A Basal Epithelial Phenotype Is More Frequent in Interval Breast Cancers Compared with Screen Detected Tumors


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Abstract

Interval breast cancer reduce the effectiveness of mammography screening programs. We studied 95 interval cancers, diagnosed between 1996 and 2001 as part of the population-based Norwegian Breast Cancer Screening Program. These cases were matched on size (±2.0 mm) to 95 screen-detected breast cancers, and the tumors were compared by immunohistochemical methods using tissue microarrays. Patients with interval cancers were more likely to be younger (odds ratio [OR], 4.7; \( P = 0.0001 \)) to have dense breasts (OR, 3.4; \( P = 0.004 \)), and to have estrogen receptor–negative and p53 expression was more frequent (OR, 4.0; \( P = 0.001 \)). Notably, interval cancers were more likely to have a basal epithelial phenotype, in that expression of cytokeratin 5/6 (OR, 2.3; \( P = 0.04 \)) and P-cadherin (OR, 2.5; \( P = 0.04 \)) was more frequent in interval cancers than in size-matched, screen-detected tumors. In a logistic regression model, p53 expression, age, and breast density were independent predictors of interval cancers. Our data suggest that breast cancers with a basal epithelial phenotype are more likely than nonbasal breast cancers to present between regular mammograms. (Cancer Epidemiol Biomarkers Prev 2005;14(5):1108–12)

Introduction

Mammography is the most commonly used method for breast cancer screening. Women who develop breast cancer between screening intervals are younger (1, 2) and have denser breasts (3, 4) than those who are diagnosed at scheduled screens. The tumors are larger and more often have a lobular (1, 3, 5) or medullary histology (2), and they are more frequently lymph node positive (6). Furthermore, these tumors tend to possess aggressive biological features, such as increased tumor cell proliferation (7, 8), estrogen receptor (ER)–negative status, and p53 expression (1, 8), and a difference in c-erbB-2 amplification has been reported between interval and screen-detected cancers (9). These results may indicate that interval cancers have a faster growth rate than cases detected by screening mammography.

Recently, it has become evident that a molecular classification, partly based on gene expression patterns, is possible in breast cancer patients (10-13). A basal-like subgroup staining positive for cytokeratin 5/6 and being associated with aggressive biological markers such as frequent TP53 mutations, has been distinguished from a larger luminal epithelial subgroup characterized by ER-positive tumors with a better outcome. We (14) and others (15) have shown that breast cancers arising in BRCA1 mutation carriers are more likely to express cytokeratin 5/6 indicating a basal epithelial phenotype. Expression of P-cadherin, another myoepithelial cell marker (16, 17) is also increased in BRCA1-related breast cancers (17) and is related to tumors that do not express ER or progesterone receptor (PR; refs. 18, 19).

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The current study was designed to establish whether, in a national mammographic screening program, different modes of detection (i.e., mammographic screening and interval presentation) select for categories of breast cancer that may represent biologically distinct subgroups, rather than representing size differences and changes in the molecular phenotype of tumors as a function of growth, known as phenotypic drift (20-22). Especially, we wanted to find out whether breast cancers exhibiting a basal-like phenotype are more likely to present as interval rather than screen-detected tumors.

Materials and Methods

Patient Series. We conducted a nested case-control study as part of the Norwegian Breast Cancer Screening Program, which started in 1996 with two-view mammography done every 24 months (23, 24). In the County of Nordland (Western Norway, 10% of the Norwegian population, ~450,000 inhabitants), a total of 80,399 patients were invited during the study period from 1996 to 2001; attendance rate was 85.4% and 84.6% during the first two screening rounds. During the first two intervals, 155 invasive interval cancers occurred (median size, 2.0 mm). A total of 317 invasive screen-detected cancers were reported during the first and second screening rounds (median diameter, 15.6 and 15.7 mm, respectively). Each of the 95 consecutive interval cancers was then matched to screen-detected invasive cancers in the same round by tumor size (±2.0 mm).

Basic Variables. Basic characteristics were recorded [i.e., age, largest tumor diameter, histologic type, histologic grade (25), lymph node metastases, and breast density]. The size of the tumors was obtained from pathology reports in 85.2% of the cases. However, if pathologic tumor size was not available (as in patients with locally advanced disease, patients presenting with systemic disease at time of diagnosis, or patients with multifocal disease), the radiologic size estimate was included (14.8%). Breast density was assigned according
to the following scale: (i) lucent, <30% glandular tissue; (ii) intermediate, 30% to 70% glandular tissue; and (iii) dense, >70% glandular tissue (23). All cases were reviewed by one experienced radiologist (A.B.); 44 cases (46.3%) were true cases (i.e., the screening mammogram was negative), but the tumor was visible at the time of diagnosis. In 12 cases (12.6%), tumors were not visible at time of screening or at time of diagnosis (transparency cases); these tumors were smaller than the interval group as a whole (median size, 11.0 mm). No significant differences were found for markers of the basal phenotype, p53, c-erbB-2, Ki-67, or histologic grade when these tumors were compared with their screen-detected counterparts (data not shown). In 20 cases (21.1%), slight or minimal changes were seen on the screening mammogram, but the findings were not diagnostic at time of screening. Fifteen cases (15.8%) were missed or overlooked. In three cases (3.2%), the tumors were outside the range of the mammogram. The median time from the last mammogram to the diagnosis of interval cancer was 17.1 months.

**Immunohistochemical Staining.** Immunohistochemistry was applied on tissue microarray slides to assess the molecular markers. The tissue-conserving tissue microarray technique has been validated in several studies (26, 27). The method has been found reliable and reproducible in breast cancers for markers such as ER, PR, and c-erbB-2 (28, 29). Tissue microarray slides were used for Ki-67, c-erbB-2, P-cadherin, P53, P-cadherin, and cytokeratin 5/6, whereas ER and PR were obtained from the routine pathology reports (based on immunohistochemistry).

The immunohistochemical staining was done on formalin-fixed and paraffin-embedded archival tissues (5-µm sections). Samples were dewaxed with xylene/ethanol before microwave antigen retrieval and antibody incubation. Regarding epitope retrieval, the conditions were optimized for each antibody.

**Ki-67.** Microwave antigen retrieval was done for 20 minutes in citrate buffer at 500 W. The slides were incubated for 1 hour at room temperature with a monoclonal rabbit Ki-67 antibody (M7240, clone MB-1, DAKO, Carpinteria, CA) at a 1:50 dilution.

**P53.** Microwave antigen retrieval was done for 20 minutes in citrate buffer at 500 W. The slides were incubated for 1 hour at room temperature with a monoclonal rabbit p53 antibody (M7001, clone DO-7, DAKO) at a 1:100 dilution.

**C-erbB-2.** The procedures for the standardized DAKO HercepTest (Code no. K5204) were followed as previously recommended (30).

**P-cadherin.** Microwave antigen retrieval was done for 15 minutes in Tris/EDTA (pH 9) at 500 W. The slides were incubated for 1 hour at room temperature with a monoclonal antibody for P-cadherin (clone 56, BD Transduction Laboratories, Lexington, KY) at a 1:400 dilution.

**Cytokeratin 5/6.** Microwave antigen retrieval was done for 15 minutes in citrate buffer. The slides were incubated for 25 minutes at room temperature with a monoclonal antibody for cytokeratin 5/6 (M7237, clone D5/16 B4) at a 1:25 dilution. For Ki-67, p53, and cytokeratin 5/6, staining was done on a DAKO TechMate 500 slide processing equipment (DakoCytomation, Copenhagen, Denmark) using the standard avidin-biotin method. The peroxidase was localized by the dianinobenzidine tetrachloride peroxidase reaction with Harris hematoxylin as counterstain. Staining for P-cadherin was done on a DAKO Autostainer. Immunoperoxidase staining was carried out using the Envision Kit (DAKO) with dianinobenzidine tetrachloride peroxidase as a substrate before counterstaining with hematoxylin.

**Evaluation of Staining.** For all markers with the exception of Ki-67 and c-erbB-2, staining was recorded by a semiquantitative and subjective grading system, considering the intensity of staining and the proportion of tumor cells showing a positive reaction. Regarding the tissue microarray slides, all cores (three, six, or nine cores depending on the amount of fibrous tissue) from each patient were evaluated. A staining index (values, 0-9) was obtained as a product of staining intensity (0-3) and proportion of immunopositive cells (10%, 1; 11-50%, 2; >50%, 3; refs. 14, 31, 32). Ki-67 staining (nuclear reactivity) was assessed according to the approach of Weidner et al. (33), counting the proportion of positive tumor cell nuclei in the most active (positive) area. C-erbB-2 immunostaining was scored according to the HercepTest criteria (34), considering the intensity and degree of membranous reaction.

For further analyses, cut points were based mainly on the distribution plots. For Ki-67 staining, the upper quartile (22.5%) was chosen as cut point. This is in accordance with Gilliland et al. defining tumors with a high proportion of proliferating cells to >20% (8). C-erbB-2 immunostaining was scored according to the HercepTest criteria (34). For p53 and P-cadherin, a positive staining was set to staining index 4 and above (>3), whereas for cytokeratin 5/6, index 0 (negative) versus index >0 (positive) was compared (14).

Staining was recorded independently by two observers (K.C. and I.S.), and the interobserver agreement (κ coefficient) between negative and positive cases was 0.90, 0.74, 0.70, and 0.76 and for p53, c-erbB-2, P-cadherin, and cytokeratin 5/6, respectively.

**Statistical Methods.** Associations between different categorical variables were assessed by Pearson’s χ² test. All statistical analyses were adapted to matched-pair data. McNemar’s test was used for hypothesis testing of the dichotomous variables. The statistical calculations were done by SPSS 12.0 with the exception of conditional logistic regression analysis for which LogXact 5.0 was used. When adjusting for age and breast density, these variables were entered as dichotomous variables (age cut point, 60 years; low to moderate density versus high); 60 years was chosen as cut point being the mean and median value. For estimation of the confidence limits for odds ratios, special programming in Maple 8 was applied (35).

**Results**

Patients with interval cancers were younger and more likely to present with high breast density than their screen-detected counterparts (Table 1). There were no significant differences between the two groups with respect to histologic grade, nodal status, locally advanced disease, or distant spread. As expected, a somewhat higher frequency of lobular carcinoma was found among interval cancers. As previously shown, positivity for ER and PR was more frequent among screen-detected cases. Expression of cytokeratin 5/6 and P-cadherin, markers of the basal-like phenotype (10, 16), had 2.3 and 2.5 times higher odds of appearing among interval cancers, respectively (Table 1). The most significant difference between interval and screen-detected cases was found for p53. Patients with interval cancers had 4.0 times higher risk for showing positive expression for p53 than screen-detected cancers. p53 (P < 0.0001), P-cadherin (P < 0.0001), and cytokeratin 5/6 expression (P < 0.0001) were all significantly associated with increased Ki-67 staining. When only true interval cases were included (44 cases), pairwise testing still revealed significant differences for p53 (P = 0.02), cytokeratin 5/6 (P = 0.02), P-cadherin (P = 0.04), ER (P = 0.03), and PR (P = 0.04; data not shown). C-erbB-2 expression was not significantly different
between the two groups, although this marker tended to be more frequently positive among interval cases. In a multiple conditional logistic regression analysis including ER, PR, Ki-67, p53, c-erbB-2, P-cadherin, and cytokeratin 5/6, age and breast density, significant and independent information was given by p53, age, and breast density. Multivariate odds ratios for these three variables are given in Table 1.

The expression of cytokeratin 5/6 and P-cadherin was significantly associated with p53 status in both screen-detected and interval cancers (Fisher’s exact test, \( P < 0.024 \) for all). No significant associations were found between breast density and cytokeratin 5/6, P-cadherin, ER, or PR.

Table 2 shows the distributions according to different markers for interval cancers clinically detected within the first year of the screening interval, compared with the second year. The majority of interval cases (78.9%) presented in the second year. However, interval cancers that expressed markers of the basal-like phenotype were as likely to present during the first as in the second year. Those that expressed c-erbB-2 and with a high breast density, presented more often in the first year after screening than in the second year.

When interval cases were compared separately with matched screen-detected cases from the first (prevalence) round of screening and the second (incidence) round cases, McNemar’s test indicated the differences to be more pronounced for the prevalence than the incidence round. However, logistic regression analysis showed the differences between the first and second round to be significant for Ki-67 only (\( P = 0.047 \)). The effect of this finding is limited by the small numbers in the subgroups.
P-cadherin is expressed in basally positioned cells in the normal breast (16), and expression of this marker in breast cancer has been associated with a poor outcome (19). It therefore seems that the basal phenotype is a distinct, biologically relevant, subgroup of breast cancer, with more aggressive and rapidly growing tumors that may present as interval cancers. As there are no correlations between breast density and cytokeratin 5/6, P-cadherin or hormone receptor status, these findings support the hypothesis that basal-like tumors seems rapidly and apparently independent of breast density.

Both cytokeratin 5/6 and P-cadherin were significantly associated with p53 status, and p53 was the strongest molecular marker to discriminate between interval and screen-detected cases, as indicated by multivariate analysis. The same was found when true interval cancers were considered separately. Thus, age, breast density, and p53 were the only variables to independently discriminate between interval and screen-detected cancers. The higher proportion of mammographic failure among younger women (1, 8) and those with dense breasts (4) have previously been emphasized. The loss of significance of cytokeratin 5/6, P-cadherin, Ki-67, ER, and PR in the multivariate model may be explained by strong associations between these covariates and p53, which is probably a major determinant of the basal-like subgroup of breast carcinomas (36). Furthermore, loss of TP53 function eliminates the growth arrest response to DNA damage and impairs the apoptotic programme. Consequently, tumors with TP53 mutations may have a higher frequency of mutations in other genes and this might also contribute to explain the higher proliferative rate of interval cancers (1, 7, 8, 43), as found in the present study. Notably, interval cases were as likely to express c-erbB-2 as screen-detected cancers, as found previously (1), but c-erb B-2-positive cases tended to occur more often in the first year of the 2-year screening interval. This is relevant, because breast tumors that do express c-erbB-2 might be classified as a distinct group (10).

This study shows the differences between interval cancers and screen-detected cases to be more pronounced for the prevalence cases than the incidence tumors; however, the differences were only significant for Ki-67, indicating more slowly growing breast cancers in the first screening round. This has, to our knowledge, not previously been reported. The basal phenotype (in contrast to markers of proliferation) thus remained an identifiable feature of interval cancers when incident, rather than prevalent screen-detected cancers were compared. This suggests that specific pathways, determined early in the life of the cancer, rather than simple growth rates, may determine whether or not a breast tumor is likely to be detected in a screening interval.

Thus, having compared tumors of equal size, our findings strongly suggest that interval cancers on average have a more aggressive molecular phenotype. Our data support the concept that different modes of detection (i.e., mammographic screening or interval presentation) selects for categories that may represent biologically distinct subgroups, rather than being an expression of phenotypic drift only (44). The identification of a basal-like profile for the interval cases further argues for the importance and clinical relevance of this breast cancer phenotype. Future efforts should be made to identify women at risk of developing basal breast cancers, and whether there are specific risk factors in addition to BRCA1 mutations. It should be considered whether these women may be offered regular surveillance with shorter interval screening periods.

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**Table 2. The distributions according to different markers for interval cancers clinically detected within the first year of the screening interval, compared with the second year, the corresponding odds ratio, first versus second year, and 95% confidence interval**

<table>
<thead>
<tr>
<th>Variable</th>
<th>First det. year (%)</th>
<th>Second det. year (%)</th>
<th>Odds ratio (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast density</td>
<td>Low/moderate 11 (55) 9 (45)</td>
<td>High 59 (79) 16 (21)</td>
<td>3.0 (1.1-8.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Histologic type</td>
<td>Ductal 17 (90) 2 (10)</td>
<td>Lobular 58 (82) 13 (18)</td>
<td>0.5 (0.1-2.5)</td>
<td>0.70*</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>3 15 (75) 5 (25)</td>
<td>0.1 (0.4-3.3)</td>
<td>0.70*</td>
<td></td>
</tr>
<tr>
<td>1 and 2</td>
<td>17 (94) 3 (16)</td>
<td>3.0 (1.1-8.5)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td>Negative 14 (74) 5 (26)</td>
<td>Positive 39 (56) 29 (44)</td>
<td>0.5 (0.2-1.6)</td>
<td>0.29*</td>
</tr>
<tr>
<td>Locally advanced disease</td>
<td>No 19 (95)</td>
<td>Yes 1 (5)</td>
<td>0.4 (0.04-2.5)</td>
<td>0.45*</td>
</tr>
<tr>
<td>Metastasis at time of diagnosis</td>
<td>No 18 (90) 2 (10)</td>
<td>72 (96) 3 (4)</td>
<td>2.5 (0.4-17.2)</td>
<td>0.28*</td>
</tr>
<tr>
<td>ER</td>
<td>Negative 6 (30) 14 (70)</td>
<td>Positive 23 (31) 52 (69)</td>
<td>1.0 (0.3-3.3)</td>
<td>0.95</td>
</tr>
<tr>
<td>PR</td>
<td>Negative 9 (45) 11 (55)</td>
<td>Positive 31 (41) 44 (59)</td>
<td>0.9 (0.3-2.3)</td>
<td>0.77</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Low 14 (70)</td>
<td>High 5 (25) 6 (30) 50 (67) 25 (33)</td>
<td>0.9 (0.3-2.5)</td>
<td>0.78</td>
</tr>
<tr>
<td>p53</td>
<td>Low 15 (75) 5 (25)</td>
<td>High 54 (72) 21 (28)</td>
<td>0.8 (0.3-2.5)</td>
<td>0.79</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td>Negative 13 (65) 7 (35)</td>
<td>Positive 63 (84) 12 (16)</td>
<td>2.8 (0.9-8.5)</td>
<td>0.06</td>
</tr>
<tr>
<td>P-cadherin</td>
<td>Negative 15 (75) 5 (25)</td>
<td>Positive 57 (76) 18 (24)</td>
<td>1.1 (0.3-3.3)</td>
<td>0.93</td>
</tr>
<tr>
<td>Cytokeratin 5/6</td>
<td>Negative 15 (75) 5 (25)</td>
<td>Positive 59 (79) 16 (21)</td>
<td>1.2 (0.4-3.9)</td>
<td>0.73</td>
</tr>
</tbody>
</table>

*Fisher’s exact two-sided test.*

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**Discussion**

This study indicates that interval breast cancers and cases detected by service mammography screening can be distinguished by markers of basal and luminal differentiation. Screening cases were more often positive for ER and PR, which is characteristic for the luminal subtype of breast carcinomas, as suggested by recent microarray studies (10, 36). Correspondingly, we found that cytokeratin 5/6, a marker of the basal-like phenotype or subtype (10, 36), was significantly increased in the interval group, after matching for the possible confounding influence of tumor size. P-cadherin, also a marker of basal or myoepithelial cells (16), showed the same pattern as cytokeratin 5/6. Expression of cytokeratin 5/6 has recently been found in subgroups of more aggressive breast cancers (37), some of which are related to germ line mutations of BRCA1 (14). Conversely, luminal cytokeratins such as cytokeratins 18 and 19 are associated with a better outcome following breast cancer (38, 39). Thus, our findings suggest that interval cancers have a different gene expression pattern than screen-detected cases. Notably, BRCA1-related breast cancers also tend to present as interval cancers (40) and contribute to false-negative mammography reports (41, 42).
References


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