ORIGINAL ARTICLE

Downregulation of nuclear expression of the p33^{ING1b} inhibitor of growth protein in invasive carcinoma of the breast

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Background/Aims: The inhibitor of growth gene 1 (ING1) is a modulator of cell cycle checkpoints, apoptosis, and cellular senescence. The most widely expressed ING1 isoform is p33^{ING1b}, which can modulate p53, a molecule that is frequently altered in breast cancer. Reduced ING1 mRNA expression has been observed in primary breast cancer expressing wild-type p53.

Methods: p33^{ING1b}, p53, oestrogen receptor (ER), and progesterone receptor (PgR) expression was studied in 86 primary invasive breast cancers using immunohistochemistry.

Results: Reduced nuclear expression of p33^{ING1b} was found in cancer cells, both in intensity and the proportion of cells staining. This was associated with enhanced cytoplasmic p33^{ING1b} expression in a proportion of cases. Analysis of several known biological factors indicated that high grade tumours were of larger size and more often negative for ER and PgR expression. However, larger tumours were more frequently p53 negative. These results provide evidence that p33^{ING1b} alterations are associated with more poorly differentiated tumours. Positive correlations were found between nuclear p33^{ING1b} expression and both ER and PgR expression.

Conclusions: Optimum function of p53 is dependent on p33^{ING1b} so that a reduction of nuclear p33^{ING1b} expression, as seen in this series, would be predicted to compromise p53 function. This study showed that p33^{ING1b} alterations were associated with more poorly differentiated tumours. Therefore, p33^{ING1b} expression could be used as a marker of differentiation in invasive breast cancer. These results support the view that loss of p33^{ING1b} may be an important molecular event in the differentiation and pathogenesis of invasive breast cancer.

Breast cancer is the most common cancer in women, affecting approximately one in nine women in Western countries, and is the second leading cause of female cancer deaths after lung cancer.¹ Although numerous molecular abnormalities have been described in breast cancer, the precise pathogenesis of the disease remains unclear.¹

The inhibitor of growth gene 1 (ING1) was first isolated in early 1996, after it was found to be preferentially expressed in a normal breast cell line but not in a panel of breast cancer cell lines.^{2–3} Since then, ING1 has been established as a modulator of cell cycle checkpoints, apoptosis, cellular senescence, and the maintenance of genomic stability.^{4–6} The most widely expressed ING1 protein isoform is p33^{ING1b}.^{3–7} Suppression of p33^{ING1b} is associated with the loss of cellular growth control and immortalisation, whereas its overexpression arrests cells in the G₀/G₁ phase of the cell cycle.⁸ Furthermore, co-immunoprecipitation studies have indicated that p33^{ING1b} interacts with p53, whereas cotransfection studies have shown that ING1 has the ability to modulate TP53 transactivation of the cyclin dependent kinase inhibitor p21^{WAF1}.⁹ Extension of these preliminary findings suggested that the association of competent protein forms of each member of the p53–p33^{ING1b} complex is essential for the optimum inhibition of cell growth and transactivation by TP53.¹⁰

"Suppression of p33^{ING1b} is associated with the loss of cellular growth control and immortalisation, whereas its overexpression arrests cells in the G₀/G₁ phase of the cell cycle"

ING1 has been mapped to the subtelomeric region of the long arm of chromosome 13 (13q33–34).¹¹ The BRCA-2

gene is located nearby (13q12).¹² High rates of 13q loss of heterozygosity have been detected in a variety of neoplasms, including breast tumours.¹³ However, recent studies have indicated that mutations in ING1 are extremely rare in breast (one in 377) and ovarian carcinomas.¹⁴ We have also shown that mutation of the ING1b gene is uncommon in several breast cancer cell lines.⁷ In contrast, reduced ING1 mRNA concentrations have been seen in breast carcinomas, particularly in tumours that had metastasised to lymph nodes.^{14–15} Recently, a mouse ING1 homologue, which shares 98% homology with human ING1, was isolated from mouse mammary epithelial cells and may have the same functions.¹⁶ The isolation of antibodies directed against ING1 gene protein products in sera taken from patients with breast cancer has provided further evidence for a role for ING1 in breast tumours.³ This ING1 immunogenicity has raised the possibility of the potential value of ING1 in diagnosis and vaccine based treatment of patients with breast cancer.

Since its discovery in 1979, TP53 (17q) has been the most extensively studied tumour suppressor gene. Loss of p53 function is thought to suppress a mechanism of protection against the accumulation of genetic alterations.¹⁷ Mutations in the tumour suppressor gene TP53 occur in about 30% of breast cancers.¹⁸ Moreover, TP53 mutations are more frequent in advanced breast cancer, suggesting that inactivation of TP53 is a late event in mammary carcinogenesis.¹⁹ Several distinct mechanisms may contribute to the inactivation of the p53

Abbreviations: ER, oestrogen receptor; ING1, inhibitor of growth gene 1; MAb, monoclonal antibody; PgR, progesterone receptor; TBS, Tris buffered saline

Figure 1 Normal and invasive breast carcinoma tissues showing p33^{ING1b} expression as detected by monoclonal antibodies GN1 (A,B) and GN2 (C-E). (A) Normal breast lobule showing strong nuclear p33^{ING1b} expression. (B) Invasive breast cancer showing reduced p33^{ING1b} nuclear expression. (C) Normal breast ducts minimally expressing p33^{ING1b} in the cytoplasm of cells. (D) Invasive breast cancer strongly expressing p33^{ING1b} in the cytoplasm of tumour cells. (E) Invasive breast cancer minimally expressing p33^{ING1b} in the cytoplasm of tumour cells.

pathway in the pathogenesis of breast cancer. Hence, the putative relation between p53 and p33^{ING1b} may contribute to tumorigenesis by affecting the function of one or both members of the p53–p33^{ING1b} complex. Therefore, our study was designed to investigate the expression of p33^{ING1b} in invasive breast cancer. This is the first report that correlates p33^{ING1b} expression with other molecular and biological factors, namely: p53 status, hormone receptors status (oestrogen receptor (ER) and progesterone receptor (PgR)), tumour grade and size, and lymph node status.

MATERIALS AND METHODS

Patients and tissues studied

The patients studied comprised a series of 86 women with invasive breast carcinoma between the ages of 38 and 80 years at diagnosis, with a mean and a median group age of 54.46 and 52 years, respectively. These patients were diagnosed at the General Hospital, Newcastle upon Tyne, UK, between 1994 and 1996. Routinely processed, formalin fixed, paraffin wax embedded blocks were retrieved from the archives. Haematoxylin and eosin stained sections were cut from each tissue block, and suitable blocks were selected for our study.

Monoclonal antibodies used

The anti-p33^{ING1b} monoclonal antibodies (MAbs) used in our study were GN1 and GN2, which were produced in our laboratories. GN1 and GN2 were generated using a full length p33^{ING1b} recombinant protein as the immunogen for the

production and selection of specific MAb secreting hybridoma cell lines. The pET recombinant protein expression system (Novogen, UK) was used as described previously.⁷

MAB GN1 recognises the p33^{ING1b} N-terminal region (amino acids 1–32), whereas MAB GN2 recognises the p33^{ING1b} C-terminal region (amino acids 33–279). On western blots they react with a band at 33 kDa. Moreover, in immunohistochemical analysis, GN1 recognises both the nuclear and cytoplasmic forms of p33^{ING1b}, whereas GN2 only recognises an epitope on p33^{ING1b} that is detectable in the cytoplasm.

MAbs used to detect p53, ER, and PgR were NCL-DO7, NCL-ER-6F11, and NCL-PgR, respectively. These MAbs were obtained from Novocastra Laboratories, Newcastle upon Tyne, UK.

Immunohistochemistry

Multiple 5 µm sections were cut from paraffin wax embedded, formalin fixed normal and tumour tissue blocks. All sections were mounted on slides pretreated with APES in the conventional manner. Immunostaining for all cases was carried out in a single batch, avoiding interbatch variation. A standard streptavidin–biotin–peroxidase complex (Dako, Ely, Cambridgeshire, UK) method was used. First, sections were dewaxed in xylene and then rehydrated by immersion in 99%, 95%, and 70% ethanol. Endogenous peroxidase was blocked by pretreatment of sections with a 0.5% solution of hydrogen peroxide in methanol for 10 minutes, and the slides were then washed under running tap water. Pressure cooking for one minute in citrate buffer (200mM citric acid, 500mM NaOH,

pH 6.0) was used for antigen retrieval. Sections were then transferred into Tris buffered saline (TBS; 140mM NaCl, 50mM Tris/HCl, pH 7.6) for five minutes. They were then blocked with 10% normal rabbit serum in TBS for 10 minutes. Excess serum was removed and the sections were covered and incubated at room temperature with the appropriate primary monoclonal antibody at the appropriate dilution for 60 minutes (MAbs GN1 and GN2 at 1/200 dilutions). The sections were washed twice with TBS for five minutes each time, and then incubated with the secondary biotinylated rabbit antimouse antibody (Dako) at a 1/500 dilution for 45 minutes at room temperature. After this, sections were washed for 2 × 5 minutes in TBS, and then covered with the tertiary streptavidin–biotin–peroxidase antibody (Dako) at a 1/100 dilution for 45 minutes at room temperature. After rinsing for 2 × 5 minutes with TBS, the peroxidase activity was developed with 1 mg/ml 3,3'-diaminobenzidine (Sigma, Poole, Dorset, UK) in sterile distilled water/H₂O₂ solution for five minutes. Sections were then washed in tap water, counterstained with haematoxylin, dehydrated, and mounted in DPX.

Scoring

Tissue scoring was carried out by two observers, one an experienced histopathologist. Tissues were visualised by conference light microscope, the cell type was identified, and the proportion and intensity of nuclear and cytoplasmic staining for p33^{ING1b} were estimated. In the case of p53, ER, and PgR only nuclear staining was assessed. Several controls were used including normal breast, stroma tissues, and negative controls lacking the primary antibodies. Intensity was assessed on a four point scale as follows: 0, negative; 1, weak staining; 2, intermediate staining; and 3, strong staining. The percentage of cells staining was assessed on a six point scale as follows: 1, 0–4%; 2, 5–19%; 3, 20–39%; 4, 40–59%; 5, 60–79%; and 6, 80–100%. The scores for intensity and proportion were then multiplied to give a composite score from 0–18 according to the method of Detre *et al.*²⁰ This scoring system was used for all proteins studied. For descriptive purposes the intensity of score values 0, 1–6, 7–12, and 13–18 were considered negative, minimal, intermediate, and strong, respectively.

Statistical analysis

To assess the relations between p33^{ING1b}, p53, ER, PgR, lymph node status, and tumour size and grade, several statistical tests were applied. Relations between the continuous variables were assessed by Spearman's test for rank correlations. In addition, the continuous variables were categorised by dividing each into two groups (high and low) around the median, and then compared with other categorical variables using Fisher's exact test. All analyses were carried out using Graph Pad Prism statistical software.

RESULTS

p33^{ING1b} expression detected by MAb GN1

The analysis of normal tissue showed strong uniform expression of p33^{ING1b} in the nuclei of normal cells in breast lobules (fig 1A). Strong nuclear expression was also evident in the nuclei of cells in the adjacent stroma. In contrast, minimal cytoplasmic p33^{ING1b} expression was evident in the cytoplasm of normal breast epithelial and stromal cells. In invasive breast cancer, however, there was reduced nuclear expression of p33^{ING1b} in cancer cells, both in intensity and in the proportion of cells staining (fig 1B). Seventeen cases showed strong expression of p33^{ING1b} in their nuclei. However, the remaining 69 cases showed reduced nuclear p33^{ING1b} expression, with two cases lacking nuclear p33^{ING1b} expression completely (fig 2). Five cases showed enhanced p33^{ING1b} cytoplasmic expression and the remaining 81 cases showed minimal cytoplasmic p33^{ING1b} expression, with 28 of these cases lacking cytoplasmic p33^{ING1b} expression completely.

Figure 2 Scattered plot graph representing the patterns of expression of p53 and p33^{ING1b}, as detected by immunohistochemistry using the monoclonal antibodies (MAbs) GN1 and GN2. The lines correspond to the median values.

p33^{ING1b} expression detected by MAb GN2

Normal breast tissue showed minimal expression of p33^{ING1b} in the cytoplasm of epithelial cells in breast lobules (fig 1C). In invasive breast cancer, however, there was enhancement of cytoplasmic expression of p33^{ING1b} in seven cases (fig 1D). The remaining 79 cases showed minimal expression of p33^{ING1b} in the cytoplasm, with 33 of these cases lacking cytoplasmic p33^{ING1b} expression completely (figs 1E and 2).

p53 expression

Strong p53 expression was seen in 15 of the 86 cases of invasive breast cancer. Of the remaining cases, 27 showed intermediate expression, 37 showed minimal expression, and seven lacked p53 expression (fig 2).

ER expression

Strong ER expression was seen in 51 of the 86 cases. Of the remaining cases, 10 showed intermediate expression, nine showed minimal expression, and 16 lacked ER expression.

PgR expression

Strong PgR expression was seen in 42 of the 86 cases. Of the remaining cases, eight showed intermediate, 16 showed minimal, and 20 lacked PgR expression.

Relations between variables

The data were analysed as both continuous and categorical variables. Positive correlations were found between p33^{ING1b} nuclear expression using MAb GN1 and both ER expression ($r = 0.3342$; $p = 0.005$) and PgR expression ($r = 0.3237$; $p = 0.0038$). A positive correlation was also found between ER expression and PgR expression ($r = 0.5223$; $p < 0.0001$).

Moreover, negative correlations were found between p33^{ING1b} nuclear expression using MAb GN1 and p33^{ING1b} cytoplasmic expression using MAb GN2 ($r = -0.2345$; $p = 0.03$). An inverse correlation was also found between p53 expression and both p33^{ING1b} cytoplasmic expression using MAb GN1 ($r = -0.2014$; $p = 0.08$) and tumour size ($r = -0.2726$; $p = 0.02$). No other significant associations were found.

DISCUSSION

In our study, we saw a reduction in nuclear expression of p33^{ING1b} in breast cancer cells, both in intensity and in the proportion of cells staining in 69 of 86 cases. There was

enhancement of the cytoplasmic expression of p33^{ING1b} in a small proportion of cases (five and seven of 86 using GN1 and GN2, respectively), and there was complete loss of cytoplasmic p33^{ING1b} expression in 28 and 33 cases (using GN1 and GN2, respectively). This last observation contrasts with findings in other tumour series described previously.^{7 21 22} This suggests two possible mechanisms related to p33^{ING1b} loss of function in invasive breast carcinomas. First, a shift in the subcellular localisation (nuclear to cytoplasmic); reduced nuclear p33^{ING1b} expression is associated with the enhancement of cytoplasmic p33^{ING1b} protein ($r = -0.2345$; $p = 0.03$). Second, complete loss of cytoplasmic p33^{ING1b} expression, perhaps mediated by enhanced degradation of the protein.

Recent work provided evidence of nuclear to nucleolar translocation of p33^{ING1b} in normal skin fibroblast cell lines after exposure to ultraviolet rays.²³ In an investigation of a wide range of other types of tumours (melanoma, childhood acute lymphoblastic leukaemia, colorectal adenocarcinoma, and papillary thyroid carcinoma) clear evidence of reduced or in some instances complete loss of nuclear p33^{ING1b} expression, in addition to a cellular compartmental shift from the nucleus to the cytoplasm, was observed.^{7 21 22} Thus, it appears that reduced nuclear expression of p33^{ING1b} may be important in several other human neoplasms.

With regard to established prognostic markers, strong p53, ER, and PgR expression was observed in 15, 51, and 42 of the 86 cases, respectively. Analysis of a variety of known biological factors in our series indicated that ER and PgR expression were positively correlated with each other ($r = 0.5223$; $p < 0.0001$), as expected. High grade tumours were of larger size ($r = 0.2882$; $p = 0.01$) and more often negative for the expression of ER ($r = -0.2729$; $p = 0.04$) and PgR ($r = -0.5355$; $p < 0.0001$), as would be predicted. However, in our series, larger tumours were more frequently p53 negative ($r = -0.2726$; $p = 0.02$), which is inconsistent with previous work.¹⁹

"It is possible that impaired p33^{ING1b} function represents an alternative mechanism for the abrogation of p53 activity in invasive breast carcinoma"

Our results provided evidence that p33^{ING1b} alterations were associated with more poorly differentiated tumours. Positive correlations were found between p33^{ING1b} nuclear expression and both ER expression ($r = 0.3342$; $p = 0.005$) and PgR expression ($r = 0.3237$; $p = 0.0038$), although there was no significant association with grade. Moreover, the optimum function of p53 is dependent on p33^{ING1b} so that a reduction in nuclear p33^{ING1b} expression, as seen in our series, would be predicted to compromise p53 function. It is possible that impaired p33^{ING1b} function represents an alternative mechanism for the abrogation of p53 activity in invasive breast carcinoma. However, no association was seen in our study between the expression of p33^{ING1b} and p53. This is in accordance with previous published work.^{23 24} It was found that tissue expression of p33^{ING1b} was similar in both p53 preserved and p53 deficient mice. This further suggests that p33^{ING1b} expression is independent of p53 status and vice versa.

TP53 mutation has been reported to be associated with poor prognosis in breast cancer.^{18 25} The accumulation of p53 protein has been reported in over 50% of primary breast cancers, including patients who do not have a TP53 mutation.²⁶ It will clearly be of interest to determine whether breast cancers without p53 alterations behave differently in terms of prognosis, dependent on the status of p33^{ING1b} expression.

In conclusion, we found that 69 of the 86 invasive breast carcinomas cases studied had reduced nuclear expression of p33^{ING1b}, which was associated with a concomitant enhancement of cytoplasmic p33^{ING1b} expression in a proportion of cases. Moreover, reduced nuclear p33^{ING1b} expression was asso-

Take home messages

- The nuclear expression of p33^{ING1b} was reduced in a large proportion (69 of 86) of invasive breast carcinomas
- This was associated with a concomitant enhancement of cytoplasmic p33^{ING1b} expression in a proportion of cases, and with complete loss of cytoplasmic p33^{ING1b} expression in 33 cases
- These alterations may cause loss of normal cellular function of the p33^{ING1b} protein
- p33^{ING1b} alterations were associated with more poorly differentiated tumours
- Loss of p33^{ING1b} may be an important molecular event in the differentiation and the pathogenesis of invasive breast carcinomas

ciated with complete loss of cytoplasmic p33^{ING1b} expression in 33 cases. This shift in subcellular localisation, in addition to the complete loss of cytoplasmic p33^{ING1b} expression, or perhaps enhanced degradation, may cause loss of normal cellular function of the p33^{ING1b} protein. Our results provided evidence that p33^{ING1b} alterations were associated with more poorly differentiated tumours. These results support the view that loss of p33^{ING1b} may be an important molecular event in the differentiation and the pathogenesis of invasive breast carcinomas.

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