Immunohistochemical Detection of c-erbB-2 and p53 in Benign Breast Disease and Breast Cancer Risk

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Background: We studied the associations between c-erbB-2 protein overexpression and p53 protein accumulation in benign breast tissue and the risk of subsequent breast cancer. Methods: We conducted a case–control study nested within the cohort of 4888 women in the National Breast Screening Study (NBSS) who were diagnosed with benign breast disease during active follow-up. Case subjects were the women who subsequently developed breast cancer (ductal carcinoma in situ [DCIS] or invasive carcinoma). Control subjects were matched to each case subject on NBSS study arm, screening center, year of birth, and age at diagnosis of benign breast disease. Histologic sections of benign and cancerous breast tissues were analyzed immunohistochemically. Information on potential confounding factors was obtained by use of a self-administered lifestyle questionnaire. Results: Accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio [OR] = 2.55; 95% confidence interval [CI] = 1.01–6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR = 0.65; 95% CI = 0.27–1.53). The findings for c-erbB-2 and p53 did not differ among strata defined by menopausal status, allocation within the NBSS, history of breast disease, and whether the benign breast disease was detected at a scheduled screen or between screens. The results were also similar after exclusion of case subjects whose diagnosis of breast cancer occurred within 1 year of their diagnosis of benign breast disease and after exclusion of subjects with DCIS. Conclusions: p53 protein accumulation, but not c-erbB-2 protein overexpression, appears to be associated with an increased risk of progression to breast cancer in women with benign breast disease. [J Natl Cancer Inst 1998;90: 1262–9]

Evidence consistent with the multistage model of carcinogenesis has come from the genetic analysis of human tumors (1). Although cross-sectional in design, these studies have suggested that cancers showing multiple genetic abnormalities evolve from earlier morphologic stages showing only a subset of the same abnormalities (2,3). For breast cancer, the relevant histologic stages are unknown, although there is some suggestion that proliferative disease without atypia progresses to atypical ductal hyperplasia and then to ductal carcinoma in situ (DCIS) and invasive cancer (4). The accompanying sequence of molecular changes is not well characterized; however, of the many candidates early-molecular events, there is some evidence that changes in c-erbB-2 and p53 protein expression might be relevant to breast cancer progression. This hypothesis is based on the high frequency of alteration of c-erbB-2 and p53 in invasive breast cancers (5), detection of c-erbB-2 (6–9) and p53 (10–17) proteins in benign breast disease, and animal models that suggest a role for these genes in the early stages of breast tumorigenesis (18,19). If this presumption is correct, individuals identified with these molecular changes in benign breast tissue might be at increased risk of progression to breast cancer. In the cohort study reported here, we investigated this possibility by studying the association between c-erbB-2 protein overexpression and p53 protein accumulation detected in benign breast tissue and the risk of subsequent breast cancer.

Subjects and Methods

The investigation was undertaken as a case–control study nested within the cohort of 4888 women in the National Breast Screening Study (NBSS) who received a histopathologic diagnosis of benign breast disease during the active follow-up phase of the NBSS.

The National Breast Screening Study

The NBSS, which has been described in detail elsewhere (20,21), is a multicenter randomized, controlled trial of screening for breast cancer in 89,835 Canadian women who were recruited during the period from 1980 to 1985 and who were followed actively until 1988 (21). Women were eligible to participate if they were 40–59 years old and had no history of breast cancer (in situ or invasive). Those who were 40–49 years old at recruitment were randomly assigned to receive either an annual mammogram and physical examination or usual care after an initial physical examination, whereas those who were 50–59 years old at recruitment were randomly assigned to receive either an annual mammogram and physical examination or an annual physical examination only. The NBSS was approved by the appropriate ethics committees, and the study described here involved the analysis of patient and data from that study in accordance with the approved study design.
Diagnosis of Breast Disease in the NBSS

When there was clinical or radiologic evidence of a lesion, patients underwent either a needle aspiration or a biopsy. In the NBSS, each histologic diagnosis was reviewed for study purposes by a reference pathologist. Our study was restricted to subjects who had no evidence of breast cancer (in situ or invasive) on their initial surgical biopsy as determined on review by the reference pathologist. Women with a history of benign breast disease were not excluded from participation.

Incident cases of breast cancer were ascertained by record linkage with the provincial cancer registries, and death clearance was performed by linkage to the Canadian National Mortality Database (21). The dates of the linkages varied by province, ranging from late 1988 to early 1991.

Definition of Case Subjects

Case subjects were the 92 women who had a histologic diagnosis of benign breast disease made by a reference pathologist during the active follow-up phase of the NBSS and who subsequently developed breast cancer. (The median interval between diagnosis of benign breast disease and subsequent breast cancer was 767 days.) For the purpose of this study, cancer was defined as any form of breast carcinoma; there were 16 case subjects with DCIS and 76 case subjects with invasive carcinoma.

Definition and Selection of Control Subjects

Control subjects were women with benign breast disease who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case subject. Five control subjects were selected randomly for each case subject from those non-case subjects available within the strata defined by screening center, NBSS study arm, year of birth, and age at diagnosis of benign breast disease. (Implicitly, therefore, control subjects were matched to case subjects on the interval between the date of diagnosis of benign breast disease and the date of diagnosis of breast cancer in the corresponding case subject.)

Questionnaire

At the time of their enrollment in the NBSS, all participants completed a questionnaire that sought identifying information, as well as data on potential breast cancer risk factors, including demographic characteristics, family history of breast cancer, and menstrual and reproductive histories.

Histopathology Review

For this study, hospitals and clinics storing the paraffin-embedded blocks of benign and malignant tissues were contacted and asked to review the histology, to send one representative block per lesion, and to indicate fixative type and whether the tissue had been frozen before fixation. Blocks or sections of paraffin-embedded benign tissue were obtained for 74 (80.4%) of the 92 case subjects and for 349 (75.9%) of the 460 control subjects; blocks or sections of malignant tissue were obtained for 62 (83.8%) of the 74 case subjects. Sections from the blocks received were reviewed and classified according to the criteria developed by Page and Anderson (22) and without knowledge of the case–control status of the study subjects.

p53 and c-erbB-2 Immunostaining

Sections (5 μm) were cut from the paraffin blocks, mounted on slides coated with aminopropyltriethoxysilane (2%; Sigma Chemical Co., St. Louis, MO), and deparaffinized. The sections for p53 immunostaining underwent antigen retrieval (microwaved in 10 mM citrate buffer [pH 6.0] for 15 minutes at a medium-high setting). In all sections, the endogenous peroxidase was inactivated with the use of 3% hydrogen peroxide, and the sections were blocked with normal goat serum (20 μL/mL; Vector Laboratories, Inc., Burlingame, CA) containing 5% crystallized bovine serum albumin (BDH Laboratory Supplies, Poole, U.K.) in phosphate-buffered saline (PBS). The sections were incubated overnight at 4 °C with antibody react with p53 (DO-7, monoclonal, dilution 1 : 40; Novocastra Laboratories, Newcastle Upon Tyne, U.K.) or c-erbB-2 (NCL-CB11, dilution 1 : 160; Novocastra Laboratories) (23). After being washed, the sections were incubated with biotinylated goat anti-mouse immunoglobulin G (dilution 1 : 200; Vector Laboratories, Inc.) for 30 minutes at room temperature (21 °C), followed by incubation with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Inc.). Immunoreactivity was visualized with 3′,3′-diaminobenzidine tetrahydrochloride (Vector Laboratories, Inc.), and the sections were counterstained briefly with hematoxylin. For p53, the positive controls were sections from a paraffin-embedded breast cancer that was known to have a p53 mutation associated with p53 protein accumulation. Positive controls for c-erbB-2 were sections of SK-BR-3 cells, a human breast cancer cell line that overexpresses c-erbB-2 (24) and that had been processed as a cell block in paraffin, and sections of a paraffin-embedded breast cancer known to overexpress c-erbB-2. The negative controls consisted of replacement of the primary antibody either with PBS or with mouse nonimmune serum.

For p53, any nuclear staining was considered a positive reaction, and cytoplasmic staining was considered nonspecific and interpreted as negative. For c-erbB-2, any staining—cytoplasmic, cytoplasmic membranous, or membranous—was considered positive. Although the protein is predominantly membranous, the significance of cytoplasmic staining is not entirely clear (24–27). For this reason, any staining was accepted as positive, but the localization of the staining was noted. The percentage of immunopositive cells was estimated and categorized into one of two groups: less than 10% or 10% to greater than 50% of epithelial cells. Staining was considered localized if one duct with its associated ductules/lobules showed immunopositivity; all other positive immunostaining was considered diffuse. The slides were reviewed without knowledge of the case–control status of the study subjects.

The immunostaining was performed on the benign tissue of 383 subjects only (74 case subjects and 309 control subjects), since 40 control subjects were eliminated because tissue for the corresponding case subject was not received. For 21 of the 383 subjects (three case subjects and 18 control subjects), there was no breast epithelium in the tissue sections, rendering them unsuitable for immunohistochemical analysis. For an additional three control subjects, there was no epithelium in the p53-immunostained sections only.

For 30 benign and 18 malignant, randomly selected biopsy specimens, the slides were reread by the same reviewer without knowledge of the results of the first reading. For both c-erbB-2 and p53, there was agreement of about 93% for the presence or absence of these changes in the benign specimens. The corresponding values of κ [a measure of agreement beyond chance (28)] were 0.72 and 0.64, respectively, indicative of substantial agreement between readings (29). For the cancers, there was 89% agreement between readings for c-erbB-2, with a κ of 0.74; for p53, there was 100% agreement, with a κ of 1.00.

Statistical Analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for the associations among c-erbB-2 protein overexpression, p53 protein accumulation, and risk of breast cancer were obtained from conditional logistic regression models (30). Adjusted OR estimates were obtained by including terms representing the following potential confounders in the regression models: history of breast cancer in a first-degree relative, age at menarche, age at first live birth, menopausal status (premenopausal, perimenopausal, or postmenopausal), body mass index [weight (kg)/height (m)2], and hyperplasia (ductal or lobular, with or without atypia). Women who reported having had a menstrual period within the last year were defined as premenopausal, as were those who who had had a hysterectomy without bilateral oophorectomy and were less than 45 years of age; those who had ceased having menstrual periods within the last 12 months without surgical intervention were defined as postmenopausal, as were those who had had a bilateral oophorectomy and those who who had had a hysterectomy only and were more than 55 years of age; the remaining women were classified as perimenopausal. For categorical variables, tests for trend (on 1 degree of freedom) in associations were performed by fitting the categorized variables as continuous variables in conditional logistic regression models. Further analyses involved within-individual comparisons of c-erbB-2 and p53 in benign breast disease and breast cancer. All statistical tests were two-sided.

Results

A summary of the numbers of paraffin blocks requested and obtained and of the subjects included in the analyses is presented in Table 1. Comparisons of those subjects for whom benign tissue was and was not obtained revealed few differences between them in their distributions by breast cancer risk factors (data not shown); similarly, there was little difference between control subjects for whom blocks were received for the corresponding case subjects and those for whom they were not.
Table 1. Summary of number of biopsy specimens sought, obtained, and analyzed

<table>
<thead>
<tr>
<th>Benign tissue</th>
<th>Case subjects</th>
<th>Control subjects</th>
<th>Malignant tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of blocks requested</td>
<td>92</td>
<td>460</td>
<td>92</td>
</tr>
<tr>
<td>No. of blocks or sections obtained</td>
<td>74</td>
<td>349</td>
<td>62</td>
</tr>
<tr>
<td>No. of sections immunostained</td>
<td>74</td>
<td>309</td>
<td>62</td>
</tr>
</tbody>
</table>

For analysis:
- c-erbB-2: 71 sections
- p53: 71 sections

*These numbers are less than those for whom immunostaining was performed because there was no breast epithelium in the tissue sections for three case subjects and 18 control subjects for both c-erbB-2 and p53 and for an additional three control subjects for p53 only. The number of case subjects and control subjects included in the matched analyses was less than the number with immunostained sections suitable for analysis because one case subject was in a stratum with no control subjects and 17 control subjects were in strata with no corresponding case subjects.

Table 2 shows the risk of breast cancer in relation to several factors for those subjects in whom immunostaining was performed. None of the variables was strongly associated with altered risk, but there was some suggestion of a reduction in risk with a family history of breast cancer, a relatively late age at menarche, a history of a live birth (regardless of the age at live birth), and postmenopausal status. There was also a small increase in risk with hyperplasia in the benign tissue. For this analysis, the one control subject with lobular hyperplasia and the three control subjects with atypical ductal hyperplasia were combined with those with ductal hyperplasia without atypia; there were no case subjects with atypical hyperplasia. When the associations shown in Table 2 were examined in all 552 subjects, they were largely unchanged, but there was less evidence for a reduction in risk with a family history of breast cancer (OR = 0.97; 95% CI = 0.55–1.73).

The tissue from nine case subjects and 40 control subjects showed immunostaining for c-erbB-2 (Fig. 1). There was a small decrease in the risk of progression from benign breast disease to breast cancer in association with c-erbB-2 protein overexpression, although the CIs included one (Table 3). ORs differed little according to the extent, location (cytoplasmic or membranous, with or without cytoplasmic staining), or distribution of immunostaining. For those subjects with hyperplasia and with c-erbB-2 immunostaining anywhere in their biopsy, the unadjusted OR was 1.13 (95% CI = 0.43–2.96) and the adjusted OR was 1.00 (95% CI = 0.34–2.97).

The tissue from 10 case subjects and 19 control subjects showed immunostaining for p53 (Fig. 2). After adjustment for breast cancer risk factors, there was a 2.5-fold increase in risk of breast cancer in association with p53 protein accumulation (Table 4). The increase in risk was evident when less than 10% of cells were immunopositive but was even greater when 10% or more were immunopositive. ORs for localized immunostaining and diffuse immunostaining were similar, but the risk was higher in those subjects where staining for p53 was observed in the same area as that for c-erbB-2 than in those subjects where it was not. In the presence of hyperplasia, p53 immunostaining anywhere in the biopsy specimen was associated with an approximately fourfold increase in risk (unadjusted OR = 4.62 [95% CI = 1.02–20.94]; adjusted OR = 3.87 [95% CI = 0.72–20.64]).

The associations for c-erbB-2 and p53 were similar after ex-
clusion of the 19 case subjects (and their matched control subjects), whose diagnosis of breast cancer was made within 1 year of their diagnosis of benign breast disease. When the analyses were restricted to the matched case–control sets containing case subjects with invasive breast cancer (i.e., after exclusion of the 14 case subjects with DCIS and their matched control subjects), the unadjusted OR for c-erbB-2 immunopositivity was 0.68 (95% CI = 0.24–1.88) and the adjusted OR was 0.60 (95% CI = 0.21–1.73), whereas the unadjusted and adjusted ORs for p53 were 2.85 (95% CI = 1.04–7.82) and 2.51 (95% CI = 0.88–7.21), respectively; with the exception of the unadjusted OR for p53, these ORs were not statistically significant. Also, when the 23 case subjects whose benign and malignant lesions occurred in opposite breasts were excluded, the unadjusted ORs for c-erbB-2 and p53 immunopositivity were 0.90 (95% CI = 0.35–2.27) and 2.11 (95% CI = 0.73–6.06), respectively. The corresponding adjusted ORs were 0.77 (95% CI = 0.29–2.04) and 1.93 (95% CI = 0.61–6.07), respectively. There was no evidence that the patterns for c-erbB-2 and p53 immunostaining differed among the strata defined by age, menopausal status, NBSS study arm, history of breast disease, and whether the benign breast disease was screen detected or interval detected.

When the analyses for c-erbB-2 and p53 were repeated according to whether individuals showed c-erbB-2 protein overexpression or p53 protein accumulation alone or in combination, the unadjusted ORs relative to those for individuals who were negative on both were 0.56 (95% CI = 0.20–1.56) for c-erbB-2 alone, 2.50 (95% CI = 0.86–7.27) for p53 alone, and 2.55 (95% CI = 0.55–11.76) for those who were immunopositive for both. The corresponding adjusted ORs were 0.51 (95% CI = 0.18–1.45), 2.43 (95% CI = 0.79–7.50), and 1.98 (95% CI = 0.40–9.90).

Table 5 shows the concordance between the immunohistochemical findings for the benign and malignant tissues for the case subjects. Of the 48 subjects who were negative for c-erbB-2 protein overexpression in their benign tissue samples, 15 showed evidence of overexpression in their malignant tissue samples; for p53 protein accumulation, the corresponding figure was 23% (10/44). Both of those subjects who showed evidence of c-erbB-2 protein overexpression in their benign tissue samples retained evidence of overexpression in their malignant tissue samples, whereas two subjects whose benign tissue samples showed p53 protein accumulation had p53-negative cancers.

**Discussion**

The results of this cohort study suggest that p53 protein accumulation as determined immunohistochemically in benign breast disease is associated with increased risk of progression to breast cancer; c-erbB-2 protein overexpression did not show this association. Since the patient characteristics were collected before 1986, there was insufficient information to determine whether any of the study subjects had Li–Fraumeni syndrome. However, it is unlikely that the presence of this syndrome accounts for our findings for p53 because the syndrome is uncom-

### Table 3. Association between c-erbB-2 protein overexpression and risk of breast cancer

<table>
<thead>
<tr>
<th>Aspect of staining</th>
<th>Level</th>
<th>No. of case subjects*</th>
<th>No. of control subjects</th>
<th>Unadjusted‡</th>
<th>Adjusted§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence</td>
<td>Absent</td>
<td>62</td>
<td>251</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>9</td>
<td>40</td>
<td>0.73 (0.31–1.68)</td>
<td>0.65 (0.27–1.53)</td>
</tr>
<tr>
<td>% cells immunopositive</td>
<td>Absent</td>
<td>62</td>
<td>251</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&lt;10</td>
<td>5</td>
<td>20</td>
<td>0.76 (0.25–2.34)</td>
<td>0.74 (0.23–2.39)</td>
</tr>
<tr>
<td></td>
<td>≥10</td>
<td>4</td>
<td>20</td>
<td>0.69 (0.22–2.17)</td>
<td>0.57 (0.18–1.83)</td>
</tr>
<tr>
<td>Location</td>
<td>Absent</td>
<td>62</td>
<td>251</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cytoplasm</td>
<td>6</td>
<td>26</td>
<td>0.74 (0.25–2.17)</td>
<td>0.59 (0.19–1.82)</td>
</tr>
<tr>
<td></td>
<td>Membrane¶</td>
<td>3</td>
<td>14</td>
<td>0.71 (0.20–2.55)</td>
<td>0.73 (0.20–2.72)</td>
</tr>
<tr>
<td>Distribution</td>
<td>Absent</td>
<td>62</td>
<td>251</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Localized</td>
<td>6</td>
<td>23</td>
<td>0.80 (0.29–2.21)</td>
<td>0.74 (0.26–2.12)</td>
</tr>
<tr>
<td></td>
<td>Diffuse</td>
<td>3</td>
<td>17</td>
<td>0.63 (0.17–2.26)</td>
<td>0.53 (0.14–1.96)</td>
</tr>
</tbody>
</table>

*Unmatched distributions (matched odds ratios cannot be calculated directly from these numbers).
†OR = odds ratio; CI = confidence interval.
‡Adjusted for matching factors only (with the use of conditional logistic regression).
§Adjusted for variables in Table 1.
¶Reference category.
¶Membranous staining only or membranous and cytoplasmic staining were combined because only one subject (a control) had purely membranous staining.
mon and immunostaining for p53 was confined to epithelial cells, which suggests that the abnormalities observed were somatic and not germline (13). Furthermore, only one of the 10 p53-immunopositive case subjects had a family history of breast cancer in a first-degree relative.

There have been two previous follow-up studies of protein changes in benign breast disease. One (16) showed no association between p53 protein accumulation and the risk of breast cancer, whereas the other (31) suggested indirectly that p53 and c-erbB-2, as part of a group of biomarkers, might be associated with risk. However, both studies were small and adjusted for confounding either incompletely (31) or not at all (16). Nevertheless, our findings are in keeping with those of Fabian et al. (31).

It is possible that our findings reflect selection bias resulting from the study of surgical biopsies only and not fine-needle aspirates. However, it seems unlikely that the mode of diagnosis would have been related to exposure (protein status). Furthermore, it is unlikely that bias arose from the selection of subjects from the benign breast disease cohort, given that both case subjects and control subjects were sampled independently of exposure and that those for whom blocks of benign tissue were and were not obtained differed little with respect to other variables. We did observe that older blocks (i.e., those collected in the early years of the NBSS) were more likely to have been discarded, but selection bias resulting from a relationship between the year of collection and immunostaining status seems unlikely. Also, although it is possible that p53 and c-erbB-2 immunopositivity might be more likely in larger lesions and that the larger the benign lesion the greater the breast cancer risk, we observed no relationship between our success in obtaining paraffin blocks and the size of the lesions from which the paraffin blocks were obtained.

### Table 4. Association between p53 protein accumulation and risk of breast cancer

<table>
<thead>
<tr>
<th>Aspect of staining</th>
<th>Level</th>
<th>No. of case subjects*</th>
<th>No. of control subjects</th>
<th>Unadjusted†</th>
<th>Adjusted§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence</td>
<td>Absent</td>
<td></td>
<td>61</td>
<td>269</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td></td>
<td>10</td>
<td>19</td>
<td>2.79 (1.16–6.73)</td>
</tr>
<tr>
<td>% cells immunopositive</td>
<td>Absent</td>
<td></td>
<td>61</td>
<td>269</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&lt;10</td>
<td>5</td>
<td>12</td>
<td>2.28 (0.74–7.04)</td>
<td>1.96 (0.60–6.47)</td>
</tr>
<tr>
<td></td>
<td>&gt;10</td>
<td>7</td>
<td></td>
<td>3.67 (1.03–13.04)</td>
<td>3.60 (0.96–13.49)</td>
</tr>
<tr>
<td>Distribution</td>
<td>Absent</td>
<td></td>
<td>61</td>
<td>269</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Localized</td>
<td>6</td>
<td>13</td>
<td>2.68 (0.86–8.32)</td>
<td>2.41 (0.73–7.93)</td>
</tr>
<tr>
<td></td>
<td>Diffuse</td>
<td>4</td>
<td>6</td>
<td>2.96 (0.78–11.28)</td>
<td>2.76 (0.69–11.07)</td>
</tr>
<tr>
<td>Same area as c-erbB-2</td>
<td>Absent</td>
<td></td>
<td>61</td>
<td>269</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Not same</td>
<td>2</td>
<td>3</td>
<td>2.27 (0.37–13.90)</td>
<td>1.48 (0.22–9.81)</td>
</tr>
<tr>
<td></td>
<td>Same</td>
<td>8</td>
<td>16</td>
<td>2.98 (1.08–8.18)</td>
<td>3.03 (1.05–8.72)</td>
</tr>
</tbody>
</table>

*Unmatched distributions (matched odds ratios cannot be calculated directly from these numbers).
†OR = odds ratio; CI = confidence interval.
‡Adjusted for matching factors only (with the use of conditional logistic regression).
§Adjusted for variables in Table 1.
||Reference category.

### Table 5. Concordance between immunostaining results for benign and malignant tissues*

<table>
<thead>
<tr>
<th>Marker</th>
<th>% with negative immunostaining of benign and malignant tissues (No.)</th>
<th>% with negative immunostaining of benign tissue and positive immunostaining of malignant tissue (No.)</th>
<th>% with positive immunostaining of benign tissue and negative immunostaining of malignant tissue (No.)</th>
<th>% with positive immunostaining of benign and malignant tissues (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-erbB-2</td>
<td>66.0 (33)</td>
<td>30.0 (15)</td>
<td>0 (0)</td>
<td>4.0 (2)</td>
</tr>
<tr>
<td>p53</td>
<td>66.7 (34)</td>
<td>19.6 (10)</td>
<td>3.9 (2)</td>
<td>9.8 (5)</td>
</tr>
</tbody>
</table>

*Percentages are percent of all subjects for whom both benign and malignant tissues are available. Of the 62 cases for whom blocks of benign and malignant tissues were obtained, two blocks of benign tissue and 10 blocks of malignant tissue were unsuitable for analysis for c-erbB-2; for p53, the corresponding numbers were two and nine.
Biased assessment of the study exposures is an unlikely source of error, given that protein status of the benign breast tissue was determined without knowledge of the patient outcome (and the slides prepared from malignant tissue were read without knowledge of the findings from the benign tissue) and that the assessments were highly repeatable. Misclassification of the immunohistochemical staining results might have arisen from various sources. First, fixative type and/or duration may affect the immunoreactivity of the p53 and c-erbB-2 proteins (32–34). However, there was little difference between case and control subjects in the distribution of their blocks by fixative type, and additional adjustment for type of fixative had little effect on the OR estimates for p53 and c-erbB-2. We were not able to control for the length of tissue fixation. Second, misclassification might have occurred also for the six case subjects and 14 control subjects for whom slides were obtained rather than paraffin blocks. For example, p53 immunoreactivity has been shown to decrease with the duration of storage of cut slides (35). However, deletion of these case subjects from the analyses had little impact on the results, and it has been shown recently that antigen retrieval, which was used in the present study, diminishes the loss of immunoreactivity (36). Third, it is possible that low levels of overexpression were not detected because of the limited sensitivity of the antibodies (33,37). Fourth, underestimation of immunopositivity might have occurred because only one block per subject was examined. Given that the most likely effect of all of these parameters on marker status is to have induced false-negatives (38) and that any misclassification arising from these sources is likely to have been nondifferential, the estimates of association would have been biased conservatively.

Misclassification of the histopathology of the initial (benign) biopsy specimen may have occurred. For example, it is possible that some case subjects had cancers that were not sampled at the time of the initial biopsy or were not represented in the block examined in this study. We addressed these issues in part by excluding those case subjects whose cancer was diagnosed within 1 year of the biopsy for benign breast disease; in so doing, the results were similar to those overall.

Although DCIS is considered by some to be a precursor of invasive breast cancer (39), we included case subjects with this diagnosis in the cancer group because the treatment (surgical resection and radiotherapy) of this condition is similar to that of invasive carcinoma (40), and we could not exclude the possibility of cancer elsewhere in the breast. Further support for differentiating DCIS from benign states and for grouping it with invasive carcinoma comes from the observations that similar proportions of DCIS and invasive carcinoma show cyclin-D overexpression and that these proportions are higher than those for various grades of benign and hyperplastic human breast lesions (41).

Confounding is a relatively unlikely explanation for the study findings, given that the ORs were adjusted for many potential confounding variables, although the possibility of residual confounding by these and other variables cannot be excluded. Nevertheless, since it is not known whether factors such as those adjusted for in the present analyses operate by altering the c-erbB-2 and p53 genes or their expression or whether they influence breast cancer risk by other means, it is not clear that the adjusted ORs are more appropriate than the unadjusted ones. In this regard, however, it should be noted that the adjusted and unadjusted associations were mostly quite similar.

The statistical power of our study was somewhat limited, given the relatively small number of individuals who had disease that progressed to breast cancer by the end of the follow-up period. Although to some extent this problem was compensated for by selecting multiple control subjects per case subject, the statistical power was compromised further by the fact that we did not obtain benign tissue for all of the potentially eligible study subjects. Indeed, given the number of study subjects included in the analyses and the observed proportion of control subjects whose tissues showed immunostaining for c-erbB-2, the statistical power of the study to detect an OR of 2 at the two-sided 5% significance level was about 58%. For p53, the corresponding statistical power was about 37%, given the observed proportion of control subjects whose tissues displayed p53 immunostaining. [These calculations were based on formulae for unmatched case–control studies, so that the power of our study was probably somewhat higher, given the matching (42).] Furthermore, our study had little power to detect differences in the associations between strata defined by menopausal status and other factors.

c-erbB-2 is a normal cellular gene present on chromosome 17q21 (43,44). It encodes a membrane protein (p185), which is tyrosine phosphorylated following interaction with its ligands (43). Overexpression of c-erbB-2 occurs either through changes in amplification and/or through messenger RNA (mRNA) overexpression (44). Its role in the development of human breast cancer is unknown (46). In the present study, there was little alteration in risk with positive immunostaining overall or by location. These results were essentially unchanged when the analyses were repeated after those with cytoplasmic staining were reclassified as negative (unadjusted OR for positive immunostaining = 0.72 [95% CI = 0.20–2.58]; adjusted OR = 0.74 [95% CI = 0.20–2.76]). The lack of association of risk with cytoplasmic staining is consistent with studies showing that breast cancer cells with cytoplasmic staining are more differentiated than those with membranous staining (47). Our results do not support a role for immunohistochemically detected c-erbB-2 protein overexpression at a relatively early stage of tumor formation. However, they do not preclude roles for c-erbB-2 gene amplification and/or mRNA overexpression or stabilization.

p53 is involved in regulating cell proliferation, inducing apoptosis, and promoting chromosomal stability. Disruption of these functions appears to have a pivotal role in carcinogenesis (48). Our findings are in keeping with a role for p53 protein accumulation in breast cancer development. However, by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer in association with p53 changes. For example, not all p53 mutations result in positive immunostaining (38). Therefore, a more complete assessment of the role of p53 in influencing breast cancer risk will come from studies combining both immunohistochemistry and p53 gene sequencing, as well as from studies of other mechanisms by which p53 can be functionally inactivated.

To a large extent, the protein changes observed in the benign tissue were evident also in the corresponding cancers. This observation provides some support for the notion that these changes might occur at a relatively early stage in the disease.
process, but it does not necessarily indicate that these changes influence the disease process. The fact that the cancers of two subjects showed evidence of apparent reversion of the p53 protein accumulation in their benign tissue could reflect methodologic problems related either to tissue fixation or to tumor sampling. However, there is evidence from cross-sectional studies for the disappearance from tumors of molecular changes present at an earlier stage. For example, high expression of the progesterone receptor has been observed in proliferative disease without atypia in the absence of expression in a concurrent DCIS (49).

In conclusion, our results suggest that p53 protein accumulation in benign breast disease is associated with increased risk of subsequent breast cancer (DCIS or invasive carcinoma). Since the investigations reported here can be done on cytologic material obtained from fine-needle aspirates of the breast (31), they could possibly be used as a screening tool in conjunction with mammography. In particular, for those women with benign breast disease who are identified as being at increased risk of progressing to breast cancer (on the basis of their status with respect to one or more of the molecular markers), closer follow-up and perhaps early intervention might be warranted.

References


Notes

Supported by the Canadian Breast Cancer Research Initiative. T. E. Rohan is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada supported with funds from the Terry Fox Run.

We thank Gaby Nagy and Mary Speagle for providing excellent technical assistance and Andrew White for preparing the data file for analysis. We are indebted to the pathologists from across Canada who provided us with tissue for the study.

Manuscript received March 17, 1998; revised June 18, 1998; accepted July 2, 1998.