p53 mutation in breast cancer. Correlation with cell kinetics and cell of origin

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Aim: Several studies have investigated the expression of the cytokeratins (CKs), vimentin, the epithelial growth factor receptor (EGFR), the oestrogen receptor (ER), and the progesterone receptor (PgR), in breast cancer, but no study has directly compared p53 mutations with these phenotypic and differentiation markers in the same case. The present study was designed to provide some of this information.
Methods: The expression of the p53 and bcl-2 proteins was evaluated by immunohistochemistry in relation to phenotypic characteristics and cellular kinetic parameters (mitotic index and apoptotic index) in 37 cases of ductal carcinoma in situ (DCIS) and 27 cases of infiltrating ductal carcinoma (IDC) of the breast. In addition, p53 gene mutation was examined by polymerase chain reaction single strand conformation polymorphism analysis (SSCP).
Results: Thirteen cases (eight DCIS and five IDC) showed expression of CK8, CK14, CK18, vimentin, and EGFR, consistent with a stem cell phenotype, whereas 44 cases (27 DCIS and 17 IDC) showed expression of CK8 and CK1, weak or negative expression of CK18, but were negative for vimentin and EGFR, consistent with a luminal cell phenotype. DCIS and IDC cases with a stem cell phenotype were ER/PgR negative and intermediately or poorly differentiated. In contrast, the cases with luminal cell phenotype were ER/PgR positive and well or intermediately differentiated. In addition, intermediately or poorly differentiated cases with a stem cell phenotype showed higher proliferative activity (per cent of MIB-l positive cells) than did intermediately or well differentiated cases with a luminal cell phenotype. Both DCIS and IDC cases with a stem cell phenotype were p53 positive and bcl-2 negative by immunohistochemistry. In IDC, p53 expression was associated with a reduction of both mitotic index and apoptotic index compared with DCIS. Most of the tumours showing a more differentiated phenotype (luminal) were p53 negative and bcl-2 positive. In these cases, cell kinetic parameters increased from DCIS to IDC. These data suggest the existence of subsets of DCIS and IDC that, because of their phenotypic characteristics, could be derived from subpopulations of normal breast cells having different control mechanisms of cell proliferation and neoplastic progression.
Conclusions: These results are compatible with the hypothesis that the phenotype of the cell of origin constrains both tumour phenotype and the choice of genetic events; however, the occurrence of p53 mutants by chance during neoplastic transformation cannot be excluded.

Abnormalities of the p53 tumour suppressor gene are among the most frequent molecular events in human and animal neoplasia.[1] It is now accepted that the inactivation of this gene, as a result of mutation, is a key step in neoplastic transformation and progression.[2 3] This effect is usually related to the loss of p53 activated cell death, growth arrest, and/or control of genomic stability.[2] However, it is not yet completely clear which of the many properties of p53 are particularly important in oncogenesis.[4 5] In addition, the p53 activation system may be influenced by cell type and specific tissue effects.[2] About 30–50% of breast cancers carry a mutant p53 gene and show p53 protein expression by immunohistochemistry.[6]

The expression of this oncoprotein in breast cancers seems to be related to poor prognosis associated with a high histological grade, epidermal growth factor receptor (EGFR) positivity, and bcl-2 and oestrogen receptor (ER) negativity.[7–16] However, it should be noted that clinical pathological studies also report the coexpression of cytokeratin (CK) and vimentin in breast cancer as a more aggressive phenotype associated with poor prognosis.[17 18] This particular phenotype has been detected in a subset of normal breast epithelial cells that grows out of mammoplasty cultures, after most of the early passage cells have senesced.[19 20] The high proliferative capacity of these cells, together with this uncommitted differentiation state, has led many observers to regard them as being derived from a small "stem cell" pool present in the basal layer of the normal

gland.[21] In contrast, most normal breast epithelial cells present in the intact gland and in early passage mammoplasty cultures express CK8, CK18 (weakly), and CK19, but not vimentin.[20]

> "It is now accepted that the inactivation of the p53 gene, as a result of mutation, is a key step in neoplastic transformation and progression"

Although several studies have investigated the expression of CKs and vimentin, EGFR, ER, and the progesterone receptor (PgR) in breast cancer,[17 18 22–30] to our knowledge, no study has directly compared p53 mutations with these phenotypic and differentiation markers in the same case. Our present study was designed to provide some of this information. We therefore analysed the expression of p53 (wild-type and mutated form) and bcl-2 proteins in relation to phenotypic characteristics in 37 cases of ductal carcinoma in situ (DCIS) and 27 cases of infiltrating ductal carcinoma (IDC) of the

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Abbreviations: AI, apoptotic index; DCIS, ductal carcinoma in situ; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; HPF, high power field; IDC, infiltrating ductal carcinoma; ISEL, in situ end labelling; MI, mitotic index; PCR, polymerase chain reaction; PgR, progesterone receptor; SSCP, single strand conformation polymorphism

Table 1 Antibodies used for immunohistochemical reactions on paraffin wax sections of formalin fixed tissue

Antigen/ antibody	Clone	Source
CK8	5D3	Menarini Diagnostics (Florence, Italy)
CK18	5D3	Menarini Diagnostics
CK14	LL002	Menarini Diagnostics
Vimentin	V9	Dako (Milan, Italy)
EGFR	31G7	DBA
ER	ER1D5	Immunotech (Marseille, France)
PgR	PR10A9	Immunotech
MIB1	MIB-1	Immunotech
p53	DO-7	Immunotech
Bcl-2	124	Dako (Milan, Italy)

CK, cytokeratin; EGFR, epidermal growth factor receptor; ER, oestrogen receptor; PgR, progesterone receptor.

breast. Because these oncoproteins are involved in the regulation of cell proliferation and cell death, we also assessed, in each case, the proliferative activity, and mitotic and apoptotic indices (MI and AI, respectively).

MATERIALS AND METHODS
Data collection
Formalin fixed, paraffin wax embedded tissue samples of 64 serial archival cases of breast carcinoma (37 cases of DCIS and 27 cases of IDC) were used. None of the DCIS cases showed any adjacent areas of invasive carcinoma. Nineteen IDC cases showed adjacent areas of in situ carcinoma. All patients were diagnosed at the Institute of Pathological Anatomy and Histology, University of Siena. The patients' ages ranged from 29 to 85 years, with a median age of 65. All patients had been treated with partial or modified radical mastectomy and axillary node dissection, with no previous chemotherapy or radiotherapy. Haematoxylin and eosin stained sections of all cases were reviewed and classified according to the common histological criteria of the World Health Organisation classification.[31] The pathological stage was assessed according to the criteria established by the International Union Against Cancer.[32]

Immunohistochemistry
Paraffin wax sections were cut to a thicknesses of 5 µm, immersed in citric acid buffer (pH 6.0) and incubated twice in a microwave oven at 750 W power for five minutes each. Table 1 details the monoclonal antibodies used. The sections were immunostained by the APAAP complex method (Dako, Glostrup, Denmark). Antibody expression was evaluated in each case by two experienced pathologists (TM, FF), independently, and consensus was reached in every case. Cases were considered positive for CK8, CK14, CK18, and vimentin when cytoplasmic staining was seen in all neoplastic cells. EGFR was only considered positive when membrane staining was seen. A semiquantitative score (±) was given for weak or focal positivity for CK18, vimentin, or EGFR.

We considered tumours to be positive for p53 or bcl-2 expression when 10% or more of the cells counted were stained, but we made no attempt to quantify the intensity of immunostaining for each cell.[33] Furthermore, we graded MIB-1, ER, and PgR expression as low or high when the percentages of positive cells were < 40% or ≥ 40%, respectively, based on the frequency histogram analysis. For negative controls, we replaced primary antibody with normal mouse serum.

In situ end labelling (ISEL)
For comparison with the morphologically registered apoptotic cells/bodies, the in situ end labelling (ISEL) technique, which visualises DNA fragmentation, was applied with minor modifications as reported previously.[34] Negative controls were obtained by omitting the DNA polymerase in the labelling mix.

Cell counts
For each case, we first assessed the cellularity by determining the number of intact epithelial cells in each high power field (HPF; median, 1200; range, 642–1872) in 30 randomly chosen HPFs (HPF, 56 000 µm^2/field), using an oil immersion objective (×l00). We then assessed the MI and AI by counting the percentages of neoplastic cells in mitosis and the number of apoptotic cells/bodies for each 100 neoplastic cells in an entire section for each case (~ 100 HPFs). With this approach, the relative number of apoptotic cells/bodies corresponded well with the percentage of cells showing ISEL of the DNA strand breaks.[34 35] The intra-observer and interobserver reproducibility of the counts was 95%.

Positively stained cells for all the antibodies used were counted in 30 randomly chosen HPFs and expressed as percentages of all epithelial cells. The slides were scored by two independent pathologists (CB, AVL), each performing repeated counts on the same section, but viewing different HPFs. To verify the reproducibility, the absence of any systematic differences between independent scorings was preliminarily tested with the Wilcoxon signed rank test for paired samples. No significant differences were found (p > 0.l). A good agreement between observers (little variance of the differences) was then shown by Pearson correlation analysis ($r = 0.95$; $p < 10^{-14}$).

Molecular analysis: screening for p53 gene mutation (PCR-SSCP)
The method used in our study for identifying mutations in the p53 gene involved DNA amplification using the polymerase chain reaction (PCR), followed by identification of the mutated products using the single strand conformation polymorphism (SSCP) technique.

DNA was extracted from paraffin wax embedded tumour tissues following the procedure described previously.[35] PCR of exons 5–10 was carried out in a 50 µl volume, consisting of 5× reaction buffer (300 mmol/litre Tris/HCl, 75 mmol/litre

Table 2 Expression of CKs, vimentin, and EGFR in DCIS and IDC

	CK8	CK14	CK18	Vimentin	EGFR	Total case
Stem cell phenotype						
DCIS	+	+	+	+	+	8
IDC	+	+	+	+	+	5
Luminal cell phenotype						
DCIS	+	+	+/–	–	–	27
IDC	+	+	+/–	–	–	17
Not classifiable						
DCIS	+	+	+	+/–	+/–	2
IDC	+	+	+	+/–	+/–	5

CK, cytokeratin; DCIS, ductal carcinoma in situ; EGFR, epidermal growth factor receptor; IDC, infiltrating ductal carcinoma.
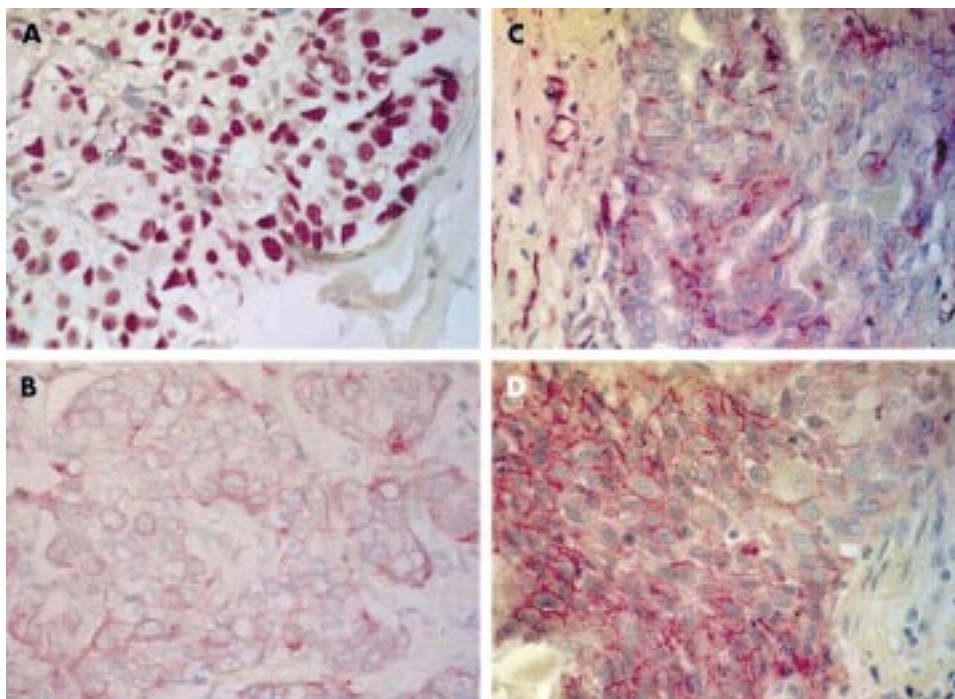
Figure 1 Expression of (A) p53 protein in relation to the stem cell phenotype: (B) CK18^{+}, (C) vimentin positive, and (D) epidermal growth factor positive.

$(NH_4)_2SO_4$, 7.5 mmol/litre MgCl$_2$ (pH 8.5)), dNTP mixture (final concentration 0.25 mmol/litre), 1.0 U cloned *Thermus aquaticus* DNA polymerase (Life Technologies, Milan, Italy), and p53 primers[21 22] (final concentration 0.4 µmol/litre). PCR amplifications were run in a programmable thermal cycler (Eppendorf, Netheler, Germany) for one minute at 94°C, one minute at 56°C (exons 5 and 8) or 59°C (exons 6, 7, 9, and 10), and one minute at 72°C, followed by a final 10 minutes at 72°C. Non-isotopic SSCP analysis of PCR products was carried out using a GenPhor electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden) on 12.5 % polyacrylamide gels (GeneGel Excel, 12.5/24 Kit; Pharmacia Biotech) at 400 V, 10°C, for 1.5 hours. Single stranded DNA fragments were stained with silver in an automated gel stainer (Hoefer Pharmacia Biotech).

Statistical analysis of scoring data

Sample mean and SD values were computed as descriptive statistics for the analysed variables (MI, AI, and MIB-1). Linear correlation analysis between these variables was performed by means of Pearson coefficient of correlation (r). A regression line of MI against AI was also computed. The Mann-Whitney test for independent samples was used to compare the differences between sample groups.

RESULTS

Expression of CK, vimentin, and EGFR in DCIS and IDC of the breast

Table 2 details the expression of CK8, CK18, CK14, vimentin, and EGFR in DCIS and IDC of the breast.

Thirteen cases (eight DCIS and five IDC) showed expression of CK8, CK14, CK18, vimentin, and EGFR (fig 1). In contrast, 44 cases (27 DCIS and 17 IDC) showed expression of CK8, CK14, weak expression or negativity for CK18, and negativity for vimentin and EGFR (fig 2). The cases of IDC with adjacent in situ areas showed the same phenotype in both components.
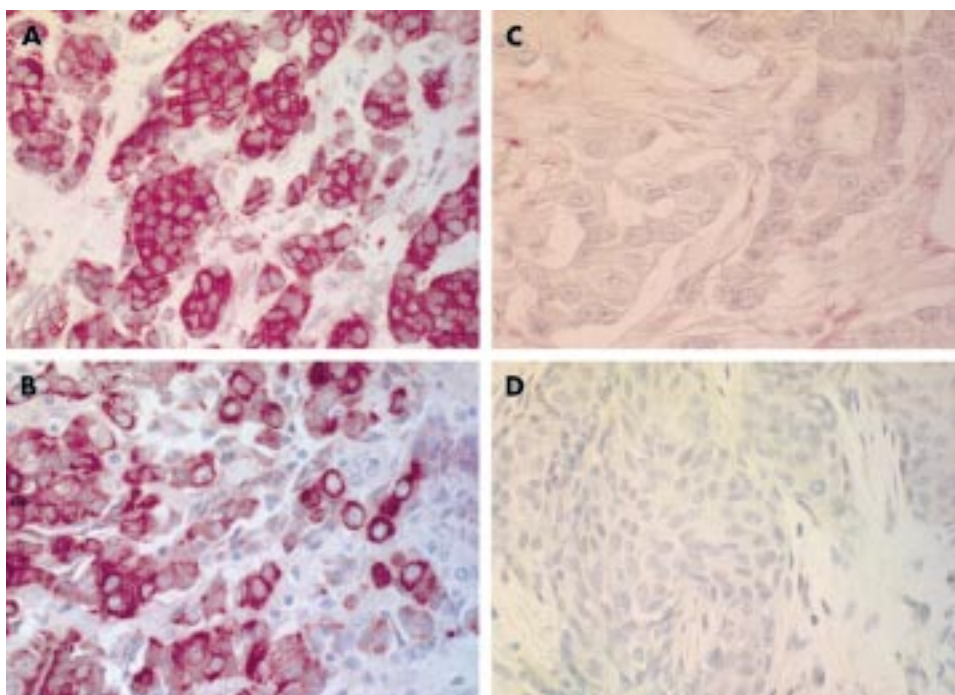

Figure 2 Expression of (A) bcl-2 protein in relation to the luminal cell phenotype: (B) CK8^{+}, (C) vimentin negative, and (D) epidermal growth factor negative.

Table 3 Expression of p53 and bcl-2 in DCIS and IDC

	Stem cell phenotype		Luminal cell phenotype		Not classifiable	
	DCIS	IDC	DCIS	IDC	DCIS	IDC
p53$^+$/bcl-2$^-$	8	5	0	2	0	0
p53$^-$/bcl-2$^+$	0	0	25	12	2	5
p53$^+$/bcl-2$^+$	0	0	2	3	0	0

DCIS, ductal carcinoma in situ; IDC, infiltrating ductal carcinoma.

According to these phenotypic characteristics, our cases were considered to be of stem or luminal cell origin, respectively.

Two DCIS and five IDC cases showed a phenotype pattern unrelated to either a luminal or stem cell origin.

Histopathological characteristics of DCIS and IDC of the breast and correlation with phenotype characteristics

The cases with a stem cell phenotype (CK8$^+$, CK14$^+$, CK18$^+$, vimentin positive, EGFR$^+$) were characterised by ER/PgR negativity; four cases (three DCIS and one IDC) were intermediately differentiated and nine cases (five DCIS and four IDC) were poorly differentiated. In contrast, the cases with a luminal cell phenotype (CK8$^+$, CK14$^+$, CK18$^{+/-}$, vimentin negative, EGFR$^-$) showed ER and PgR positivity; seven cases (four DCIS and two IDC) were well differentiated, 31 cases were intermediately differentiated (18 DCIS and 12 IDC), and one IDC was poorly differentiated. Furthermore, DCIS and IDC cases with a stem cell phenotype had much higher values of MIB-1 positive cells (mean, 63.5% and 69.95%, respectively) than cases with a luminal cell phenotype (mean, 14.96% and 18.9%, respectively; $p < 0.00l$).

Expression of p53 and bcl-2 in DCIS and IDC of the breast and correlation with phenotype characteristics

p53$^+$ and bcl-2$^+$ cells showed nuclear and cytoplasmic positivity, respectively, in a variable number of neoplastic cells in both DCIS and IDC. A significant inverse correlation between bcl-2 and p53 expression was detected—15 cases (eight cases of DCIS and seven cases of IDC) were p53$^+$/bcl-2$^-$, and 44 cases (27 cases of DCIS and 17 cases of IDC) were, on the contrary, p53$^-$/bcl-2$^+$. Only five cases were bcl2$^+$ and p53$^+$. No p53$^-$/bcl-2$^-$ cases were observed.

Table 3 summarises the expression of p53 and bcl-2 in DCIS and IDC in relation to phenotypic characteristics. Thirteen cases (eight DCIS and five IDC cases) were p53$^+$/bcl-2$^-$ and showed a phenotype consistent with a stem cell origin (CK8$^+$, CK14$^+$, CKl8$^+$, vimentin positive, EGFR$^+$). Only two p53$^+$/bcl-2$^-$ cases showed a luminal cell phenotype. Forty four cases were p53$^-$/bcl-2$^+$ (27 DCIS and 17 IDC cases) and 37 showed a phenotype consistent with a luminal cell origin (CK8$^+$, CK14$^+$, CK18$^{+/-}$, vimentin negative, EGFR$^-$). The five p53$^+$/bcl-2$^+$ cases also showed a phenotype consistent with a luminal cell origin.

Mutational screening of p53

Thirty seven DCIS and 27 IDC samples were examined for mutation in exons 5 to 10 of the p53 gene by PCR-SSCP. By this method, no band shifts were observed in DCIS, either in p53$^-$ cases or in p53$^+$ cases evaluated by immunohistochemistry; conversely, band shifts suggestive of point mutation were observed only in the 10 cases of IDC that were also positive for p53 protein by immunohistochemistry. No band shifts were detected in IDCs that were negative for p53 protein by immunohistochemistry.

Cellular kinetics of DCIS and IDC of the breast and correlation with phenotype characteristics

Figure 3 illustrates the cellular kinetic characteristics (MI and AI) of DCIS and IDC of the breast. In p53$^+$/bcl-2$^-$ cases with a stem cell phenotype, there was a reduction of both MI and AI in IDC when compared with DCIS ($p < 0.00l$). The converse

Stem cell phenotype and p53+/bcl2–
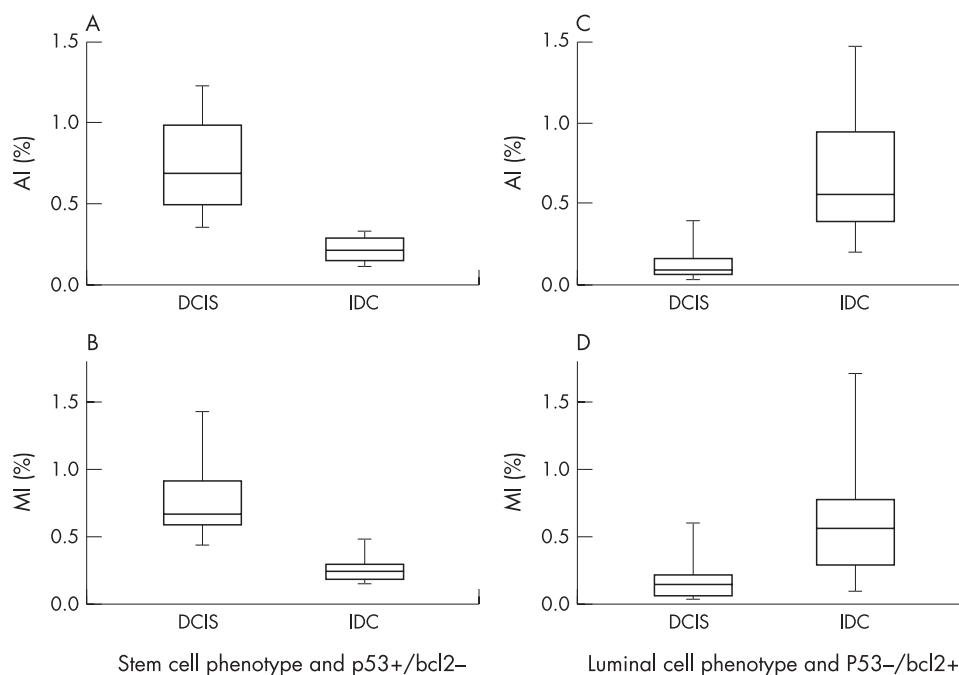
Luminal cell phenotype and P53–/bcl2+

Figure 3 Cellular kinetic characteristics (apoptotic index (AI) and mitotic index (MI)) of ductal carcinoma in situ (DCIS) and infiltrating ductal carcinoma (IDC): in p53$^+$/bcl-2$^-$ cases with a stem cell phenotype there is a reduction of both AI (A) and MI (B) in IDC compared with DCIS ($p < 0.001$). In p53$^-$/bcl-2$^+$ cases with a luminal cell phenotype, AI (C) and MI (D) increased from DCIS to IDC ($p < 0.001$).

was seen for the p53⁻/bcl-2⁺ cases with a luminal cell phenotype (p < 0.001).

DISCUSSION

In our study we analysed the expression of p53 (wild-type and mutated form) and bcl-2 proteins in relation to the phenotypic and cellular kinetic characteristics of 37 DCIS and 27 IDC samples from the breast. The phenotypic characteristics of our cases suggest a different cell origin among breast cancers. In fact, 13 cases (eight DCIS and five IDC cases) showed expression of CK8, CK14, CK18, vimentin, and EGFR, consistent with a stem cell origin; 44 cases (27 DCIS and 17 IDC) showed expression of CK8 and CK14, weak expression or negativity for CK18, and negativity for vimentin and EGFR, consistent with a luminal cell origin. Two DCIS and five IDC cases showed a phenotypic pattern unrelated to either a luminal cell or stem cell origin. The interpretation of these results is of course speculative, because these cases may have lost their phenotype or may be derived from yet another precursor cell.

> "Our results nevertheless suggest that p53 was wild-type in ductal in situ carcinoma cases and mutated in infiltrating ductal carcinoma cases because the SSCP technique has a sensitivity and specificity of approximately 90%

Both DCIS and IDC cases with a stem cell phenotype were p53⁺/bcl-2⁻ by immunohistochemistry. Among these cases, the screening for p53 mutations with SSCP analysis showed a band shift suggesting a point mutation in IDC, whereas no band shift was detected in DCIS. Without sequencing the PCR products we cannot completely exclude either false negative cases, because we have examined only a limited number of p53 exons, or false positive cases for the presence of polymorphisms.[36] With this restriction in mind, our results nevertheless suggest that p53 was wild-type in DCIS cases and mutated in IDC cases because the SSCP technique has a sensitivity and specificity of approximately 90%.[37] Interestingly, when compared with DCIS, p53 expression in IDC was associated with a reduction of both MI and AI. Abundant apoptosis in DCIS has been identified independent of p53 immunostaining.[38] In this last group, the expression of p53 might well reflect the presence of a wild-type protein, as a physiological response to DNA damage.[2–4] Conversely, the p53 protein in IDC has probably lost its physiological function to induce cell death. It is well known that reduced apoptosis may lead to a shift in tissue kinetics towards increased cell numbers, and also to the preservation of genetically aberrant cells, thereby favouring neoplastic progression.[39 40] In particular, this model has been observed in poorly differentiated carcinomas of the breast.[41] In our series, the IDC cases with p53 mutation were poorly or moderately differentiated tumours, ER and PgR negative, and showed significantly higher numbers of MIB-1 positive cells. The high proliferative activity of these tumours, as compared with the reduction of MI, may also be linked to inactivation of p53, which leads to the abrogation of the G1/S block and/or the inhibition of G2 exit.[42] The presence of "proliferation associated" proteins is not necessarily an indication that the respective cells will successfully terminate the cell cycle and divide. An alteration of cell cycle progression may be present in such cases and the neoplastic growth may be the result of a reduction of cell death rather than increased cell division. The identification of such a kinetic pattern may be of some prognostic value because high metastatic potential has been shown to be associated with increased resistance to apoptosis.[43]

Most of the tumours showing a more differentiated phenotype (luminal) were p53⁻/bcl-2⁺. In these cases, cell kinetic parameters increased from DCIS to IDC. Similar kinetic characteristics have been observed in well differentiated breast

Take home messages

- Thirteen cases (eight ductal carcinoma in situ (DCIS) and five infiltrating ductal carcinoma (IDC)) showed expression characteristics consistent with a stem cell phenotype, whereas 44 cases (27 DCIS and 17 IDC) showed characteristics consistent with a luminal cell phenotype
- Most of the tumours showing a more differentiated phenotype (luminal) were p53⁻/bcl-2⁺. In these cases, cell kinetic parameters increased from DCIS to IDC
- These data suggest the existence of subsets of DCIS and IDC that, because of their phenotypic characteristics, could be derived from subpopulations of normal breast cells having different control mechanisms of cell proliferation and neoplastic progression
- However, the occurrence of p53 mutants by chance during neoplastic transformation cannot be excluded and further studies are warranted

tumours.[41] The increased values of AI and MI from DCIS to IDC in such cases could be related to an alteration of cell growth control, probably as a result of other genetic lesions involving the retinoblastoma pathway.[44] The coexistence of high percentages of bcl-2⁺ cells and increased AI could tentatively be explained by bcl-2 independent pathways to programmed cell death.[45]

In conclusion, our data suggests the existence of subsets of DCIS and IDC that, because of their phenotypic characteristics, could be derived from subpopulations of normal breast cells, which probably use different control mechanisms of cell proliferation and neoplastic progression—p53 dependent in the cases with a stem cell phenotype and p53 independent in the cases with a luminal cell phenotype. In fact, the liability of a given tumour to exhibit p53 mutation seems to be predetermined by the nature of its cell of origin, depending more specifically to what extent wild-type p53 forms a rate limiting step in the control of the proliferating life span in that cell.[46 47]

> "The coexistence of high percentages of bcl-2⁺ cells and increased apoptotic index could tentatively be explained by bcl-2 independent pathways to programmed cell death"

Our results are compatible with the hypothesis that the cell of origin constrains both tumour phenotype and choice of genetic events.[47] However, the occurrence of p53 mutants by chance during neoplastic transformation cannot be excluded, as is suggested by the finding of p53⁺/bcl-2⁺ cases. However, it must be remembered that in situ and invasive breast lesions were taken from different patients, and that none of the patients with an in situ lesion had adjacent invasive carcinoma. Therefore, our results should be interpreted with caution. More direct evidence of our hypothesis could be obtained from the study of patients with invasive breast cancer who had a previous breast biopsy that showed carcinoma in situ. We are presently gathering these cases and performing kinetic and molecular studies by capture laser microdissection.

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