Journal of Pathology
J Pathol 2003; **201**: 573–580.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1486

Original Paper

Expression of the cell death genes BNip3 and NIX in ductal carcinoma *in situ* of the breast; correlation of BNip3 levels with necrosis and grade

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Received: 7 May 2003
Revised: 3 July 2003
Accepted: 17 September 2003

Abstract

Ductal carcinoma *in situ* (DCIS) of the breast is an early, non-invasive lesion and the prognosis is associated with the extent of necrosis and cell death within the tumour. Two cell death genes, BNip3 and NIX, are up-regulated in response to hypoxia in breast carcinoma cells, although any involvement of either gene in disease progression is currently unknown. This study has analysed the expression of BNip3 and NIX in 56 samples of breast DCIS, as well as in adjacent benign and invasive breast tissue. Both genes are strongly expressed in the epithelial component of a subset of DCIS and invasive disease. The data show a correlation between high expression of BNip3 in the DCIS cells and a high-grade, necrotic lesion that is likely to be associated with invasive tumour. BNip3 was present in tumour-associated macrophages and in apocrine metaplastic lesions. Expression of NIX did not correlate with any of the parameters investigated Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: **BNip3; hypoxia; ductal carcinoma *in situ* of the breast; necrosis; cell death**

Introduction

The treatment and management of patients with ductal carcinoma *in situ* (DCIS) of the breast is a controversial subject, due to the heterogeneity of the tumour biology and uncertainty as to the natural history of the disease. DCIS treated with excision alone can recur in the region of the initial biopsy and approximately 50% of these recurrences have an invasive component [1]. The presence of necrosis, an extreme manifestation of hypoxia, in the initial biopsy correlates with recurrence and poor outcome [2]. Although the mechanism linking necrosis and poor prognosis is unknown, cells in culture are able to escape hypoxia-induced cell death and acquire sustained resistance to apoptotic signals [3]. Most tumour cells retain the ability to die in response to hypoxic stress [4] and it could be that this loss of cell death signal-sensitive cells leads to the selection of viable cells which are more resistant to treatment and contribute to tumour relapse [5]. High-grade DCIS with necrosis is also associated with increased levels of DNA fragmentation compared to the low-grade, non-comedo type, indicating that these tumours are associated with an increased rate of cell death [6]. The cell death genes BNip3 and NIX encode proteins associated with the mitochondria that cause cell death within 48 h of transient overexpression [7]. Both genes contain hypoxia response elements and are induced in carcinoma cell lines cultured in a low oxygen environment [8,9]. We have previously shown that BNip3 and NIX mRNAs are expressed at higher levels in breast carcinoma tissue than in normal breast adjacent to the tumour and that in invasive breast carcinoma, expression of both genes is often localized to peri-necrotic regions [9]. This study aimed to define the location of BNip3 and NIX expression in breast tissue and to determine whether the presence in DCIS of either factor was related to the degree of necrosis and grade, VEGF expression, and association with invasive carcinoma.

Materials and methods

Human breast tumour samples

Formalin-fixed, paraffin-embedded surgical resection specimens of pure DCIS (DCIS−) and DCIS associated with invasive carcinoma in the same biopsy (DCIS+) were collected from the John Radcliffe and Churchill Hospitals between 1997 and 1999. Of the DCIS− samples, four were low grade, 16 intermediate grade, and 11 high grade. The DCIS+ cohort contained five intermediate-grade and 20 high-grade samples; details of oestrogen receptor (ER) status and

grade are given in Table 1. The cohort was previously used in a study of hypoxia-inducible carbonic anhydrases [10] and the lesions originally classified into histological grades according to the Van Nuys scoring system [11] were regraded for this study using the same system to allow for any variations due to deeper cutting of the paraffin blocks; some minor adjustments were made accordingly. In addition, intraductal necrosis was graded on haematoxylin and eosin-stained sections using the following system: 0 = no necrosis; 1 = minimal necrosis (1–2 ducts); 2 = moderate necrosis (50% of ducts necrotic); and 3 = extensive necrosis (majority of ducts necrotic).

In situ hybridization

The *in situ* hybridization riboprobes for BNip3, NIX, and the corresponding sense controls have been described previously, as has the protocol used in this study [9]. The template used for the VEGF riboprobe consisted of nucleotides −16 to +217 of VEGF 121 and also recognized VEGF isoforms 165, 189, and 206 [13]. Briefly, the ^{33}P-labelled riboprobes were diluted to 30 000–40 000 cpm/μl in hybridization buffer and incubated on the sections for 18 h at 55 °C. The slides were then washed and treated with RNAse A before being coated with autoradiographic emulsion and exposed for 21 days at 4 °C.

Immunohistochemistry

Sections serial to those used for *in situ* hybridization were dewaxed and rehydrated and those to be subjected to BNip3 immunohistochemistry were treated with 1 mM EDTA (pH 8.0) overnight at 60 °C. After washing, endogenous peroxidase activity was quenched by incubating the sections with 0.3% hydrogen peroxide for 10 min, before treatment with 0.2% Triton X-100 for 10 min. A 1 : 1000 dilution of mouse monoclonal antibodies to human CD34 (Serotec, Oxford, UK) or CD68 [14], or a 1 : 50 dilution of a rabbit polyclonal antibody to human BNip3 [15], was applied to the sections for 3 h at room temperature. Staining was visualized using the Envision anti-mouse or anti-rabbit DAB system (Dako Ltd, High Wycombe, Bucks, UK) and sections were counterstained in Mayer's haemalum (BDH, Poole, Dorset, UK). Replacement of the primary antibody with PBS was used as a negative control.

Table 1. Breakdown of breast DCIS samples used in the study. The absence or presence of associated invasive disease is designated by DCIS− and DCIS+, respectively. Oestrogen receptor (ER) status was determined using the immunohistochemical method described by Leake *et al* [12]

	DCIS−	DCIS+
DCIS grade		
Low	4	0
Intermediate	16	5
High	11	20
Invasive grade		
I	N/A	2
II	N/A	11
III	N/A	12
Nodes positive		
0	N/A	12
1–3	N/A	9
> 3	N/A	1
Not known	N/A	3
ER status		
Negative	9	6
Positive	16	12
Not known	6	7

N/A = not applicable.

Assessment of *in situ* hybridization

Assessment of signal strength and localization was performed independently by HMS and a pathologist (MF), using a dark field microscope. Scoring was as follows: 0: no hybridization in any ducts; 1: weak hybridization in some or all ducts; 2: moderate hybridization in some or all ducts; and 3: strong hybridization in all ducts. When a structure acting as an internal control was not available, apocrine metaplasia provided a consistent reference for 'strong', whereas 'weak' constituted the minimum reactivity that allowed a structure to be identified from the *in situ* slide alone. VEGF reactivity provided a positive internal control for mRNA integrity and a reference slide of known signal strength was included in each run to control for hybridization efficiency. *In situ* hybridization scores were analysed as contingency tables using Pearson's χ^2 test.

Results

Correlation of *in situ* hybridization and immunohistochemistry

In situ hybridization for BNip3 and NIX was carried out on a series of 56 breast resection specimens containing DCIS. Tissue sections serial to those used for *in situ* hybridization were subjected to immunohistochemistry for BNip3; however, NIX protein could not be analysed, as a suitable antibody was not available. We found that BNip3 protein corresponded to sites of BNip3 mRNA expression (Figure 1). Sense controls for BNip3 and NIX mRNAs showed no specific hybridisation, and omission of the primary BNip3 antibody resulted in a complete loss of signal (Figure 1). For comparison between BNip3 and NIX expression, most of the figures show *in situ* hybridization images.

BNip3 and NIX mRNA expression in benign breast lesions

Individual cases showed a variable number of morphologically normal ducts and lobules. In addition,

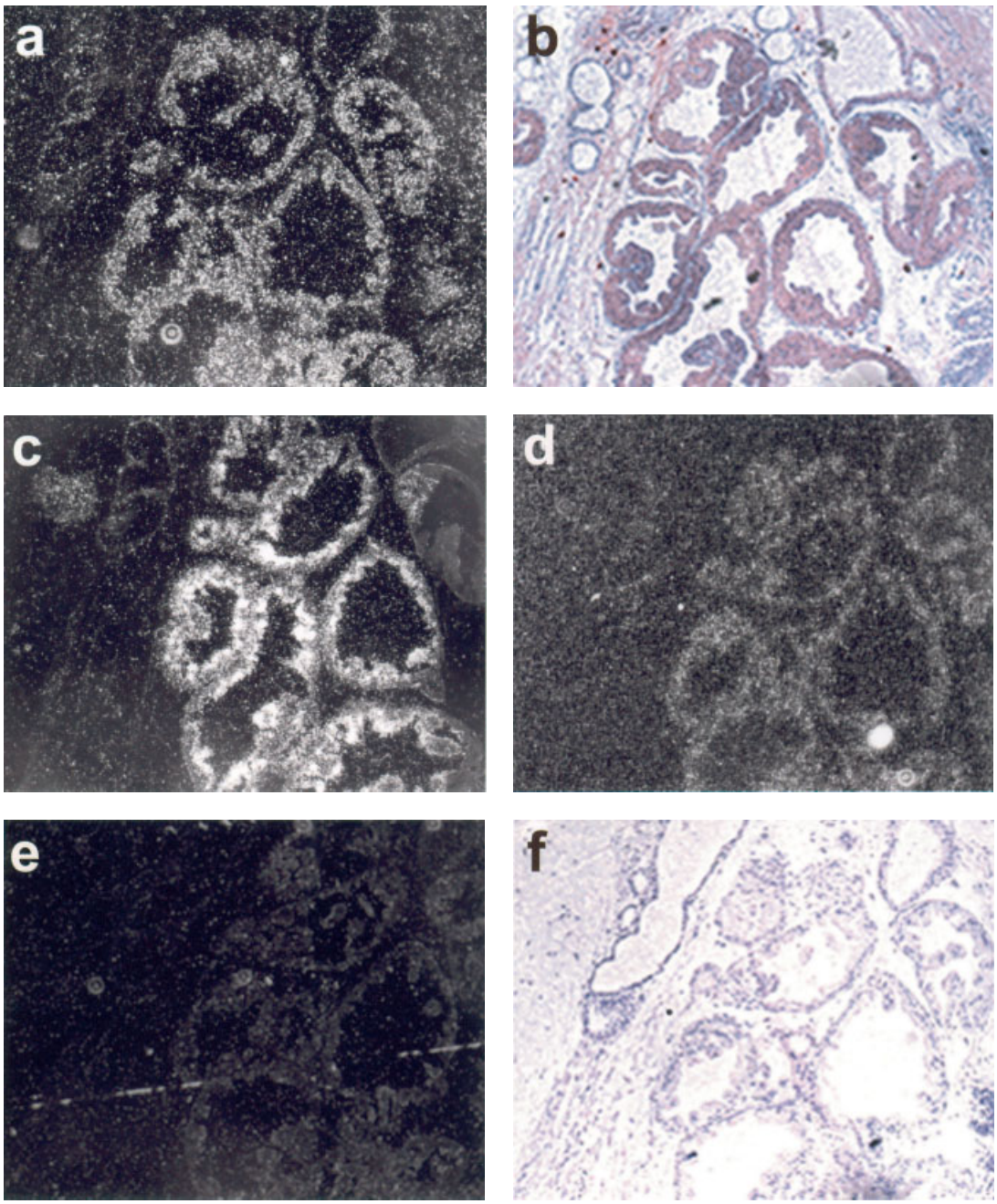

Figure 1. *In situ* hybridization (ISH) analysis of breast tissue containing an area of apocrine metaplasia. The abnormal cells of the apocrine metaplasia are strongly positive for mRNA encoding BNip3 (a; silver grains) and BNip3 protein (b; brown staining). The same cells are also strongly positive for NIX mRNAs (c), whereas VEGF mRNA is only weakly expressed (d). The sense control riboprobe shows no specific hybridization (e) and omitting the primary antibody during the immunohistochemical procedure resulted in loss of staining (f)

several cases showed features of benign breast disease including cysts, adenosis, apocrine metaplasia, and ductal hyperplasia. Normal breast tissue and overlying skin, where present, showed no BNip3 or NIX expression (data not shown). The benign breast lesions showed varying positivity for both BNip3 and NIX: cyst lining epithelium was negative (1/1); hyperplasia showed borderline positivity (1/2) or was negative (2/2); and two cases of sclerosing adenosis showed weak positivity (2/2), while apocrine metaplasia was strongly positive both for BNip3 (2/3 cases) and for NIX (3/3 cases; Figure 1). VEGF mRNA did not co-localize with BNip3 and NIX mRNA in benign tissues. Epithelial hyperplasia of the usual type was often strongly VEGF-positive (data not shown), whilst apocrine metaplasia only weakly expressed VEGF (Figure 1).

BNip3 and NIX mRNA expression in tumour-associated macrophages (TAMs)

TAMs (confirmed by CD68 immunohistochemical staining) were present in 37/59 cases examined and no case contained both negative and positive TAMs. Groups of BNip3 (Figure 2) and NIX (Figure 3)

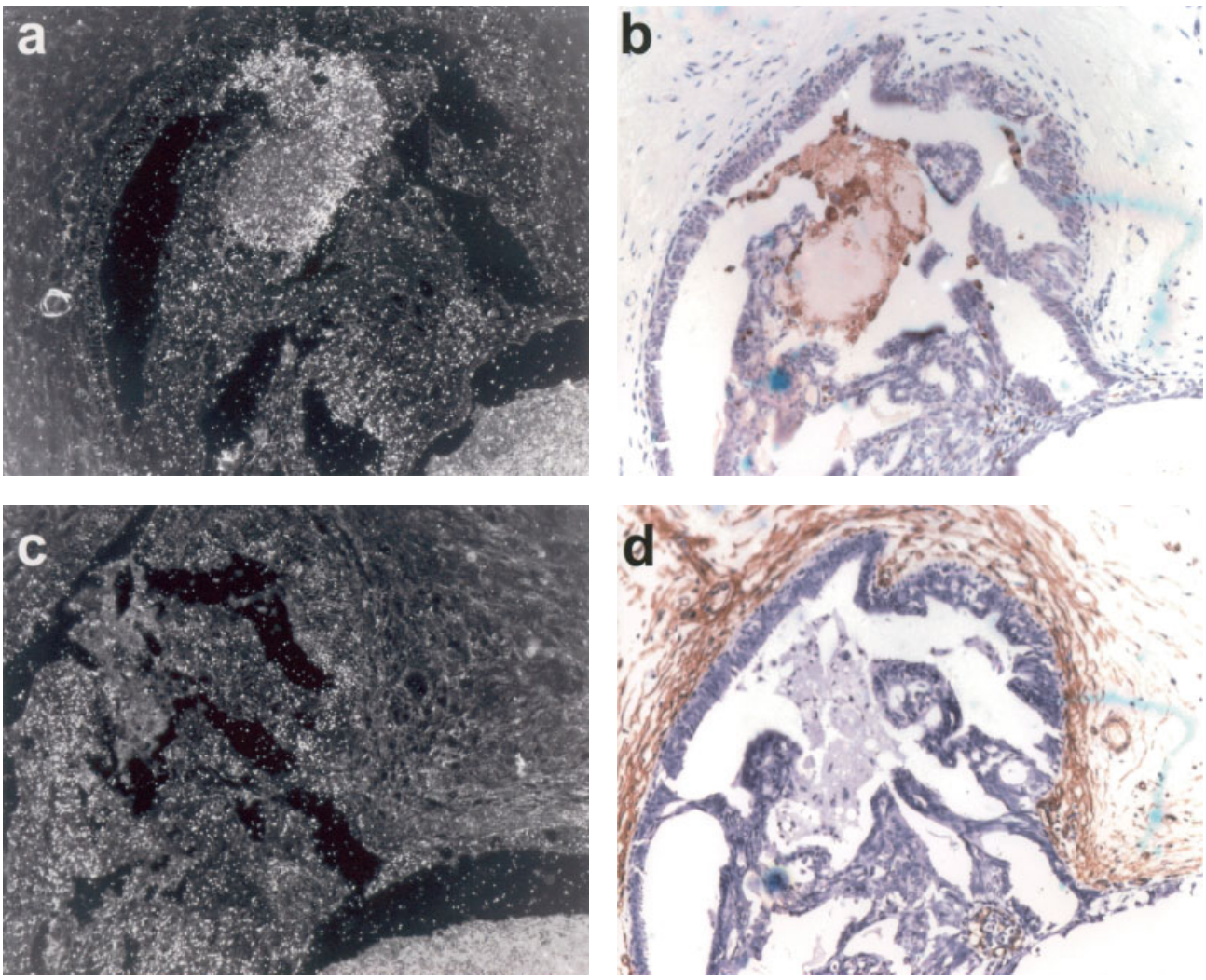

Figure 2. ISH analysis of tumour-associated macrophages in a necrotic region of a breast DCIS. (a) mRNA encoding BNip3 is expressed strongly by tumour-associated macrophages, the presence of which is confirmed by immunohistochemistry for CD68 on a serial section (b). VEGF mRNA is not expressed by the same macrophages on a serial section (c). Staining for CD34 reveals local blood vessels, none of which is near the area of macrophages (d)

positive macrophages were identified in 26/37 and 34/37 cases, respectively. These were often, but not invariably, associated with necrosis and NIX mRNA expression was at higher levels than that of BNip3 in macrophages (Figure 3). VEGF mRNA did not co-localize with BNip3 and NIX mRNA in TAMs, which were VEGF-negative (Figures 2 and 3).

BNip3 and NIX mRNA expression in breast DCIS

BNip3 and NIX mRNA expression occurred in the epithelial cells of the DCIS tissue and always co-localized with VEGF expression (Figure 3), although DCIS samples negative or weakly positive for BNip3 or NIX were often strongly VEGF-positive (data not shown). BNip3, but not NIX, hybridization varied significantly with the grade of DCIS, with high-grade DCIS correlating with strong BNip3 hybridization ($p = 0.01$; Figures 4A and 4B and Tables 2 and 3).

In DCIS with a high necrosis score, BNip3 mRNA showed a markedly peri-necrotic location, with a gradient of positivity towards the necrotic centre. Strong BNip3 hybridization corresponded to those cases having a high necrosis score ($p < 0.001$) (Table 2) and the signal intensified as the level of necrosis increased (Figure 4C). NIX hybridization was generally weaker than BNip3 hybridization and did not

Table 2. Results of *in situ* hybridization analysis of BNip3 expression in breast DCIS samples

	BNip3		
	Low	**High**	***p*-value**
Necrosis			
0–1	16	2	< 0.001
2–3	10	21	
Grade			
Intermediate/low	17	8	
High	9	15	< 0.02
Type			
− invasion	20	8	< 0.002
+ invasion	6	15	

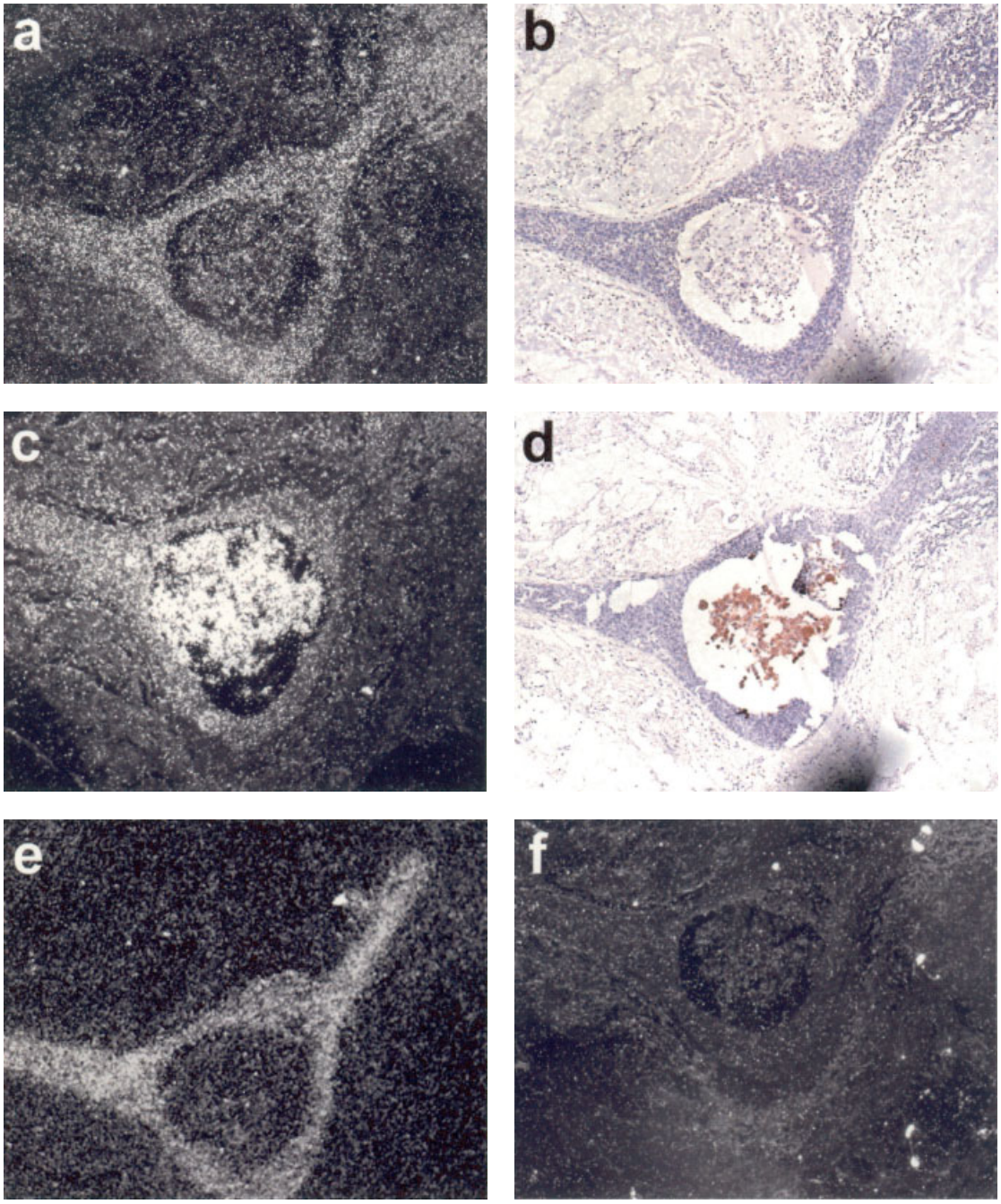

Figure 3. ISH analysis of breast tissue containing a high-grade DCIS. The epithelial cells of the DCIS express BNip3 mRNA (dark field view is shown in a; bright field view in b). In this tumour, NIX mRNAs are expressed by the same cells, as well as by tumour-associated macrophages (c), the presence of which is confirmed by immunohistochemistry for CD68 on a serial section (d). VEGF mRNA expression co-localizes with BNip3 and NIX expression in epithelial DCIS cells, but is not expressed by tumour-associated macrophages (e). No specific hybridization was seen with the sense control (f)

correlate significantly with the amount of necrosis (Figure 4D and Table 3). When the DCIS tumours were grouped according to presence or absence of invasive carcinoma, strong BNip3 expression was correlated significantly with DCIS associated with invasion ($p = 0.0016$; Figure 4E and Table 2). Conversely, NIX expression did not correlate with DCIS associated with invasion (Figure 4F). Neither BNip3 nor NIX level of expression correlated with ER status of the DCIS or with size of lesion (data not shown). In DCIS+ cases, there was also no significant correlation between lymph node involvement or the grade of the invasive component and high levels of expression of BNip3 or NIX (data not shown).

BNip3 and NIX mRNA expression in invasive carcinoma associated with DCIS

The sample group contained 25 cases of DCIS that were associated with invasive carcinoma. Owing to an insufficient invasive component on some of the sections, analysis was limited to 19 cases. Invasive tumour was BNip3-negative in 11/19 cases, weakly

Table 3. Results of *in situ* hybridization analysis of NIX expression in breast DCIS samples

	NIX		
	Low	**High**	***p*-value**
Necrosis			
0–1	10	8	NS
2–3	15	16	
Grade			
Intermediate/low	13	12	NS
High	12	12	
Type			
− invasion	15	13	NS
+ invasion	10	11	

NS = not significant.

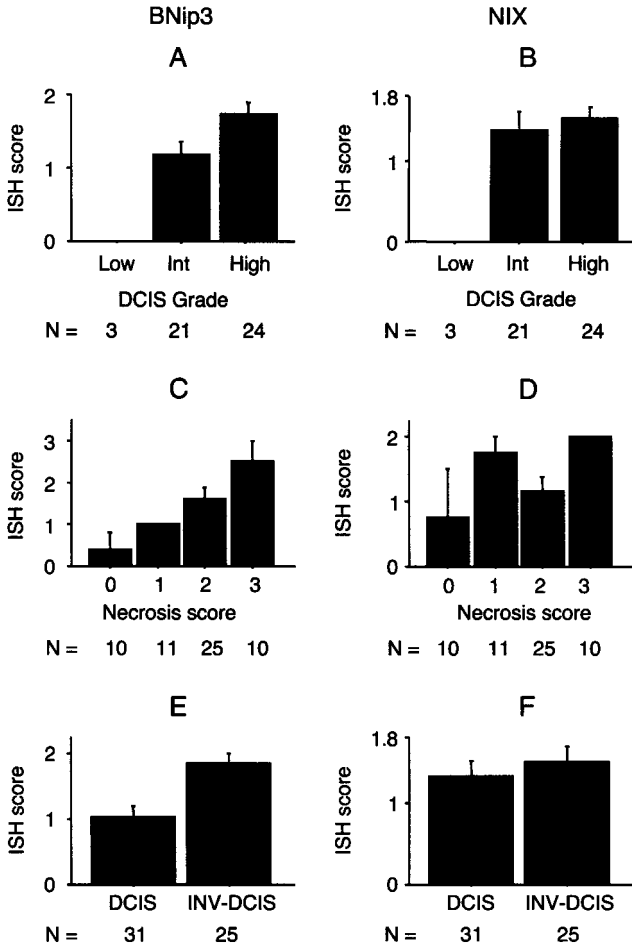

Figure 4. Bar graphs of all DCIS samples, showing the number of samples, mean, and standard errors for each group. (A) Strong expression of BNip3 mRNA is associated with high-grade DCIS, compared with low and intermediate (Int) grade DCIS ($p < 0.02$). (B) NIX mRNA is not expressed by low-grade DCIS and is not associated with high-grade rather than intermediate-grade DCIS. (C) Strong expression of BNip3 mRNA is associated with high necrosis (2–3) compared with low necrosis (0–1; $p < 0.005$). (D) The level of NIX mRNA does not correlate with the level of necrosis in the DCIS. (E) BNip3 mRNA expression is higher in DCIS samples associated with invasive disease than in DCIS only ($p < 0.005$). (F) The level of NIX mRNA in DCIS does not correlate with the presence of invasive disease

positive for BNip3 in 4/19 cases, and strongly positive in 4/19 cases. Invasive tissue was negative, weakly positive, and strongly positive for NIX in 9/19, 5/19, and 5/19 cases, respectively. Gene expression in the invasive tissue was localized to tumour epithelial cells. Figure 5 shows invasive tissue strongly positive for BNip3 and negative for NIX, and VEGF mRNA was expressed in all the invasive components analysed (data not shown). There was no correlation between DCIS expressing high levels of BNip3 and/or NIX and the associated invasive component also expressing high levels of the gene (data not shown), although the invasive component was never positive for BNip3 or NIX if the associated DCIS was negative. There was also no correlation of grade or necrosis score of the DCIS with BNip3 or NIX expression in the associated invasive component (data not shown).

Discussion

We have shown that BNip3 expression in breast DCIS is associated with a high-grade, necrotic phenotype and with the presence of invasive disease. Conversely, NIX expression in breast DCIS is not associated with grade, the amount of necrosis or the presence of invasive disease. We have reported previously that a range of cell lines, including endothelial, macrophage, and carcinoma-derived lines, increase their levels of BNip3 and NIX mRNA in response to hypoxia [9]. This study confirms that, *in vivo*, BNip3 is highly associated with areas of hypoxia, but suggests that NIX may be regulated by other mechanisms in DCIS. Previous evidence has suggested that BNip3 overexpression promotes a necrosis-like programmed cell death, which is caspase-independent and does not involve the release of cytochrome *c* [16]. The mechanism of cell death that results from NIX overexpression has not been investigated in the same system, although NIX does induce cytochrome *c* release from isolated mitochondria [17]. The marked up-regulation and correlation of BNip3 expression with necrosis suggests that this may be a key driving mechanism for this phenotype in DCIS. This does not explain the origin of the hypoxia, but rather the mechanism of cell death. The causes of hypoxia may be increased oxygen consumption by a rapidly proliferating neoplasm, or outgrowth of blood supply at the periphery of the lesion. In our original paper, we used northern blot analysis to identify the up-regulation of BNip3 and NIX in breast carcinoma tissue when compared with benign breast tissue and using this technique, NIX expression was higher than BNip3 expression [9]. In this *in situ* hybridization study, NIX expression was often lower than BNip3 expression in the epithelial component of the DCIS, but higher in the tumour-associated macrophages. This could explain why the original northern blot analysis of whole breast carcinoma tissue showed a higher overall level of NIX.

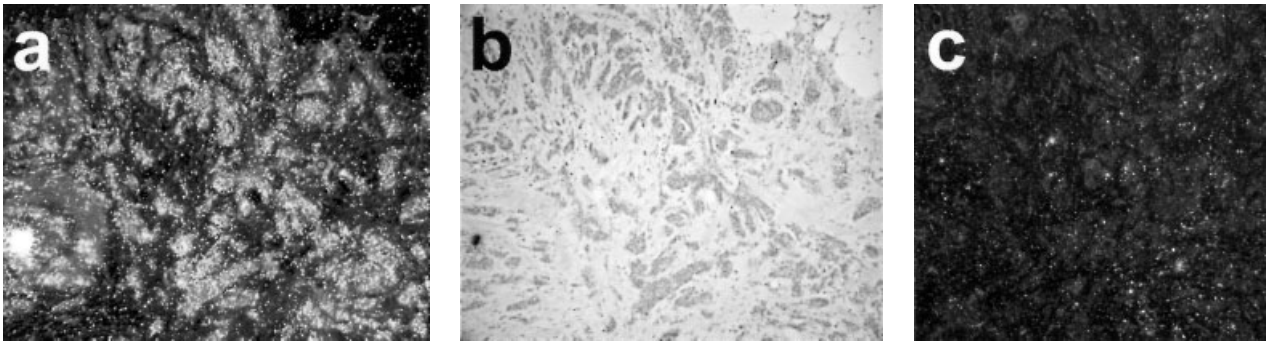

Figure 5. Serial sections of invasive breast carcinoma associated with high-grade DCIS. ISH for BNip3 reveals strong expression in the epithelial cells of the invasive tumour (dark field view is shown in a; bright field in b), whereas ISH for NIX gives no signal (c)

BNip3 and NIX, but not VEGF mRNAs, were highly expressed in regions of apocrine metaplasia where this could be assessed, while the opposite was seen in benign adenosis and hyperplasia. This suggests that, unlike VEGF, BNip3 and NIX may be up-regulated in response to metaplasia rather than hyperplasia. Interestingly, metaplastic apocrine cells in the breast are characterized by large numbers of mitochondria [18], the main expression site of BNip3 and NIX during cell death [7]. A high proportion of tumour-associated macrophages expressed BNip3 and NIX mRNA but not VEGF. In these cases, NIX mRNA expression occurred more frequently and with a higher intensity than BNip3 mRNA expression. It is known that macrophages are long-lived cells resistant to many apoptotic stimuli [19] and that differentiated macrophages constitutively express survival factors that may provide resistance to cell death [20]. It could be, therefore, that expression of BNip3 and NIX by tumour-associated macrophages is a result of the hypoxic environment towards which they are attracted [21] and that neither gene causes cell death, due to the presence of survival factors in the differentiated macrophages. Also, metaplasia is an alteration or transformation of one normal cell phenotype to another in response to a chronic stress. It is interesting that aberrant expression of BNip3 and NIX occurs in both apocrine cells and macrophages without subsequent cell death and raises the question of whether common survival factors exist in these cells to abrogate the cell death stimuli.

All of the epithelial components of the DCIS cases that expressed BNip3 and NIX also expressed VEGF, indicating that these tumours are subject to conflicting signals, survival versus death. Hypoxia induces a complex gene programme and BNip3 is a major factor in the response producing necrosis. However, VEGF is also induced, as is the migration factor hepatocyte growth factor [22]. Also, dedifferentiation associated with a more primitive cell phenotype capable of migration is induced [23]. As time progresses, there will be selection for these other features as long as cell death is not 100% and it is likely that there will be accumulation of mutations allowing cells to survive BNip3 expression and hence selecting for progression. Some BNip3- and NIX-negative tumours were also positive for VEGF, suggesting that distinct populations of DCIS exist with regard to the response to hypoxia, and this heterogeneity may represent microenvironmental differences that would provide selective advantages for clonal evolution.

The identification of molecular factors that highlight a DCIS patient population with a high risk for invasive recurrence would be very desirable when treating this unpredictable disease. Changes in the cell death threshold of tumour cells may also give insight into the mechanisms of radio- or chemotherapy resistance. We have shown that high expression of the cell death gene BNip3 correlates significantly with high-grade, necrotic DCIS and with DCIS adjacent to invasive disease. This study was designed to assess expression patterns and relationships to other variables, but a much larger study with a 5- to 10-year follow-up and assessment of DCIS with occult areas of invasion should be able to assess whether the expression of BNip3 is of value in clinical assessment of risk of relapse of DCIS.

Acknowledgements

We would like to thank Professor Peter Ratcliffe for helpful discussions throughout this work. This work was supported by Cancer Research UK.

References

1. Fonseca R, Hartmann LC, Petersen IA, Donohue JH, Crotty TB, Gisvold JJ. Ductal carcinoma *in situ* of the breast. *Ann Intern Med* 1997; **127**: 1013–1022.
2. Fisher ER, Dignam J, Tan-Chiu E, *et al*. Pathologic findings from the National Surgical Adjuvant Breast Project (NSABP) eight year update of Protocol B17: intraductal carcinoma. *Cancer* 1999; **86**: 429–438.
3. Graeber TG, Osmanian C, Jacks T, *et al*. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours [see comments]. *Nature* 1996; **379**: 88–91.
4. Shimizu S, Eguchi Y, Kamiike W, *et al*. Induction of apoptosis as well as necrosis by hypoxia and predominant prevention of apoptosis by Bcl-2 and Bcl-XL. *Cancer Res* 1996; **56**: 2161–2166.
5. Schmaltz C, Hardenbergh PH, Wells A, Fisher DE. Regulation of proliferation-survival decisions during tumor cell hypoxia. *Mol Cell Biol* 1998; **18**: 2845–2854.

6. Zaugg K, Bodis S. Is there a role for molecular prognostic factors in the clinical management of ductal carcinoma (DCIS) of the breast. *Radiother Oncol* 2000; **55**: 95–99.
7. Chen G, Cizeau J, Vande Velde C, *et al*. NIX and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. *J Biol Chem* 1999; **274**: 7–10.
8. Bruik RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci U S A* 2000; **97**: 9082–9087.
9. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumours. *Cancer Res* 2001; **61**: 6669–6673.
10. Wykoff CC, Beasley NJP, Watson PH, *et al*. Hypoxia inducible expression of tumor-associated carbonic anhydrases. *Cancer Res* 2000; **60**: 7075–7083.
11. Silverstein MJ, Lagois MD, Craig PH, *et al*. A prognostic index for ductal carcinoma *in situ* of the breast. *Cancer* 1996; **77**: 2267–2274.
12. Leake R, Barnes D, Pinder S, *et al*. Immunohistochemical detection of steroid receptors in breast cancer: a working protocol. *J Clin Pathol* 2000; **53**: 634–635.
13. Zhang HT, Scott PA, Ziche M, Weich HA, Harris AL, Bicknell R. Enhancement of tumour growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cells. *J Natl Cancer Inst* 1995; **87**: 219–231.
14. Talks KL, Turley H, Gatter KC, *et al*. The expression and distribution of the hypoxia-inducible factors HIF-1a and HIF-2a in normal human tissues, cancers and tumour-associated macrophages. *Am J Pathol* 2000; **157**: 411–421.
15. Ray R, Chen G, Vande Velde C, *et al*. BNIP3 heterodimerizes with Bcl-2/Bcl-XL and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. *J Biol Chem* 2000; **275**: 1439–1448.
16. Vande Velde C, Cizeau J, Dubik D, *et al*. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* 2000; **20**: 5454–5468.
17. Imazu T, Shimizu S, Tagami S, *et al*. Bcl-2/E1B 19 kDa-interacting protein 3-like protein (Bnip3L) interacts with Bcl-2/Bcl-xl and induces apoptosis by altering mitochondrial membrane permeability. *Oncogene* 1999; **18**: 4523–4529.
18. Ahmed A. Apocrine metaplasia in cystic hyperplastic mastopathy. Histochemical and ultrastructural observations. *J Pathol* 1975; **115**: 211–214.
19. Kiener HR, Davis PM, Starling GC, *et al*. Differential induction of apoptosis by Fas–Fas ligand interactions in human monocytes and macrophages. *J Exp Med* 1997; **185**: 1511–1516.
20. Xaus J, Comalada M, Valledor AF, *et al*. Molecular mechanisms involved in macrophage survival, proliferation, activation or apoptosis. *Immunology* 2001; **204**: 543–550.
21. Lewis JS, Lee JA, Underwood JCE, Harris AL, Lewis CE. Macrophage responses to hypoxia: relevance to disease mechanisms. *J Leukocyte Biol* 1999; **66**: 889–900.
22. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell* 2003; **3**: 347–361.
23. Helczynska K, Kronblad A, Jogi A, *et al*. Hypoxia promotes a dedifferentiated phenotype in ductal breast carcinoma *in situ*. *Cancer Res* 2003; **63**: 1441–1444.

