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Immunoreactive p53 and metallothionein expression in duct carcinoma in situ of the breast

No correlation

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Abstract Immunocytochemically detectable MT and p53 have been found more commonly in comedo DCIS of the breast with high-grade cytology. The aim of this study is to confirm these findings and to investigate the relationship between MT and p53 in a single large series of cases of DCIS of the breast. To this end, 127 cases of DCIS were classified histologically according to architecture, cytonuclear differentiation (grade), presence and extent of intraduct necrosis, and using the Van Nuys system. Sections were immunostained for p53 and MT (E9) using established techniques, and the extent and intensity of staining were assessed semi-quantitively. The results confirmed that there was generally more MT and p53 positivity in poorly differentiated (grade 3) DCIS with extensive necrosis and that MT expression was greater in grade 2 lesions than p53 expression. However, overall there was no statistically significant correlation between p53 and MT staining. The results indicate that MT and p53 overexpression may arise from independent mechanisms in early breast neoplasia.

Key words p53 · Metallothionein · Duct carcinoma of breast · DCIS · Immunohistochemistry

Introduction

Metallothionein (MT) is a low-molecular-weight cysteine-rich protein, which has the ability to bind and sequestrate heavy metal ions. It has recently been shown that immunocytochemically detectable increased levels of MT expression in invasive breast carcinomas are associated with histological and clinical markers of tumour progression and with worse prognosis [6, 7, 23]. Although normal and hyperplastic breast ductal epithelia have not previously been reported to express MT in detectable amounts [3], MT is variably expressed in duct carcinoma in situ (DCIS), with consistently greater amounts in the comedo type, in cytologically high-grade lesions and associated with extensive intraduct necrosis [5].

The protein p53 is a nuclear phosphoprotein which, in "wild type" form, is usually immunocytochemically undetectable in normal unprovoked breast epithelium. It has been postulated that increased expression of wild type functional p53 protein inhibits progression through the cell cycle in situations of ongoing DNA damage. This may allow time for DNA repair processes to occur before mitosis, a mechanism by which the cell is able to prevent the accumulation of DNA mutations [15, 19]. Raised levels of immunocytochemically detectable p53 protein have been noted as a physiological response to experimentally induced DNA damage (e.g. by UV radiation) [10, 14]. Mutant p53 protein resistant to intracellular degradation has also been found in raised amounts in neoplastic cells. The mutant form is also considered to be ineffective in responding to DNA damage and is therefore associated with cells with greater genomic instability and a greater malignant potential. However, immunocytochemically it is not possible to be certain in an individual case whether the p53 protein accumulating in the cells is wild type or mutated [9, 27]. Immunohistochemically detected p53 protein in infiltrating mammary carcinomas has nevertheless been shown to be consistently associated with a worse prognosis [1] and has also been shown to be present in DCIS, where it is associated with large cell size and intraluminal necrosis [22, 25].

Given these parallel observations the purpose of the present study was to check for a possible correlation between p53 and MT immunoreactivity in a series of cases of DCIS of the breast and to explore possible functional links between the two proteins in preinvasive neoplastic breast lesions.

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Materials and methods

Case material

The 127 cases of DCIS of breast were retrieved from the files of the Departments of Pathology at Llandough Hospital Trust and the University Hospital of Wales, Cardiff.

A representative block from each case, containing DCIS with background breast tissue, was selected for immunocytochemical staining of p53 and MT. The morphological charactaristics of nuclear grade and extent of necrosis were assessed by observation of the material on an adjacent serial section stained with haematoxylin and eosin.

Morphological classification of lesions

According to the duct architecture [2]

The architectural pattern of the DCIS was determined in the following way. If more than 70% of the structures present were of a single architectural type, the case was classified according to the predominent architectural type. Any case in which there were significant numbers of ducts (more than 10%) showing any degree of intraduct necrosis (defined as 5 or more pyknotic epithelial nuclei with debris in the lumen of a duct) was recorded as "with comedo".
The following categories were observed:

PC: pure comedo (>70% of ducts present show intraduct necrosis)
S: solid (<10% of ducts present show intraduct necrosis)
SC: solid with comedo (>10% but<70% of ducts present show intraduct necrosis)
C: cribriform (<10% of ducts present show intraduct necrosis)
CC: cribriform with comedo (>10% but<70% of ducts present show intraduct necrosis)
M: micropapillary
In this classification the cytological features of the tumour were ignored.

According to cytonuclear and architectural features as described by Holland et al. [11]

This classification is based on nuclear size and pleomorphism and polarity of the cells (architectural features). Well-differentiated DCIS showed uniform rounded regular nuclei with uniform chromatin staining and polarity of cells towards lumina within the architecture of the malignant intraduct proliferation. Moderately differentiated DCIS showed moderate nuclear pleomorphism with loss of polarity but some residual orientation of tumour cells. Poorly differentiated DCIS showed marked nuclear pleomorphism, coarse chromatin, hyperchromasia, and epithelial mitotic activity with loss of polarity of cells. Cytonuclear differentiation of DCIS was generally uniform within each individual case. If the cytonuclear features showed variation the case was classified according to the least differentiated area.

According to extent of intraduct necrosis

A third type of analysis based on a case-by-case comparison was performed on the basis of categorising the lesion/case as a whole as either pure comedo (i.e.>90% of ducts containing necrosis), DCIS with necrosis (DN+, i.e.>10% but<90% of ducts containing necrosis) or DCIS without necrosis (DN–, i.e.<10% of ducts containing necrosis).

Using the classification proposed by Silverstein et al. (Van Nuys) [25]

Lesions with high-grade (grade 3) nuclear features with or without necrosis were placed in the high-grade group. Non-high-grade DCIS was divided by the presence (group 2) or absence (group 1) of comedo-type necrosis. Although not defined in the original description, the observers in this analysis defined necrosis as the presence of at least 5 pyknotic epithelial cell nuclei with debris in the lumen of a duct. One duct containing necrosis as defined was enough to move a case from group 1 to group 2. Occasional desquamated or individually necrotic cells were ignored and were not scored as comedo type necrosis.

Immunocytochemical staining of p53 and MT

Paraffin sections 5 μm thick were cut from each block. For p53 staining after dewaxing and blocking, the endogenous peroxidase sections were then bathed in citrate buffer solution 0.01 M, pH 6 and autoclaved at 120°C, 15 psi for 10 min. After cooling in running tap water the sections were then transferred to PBS and immunostained. Monoclonal mouse antibody to human p53 protein was obtained from Dako: DO-7 at 1:100 dilution. The antibody reacts with the wild type and the mutant type of the p53 protein. The technique was validated on normal mucosa and tumour tissue from 10 cases of colon adenocarcinoma using frozen and paraffin-embedded sections and a panel of antibodies against p53 including PAB 421 [16].

For MT, monoclonal antibody to MT at 1:10,000 dilution was applied overnight at 4°C. The monoclonal anti-MT antibody, E9, was used in its unfractionated ascites form. The antibody is now commercially available in its purified form from Dako [13].

The tissue binding of the primary antibody in each case was revealed by the double immunoperoxidase method involving rabbit anti-mouse peroxidase (Dako) at 1:100 dilution for 1 h at 20°C), followed by secondary conjugate of swine anti-rabbit peroxidase (Dako) at 1:100 dilution for 1 h at 20°C). The sections were then rinsed with three changes of PBS and incubated in DAB/H_2O_2 substrate for 5 min at 20°C. They were finally counterstained in haematoxylin before being dehydrated and mounted.

Analysis of immunopositivity: intensity distribution Scoring

Within an individual ductal structure containing malignant epithelium, the total percentage of positive cells was assessed. Then the percentages of weakly, moderately and strongly staining cells were assessed so that the sum of these categories equated with the overall percentage positivity. A staining score was then calculated as follows:

Score (out of maximum of 300)=1 × percentage of weak (+)
2 × percentage of moderate (++)
3 × percentage of strong (+++)

The assessment was performed on a consensus basis by two pathologists (A.G. D-J. and B.J.) using a double-headed microscope. Initially, an overall decision on whether or not an individual case showed homogeneous staining was made. If all the ducts present showed similar levels of staining (homogeneous) three ducts were chosen at random for assessment. If staining appeared to be different in different ducts (heterogeneous) the three most strongly staining ducts and the three most weakly staining ducts were assessed and the corresponding mean values calculated. An average value was calculated from these means to represent the overall score for the lesion.

Background ducts and lobules showed no p53 positivity in either myoepithelial or epithelial cells. Immunopositivity for p53 in DCIS was almost exclusively intranuclear and was in many cases undetectable, resulting in an intensity distribution (ID) score of 0. Immunopositivity for MT in DCIS was seen in both nuclei and cytoplasm and the overall degree of positivity was assessed with no

attempt to score the two compartments separately. In this study background ducts and lobular epithelium showed some degree of positivity for MT. The degree of this positivity in background epithelial cells was assessed using the same ID scoring system described above in all cases in the same sections as the DCIS, but on a different occasion and without reference to the ID of the DCIS component.

Myoepithelial cells around background ducts and lobules stained strongly positive for MT, and these cells were disregarded during ID scoring.

Statistical analysis

Although the arithmetic mean and standard deviations of the observed ID values are given, the data were not normally distributed and so a non-parametric procedure for multiple comparisons between grouped data sets was applied with a standard error corrected for tied ranks. Such a test allows for zero values in one data set and also for non-normal frequency distributions within the data sets. Critical values for this test, Q(a), (k), are given by Zar [29].

Spearman's ranked correlation test was applied for comparison of p53 versus MT with paired data sets. Parametric procedures were inapplicable owing to the non-normal frequency distribution of the p53 data set (many lesions showed no positivity for p53, thus scoring ID of 0). Correlation coefficients, *r*s, were calculated with corrections for tied ranks.

Results

An example of positive immunostaining for MT in DCIS is shown in Fig. 1a, and an example of positive immunostaining for p53 in Fig. 1b. Analysis of the data was performed using the four different approaches to the classification of the DCIS, as outlined previously.

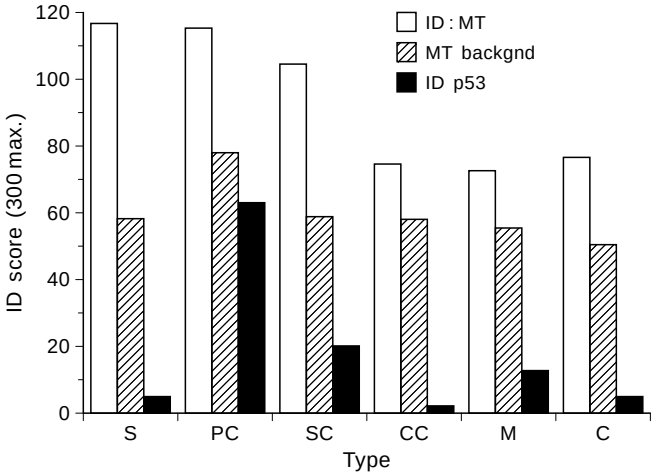


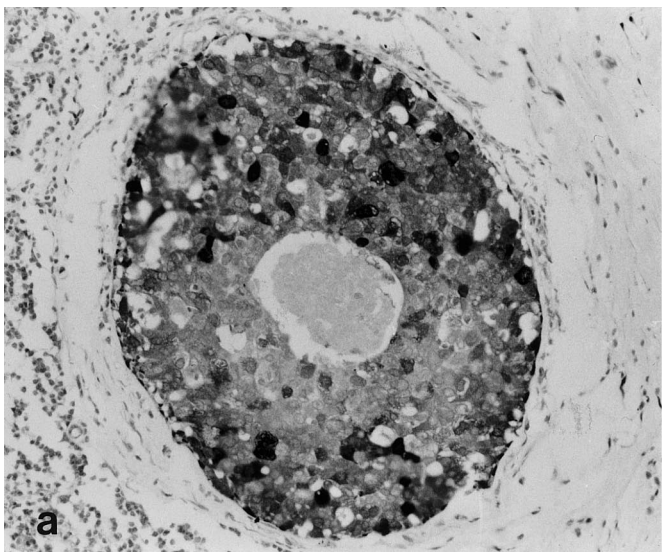
Fig. 2 Immunostaining of DCIS for p53 and MT according to architectural type. Mean ID scores (out of a maximum of 300) are plotted [*S* solid, *PC* pure comedo (>70% ducts containing necrosis), *SC* solid with comedo, *CC* cribriform with comedo, *M* micropapillary, *C* cribriform]

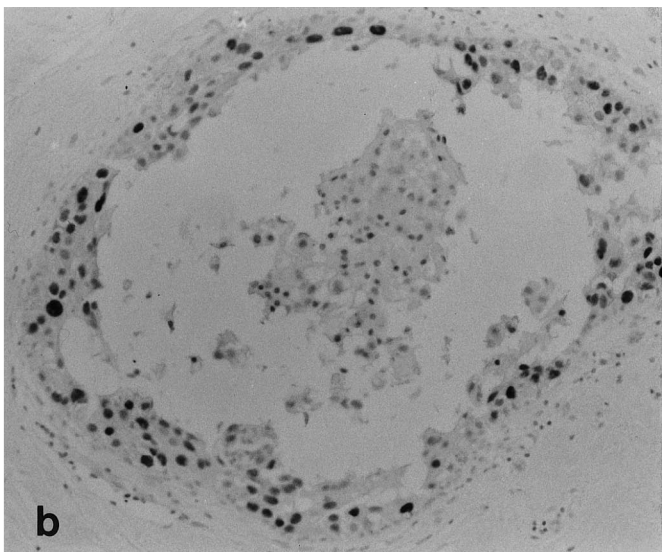
Analysis according to duct architectural type

The ID scores for p53 in relation to type of DCIS are shown in Fig. 2. The results show low expression of p53 in cribriform (mean: 4.6, SD: 9.8, *n*=15) micropapillary (mean: 12.9, SD: 2.4, *n*=15) and cribriform with focal necrosis (mean: 1.9, SD: 2.1, *n*=6) . There is more p53 expression in solid-type DCIS (mean: 4.8, SD: 8.4, *n*=11), and this expression is increased if more than 10% of ducts contain necrosis (SC; mean: 19.9, SD: 30.6, *n*=27). The highest p53 immunopositivity was seen in the pure comedo type of DCIS (mean: 62.9, SD: 78.8, *n*=31). The difference between micropapillary and pure comedo is highly significant ($P<0.01$) and the difference between micropapillary and solid with comedo (SC) is also significant ($P<0.05$).

ID scores for MT according to type of DCIS are also shown in Fig. 2, and the results show an overall higher

Fig. 1a Duct containing duct carcinoma in situ (DCIS) showing immunopositivity for metallothionein (MT). This duct was given an intensity distribution (ID) score of 118 (out of a possible maximum of 300). **b** Duct containing DCIS showing nuclear immunopositivity for p53. This duct was given an ID score of 155 (out of a possible maximum of 300). ×200





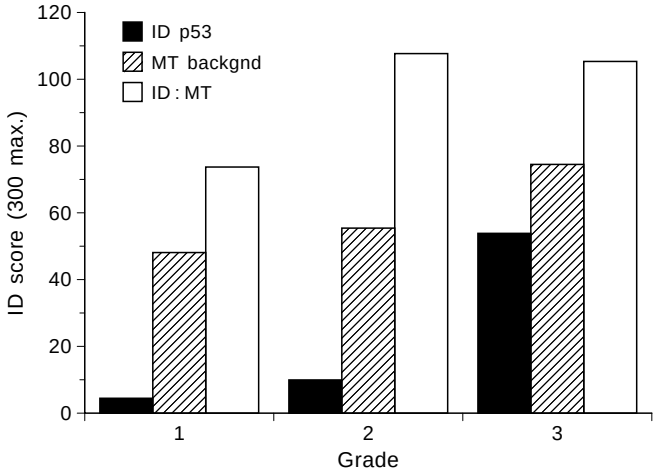


Fig. 3 Immunostaining of DCIS for p53 and MT according to cytonuclear differentiation (grade) [11]. Mean ID scores (out of a maximum of 300) are plotted (*1* well differentated, *2* moderately differentiated, *3* poorly differentiated)

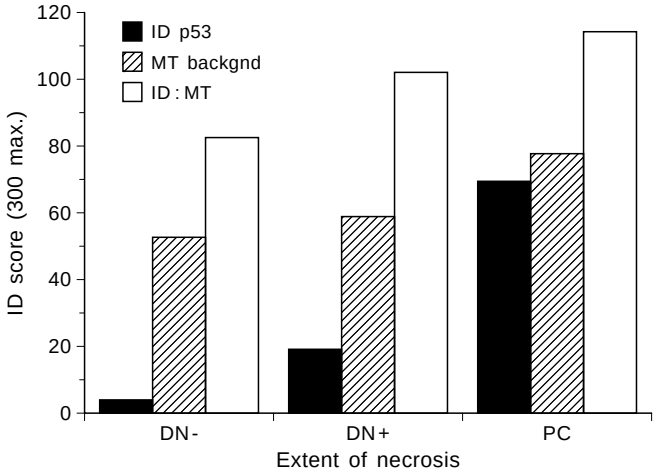


Fig. 4 Immunostaining of DCIS for p53 and MT according to extent of necrosis (disregarding architecture and cytology). Mean ID scores (out of a maximum of 300) are plotted [*DN–* no necrosis (<10% of ducts contain necrosis, *DN+* with necrosis (>10% and<90% of ducts contain necrosis), *PC* pure comedo (>90% of ducts contain necrosis)]

level of positive staining but with a pattern similar to that for p53. There is lower MT ID in cribriform DCIS (mean: 76.7, SD: 48.1, *n*=15), cribriform with more than 10% of ducts containing necrosis (mean: 74.8, SD: 30.7, *n*=6), and micropapillary (mean: 72.4, SD: 41.6, *n*=15). There is significantly more MT expression in solid DCIS (mean: 117.6, SD: 29.1, *n*=11), solid DCIS with ducts containing necrosis (mean: 104.6, SD: 44.1, *n*=27) and pure comedo-type DCIS (mean: 118.2, SD: 43.6, *n*=31). The differences between micropapillary and pure comedo is significant ($P<0.05$) and that between micropapillary and solid (S) is significant ($P<0.05$).

Analysis according to cytological differentiation and cellular polarity (cytological grade)

ID scores for p53 in relation to cytonuclear differentiation (grade) are shown in Fig. 3. There is little p53 positivity in well-differentiated lesions, i.e. grade 1 (mean: 5.0, SD: 9.2, *n*=27) and moderately differentiated lesions, i.e. grade 2 (mean: 10.3, SD 13.7, *n*=35) and significantly ($P<0.05$) increased expression of p53 in poorly differentiated, i.e. grade 3 DCIS lesions (mean: 54.4, SD: 72.2, *n*=40) . The ID scores for MT in relation to p53 expression are also shown in Fig. 3, and there is significantly ($P<0.05$) more MT expression in lesions of grades 2 (mean: 107.7, SD: 48.2, *n*=34) and 3 (mean: 105.4, SD: 44.5, *n*=74) than in grade 1 lesions (mean: 73.7, SD: 41.4, *n*=36). Interestingly, there is a marked difference between p53 expression and MT expression in grade 2 lesions in which there is raised expression of MT but not of p53.

Analysis according to extent of intraduct necrosis

ID scores for p53 in relation to the extent of intraduct necrosis is shown in Fig. 4. There is very low p53 expression in ducts containing no necrosis (mean: 3.3, SD:7.3, *n*=35), with higher expression in ducts with necrosis (mean: 18.7, SD: 28.8, *n*=42), but much higher levels are seen in pure comedo cases (mean: 69.0, SD:79.5, *n*=30). ID scores for MT expression in relation to necrosis are shown in Fig. 4 and the data mirror those for p53 in that lowest expression of MT is seen in cases without intraduct necrosis (mean: 82.1, SD: 47.7, *n*=35), whereas significantly higher expression is seen in cases of pure comedo DCIS (mean: 113.6, SD: 47.4, *n*=29) .

Analysis according to Van Nuys classification

ID scores for p53 and MT in relation to the Van Nuys classification are shown in Fig. 5. The results are very similar to those analysed by the Holland classification (cytology and polarity). In both analyses p53 and MT expression in low-grade DCIS is significantly ($P<0.05$) different from expression in high-grade lesions.

There was significantly more MT in background breast containing grade 3 DCIS than in background breast containing grade 1 DCIS (Holland and Van Nuys classifications).

Overall the results show a parallelism between expression of p53 and metallothionein in relation to each of the classification systems analysed. The relationship between p53 and MT ID scores is shown in Fig. 6. There is no significant correlation between the levels of the two proteins (Spearmans' ranked correlation test).

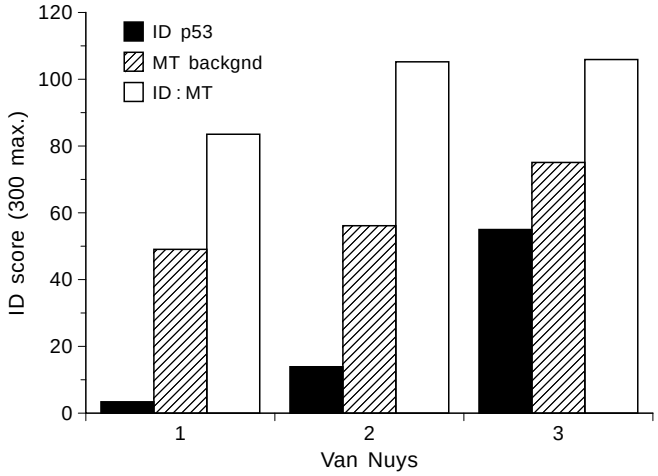


Fig. 5 Immunostaining of DCIS for p53 and MT [25]. Mean ID scores (out of a maximum of 300) are plotted [*1* low-grade cytology, no necrosis, *2* low-grade cytology with necrosis, *3* high grade cytology (with or without necrosis)]

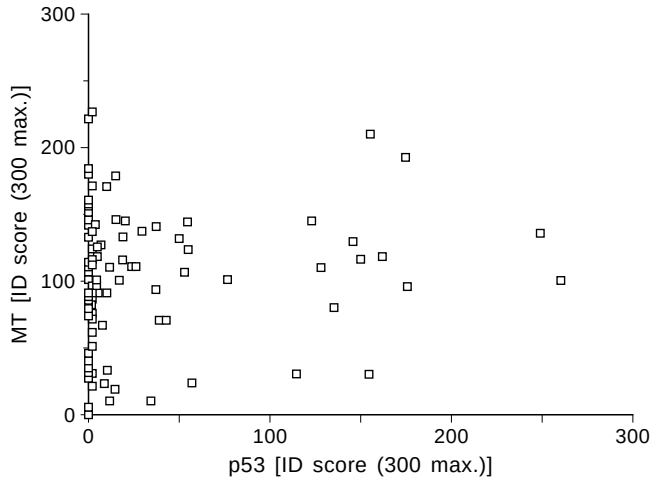


Fig. 6 ID scores of immunostaining for p53 and MT in DCIS cases are plotted. No relationship between the expression of the two proteins can be demonstrated

Discussion

In invasive breast carcinomas, increased MT expression and p53 expression by tumour cells are associated with a worse prognosis [1, 6, 23]. These observations are reflected in the pre-invasive stage of the disease.

This larger series confirms that the expression of MT is greater in grade 2 and 3 DCIS and those with extensive necrosis (>90% of ducts showing intraluminal necrosis), as previously observed, but grade 1 lesions and DCIS without necrosis also show MT expression [5]. In this study background morphologically 'normal' breast epithelial cells were found to contain immunocytochemically detectable MT. Previous studies of MT expression in benign breast disease have not shown this phenomenon [3]. There are several possible explanations for this observation in this study. The assay may be particularly sensitive so that previously undetectable levels of MT have been immunostained. This is unimportant in that all the sections were immunostained as a 'batch' and although all ID scores may be raised the differences between the grades of DCIS will remain. Alternatively, the background breast epithelium in these cases may not be 'normal' but may show a genuine increase in MT levels above those in normal breast epithelium not associated with morphological change.

Of particular interest is the observation that there is more immunocytochemically detectable MT in background epithelium from breasts containing grade 3 DCIS than in background epithelium from breasts containing grade 1 DCIS (Figs. 3–5). If there was a general increase in threshold, this finding would not be expected. In view of the possibility that MT expression may be an early event in breast carcinogenesis, it would be of considerable interest to explore this possibility further by comparing MT expression in genuinely normal breast epithelium directly with that in background morphologically "normal" epithelium from breasts containing malignant disease. A more sensitive assay than standard immunocytochemistry would probably be required to resolve the question.

Early studies investigating the expression of p53 protein in DCIS were limited because the small numbers of pure DCIS were included as part of a larger group of infiltrating breast carcinomas. Schmitt et al. found no correlation between p53 positivity (defined as 5% or more of tumour cell nuclei showing p53 immunostaining), but only 23 cases were studied and only an architectural classification was used [24]. Umekita et al. found only 1 of 13 cases of pure DCIS expressing p53, but in invasive tumours with an in situ component there was a significant correlation between p53 expression and histological grade and comedo subtype (but the method of grading and the definition of comedo are not given in this paper) [26]. The study presented here is the first to document the expression of immunocytochemically detectable p53 in DCIS classified by a number of different published systems. The results confirm that there are higher levels of expression of p53 protein in high-grade DCIS with necrosis [22, 25, 28]. Detection of p53 immunocytochemically is virtually confined to grade 3 DCIS with pure "comedo" architecture (defined as more than 90% of ducts present showing intraluminal necrosis), as shown in Figs. 2–4. All immunocytochemical studies on p53 accumulation must be interpreted with caution, in that raised intracellular p53 levels do not necessarily imply mutated protein product [9, 27].

The raised expression of p53 in high-grade comedo DCIS does not therefore mean that *p53* mutation has occurred. Early attempts to demonstrate *p53* gene mutations in p53-immunopositive DCIS have had limited success [21].

There are a number of mechanisms other than mutation by which p53 may be functionally inactivated. Loss of function could be due to a sequestration of p53 in the cytoplasmic compartment away from its intranuclear site

of action [20]. More recently, enzymatic modification of the carboxy-terminal regulatory site by protein kinases has been shown to alter binding of wild type p53 to DNA [12]. There are theoretical mechanisms by which MT and p53 may interact in the control of cell division. p53 binds to DNA (preventing transcription) through a zinc-dependent binding mechanism. Binding of zinc finger proteins, which act as transcription factors, can be weakened in vitro by the presence of metal chelating agents [4]. In addition, it has been shown in vitro that exposure to a metal-chelating agent induces a reversible conformational change in "wild type" p53 to the mutant form. It is thought that binding of zinc ions to cysteinyl residues stabilises the tertiary structure of p53. [8]. MTs also have high affinity for zinc ions and so could act as intracellular sequestraters of zinc . Cells containing MT in sufficient quantity to reduce intranuclear zinc ion levels and functionally inactivate p53 would be at a growth advantage and be able to proliferate, accumulating mutational events. The data presented here show that there is expression of MT in grade 1 and grade 2 DCIS (and even the suggestion of expression in background epithelium in breast tissue containing DCIS) but minimal p53 accumulation, indicating that the accumulation of MT may be an important event in carcinogenesis of "low and intermediate grade" breast intraduct neoplasia without the appearance of immunocytochemically detectable p53. This finding would be in keeping with the hypothesis outlined above, in that over-expression of MT as an initial event could lead to sequestration of zinc ion and functional inactivation of p53, either through inability to bind DNA or through conformational change to mutant form, because of low zinc ion concentrations. By contrast, "high grade" DCIS is more likely to involve inactivation of p53 by mutation, making MT levels irrelevant.

The cytonuclear differentiation (grade) of DCIS as classified using cytological and polarity features is correlated with the grade of the invasive carcinoma arising from it [18]. It is likely therefore that the p53/MT profile of the DCIS will be perpetuated into the invasive stage of the disease.

Although long periods of follow-up are required and there are relatively small numbers of cases for study, high cytonuclear grade and the presence of necrosis have been linked to local recurrence of DCIS and development of invasive breast carcinoma [17, 25]. As levels of p53 and MT are raised in high-grade DCIS with necrosis it is likely that they are also linked to more aggressive biological behaviour in DCIS, but there is little direct evidence to support this. The rise in both p53 and MT expression in high cytonuclear grade DCIS with necrosis suggested a possible link between the expression of these two proteins, but there is no statistical correlation between the ID scores in this series. This indicates that the expression of MT and p53 is not linked in any simple direct way in early breast neoplasia. This finding would be consistent with the hypothesis that neoplastic progression in early breast cancer could occur by at least three independent pathways. The first pathway (possibly active in grade 1 and 2 DCIS) would involve initial increased expression of MT, sequestration of intracellular zinc and functional inactivation of normal wild type p53 through inhibition of intranuclear zinc dependent binding. The second pathway (in grade 3 DCIS) would involve inactivation and accumulation of p53 by other independent mechanisms (one of which would be *p53* gene mutation), and in these circumstances the level of MT expression in the cell would be irrelevant. The third pathway would involve accumulation of neither MT nor p53. In keeping with these postulates, some cases of high-grade comedo DCIS show high levels of expression of MT and p53, whilst others show either MT or p53 expression alone, and in addition there are cases that are negative for both MT and p53 immunostaining.

In conclusion, the results presented here confirm previous findings that there are higher levels of expression of p53 and MT protein in high-grade DCIS with necrosis whichever classification system of DCIS is applied. In this study no correlation was found between p53 and MT, suggesting that raised expression of these proteins in high-grade DCIS comes about by independent pathways.

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