ORIGINAL ARTICLE

Relationship of p53, Bcl-2, Ki-67 index and E-cadherin expression in early invasive breast cancers with comedonecrosis as an accelerated apoptosis

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Aims: To study the relationship between comedonecrosis formation and morphology, apoptosis, and p53, Bcl-2, Ki-67 index and E-cadherin expression in early invasive breast cancer.

Experimental design: Early invasive breast cancers were first divided into two groups according to the presence (CN+ tumours) or absence (CN– tumours) of comedonecrosis. The histological grade, apoptosis, and expression of E-cadherin, Ki-67, p53 and Bcl-2 in the cancer-affected area, and in normal ducts from the specimen, were then examined.

Results: Less tubule and gland formation was seen in CN+ tumours than in CN– tumours, although the histological grade between the groups was not different. During early comedonecrosis, cells undergo apoptosis and subsequent necrosis. p53 was higher in CN+ tumours than in CN– tumours and normal ducts, whereas Bcl-2 was lower in CN+ tumours than in CN– tumours and normal ducts. Both tumours had higher Ki-67 than in normal ducts, but no difference was evident between the tumours. CN+ tumours had slightly higher E-cadherin than that in CN– tumours, but lower than that in normal ducts. The level of comedonecrosis was positively correlated with p53, but inversely correlated with Bcl-2 in all tumours, and p53 and Bcl-2 were inversely correlated with each other. Furthermore, comedonecrosis and p53 were correlated with Ki-67 in CN+ tumours, and Bcl-2 was correlated with Ki-67 in CN– tumours.

Conclusion: Comedonecrosis may be actively regulated through an apoptotic procedure in massive cancers for their survival and progression, and the above proteins may be associated cooperatively in this process. CN+ and CN– tumours may have opposite proliferative systems under the p53–Bcl-2 pathway.

Solid breast cancers often exhibit comedonecrosis, comprising massive tumour cell death in the central regions of the tumour. Although comedonecrosis helps maintain a stable tumour volume and nutritional condition,¹ the significance of comedonecrosis and the mechanism by which it is effected remain unclear.

Apoptosis is one type of cell death, and is induced by suicide mechanisms to maintain homeostasis.² This process is strictly controlled by several genes. Tumour suppressor gene p53 induces cell cycle arrest and apoptosis.³ Mutations of p53 represent the most common genetic alteration in human cancers. Nuclear overexpression of p53 is frequently observed in many cancers, and is induced by accumulation of point mutations of the gene.⁴ Bcl-2 is one of the Bcl-2 family of proteins and provides protection from apoptosis through various stimuli.⁵ The expression of Bcl-2 family proteins is also observed in several cancers, including breast cancer.^{6–7} E-cadherin is a cell adhesion molecule between epithelial cells.⁸ This protein is also known to be a suppressor of cancer progression caused by adhesive effects.^{9–10}

We analysed the significance of comedonecrosis formation in early invasive breast cancers, which are limited to primary areas without metastasis. Our findings indicate that comedonecrosis may be actively regulated through apoptosis, with the above-mentioned proteins being used for survival and progression.

MATERIALS AND METHODS

Samples

Pathological samples of early invasive breast cancer tissues were obtained from the Department of Clinical Pathology,

Toyooka Hospital, Hyogo, Japan. A total of 46 invasive ductal cancers <5 cm in diameter, without tumour-positive regional lymph nodes or distant metastases (tumour, node and metastasis (TNM) classification, T1 or T2, N0, M0: stage I or II), were examined. Of these, 23 cases represented carcinomas with some degree of comedonecrosis (CN+ tumour); the other 23 cases were categorised as carcinomas without comedonecrosis (CN– tumour). Neither the age of the patient nor stage of the tumour differed markedly between the two categories of comedonecrosis (data not shown). These processes were carried out ethically under the provisions of the Declaration of Helsinki.

Histology and immunohistochemical studies

Samples of breast cancer tissue were cut into serial tissue blocks, 5-mm thick, after being fixed in 10% formaldehyde solution. These were then embedded in paraffin wax. Tissue specimens were sectioned at 4-µm intervals and stained with haematoxylin and eosin. Immunohistochemical examinations were also carried out with an Envision kit (Dako, Kyoto, Japan). Primary antibodies used in this study included anti-single-strand (ss) DNA (×200; Dako), p53 (prediluted; Dako), Ki-67 (MIB-1, ×50; Dako), Bcl-2 (prediluted; Dako), E-cadherin (×1000; Transduction Laboratories, Heidelberg, Germany) and M30 (antibody for the detection of caspase cleavage product of cytokeratin 18, ×10; Roche, Mannheim, Germany).¹¹ Oestrogen receptor, progesterone receptor and

Abbreviations: HER2, human epidermal growth factor receptor 2; TUNEL, terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end-labelling; ssDNA, single-strand DNA

Figure 1 Histological finding of breast cancers in CN+ and CN- tumours (haematoxylin and eosin stain, $\times 100$). (A) Representative histological findings of early breast cancer in CN+ tumours. Massive cancer nodules had some amount of comedonecrosis (arrows; tubule and gland formation score, 3; comedonecrosis score, 3). (B) Representative histological findings of early breast cancer in CN- tumours. Small cancer nodules had invaded diffusely (tubule and gland formation score, 1).

human epidermal growth factor receptor 2 (HER2) staining was also carried out by a commercial company (SRL, Tokyo, Japan).

Histological grade, level of comedonecrosis and immunohistochemical expression

Histological grade was determined by the World Health Organization (WHO) system as follows:^{12,13} tubule and gland formation score—most tumours ($>75\%$), 1; moderate degree (10–75%), 2; and little or none ($<10\%$), 3; nuclear pleomorphism score—small, regular uniform, 1; moderate increase in size and variability, 2; and marked variation, 3; mitotic counts in 10 high-power fields ($\times 400$)—0–7, 1; 8–14, 2; and ≥ 15 , 3. The total values are added together, producing scores of 3–9, to which the grade is assigned as follows: 3–5 points, grade 1; 6–7 points, grade 2; and 8–9 points, grade 3.

Level of comedonecrosis was scored as follows: no comedonecrosis, 0; comedonecrosis comprising 1–9% of total tumour area in all cancer nodules, 1; comedonecrosis comprising 10–33% of total tumour area in all cancer nodules, 2; and comedonecrosis comprising $>33\%$ of total tumour area in all cancer nodules, 3. Immunohistochemical expression of p53, Bcl-2, E-cadherin, HER2, oestrogen and progesterone receptors was scored as follows: negative, 0; mild positive, 1; moderate positive, 2; and strong positive, 3. The score that was most dominant in the specimen was used for all the above grades. Expression of Ki-67 was evaluated as a Ki-67 index (%).

Identification of apoptotic cells

Apoptotic cells were identified by three different staining processes: in situ terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick

Figure 2 Apoptotic findings in early-phase comedonecrosis (CN). (A) In early-phase comedonecrosis, cancer cells show reduced cytoplasm with eosinophilic changes and condensed nuclei (arrow). In addition, several apoptotic bodies are apparent (closed arrowhead). Towards the central region of the tumour, tumour cells increasingly show massive autolytic changes, eventually being reduced to cellular debris and necrosis (open arrowhead; haematoxylin and eosin stain, $\times 200$). (B) M30 staining ($\times 400$), is positive in cytoplasm (arrows). (C, D) Staining for single-strand DNA (C; $\times 400$) and terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end-labelling (TUNEL) (D; $\times 400$) are also positive for nuclei (arrows) in cancer cells during early-phase comedonecrosis.

end-labelling (TUNEL), M30¹² and ssDNA. TUNEL methods were carried out with an in situ Apoptosis Detection Kit (Takara, Shiga, Japan), as described previously.¹⁴ M30 stainings were carried out with M30 CytoDEATH (Roche). Cells with TUNEL-positive nuclei were interpreted as apoptotic cells. Similarly, cells that were immunohistochemically positive for M30 in cytoplasm and ssDNA in nuclei were identified as apoptotic cells.

Statistical analysis

Non-parametric analyses (Mann–Whitney U test or Spearman's rank correction) were carried out using StatView software. Values of $p < 0.05$ were considered to be significant.

RESULTS

Histological features of CN+ and CN– tumours

Our cases were categorised into two tumour categories: those with some degree of comedonecrosis (CN+ tumours, $n = 23$; fig 1A) and those without CN (CN– tumours, $n = 23$; fig 1B). In the histological analysis, CN+ tumours showed less tubule and gland formation than CN– tumours ($p = 0.003$; table 1), indicating that, although CN+ tumours frequently grew massively compared with CN– tumours (fig 1),¹² the total grade, including nuclear pleomorphism and mitotic counts, was no different between the groups.

Induction of apoptosis in early-phase comedonecrosis

The pathological change underlying comedonecrosis was examined next. In early-phase comedonecrosis, cancer cells displayed reduced cytoplasm volume with eosinophilic changes and condensed nuclei (fig 2A). Some apoptotic bodies were also found. Towards the central region of the tumour, cells gradually showed massive autolytic changes, finally being reduced to cell debris and necrosis. Staining for M30, ssDNA and TUNEL (fig 2B–D), identifying apoptotic cells, was positive on cells during early-phase comedonecrosis.

Expression of p53, Bcl-2, Ki-67 and E-cadherin in CN+ and CN– tumours

To determine which proteins participate in the apoptotic process of comedonecrosis, expressions of E-cadherin, Ki-67 index, p53 and Bcl-2 were examined immunohistochemically in CN+ and CN– tumours (figs 3, 4). Interestingly, expression of p53 was markedly raised in CN+ tumours compared with that in normal ducts and CN– tumours (mean 1.4 (SD 1.2) in CN+ tumours; mean 0.1 (SD 0.3) in CN– tumours; mean 0.0 (SD 0.1) in normal ducts; $p < 0.0001$ in CN+ tumours ν normal ducts, $p = 0.003$ in CN+ tumours ν CN– tumours, respectively; figs 3A–C, 4A). By contrast, the expression of Bcl-2 was significantly lower in CN+ tumours than in normal ducts and CN– tumours (mean 0.5 (SD 0.5) in CN+ tumours; mean 2.0 (SD 1.0) in CN– tumours; mean 1.0 (SD 0.3) in normal ducts; $p = 0.002$ in CN+ tumours ν normal ducts, $p < 0.0001$ in CN+ tumours ν CN– tumours, respectively; figs 3D–F, 4B). CN+ tumours showed a wide range of Ki-67, but no significant difference was identified compared with CN– tumours, although both CN+ and CN– groups showed a higher index than normal ducts (mean 7.2 (SD 8.4) in CN+ tumours; mean 3.8 (SD 1.4) in CN– tumours; mean 0.4 (SD 0.5) in normal ducts; $p < 0.0001$ in CN+ or CN– tumours ν normal ducts, not significant in CN+ tumours ν CN– tumours, respectively; figs 3G–I, 4C). E-cadherin expression in CN+ tumours was also significantly lower than in normal ducts, but higher than in CN– tumours (mean 2.4 (SD 0.6) in CN+ tumours; mean 1.8 (SD 0.6) in CN– tumours; mean 2.9 (SD 0.3) in normal ducts; $p = 0.005$ in CN+ tumours ν normal ducts, $p = 0.003$ in CN+ tumours ν

CN– tumours, respectively; figs 3J–L, 4D). Although expression of HER2, oestrogen and progesterone receptors for breast cancer-specific growth factors and hormones was also evaluated,^{15–16} no marked differences were identified between tumours (data not shown).

Correlation of comedonecrosis, p53, Bcl-2 expressions and Ki-67 index in CN+ and CN– tumours

Lastly, correlations between comedonecrosis levels and protein expression were investigated in all tumours (fig 5). A major correlation was observed between comedonecrosis level and p53, and an inverse correlation between comedonecrosis level and Bcl-2 ($p < 0.0001$ for both; fig 5A,B). In addition, there was also an inverse correlation between p53 and Bcl-2 ($p < 0.0001$; fig 5C). CN+ tumours alone also showed similar results (data not shown). In addition, there was a significant correlation between comedonecrosis level and p53 and Ki-67 in CN+ tumours (CN: $p = 0.0004$; p53: $p = 0.0009$; fig 6A,B). In contrast, there was a significant correlation between Bcl-2 and Ki-67 in CN– tumours ($p = 0.028$; fig 6C). No apparent correlation was, however, found between the expression of E-cadherin, HER2, oestrogen or progesterone and comedonecrosis or apoptosis-related proteins (data not shown).

DISCUSSION

In this study, we examined the relevance of comedonecrosis formation in early invasive breast cancer by investigating the morphology, apoptosis and its related proteins. At first, the CN+ tumours showed less tubule and gland formation than CN– tumours, indicating that CN+ tumours grew more massively than CN– tumours, although the histological grade between the tumours was not different. In this respect, comedonecrosis formation leads to homeostasis in the remaining cells, facilitating further growth and invasion.¹ In addition, cancer cells in early-phase comedonecrosis underwent apoptosis and subsequently become necrotic.¹⁷ These findings suggest that comedonecrosis is predominantly induced in massive cancer for the survival and progression of the cancer, and that active cell death by apoptosis may be associated with this process.

Next, we evaluated the expression of various apoptosis-related proteins. Although p53 in CN+ tumours was markedly higher than in CN– tumours and in normal ducts, Bcl-2 was notably lower in CN+ tumours than in CN– tumours and in normal ducts. In addition, the level of comedonecrosis was positively correlated with p53, but inversely correlated with Bcl-2. Furthermore, p53 and Bcl-2 were inversely correlated with each other. In this respect, p53 is a negative regulator for Bcl-2, increasing Bax for induction of apoptosis.^{18–20} Also, the accumulation of either mutant or wild-type p53 protein leads to a reduction in Bcl-2.¹⁸ Although these findings are comparable to ours, why upregulation of Bcl-2 in CN– tumours is greater than that in normal ducts, even at the same low level of p53, is still unclear. Most breast cancers without p53 expression, but with mutation of the gene, have a frameshift or nonsense mutation.²¹ Such tumours may lead to a further increase in Bcl-2, less by the p53-suppressing mechanism than in normal ducts. Although we did not carry out gene analysis, it may at least play a part in the further increase in Bcl-2 in CN– tumours.

The Ki-67 index did not differ noticeably between CN+ and CN– tumours, but a correlation with comedonecrosis in CN+ tumours was observed. This indicates that the proliferative activity of the cell itself may not be directly related to the induction of comedonecrosis, but that, when a massive cancer is involved, high levels of proliferative activity may lead to the initiation of comedonecrosis. Although there is a correlation between the Ki-67 index and p53 in

Figure 3 Representative immunohistochemical expression of p53, Bcl-2, Ki-67 and E-cadherin in CN+ and CN- tumours and normal ducts ($\times 400$). Expression of p53 in CN+ group, score 3 (A); in CN- group, score 0 (B); and in normal duct, score 0 (C). Bcl-2 expression in CN+ group, score 0 (D); in CN- group, score 3 (E); and in normal ducts, score 1 (F). Ki-67 index in CN+ group, 20% (G); in CN- group, 4% (H); and in normal ducts 0% (I). E-cadherin expression in CN+ group, score 2 (J); in CN- group, score 1 (K); and ND, score 3 (L). CN, comedonecrosis.

Table 1 Histological grade in the CN+ and CN- groups

Feature	CN		p Value*
	+	-	
Tubule and gland formation	2.3 (0.6)	1.7 (0.8)	0.003
Nuclear pleomorphism	1.3 (1.2)	1.2 (1.1)	Not significant
Mitotic counts	1.5 (0.7)	1.3 (0.5)	Not significant
Grade	1.7 (0.8)	1.4 (0.5)	Not significant

Values are mean (SD).
n=23 in both CN+ and CN- groups.
*Unpaired Mann-Whitney U test.

Figure 4 Comparison of p53, Bcl-2, Ki-67 index and E-cadherin expression in CN+ and CN- tumours and in normal ducts. Expression of p53 (A), Bcl-2 (B), Ki-67 index (C) and E-cadherin (D) was compared by using the grading system in CN+ and CN- tumours and in normal ducts. Data are shown as mean score (SD). CN+ tumours, $n = 23$; CN- tumours, $n = 23$; normal ducts, $n = 46$. Mann-Whitney U test was carried out for statistical analysis. NS, not significant.

CN+ tumours, there is also a comparable correlation between the Ki-67 index and Bcl-2 in CN- tumours, suggesting that both tumours use different or opposite proliferative processes through the p53-Bcl-2 pathway.

E-cadherin expression was higher in CN+ tumours than in CN- tumours, but lower than in normal ducts, which is consistent with the conception that massive tumour nodules in CN+ tumours show relatively strong cell adhesion, whereas small and diffuse (non-massive) nodules in CN- tumours show relatively weak cell adhesion.⁹ The lower

expression in both CN+ and CN- groups than in normal ducts is one of the intrinsic characteristics of invasive carcinoma.¹⁰ It should also be noted that E-cadherin and Bcl-2 negatively regulate each other,^{22, 23} suggesting that E-cadherin also plays a part in the control of comedonecrosis by regulation of Bcl-2. The reverse may be true, although no major correlation of p53, Bcl-2 or comedonecrosis with E-cadherin was found in this study.

Relationships of CN with HER2, oestrogen and progesterone expression were also examined. The expressions of these

Figure 5 Correlation between comedonecrosis, p53 and Bcl-2 expression in all tumours. Correlation of CN with p53 (A) or Bcl-2 (B), and of p53 with Bcl-2 expression (C) in all tumours. The box shows 75th and 25th centiles surrounding the horizontal median line. Additional upper and lower lines indicate the 90th and 10th centiles. The median Bcl-2 score is 1 in the group with CN score 1 and 0 in the group with CN score 2. These scores are included in the upper and lower lines of the boxes. All tumours, $n = 46$ (CN: score 0, $n = 23$; score 1, $n = 6$; score 2, $n = 11$; score 3, $n = 6$; p53: score 0, $n = 27$; score 1, $n = 9$; score 2, $n = 5$; score 3, $n = 5$). Statistical analysis was carried out with the Spearman's rank correlation. *Group with CN score of 3, described in text.

Figure 6 Correlation between comedonecrosis (CN), p53 and Bcl-2 expressions and Ki-67 index in CN+ tumours and CN- tumours. Correlation of CN with Ki-67 (A), of p53 with Ki-67 (B) in CN+ tumours and of Bcl-2 with Ki-67 in CN- tumours (C). The box shows the 75th and 25th centiles surrounding the horizontal median line. Additional upper and lower lines indicate the 90th and 10th centiles. CN+ tumour, n=23 (CN: score 1, n=6; score 2, n=11; score 3, n=6. p53: score 0, n=7; score 1, n=6; score 2, n=5; score 3, n=5). CN- tumours, n=23 (Bcl-2: score 0, n=2; score 1, n=4; score 2, n=9; score 3, n=8). Statistical analysis was carried out with the Spearman's rank correlation. *Group with CN score of 3, described in text.

proteins, however, did not differ between the CN+ and CN- groups. In addition, no correlation was found between expression and apoptotic proteins. Although we did not investigate the ligands for these receptors, our findings suggest that breast cancer-specific factors may not be considerably associated with the induction of comedonecrosis.

A previous report shows that the presence of comedonecrosis indicates poor prognosis in breast cancer.²⁴ Although we did not examine the prognosis in this study, we found that one group had a comedonecrosis score of 3, with a p53 score of 3 and a Bcl-2 score of 0 (fig 5A,B, marked by *), which showed the highest Ki-67 index of all groups (fig 6A, marked by *), possibly having a high potential for malignancy. Also, the group contained all the four people with grade 3, which represents the worst prognosis in this study (data not shown).²⁵

Overall, our data suggest that the general apoptosis-related proteins, p53, Bcl-2 and E-cadherin, rather than breast cancer-specific factors play important and cooperative roles in comedonecrosis formation in early invasive breast cancers. Further functional analysis is, however, needed to determine whether this is the case. In addition, there may be two opposed proliferative mechanisms associated with the p53-Bcl-2 pathway. From the point of view of treatment, controlling comedonecrosis with these proteins in either CN+ or CN- tumours may delay cancer progression.²⁶

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Take-home messages

- Comedonecrosis formation is predominantly observed in massive cancers, with the apoptotic process in early invasive breast cancers.
- A positive correlation exists between comedonecrosis formation and p53, but a negative correlation is observed between comedonecrosis formation and Bcl-2 expression in all subjects.
- Bcl-2 expression was also markedly different from that in normal ducts.
- The survival and progression of comedonecrosis formation may be actively regulated through the apoptotic process in massive cancers.

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