[CANCER RESEARCH 62, 1000–1003, February 15, 2002]

Advances in Brief

Increased Risk of Local Recurrence Is Associated with Allelic Loss in Normal Lobules of Breast Cancer Patients[1]

Zheng Li, Dan H. Moore, Zhen Hang Meng, Britt-Marie Ljung, Joe W. Gray, and Shanaz H. Dairkee[2]

Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, California 94115 [Z. L., D. H. M., Z. H. M., S. H. D.], and Department of Pathology [B-M. L.] and Cancer Center [J. W. G.], University of California, San Francisco, California 94143

Abstract

Allelic losses characteristic of tumor cells, when displayed by morphologically normal terminal ductal lobular units (TDLUs) adjacent to carcinoma [G. Deng *et al.*, Science (Wash. DC), *274:* 2057–2059, 1996], may indicate an extended field of increased cancer susceptibility within the affected breast tissue. We investigated this possibility by asking whether the presence of loss of heterozygosity (LOH) at chromosome 3p11–26 in histologically normal TDLUs (*3pLOHn*) could lead to an increased risk of local tumor recurrence. We assessed LOHs in normal TDLUs adjacent to 48 informative cases of early-stage invasive breast cancer samples and found *3pLOHn* in ~25% (13 of 48) of patients whose tumors had 3pLOH in this region. Our analyses suggest that the most frequent region of LOH is localized at 3p24.3. We also demonstrate, using a Cox proportional hazards regression model, that the presence of *3pLOHn* was the only variable significantly related to local tumor recurrence, leading to a 3.9–5.2-fold increase in the hazard ratio ($P < 0.05$). The time to recurrence was longer in such cases than in those without *3pLOHn*, suggesting *de novo* tumor development. These data provide a strong rationale to assess histologically normal breast tissue at the margins of surgically excised cancers for molecular predictors of local recurrence after breast-conserving treatment.

Introduction

Advances in mammographic detection of breast tumors have allowed earlier detection and better localization, thereby offering the possibility of minimal surgical resection. Despite the increasing acceptance of breast conservation in the treatment of early-stage breast cancer, concerns regarding excision width have not been fully addressed (1). The effectiveness of wide resection margins in reducing the rate of local recurrence in early-stage patients (2–4) remains to be established. In randomized trials designed to test the efficacy of postlumpectomy adjuvant therapies, margin status did not affect clinical outcome (5, 6). This suggests that some ipsilateral recurrences arise *de novo* from clonal precursors within a localized "field" of increased susceptibility. This possibility is further supported by high rates of local recurrence observed in younger patients with histologically clear surgical margins (7). Molecular indicators that enable early detection of such high-susceptibility, histologically normal regions would be an important clinical adjunct. LOH[3] in morphologically normal lobules adjacent to breast cancer at 3p22–25 has been suggested as one event that could increase susceptibility to breast cancer (8). We demonstrate here that allele-specific LOH within this region in morphologically normal TDLUs adjacent to carcinoma (*3pLOHn*) is a high-risk marker of local recurrence in early-stage breast cancer patients. In addition, we have narrowed the region of LOH to 3p24.3, a chromosomal region not reported previously to harbor genes causally associated with the suppression of malignancy. This pilot study provides a strong rationale for examining *3pLOHn* in a larger patient cohort for further validation and clinical application.

Materials and Methods

Acquisition of Archival Tissue Samples. Because the primary goal of this study was to investigate the role of morphologically normal TDLUs adjacent to cancerous breast tissue in the development of locally recurrent breast disease, the most important criteria for sample selection were: (*a*) early-stage patients treated with breast-conserving surgery; and (*b*) the presence of TDLUs, composed of ≥500 cells, within the tumor sample (for optimal DNA yield). Under Institutional Review Board-approved guidelines, we identified 64 cases of stage I and II patients diagnosed with invasive ductal breast carcinoma at the California Pacific Medical Center, San Francisco, and the University of California, San Francisco Medical Center between 1986 and 1993 for which archived samples and information regarding local recurrence or disease-free status were available. The median age of the patients was 53 years (range, 25–87 years), and the median follow-up was 76 months (range, 11–134 months).

Microdissection and LOH Analysis. Serial 5-μm sections of paraffin-embedded tumor and adjacent normal tissue were used for manual microdissection. To maximize the DNA yield, the same TDLUs or group of tumor cells was microdissected and pooled from two to five sequential sections. The criterion for selection of morphologically normal TDLUs, reviewed by two pathologists (B. M. L. and Z. H. M.), was the presence of several acini displaying prominent lumens. Each acinus within microdissected TDLUs consisted of a single continuous myoepithelial or basal layer surrounding a single layer of luminal epithelial cells with uniformly small nuclei containing evenly distributed chromatin.

Nonmalignant skin DNA from each case was isolated and assessed for the presence of heterozygosity at the following RFLP and microsatellite loci between 3p11–26: *D3S2397, 71G12, D3S2405, D3S1597, D3S2414, D3S1244, D3S3038, D3S1255, TRβ1, D3S2423, D3S2396, D3S1768, D3S1766, 2C06,* and *D3S2438.* Informative cases were microdissected and analyzed for LOH. Nonmalignant skin and tumor DNAs were compared. Samples of tumors that displayed allelic loss at any locus were further tested for LOH in morphologically normal TDLUs adjacent to tumor.

We used a robust PCR-based assay for LOH analysis to effectively use the small amounts of DNA obtained by microdissection of individual TDLUs. Microdissected cells were incubated in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Tween 20, and 400 mg/ml proteinase K) at 50°C until the sample was clear. DNA was amplified in a 10-μl reaction mixture for a total of 40 cycles, as described previously (8). To minimize the chance of over interpretation of PCR results because of technical artifacts often associated with LOH analysis, the following measures were adopted: (*a*) the number of cells was determined before microdissection and solubilization in a standard volume:cell ratio; (*b*) a minimum DNA template volume containing the equivalent of 50 cells was amplified by PCR; and (*c*) allelic intensity was compared within multiple runs of a sample in a single PCR assay and between independent assays. Depending on the test locus, the expected constitutional allelic ratio of 1:1 varied by an average of 5% in control samples of nonmalignant skin from different individuals. LOH was recorded only if we observed a >30% reduc-

Received 7/12/01; accepted 1/14/02.

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 by NIH Grant P50 CA-58207.

[2] To whom requests for reprints should be addressed, at Geraldine Brush Cancer Research Institute, 2330 Clay Street, San Francisco, CA 94115. Phone: (415) 561-1653; Fax: (415) 561-1390; E-mail: shanaz@cooper.cpmc.org.

[3] The abbreviations used are: LOH, loss of heterozygosity; TDLU, terminal ductal lobular unit.

tion in the allelic ratio of test sample compared with control and a <5% difference between parallel duplicate reactions of test samples showing reduced allelic ratios.

Statistical Analysis of Recurrence. Statistical analyses of events associated with recurrence were limited to 48 patients with stage I and II breast cancer whose tumors displayed 3pLOH. Clinical data in the correlative analyses included: patient status (alive, with local recurrence, or no evidence of disease), age, lymph node positivity, tumor grade, tumor size, tumor margins (negative or positive), chemotherapy (yes or no), and hormonal therapy (yes or no). Information regarding patient outcome was obtained by chart review. Molecular analyses were blinded to patient outcome until allelic ratios were determined. Multiple retrospective statistical analyses were carried out.

We first tested whether the prevalence of *3pLOHn* was the same in recurrent and nonrecurrent cases by Fisher's exact test. In a second analysis, a Cox proportional hazards model was used to assess the risk of recurrence in the presence or absence of *3pLOHn*. Patients with distant recurrence but without detectable local recurrence were censored at the time of distant recurrence. Factors such as grade, stage, nodal status, age, and treatment modality were included in the model. Finally, we compared time to local recurrence in cases with and without *3pLOHn* using a Wilcoxon rank sum test.

In addition, a χ^2 test was used to determine whether LOH occurred more frequently in tumors at the *TRβ1* locus compared with any other locus and whether adjacent normal TDLUs were more likely to display LOH at locus *TRβ1* in such tumors than in tumors showing LOH at other loci. Each table compared the number of tumors (or adjacent normal TDLUs) with LOH at that locus *versus* the number at all other loci combined. Two-sided tests of significance were used for all analyses.

Results

Prevalence and Chromosomal Extent of *3pLOHn*. Stage I and II tumors were selected for this LOH study to ensure that normal adjacent TDLUs would not be overgrown by aggressively proliferating malignant populations, as is often the case in late-stage tumors. Up to 15 loci were analyzed for LOH to define the most commonly deleted region. The *TRβ1* locus was included because it was reported to be deleted in an earlier study of normal TDLUs adjacent to carcinoma (8). LOH involving at least one locus on 3p was present in 48 of 64 of the tumors, and the prevalence ranged from 46 to 67% at loci between 3p24.2 and 3p25. Importantly, *3pLOHn* was present at ≥1 locus between 3p11–26 in adjacent morphologically normal TDLUs in 13 of the 48 breast tumors that displayed 3pLOH (Fig. 1). Variations observed in the degree of LOH between microdissected lobular units reflected the ratio of intralobular fibroblasts to epithelial component of TDLUs. Similarly, in some tumors, intratumor fibroblasts comprised a noticeable fraction, whereas in other cases such contamination was minimal.

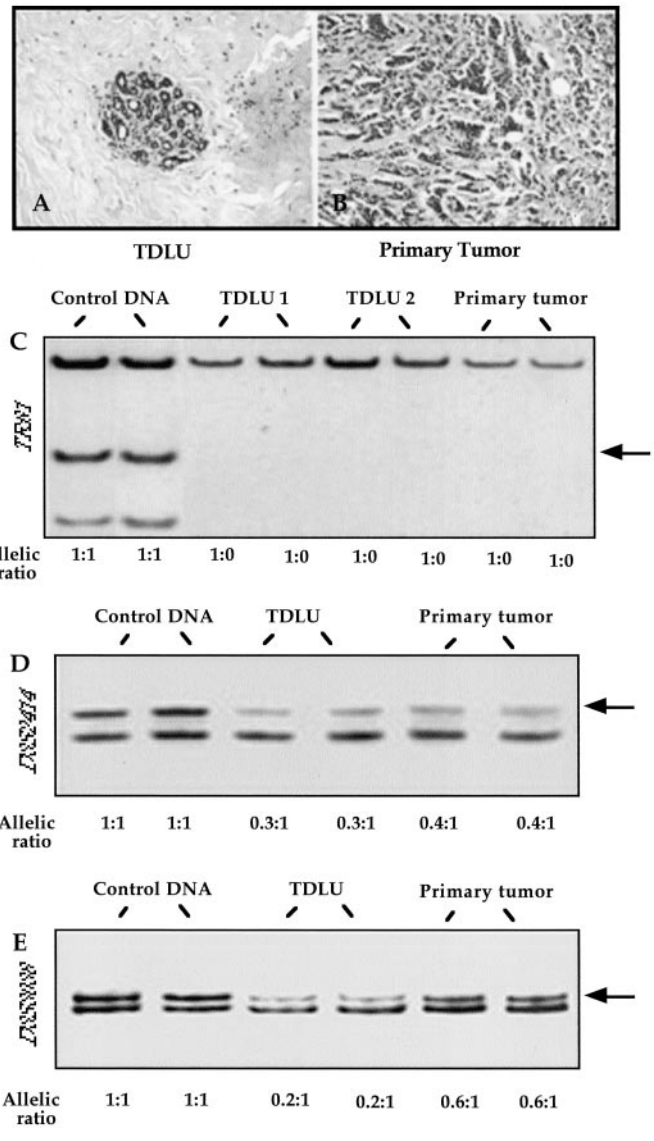

Fig. 1. Representative histology of microdissected area in H&E-stained sections of archival breast tumor tissue and PCR-based LOH analysis. *A*, morphologically normal TDLUs. Note relatively limited cell yields from individual breast lobules in comparison with primary invasive tumor (*B*). *C–E*, examples of allele-specific LOH at polymorphic loci within chromosome 3p11–26 in normal adjacent TDLUs and primary invasive tumor. All samples were run in parallel duplicates for data reproducibility. *Arrows*, deleted allele. The constitutional genotype at each locus is shown as control DNA isolated from nonmalignant skin. Allelic ratios determined from gel scans are given for each PCR run. *C*, RFLP locus, *TRβ1* (case 15). Two separate sites of normal adjacent TDLUs and invasive tumor display loss of allele 2. *D*, microsatellite locus, *D3S2414* (case 10). Normal TDLUs and tumor show loss of allele 1. *E*, microsatellite locus, *D3S3038* (case 16). Normal TDLUs and tumor show loss of allele 1.

We analyzed matched sets of primary tumor and normal adjacent TDLUs to determine the most common region of early allelic loss. As shown cumulatively in Fig. 2, LOH was detected in 113 of 172 allelic ratios of 48 informative tumors. The *TRβ1* locus at 3p24.3 was most commonly involved in allelic loss in normal adjacent TDLUs. Seven of 18 LOH events at *TRβ1* in contrast to only 14 of 95 LOH events at other loci were displayed by normal adjacent TDLUs ($P = 0.02$, two-sided Fisher's exact test). Interestingly, in the tumor itself the frequency of loss at *TRβ1* was not significantly different from the frequency of LOH at other loci, 18 of 24 at *TRβ1 versus* 95 of 138 at other loci.

Associations with *3pLOHn*. Several tests demonstrated an association between the presence of *3pLOHn* and local tumor recurrence in 48 patients whose tumors displayed 3pLOH. The prevalence of *3pLOHn*, which showed that 6 of 11 (55%) locally recurrent cases, displayed *3pLOHn* compared with 7 of 37 (27%) cases that did not recur locally. This difference is statistically significant ($P = 0.0475$ by two-sided Fisher's exact test).

A Cox proportional hazards model was used to determine the association between *3pLOHn* and risk of local recurrence. Table 1 shows the ratios of the risk of recurrence for cases with *3pLOHn* to risk of recurrence for cases without *3pLOHn* (defined as the hazards ratio), taking into account several other factors associated previously with recurrence. The hazard ratios for local recurrence in the presence of *3pLOHn* ranged from 3.9 to 5.2 and were statistically significant (Table 1), even in the presence of the other confounding factors, *e.g.*, grade, tumor margin, and others. The increased freedom from recurrence in patients without *3pLOHn* is summarized in the Kaplan-Meier plot in Fig. 3.

We also compared the time to local recurrence for patients with and without *3pLOHn*. The mean time to recurrence for those with *3pLOHn* was 62.2 months, whereas without *3pLOHn* was 38.6

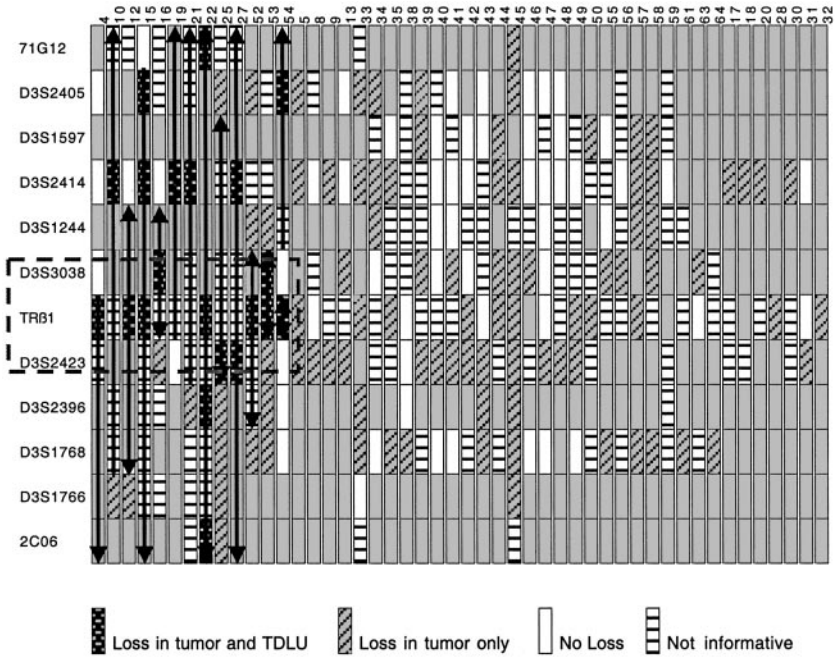

Fig. 2. Schematic representation of deletion mapping at chromosome 3p11–26 in matched pairs of microdissected tumor and morphologically normal adjacent TDLUs. The data shown are based on >300 independent allelic ratios in 48 cases of primary invasive breast cancer (1–3 DNA samples/case). The order of 3p loci is shown from telomere (*top*) to centromere (*bottom*). Data points represent results of ≥2 independent assays, each comprised of duplicate PCR reactions. Each column represents results of LOH analysis at multiple loci (indicated at the *left* of the diagram) on a single case (identified by a number at the *top*). The first 13 columns are cases, which displayed *3pLOHn*. *Arrows*, potential extent of *3pLOHn*. The *dashed rectangle* defines the boundary of *3pLOHn* encompassing the *TRβ1* locus.

Table 1 *Association between local recurrence and 3pLOHn*[a]

Factors in model	*n*	Hazard ratio	95% CI[b]	*P*	
				3pLOHn	Other
3pLOHn alone	48	4.00	(1.19–13.4)	0.025	N/A
3pLOHn + stage	47	4.53	(1.34–15.3)	0.015	0.54
3pLOHn + grade	48	4.03	(1.18–13.7)	0.026	0.22
3pLOHn + pos. nodes	47	5.22	(1.51–18.2)	0.009	0.39
3pLOHn + margin	48	4.10	(1.22–13.7)	0.022	0.59
3pLOHn + rad. Tx	47	3.94	(1.18–13.1)	0.026	0.60
3pLOHn + chemo. Tx	48	3.90	(1.16–13.1)	0.028	0.52
3pLOHn + hormo. Tx	47	4.56	(1.23–16.9)	0.023	0.92
3pLOHn + age	48	4.01	(1.20–13.4)	0.024	0.81

[a] Cox proportional hazards model.
[b] CI, confidence interval; pos., positive; rad. Tx, radiation therapy; chemo. Tx, chemotherapy; hormo. Tx, hormonal therapy.

months ($P = 0.068$ based on Wilcoxon rank sum test, two-sided). This suggests *de novo* tumor development in recurrences of patients with *3pLOHn*, which would ostensibly require a greater time interval.

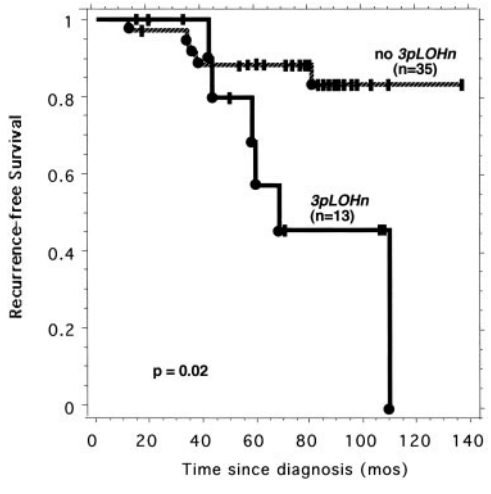

Fig. 3. Local disease-free survival in stage I and II lumpectomy patients by *3pLOHn* status. *Dashed line*, patients with no *3pLOHn*. *Solid line*, patients who displayed *3pLOHn*. *Solid circles*, local recurrences. *Vertical hashes*, cases with local disease-free status at last date of follow-up.

Because both curves were of cases known to have recurred, the average recurrence-free survival estimates will be less than those of unselected stage I and II patients.

Discussion

Increased risk of tumor recurrence has been associated with dissemination of tumor cells beyond the surgical margins, tumor size and grade, and age at diagnosis (reviewed in Ref. 9). Deng *et al.* (8) have suggested that the existence of localized LOH involving chromosome 3p22–26 in morphologically normal breast TDLUs surrounding cancers might predispose individuals to local recurrence. Evidence reported in this paper supports that hypothesis and narrows the region of most frequent loss to 3p24.3. Patients with *3pLOHn* in this region were at 4–5-fold higher risk of recurrence than in those patients where *3pLOHn* was not detected. Furthermore, *3pLOHn* remained a significant predictor of local recurrence even when considered with other risk factors such as age, grade, positive nodes, tumor margins, and radiation therapy often found to be significantly associated with outcome (10). This observation is consistent with the hypothesis that breast epithelial cells carrying LOH at 3p24.3 have increased susceptibility to additional "hits" that are necessary for tumor development. This model also explains our observation that the mean time to recurrence for patients with *3pLOHn* was significantly longer than in patients without *3pLOHn* (62.2 months *versus* 38.6 months) because tumors arising *de novo* would be expected to take longer to develop than those resulting from regrowth of residual tumor cells. Interestingly, in close agreement with our findings but using the approach of analyzing the local recurrences themselves, Haffty *et al.* (11) found that the mean time to recurrence in patients thought to have a new primary tumor was 67 months *versus* 42 months in those representing residual disease. More recent studies have shown a better overall survival for patients with new primary tumors than those with true recurrences (12).

These findings implicate a tumor suppressor gene in the region of LOH at 3p24.3 defined in this study. Known tumor suppressor genes on 3p include *FHIT* at 3p14.2 (13, 14) and *VHL* at 3p25 (15). However, neither of these genes is in the narrowly defined region of common deletion at 3p24.3 and thus unlikely to be involved in

3pLOHn-related risk. Candidate genes in the 3p24.3 region include *TRβ1* and *RARβ*. Notably, all cases of local recurrence associated with *3pLOHn* displayed LOH encompassing the *TRβ1* locus.

We speculate that the *3pLOHn* observed in this and a previous study (8) occurs early in breast development and is propagated throughout one or more branches of the mammary tree during breast development and maturation. The extent of breast tissue harboring LOH will depend on the timing of the initial genetic insult. For example, the acquisition of LOH before puberty could result in wide distribution throughout the breast tissue. In contrast, LOH occurring in the adult breast might be expanded during limited cycles of breast proliferation such as pregnancy and thus remain relatively confined. This is likely to vary among patients and remains to be determined. In this model, patients with *3pLOHn* would be at increased risk of developing breast cancer and would be at increased risk of local recurrence. Propagation of genetic aberrations conferring increased susceptibility during breast development could partially explain the increased incidence of breast cancer in atomic bomb survivors who were at a prepubertal stage when exposed (16). Although, the size of the present study is too small to immediately impact clinical practice, it provides a strong rationale for validation in larger independent patient populations. Simultaneously, technical improvements, which enable rapid detection of such deletions, for example in ductal lavage or nipple aspirates, must be sought. Concurrently, an understanding of the biological characteristics of tumors, which originate from the pathway involving allelic loss at chromosome 3p24.3, could provide improvements in prevention and control for this patient subset.

Acknowledgments

We thank Drs. Debu Tripathy, Mark Rounsaville, Kathleen Lamborn, and Gary Clark for critical reading of the manuscript.

References

1. Veronesi, U. The current status of breast-conserving therapy. Breast J., *2:* 100–102, 1996.
2. Veronesi, U., Luini, A., Galimberti, V., and Zurrida, S. Conservation approaches for the management of stage I/II carcinoma of the breast: Milan Cancer Institute trials. World J Surg., *18:* 70–75, 1994.
3. Ghossein, N. A., Alpert, S., Barba, J., Pressman, P., Stacey, P., Lorenz, E., Shulman, M., and Sadarangani, G. J. Breast cancer. Importance of adequate surgical excision prior to radiotherapy in the local control of breast cancer in patients treated conservatively. Arch. Surg., *127:* 411–415, 1992.
4. Silverstein, M. J., Lagios, M. D., Groshen, S., Waisman, J. R., Lewinsky, B. S., Martino, S., Gamagami, P., and Colburn, W. J. The influence of margin width on local control of ductal carcinoma *in situ* of the breast. N. Engl. J. Med., *340:* 1455–1461, 1999.
5. Fisher, E. R., Dignam, J., Tan-Chiu, E., Costantino, J., Fisher, B., Paik, S., and Wolmark, N. Pathologic findings from the National Surgical Adjuvant Breast Project (NSABP) eight-year update of Protocol B-17: intraductal carcinoma. Cancer (Phila.), *86:* 429–438, 1999.
6. Fisher, B., Dignam, J., Wolmark, N., Wickerham, D. L., Fisher, E. R., Mamounas, E., Smith, R., Begovic, M., Dimitrov, N. V., Margolese, R. G., Kardinal, C. G., Kavanah, M. T., Fehrenbacher, L., and Oishi, R. H. Tamoxifen in treatment of intraductal breast cancer: National Surgical Adjuvant Breast and Bowel Project B-24 randomized control trial. Lancet, *353:* 1993–2000, 1999.
7. Wazer, D. E., Schmidt-Ullrich, R. K., Ruthazer, R., Schmid, C., Graham, R., Safaii, H., Rothschild, J., and McGrath, J. Factors determining outcome for breast conserving irradiation with margin-directed dose escalation to the tumor bed. Int. J. Radiat. Oncol. Biol. Phys., *40:* 851–858, 1998.
8. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science (Wash. DC), *274:* 2057–2059, 1996.
9. Macmillan, R. D., Purushotham, A. D., and George, W. D. Local recurrence after breast-conserving surgery for breast cancer. Br. J. Cancer, *83:* 149–155, 1996.
10. Clark, G. M. Prognostic and predictive factors. *In:* J. R. Harris, M. E. Lippman, M. Morrow, and S. Hellman (eds), Diseases of the Breast, pp. 461–485. Philadelphia: Lippincott-Raven, 1996.
11. Haffty, B. G., Carter, D., Flynn, S. D., Fischer, D. B., Brash, D. E., Simons, J., Ziegler, A. M., and Fischer, J. J. Local recurrence *versus* new primary: clinical analysis of 82 breast relapses and potential applications for genetic fingerprinting. Int. J. Radiat. Oncol. Biol. Phys., *20:* 575–583, 1993.
12. Smith, T. E., Lee, D., Turner, B. C., Carter, D., and Haffty, B. G. True recurrence *vs.* new primary ipsilateral breast tumor relapse: an analysis of clinical and pathologic differences and their implications in natural history, prognoses, and therapeutic management. Int. J. Radiat. Oncol. Biol. Phys., *48:* 1281–1289, 2000.
13. 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.
14. Campiglio, M., Pekarsky, Y., Menard, S., Tagliabue, E., Pilotti, S., and Croce, C. M. FHIT loss of function in human breast cancer correlates with advanced stage of the disease. Cancer Res., *59:* 3866–3869, 1999.
15. Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F. M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., and Geil, L. Identification of the von Hippel-Lindau disease tumor suppressor gene. Science (Wash. DC), *260:* 1317–1320, 1993.
16. Pierce, D. A., and Mendelsohn, M. L. A model for radiation-related cancer suggested by atomic bomb survivor data. Radiat. Res., *152:* 642–654, 1999.