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BNIP3 as a Progression Marker in Primary Human Breast Cancer; Opposing Functions in *In situ* Versus Invasive Cancer

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Abstract

Purpose: BNIP3 is involved in cell death and cell survival via autophagy. Its perinecrotic localization within ductal carcinoma *in situ* (DCIS) suggests an involvement in neoplastic cellular adaptation to low oxygen tension. This study has investigated the role of BNIP3 in normal and neoplastic breast.

Experimental Design: Whole sections from 11 normal breast and microarrayed tissue cores from 81 DCIS and 251 invasive carcinomas were stained for BNIP3 and hypoxia-inducible factor-1α. The pattern and level of BNIP3 expression were correlated with clinicopathologic variables and hypoxia-inducible factor-1α.

Results: BNIP3 expression was significantly up-regulated in the cytoplasm of DCIS and invasive carcinoma compared with normal breast ($P = 0.0005$ and $P < 0.0001$, respectively). Nuclear BNIP3 expression was associated with smaller tumor size ($P = 0.04$), low tumor grade ($P = 0.005$), and estrogen receptor positivity ($P = 0.008$) in invasive tumors. Nuclear BNIP3 expression was also associated with a longer disease-free survival among low-grade and estrogen receptor – positive tumors. ($P = 0.03$ and 0.04, respectively). Conversely, nuclear BNIP3 expression in DCIS was associated with a 3-fold increase in recurrence and a shorter disease-free survival ($P = 0.03$).

Conclusions: Up-regulation of BNIP3 expression in DCIS and invasive carcinoma suggests a significant role in breast tumor progression. Its association with good survival outcome in invasive carcinoma but with an increased risk of recurrence and shorter disease-free survival in DCIS may suggest a pivotal switch from a cell death to survival function during the transition from preinvasive to invasive breast cancer.

Hypoxia is now recognized to be a poor prognostic indicator being associated with resistance to radiation (1) and chemotherapy (2). Hypoxia occurs early in tumor development with even small tumors rapidly outgrowing their oxygen requirements but through a combination of co-option of preexisting blood vessels (3) and cellular adaptive responses, neoplastic cells are able to survive under such low oxygen tension. The key regulatory molecule central to the hypoxic adaptive response is hypoxia-inducible factor (HIF)-1α. HIF is a transcription factor composed of an inducible HIF-1α and the constitutively expressed HIF-1 β (HIF-1β, also known as aryl hydrocarbon nuclear translocator). In the presence of molecular oxygen, HIF-1α is hydroxylated by three prolyl hydroxylases, which via the von Hippel-Lindau protein and ubiquitin pathway rapidly targets HIF-1α protein for proteolytic degradation. However, in hypoxia, because oxygen is not available, hydroxylation does not occur and the HIF-1α subunit is stabilized, translocated to the nucleus where it associates with HIF-1β to form a transcription complex (4). The complex then binds to specific hypoxia response elements in the promoters of genes that regulate angiogenesis, glycolysis, and cell survival, all beneficial to a tumor in a hypoxic microenvironment.

BNIP3 is a prodeath member of the Bcl-2 family of apoptotic proteins that is also induced by hypoxia through HIF-1α binding to the HRE-2 site of the BNIP3 promoter (5). Its mechanism of action is reported to be through insertion into the mitochondrial membrane, which leads to the opening of the mitochondrial permeability pore with resultant membrane depolarization and generation of reactive oxygen species. This rapid and profound mitochondrial dysfunction is accompanied by an increase in plasma membrane permeability, extensive cytoplasmic vacuolation, and DNA strand breaks and results ultimately in cell death. This nonapoptotic mechanism of cell death is independent of cytochrome *c* release from the mitochondria, caspase activation, or the nuclear translocation

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of apoptosis-inducing factor (6). Although BNIP3 overexpression is reported to induce cell death in breast and renal carcinoma cell lines (5), not all investigators have shown that BNIP3 is involved in this process. Indeed, a growing body of evidence suggests that BNIP3 is also involved in autophagy (7, 8), a complex process that can either promote cellular survival and adaptation in conditions of hypoxic or metabolic stress or result in autophagic death by excessive self-digestion.

BNIP3 expression is restricted to few normal tissues, such as skeletal muscle and brain (6, 9, 10). We have examined previously the mRNA expression of BNIP3 in human breast tissues, and in contrast to normal breast where BNIP3 mRNA was not identified, we observed up-regulation of BNIP3 in ductal carcinoma *in situ* (DCIS) and invasive carcinoma (11). Furthermore, its localization to areas of necrosis, presumed regions of hypoxia, within the tumor strongly suggested involvement in neoplastic cellular adaptation to low oxygen (11). Our *in vitro* studies confirmed that BNIP3 is up-regulated in MCF-7 breast cancer cells treated with hypoxia and that the BNIP3 increase is HIF-1α mediated (12). In this study, to further evaluate the role of BNIP3 in breast cancer, we have examined the level of expression of BNIP3 in a large characterized series of DCIS and invasive carcinomas and correlated BNIP3 expression with standard clinicopathologic variables together with HIF-1α and survival. Because we observed both nuclear and cytoplasmic localization in our pilot study, we analyzed BNIP3 expression in these cellular compartments.

Materials and Methods

Patient and tumor characteristics. Whole tissue sections from 11 histologically normal breast tissues obtained from reduction mammoplasties together with microarrayed tissue cores from 81 pure DCIS (2-mm cores) as well as 251 invasive breast carcinomas (1-mm cores) were collected from patients who underwent surgery at the John Radcliffe Hospital (Oxford, United Kingdom). This study has Ethical Committee approval (number C02.216). Information of the characteristics of patients with DCIS and invasive carcinoma, including patient's age, tumor size, grade, histology, nodal status, estrogen receptor (ER) status, and HER-2 status were collected from clinical and pathologic records. The median age of patients with DCIS and invasive carcinomas was 56 years (range, 32-75 years) and 58 years (range, 29-90 years), respectively. Seventy-three percent of invasive tumors were classified as invasive ductal of no specific type, 6% as invasive lobular carcinoma, and 7% as other histologic types. Data were unavailable for 14%. The clinicopathologic characteristics of the invasive series is presented in Table 1 and those of the DCIS series in Table 2. Forty-four percent of invasive carcinomas were associated with nodal disease, and 67% were ER positive. Median follow-up period for patients with invasive and DCIS tumors was 89.4 months (range, 3.9-137.4 months) and 114.2 months (range, 10.3-207.8 months), respectively. In patients with invasive tumors who were <50 years of age, adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil were administered if the tumors were node positive or ER negative and/or >3 cm. Patients >50 years of age with ER-negative, node-positive tumors also received cyclophosphamide, methotrexate, and 5-fluorouracil. There were 27 relapses and no deaths among those with DCIS and 113 relapses and 107 deaths among those with invasive carcinoma.

BNIP3 immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections (4 μm) of whole sections of normal breast and the tissue microarrays of DCIS and invasive carcinomas were dewaxed in citroclear and rehydrated with graduated ethanol solutions. We have shown previously the validity using the approach (13). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 min and nonspecific binding with 2.5% normal horse serum for 20 min. A 1:400 dilution of monoclonal BNIP3 antibody (Sigma, St. Louis, MO) was then applied for 2.5 h at room temperature. Substitution of the primary antibody with PBS served as a negative control. Normal skeletal muscle tissue was used as a positive control. Primary antibody incubation was followed by application of the EnVision kit secondary antibody (DAKO A/S, Glostrup, Denmark) for 30 min and the peroxidase reaction was developed using diaminobenzidine provided in the kit. The slides were then counterstained in hematoxylin and mounted in aqueous mountant.

Table 1. Clinicopathologic characteristics of patients with invasive tumors together with univariate analysis of BNIP3 expression

	Nuclear BNIP3 negative	Nuclear BNIP3 positive	*P*	Cytoplasmic BNIP3 negative	Cytoplasmic BNIP3 positive	*P*
Median age (y)	58.0	56.5	0.49	56.0	58.0	0.91
Median size (mm)	22.0	18.5	0.04	21.5	20.0	0.31
Grade						
1	22	21	0.005	12	31	0.37
2	50	23		17	56	
3	52	15		23	44	
ER						
Negative	64	17	0.008	26	55	0.35
Positive	104	63		44	123	
HER-2						
Negative	129	59	0.42	51	137	0.51
Positive	19	12		6	25	
Nodal Status						
Negative	92	46	0.65	40	98	0.73
Positive	77	34		30	81	
HIF-1α						
Negative	32	14	0.83	11	35	0.59
Positive	97	46		40	103	
Recurrence						
Negative	99	57	0.05	43	113	0.80
Positive	70	23		27	66	

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Table 2. Clinicopathologic characteristics of patients with DCIS together with univariate analysis of BNIP3 expression

	Nuclear			Cytoplasmic		
	BNIP3 negative	**BNIP3 positive**	***P***	**BNIP3 negative**	**BNIP3 positive**	***P***
Nuclear grade						
Low	12	1	0.51	4	9	0.33
Intermediate	14	12		7	19	
High	26	10		15	21	
HIF-1α						
Negative	6	1	0.66	4	3	0.18
Positive	38	18		15	41	
Recurrence						
Negative	41	12	0.02	18	35	0.96
Positive	12	12		8	16	

Scoring was done by two observers simultaneously. The level of BNIP3 staining was scored with respect to the percentage of cells expressing the protein and the intensity of staining in both the nucleus and the cytoplasm. The scoring system for intensity was as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining; and the scoring system for percentage was as follows: 0, no cells staining positive for BNIP3; 1, ≤10% cells staining positive; 2, 11% to 50% positive cells; 3, 51% to 80% positive cells; and 4 ≥80% positive cells. A final score was obtained from the product of the staining intensity and percentage of cells in both nuclear and cytoplasmic compartments, a range from 0 to 12. To stratify patients for analysis, a score of 2 or more was considered positive for cytoplasmic or nuclear BNIP3 in the statistical analysis.

HIF-1α immunohistochemistry. Tissues sections were also stained with the monoclonal antibody ESEE 122 as described previously (dilution of 1:40; ref. 14) and visualized with the EnVision-horseradish peroxidase kit (DAKO A/S). The intensity of nuclear staining was assessed; 0, no staining: 1, weak staining; and 2, strong staining as reported previously; tumors showing strong staining were considered positive for the analysis (15).

Statistical methods. χ^2 test for trend was used to evaluate the relationship of cytoplasmic and nuclear BNIP3 positivity in normal breast, DCIS, and invasive carcinoma specimens. Correlation between BNIP3 and various clinicopathologic variables were evaluated using either the χ^2 test or Fisher's exact test where appropriate. Stratification for grade was low (grade 1 and 2) versus high (grade 3). Kaplan-Meier survival curves were calculated using tumor recurrence (defined as the first reappearance of tumor at any site following definitive treatment) as the end point. The difference in the time interval to recurrence (disease-free survival) was calculated by means of a log-rank

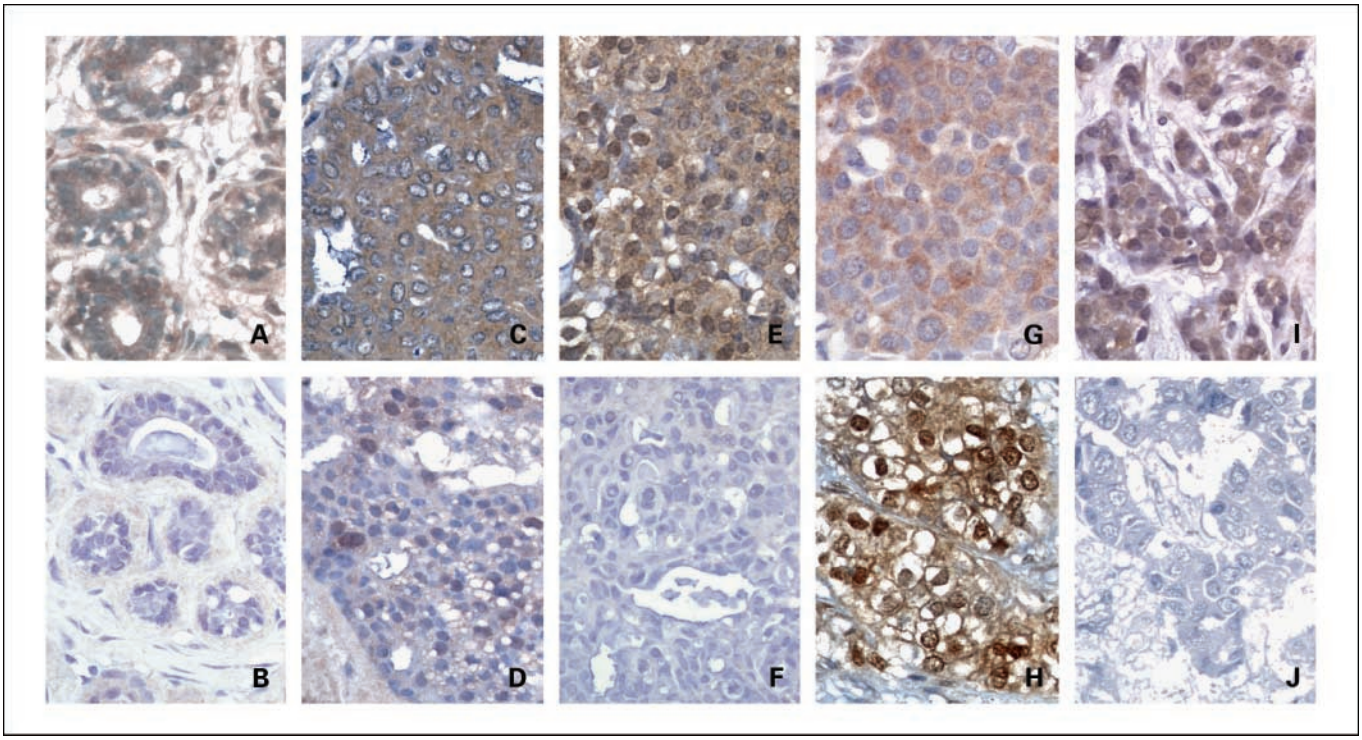

Fig. 1. Immunohistochemical staining of BNIP3 in normal breast tissue (*A* and *B*), DCIS (*C-F*), and invasive carcinoma (*G-J*). *A,* weak cytoplasmic and nuclear staining within the epithelial luminal cells of normal breast with some stromal cell staining. *B,* negative staining in normal breast; *C,* strong cytoplasmic staining in neoplastic cells of intermediate nuclear grade DCIS; *D,* heterogeneous strong nuclear staining in intermediate nuclear grade DCIS; *E,* strong cytoplasmic and nuclear staining in intermediate nuclear grade DCIS; *F,* negative staining in DCIS; *G,* weak with focally moderate cytoplasmic staining in invasive ductal carcinoma; *H,* strong predominantly nuclear staining in invasive carcinoma; *I,* strong cytoplasmic and nuclear staining in invasive carcinoma; *J,* negative staining in invasive carcinoma.

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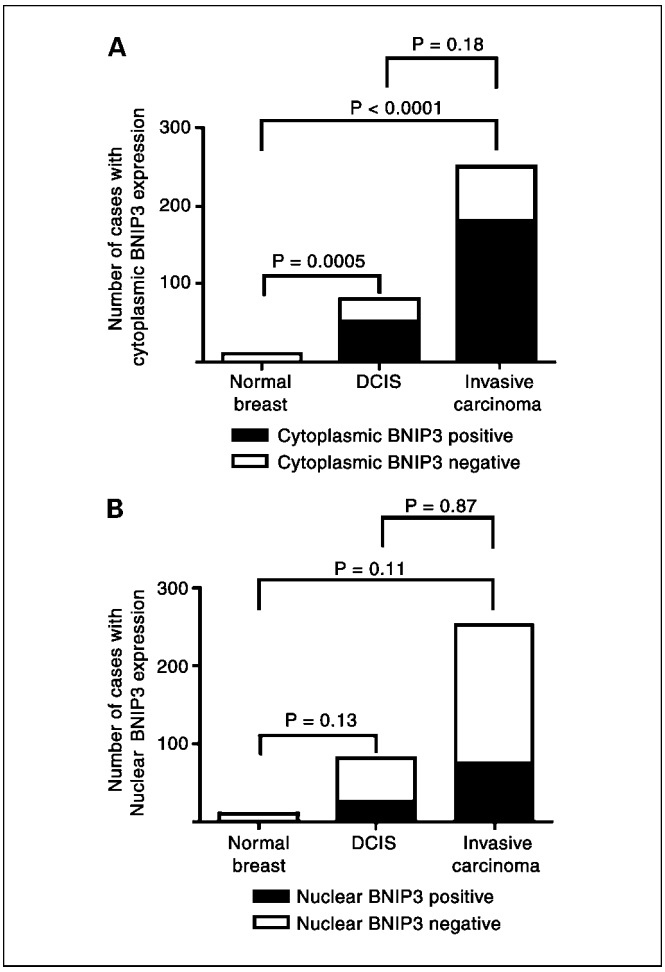

Fig. 2. *A,* frequency of cytoplasmic BNIP3 expression in normal breast tissue, DCIS, and invasive carcinomas. *B,* frequency nuclear BNIP3 expression in normal breast tissue, DCIS, and invasive carcinomas.

test. All univariate and survival analyses were done with GraphPad Prism version 4 (GraphPad Software, Inc., San Diego CA). In addition, the Cox proportional hazard regression model was used to identify independent prognostic factors for disease-free survival. This was carried out using the Stata package release 8.1 (Stata Corp., College Station, Texas). A two-tailed *P* value test was used in all analyses and a *P* value of <0.05 was considered statistically significant.

Results

BNIP3 expression in normal breast tissue, DCIS, and invasive carcinomas. In normal breast tissues, only 1 of the 11 specimens displayed focal weak to moderate staining for BNIP3 in the cytoplasm and in the nucleus (Fig. 1A). Weak focal BNIP3 expression was occasionally observed in nonepithelial elements, including a small proportion of the nuclei in arteriolar smooth muscle and in the intralobular stroma but no BNIP3 expression was observed in the cytoplasm or nucleus of the other normal breast tissues (Fig. 1B). There was also no expression in areas of columnar cell change that was present in some nonneoplastic tissue sections.

In contrast, BNIP3 was expressed in the cytoplasm of 52 of 81 (64.2%) DCIS and in 181 of 251 (72.1%) invasive carcinomas. BNIP3 expression in the cytoplasm was significantly up-regulated in DCIS and invasive carcinomas compared with normal breast tissue ($P = 0.0005$ and $P < 0.0001$, respectively) but there was no significant difference in BNIP3 expression between DCIS and invasive carcinomas ($P = 0.18$; Fig. 2A). Thirty-one percent (25 of 81) of DCIS and 32% (80 of 251) of invasive tumors were positive for BNIP3 within the nucleus. BNIP3 expression in the nucleus was up-regulated in DCIS and invasive carcinoma compared with normal breast tissue but this was not statistically significant ($P = 0.13$ and 0.11, respectively). There was also no significant difference between DCIS and invasive carcinomas ($P = 0.87$) with regards to BNIP3 expression in the nuclear compartment (Fig. 2B). BNIP3 expression within the cytoplasm alone was observed in 40% (32 of 81) and 46% (115 of 251) of DCIS and invasive carcinomas, respectively, whereas only 6% (5 of 81) of DCIS and 6% (14 of 251) of invasive tumors expressed BNIP3 exclusively within the nucleus. One quarter of the tumors [25% (20/81) of DCIS and 26% (66 of 251) of invasive carcinomas] expressed BNIP3 in both the cytoplasm and the nucleus. Thirty percent (24 of 81) of DCIS and 22% (56 of 251) of invasive carcinomas showed no BNIP3 in either the cytoplasm or the nucleus (Fig. 1C-J; Table 3).

Correlation of BNIP3 expression with clinicopathologic variables, HIF-1α and survival. Nuclear expression of BNIP3 in invasive carcinomas was significantly associated with a smaller tumor size ($P = 0.04$), low tumor grade ($P = 0.005$), and ER positivity ($P = 0.008$) but showed no association with patient age, tumor size, nodal disease, HER-2 status, local recurrence, or HIF-1α status ($P > 0.05$; Table 1). There was no significant difference in disease-free survival ($P = 0.06$) or overall survival ($P = 0.34$) among all patients with nuclear BNIP3-positive compared with nuclear BNIP3-negative invasive carcinomas (Fig. 3A), which was confirmed in the multivariate model (Table 4A). However, there was a significantly longer disease-free survival in those patients with ER-positive BNIP3 nuclear-positive invasive tumors ($P = 0.04$; Fig. 3B; Table 3B) and in low-grade (grade 1 and 2) but not in high-grade (grade 3) BNIP3 nuclear-positive invasive tumors ($P = 0.03$; Fig. 3C).

Table 3. Subcellular location of BNIP3 in normal breast tissue, DCIS, and invasive carcinoma

	Normal breast tissue, *n* = 11	DCIS (%) *n* = 81	Invasive carcinoma (%) *n* = 251
No expression	10	24 (29.6)	56 (22.3)
Exclusive cytoplasmic expression	0	32 (39.5)	115 (45.8)
Exclusive nuclear expression	0	5 (6.2)	14 (5.6)
Cytoplasmic and nuclear expression	1	20 (24.7)	66 (26.3)

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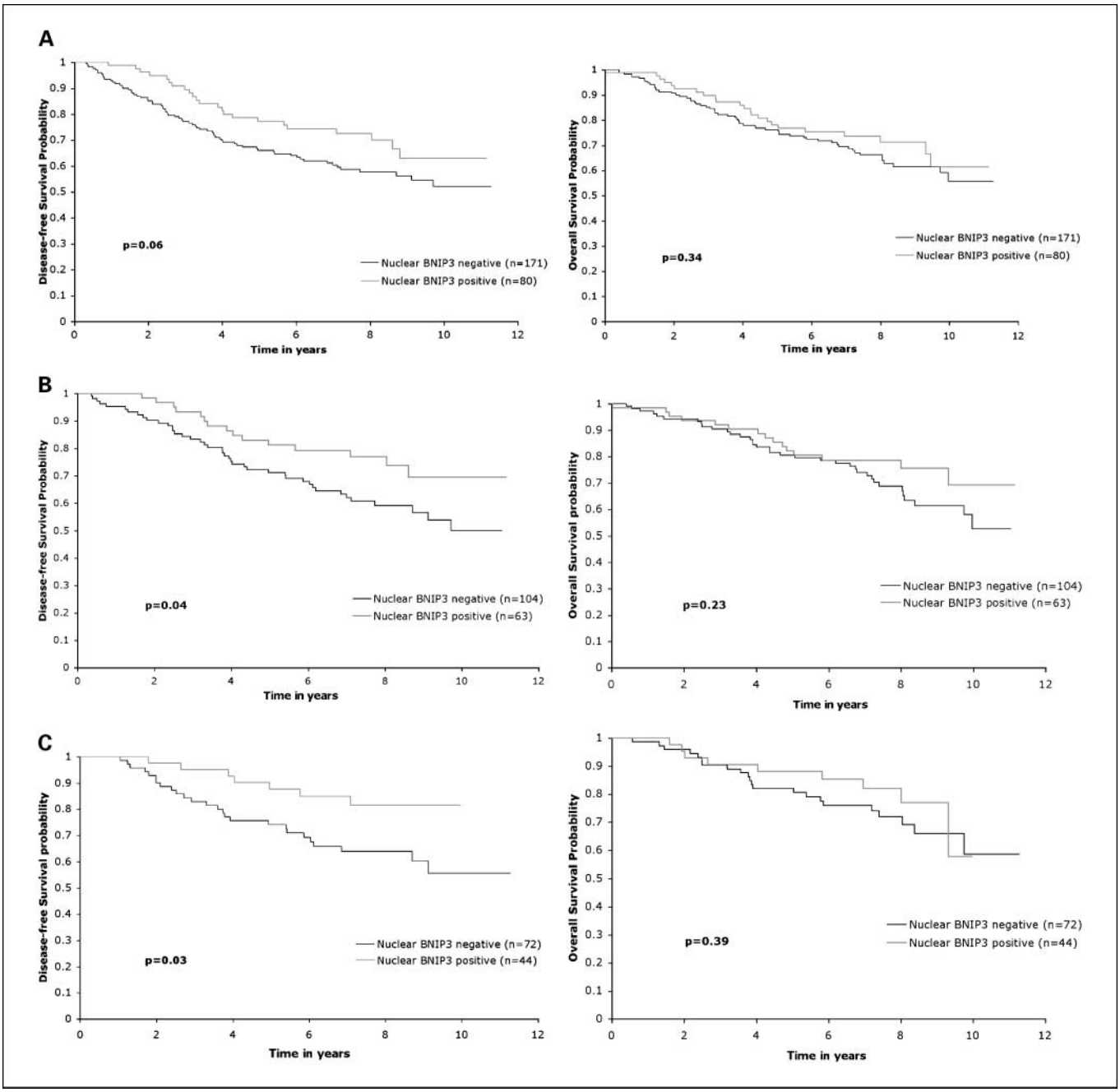

Fig. 3. *A,* Kaplan and Meier curves of disease-free (*left*) and overall survival (*right*) stratifying patients by nuclear BNIP3 expression in invasive carcinoma (all $n = 251$). *B,* Kaplan and Meier curves of disease-free (*left*) and overall survival (*right*) stratifying patients by nuclear BNIP3 expression among ER-positive invasive carcinomas ($n = 167$). *C*, Kaplan and Meier curves of disease-free (*left*) and overall survival (*right*) stratifying patients by nuclear BNIP3 expression in low-grade (Grades 1 and 2) invasive carcinomas ($n = 116$).

BNIP3 expression in the cytoplasm was not associated with patient age, tumor size, histologic type, nodal status, ER status, or HER-2 status nor was it associated with the risk of recurrence or disease-free survival in invasive carcinomas ($P > 0.05$; Table 1).

Nuclear expression of BNIP3 in DCIS was associated with a 3-fold increase in the risk of disease recurrence (odds ratio, 3.4; 95% confidence interval, 1.224-9.541; $P = 0.02$; Table 2) and a shorter disease-free survival in DCIS ($P = 0.03$; Fig. 4) but this was of borderline significance on multivariate analysis ($P = 0.05$; Table 5).

There was however no association between HIF-1α and BNIP3 expression in the nucleus or cytoplasm in DCIS ($P = 0.66$ and 0.18, respectively) or in invasive carcinomas ($P = 0.83$ and 0.59, respectively).

Discussion

Using immunohistochemistry in a series of normal and neoplastic tissues, we have confirmed that BNIP3 is expressed at significantly higher levels in the cytoplasm and nucleus of

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neoplastic cells in DCIS and in invasive breast carcinoma than in normal breast tissue (11, 12). Although we observed no association between BNIP3 and HIF-1α levels in our tumor samples, this is likely to be due to the difference in protein half-lives, which for HIF-1α is measured in minutes compared with that of BNIP3, which is ~4 h (data not shown). A further factor for this discordance is the differing oxygen tensions required for HIF induced proteins (15) but we cannot exclude the possibility that the relationship between HIF-1α and BNIP3 is more complex *in vivo* than *in vitro*, with other mechanisms modulating BNIP3 expression. Although BNIP3 was not identified in epithelial elements of the normal breast, it would be of interest to examine BNIP3 expression in detail in the epithelium throughout the cyclical changes that occur in premenopausal tissues to assess its role in normal physiology.

Hypoxia has been shown to activate many genes that confer a growth benefit, including those responsible for angiogenesis (16–18), up-regulation of glycolytic enzymes (19), and maintenance of telomerase activity (20). However, hypoxia has also been shown to activate genes that inhibit cell growth and promote cell death (21, 22). Although the expression of putative cell death genes, such as BNIP3, might seem to disadvantage the tumor, there is accruing data to suggest that BNIP3 may not result in cell death but through autophagic sequestration may lead to tumor survival when under stress. Thus, overexpression of BNIP3 in MCF-7 breast cancer cells does not result in cell death and constitutive BNIP3 expression is not toxic (23). The BNIP3-mediated autophagy degradation of cell proteins would allow recycling of essential constituents to aid cellular survival and allow the cell to adapt to hypoxic and metabolic stresses (24–26). Indeed, autophagy has been reported to facilitate the survival of human colonic cancer cells during prolonged periods of nutrient deprivation (27) and the survival of breast, prostate, and colonic cancer cells that are exposed to low doses of irradiation (28). Nevertheless, some studies have suggested that malignant cells show lower levels of autophagy, although many of these investigations have relied on snap shots in animal models or transformed cell lines (29). More dynamic studies examining autophagy at different time points in tumorigenesis suggest that the rate of autophagy varies with malignant progression with autophagy capacity being highest in noninvasive tumors with a reduction in capacity in invasive carcinomas (30). It is of interest that genetic alterations have been identified that link a loss of autophagic capacity to cancer development. Thus, beclin-1 is monoallelically deleted in 50% of breast cancers (31, 32), suggesting that it may be a tumor suppressor gene. Furthermore, heterozygous disruption of beclin-1 increases the frequency of spontaneous malignancies (33). However, the observation that other copy of beclin-1 is maintained in breast cancers and that beclin-1 haploinsuffcient mice do not show depletion of beclin-1 protein implies an important beneficial role for beclin-1 and autophagy in regulating the delicate balance between cell death and survival. Beclin-1 has been shown to promote autophagy in MCF-7 human breast carcinoma cells in low serum conditions (33) and there is increased autophagy without increasing cell death in MCF-7 breast cancer cells that overexpress beclin-1, arguing for an important role for autophagy in cancer cell survival. Unfortunately, we have not been able to assess autophagy in this series of breast tumors because, currently, there are no antibodies to beclin-1 that are reliable in formalin-fixed paraffin embedded material.

Where expression of BNIP3 was retained in the tumor we, as others, also observed BNIP3 in the nucleus as well as the cytoplasm. BNIP3 in human glial cells is primarily localized to the nucleus whereas, in hypoxic conditions, BNIP3 is present mostly in the cytoplasm (10). However, in glial tumors, BNIP3 is located in the nucleus in areas of hypoxia (10). In our studies in breast cancer cells, we observed an increase in autophagy that

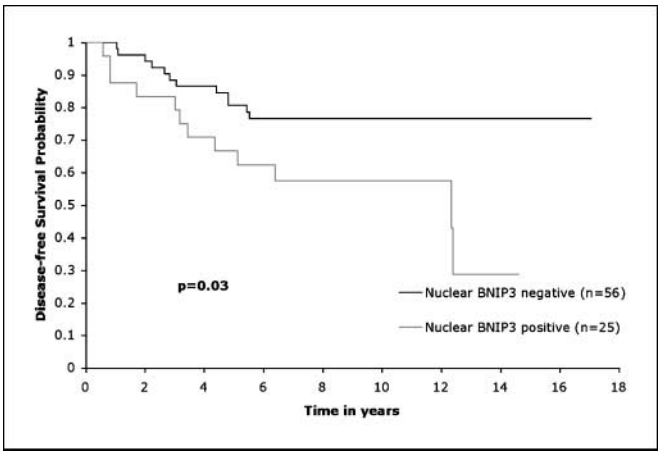

Fig. 4. Kaplan and Meier curves of disease-free survival stratified by nuclear BNIP3 expression in DCIS (n = 81).

Table 4. Multivariate analysis (Cox regression model)

	Hazard ratio (95% CI)	*P*
A. Multivariate analysis (Cox regression model) of disease-free survival for standard clinicopathologic variables and nuclear BNIP3 in invasive carcinomas (n = 157)		
Nuclear BNIP3	0.63 (0.33-1.21)	0.16
Tumor grade	1.68 (1.13-2.51)	0.01
Tumor size	2.90 (1.59-5.30)	0.001
Nodal status	2.91 (1.65-5.12)	<0.001
HIF-1α status	1.22 (0.67-2.23)	0.51
B. Multivariate analysis (Cox regression model) of disease-free survival for standard clinicopathologic variables and nuclear BNIP3 in ER-positive invasive carcinomas (n = 110)		
Nuclear BNIP3	0.38 (0.15-0.96)	0.04
Tumor grade	2.02 (1.18-3.44)	0.01
Tumor size	4.18 (1.84-9.45)	0.001
Nodal status	3.31 (1.53-7.18)	0.002
HIF-1α status	2.47 (1.14-5.38)	0.02
C. Multivariate analysis (Cox regression model) of disease-free survival for standard clinicopathologic variables and nuclear BNIP3 in ER-negative invasive carcinomas (n = 56)		
Nuclear BNIP3	1.19 (0.50-2.85)	0.70
Tumor grade	1.02 (0.54-1.96)	0.94
Tumor size	2.53 (1.13-5.68)	0.03
Nodal status	1.96 (0.89-4.36)	0.10

Abbreviation: 95% CI, 95% confidence interval.

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Table 5. Multivariate analysis (Cox regression model) of nuclear grade and nuclear BNIP3 expression with regards to disease-free survival DCIS (n = 73)

	Hazard ratio	95% CI	*P*
Nuclear BNIP3	2.26	1.01-5.05	0.05
Nuclear grade	1.58	0.86-2.89	0.14

is mediated via BNIP3 under hypoxic conditions.[4] These findings are in accordance with our observation that cytoplasmic BNIP3 in human breast tumors may enable autophagy leading to enhanced cell survival. As a corollary of this, its nuclear location would imply its sequestration and interruption of the autophagic process leading to a less aggressive tumor phenotype. This is in keeping with our findings of a longer survival in patients with BNIP3 nuclear expression in invasive carcinomas, an effect that is pronounced in tumors already recognized to have a good prognosis (i.e., those that are ER positive and/or of low-grade). This suggests that targeting and inhibiting the autophagy pathway may be useful in the treatment (34) of breast cancer and that this may be effective even in tumors thought to have a good outcome. It is of interest that autophagy occurs in MCF-7 human breast carcinoma cells treated with tamoxifen (35), but this may be a stress response and it will be of interest to inhibit this and assess how it modifies tamoxifen response.

It is still not understood which mechanisms determine whether the cell-protective or cell-destructive effect of autophagy predominates but it is likely that autophagy results in a different outcome in different tumors depending on the stress stimuli. With regard to the balance of cell death or survival through the autophagy pathway in human breast cancer, we observed a striking difference in survival association between DCIS and invasive carcinomas. Thus, in contrast to invasive carcinomas as outlined above, nuclear BNIP3 in DCIS is associated with patients having a shorter disease-free survival. This finding agrees with our previous data showing a positive relationship between BNIP3 mRNA levels and DCIS grade, necrosis score, and the presence of invasive breast carcinoma (11). This suggests a switch during progression from in situ to invasive disease from BNIP3-mediating apoptosis and cell death to autophagy, such that any cell death effects of BNIP3 are bypassed. One potential mechanism of the different effects of BNIP is through the generation of a truncated form. These have been shown to behave as a dominant negative (10) and may explain the mechanism by which the differing effects of BNIP3 are mediated. However, in other tumor types, such as non–small cell lung cancer, patients with tumors showing nuclear BNIP3 seem to have a shorter survival (36). This may reflect a different balance of the autophagic pathway in lung cancer resulting in tumor cell death rather than survival possibly due to an intact beclin-1 (to the best of our knowledge, currently, there are no data on beclin-1 in lung cancer). Thus, it would be of great interest to measure the basal level of autophagy, beclin-1, and BNIP3 in lung cancers compared with that of *in situ* and invasive breast cancer.

In summary, we have shown significant increase in cytoplasmic BNIP3 expression in DCIS and invasive carcinoma compared with normal breast tissue, suggesting an important role in neoplastic progression in breast cancer. We also observed that nuclear BNIP3 expression is associated with a higher risk of recurrence and shorter disease-free interval in DCIS but associated with a less aggressive phenotype in invasive breast carcinomas. Because BNIP3 is known to play a role in autophagy and cell death and survival, this suggests a pivotal role for this protein during the transition from *in situ* to invasive disease. The complex role of BNIP3 in breast cancer warrants further work to gain a better understanding of the role of BNIP3 in breast tumor progression.

[4] Unpublished data.

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