonstrated aberrant transcripts deleted similarly for various exons (data not shown). We concluded from these studies that the great majority of breast cancers and all normal breast cell lines express wt *FHIT* transcripts, and only in rare cases is this disrupted by homozygous deletion of *FHIT* coding exons. Faint aberrant bands can also be identified by sensitive nested RT-PCR, and most of these transcripts, when sequenced, represent precise exonic deletions.

Screening for Point Mutations in Sporadic Breast Cancers and Families at High Risk for Developing Breast Cancer. To detect *FHIT* point mutations, heminested RT-PCR SSCP analysis of the entire *FHIT* open reading frame was performed on cDNAs from 32 breast carcinomas and 18 normal breast cell lines (data not shown). Four silent polymorphisms were found: 524 A/G (exon 6), 545 G/A (exon 6), 626 C/T (exon 7), and 656 T/C (exon 8). These polymorphisms have been reported by us and others previously from different samples (8, 13, 14, 19). A 651 G→T point mutation, changing valine to phenylalanine at Fhit position 97, was identified in tumor line HCC1569 and its corresponding normal stromal cell line, HMS79. These cell lines each expressed mutant as well as wt *FHIT* transcripts. We confirmed that these point mutations occurred at the genomic level by analyzing genomic DNA from the corresponding primary breast tumor, with DCIS and lymphocytes microdissected from archival paraffin-embedded sections (Fig. 3). Samples were subjected to both direct sequencing and a customized PCR-RFLP assay, whereby the 651 G→T mutation abolished a PCR-generated *Afl*III site at this site (Fig. 3*B*). Both methods demonstrated the same point mutation in the genomic DNA from these samples and indicated that the patient contained a copy of wt *FHIT* along with the mutated allele (Fig. 3*B*). Furthermore, analysis of the peripheral blood lymphocyte DNA from two unaffected daughters of the patient showed that one daughter had inherited this mutation (Fig. 3, *A* and *C*). The functional effect of this mutation on Fhit function is unclear, although it has been shown that Fhit 5′,5′′′-P_1P_3-triphosphate hydrolase activity is dependent on residues in the histidine triad, suggesting a connection between dinucleotide metabolism and tumorigenesis (20). Of interest, a similar phenylalanine residue is found in the histidine triad region of Yhit, a yeast homologue of human Fhit (7). This finding and the presence of wt transcripts in the affected cell lines suggest that this substitution may not affect gene function.

To investigate whether this 651 G→T mutation is associated with familial breast cancers, we used the *Afl*III site PCR-RFLP assay to analyze 280 peripheral blood lymphocyte DNAs from familial breast cancer probands from our familial breast cancer repository but did not identify any similar mutations. Because the valine encoded by the wt codon at this site is within the Fhit histidine triad that forms a putative critical functional domain (20), we examined the remainder of exon 8 of these familial breast cancer samples by genomic SSCP to test the possibility that other histidine triad point mutations may be present. Only one other DNA sequence alteration was observed (breast cancer proband family 154, Fig. 4) in intron 7, 13 bp upstream of the exon 8 boundary. RT-PCR analysis of the cDNA from a BL cell line derived from this proband, however, only showed the wt and exon 8-deleted variant and no other aberrant transcripts (Fig. 2*B*). For family 154 with the intronic mutation, the unavailability of original tumor blocks prevented us from testing whether this mutation segregated with tumors.

These data led us to conclude that *FHIT* histidine triad mutations are rare in sporadic and familial breast cancer. The functional signif-

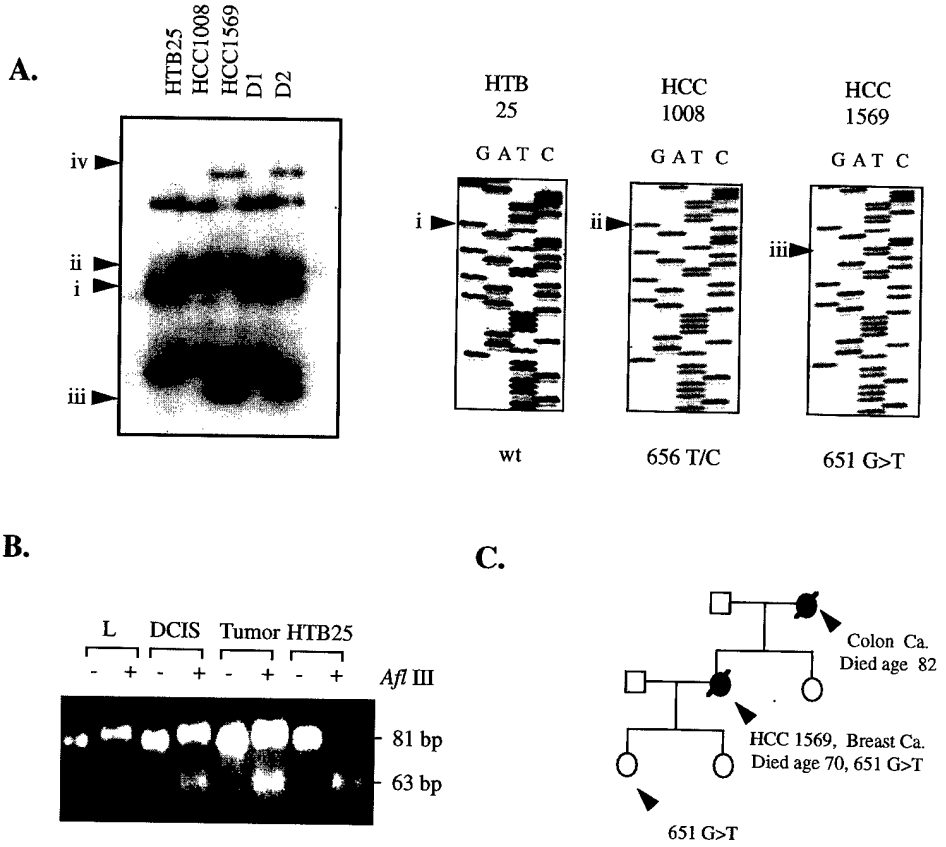

Fig. 3. Germ-line mutation within the *FHIT* histidine triad motif. *A,* genomic SSCP analysis of *FHIT* exon 8 demonstrating the wt pattern in HTB25, 656 T/C polymorphism in HCC1008 (*arrowhead ii*), and mutant band (*arrowheads iii* and *iv*) plus 656 T/C polymorphism in HCC1569. One of the two daughters (*D1*) of patient HCC1569 has one wt allele and one allele with the 656 T/C polymorphism. The other daughter (*D2*) contains one wt allele and one allele with the 651 G→T mutation. The wt allele was paternal in both daughters. Daughters D1 and D2 inherited the polymorphic allele or mutant allele, respectively, from their mother. The polymorphic band (*arrowhead ii*) actually contains the polymorphic sequence 656 T/C and the mutant 651 G→T sequence (*HCC1569*). *Arrowheads, right three panels,* sequence of selected bands. Note the order of the lanes (from left to right) on each sequencing panel: G, A, T, and C. *B,* PCR-RFLP, as described in "Materials and Methods," confirms the presence of germ-line *FHIT* mutation at nt 651 in archival microdissected lymphocytes (*L*), DCIS, and invasive tumor (*Tumor, HTB25*). *C,* pedigree of the patient from whom HCC1569 was derived.

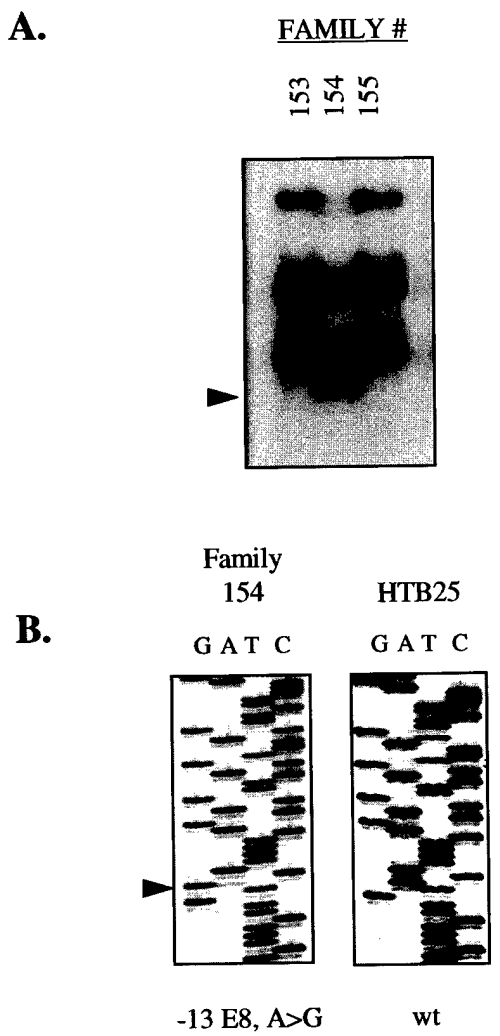

Fig. 4. Germ-line mutation of *FHIT* intron 7. *A,* genomic SSCP analysis of *FHIT* exon 8 demonstrating the mutant pattern in the peripheral blood lymphocyte DNA from the proband of family 154. *Arrowheads,* the sequence of the abnormal SSCP band in the proband DNA. *B,* the sequence (*A, arrowheads*) was due to an intronic A→G substitution, on the sense strand, 13 bp upstream of exon 8 boundary. Note the order of the lanes on the sequencing gel from left to right: G, A, T, and C.

icance of such mutations remains to be determined. It should be noted, however, that others have described *FHIT* exonic deletions and 3p14 rearrangements in the benign breast lesions from two women belonging to breast cancer families, one of which also had an aberrant *FHIT* transcript in a karyotypically normal fibroadenosis specimen (10). However, deletions were also observed in another 3p14.2 gene, *PTPRG,* which is adjacent to *FHIT,* and thus *FRA3B* abnormalities may not specifically target *FHIT.* In this context, it has been shown that some homozygous deletions (*e.g.,* in CC19 and KatoIII cell lines) do not involve *FHIT* coding exon deletions (7, 21). Moreover, these types of deletions can also be found in nontumor DNA, albeit from potentially unstable hybrid clones or yeast artificial chromosomes (21).

In summary, LOH at 3p14.2 is a frequent and early event in breast cancer, which occurs at *FHIT* and the *FRA3B* common fragile site. The presence of homozygous deletions of *FHIT* exons in breast cancer cell lines and the LOH in invasive and preneoplastic lesions implicate abnormalities in the *FHIT* region as important early events in breast pathogenesis, perhaps leading to destabilization of the remainder of the 3p arm. Arguing against *FHIT* being the target gene is the infrequency of *FHIT* point mutations, a characteristic expected of classical TSGs, and the uncertain role of the low-abundance aberrant *FHIT* transcripts, particularly as wt transcripts are nearly always coexpressed even in pure tumor cell populations. Thus, the issue of whether *FHIT* is the critical TSG at the 3p14.2 *FRA3B* fragile site needs to be resolved by functional analysis and exclusion of other TSGs in this large genomic region.

Acknowledgments

We thank Jerry McCoy and Kristin Shelby for sample collections. We also thank John Calder for his work during summer rotation in the laboratory, and Eric Biesterveld for expert assistance.

References

1. Bieche, I., and Lidereau, R. Genetic alterations in breast cancer. Genes Chromosomes Cancer, *14:* 227–251, 1995.
2. Cox, L. A., Chen, G., and Lee, E. Y. Tumor suppressor genes and their roles in breast cancer. Breast Cancer Res. Treat., *32:* 19–38, 1994.
3. Devilee, P., and Cornelisse, C. J. Somatic genetic changes in human breast cancer. Biochim. Biophys. Acta, *1198:* 113–130, 1994.
4. Callahan, R., Gallahan, D., Smith, G., Cropp, C., Merlo, G., Diella, F., Liscia, D., and Lidereau, R. Frequent mutations in breast cancer. Ann. NY Acad. Sci., *698:* 21–30, 1993.
5. Chen, L. C., Matsumura, K., Deng, G., Kurisu, W., Ljung, B. M., Lerman, M. I., Waldman, F. M., and Smith, H. S. Deletion of two separate regions on chromosome 3p in breast cancers. Cancer Res., *54:* 3021–3024, 1994.
6. Devilee, P., van den Broek, M., Kuipers-Dijkshoorn, N., Kolluri, R., Khan, P. M., Pearson, P. L., and Cornelisse, C. J. At least four different chromosomal regions are involved in loss of heterozygosity in human breast carcinoma. Genomics, *5:* 554–560, 1989.
7. Ohta, M., Inoue, H., Cotticelli, M. G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., Croce, C. M., and Huebner, K. The *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Cell, *84:* 587–597, 1996.
8. Thiagalingam, S., Lisitsyn, N. A., Hamaguchi, M., Wigler, M. H., Willson, J. K., Markowitz, S. D., Leach, F. S., Kinzler, K. W., and Vogelstein, B. Evaluation of the *FHIT* gene in colorectal cancers. Cancer Res., *56:* 2936–2939, 1996.
9. Negrini, M., Monaco, C., Vorechovsky, I., Ohta, M., Druck, T., Baffa, R., Huebner, K., and Croce, C. M. The *FHIT* gene at 3p14.2 is abnormal in breast carcinomas. Cancer Res., *56:* 3173–3179, 1996.
10. Panagopoulos, I., Pandis, N., Thelin, S., Petersson, C., Mertens, F., Borg, A., Kristoffersson, U., Mitelman, F., and Aman, P. The *FHIT* and *PTPRG* genes are deleted in benign proliferative breast disease associated with familial breast cancer and cytogenetic rearrangements of chromosome band 3p14. Cancer Res., *56:* 4871–4875, 1996.
11. Hung, J., Kishimoto, Y., Sugio, K., Virmani, A., McIntire, D. D., Minna, J. D., and Gazdar, A. F. Allele-specific chromosome 3p deletions occur at an early stage in the pathogenesis of lung carcinoma. J. Am. Med. Assoc., *273:* 558–563, 1995.
12. Wistuba, I. I., Montellano, F. D., Milchgrub, S., Virmani, A. K., Behrens, C., Chen, H., Ahmadian, M., Novak, J., Muller, C., Minna, J. D., and Gazdar, A. F. Deletion of chromosome 3p are frequent and early events in the pathogenesis of uterine cervical carcinoma. Cancer Res., *57:* 3154–3158, 1997.
13. Druck, T., Hadaczek, P., Fu, T. B., Ohta, M., Siprashvili, Z., Baffa, R., Negrini, M., Kastury, K., Veronese, M. L., Rosen, D., Rothstein, J., McCue, P., Cotticelli, M. G., Inoue, H., Croce, C. M., and Huebner, K. Structure and expression of the human *FHIT* gene in normal and tumor cells. Cancer Res., *57:* 504–512, 1997.
14. Fong, K. M., Biesterveld, E. J., Virmani, A., Wistuba, I., Sekido, Y., Bader, S. A., Ahmadian, M., Tiong Ong, S., Rassool, F. V., Zimmerman, P. V., Giaccone, G., Gazdar, A. F., and Minna, J. D. *FHIT* and FRA3B 3p14.2 allele loss are common in lung cancer and preneoplastic bronchial lesions and are associated with cancer-related *FHIT* cDNA splicing aberrations. Cancer Res., *57:* 2256–2267, 1997.
15. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science (Washington DC), *274:* 2057–2059, 1996.
16. Man, S., Ellis, I., Sibbering, R., Blamey, R., and Brook, D. High levels of allele loss at *FHIT* and *ATM* genes in non-comedo ductal carcinoma *in situ* and grade I tubular invasive breast cancers. Cancer Res., *56:* 5484–5489, 1996.
17. Wilke, C., Hall, B., Hoge, A., Pardee, W., Smith, D., and Glover, T. *FRA3B* extends over a broad region and contains a spontaneous HPV16 integration site: direct evidence for the coincidence of viral integration sites and fragile sites. Hum. Mol. Genet., *5:* 187–196, 1996.
18. Zimonjic, D., Druck, T., Ohta, M., Kastury, K., Croce, C., Popescu, N., and Huebner, K. Positions of chromosome 3p14.2 fragile sites (*FRA3B*) within the *FHIT* gene. Cancer Res., *57:* 1166–1170, 1997.
19. Mao, L., Fan, Y. H., Lotan, R., and Hong, W. K. Frequent abnormalities of *FHIT,* a candidate tumor suppressor gene, in head and neck cancer cell lines. Cancer Res., *56:* 5128–5131, 1996.
20. Barnes, L. D., Garrison, P. N., Siprashvili, Z., Guranowski, A., Robinson, A. K., Ingram, S. W., Croce, C. M., Ohta, M., and Huebner, K. Fhit, a putative tumor suppressor in humans, is a dinucleoside 5′,5‴-P1,P3-triphosphate hydrolase. Biochemistry, *35:* 11529–11535, 1996.
21. Boldog, F., Gemmill, R. M., West, J., Robinson, M., Robinson, L., Li, E. F., Roche, J., Todd, S., Waggoner, B., Lundstrom, R., Jacobson, J., Mullokandov, M. R., Klinger, H., and Drabkin, H. A. Chromosome 3p14 homozygous deletions and sequence analysis of FRA3B. Hum. Mol. Genet., *6:* 193–203, 1997.

Telomerase Enzyme Activity and RNA Expression during the Multistage Pathogenesis of Breast Carcinoma[1]

Kazuo Yashima, Sara Milchgrub, Lauren S. Gollahon, Anirban Maitra, M. H. Saboorian, Jerry W. Shay, and Adi F. Gazdar[2]

Hamon Center for Therapeutic Oncology Research [K. Y., A. F. G.] and Departments of Pathology [S. M., A. M., M. H. S., A. F. G.] and Cell Biology and Neuroscience [L. S. G., J. W. S.], University of Texas Southwestern Medical Center, Dallas, Texas 75235-8593

ABSTRACT

Telomerase, an RNA-containing enzyme, is associated with cellular immortality and malignancy. We investigated the role of telomerase during the multistage pathogenesis of breast cancer. We used the semiquantitative, PCR-based telomeric repeat amplification protocol assay for enzyme activity (42 specimens from 42 patients) and a radioactive *in situ* assay for expression of its RNA component (human telomerase RNA; hTR) for the identification of telomerase-positive cells in archival resection samples ($n = 67$ from 39 patients). Low telomerase activity was detected in 1 (14%) of 7 samples of benign breast disease, in 4 (67%) of 6 fibroadenomas, in 11 (92%) of 12 carcinoma *in situ* (CIS) lesions, and in 16 (94%) of 17 invasive breast cancers. There was a progressive increase in the mean telomerase levels with progressive increase in severity of histopathological change ($P < 0.05$). Almost all of 67 resection samples expressed hTR, irrespective of histology. Expression was low to moderate in some samples of normal epithelium and nonproliferative fibrocystic changes. hTR expression was limited to epithelial cells; expression in stromal cells, including those in fibroadenomas, was negative. Increased hTR expression was observed in some foci of apocrine metaplasia and atypical hyperplasia. Increased hTR expression was also observed in all CIS and invasive lesions, although considerable heterogeneity was noted. Focal up-regulation was frequently noted in CIS lesions in the vicinity of invasive tumors. Thus, up-regulation of hTR may be a predictive marker for invasive tumor development.

INTRODUCTION

Carcinogenesis is a multistep process characterized by multiple genetic changes, and epithelial cancers in adults are preceded by a series of morphologically recognizable preneoplastic lesions that develop and progress over a period of several years. For breast cancer, the morphological steps involve epithelial hyperplasia, atypical hyperplasia, CIS,[3] and invasive carcinoma (1). It is believed that the initial transformation event involves epithelial cells in the terminal duct lobular unit. Elucidation of the molecular mechanisms of carcinogenesis is likely to be necessary for an understanding of tumor pathogenesis, for the identification of useful molecular markers for diagnosis, and ultimately for the development of therapeutic strategies.

Recent findings support the concept that activation of telomerase may be a critical, if not obligate, step in the development of cancer (2, 3). Telomerase is a complex enzyme containing both a catalytic protein component and a RNA component. The RNA component contains a small template region that can bind to telomeres from which telomerase synthesizes telomeric DNA repeats (TTAGGG; Refs. 4 and 5). In humans, telomerase is expressed in most cancers and immortal cell lines, including breast cancer, but is inactive in normal somatic cells except for germ cells, activated lymphocytes, and proliferating cells of renewal tissues (2, 3, 6, 7).

More recently, it has been reported that some preneoplastic lesions also express weak telomerase activity, as determined by the PCR-based TRAP assay (3, 8). In breast samples, telomerase activity was present not only in invasive lesions but also in CIS lesions, fibroadenomas, and even normal epithelium (9–12). In addition, heterogeneous expression of telomerase activity was observed in invasive breast cancers (12). However, because the TRAP assay uses whole-tissue extracts, the fraction of tumor cells in a heterogeneous biopsy and the identity of the telomerase-expressing cells is unknown.

A major advance in the telomerase field came with the cloning of the gene coding for the RNA component of human telomerase (*hTR*; Ref. 5). Although Northern analyses of hTR levels may not always parallel telomerase enzyme activity (13), we have observed good concordance of hTR (as detected by ISH) and telomerase enzyme activity (as detected by the TRAP assay) in adult tissues and tumors (8). The *in situ* method has some obvious advantages, compared with using a Northern blot hybridization for hTR or a PCR-based assay for telomerase activity: (*a*) it allows the use of archival materials, as opposed to fresh or fresh frozen tissues; and (*b*) because telomerase may be expressed in some surrounding normal tissues such as activated

Received 6/11/97; revised 10/3/97; accepted 10/6/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by grants from the Geron Corp. (Menlo Park, CA) and the Susan G. Komen Breast Cancer Foundation.

[2] To whom requests for reprints should be addressed, at Hamon Center for Therapeutic Oncology Research, UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8593. Phone: (214) 648-4921; Fax: (214) 648-4924; E-mail: gazdar@simmons.swmed.edu.

[3] The abbreviations used are: CIS, carcinoma *in situ*; TRAP, telomeric repeat amplification protocol; hTR, human telomerase RNA; ISH, *in situ* hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

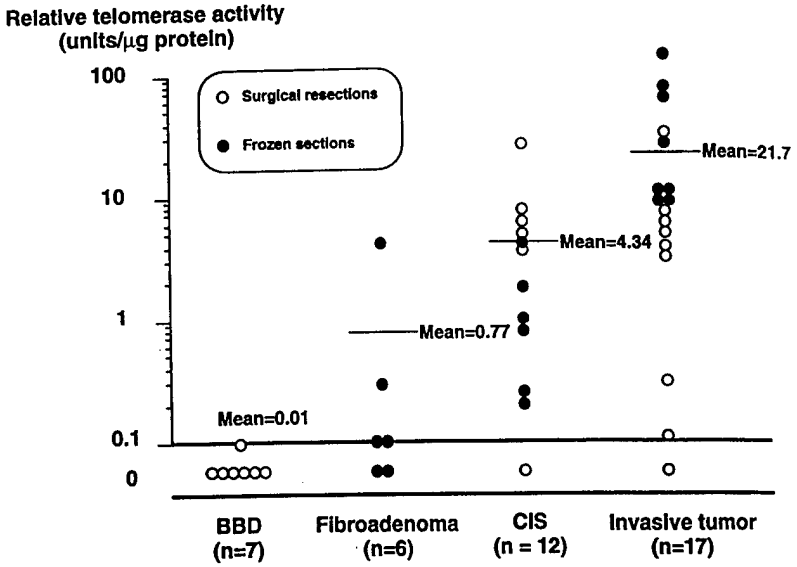

Fig. 1 Relative telomerase activity (units/μg protein) in frozen breast samples.

lymphocytes and proliferating epithelial stem cells, it identifies the cellular origin of telomerase expression.

To further understand the role of telomerase in the normal, abnormal, and malignant breast, we studied both enzyme activity, using the semiquantitative TRAP assay, and hTR expression using a radioactive ISH method.

MATERIALS AND METHODS

Tissue Samples. A total of 42 surgically resected samples (20 frozen sections and 22 fresh frozen samples) for TRAP assay were obtained from patients with invasive breast cancer ($n = 17$), CIS ($n = 12$), fibroadenoma ($n = 6$), or benign breast disease ($n = 7$) from Parkland Memorial Hospital (Dallas, TX). These samples were stored at −80°C until extraction. Five of the six fibroadenoma cases were also used for ISH.

We analyzed hTR expression in breast cancers and their normal epithelium and premalignant lesions and fibroadenomas. The cases were selected because they contained multiple histological features representing the spectrum of pathological changes in the multistage pathogenesis of breast cancer, both ductal and lobular carcinomas. We examined multiple microslides ($n = 67$) from 39 patients (34 with carcinoma and 5 with fibroadenomas). Of the carcinomas, 24 were ductal carcinomas, 2 were lobular carcinomas, and 8 had mixed patterns. CIS lesions were present in 31 of the carcinoma cases, of which 6 were of the comedo type. Patients ranged in age from 20 to 78. All diagnoses of pathological specimens were verified by experienced pathologists (S. M., M. H. S., and A. F. G.). Appropriate Institutional Review Board permission was obtained for the purposes of the study.

Telomerase Assay. Extracts of tissue specimens and assay of telomerase activity were processed as described earlier (2, 14, 15). For quantitation, the signal intensity of the telomerase-specific, six-base repeat ladder was determined by area integration, and this value was normalized to the signal obtained for the internal standard present in analyzed samples. The normalized

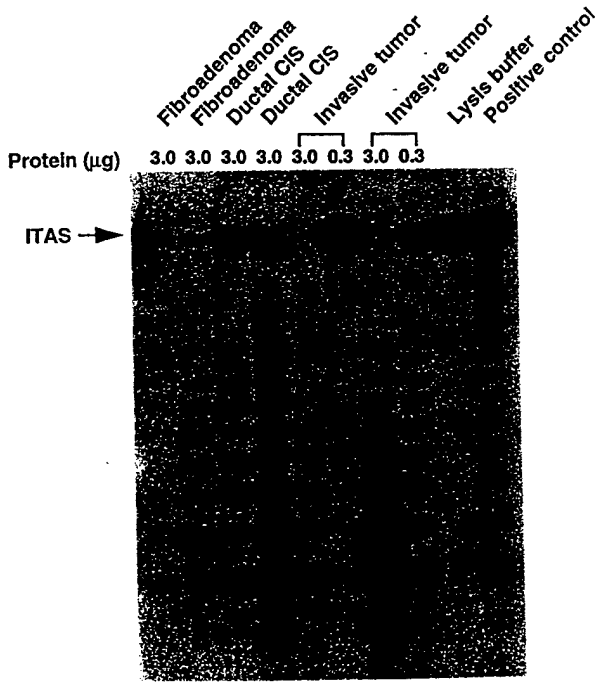

Fig. 2 TRAP assay results in fibroadenoma, ductal CIS, and invasi carcinoma, using the internal telomerase assay standard (*ITAS*).

values of telomerase activity so obtained were expressed arbitrary units. The activity of each sample was normalized that of 1 μg of total cellular protein.

ISH for hTR. ISH for hTR was performed as describ previously (15) but with some modifications in the washi steps. After hybridization, the tissue was subjected to string washing at 50°C in 5× SSC, 10 mM DTT for 30 min, at 65 in 50% formamide, 2× SSC, 10 mM DTT for 20–40 min, a

Fig. 3 Heterogeneity of hTR expression in normal duct (*D*) and in ductal hyperplasia (*H*). Both bright-field (*a*) and dark-field (*b*) images are presented.

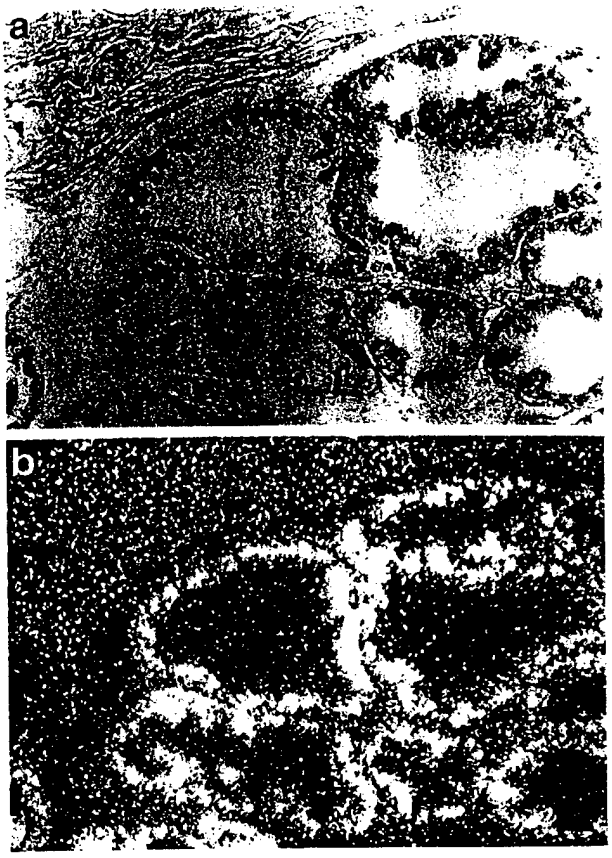

Fig. 4 hTR expression in apocrine metaplasia. Both bright-field (*a*) and dark-field (*b*) images are presented.

washed twice at 37°C in 0.4 M NaCl, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA for 10 min before treatment with 20 μg/ml RNase A at 37°C for 30 min. Following washes in 2× SSC for 10–20 min and 0.1× SSC twice for 10–20 min at 37–50°C, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for 3 weeks in photoresistant boxes with a desiccant ~~at~~ 4°C. To confirm the presence of intact RNA, replicate slides from each sample used for hTR expression were also tested for expression of a housekeeping gene, *GAPDH*. The Zba/*Hin*dIII fragment from the *GAPDH* cDNA in pBR322 obtained from the American Type Culture Collection (Rockville, MD) was subcloned into pBluescript. Intense GAPDH hybridization signals were present in all sections studied.

Following hybridization, the slides were examined by both bright-field and dark-field microscopy, the latter being more sensitive for the detection of weak signals. hTR expression was scored as follows: weak, faint expression, detected by bright-field examination at high magnification, but which was detected at low magnification by dark-field examination; moderate, expression readily recognized by bright-field examination at low magnification; high, bright-field examination expression at a level considerably greater than those usually present in normal epithelium or fibrocystic disease.

RESULTS

Telomerase Enzyme Activity in Breast Samples. Telomerase activity was detected in one of seven samples of benign breast disease (which included mixtures of normal and nonproliferative fibrocystic disease), four (67%) of 6 fibroadenomas, 11 (92%) of 12 CIS, and 16 (94%) of 17 invasive breast cancers. In benign breast disease and fibroadenomas, with one exception, all positive samples had very low levels of telomerase activity, less than 1.0 unit/μg protein (Fig. 1). In CIS and invasive tumors, mean values of relative telomerase activity were 4.34 and 21.7 units/μg protein, respectively. The mean level of the invasive tumors was similar to those present in invasive lung tumors (8). There was a progressive increase in the mean telomerase levels with progressive increase in severity of histopathological change ($P < 0.05$, as determined by the Kruskall-Wallis test for rank sums). Examples of the TRAP assay are illustrated in Fig. 2.

hTR Expression in Surgical Resection Samples. Whereas in 17 (44%) of 39 patients, histologically normal epithelium and nonproliferative fibrocystic disease samples had only background levels of hTR, the samples from 22 (56%) patients had weak to moderate hTR expression (Fig. 3). Moderate to high hTR expression was present in samples of apocrine metaplasia (Fig. 4). In both examples of atypical hyperplasia examined focally, high hTR expression was observed (the only

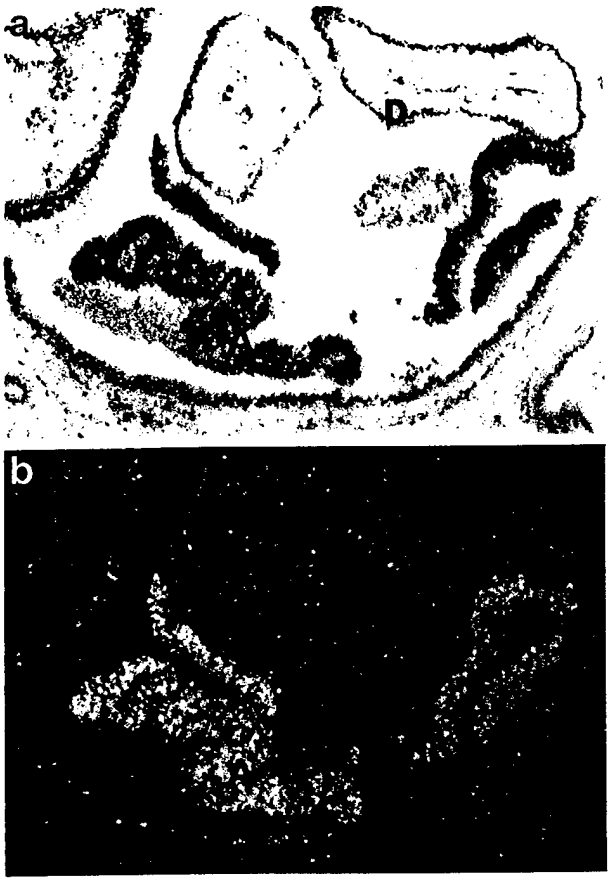

Fig. 5 hTR expression in atypical ductal hyperplasia. Both bright-field (*a*) and dark-field (*b*) images are presented. There is considerable up-regulation of hTR expression in the atypical epithelium. The adjacent ductal epithelium (*D*) is negative for expression.

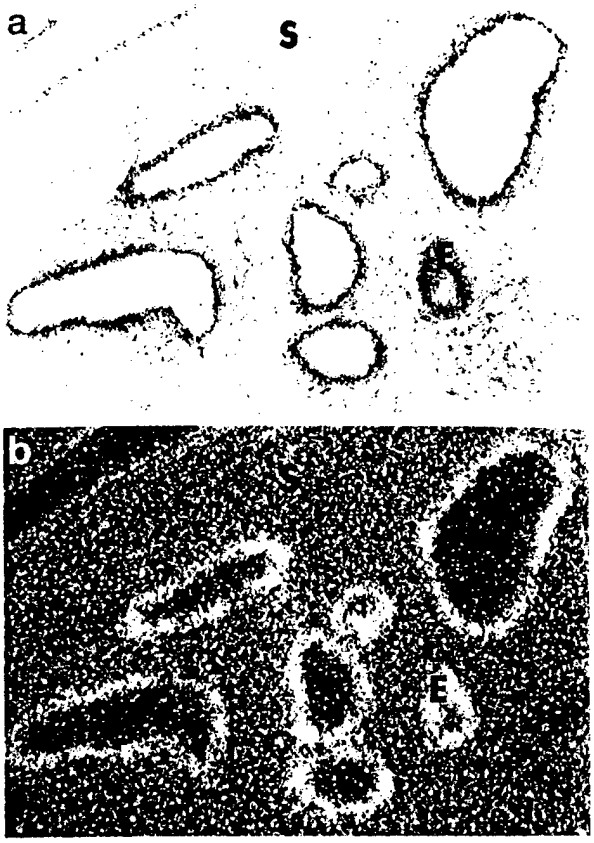

Fig. 6 hTR expression in fibroadenoma of the breast. Weak expression is limited to the epithelial elements (*E*), whereas the stromal elements (*S*) are negative. The weak expression is better visualized on the dark-field image (*b*) than on the bright-field image (*a*).

nonmalignant samples in which high expression was found; Fig. 5). All five fibroadenomas expressed moderate levels of hTR exclusively in the epithelial cells but not in stromal cells (Fig. 6). Expression was limited to epithelial cells and germinal centers of lymphoid follicles (when present). Stromal cells and mature lymphocytes were negative. All CIS and invasive lesions from 34 patients expressed telomerase; expression varied from weak to strong.

Heterogeneity of hTR Expression in Breast Carcinomas. In all examples of benign disease and fibroadenomas and in most examples of CIS and invasive carcinoma, hTR expression in the epithelial cells was relatively uniform. However, considerable heterogeneity of hTR expression was noted in morphologically similar areas of CIS and invasive lesions in about one-third of the cases (13 of 43 samples, 30%; Figs. 7 and 8). The intrasample heterogeneity varied from weak expression in some cells to intense expression in almost all cells. In addition, intense focal up-regulation was noted in 21 of 43 samples of CIS and invasive lesions. When this was noted in CIS samples, it occurred adjacent to areas of invasive carcinoma that showed the same intense expression. The heterogeneity was reproducible when replicate slides were used for expression studies, demonstrating that it did not represent an artifact.

DISCUSSION

In breast disease, telomerase activity has been reported to be present not only in CIS and invasive carcinomas but also in fibroadenomas and occasional samples of histologically normal breast (9–12). To clarify these results and understand the role of telomerase during the multistage pathogenesis of breast carcinoma, the precise identification of the telomerase-positive cells is essential. We examined normal, benign, and malignant breast lesions; we used the semiquantitative TRAP assay to determine telomerase activity in frozen samples and used hTR expression for cellular localization in archival, paraffin-fixed tissues.

The TRAP assay detected weak telomerase activity in one sample of benign breast disease and in four (67%) of six fibroadenomas. Significantly higher levels of activity were present in almost all examples of *in situ* and invasive carcinomas. Whereas the semiquantitative TRAP assay demonstrated considerable variation in enzyme activity, the levels in invasive carcinomas were significantly higher than in CIS cases. The mean level in invasive breast carcinomas (21.7 units/μg protein) was similar to the mean level present in invasive lung carcinomas (34 units/μg protein; Ref. 8). Of interest, the mean levels in squamous *in situ* carcinomas of the bronchus were considerably lower than in invasive lung carcinomas (8). As in the multistage pathogenesis of lung cancers, progressively increasing severity

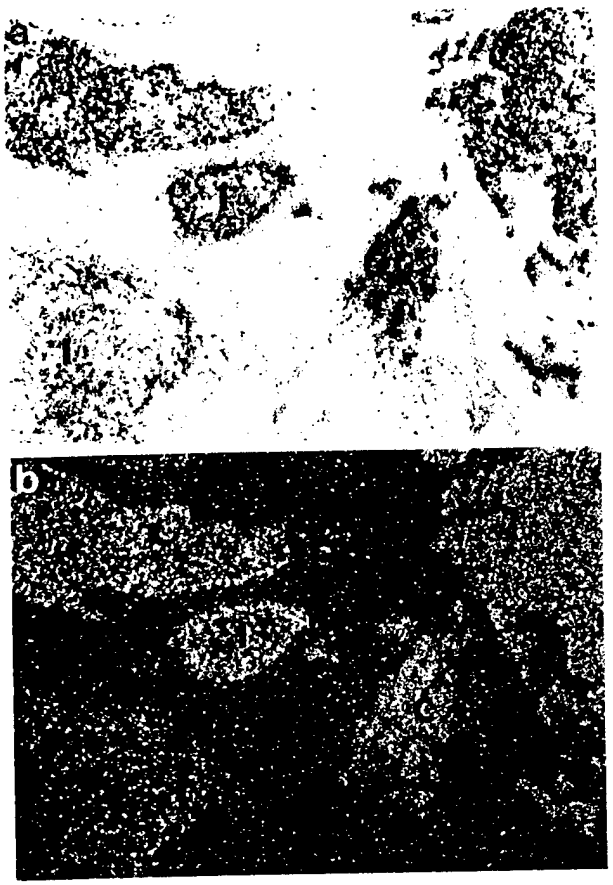

Fig. 7 hTR expression in CIS (*I*) and invasive carcinoma (*C*). Moderate to high expression is present in the invasive carcinoma, whereas there is considerable variation of expression in the CIS foci. Both bright-field (*a*) and dark-field (*b*) images are presented.

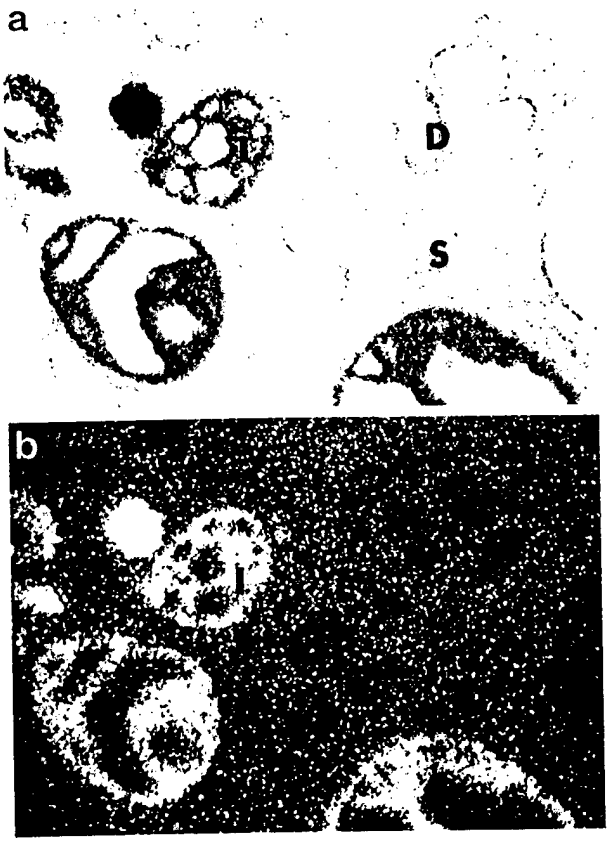

Fig. 8 hTR expression in ductal CIS (*I*). Adjacent ducts (*D*) and stroma (*S*) are negative. Both bright-field (*a*) and dark-field (*b*) images are presented. The CIS foci demonstrate considerable variation of expression, with one focus showing marked up-regulation.

of pathological change is accompanied by increasing frequency and degree of telomerase enzyme activity, and the levels in invasive carcinomas were higher than in CIS lesions.

We used an ISH assay for hTR to precisely identify the telomerase-positive cells. We detected weak to moderate hTR expression in over one-half of the samples of histologically normal and benign breast diseases, including fibroadenomas. Surprisingly, apocrine metaplasia expressed moderate to high levels of hTR, and focal high expression was present in both samples of atypical ductal hyperplasia. Expression was limited to epithelial cells and to germinal centers of lymphoid follicles (which were rarely present). Expression was absent in stromal cells, including the stromal component of fibroadenomas. Because weak telomerase activity is expressed by most proliferating cells of regenerative epithelia (3), the finding of low levels of telomerase activity and low to moderate hTR expression in some samples of histologically normal and benign breast disease samples probably represents the presence of proliferating regenerative cells in these samples. The relatively high expression of hTR in apocrine metaplasia, usually considered to be a form of nonproliferative fibrocystic disease, cannot be readily explained.

Moderate to high hTR expression was present in almost all invasive carcinomas and CIS lesions. In most samples, expression was relatively uniform in the positive epithelial cells. However, heterogeneity was observed in about one-third of the carcinomas. In these cases, heterogeneity of expression was noted in morphologically similar areas of CIS and invasive lesions, which, presumably, could not be explained on the basis of proliferation. In addition, intense focal up-regulation was noted in a subset of CIS in the vicinity of invasive carcinomas and in adjacent foci of corresponding invasive tumors. We have noted similar up-regulation in squamous CIS lesions adjacent to invasive squamous carcinomas of the lung (8). We have postulated that such intense up-regulation in CIS lesions may precede invasion and, thus, may be a predictor of imminent invasion (8). This up-regulation appeared independent of cell proliferation and may represent loss of a telomerase suppressor (16) during the later stages of carcinogenesis. Consistent with this theory, telomerase enzyme activity is significantly higher in invasive lung (8) and breast carcinomas than in their respective CIS lesions.

Thus, telomerase control in breast carcinomas (and possibly in other epithelial tumors) appears to be a two-tiered phenomenon. Low levels of activity and expression are present in many samples of normal and benign breast disease. This activity may reflect "physiological" expression in proliferating regener-

ative cells. In CIS, and possibly in atypical hyperplasia, dysregulated expression of hTR occurs, although there is considerable heterogeneity in some cases. Telomerase dysregulation in preinvasive lesions tissues may represent a method to overcome or abrogate cellular senescence, resulting in a commitment to indefinite cell growth, eventually leading to malignancy. Focal up-regulation was frequently noted in CIS foci in the vicinity of invasive tumors. Whether such up-regulation is a predictive marker for tumor development is presently under investigation.

REFERENCES

1. Allred, D. C., O'Connell, P., and Fuqua, S. A. Biomarkers in early breast neoplasia. J. Cell. Biochem. Suppl., *17G:* 125–131, 1993.

2. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer [see comments]. Science (Washington DC), *266:* 2011–2015, 1994.

3. Shay, J. W., and Gazdar, A. F. Telomerase in the early detection of cancer. J. Clin. Pathol., *50:* 106–109, 1997.

4. Blackburn, E. H. Structure and function of telomeres. Nature (Lond.), *350:* 569–573, 1991.

5. Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., *et al.* The RNA component of human telomerase. Science (Washington DC), *269:* 1236–1241, 1995.

6. Hiyama, E., Tatsumoto, N., Kodama, T., Hiyama, K., Shay, J. W., and Yokoyama, T. Telomerase activity in human intestine. Int. J. Oncol., *9:* 453–458, 1996.

7. Taylor, R. S., Ramirez, R. D., Ogoshi, M., Chaffins, M., Piatyszek, M. A., and Shay, J. W. Telomerase activity in malignant and nonmalignant skin conditions. J. Invest. Dermatol., *106:* 759–765, 1996.

8. Yashima, K., Litzky, L. A., Kaiser, L., Rogers, T., Lam, S., Wistuba, I. I., Milchgrub, S., Srivastava, S., Piatyszek, M. A., Shay, J. W., and Gazdar, A. F. Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. Cancer Res., *57:* 2373–2377, 1997.

9. Hiyama, E., Gollahon, L., Kataoka, T., Kuroi, K., Yokoyama, T., Gazdar, A. F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors. J. Natl. Cancer Inst., *88:* 116–122, 1996.

10. Sugino, T., Yoshida, K., Bolodeoku, J., Tahara, H., Buley, I., Manek, S., Wells, C., Goodison, S., Ide, T., Suzuki, T., Tahara, E., and Tarin, D. Telomerase activity in human breast cancer and benign breast lesions: diagnostic applications in clinical specimens, including fine needle aspirates. Int. J. Cancer, *69:* 301–306, 1996.

11. Bednarek, A. K., Sahin, A., Brenner, A. J., Johnston, D. A., and Aldaz, C. A. Analysis of telomerase activity levels in breast cancer: positive detection at the *in situ* breast carcinoma stage. Clin. Cancer Res., *3:* 11–16, 1997.

12. Tsao, J-L., Zhao, Y., Lukas, J., Yang, X., Shah, A., Press, M., and Shibata, D. Telomerase activity in normal and neoplastic breast. Clin. Cancer Res., *3:* 627–631, 1997.

13. Avilion, A. A., Piatyszek, M. A., Gupta, J., Shay, J. W., Bacchetti, S., and Greider, C. W. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. Cancer Res., *56:* 645–650, 1996.

14. Piatyszek, M. A., Kim, N. W., Weinrich, S. L., Hiyama, K., Hiyama, E., Wright, W. E., and Shay, J. W. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). Methods Cell Sci., *17:* 1–15, 1995.

15. Yashima, K., Piatyszek, M. A., Saboorian, H. M., Virmani, A. K., Brown, D., Shay, J. W., and Gazdar, A. F. Telomerase activity and *in situ* telomerase RNA expression in malignant and non-malignant lymph nodes. J. Clin. Pathol., *50:* 110–117, 1997.

16. Ohmura, H., Tahara, H., Suzuki, M., Ide, T., Shimizu, M., Yoshida, M. A., Tahara, E., Shay, J. W., Barrett, J. C., and Oshimura, M. Restoration of the cellular senescence program and repression of telomerase by human chromosome 3. Jpn. J. Cancer Res., *86:* 899–904, 1995.

[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.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[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.).

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

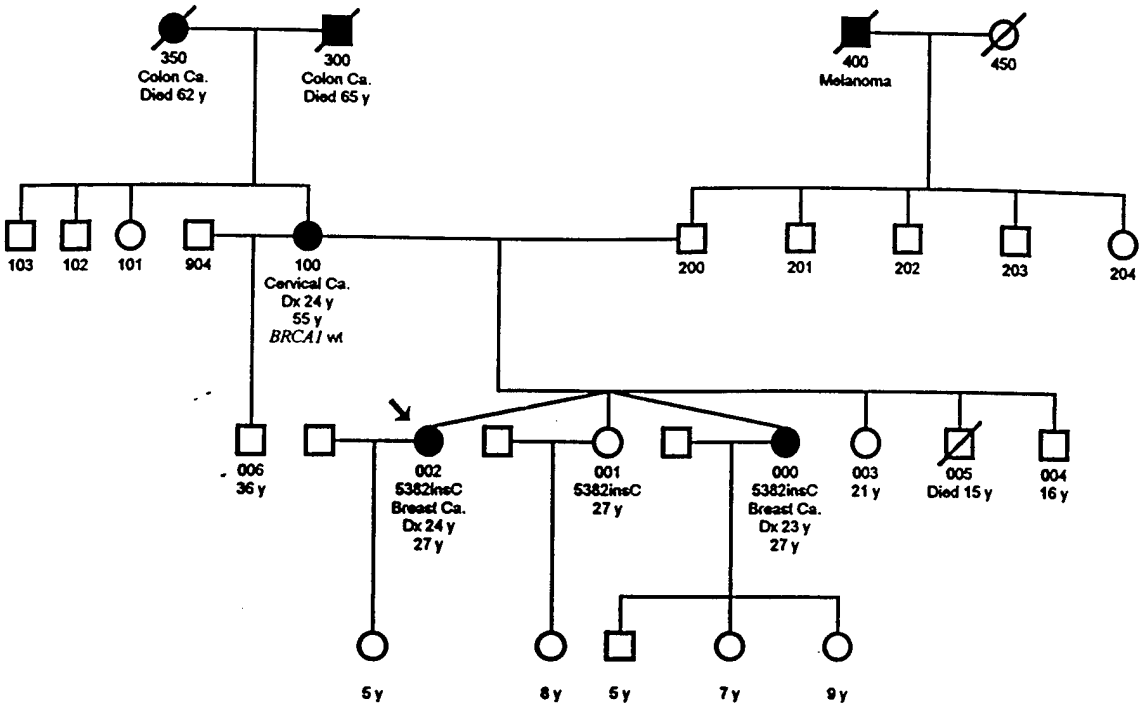

Fig. 1. Pedigree of the family from which the HCC1937 cell line was derived. The patient from which the tumor cell line is derived is indicated by the *arrow*. Germ-line DNA from the patient as well as the affected and one unaffected sister was heterozygous for the *BRCA1* mutation, 5382insC. The patient's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

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

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

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

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

Results

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

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

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

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

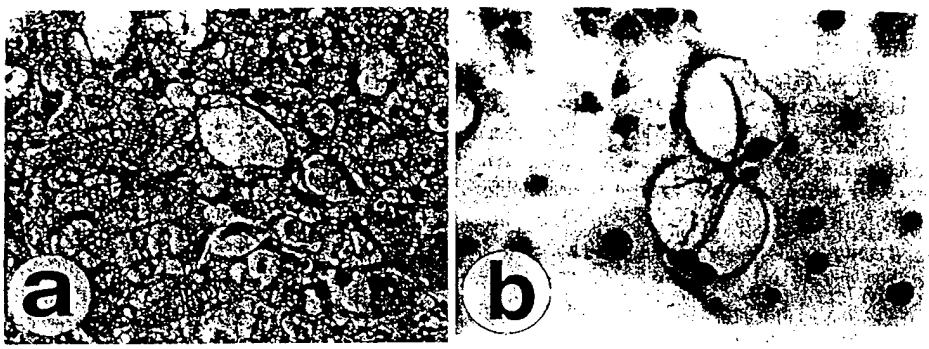

Fig. 2. Morphology of the breast cancer primary tumor and cell line, H&E stain. *a*, the primary breast carcinoma from which HCC1937 was derived. *b*, HCC1937 tumor cell line, cytospin preparation. Giant vacuolated mono- and dinucleated cells are present in both the tumor and cell line. The nonvacuolated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

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

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

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

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

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

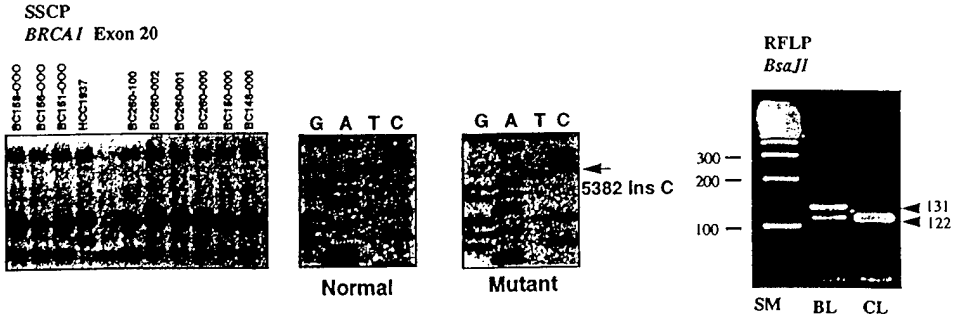

Fig. 3. Molecular analysis of *BRCA1*. Single-strand conformation analysis (*left*) revealed an aberrant band in lymphocyte DNA from the patient (*BC260-002*) and each of her two sisters analyzed (*BC260-001* and *BC260-000*). The tumor cell line demonstrated the mutant band as well as the absence of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

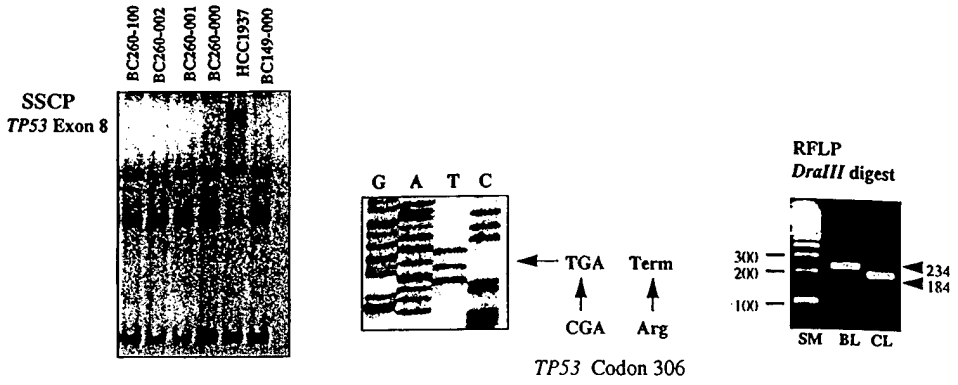

Fig. 4. Molecular analysis of *TP53*. Single-strand conformation analysis of the *TP53* gene revealed an abnormality in exon 8. Sequence analysis demonstrated a point mutation leading to a termination at codon 306. This mutation is also demonstrated by designed restriction fragment length polymorphism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

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

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

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

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

Discussion

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

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

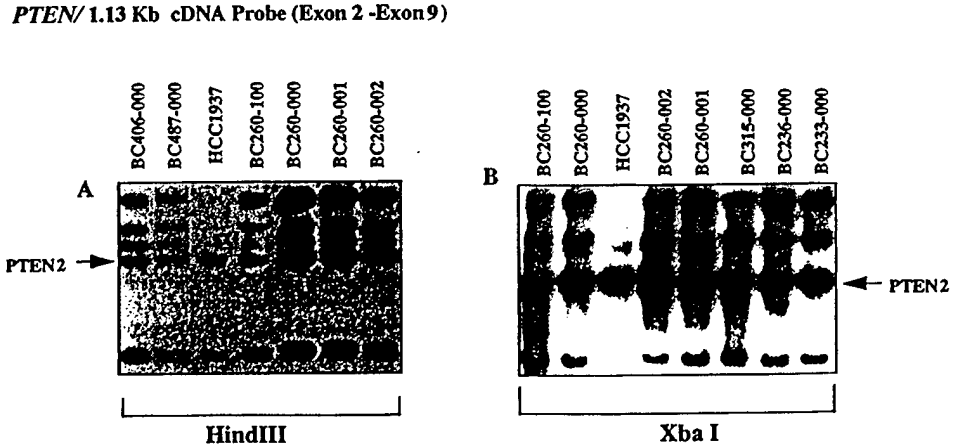

Fig. 5. Southern blot demonstrating absence of the *PTEN* coding sequence in HCC1937. DNA was digested with *Hin*dIII (*left*) or *Xba*I (*right*). The 1.13Kb probe used was prepared from *PTEN* cDNA and contains exons 2–9 (22). Absent bands were observed in the lane containing HCC1937 DNA. Similar results 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]
1q21	*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
5q35	*mfd154*		LOH	Paternal
5q13–q14	*CRTL*		LOH	Paternal
5cen–5q11.2	*D5S76*		LOH	ND
6p21.3	*TAP1*	LOH	LOH	Paternal
6q13	*D6S280*	LOH	LOH	Paternal
7q31.1–q31.2	*D7S522*		RH	
7q31	*WNT2*		RH	
8q11.2–q12	*D8S285*		RH	
8p21–22	*D8S602*	LOH	LOH	Paternal
8p21–22	*D8S254*	LOH	LOH	Paternal
9p21	*IFNA*	LOH	LOH	Maternal
9p21	*D9S1748*		LOH	ND
9p21	*D9S171*		LOH	Maternal
9p21	*IFNA2*		LOH	ND
9p21	*D9S1747*		LOH	Maternal
9p13	*PAL127*		RH	
9p12	*IF6*		RH	
9q22.3–q31	*9S58*		RH	
9q21.1–q13	*9S146*		RH	
9q31	*9S109*		RH	
9q22	*9S196*		RH	
10q23–q24	*D10S185*	LOH	LOH	Paternal
11p15.5	*TH3.1*		RH	
11p15.5	*IGF2*		RH	
11q	*INT-2*	NI	NI	
11q	*PYGM*	RH	RH	
13q12.3–q13	*D13S267*		LOH	Maternal
13q12.3–q13	*D13S171*		LOH	ND
13q14	*RB*	RH	RH	
17p13.1	*TP53AAAAT*	LOH	LOH	Maternal
17q21	*D17S1322*		LOH	Maternal
19p12	*D19S433*	RH	RH	
19p13.2	*D19S391*	RH	RH	

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

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

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

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

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

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

Acknowledgments

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.

References

1. Easton, D., Bishop, D., Ford, D., Crockford, G., and Breast-Cancer-Linkage-Consortium. Genetic linkage analysis in familial breast and ovarian cancer: linkage from 214 families. Am. J. Hum. Genet., *52:* 678–701, 1993.
2. Fitzgerald, M. G., MacDonald, D. J., Krainer, M., Hoover, I., O'Neil, E., Unsal, H., Silva-Arrierto, S., Finkelstein, D. M., Beer-Romero, P., Englert, C., Scroi, D. C., Smith, B., Younger, J. W., Garber, J. E., Duda, R. B., Mayzel, K. A., Isselbacher, K. J., Friend, S. H., and Haber, D. A. Germ-line *BRCA1* mutations in Jewish and non-Jewish women with early-onset breast cancer. N. Engl. J. Med., *334:* 143–149, 1996.
3. Langston, A., Malone, K., Thompson, J., Daling, J., and Ostrander, E. *BRCA1* mutations in a population-based sample of young women with breast cancer. N. Engl. J. Med., *334:* 137–142, 1996.
4. Tirkkonen, M., Johannsson, O., Agnarsson, B. A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R. B., Borg, A., and Kallioniemi, O-P. Distinct somatic genetic changes associated with tumor progression in carriers of *BRCA1* and *BRCA2* germline mutations. Cancer Rés., *57:* 1222–1227, 1997.
5. Scully, R., Chen, J., Ochs, R., Keegan, K., Hoekstra, M., Jeunteun, J., and Livingston, D. M. Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. Cell, *90:* 425–435, 1997.
6. Monteiro, A. N. A., August, A., and Hanafusa, H. Evidence for a transcriptional activation function of BRCA1 C-terminal region. Proc. Natl. Acad. Sci. USA, *93:* 13595–13599, 1996.
7. Jensen, R. A., Thompson, M. E., Jetton, T. L., Szabo, C. I., van der Meer, R., Helou, B., Tronick, S. R., Page, D. L., King, M-C. and Holt, J. T. BRCA1 is secreted and exhibits properties of a granin. Nat. Genet., *12:* 303–308, 1996.
8. Futreal, P., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L., Haugen-Strano, A., Swensen, J., Miki, Y., Eddington, K., McClure, 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 (Washington DC), *266:* 120–122, 1994.
9. Chen, Y., Chen, C-F., Riley, D. J., Allred, C., Chen, P-L., Von Hoff, D., Osborne, C. K., and Lee, W-H. Aberrant subcellular localization of BRCA1 in breast cancer. Science (Washington DC), *270:* 789–791, 1995.
10. Hiyama, K., Hiyama, E., Ishioka, S., Yamakido, M., Inai, K., Gazdar, A. F., Piatyszek, M. A., and Shay, J. A. Telomerase activity in small-cell and non-small-cell lung cancers. J. Natl. Cancer Inst., *87:* 895–902, 1995.
11. Barch, M. J. The ACT Cytogenetics Laboratory Manual, pp. New York: Raven Press, 1991.
12. Miller, G., and Lipman, M. Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc. Natl. Acad. Sci. USA, *70:* 190–194, 1973.
13. Sambrook, J., Fritsch, E., and Maniatis, T. Molecular Cloning: A Laboratory Manual, pp. Plainview, NY: Cold Spring Harbor Laboratory, 1989.
14. Hung, J., Kishimoto, Y., Sugio, K., Virmani, A., McIntire, D., Minna, J., and Gazdar, A. Allele-specific chromosome 3p deletions occur at an early stage in the pathogenesis of lung carcinoma. J. Am. Med. Assoc., *273:* 558–563, 1995.
15. Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc. Natl. Acad. Sci. USA, *86:* 2766–2770, 1989.
16. Buchman, V., Chumakaov, P., Ninkina, N., Samarina, O., and Georgiev, G. A variation in the structure of the protein-coding region of the human *p53* gene. Gene (Amst.), *70:* 245–252, 1988.
17. Kamb, A., Gruis, N., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S., Stockert, E., Day, R., Johnson, B., and Skolnick, M. A cell cycle regulator potentially involved in genesis of many tumor types. Science (Washington DC), *264:* 436–440, 1994.
18. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S., Giovanella, B., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M., and Parsons, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science (Washington DC), *275:* 1943–1947, 1997.
19. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, A., Harshman, K., Tavtigian, S. Liu, Q., Cochran, C., Bennett, L. M., Ding, W., Bell, R., Rosenthal, J., Hussey, C. Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R. Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P. K., Norris, F. H., Helvering L., Morrison, P., Rosteck, P., Lai, M., Barrett, C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A., and Skolnick, M. H. A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. Science (Washington DC), *266:* 66–71, 1994.
20. Friedman, L. S., O. E., Szabo, C. I., Dowd, P., Lynch, E. D., and Rowell, S. E., K. M.-C. Confirmation of *BRCA1* by analysis of germline mutations linked to breast and ovarian cancer in ten families. Nat. Genet., *8:* 399–404, 1994.
21. Wooster, R., Signold, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N. Gregory, S., Gumba, C., Micklem, G., Barfoot, R., Hamoud, R., Patel, S., Rice, C. Biggs, P., Hashim, Y., Smith, A., Connor, F., Arason, A., Gudmundson, J., Floenec D., Kelsell, D., Ford, D., Tonin, P., Bishop, D. T., Spurr, N. K., Ponder, B. A. J. Ecles, R., Peto, J., Deviolee, P., Cornelisse, C., Lynch, H., Narod, S., Lenoir, B. Eglisson, V., Barkadottir, R. B., Easton, D. F., Bentley, D. R., Futreal, P. A. Ashworth, A., and Stratton, M. R. Identification of the breast cancer susceptibility gene *BRCA2*. Nature (Lond.), *378:* 789–782, 1995.
22. Forgacs, E., Biesterveld, E., Sekido, Y., Fong, K., Muneer, S., Wistuba, I., Milchgrub S., Brezinschek, R., Virmani, A., Gazdar, A. F., and Minna, J. D. Mutation analysis of the *PTEN/MMAC1* gene in lung cancer. Oncogene, in press, 1998.
23. Tavassoli, F. A., and Norris, H. J. Secretory carcinoma of the breast. Cancer (Phila.) *45:* 2404–2413, 1980.
24. Rosen, P. P., and Cranor, M. L. Secretory carcinoma of the breast. Arch. Pathol. Lab Med., *115:* 141–144, 1991.
25. Shinagawa, T., Tadokoro, M., Takeuchi, E., Oikawa, K., Kanasugi, K., and Kataba, Y. Aspiration biopsy cytology of secretory carcinoma of the breast. A case report. Acta Cytol., *36:* 189–193, 1992.
26. Johannsson, O. T., Idvall, I., Anderson, C., Borg, A., Barkardottir, R. B., Egilsson, V., and Olsson, H. Tumour biological features of BRCA1-induced breast and ovarian cancer. Eur. J. Cancer, *33:* 362–371, 1997.
27. Johannsson, O., Ranstam, J., Borg, A., and Olsson, H. Survival of BRCA1 breast and ovarian cancer patients: a population-based study from southern Sweden. J. Clin. Oncol., *16:* 397–404, 1998.
28. Couch, F., and Weber, B. Breast Cancer Information Core. Mutations and polymorphisms in the familial early-onset breast cancer (*BRCA1*) gene. Hum. Mutat., *8:* 8–18, 1996.
29. Yuan, Y., Kim, W-H., Han, H. S., Lee, J-H., Park, H-S., Chung, J-K., Kang, S-B., and Park, J-G. Establishment and characterization of human ovarian carcinoma cell lines. Gynecol. Oncol., *66:* 378, 1997.
30. Oh, J., Noh, D., Choe, K., Kang, S., Kim, L., Ro, M., Paik, N., Yang, D., Oh, S., Lee, S., and Park, J. Germline mutation of *BRCA1* gene in Korean breast and ovarian cancer patients. J. Kor. Cancer Assoc., *27:* 1061–1069, 1995.
31. Merajver, S. D., Pham, T. M., Caduff, R. F., Chen, M., Pay, E. L., Cooney, K. A. Weber, B. L., Collins, F. S., Johnston, C., and Frank, T. S. Somatic mutations in the *BRCA1* gene in sporadic ovarian tumours. Nat. Genet., *9:* 439–443, 1995.
32. Marcus, J. N., Watson, P., Page, D. L., Narod, S. A., Lenoir, G. M., Tonin, P., Linder-Stephenson, L., Salerno, G., Conway, T. A., and Lynch, H. T. Hereditary breast cancer: pathobiology, prognosis and *BRCA1* and *BRCA2* gene linkage. Cancer (Phila.), *77:* 697–709, 1996.
33. Struewing, J. P., Hartge, P., Wacholder, S., Baker, S. M., Berlin, M., McAdams, M., Timmerman, M. M., Brody, L. C., and Tucker, M. A. The risk of cancer associated with specific mutations of *BRCA1* and *BRCA2* among Ashkenzi Jews. N. Engl. J. Med., *336:* 1401–1408, 1997.
34. Ramus, S. J., Friedman, L. S., Gayther, S. A., Ponder, B. A. J., Bobrow, L. G., van der Looji, M., Papp, J., and Olah, E. A breast/ovarian cancer patient with germline mutations in both *BRCA1* and *BRCA2*. Nat. Genet., *15:* 14–15, 1997.
35. Stoppa-Lyonnet, D., Fricker, J. P., Essioux, L., Pages, S., Limacher, J. M., Sobol, H., Laurent-Puig, P., and Thomas, G. Segregation of two *BRCA1* mutations in a single family. Am. J. Hum. Genet., *59:* 479–481, 1996.
36. Holt, J. T., Thompson, M. E., Szabo, C., Robinson-Benion, C., Arteaga, C. L., King, M-C. and Jensen, R. A. Growth retardation and tumour inhibition by BRCA1. Nat. Genet., *12:* 298–302, 1996.

Int. J. Cancer: 78, 766–774 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

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

Adi F. GAZDAR[1,2]*, Venkatesh KURVARI[1,3], Arvind VIRMANI[1,2], Lauren GOLLAHON[3], Masahiro SAKAGUCHI[1], Max WESTERFIELD[1], Duli KODAGODA[1], Victor STASNY[1], H. Thomas CUNNINGHAM[1], Ignacio I. WISTUBA[1], Gail TOMLINSON[1,4], Vijay TONK[1], Raheela ASHFAQ[1,2], A. Marilyn LEITCH[7], John D. MINNA[1,5,6] and Jerry W. SHAY[3]

[1]*Hamon Center for Therapeutic Oncology Research, Dallas, TX, USA*
[2]*Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[3]*Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[4]*Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[5]*Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[6]*Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, USA*
[7]*Department of Surgery, University of Texas Southwestern Medical Center, Dallas, TX, USA*

The goal of our study 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 19 tumor cell lines, we also established one or more corresponding non-malignant cell strains or B lymphoblastoid (BL) lines, which included 16 BL 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 the following features: they were derived from large tumors with or without axillary node metastases; were aneuploid and exhibited a moderate to poorly differentiated phenotype; were estrogen receptor (ER)- and progesterone receptor (PR)-negative; and overexpressed p53 and HER2/neu proteins. Of 13 patients with primary breast cancers receiving curative intent mastectomies, 7 were dead after a mean period of 10 months. Our panel of paired tumor and non-malignant cell lines should provide important new reagents for breast cancer research.
***Int. J. Cancer* 78:766–774, 1998.**

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 that can be widely distributed to facilitate comparative studies. The usefulness of the modest number of breast cancer cell lines being used 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 and Ozzello, 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 *et al.*, 1984, 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 50–70 human breast cancer lines have been described in publications, 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 and Jordan, 1997), whereas other cell lines that 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 available breast tumor cell lines.

MATERIAL 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 (FBS), 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% FBS. 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

Grant sponsor: U.S. Army; Grant number: DAMD 17–94-J-4077.

*Correspondence to: Hamon Center for Therapeutic Oncology Research, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235–8593, USA. Fax: (214) 648–4940.
E-mail: gazdar@simmons.swmed.edu

Received 27 April 1998; Revised 3 July 1998

189 tumor samples were used 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 5-year period (1991–1996), and included patients from diverse age and ethnic groups, representing various stages of disease progression as determined by tumor, node, metastasis (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 hr) 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 cryo-preserved in MCDB170 (GIBCO-BRL) medium containing 10% dimethyl sulfoxide (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 Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% iron-fortified serum (HyClone, Logan, UT) for stromal cell growth (Hammond *et al.*, 1984).

Epstein-Barr virus (EBV) lymphocyte transformation

B lymphoblastoid (BL) 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, St. Louis, MO), washed twice in RPMI-1640 and resuspended in initiation medium (RPMI-1640 with 15% FBS, 25 mM Hepes and 1 mM sodium pyruvate). The cell suspension (approx. 1.0–5.0 × 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 an 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, human lymphocyte antigen (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 7 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 (PBS) and bovine serum albumin (BSA). Cell aggregates were disrupted gently using a pipette, and the resulting single cell suspension was adjusted to 1 × 10^7 cells/ml. Then, 1 × 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 fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma) in the dark on ice. Cells were washed once with PBS-BSA, fixed in 1% paraformaldehyde and analyzed using a FACScan Flow Cytometer (Becton-Dickinson, 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 Pathology work station

TABLE I – CLINICAL AND PATHOLOGICAL FEATURES OF BREAST TUMORS USED FOR INITIATION OF CELL LINES

Cell line	Patient age/ ethicity	Current status (months)[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
HCC1007[1]	~~67/B~~	Dead (10)	Pr.breast	Ductal ca	IIA	2	3	12/12		6/7/94	9	BL
HCC1008[1]	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

Abbreviations: 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 cm.

(Roche, Elon College, NC). A minimum of 200 tumor cells was analyzed for ploidy using stromal cells as an internal control.

Immunostaining

Immunostaining analyses were performed as described earlier (Yu *et al.*, 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, Carpenteria, CA; dilution 1:160); progesterone receptor (Zymed, South 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 II): 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.

Reverse transcription-polymerase chain reaction (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 2 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 (HRP)-conjugated antibody. The receptor concentration was determined from a standard curve.

HER2/neu expression assays

HER2/neu expression was determined by a quantitative enzyme-linked immunosorbent assay (ELISA) assay (Calbiochem, La Jolla, CA) 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 2 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 5-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 or growth period for establishment of tumor cell lines (*i.e.*, the time before sufficient tumor cell growth occurred for initial cell passage) ranged from 4 to 44 months, with a median time of 12 months. This period was 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 most cell lines (15) were initiated in RPMI1640 (GIBCO-BRL) medium supplemented with 5% FBS, ACL4 medium (GIBCO-BRL) supplemented with 5% serum was used for initiating 6 cell lines. After establishment, the 2 media were generally interchangeable, and most cell lines grew equally well in either medium, with approximate doubling periods of 24–72 hr.

We established paired, non-malignant BL 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 BL 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.

TABLE II – 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

Abbreviations: 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. Normal epithelial lines had a mean value of 0.5 fmol/μg protein which was set as a relative value of 1.

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 of 40 through 60 years (Table I). These patients represented diverse ethnic backgrounds: 10 white, 6 African-American, 2 Hispanic, 1 Asian and 1 East Indian. Most patients (n = 18) had primary invasive carcinomas, of which 17 were ductal (one with metaplasia), whereas 1 primary tumor had a diagnosis of acantholytic squamous carcinoma. Three tumor cell lines were derived from metastases (1 from a lymph node with metastatic ductal carcinoma, and 2 from malignant pleural effusions [1 from a metastatic lobular carcinoma and 1 from adenocarcinoma, not otherwise specified]). Analysis of the TNM staging indicated that these patients had 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; 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 2 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 (range, from 5 to 22 months).

Of 15 primary tumors successfully cultured whose nodal status was determined, 10 (67%) had lymph node metastases, whereas 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, 5 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 had 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 4 patients follows:

HCC38 was initiated from a 50-year-old woman with a primary breast tumor as well as a second primary leiomyosarcoma (Fig. 1*a*). 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. 1*b*) was initiated from a 32-year-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 loss of heterozygosity (LOH) in breast cancers (Sekido *et al.*, 1998).

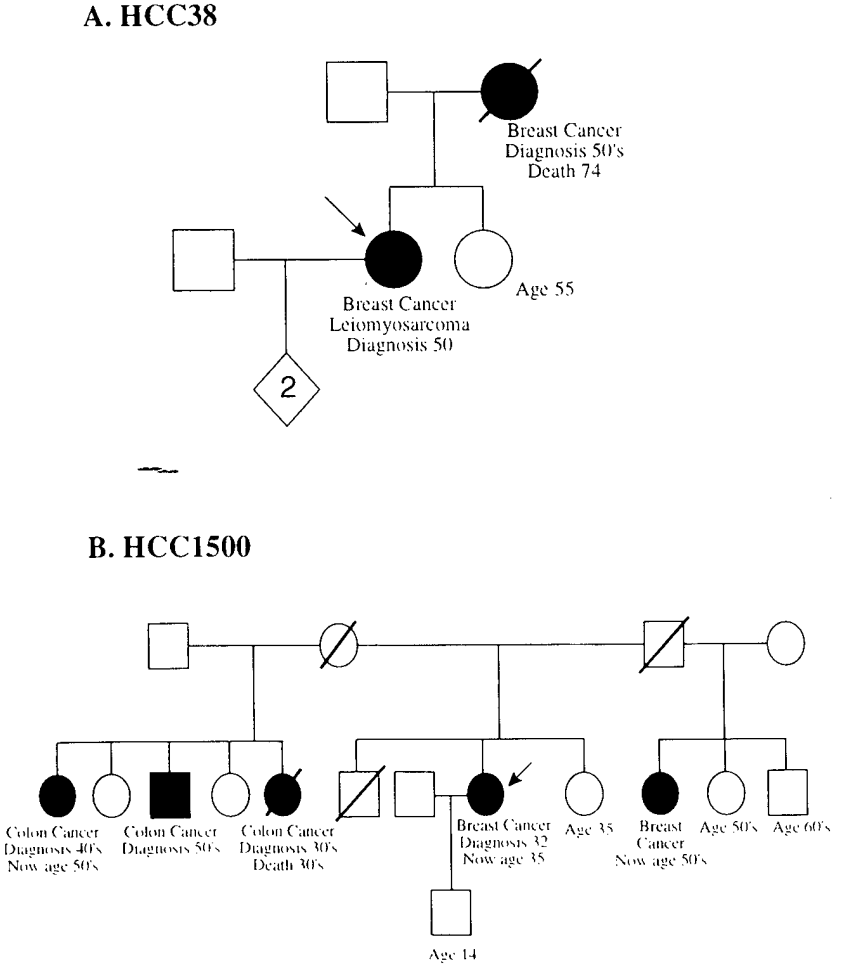

FIGURE 1 – Pedigrees of 4 of the 5 patients with known genetic predisposition (see Results section for more information). (*a*) Pedigree of a woman with a primary breast tumor (corresponding to cell line HCC38) with prior history of sarcoma, whose mother had died of breast cancer. (*b*) Pedigree of a young woman with a significant family history of cancer. Cell line HCC1500 was initiated from this patient.

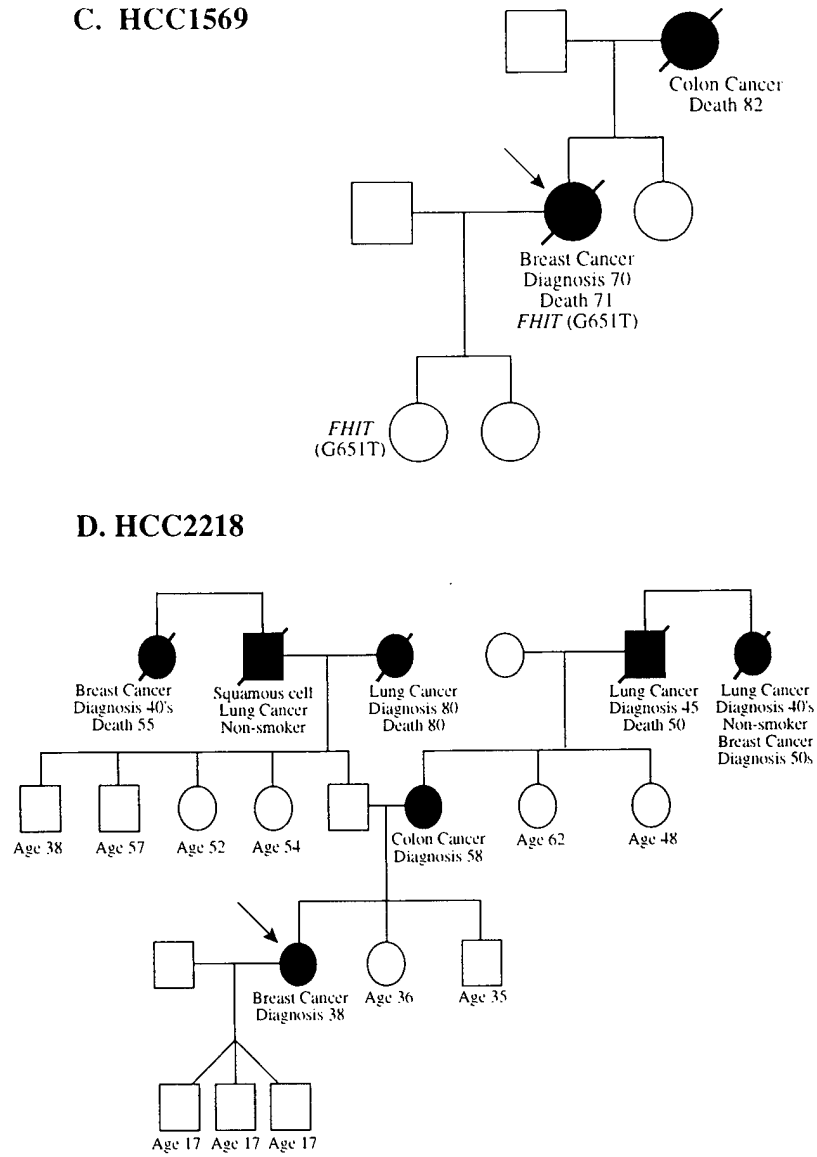

FIGURE 1 (*Continued*) – (*c*) Pedigree of an older patient, corresponding to cell line HCC1569, with a germline mutation of *FHIT* gene. (*d*) Pedigree of the 38-year-old patient from whom cell line HCC2218 was initiated.

One cell line, HCC1569, was found to have a mutation of the *FHIT* gene that 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. 1*c*). Further details of the mutation have been published elsewhere (Ahmadian *et al.*, 1997). It is unclear whether the germline alteration that 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 aged 38 years at diagnosis (Fig. 1*d*). 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 *BRCA1* mutation in this family.

As reported separately in greater detail, HCC1937 was derived from a 24-year-old woman with a germline *BRCA1* mutation, insertion C at nucleotide 5382 (Tomlinson *et al.*, 1998). This mutation was present in 2 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 Figure 2. Most ($n = 16$) of the cell lines (Table III) exhibited partial ($n = 8$) or complete ($n = 8$) substrate attachment (Figs. 2*a–c*). However, 5 lines lacked substrate adherence (Fig. 2*d*). 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. 2*c,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 than in 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 epithelioid "cobblestone" morphology and were occasionally multi-nucleated or vacuolated. At confluence, adherent cell line HCC1937 exhibited a considerable sub-population of giant vacuolated cells.

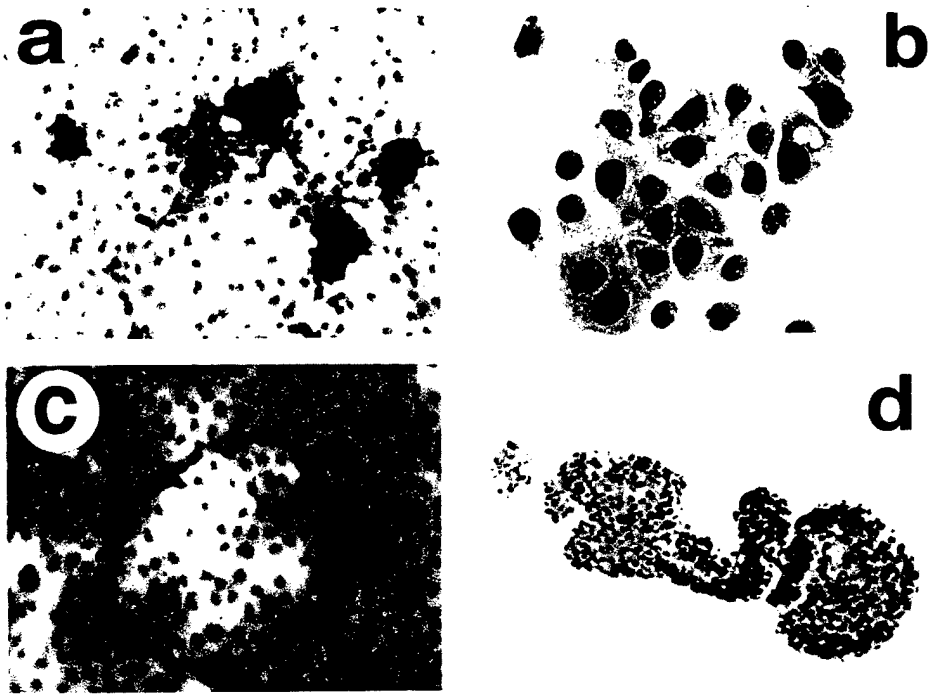

FIGURE 2 – Morphological appearances of breast carcinoma cell lines. For adherent or partially adherent cell lines (Figs. 1*a–c*), cells were cultured on acid-cleaned glass slides, fixed in alcohol and stained with hematoxylin and eosin. For purely non-adherent cultures (Fig. 1*d*), cytospin preparations were handled as described for adherent cultures. (*a*) Cell line HCC1395. Adherent epitheloid cells and partially adherent or non-adherent sub-populations (darkly staining cell masses) are present. (*b*) Cell line HCC1428. Large epitheloid cells with occasional vacuole formation. (*c*) Cell line HCC712. Adherent sub-population 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 III – CHARACTERIZATION OF BREAST TUMOR CELL LINES

Cell line	ATCC number	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.

Non-malignant cell strains

Non-malignant cell strains were initiated as described in the Material and Methods section, and evaluated for their growth patterns and morphological features in culture. Immortalized BL 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 life span (average of 50 population doublings, range from 40 to 90), and lacked telomerase enzyme activity (data not shown). When breast epithelial cell strains became senescent, they enlarged, flattened, became multi-nucleated and vacuolated, and remained attached to the culture dish for several months before detaching. Mammary stromal cell strains growing in serum-containing medium exhibited fusiform fibroblast morphology, and grew in monolayers with a 20–24 hr population doubling period. They lacked expression of CD45, CK19 and EGP2, had a finite life span (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 Material and Methods section. All of the 21 cell lines were positive for EGP2 expression as analyzed by FACS (Table III). These results were confirmed by RT-PCR for all 15 of the cell lines tested, with complete concordance between the data from the 2 methods. In addition, the tumor cell lines were also tested for K19 expression by RT-PCR. As shown in Table III, 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 Material and Methods section. DNA fingerprinting analysis of the 16 tumor cell lines that had 16 corresponding non-malignant lymphocyte cultures demonstrated 100% correlation for the 6 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 III). Most 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 III). 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 to 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 Material and Methods section. 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 Material and Methods section. Table II 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, whereas 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 has not been determined. The expression of p53, however, was up-regulated (indicated by ++ or +++ in Table II) in 9 of 20 (45%) cell lines, whereas the overexpression of HER2/neu (as determined by immunostaining) was also frequent (8/20 or 40%; indicated by ++ or +++ in Table II). 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 II). Relative to its expression in control non-malignant breast epithelial strains, HER2/neu expression was highly up-regulated (10- to 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- to 4-fold in others such as HCC2157. A comparison of the data from the 2 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 2 methods was not possible, all 8 cell lines that showed HER2/neu overexpression by immunostaining also showed several-fold overexpression levels by ELISA, indicating concordance between the 2 methods. Seven of these 8 cell lines showed HER2/neu expression levels 10- to 30-fold higher than the levels in non-malignant epithelial cell strains (Table II). HER2/neu expression levels were similar in the paired tumor cell lines HCC1007 and HCC1008 derived from the same individual.

DISCUSSION

Our 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, whereas our success rate for metastatic tumors was 3 (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. Although 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 these 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 and Ozzello, 1958), and only a modest number of cell lines have since been established (Meltzer *et al.*, 1991). Caillieau *et al.* (1978) reported a culture success rate of approximately 10% from metastatic tumors, while culture attempts from approximately 300 primary breast carcinomas were completely unsuccessful. In another study, only one of 136 (0.7%) primary tumors was successfully cultured (Amadori *et al.*, 1993). 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 used 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 most (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 differentiation; (d) steroid receptor negative; (e) HER2/neu overexpression; 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 overexpression 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 with 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 2- to 30-fold greater than the values measured in non-malignant epithelial cell strains. In 11 cell lines, this increase was modest (2- to 4-fold), whereas in 10 cell lines it was considerable (7- to 30-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 5 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), 4 homozygous deletions at 3 chromosomal 3p regions were identified in the tumor cell lines. These regions are 3p12 (cell line HCC38) (Sundaresan *et al.*, 1998), *FHIT* gene at 3p14.2 (cell lines HCC1428 and HCC1806) (Ahmadian *et al.*, 1997) and 3p21.3 (cell line HCC1500) (Sekido *et al.*, 1998). Because the putative recessive oncogenes at 2 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 deposited with the American Type Culture Collection (ATCC; Rockville, MD) for broad distribution to the scientific community.

ACKNOWLEDGEMENTS

We thank Drs. J. Hutch, D. Euhus, E. Frenkel and G. Peters for their help in making the normal and tumor tissues available for this study. We also thank Ms. Y.-Y. Liu and Ms. B. Thomas for tissue collection and technical assistance.

REFERENCES

AHMADIAN, M., WISTUBA, I.I., FONG, K.M., BEHRENS, C., KODAGODA, D.R., SABOORIAN, M.H., SHAY, J., TOMLINSON, G.E., BLUM, J., MINNA, J.D. and GAZDAR, A.F., Analysis of the FHIT gene and FRA3B region in sporadic breast cancer, preneoplastic lesions, and familial breast cancer probands. *Cancer Res.*, 57, 3664–3668 (1997).

AMADORI, D., BERTONI, L., FLAMIGNI, A., SAVINI, S., GIOVANNI, C.D., CASANOVA, S., PAOLA, F.D., AMADORI, A., GIULOTTO, E. and ZOLI, W., Establishment and characterization of a new cell line from primary human breast carcinoma. *Breast Cancer Res. Treat.*, 28, 251–260 (1993).

BAND, V. and SAGER, R., Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc. nat. Acad. Sci. (Wash.)*, 86, 1249–1253 (1989).

BAND, V., ZAJCHOWSKI, D., SWISSHELM, K., TRASK, D., KULESA, V., COHEN, C., CONNOLLY, J. and SAGER, R., Tumor progression in four mammary epithelial cell lines derived from the same patient. *Cancer Res.*, 50, 7351–7357 (1990).

CAILLEAU, R., OLIVE, M. and CRUCIGER, Q.V., Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. *In Vitro*, 14, 911–915 (1978).

ETHIER, S.P., MAHACEK, M.L., GULLICK, W.J., FRANK, T.S. and WEBER, B.L., Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. *Cancer Res.*, 53, 627–635 (1993).

GAZDAR, A.F. and OIE, H.K., Cell culture methods for human lung cancer. *Cancer Genet. Cytogenet.*, 19, 5–10 (1986*a*).

GAZDAR, A.F. and OIE, H.K., Growth of cell lines and clinical specimens of human non-small cell lung cancer in a serum-free defined medium [letter to the editor]. *Cancer Res.*, 46, 6011 (1986*b*).

HAMMOND, S.L., HAM, R.G. and STAMPFER, M.R., Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. *Proc. nat. Acad. Sci. (Wash.)*, 81, 5435–5439 (1984).

ITO, I., YOSHIMOTO, M., IWASE, T., WATANABE, S., KATAGIRI, T., HARADA, Y., KASUMI, F., YASUDA, S., MITOMI, T., EMI, M. and NAKAMURA, Y., Association of genetic alterations on chromosome 17 and loss of hormone receptors in breast cancer. *Brit. J. Cancer*, 71, 438–441 (1995).

LASFARGUES, E.Y. and OZZELLO, L., Cultivation of human breast carcinoma. *J. nat. Cancer Inst.*, 21, 1131–1147 (1958).

LATZA, U., NIEDOBITEK, G., SCHWARTING, R., NEKARDA, H. and STEIN, H., Ber-EP4: new monoclonal antibody which distinguishes epithelia from mesothelia. *J. clin. Pathol.*, 43, 213–239 (1990).

LEIBOVITZ, A., Cell lines from human breast. *In:* R. Hay, J.-G. Park and A.F. Gazdar (eds.), *Atlas of human tumor cell lines*, pp. 161–184, Academic, Philadelphia (1994).

LEVENSON, A.S. and JORDAN, C.V., MCF-7: The first hormone-responsive breast cancer cell line. *Cancer Res.*, 57, 3071–3078 (1997).

LOUHE, L.G. and KING, M.C., A novel approach to establishing permanent lymphoblastoid cell lines: Epstein-Barr virus transformation of cryopreserved lymphocytes [letter] [see comments]. *Amer. J. hum. Genet.*, 48, 637–638 (1991).

MAHACEK, M.L., BEER, D.G., FRANK, T.S. and ETHIER, S.P., Finite proliferative lifespan *in vitro* of a human breast cancer cell strain isolated from a metastatic lymph node. *Breast Cancer Res. Treat.*, 28, 267–276 (1993).

MCCALLUM, H.M. and LOWTHER, G.W., Long-term culture of primary breast cancer in defined medium. *Breast Cancer Res. Treat.*, 39, 247–259 (1996).

MELTZER, P., LEIBOVITZ, A., DALTON, W., VILLAR, H., KUTE, T., DAVIS, J., NAGLE, R. and TRENT, J., Establishment of two new cell lines derived from human breast carcinomas with HER-2/neu amplification. *Brit. J. Cancer*, 63, 727–735 (1991).

OIE, H.K., RUSSELL, E.K., CARNEY, D.N. and GAZDAR, A.F., Cell culture methods for the establishment of the NCI series of lung cancer cell lines. *J. cell. Biochem.*, (Suppl. 24), 24–31 (1996).

PETERSEN, O.W., VAN DEURS, B., NIELSEN, K.V., MADSEN, M.W., LAURSEN, I., BALSLEV, I. and BRIAND, P., Differential tumorigenicity of two autologous human breast carcinoma cell lines, HMT-3909S1 and HMT-3909S8, established in serum-free medium. *Cancer Res.*, 50, 1257–1270 (1990).

SAKAGUCHI, M., VIRMANI, A.K., ASHFAQ, R., ROGERS, T.E., RATHI, A., LIU, Y., KODAGODA, D., CUNNINGHAM, H.T. and GAZDAR, A.F., Development of a sensitive, specific PCR-based assay for epithelial tumor cells in effusions. *Brit. J. Cancer* (1998) (In press.).

SEKIDO, Y., AHMADIAN, M., WISTUBA, I.I., LATIF, F., BADER, S., WEI, M.-H., GAZDAR, A.F., LERMAN, M.I. and MINNA, J.D., Cloning of a breast cancer homozygous deletion junction narrows the region of search for a 3p21.3 tumor suppressor gene. *Oncogene*, **16,** 3151–3157 (1998).

SHAY, J.W., TOMLINSON, G., PIATYSZEK, M.A. and GOLLAHON, L.S., Spontaneous *in vitro* immortalization of breast epithelial cells from a patient with Li–Fraumeni syndrome. *Mol. cell. Biol.*, 15, 425–432 (1995).

SMITH, H.S., WOLMAN, S.R., DAIRKEE, S.H., HANCOCK, M.C., LIPPMAN, M., LEIF, A. and HACKETT, A.J., Immortalization in culture: occurrence at a late stage in the progression of breast cancer. *J. nat. Cancer Inst.*, 78, 611–615 (1987).

SMITH, H.S., WOLMAN, S.R. and HACKETT, A.J., The biology of breast cancer at the cellular level. *Biochem. biophys. Acta*, 738, 103–123 (1984).

STAMPFER, M.R. and YASWEN, P., Culture systems for study of human mammary epithelial cell proliferation, differentiation and transformation [see comments]. *Cancer Surv.*, 18, 7–34 (1993).

SUNDARESAN, V., CHUNG, G., HEPPELL-PARTON, A., XIONG, J., GRUNDY, C., ROBERTS, I., JAMES, L., CAHN, A., DOUGLAS, J., MINNA, J., SEKIDO, Y., LERMAN, M., LATIF, F., BERGH, J., LI, H., LOWE, N., OGILVIE, D. and RABBITTS, P., Homozygous deletions at 3p12 in breast and lung cancer. *Oncogene*. (1998) (In press).

TOMLINSON, G.E., CHEN, T.T.-L., STASNY, V.A., VIRMANI, A.K., SPILLMAN, M.A., TONK, V., BLUM, J.L., SCHNEIDER, N.R., WISTUBA, I.I., SHAY, J.W., MINNA, J.D. and GAZDAR, A.F., Characterization of a breast cancer cell line derived from a germline *BRCA1* mutation carrier. *Cancer Res.* **58,** 3237–3242 (1998).

VIRMANI, A.K., FONG, K.M., KODAGODA, D., MCINTIRE, D., HUNG, J., TONK, V., MINNA, J.D. and GAZDAR, A.F., Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. *Genes Chromosomes Cancer*, 21, 1–12 (1998).

YU, C.C., WOODS, A.L. and LEVISON, D.A., The assessment of cellular proliferation by immunohistochemistry: a review of currently available methods and their applications. *Histochem. J.*, 24, 121–131 (1992).

Advances in Brief

Comparison of Features of Human Breast Cancer Cell Lines and Their Corresponding Tumors[1]

Ignacio I. Wistuba, Carmen Behrens, Sara Milchgrub, Salahuddin Syed, Mohsen Ahmadian, Arvind K. Virmani, Venkatesh Kurvari, Thomas H. Cunningham, Raheela Ashfaq, John D. Minna, and Adi F. Gazdar[2]

Hamon Center for Therapeutic Oncology Research [I. I. W., C. B., S. S., M. A., A. K. V., V. K., T. H. C., J. D. M., A. F. G.], and Departments of Pathology [S. M., A. K. V., R. A., A. F. G.], Pharmacology [J. D. M.], and Internal Medicine [J. D. M.], University of Texas Southwestern Medical Center, Dallas, Texas 75235, and Department of Pathology, Pontificia Universidad Católica de Chile, Santiago, Chile [I. I. W.]

Abstract

Although human tumor-derived cell lines play an important role in the investigation of cancer biology and genetics, there is no comprehensive study comparing tumor cell line properties with those of the individual tumors from which they were derived. We compared the properties of a series of 18 human breast cancer cell lines that were cultured for a median period of 25 months (range, 9–60 months) and their corresponding archival tumor tissues. We compared morphological characteristics, ploidy, and immunohistochemical expression of estrogen receptors, progesterone receptors, and HER2/neu and p53 proteins. For 17 of these cases, we also tested for allelic losses at 18 chromosomal regions frequently deleted in breast tumors using 51 polymorphic microsatellite markers, and we determined the *TP53* gene mutation status in exons 5 to 10. There was an excellent correlation between the breast tumor cell lines and their corresponding tumor tissues for morphological features (100%); presence of aneuploidy (87%); immunohistochemical expression of estrogen receptors (87%), progesterone receptors (73%), and HER2/neu (93%) and p53 proteins (100%); allelic loss at all of the chromosomal regions analyzed (82–100% concordance); and *TP53* gene mutations (75%). The same parental allele was lost in 279 (99%) of 281 of the comparisons of allelic losses. The fractional allelic loss indices (a reflection of the total allelic loss) of the cell lines and their corresponding tumor tissues were identical or similar in 15 (88%) of 17 paired comparisons. Although our previous studies (A. Gazdar *et al.*, Int. J. Cancer, in press) indicated that only a subset of primary breast carcinomas that have several features indicative of advanced tumors with poor prognosis can be successfully cultured, the cell lines retain the properties of their parental tumors for lengthy culture periods and, thus, provide suitable model systems for biomedical studies.

Introduction

Cell lines established from human tumors provide an unlimited, self-replicating source of malignant cells free of contaminating stromal cells and can be studied by investigators throughout the world. Ever since the description of the HeLa tumor cell line in 1952 (1), which was established from a patient with cervical carcinoma, permanent cultures derived from human tumors have been widely used to investigate almost every aspect of cancer biology. A search of the Medline database for the years 1991–(November) 1997 using the MESH terms "cell cultures, tumor" and "human" yielded a yearly average of >7600 citations, and this figure is almost certainly a considerable underestimate of the true number of published scientific studies that used human tumor cell lines.

Despite the pivotal role played by human tumor cell lines in biomedical research, there is a widespread belief in the scientific community that they are not representative of the tumors from which they were derived. Tumor cell lines, including breast carcinomas cell lines, have extensive chromosomal rearrangements, oncogene mutations, and multiple sites of allelic loss and gene amplification (2–5). Thus, many investigators presume that the loss of phenotypic properties and additional molecular changes develop during the prolonged time required for cell culture establishment and subsequent passage. However, as far as we could determine, no detailed comparison of the properties of human cell lines with those of the tumors from which they were derived has been published for any cancer type.

The establishment of permanent cell cultures from breast cancers is difficult, and relatively few cell lines have been established and characterized (6, 7). Almost all of the widely used cultures were established from malignant effusions and thus were from patients with an advanced stage of the disease (8). Recently, we have established 21 new human breast carcinoma cell lines, most of them derived from primary tumors (9). We compared the morphological, phenotypic, and genetic changes in these cell lines with those present in their corresponding tumor tissues.

Received 8/24/98; revised 10/14/98; accepted 10/14/98.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by the United States Army Grant DAMD17–94-J-4077 and the Komen Foundation.
[2] To whom requests for reprints should be addressed, at Hamon Center for Therapeutic Oncology Research, NB8.106, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8593. Phone: (214) 648-4921; Fax: (214) 648-4924; E-mail: gazdar@simmons.swmed.edu.

Materials and Methods

Cell Line and Tissue Samples. Eighteen human breast cancer lines (prefix HCC[3] for Hamon Cancer Center) established in our laboratory (1992–1996) from invasive ductal breast carcinomas and their corresponding archival paraffin-embedded tumor tissue were studied (9). All of the cell lines from which corresponding archival materials were available were used. Of these, 17 cell lines were established from primary breast tumors and 1 from an axillary lymph node metastasis. The breast cancer patients ranged in age from 24 to 82 years, although most (n, 11) were between the ages 40 and 60. Analysis of the TNM (tumor-node-metastasis) staging indicated that these patients were suffering from varying degrees of tumor extent, ranging from stage I (2 patients) through stage IV (1 patient). Most of the breast cancers were bulky tumors, and 14 (82%) of them were larger than 2 cm in size. Four of the patients had received prior chemotherapy. The median time period from date of initial culture before the cell lines were studied was 26 months (range, 9–60 months; Table 1). Because the quality of DNA obtained from one archival tumor sample was unsatisfactory for PCR-based genetic analyses, only 17 paired tumor samples could be studied for allelic deletions. Ploidy and immunohistochemical analyses were performed on the 15 paired samples from which adequate numbers of microslides were available.

Morphological, Ploidy, and Immunohistochemical Analyses. For morphological, ploidy, and immunohistochemical analyses, suspension cultures were harvested during log phase, and adherent cultures were harvested when semiconfluent, fixed briefly in formaldehyde, pelleted, and suspended in agar (1.5%) and then embedded in paraffin. Five-μm sections were prepared from paraffin-embedded cell lines and corresponding archival tumor tissues. The histological sections of the cell pellets and their corresponding archival tumor tissues were analyzed for structural features including cell morphology, nuclear atypia, and presence of duct-like structures. Because the monolayer cultures lacked distinctive morphological structures (other than dome formations), the cytological features of the cells were studied. For ploidy analysis, 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 cells were analyzed for ploidy using stromal cells as an internal control. The PI was calculated for each cell line and tumor tissue sample. The ploidy status was categorized as diploid (PI = 0.90–1.1) or aneuploid (PI > 1.1).

Immunohistochemical analyses using primary mouse monoclonal antibodies for ER (DAKO Corp., Carpenteria, CA; dilution 1:160), PR (Zymed Laboratories Inc., South San Francisco, CA; dilution 1:200), HER2/neu (cerb-2, DAKO; dilution 1:14,000), and p53 protein (DAKO; dilution 1:80) expression were performed. Immunostaining was performed using a standard avidin-biotin immunoperoxidase method. A minimum of 15 microscopy fields or up to 30,000 μm^2 of nuclear area was quantified. Immunostained sections were scored by a semiquantitative method evaluating the incidence of positive-stained cells as follows: (a) negative (−); (b) low positivity (+) if expression was restricted to 0–30% of the cells; (c) moderate positivity (++) for 30–70% positive cells; and (d) strong positivity (+++) if >70% of the cells were positive.

LOH Analysis. PCR-LOH analyses were performed on genomic DNA extracted from cell lines. Because breast cancers frequently contain large numbers of stromal and inflammatory cells, we used DNA from microdissected archival tissues for molecular assays. From archival paraffin-embedded tumor tissue samples, invasive breast carcinoma areas were identified and precisely dissected under microscopic visualization from noncoverslipped H&E-stained slides, and DNA extraction were performed as described previously (10). From multiple sections of each tissue sample, 1000–2000 sectioned cells were microdissected. Lymphocytes obtained from corresponding resected metastasis-free lymph nodes provided a source for constitutional DNA. Five μl of the proteinase K-digested samples, containing DNA from at least 100 sectioned cells, were used for each multiplex PCR reaction.

To evaluate LOH, we used primers flanking 51 microsatellite repeat polymorphisms spanning chromosomal regions at: (a) 3p (n, 21); (b) 5q22 in the *APC-MCC* region (n, 1); (c) 6p (n, 1); (d) 6q (n, 8); (e) 8p (n, 5); (f) 9p21 (n, 2); (g) 10q23–24 at *PTEN/MMCA1* gene region (n, 4); (h) 11q13 (n, 2); (i) 13q14 at *RB* gene (n, 1); (j) 17p13 at *TP53* gene (n, 2); and (k) 17q21 at *BRCA1* gene region (n, 4). The microsatellite markers and the chromosomal regions analyzed were as follows: (a) 3p12 (D3S1274, D3S1284, and D3S1511); (b) 3p14.2 (D3S4103 and D3S1300 at the *FHIT* gene); (c) 3p14–21 (D3S1766); (d) 3p21 (D3S1573, D3S1447, K1.CA, *ITIH-1*, D3S1478, and D3S1029); (e) 3p22–24 (D3S1582, D3S1612, D3S2432, D3S1351, D3S1537, and D3S1244); (f) 3p25 (D3S1293, D3S1597, and D3S1110); (g) 5q22 (L5.71 in the *APC-MCC* region); (h) 6p (D6S1270); (i) 6q13–14 (D6S280 and D6S300); (j) 6q16–21 (D6S249, D6S1021), 6q22–27 (D6S262, D6S415, D6S264, and D6S305); (k) 8p12-ter (D8S1130, D8S1068, D8S549, D8S602, and NEFL); (l) 9p21 (*IFNA* and D9S1478); (m) 10q23–24 (D10S185, D10S2491, D10S2492, and AFMa086wg9 spanning the *PTEN/MMCA1* gene); (n) 11q13 (INT-2 and PYGM); (o) 13q14 (dinucleotide repeat at the *RB* gene); (p) 17p13 (*TP*53 dinucleotide and pentanucleotide repeats); and (q) 17q21 (D17S855, D17S969, D17S1322, and D17S1323 flanking the *BRCA1* gene). Primer sequences can be obtained from the Genome Data Base, with five exceptions (*ITIH-1*, pentanucleotide, and dinucleotide repeats in the *TP*53 gene; dinucleotide repeat in the *RB* gene; and the K1.CA marker) previously published and referenced (11). For all of the samples, multiplex PCR (up to 6 markers) was performed in the first amplification, followed by uniplex PCR for individual microsatellite markers as described previously (11).

***TP53* Gene Mutation Analysis.** We examined the breast cancer cell lines for mutations in exons 5–10 of the *TP53* gene by sequencing both strands by automated cycle sequencing using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer). For those cell lines that exhibited *TP53* gene mutations, they and their corresponding tumor and normal tissues were analyzed by nested PCR methodology followed by SSCP analysis as de-

[3] The abbreviations used are: HCC, Hamon Cancer Center; PI, ploidy index; SSCP, single-strand conformational polymorphism; FAL, fractional allelic loss; ER, estrogen receptor; PR, progesterone receptor.

Table 1 Comparison of ploidy, immunohistochemistry, and LOH frequencies between breast tumor cell lines and their corresponding tumor tissues

Feature	Frequency (%) tumor tissue/cell line[a]	Tumor tissue/cell line +/+	+/−	−/+	−/−	Concordance *n* (%)
Morphology						
Poor differentiations	83/83	15	0	0	0	15 of 15 (100)
Moderate differentiation	17/17	3	0	0	0	3 of 3 (100)
Aneuploidy	80/93	12	0	2	1	13 of 15 (87)
Protein expression[b]						
Estrogen receptor	7/20	1	0	2	12	13 of 15 (87)
Progesterone receptor	20/20	1	2	2	10	11 of 15 (73)
HER2/neu	33/40	5	0	1	9	14 of 15 (93)
p53 protein	73/73	11	0	0	4	15 of 15 (100)
LOH frequencies by chromosomal regions[c]						
3p25	70/64	11	1	0	5	16 of 17 (94)
3p22–24	53/53	9	0	0	8	17 of 17 (100)
3p21.3	58/64	10	0	1	6	16 of 17 (94)
3p14–21	58/58	7	0	0	5	12 of 12 (100)
3p14.2 (*FHIT* gene)	53/67	8	0	2	5	13 of 15 (87)
3p12	57/57	8	0	0	6	14 of 14 (100)
Any 3p	71/71	12	0	0	5	17 of 17 (100)
5q22 (*APC–MCC* region)	63/63	7	0	0	4	11 of 11 (100)
6p	29/29	2	0	0	5	7 of 7 (100)
6q13–14	56/44	7	2	0	7	14 of 16 (88)
6q16–21	46/46	6	0	0	7	13 of 13 (100)
6q22–27	47/65	8	0	3	6	14 of 17 (82)
Any 6q	77/82	13	0	1	3	16 of 17 (94)
8p12–pter	75/81	12	0	1	3	15 of 16 (94)
9p21 (*CDRN2a* gene)	46/46	6	0	0	7	13 of 13 (100)
10q23–24 (*PTEN/MMCA1* gene)	50/50	8	0	0	8	16 of 16 (100)
11q13	50/50	7	0	0	7	14 of 14 (100)
13q (*RB* gene)	46/46	5	0	0	6	11 of 11 (100)
17p (*TP53* gene)	79/79	11	0	0	3	14 of 14 (100)
17q (*BRCA1* gene)	88/88	14	0	0	2	16 of 16 (100)

[a] All 18 paired cell line/tumor tissue cases were used for morphology comparison, 15 paired cell line/tumor tissue cases were used for immunohistochemistry analyses, and 17 paired cases were used for LOH analyses.

[b] As determined by immunostaining.

[c] For LOH studies +, LOH; −, retention of heterozygosity.

scribed previously (12) of the exon with the mutation. If identical bandshifts were present in the tumor and cell line, but not in the corresponding normal tissue, we concluded that the mutation detected in the cell line by sequencing was also present in the original tumor.

Data Analysis. To compare the total frequencies of LOH between microdissected tumor tissue samples and cell lines, we used the FAL index. The FAL index is a measure of LOH for all of the informative chromosomal loci (maximum 51 loci) per tumor or cell line sample. The FAL index was calculated as follows:

$$\text{FAL index} = \frac{\text{Total number of chromosomal loci with LOH}}{\text{Total number of informative loci examined}}$$

Results

Morphological Features of the Cell Lines and Their Corresponding Tumor Tissues. There was an excellent correlation between the morphological features of the primary tumors and their corresponding cell lines (Table 1). Most of the tumors (15 of 18, 83%) were poorly differentiated, and the corresponding cell lines grew as monolayers of epitheloid cells devoid of obvious organization or secretory activity. These cultures consisted of medium- or large-sized cells with high-grade nuclear atypia and the presence of occasional multinucleated cells. Three (17%) of the tumors were moderately differentiated and demonstrated duct-like structures (Fig. 1). The corresponding cell lines also were moderately differentiated and formed hollow or solid duct-like structures as well as hollow spherical fluid-filled morula-like structures lined by a single layer of epitheloid cells. The nuclei of the moderately differentiated cell lines demonstrated considerably less nuclear atypia than cell lines derived from poorly differentiated tumors.

Ploidy and Immunohistochemical Profile of the Cell Lines and Their Corresponding Tumor Tissues. The ploidy status and the immunohistochemical expression of ER, PR, HER2/neu, and p53 protein were compared for the cell lines and tumor tissues (Table 1). Although a poor correlation was noted in the PIs of the cell lines and corresponding tumor tissues (data not shown), a high correlation was detected in the ploidy categories (Table 1). For example, the cell line HCC70 had a PI of 2.0, whereas that of its corresponding tumor was 2.7, but both

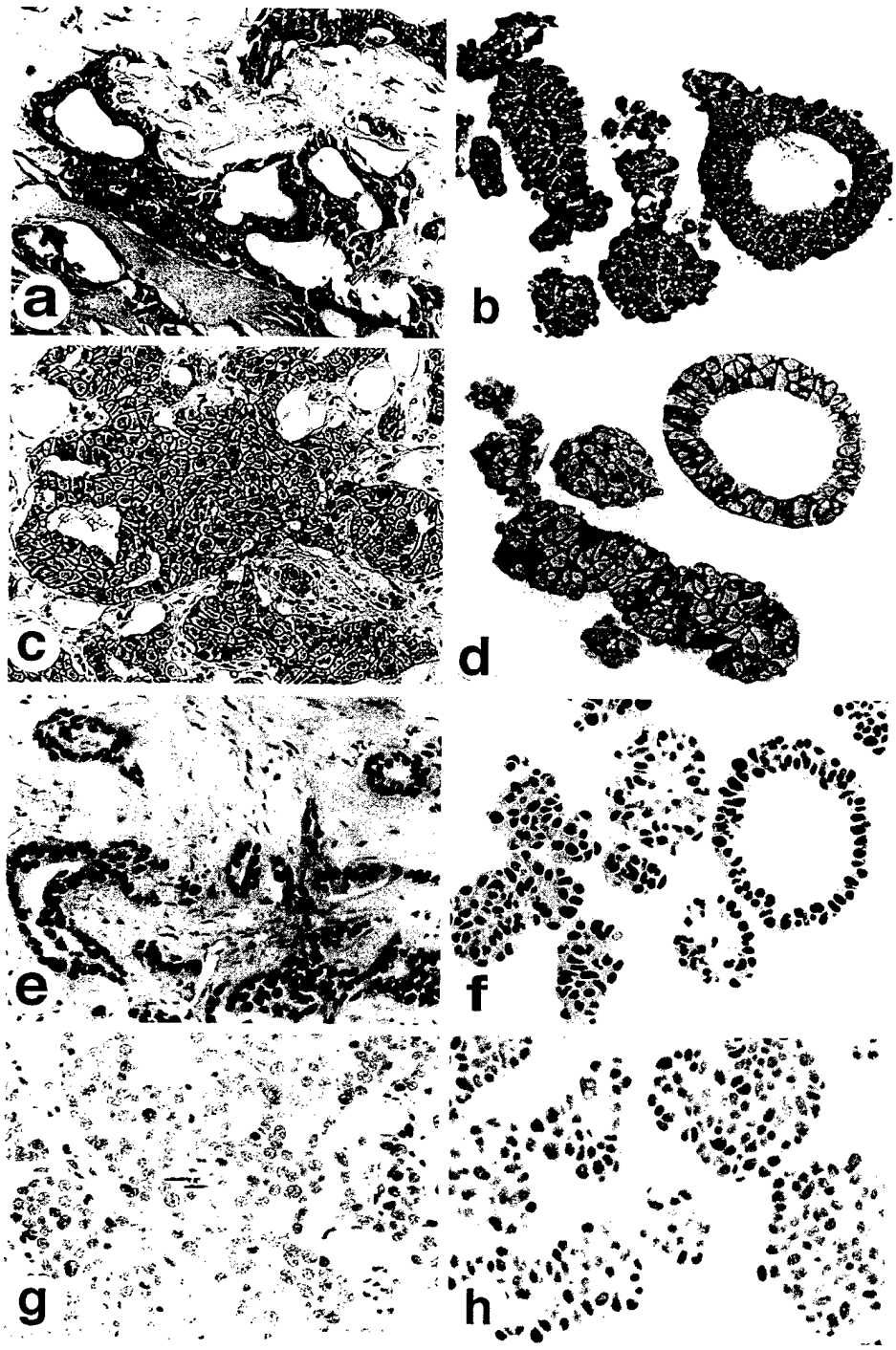

Fig. 1 Comparison of phenotypic properties between breast cancer cell lines and their corresponding tumor tissues. Representative examples of morphology (*a* and *b*), and immunostaining (*c*, *d*, *e*, *f*, *g*, and *h*) of established breast tumor cell line HC1007 and its corresponding tumor tissue. Tumor tissue (*a*) and cell line (*b*) histologies showing moderately differentiated invasive ductal carcinoma (H&E, ×40). Note the gland-like and duct-like structures in both tumor tissue and cell lines. HER2/neu immunostaining demonstrating overexpression in tumor tissue (*c*) and cell line (*d*). Immunostaining is mainly present on the outer cell membrane. p53 protein immunostaining demonstrating nuclear over-expression in tumor tissue (*e*) and cell line (*f*). ER immunostaining demonstrating negative expression in tumor tissue (*g*) and corresponding cell line (*h*).

were categorized as being aneuploid. Thirteen (87%) of 15 cell lines demonstrated the same ploidy categories as their corresponding tumor tissues (Table 1).

Although the frequencies of immunohistochemical detection of ER and PR expression (Fig. 1) were low in both the cell lines and the tumor tissues (Table 1), a high correlation (87% and 73%, respectively) was present between the two types of tumor samples. One of the three cell lines derived from moderately differentiated tumors expressed ER, and two expressed PR. Immunohistochemical expressions of the HER2/neu and p53 proteins (Fig. 1) demonstrated a high degree of correlation between tumors and cell lines for both markers (93% and 100%, respectively; Table 1). For both the tumors and the cell lines, overexpression of the p53 protein was localized in all of the cases to the cell nucleus, whereas HER2/neu expression was localized predominantly to the outer cell membrane.

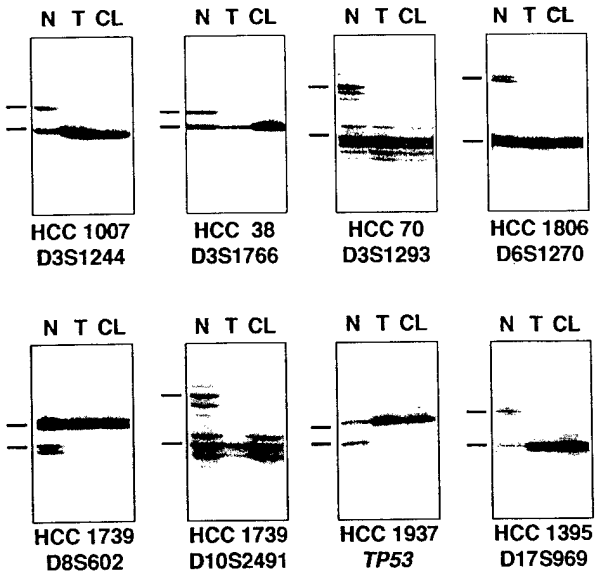

Fig. 2 Comparison of allelic loss between breast cancer cell lines and their corresponding tumor tissues. Eight representative examples of LOH in paired breast cancer cell lines and their corresponding invasive tumors. Each panel demonstrates microsatellite analysis of nonmalignant tissue (*N*), microdissected DNA of invasive breast carcinoma (*T*), and genomic DNA extracted from the corresponding cell line (*CL*). LOH in both sample types was detected by the complete absence of one of the two alleles present in constitutional normal DNA. *Bars*, the positions of the major allelic bands.

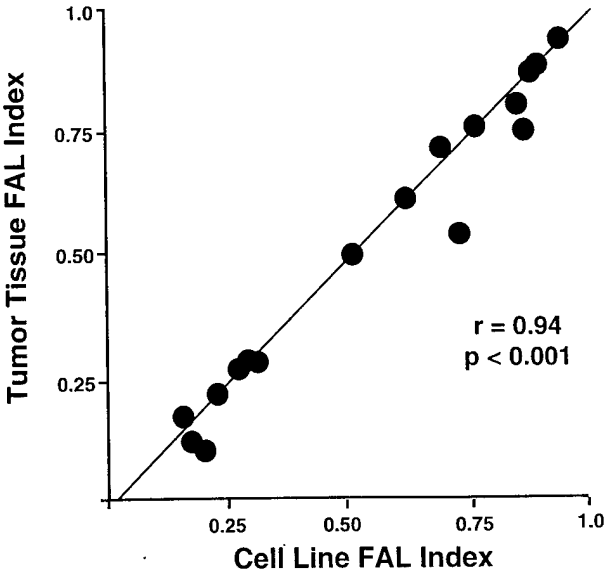

Fig. 3 Correlation between LOH in established breast tumor cell lines and their corresponding tumor tissues using the FAL index (an indicator of LOH at all of the informative loci analyzed per case).

LOH Analysis of Cell Lines and Their Corresponding Tumor Tissues. Because analyses were performed on archival tumor tissue samples that had been carefully microdissected and on cultures that were pure tumor-cell populations, allelic losses for both sample types were detected as complete loss of one of the two alleles present in constitutional DNA of informative cases (Fig. 2). High frequencies (range 44–88%) of allelic loss were detected at all but 1 (6p, 29%) of the 18 chromosomal regions analyzed in the archival tumor specimens (Table 1). The highest frequencies were for allelic losses at one or more 3p regions (71%), 8p regions (75%), one or more 6q regions (77%), *TP53* gene on 17p.13 (79%), and 17q at the *BRCA1* gene region (88%). The LOH frequencies in the cell lines and their corresponding tumor tissues were identical for 12 (67%) and similar at 5 (28%) of the 18 chromosomal regions analyzed (Table 1). Only one chromosomal region (6q22–27) demonstrated a modest difference in the LOH frequency between the cell lines (65%) and their corresponding archival tumors (47%; Table 1). There was an excellent correlation between the FAL index (representing the overall incidence of LOH at the 51 microsatellite markers analyzed) of the cell lines and their corresponding tumor tissues ($r = 0.94$; $P < 0.001$; Fig. 3). The FAL index values of cell lines and their corresponding tumor tissues were identical in eight (47%) or similar in seven (41%) of the 17 pairs. Of the seven cases in which the FAL index values were similar but not identical, the indices of the cell lines were higher than those of the corresponding tumors in five instances and lower in two cases. Only in two (12%) of the tumor-cell-line comparisons was there an important difference. In specimen HCC38, the cell line FAL index value (0.74) was 37% greater than the FAL of the corresponding tumor (0.54). However, there was a greater discrepancy in specimen HCC2218, in which the tumor had lost 3 of 27 informative loci (FAL index, 0.11), whereas the corresponding cell lines had lost 6 loci (FAL index, 0.22).

We compared the patterns of allelic loss in cell lines and tumor tissues for the 51 polymorphic markers examined (Table 2). The overall concordance was 96% (range, 78–100%). For eight pairs the concordance was 100%, and for seven of the pairs, the concordance was 91–97%. When allelic losses of individual markers were present in both the cell lines and the corresponding tumors, we determined whether the same parental allele was lost in both samples. The same allele was lost in 279 (99%) of 281 possible comparisons (Table 2).

***TP53* Gene Mutation Analysis.** *TP53* gene mutations were detected in 8 (47%) of the 17 breast cancer cell lines analyzed. These mutations are detailed in Table 3. We performed SSCP analysis of the involved exon using DNA from the cell line and corresponding normal and tumor tissues. Identical band shifts were detected in six cell lines and their corresponding tumor tissues (75% correlation), but only wild-type forms were present in the corresponding normal tissues. There was a good correlation between the presence of *TP53* gene mutation in cell lines and p53 immunostaining (Table 3). All eight cell lines with *TP53* gene mutations also immunostained for p53. Of these, the five cell lines that overexpressed p53 demonstrated point mutations, and the remaining three cell lines that were p53-immunostaining-negative had a frame shift or stop mutations.

Effect of Culture Time. The median time in continuous culture of the cell lines before testing was 26 months (range, 9–60 months; Table 2). No correlation between the culture

Table 2 Concordance for retention of heterozygosity or LOH

Cell line no.	Cell line time period of culture (mo)[a]	Heterozygous markers,[b] *n*	Retention of heterozygosity[c]	LOH tumor/cell line	Concordance[d] *n* (%)	Loss of the same allele[e] *n* (%)
HCC38	60	35	9	19/26	28 of 35 (80)	19 of 19 (100)
HCC70	58	31	23	8/8	31 of 31 (100)	8 of 8 (100)
HCC712	56	26	20	6/6	26 of 26 (100)	6 of 6 (100)
HCC1007	34	32	9	23/22	31 of 32 (97)	22 of 22 (100)
HCC1143	32	30	2	27/28	29 of 30 (97)	27 of 27 (100)
HCC1187	31	28	7	21/21	28 of 28 (100)	21 of 21 (100)
HCC1395	28	28	5	21/23	26 of 28 (93)	21 of 21 (100)
HCC1419	27	33	26	7/6	32 of 33 (97)	6 of 6 (100)
HCC1500	26	41	16	25/25	41 of 41 (100)	25 of 25 (100)
HCC1569	25	32	25	4/7	29 of 32 (91)	4 of 4 (100)
HCC1685	24	26	18	7/8	25 of 26 (96)	25 of 25 (100)
HCC1739	22	25	3	22/22	25 of 25 (100)	22 of 22 (100)
HCC1806	20	32	4	28/28	32 of 32 (100)	28 of 28 (100)
HCC1937	18	32	5	24/27	29 of 32 (91)	24 of 24 (100)
HCC2157	13	27	20	7/7	27 of 27 (100)	7 of 7 (100)
HCC2218	12	27	18	3/9	21 of 27 (78)	3 of 3 (100)
HCC2389	9	26	13	13/13	26 of 26 (100)	11 of 13 (85)
Total				265/286 (92%)	486 of 509 (96)	279 of 281 (99)

[a] Ranked by cell line time period of culture.
[b] 51 total markers tested.
[c] Number of loci with retention of heterozygosity in both tumors and cell lines.
[d] Concordance for both retention of heterozygosity and LOH.
[e] For comparisons when both tumor and cell line demonstrated LOH for an individual marker.

Table 3 Concordance for *TP53* gene mutations between breast cancer cell lines and corresponding tumor tissues

Cell no.	Cell line *TP53* gene mutation	Base substitution/amino acid change	p53 immunohistochemistry tumor/cell line	Band shift in SCCP analysis[a,b]		
				Normal tissue	Tumor tissue	Cell line
HCC38	Exon 8/Codon 273	CGT to CTT /Arg to Leu	+/+	−	−	+
HCC70	Exon 7/Codon 248	CGG to CAG /Arg to Gln	++/+++	−	+	+
HCC1007	Exon 8/Codon 281	GAC to CAC /Asp to His	++/+++	−	+	+
HCC1395	Exon 5/Codon 175	CGC to CAC /Arg to His	++/++	−	+	+
HCC1569	Exon 8/Codon 294	GAG to TAG /Glu to Term[c]	−/−	−	−	+
HCC1806	Exon 7/Codon 256	2 bp insertion (AA)[d]	−/−	−	+	+
HCC1937	Exon 8/Codon 306	CGA to TGA /Arg to Term[c]	ND[e]/−	−	+	+
HCC2218	Exon 8/Codon 283	CGC to TGC /Arg to Cys	+/+	−	+	+

[a] Of exon with mutations in cell lines.
[b] Identical band shift was detected in the six pairs demonstrating band shift in both tumor and cell lines.
[c] Stop mutation.
[d] Resulting in a frame shift mutation.
[e] ND, not done.

period and theconcordance of allelic loss was demonstrated. The cell lines with the greatest discordance of allelic loss when compared to their parental tumors were HCC38 (concordance of 80%) and HCC2218 (concordance of 78%) with 60 and 12 months in culture, respectively. However, two other cell lines (HCC70 and HCC712) that had been cultured for 56 and 58 months, respectively, demonstrated 100% concordance.

Discussion

Recently, we described the establishment and characterization of 21 new human breast carcinoma cell lines, most of them derived from primary breast carcinomas (9). Using this panel of breast tumor cell lines and their corresponding archival breast tumor tissues, we determined whether the morphological, phenotypic, and genetic changes detected in the cell lines represented those present in their corresponding tumor tissues.

There was an excellent correlation between the morphological features of the primary tumors and their corresponding cell lines. The poorly differentiated tumors grew as epitheloid monolayers lacking obvious morphological evidence of differentiation and exhibiting high-grade nuclear atypia. In comparison, the three cell lines derived from moderately differentiated tumors grew as floating cells organized into duct-like and hollow morula-like structures. Their nuclei demonstrated considerably less nuclear atypia than cell lines derived from poorly differentiated tumors.

Aneuploidy (defined as an abnormal nuclear content of DNA) has been considered evidence of widespread genetic

damage and DNA instability (13). The majority of reports indicate that approximately two-thirds of breast cancers are aneuploid (6); however, almost all of the long-established human breast cancer cell lines were aneuploid (6). In our series, most of the tumors (12 of 15, 80%) and cell lines (14 of 15, 93%) demonstrated one or more aneuploid populations, but the correlation between the specific degree of aneuploidy of the tumors and their corresponding cell lines was variable.

The hormone-dependent nature of some human breast cancers has been recognized for a long time. Approximately 45–65% of primary breast carcinomas are ER- and/or PR-positive (14). Expression of steroid receptors by breast tumors correlates well with a low histological grade and responsiveness to hormonal manipulations (15). However, human breast cancer cell lines with steroid receptor expression are rare, and MCF-7 is perhaps the best and most widely studied breast carcinoma cell line because of its steroid receptor status and estrogen sensitivity (16). As determined by an immunohistochemical method, only 20% of our cell lines (derived mainly from poorly differentiated tumors) demonstrated ER or PR expression. Of the three cell lines derived from moderately differentiated tumors, one expressed ER and two expressed PR. The correlation between the paired tumors and cell lines was 87% for ER expression and 73% for PR expression.

HER2/neu oncogene abnormalities have been extensively studied in breast carcinomas by measuring both gene amplification and HER2/neu oncoprotein overexpression (17, 18). HER2/neu amplification/overexpression has been reported to occur in 10–30% of breast carcinomas, especially in the more aggressive, poorly differentiated carcinomas that are ER-negative, with lymph node metastases and aneuploidy (18). HER2/neu immunohistochemical expression was detected in 6 (40%) of 14 of the breast cancer cell lines and in 5 (33%) of 15 of their corresponding tumors, showing a very high correlation (93%) between tumor samples and their corresponding cell lines.

Point mutations in the *TP53* gene may result in variant p53 proteins that have an increased half-life and thus can be detected by immunohistochemical techniques that fail to immunostain the low amounts of wild-type p53 protein in cells without *TP53* mutations (19). *TP53* gene mutations have been detected in about 40% of breast carcinomas, and p53 protein overexpression has been reported in about 30% of tumors and is associated with shortened survival in breast carcinoma patients (20). p53 protein expression, as demonstrated by an immunohistochemical method, was detected in 80% of the breast tumor cell lines and their corresponding tumor tissues, which indicates complete correlation (100%). However, *TP53* gene mutations in exons 5–10 were detected only in 47% of the tumor cell lines. Of interest, six of those corresponding tumor tissues exhibited the identical *TP53* gene mutation (75% correlation).

We determined allelic loss at 18 chromosomal regions frequently deleted in breast cancers using 51 polymorphic microsatellite markers. Nearly identical, high LOH frequencies at all of the chromosomal regions analyzed were detected between tumor and their corresponding cell lines. The FAL indices (an indicator of total allelic loss) were similar or identical in all but two tumor/cell line comparisons. For all of the individual markers, there was an excellent correlation between tumors and cell lines (mean concordance of 96%). In all but two of the 275 (99%) comparisons, when allelic loss of a particular microsatellite was present in both the tumor and corresponding cell line, the identical parental allele was lost in both, confirming that the allelic loss originated in the original tumor tissue.

The LOH frequencies detected at chromosomal regions 9p21 (*CDKN2a* gene, 46%), 13q.14 (*RB* gene, 46%), 10q23–24 (*PTEN/MMCA1* gene, 50%) and 6q (77%), in our breast tumors are similar to the LOH frequencies reported previously in sporadic breast cancers (21–26). However, our tumors (and their corresponding cell lines) demonstrated a higher incidence of allelic loss compared to the literature for the *BRCA1* gene (17q21, 88 *versus* 35–45%), 8p (75 *versus* 46%), *TP53* gene (17p.13, 79 *versus* 48–64%), 5q (*APC-MCC* genes, 63 *versus* 28%), 3p (71 *versus* 30–50%), and 11q13 (50 *versus* 23%) regions than reported in the literature (27–32). These findings suggest that tumors that can be successfully cultured have a higher incidence of genetic changes than sporadic tumors.

Other than differences in the degree of aneuploidy, the other properties studied demonstrated a remarkable degree of concordance between tumors and their corresponding cancer cell lines. These features included morphological characteristics; presence of aneuploidy; immunohistochemical expression profile for ER, PR, HER2/neu, and p53 protein; and a similar allelic loss pattern for multiple loci frequently deleted in breast carcinoma. Our studies were performed on cell lines cultured for a median period of 26 months. The concordance between tumors and cell lines for all of the comparisons was 100% for two of the three pairs cultured for 56 months or longer, which indicates that the properties of cell lines usually closely resemble those of their parental tumors for periods of up to 5 years. Although only a relatively small subset (11%) of breast cancers can be successfully cultured, data presented here and elsewhere (9) indicate that breast tumors that can be successfully cultured consist of a subset of primary carcinomas that have several features suggestive of a poor prognosis. These features include bulky, poorly differentiated, aneuploid tumors lacking ER and PR expression and having high frequencies of nodal metastases, HER2/neu and p53 protein overexpression, *TP53* gene mutations, and multiple allelic losses involving 3p, 5q, 8p, 11q, 17p13 (*TP53* gene), and 17q21 (*BRCA1* gene) chromosomal regions. Of interest, more than one-half of the patients whose tumors were successfully cultured died within 10 months of their surgery (9).

Genomic instability is a characteristic feature of many tumors, and it may develop early during pathogenesis (13). Genomic instability persists after tumor development and results in the frequent appearance of multiple subclonal populations (33); presumably, the instability continues during culture life. Because cell cultures frequently have population-doubling times considerably shorter than those of *in vivo* tumors, the frequency of mutational change in cultures may be more rapid than in their corresponding tumors. However, our data indicate that many of the phenotypic and genotypic properties of breast carcinoma cultures and their corresponding tumors are similar for culture times of up to 60 months. Thus, breast carcinoma cell lines are useful models for studying at least one major form of breast cancer.

References

1. Scherer, W. F., Syverton, J. T., and Gey, G. O. Study on the propagation in vitro of poliomyelitis viruses: viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exp. Med., *97:* 695–709, 1953.

2. Ahmadian, M., Wistuba, I. I., Fong, K. M., Behrens, C., Kodagoda, D. R., Saboorian, M. H., Shay, J., Tomlinson, G. E., Blum, J., Minna, J., and Gazdar, A. F. Analysis of the *FHIT* gene and FRA3B region in sporadic breast cancer, preneoplastic lesions, and familial breast cancer probands. Cancer Res., *57:* 3664–3668, 1997.

3. Virmani, A. K., Fong, K. M., Kodagoda, D., McIntire, D., Hung, J., Tonk, V., Minna, J. D., and Gazdar, A. F. Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. Genes Chromosomes Cancer, *21:* 308–319, 1998.

4. Buckley, M. F., Sweeney, K. J., Hamilton, J. A., Sini, R. L., Manning, D. L., Nicholson, R. I., deFazio, A., Watts, C. K., Musgrove, E. A., and Sutherland, R. L. Expression and amplification of cyclin genes in human breast cancer. Oncogene, *8:* 2127–2133, 1993.

5. Muleris, M., Almeida, A., Gerbault-Seureau, M., Malfoy, B., and Dutrillaux, B. Detection of DNA amplification in 17 primary breast carcinomas with homogeneously staining regions by a modified comparative genomic hybridization technique. Genes Chromosomes Cancer, *10:* 160–170, 1994.

6. Smith, H. S., Wolman, S. R., and Hackett, A. J. The biology of breast cancer at the cellular level. Biochim. Biophys. Acta, *738:* 103–123, 1984.

7. McCallum, H. M., and Lowther, G. W. Long-term culture of primary breast cancer in defined medium. Breast Cancer Res. Treat., *39:* 247–259, 1996.

8. Leibovitz, A. Cell lines from human breast. *In:* R. Hay, J.-G. Park, and A. F. Gazdar (eds.), An Atlas of Human Tumor Cell Lines, pp. 161–184. Philadelphia: Academic Press, 1994.

9. Gazdar, A. F., Kurvari, V., Virmani, A., Gollahon, L., Sakaguchi, M., Westerfield, M., Kodagoda, D., Stasny, V., Cunningham, T., Wistuba, I. I., Tomlinson, G., Tonk, V., Ashfaq, R., Minna, J. D., and Shay, J. W. Characterization of paired tumor and non-tumor cell lines established from patients with breast cancer. Int. J. Cancer, in press, 1998.

10. Hung, J., Kishimoto, Y., Sugio, K., Virmani, A., McIntire, D. D., Minna, J. D., and Gazdar, A. F. Allele-specific chromosome 3p deletions occur at an early stage in the pathogenesis of lung carcinoma. J. Am. Med. Assoc., *273:* 558–563, 1995.

11. Wistuba, I. I., Behrens, C., Milchgrub, S., Virmani, A., Jagirdar, J., Thomas, B., Ioachim, H. L., Litzky, L. A., Brambilla, E. M., Minna, J. D., and Gazdar, A. F. Comparison of molecular changes in lung cancers in HIV-positive and HIV-indeterminate subjects. J. Am. Med. Assoc., *279:* 1554–1559, 1997.

12. Wistuba, I. I., Montellano, F. D., Milchgrub, S., Virmani, A. K., Behrens, C., Chen, H., Ahmadian, M., Nowak, J. A., Muller, C., Minna, J. D., and Gazdar, A. F. Deletions of chromosome 3p are frequent and early events in the pathogenesis of uterine cervical carcinoma. Cancer Res., *57:* 3154–3158, 1997.

13. Loeb, L. A. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res., *51:* 3075–3079, 1991.

14. Tavassoli, F. A. General Considerations: Pathology of the Breast, pp. 46–47. East Norwalk, CT: Appleton & Lange, 1992.

15. McGuire, W. L., and Clark, G. M. Prognostic factors and treatment decisions in axillary node-negative breast cancer. N. Engl. J. Med., *326:* 1756–1761, 1992.

16. Levenson, A. S., and Jordan, C. V. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res., *57:* 3071–3078, 1997.

17. Slamon, D. J., and Clark, G. M. Amplification of c-*erb*B-2 and aggressive human breast tumor. Science (Washington DC), *240:* 1795–1798, 1988.

18. Press, M. F., Pike, M. C., Chazin, V. R., Hung, G., Udove, J. A., Markowicz, M., Danyluk, J., Godolphin, W., Sliwkowski, M., Akita, R., Paterson, M. C., and Slamon, D. J. Her-2/*neu* expression in node-negative breast cancer: direct tissue quantitation by computarized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res., *53:* 4960–4970, 1993.

19. Baas, I. O., Mulder, J. W., Offerhaus, G. J., Vogelstein, B., and Hamilton, S. R. An evaluation of six antibodies for immunohistochemistry of mutant *p53* gene product in archival colorectal neoplasms. J. Pathol., *172:* 5–12, 1994.

20. Silvestrini, R., Benini, E., Daidone, M. G., Veneroni, S., Boracchi, P., Cappellend, V., DiFronzo, G., and Veronesi, U. p53 as an independent prognostic marker in lymph node-negative breast cancer patients. J. Natl. Cancer Inst., *85:* 965–970, 1993.

21. An, H. X., Niederacher, D., Picard, F., van Roeyen, C., Bender, H. G., and Beckmann, M. W. Frequent allele loss on 9p21–22 defines a smallest common region in the vicinity of the *CDKN2* gene in sporadic breast cancer. Genes Chromosomes Cancer, *17:* 14–20, 1996.

22. Andersen, T. I., Gaustad, A., Ottestad, L., Farrants, G. W., Nesland, J. M., Tveit, K. M., and Borresen, A. L. Genetic alterations of the tumour suppressor gene regions 3p, 11p, 13q, 17p, and 17q in human breast carcinomas. Genes Chromosomes Cancer, *4:* 113–121, 1992.

23. Devilee, P., van den Broek, M., Kuipers-Dijkshoorn, N., Kolluri, R., Khan, P. M., Pearson, P. L., and Cornelisse, C. J. At least four different chromosomal regions are involved in loss of heterozygosity in human breast carcinoma. Genomics, *5:* 554–560, 1989.

24. Li, J., Yen, C., Liaw, d., Podyspanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast and prostate cancer. Science (Washington DC). *275:* 1943–1947, 1997.

25. Fujii, H., and Gabrielson, E. Detection of frequent allelic loss of 6q23–q25.2 in microdissected human breast cancer tissues. Genes Chromosomes Cancer, *6:* 35–39, 1996.

26. Orphanos, V., McGown, G., Hey, Y., Boyle, J. M., and Santibanez-Koref, M. Proximal 6q, a region showing allele loss in primary breast cancer. Br. J. Cancer, *71:* 290–293, 1995.

27. Nagai, M. A., Yamamoto, L., Salaorni, S., Pacheco, M. M., Brentani, M. M., Barbosa, E. M., Brentani, R. R., Mazoyer, S., Smith, S. A., Ponder, B. A-J., and Mulligan, L. M. Detailed deletion mapping of chromosome segment 17q-12–21 in sporadic breast tumours. Genes Chromosomes Cancer, *11:* 58–62, 1994.

28. Kirchweger, R., Zeillinger, R., Schneeberger, C., Speiser, P., Louason, G., and Theillet, C. Patterns of allele losses suggest the existence of five distinct regions of LOH on chromosome 17 in breast cancer. Int. J. Cancer, *56:* 193–199, 1994.

29. Yaremko, M. L., Kutza, C., Lyzak, J., Mick, R., Recant, W. M., and Westbrook, C. A. Loss of heterozygosity from the short arm of chromosome 18 is associated with invasive behavior in breast cancer. Genes Chromosomes Cancer, *16:* 189–195, 1996.

30. Singh, S., Simon, M., Meybohm, I., Jantke, I., Jonat, W., Maass, H., and Goedde, H. W. Human breast cancer: frequent *p53* allele loss and protein overexpression. Hum. Genet., *90:* 635–640, 1993.

31. Thompson, A. M., Morris, R. G., Wallace, M., Wyllie, A. H., Steel, C. M., and Carter, D. C. Allele loss from 5q21 (*APC/MCC*) and 18q21 (*DCC*) and *DCC* mRNA expression in breast cancer. Br. J. Cancer, *68:* 64–68, 1993.

32. Gudmundsson, J., Barkardottir, R. B., Eiriksdottir, G., Baldursson, T., Arason, A., Egilsson, V., and Ingvarsson, S. Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations. Br. J. Cancer, *72:* 696–701, 1995.

33. Nowell, P. C. The clonal evolution of tumor cell populations. Science (Washington DC), *194:* 23–28, 1976.