Advances in Brief

Differences in Ki67 and c-erbB2 Expression between Screen-detected and True Interval Breast Cancers

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Abstract

Breast cancer screening facilitates the early detection of breast cancer, although a significant number of tumors still arise in the interval between screening. The objective of this study was to measure the expression of five markers of proven prognostic significance in symptomatic breast cancer (estrogen receptor, progesterone receptor, p53, Ki67, and c-erbB2) in screen-detected and interval breast cancers to identify biological markers that may be associated with the emergence of symptomatic breast cancer in the screening interval. The expression of estrogen receptor, progesterone receptor, p53, Ki67, and c-erbB2 was assessed in a series of 51 true interval and 84 screened-detected invasive tumors by immunohistochemistry. Interval cancers tended to be of higher histological grade and were of larger pathological size than screen-detected cancers. Expression of estrogen receptor was 1.7-fold lower ($P < 0.001$), whereas expression of p53 was 2.5-fold ($P < 0.01$), Ki67 2.4-fold ($P < 0.001$), and c-erbB2 3.6-fold higher ($P < 0.01$) in true interval cancers compared with screen-detected invasive cancers. There was no significant difference in progesterone receptor expression. The most important differences identified by multiple logistic regression analysis were in the expression of Ki67 and c-erbB2. The differences in the expression of these markers were more important than clinical features such as pathological grade and size. Using the logistic regression model, 83% of the tumors analyzed in this study could be correctly assigned as interval or screen-detected tumors on the basis of Ki67 and c-erbB2 expression. The importance of high expression of Ki67 in interval cancers compared with screen-detected cancers suggests that tumors may become symptomatic in the screening interval as a result of increased levels of cell proliferation. The inclusion of c-erbB2 in the regression equation suggests that this growth factor receptor may play a significant role in stimulating the rapid growth of interval cancers.

Introduction

Breast cancer screening has been instigated in a number of countries in response to evidence that the early diagnosis and treatment of breast cancer results in a significant reduction in mortality. In the United Kingdom, screening is offered to women between the ages of 50 and 64 at intervals of 3 years (1).

The tumors that become symptomatic in the interval between screening are referred to as interval cancers. Early studies on interval cancers did not classify them into separate categories. In the United Kingdom, breast screening program, interval cancers have been divided into four groups (2). True interval cancers show a mammographic abnormality at the time of diagnosis but no abnormality on the screening mammogram. False-negative interval cancers show an abnormality on the screening mammogram at the same site at which the tumor arises. Occult tumors have no radiological symptoms at diagnosis; therefore, it is not possible to assess whether they were present at the last screen. The remainder are unclassified (2).

Early studies on interval cancers suggested that they represent a particularly virulent form of breast cancer. DeGroote *et al.* (3) showed that interval cancers detected within 12 months of a screen had a higher incidence of positive lymph nodes, higher overall mortality, and lower 6-year survival than screen-detected tumors. Similar findings have been reported by others (4–9). In contrast to the view that interval cancers are particularly virulent, Holmberg *et al.* (10), Peeters *et al.* (11), and Brekelmans *et al.* (12) compared the survival of women with interval cancers to those with symptomatic cancers detected independently of screening and concluded that interval cancers and symptomatic cancers have a similar prognosis. Burrell *et al.* (13) concluded that interval cancers have prognostic features similar to those of breast cancers in a nonscreened population and worse than those of screen-detected cancers. In contrast to the other studies, Frisell *et al.* (14) found that interval cancers have a better prognosis than control cancers.

In contrast to the view that interval cancers represent a more virulent form of breast cancer, a recurrent theme is that they are more difficult to diagnose and therefore present later. In recognition of this, the United Kingdom breast screening program recognizes the category of occult interval cancers that are mammographically invisible at diagnosis. Ikeda *et al.* (5) concluded that interval cancers were dominated by comedo, medullary, and mucinous carcinomas that often had a nonspecific appearance on prior screening mammograms, and others (4, 11, 15) found that interval cancers were associated with a lack of calcification and dense breast parenchyma (Wolfe patterns P2 and DY), all of which would reduce the likelihood of diagnosis by mammography. These observations are consistent with other studies (6, 16), which have shown that interval cancers are more

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frequent in younger women who tend to have higher parenchymal density.

A number of prognostic factors for symptomatic breast cancer have been identified. The important pathological features of prognostic significance are tumor size, grade, and the presence of lymph node metastases. In symptomatic breast cancer, the presence of lymph node metastases is generally considered to be the most important. In screen-detected cancers, however, the incidence of tumors with lymph node involvement is low as a result of an earlier diagnosis and the smaller size of the tumor and is therefore of limited prognostic value.

The expression of a number of proteins has been measured in symptomatic breast cancer in an attempt to identify prognostic factors with which to predict the biological behavior of individual tumors. Estrogens are important in controlling the growth of breast cancer cells, and the estrogen receptor mediates their effects. The estrogen receptor is a marker of good prognosis and is also predictive of a response to endocrine therapy (17). The progesterone receptor is induced by estrogen in a variety of tissues and is a marker of good prognosis and hormone responsiveness (18). *p53* is one of the most commonly mutated genes in cancer and its expression in cancer cells normally reflects mutations that stabilize the protein. c-erbB2 is one of a family of membrane growth factor receptors related to the receptor for epidermal growth factor. Overexpression of p53 or c-erbB2 is a marker of poor prognosis (19–24). Ki67 expression is limited to the G_1 and S phases of the cell cycle and is a widely used marker of cell proliferation. High cell proliferation, as reflected by Ki67 staining as well as other techniques, such as flow cytometry and thymidine labeling, is also indicative of a poor prognosis (25–28).

Materials and Methods

Patients. All patients were under the care of the Breast Screening Unit, Newcastle General Hospital. The interval cancers analyzed had arisen in women who had been screened and treated at the same center. The vast majority (97%) of the patients were 50–65 years of age (mean, 57.6 years). Two women in the interval group were younger than 50, and two women in the screen-detected group were older than 65.

Interval cancers are classified according to the reason for their nondiagnosis at screening. The true interval cancers in this study were defined by the criteria of Simpson *et al.* (2), which requires that at least two members of an audit panel identify the tumor on the previous screening film when presented with a set of films containing both interval cancers and controls from centers other than their own. This method avoids classifying tumors as "false-negative" or "missed" on the basis of retrospective searching for a trace of tumor that is unlikely to be detected in any other way.

The size, grade, and lymph node status of the tumors were obtained from pathology reports. Tumors were graded by the modified Bloom and Richardson method (29). The pathological size was the maximum diameter.

Immunohistochemistry. Three-μm sections were cut from representative blocks of formalin-fixed, paraffin-embedded tumors onto slides coated with 3-aminopropyl-triethoxysilane. The sections were dried in an incubator at 37°C overnight and then heated at 60°C for 15 min.

Sections were dewaxed in xylene and graded alcohol/water mixtures, immersed in a 0.5% hydrogen peroxide/methanol mixture for 10 min to block endogenous peroxidase activity, and then rinsed in distilled water. Sections to be stained for estrogen receptor, progesterone receptor, p53, and Ki-67 were microwaved two times for 5 min each time in 10 mM sodium citrate (pH 6) and then left to stand in hot buffer for 20 min. Sections to be stained for c-erbB2 were not pretreated. After the pretreatment, slides were rinsed in distilled water, 5 mM Tris-buffered saline (pH 7.6; TBS) and then incubated in either normal rabbit serum or normal swine serum (diluted 1:10 in TBS) for 10 min. The following antibodies were used: estrogen receptor, NCL-ER-LH2 (1:10 dilution); progesterone receptor, NCL-PGR (1:10 dilution); p53, NCL-p53–1801 (1:40 dilution); Ki67, NCL-Ki67-MM1 (1:100 dilution); and c-erbB2, NCL-CB11 (1:40 dilution). All antibodies were obtained from Novocastra Laboratories (Newcastle upon Tyne, United Kingdom). The sections were incubated with primary antibody, diluted in either normal rabbit or swine serum for 1 h, rinsed in TBS two times for 5 min each time, and then incubated with biotinylated rabbit antimouse immunoglobulin (diluted 1:500) in normal rabbit serum for 30 min. The sections were then washed twice in TBS, incubated with avidin-biotin immunoperoxidase complex, and then developed using nickel-modified diaminobenzidine, followed by intensification using 0.5% cobalt chloride. Slides were washed in distilled water, counterstained in 0.1% nuclear fast red in 5% aluminum sulfate for 2 min, washed, dehydrated in graded alcohols, cleared in xylene, and mounted in distrene/phthalein/xylol. Positive and negative controls were included in each run. Tumor sections that were positive for each of the markers were included as positive controls. Sections from the same tumors incubated without primary antibody were used as negative controls. All slides were scored by one person. The scoring was validated on ~5% of slides by a second person. The scoring by the two observers was in close agreement.

The level of expression was assessed as the percentage of cells showing specific staining. One thousand cells were counted from random fields.

Data Analysis. Data were analyzed using the SPSS statistical package. The importance of predictive markers for discriminating screen-detected and interval cancers was assessed by logistic regression analysis. Lymph node status was excluded as an independent variable because it was known for only ~50% of cases. Forwards and backward regression gave the same regression model.

Results

Clinical Characteristics of the Screen-detected and True Interval Cancers. In this study, the expression of five biological markers was compared in 84 invasive screen-detected and 51 true interval breast carcinomas.

Information on tumor grade was available for all 84 screen-detected cancers and 50 of the true interval cancers; one was a ductal carcinoma *in situ* (Table 1). For the screen-detected invasive cancers, 48% were of low grade (grade 1), whereas only 7% were grade 3. In contrast, 42% of the true interval

Table 1 Pathological features of screen-detected and true interval cancers

Pathological data	Screen-detected invasive cancers	True interval cancers	*P*
Total no. of cases 135	84	51	
Grade	$n = 84$	$n = 50$[a]	
1	40 (48%)	5 (10%)	
2	38 (45%)	24 (48%)	$P < 0.001$
3	6 (7%)	21 (42%)	
Size	$n = 82$[b]	$n = 49$[c]	
Mean	17.7 mm	20.5 mm	
Smaller than mean	55 (67%)	29 (59%)	$P < 0.01$
Larger than mean	27 (33%)	20 (41%)	
<10 mm	21 (26%)	5 (10%)	
10 mm–15 mm	23 (28%)	11 (22%)	$P < 0.05$
15 mm–20 mm	22 (27%)	13 (27%)	
>20 mm	16 (19%)	20 (41%)	
Node status	$n = 44$	$n = 29$	
Negative	26 (59.1%)	14 (48.3%)	
Positive	18 (40.9%)	15 (51.7%)	

[a] One true interval cancer was a ductal carcinoma *in situ* and was not analyzed further.

[b] The sizes of two screen-detected invasive cancers could not be determined because they were multifocal.

[c] The size of one true interval cancer could not be determined because it was multifocal.

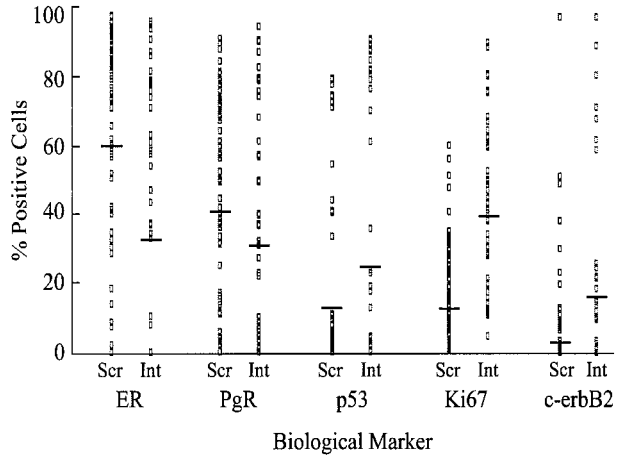

Fig. 1 Scatterplot of expression of biological markers in screen-detected (*Scr*) and true interval (*Int*) breast cancers. Each box represents the percentage of positive cells in one case. For markers with a large number of negative cases, these negative cases are not visible. *Bar*, the mean value for all tumors. *ER*, estrogen receptor; *PgR*, progesterone receptor.

cancers were grade three, whereas only 10% were grade one. The distribution of grade within the two groups of tumors was significantly different ($P < 0.001$, χ^2 test).

The true interval cancers (mean diameter, 20.5 mm) were significantly larger than the screen-detected cancers (mean diameter, 17.7 mm; $P < 0.01$, Mann Whitney Test). Although the difference between the mean diameters of the two groups of tumors was not large (2.8 mm), 67% of screen-detected cancers but only 59% of the true interval cancers were smaller than the mean diameter of the screen-detected invasive cancers ($P < 0.01$, χ^2 test).

As a result of the policy for the sampling of nodes, the nodal status was known for only 29 (57%) true interval cancers and 44 (52%) screen-detected cancers. Analysis of the cancers where node status was known showed that there was no statistically significant difference in the frequency of node positivity between the two groups of tumors.

Relationships between the Expression of the Biological Markers in Screen-detected and True Interval Cancers. Estrogen receptor, progesterone receptor, p53, Ki67, and c-erbB2 were detected in tissue sections using immunohistochemistry as described in "Materials and Methods." The level of expression of each protein was defined as the percentage of cells stained. In contrast to many immunohistochemical studies in which data are dichotomized using cutoff points, the quantitation of staining as a continuous variable allowed the use of simple and logistic regression in the analysis of the data.

In the majority of cases, estrogen receptor was localized in the cell nuclei only. In a small minority of cases, cytoplasmic staining was observed. Because of the small proportion of cells showing cytoplasmic staining, nuclear staining only was quantitated. Estrogen receptor expression was lower (1.7-fold; $P < 0.001$) in interval cancers (mean, 35% positive cells) than in screen-detected cancers (mean, 60% positive cells; Fig. 1). The distribution of staining was also significantly different ($P < 0.001$) in the screen-detected and interval cancers. Forty-four % of interval cancers showed no staining as compared with 18% of screen-detected cancers, whereas 57% of screen-detected cancers had a high proportion (>67%) of stained cells (Fig. 2).

Staining of the progesterone receptor was also predominantly nuclear. Neither the mean level of expression (Fig. 1) nor the distribution of the proportion of cells stained (Fig. 2) was significantly different in the screen-detected and interval cancers.

p53 protein was only detected within cell nuclei. p53 expression was 2.5-fold higher ($P < 0.01$) in interval cancers (mean, 26% positive cells) than in screen-detected cancers (mean, 10% positive cells; Fig. 1). The distribution of the proportion of cells stained was significantly different ($P < 0.05$, χ^2 test) in the screen-detected and interval cancers (Fig. 2). Although the proportion of cases showing no, weak (1–33% positive cells), and intermediate (34–66% positive cells) staining was similar, a much higher proportion (26% compared with 8%) of the interval cancers had a high proportion of stained cells.

Ki67 staining was exclusively nuclear. Ki67 staining was higher (2.4-fold; $P < 0.001$) in interval cancers (mean, 40% positive cells) than in screen-detected cancers (mean, 16% positive cells; Fig. 1). The distribution in the proportion of cells stained was also significantly different in the screen-detected and invasive cancers ($P < 0.001$). The main difference was the reduced number of interval cancers that had a low proportion (1–33%) of stained cells and the increased number that had a moderate (34–66%) or high (67–100%) proportion of stained cells (Fig. 2).

For c-erbB2, cells showing strong membrane staining were scored as positive, although cytoplasmic staining was occasionally observed in cases showing membranous staining. The pro-

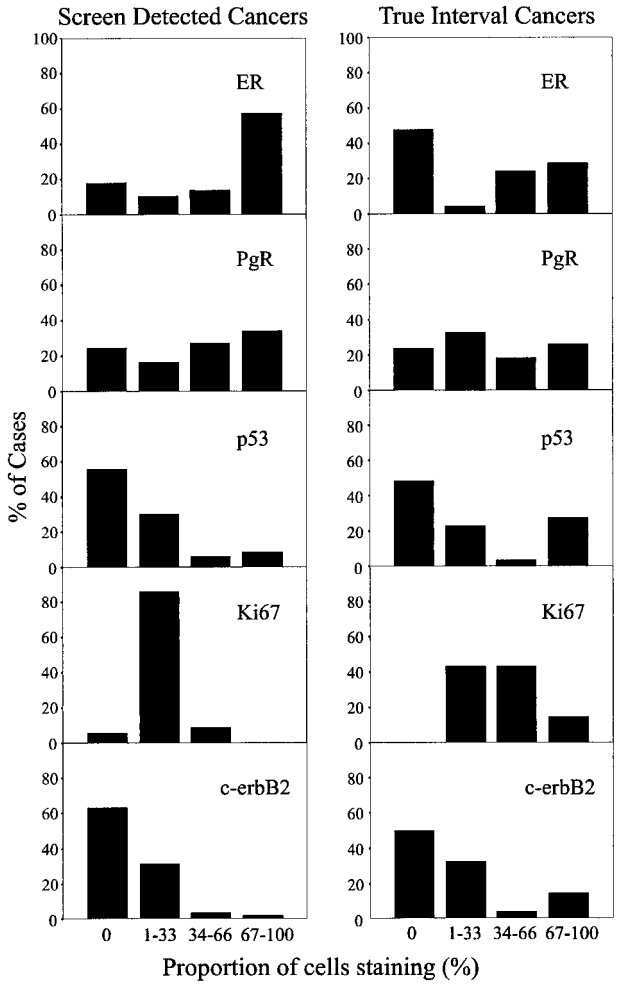

Fig. 2 Expression of biological markers in screen-detected and true interval cancers. Tumors were scored for the expression of biological markers and categorized as negative (0%), weak (1–33%), moderate (34–66%), and strong (67–100%). The percentage of cases in each category is plotted. *ER*, estrogen receptor; *PgR*, progesterone receptor.

portion of cells staining for c-erbB2 was higher (3.6-fold; $P < 0.01$) in the interval cancers (mean, 19% stained cells) than in screen-detected cancers (mean, 5% stained cells; Fig. 1). The distribution of cases showing positive staining was significantly different ($P < 0.05$) between the two groups of tumors (Fig. 2). The principle difference was the increased number of interval cancers that had a high proportion (>67%) of stained cells.

Simple logistic regression analysis confirmed the differences between the screen-detected and interval cancers. High estrogen receptor expression was found in screen-detected cancers($P < 0.001$), whereas high p53 ($P < 0.01$), Ki67 ($P < 0.001$), and c-erbB2 ($P < 0.001$) expression and pathological grade ($P < 0.001$) were found in interval cancers. Progesterone receptor and size were not different between the two groups of tumors.

Correlation between Expression of Biological Markers in Screen-detected and True Interval Cancers. The relationships between the expression of the prognostic markers in the screen-detected and interval cancers are summarized in Table 2. In general, if the expression of two markers showed a significant correlation in one group of tumors, it was also found to be correlated in the other. For both groups of tumors, there was a highly significant positive correlation between estrogen and progesterone receptor expression and a significant negative correlation between estrogen receptor expression and p53, Ki67, and grade. There was a significant positive correlation between p53 expression and Ki67 expression and histological grade and a significant negative correlation between p53 and estrogen receptor expression. Ki67 was positively correlated with p53 expression and grade and negatively correlated with estrogen receptor.

There were, however, some differences between the groups of tumors. There was a significant negative correlation between progesterone receptor and Ki67 expression and histological grade in the interval cancers but not in the screen-detected cancers. In addition, Ki67 was correlated with size in the interval cancers but not the screen-detected cancers. However, the correlation between c-erbB2 and the other prognostic markers differed most between the two groups of tumors. c-erbB2 expression was significantly positively correlated with p53 and Ki67 expression and histological grade and negatively correlated with estrogen and progesterone receptor expression in screen-detected cancers. c-erbB2 expression was not correlated with the expression of any biological or pathological marker in the interval cancers.

Logistic Regression Analysis. Because there were significant differences between the screen-detected and interval cancers in the distribution of tumor grade, size, and the expression of four of the five prognostic markers, logistic regression was used to derive a model to identify the most important factors. The seven variables included in the analysis were pathological grade and size, together with the expression of the five biological markers. The same model was obtained using forward and backward stepwise logistic regression analysis. The regression equation retained only two of the seven variables, Ki67 expression and c-erbB2 expression, of which Ki67 was the most important factor.

The regression equation was:

$$Z = -2.627 + 0.0682\ (\text{Ki67 expression}) + 0.0202\ (\text{c-erbB2 expression})$$

The estimated probability given by the equation:

$$\textit{Estimated probability} \equiv \frac{1}{1 + e^{-z}}$$

of each tumor being a screen-detected or an interval cancer was calculated using the value of Z from the regression equation. Fig. 3 shows the histogram for the estimated probabilities with the screen-detected tumors above the line and the true interval tumors below the line. Using the standard value of 0.5 for the probability as the cutoff to predict screen-detected and interval cancers, 92% of the screen detected and 65% of the interval cancers were predicted correctly. Overall, 83% of cases were assigned to the correct group of tumor on the basis of Ki-67 and c-erbB2 expression.

Table 2 Correlation between the expression of biological markers within the groups of screen-detected and true interval cancers
The table lists the markers for which there is a significant positive or negative correlation and the level of significance.

	Screen-detected tumors[a]				True interval cancers[a]			
	+ve correlation		−ve correlation		+ve correlation		−ve correlation	
ER	PR	<0.001	p53	<0.05	PR	<0.001	p53	<0.01
			Ki67	<0.05			Ki67	<0.001
			Grade	0.001			Grade	<0.001
			c-erbB2	0.001				
PR	ER	<0.001	c-erbB2	<0.01	ER	<0.001	Ki-67	<0.001
							Grade	<0.01
p53	Ki67	<0.001	ER	<0.05	Ki67	<0.01	ER	<0.01
	Grade	<0.001			Grade	<0.01		
	c-erbB2	<0.01						
Ki67	p53	<0.001	ER	<0.05	p53	<0.01	ER	<0.001
	Grade	<0.001			Grade	<0.001	PR	<0.001
	c-erbB2	<0.01			Size	<0.05		
c-erbB2	p53	<0.01	ER	<0.001				
	Ki67	<0.01	PR	<0.01				
	Grade	<0.001						

[a] +ve, positive; −ve, negative; ER, estrogen receptor; PR, progesterone receptor.

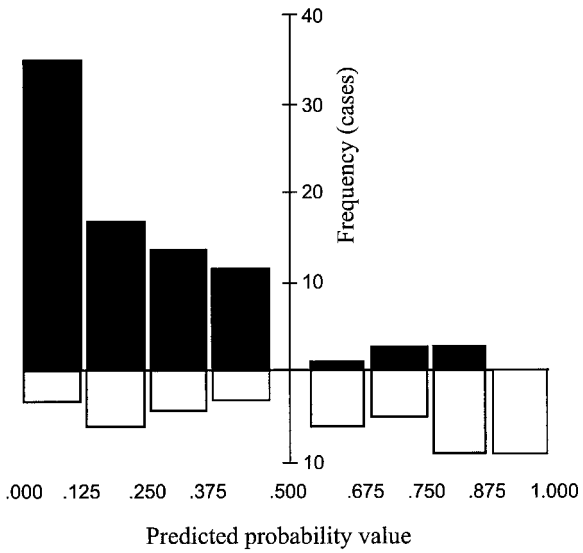

Fig. 3 Predicted probability of tumors being classified as screen-detected cancers or true interval cancers based on their expression of Ki-67 and c-erbB2. ■ (above the *line*), number of screen-detected cancers within each probability group; □ (below the *line*), number of interval cancers within each probability group.

Discussion

In this retrospective study, the expression of the five biological markers was found to be associated with pathological features and with the expression of other biological markers. Estrogen receptor, which is a marker of good prognosis in symptomatic breast cancer, was expressed at higher levels in screen-detected cancers as compared with interval cancers, whereas Ki67, p53, and c-erbB2, which are markers of poor prognosis in symptomatic breast cancer, were expressed at higher levels in interval cancers as compared with screen-detected cancers. This study reinforces the view that interval cancers are biologically different from screen-detected cancers, rather than being more difficult to detect. In addition, the interval cancers appeared to be of worse prognosis on the basis of the expression of biological markers than screen-detected cancers.

In addition, the two groups of tumors also differed clinically. The most marked difference was in the grade of the tumors, with the interval cancers having a significantly higher proportion of high-grade tumors than the screen-detected cancers. Because the expression of prognostic markers is associated with tumor grade, this could account for the differences in the expression of the biological markers. Multiple regression analysis was, therefore, used to identify the most important differences between the two groups of tumors.

Surprisingly, this analysis identified cell proliferation, as measured by Ki67 staining, to be the most important factor. No pathological features were significant in this analysis, and only c-erbB2 expression was significant in addition to Ki67.

Few studies have measured the expression of biological markers of prognosis. In this study, the expression of five biological markers of prognosis was compared in screen-detected and interval cancers. A number of studies have measured estrogen receptor levels in interval cancers (4, 9, 11). They reported that estrogen receptor expression is lower in interval cancers than in screen-detected cancers, and this is consistent with the data in this study. Only one study (15) has reported the expression of other biological markers of prognosis. This study included c-erbB2, p53, and cathepsin D and concluded that there is no difference in their expression in these two groups of tumors. In contrast to our study, Koivunen *et al.* (15) measured the expression of these markers using a semiquantitative scoring method and then compared these values to those quoted in the literature, rather than a control group of tumors using the same methodology. Given that the level of positivity of most biological markers cited in the literature is variable, it was not surprising that Koivunen *et al.* (15) found that interval cancers showed no differences to the control group.

The observation that Ki67 expression showed the greatest difference between screen-detected and interval cancers clearly suggests that the rate of cell proliferation is important in the

genesis of breast tumors in the interval between screening. Grade is a composite measure of mitosis, differentiation, and nuclear pleomorphism, and the observation that Ki67 staining rather than grade is the most important feature suggests that cell proliferation may show the largest differences of the three features between the two groups of tumors.

The identification of c-erbB2 expression as the only other significant factor in the regression equation suggests that the expression of this growth factor receptor may be important in the development of interval cancers. The erbB family of receptors (erbB1–4) interact with a number of ligands and form homo- and heterodimers (30) of differing ligand specificity. Our results, therefore, suggest that the erbB family of receptors and their ligands may be important in driving the proliferation of tumors that become symptomatic in the screening interval.

Despite the fact that Ki67 expression differed considerably between screen-detected and interval breast cancers, it is unclear whether this has any impact on prognosis. There are relatively few studies on the prognosis of women with interval cancers, and all are on relatively small numbers of patients. However, the existing data does not support the view that interval cancers are particularly malignant. This suggests that although the higher rate of proliferation may help to explain how the tumor becomes symptomatic, other features such as the ability of cells to metastasize and degrade the function of other vital organs are more important in determining the survival of breast cancer patients.

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