High Expression of the Trefoil Protein TFF1 in Interval Breast Cancers

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Breast cancer screening is important for the early detection of breast cancer. Tumors that become symptomatic in the screening interval are known as interval cancers but the reasons for their rapid progression are unknown. Estrogen receptor expression is lower in interval cancers suggesting that they may have reduced hormonal responsiveness. To investigate this hypothesis we have measured the expression of the estrogen receptor and three estrogen-responsive genes (cathepsin D, progesterone receptor, and TFF1) in screen-detected and interval breast cancers. The expression of the protease cathepsin D was not associated with estrogen receptor in either group of tumor. Progesterone receptor expression was highly correlated with that of the estrogen receptor in both groups of tumors but it was not expressed at significantly different levels in the two groups of tumors. Expression of TFF1, a cellular motogen, was correlated with estrogen receptor in screen-detected but not interval cancers and was expressed at markedly higher levels in interval breast tumors, the group that expresses lower levels of estrogen receptor. Interval cancers are characterized by high levels of expression of TFF1 and/or Ki67 suggesting that cell migration and cell division play important roles in the rapid progression of interval cancers. The observation that TFF1 expression in interval cancers tends to be estrogen-independent and that interval cancers have reduced estrogen receptor expression suggests they may have a reduced response to hormone therapy. (*Am J Pathol* 2001, 159:215–221)

Women between the ages of 50 and 64 years of age are invited for breast cancer screening every 3 years in the United Kingdom.¹ Cancers that become symptomatic in the interval between screening are referred to as interval cancers. True interval cancers² show a mammographic abnormality at the time of diagnosis but no abnormality on the previous screening mammogram and are there-

fore thought to have arisen and become symptomatic during the screening interval. In contrast, other groups of interval cancer are assumed to result from a failure to detect the tumor at the time of screening (false-negative) or a lack of radiological signs both at screening and at diagnosis (occult).

The biology of interval cancers is poorly understood. An appreciation of the factors responsible for their presentation within the screening interval may lead to the identification of factors responsible for the rapid progression of breast cancer. Early studies of interval cancers suggested that they represent a particularly virulent form of breast cancer with a bad prognosis,³ however more recent studies have failed to show that women with interval cancers have a worse survival than unscreened women^{4,5} despite their rapid progression within the screening interval.

Trefoil proteins are a recently-discovered group of peptides that have a high degree of sequence and structural homology within 43 to 44 amino acids termed the trefoil domain.⁶ They are normally expressed at highest levels in the mucosa of the gastrointestinal tract, however they are often expressed ectopically in primary tumors of other tissues.⁷ The biological function of trefoil proteins is not entirely clear, however one important function is thought to be the control of cell motility.^{8,9} The observation that trefoil proteins can be expressed at high levels in tumors has led to speculation that they may facilitate tumor cell dissemination. There are three human trefoil proteins (TFF1, TFF2, and TFF3). Two, TFF1 and TFF2, are expressed predominantly in the gastric mucosa whereas the third, TFF3, is expressed predominantly in the small intestine.¹⁰

Two of the trefoil proteins, TFF1 and TFF3, are frequently expressed at high levels in breast cancer, whereas TFF2 is expressed infrequently.^{11–13} The expression of TFF1 and TFF3 is regulated by estrogen in estrogen-responsive breast cancer cells in culture. Their expression is associated with that of the estrogen receptor and TFF1 is a marker of hormone responsiveness in tumors.^{12,14–16}

Cathepsin D is an aspartyl protease involved in protein catabolism. Cathepsin D was identified as an estrogen-

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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			
1	48%	10%	<0.001
2	45%	48%	
3	7%	42%	
Size			
Mean	17.7 mm	20.5 mm	<0.01
Node status			
Negative	59.1%	48.3%	NS
Positive	40.9%	51.7%	

regulated protein in estrogen-responsive breast cancer cell lines.¹⁷ It is generally considered to be a marker of poor prognosis in breast cancer.¹⁸

Few studies have analyzed the expression of biological markers in interval cancers although this may help to understand the factors leading to them becoming symptomatic in the screening interval. We have previously compared the expression of biological markers in screen-detected and interval breast cancers and found that the greatest differences between the two groups was in the expression of the proliferation marker Ki67 and c-erbB2.¹⁹ The expression of the estrogen receptor was also lower in the interval compared to the screen-detected invasive cancers. As the estrogen receptor status reflects the hormone responsiveness of the tumor, we have now measured the expression of three estrogen-regulated proteins to investigate whether interval cancers are less estrogen responsive than screen-detected cancers.

Materials and Methods

Patients and Tumors

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 at the same center. The study was performed on 51 true interval cancers and 84 screen-detected invasive breast cancers. The true interval cancers in this study were defined by the criteria of Simpson and colleagues,² which requires that at least two members of an audit panel do not identify a tumor on the previous screening film.

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.²⁰ The pathological size was the maximum diameter.

The clinical characteristics of the two groups of tumors are shown in Table 1. There was a statistically significant difference in the distribution of grade ($P < 0.001$, chi-square test) with the interval cancers having a greater proportion of high-grade tumors. The mean maximum diameter of the interval cancers (20.5 mm) was significantly ($P < 0.01$) greater than that of the screen-detected

cancers (17.7 mm) although this difference was small. The proportion of node-negative and node-positive tumors in the two groups was not significantly different.

Immunohistochemistry

Three- μ m 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 minutes.

Sections were dewaxed in xylene and graded alcohol/water mixtures, immersed in a 0.5% hydrogen peroxide/methanol mixture for 10 minutes to block endogenous peroxidase activity and then rinsed in distilled water. Sections to be stained for estrogen and progesterone receptor were microwaved for 2 $\times$ 5 minutes in 10 mmol/L sodium citrate (pH 6) and then left to stand in hot buffer for 20 minutes. After the pretreatment, slides were rinsed in distilled water, Tris-buffered saline (pH 7.6,) and then incubated in either normal rabbit serum or normal swine serum (diluted 1:10 in Tris-buffered saline) for 10 minutes. The following antibodies were used: estrogen receptor, mouse monoclonal NCL-ER-LH2 (1:10 dilution); progesterone receptor, mouse monoclonal NCL-PGR (1:10 dilution); TFF1, rabbit polyclonal antibodies raised against the 31 C-terminal amino acids (1:100 dilution); cathepsin D, mouse monoclonal NCL-CDm (1:100 dilution); p53, mouse monoclonal NCL-p53-1801 (1:40 dilution); Ki67, mouse monoclonal NCL-Ki67-MM1 (1:100 dilution), c-erbB2, mouse monoclonal NCL-CB11 (1:40 dilution). All mouse monoclonal antibodies were obtained from Novocastra Laboratories (Newcastle on Tyne, United Kingdom). The sections were incubated with primary antibody, diluted in either normal rabbit serum (for mouse monoclonal antibodies) or swine serum (for rabbit polyclonal antibodies), for 1 hour and then rinsed in Tris-buffered saline for 2 $\times$ 5 minutes. They were then incubated with biotinylated rabbit anti-mouse immunoglobulin (diluted 1:500) in normal rabbit serum (for the mouse monoclonal antibodies) or biotinylated swine anti-rabbit immunoglobulins (diluted 1:1000) in normal rabbit serum (for the rabbit polyclonal TFF1 antibody) for 30 minutes. The sections were then washed twice in Tris-buffered saline, 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 minutes, 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.

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 (SPSS, Inc., Chicago, IL, USA). The importance of predictive markers for discriminating screen-detected and interval cancers was assessed by logistic regression analysis. Tumor size and grade and the expression of c-erbB2 and p53¹⁹ were also included in the analysis. Forwards and backwards regression gave the same regression model.

Results

Expression of Estrogen Receptor, Progesterone Receptor, Cathepsin D, and TFF1 in Interval and Screen-Detected Breast Cancers

Expression of the biological markers analyzed in this study was measured using immunohistochemistry. Figure 1 shows an example of an interval breast tumor stained for estrogen receptor (Figure 1B), TFF1 (Figure 1C), and Ki67 (Figure 1D). Staining for estrogen receptor was predominantly nuclear, staining for TFF1 was cytoplasmic, and staining for Ki67 was exclusively nuclear.

Figure 2 shows scatterplots for the expression of estrogen receptor, progesterone receptor, cathepsin D, and TFF1 in screen-detected and interval breast cancers. Estrogen receptor was expressed at lower levels in the interval cancers than in the screen-detected cancers (median 34% compared to 77% positive cells, $P < 0.001$) suggesting that interval cancers are less estrogen responsive. To assess the estrogen responsiveness of these two groups of tumors, the expression of three proteins (progesterone receptor, cathepsin D, and TFF1) that are regulated by estrogen in estrogen-responsive breast cancer cell lines was measured. The levels of expression of the progesterone receptor and cathepsin D were lower in the interval cancers (median 31% compared to 42% positive cells for progesterone receptor; 39% compared to 56% for cathepsin D), however the differences between the median values were not statistically significant. In contrast to the other estrogen-responsive proteins, TFF1 expression was dramatically increased in interval cancers (median 55.5% compared to 15% positive cells, $P < 0.001$).

Correlation between Expression of Estrogen Receptor, Progesterone Receptor, Cathepsin D, and TFF1

To explore the relationship between the expression of the estrogen receptor and the estrogen-regulated proteins in individual tumors, estrogen receptor expression was correlated with that of progesterone receptor, cathepsin D, and TFF1 in both the screen-detected and interval cancers.

Progesterone receptor expression was highly correlated with that of the estrogen receptor in both groups of tumors ($P < 0.0001$) consistent with its well-documented

role as an estrogen-regulated protein in breast cancer (Table 2). This was in contrast to cathepsin D, the expression of which was not correlated with that of estrogen receptor in either group of tumors. The expression of TFF1 was significantly correlated with that of the estrogen receptor in the screen-detected tumors ($P = 0.02$), however there was no correlation ($P = 0.35$) in the group of interval cancers.

Lack of Estrogen Regulation of TFF1 in Interval Cancers

To explore whether the increased TFF1 expression in interval cancers and lack of a correlation between estrogen receptor and TFF1 expression in this group of tumors may reflect a loss of control by estrogen, the levels of expression of the two proteins were investigated in estrogen receptor-negative and estrogen receptor-positive subgroups of interval and screen-detected breast cancers. Figure 3 shows scatterplots for estrogen receptor and TFF1 expression in the two groups of tumors.

A higher proportion of estrogen receptor-negative interval cancers than invasive screen-detected cancers expressed TFF1 (44% compared to 17.9%, $P < 0.01$) strongly suggesting that the increased levels of TFF1 in this subgroup of interval cancers results from regulation by factors other than estrogen.

TFF1 levels were also compared between the two groups of estrogen receptor-positive tumors using the median value of TFF1 (27.3%) to define TFF1-positive and TFF1-negative tumors. A significantly higher proportion of interval cancers were TFF1-positive (75% compared to 43% of screen-detected tumors, $P < 0.001$).

Logistic Regression Analysis

Logistic regression modeling is a multivariate technique that can be used to estimate the probability that a tumor is an interval or screen-detected cancer by analysis of the expression of molecular markers. This was used to identify the most significant differences between the two tumor types. Tumor size and grade and the expression of seven biological markers (estrogen receptor, progesterone receptor, cathepsin D, TFF1, c-erbB2, Ki67, p53) were included in the regression model. The regression equations shown below did not include lymph node status as this was known for only 54% of cases. This would have excluded 46% of cases from the analysis. However, analysis of the subset of cases for which node status was known identified the same factors in the regression equation as the analysis of all cases.

TFF1 and Ki67 expression were the only two variables retained of which Ki67 was the slightly more important factor. The regression equation was: $Z = -3.88 + 0.086$ (Ki67 expression) $+ 0.05$ (TFF1 expression).

The estimated probability of each tumor being a screen-detected or an interval cancer was calculated using the value Z from the regression equation. Figure 4A shows the histogram for the estimated probabilities with the screen-detected tumors above the line and the inter-

Figure 1. Immunohistochemical detection of estrogen receptor, TFF1, and Ki67. Sections of an interval breast cancer were stained for estrogen receptor (B), TFF1 (C), and Ki67 (D) as described in the Material and Methods. A: The control with no primary antibody.

Figure 2. Expression of estrogen receptor, progesterone receptor, TFF1, and cathepsin D in screen-detected and true interval cancers. The level of expression of estrogen receptor (ER), progesterone receptor, TFF1, and cathepsin D was measured as described in the Materials and Methods. The horizontal bar shows the median value. ER, estrogen receptor; PGR, progesterone receptor; Scr, screen-detected tumors; Int, true interval cancers.

val cancers below. Using the value of 0.5 for the probability as the cutoff to predict screen-detected and interval cancers, 93% of screen-detected and 78% of interval cancers were correctly assigned. Overall, 87% of cases were assigned to the correct group of tumors on the basis of TFF1 and Ki67 expression.

Figure 4B shows the predicted probability when TFF1 and cathepsin D were not included as variables. Only two variables were retained. In this case the two variables retained were Ki67 and c-erbB2 of which Ki67 was the more important. The regression equation was: $Z = -2.627 + 0.0682 (\text{Ki67 expression}) + 0.0202 (\text{c-erbB2 expression})$.

Ninety-two percent of screen-detected but only 65% of interval cancers were correctly predicted using 0.5 for the probability as the cutoff to predict screen-detected and interval cancers when TFF1 was excluded from the regression equation (Figure 4B). Comparison of the regression models with and without TFF1 included as a variable suggested that inclusion of TFF1 as a variable is particularly helpful in correctly predicting those tumors that are interval cancers.

Table 2. Correlation between Expression of Estrogen Receptor and Progesterone Receptor, Cathepsin D, and TFF1 in Screen-Detected and Interval Breast Cancers

	Screen detected		Interval	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Progesterone receptor	0.54	<0.0001*	0.66	<0.0001*
Cathepsin D	0.01	0.96	0.000	0.99
TFF1	0.25	0.02*	0.135	0.35

The correlation coefficient (*r*) and level of significance (*P*) are shown.

*Indicates a statistically significant correlation.

Figure 3. Scatterplot of estrogen receptor and TFF1 expression in screen-detected (A) and true interval (B) breast cancers. Estrogen receptor and TFF1 expression were measured as the proportion of positively staining cells as described in the Materials and Methods. Estrogen receptor-negative tumors were defined as having 0% positive cells.

Relationship between TFF1 and Ki67 Expression in Interval and Screen-Detected Breast Cancers

As TFF1 and the cell proliferation marker Ki 67 were the only two variables retained in the logistic regression analysis, and because TFF1 has been reported to inhibit cell proliferation in some experimental systems,²¹ we investigated the relationship between TFF1 and Ki67 expression.

Figure 5 shows scatterplots of the expression of TFF1 and Ki67 in the two groups of tumors. The pattern of the distribution of TFF1 and Ki67 expression in the two groups of tumors is distinct. There are significantly ($P < 0.01$) fewer Ki67-negative, TFF1-negative, and significantly ($P < 0.01$) more Ki67-positive, TFF1-positive interval cancers than screen-detected cancers.

Although interval cancers are characterized by high levels of expression of both Ki67 and TFF1 (Figure 5B), there was a significant inverse correlation between TFF1 and Ki67 expression in this group ($r = -0.038$, $P = 0.01$). There was no significant correlation for the screen-detected cancers.

Figure 4. Predicted probability of tumors being screen detected (filled columns) or interval (unfilled columns) cancers based on their expression of Ki67 and TFF1 (A) or Ki67 and c-erbB2 (B). The vertical axis shows the number of tumors in each probability group. The probability groups (from 0 to 1 in 0.125 increments) are on the horizontal axis.

Discussion

Interval breast cancers are of considerable biological interest because they progress rapidly in the interval between screening. There have been few studies on the expression of biological markers in interval cancers although such studies could be useful in identifying features responsible for the rapid progression of both screened and symptomatic breast cancers.

We have shown previously that the expression of a number of biological markers differ markedly between screen-detected and interval cancers. The expression of the cell proliferation marker Ki-67 and c-erbB2 differed most between the two groups of tumors. Increased cell proliferation would provide a rationale for interval tumors becoming symptomatic in the screening interval and the observation that c-erbB2 expression was the second most important factor in the regression equation suggested that the increased proliferation could be driven by

Figure 5. Scatterplot of TFF1 Ki67 expression in screen-detected (A) and true interval (B) breast cancers. Estrogen receptor and TFF1 expression were measured as the proportion of positively staining cells as described in the Materials and Methods. The vertical (Ki67) and horizontal (TFF1) dotted lines show the median value for the proportion of positively stained cells that was used as the cut-off to define positive and negative tumors (27.3% for TFF1 and 19.8% for Ki67).

ligands for the c-erbB2 family of tyrosine kinase receptors with which c-erbB2 heterodimerizes.²² We also found that the expression of the estrogen receptor was markedly different between interval and screen-detected tumors.¹⁹ In the present study we have therefore measured the expression of estrogen-regulated proteins to investigate the hypothesis that interval cancers have reduced estrogen responsiveness.

We found that the expression of the estrogen-regulated proteins was not always associated with that of the estrogen receptor. Cathepsin D expression was not associated with that of the estrogen receptor in either screen-detected or interval cancers. Although cathepsin D expression is clearly regulated by estrogens in estrogen-responsive breast cancer cell lines,¹⁷ a majority of studies have failed to show an association between estrogen receptor and cathepsin D expression *in vivo*¹⁸ and our findings are therefore in agreement with other studies. Expression of the progesterone receptor was highly

correlated with that of estrogen receptor in both groups of tumors and this is consistent with the majority of the data in the literature. However, unlike estrogen receptor expression, progesterone receptor expression was not significantly lower in the interval cancers.

In contrast to the correlation between the expression of the estrogen receptor and progesterone receptor and the lack of correlation between the expression of the estrogen receptor and cathepsin D that was found in both groups of tumors, the correlation between TFF1 expression and estrogen receptor expression was significant for the screen-detected cancers only.

The increased expression of TFF1 in interval cancers was particularly surprising given that they have lower levels of expression of estrogen receptor. This suggests that TFF1 expression is not regulated by estrogen in this group of tumors and the observation that estrogen receptor-negative interval cancers express TFF1 is consistent with this. In normal tissues, TFF1 is expressed at highest levels in the gastric mucosa²³ where its expression is thought to be independent of estrogen and therefore the high levels of constitutive TFF1 expression in the interval cancers may be driven by the same factors responsible for the high levels of expression in the gastric mucosa. The TFF1 promoter is known to be regulated by a number of factors apart from estrogen^{24,25} and it is therefore possible that, in contrast to the screen-detected cancers, TFF1 expression is driven by factors other than estrogen in the interval cancers.

The increased expression of TFF1 may be of biological significance in the development of interval cancers. A number of biological properties have been ascribed to trefoil proteins including interactions with mucus²⁶ and the ability to increase cell migration. An increase in cell migration may be an important feature that facilitates the development of tumors in the screening interval.

The relationship between TFF1 and Ki67 was markedly different in the screen-detected and interval cancers. In the screen-detected cancers almost all of the tumors expressing high levels of TFF1 had low levels of Ki67. It has been reported that high concentrations of TFF1 inhibit cell proliferation²¹ and this is consistent with its putative role as a tumor suppressor gene.²⁷ In contrast, in the group of interval cancers most tumors have high levels of expression of TFF1 independent of the expression of Ki67. The observation that all of the interval cancers express either high levels of TFF1 or Ki67 or both suggests that one or both of these factors could be important in the development of interval cancers.

Overall the expression of the estrogen-regulated genes in the two groups of tumors strongly suggests that interval breast cancers have reduced estrogen responsiveness. The clinical treatment of interval cancers does not currently differ from symptomatic or screen-detected cancers because although, by definition they are a group of rapidly growing tumors they are not thought to have a particularly poor prognosis.^{4,5} Our data, however, suggest that adjuvant hormone therapy may be relatively ineffective in this group of tumors.

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