Expression and Functional Characterization of the BNIP3 Protein in Renal Cell Carcinomas

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Abstract
BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) is a BH3-only protein that regulates apoptosis and autophagy. BNIP3 plays also an important role in hypoxia-induced cell response and is regulated by HIF1. Here, we studied a possible association of BNIP3 expression and the prognosis of patients with renal cell carcinomas (RCCs) and examined the functional relevance of BNIP3 in the regulation of cell survival and apoptosis of renal carcinoma cells. BNIP3 expression was determined by immunohistochemistry in RCC tumor tissue samples of 569 patients using a tissue microarray. Functional characterization of BNIP3 in renal carcinoma cells indicates prosurvival effects. In human RCC tumor samples, high cytoplasmic BNIP3 expression was associated with high-grade RCCs and regional lymph node metastasis. BNIP3 expression correlated negatively with disease-specific survival. Multivariate Cox regression analysis retained BNIP3 expression as an independent prognostic factor in patients without distant metastasis. Together, our studies imply that BNIP3 regulates cell survival in RCCs and its expression is an independent prognostic marker in patients with localized RCCs.

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Introduction
B-cell leukemia/lymphoma 2 (BCL-2)/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) is an atypical BCL-2 homology domain 3-only (BH3-only) protein involved in cell death, mitochondrial clearance, and autophagy [1,2]. Moreover, BNIP3 overexpression was reported to induce delayed cell death in cell lines [3,4]. Although this cell death was initially referred to as apoptotic, subsequent studies demonstrated that BNIP3 causes a necrosis-like cell death independent of Apaf-1, caspase activation, or cytochrome c release [5]. Further studies link BNIP3 to autophagic cell death characterized by extensive cytoplasmic vacuoles resembling autophagosomes and microtubule-associated protein light chain 3 (LC3) conversion [6,7]. However, BNIP3-induced autophagy can also trigger prosurvival effects in response to hypoxic stress in both normal and tumor cells [8] and provokes a protective removal of damaged mitochondria in myocytes upon ischemic injury [9]. This indicates that, depending on the context, BNIP3 has the potential to promote cell survival or to induce cell death. Interestingly, a recent study has shown that phosphorylation of serine residues determines prosurvival or prodeath activity of BNIP3 [10].

Overexpression of BNIP3 is frequently observed in human malignancies and association with aggressive tumor biology, and poor clinical outcome has been described in salivary gland adenoid cystic carcinoma [11], endometrial cancer [12], ductal carcinoma in situ of the breast [13], and cervical cancer [13]. In contrast, high BNIP3 expression is associated with favorable outcome in invasive human breast cancer [14] and laryngeal cancer [15]. Furthermore, downregulation of BNIP3 has been described in pancreatic cancer [16], and silencing of BNIP3 by promoter hypermethylation has been shown in colorectal and gastric cancer cell lines [17] as well as in hematopoietic tumors [18]. These varying results might reflect
cell-type–specific and context-depending functions of BNIP3 in cancer [19].

BNIP3 is a hypoxia-responsive gene [20] and a direct target of hypoxia-inducible factor (HIF) [21]. Accordingly, in cell lines devoid of von Hippel–Lindau (VHL) protein, BNIP3 is constitutively upregulated under normoxic conditions. Interestingly, comprehensive systematic analyses of BNIP3 expression in renal cell carcinoma (RCC), including clear-cell RCC (cRCC), the most frequent VHL loss–associated malignancy, are missing. In this study, we took advantage of a large, hospital-based series of RCCs with long-term follow-up information to examine BNIP3 expression and to systematically assess the clinical properties. Furthermore, we performed in vitro studies to functionally characterize BNIP3 in RCC cell lines.

Patients and Methods

Patients

Tissue samples from 932 patients with primary RCC treated at the Department of Urology at the University of Heidelberg between 1987 and 2005 were collected. The human tissue samples were provided by the Tissue Bank of the National Center for Tumor Diseases Heidelberg after approval by the ethics committee of the University of Heidelberg. Clinical follow-up was available for 912 cases. Patient treatment and evaluation were performed as described previously [22]. Survival was calculated from the date of surgery until last visit or death. All tissue samples were reviewed by at least two pathologists experienced in urologic pathology (S.M.G., W.R.). Tumor classification and grading were performed according to the World Health Organization [23]; for staging, the seventh edition of the TNM classification [24] was used.

Materials and Cells

Antibodies were obtained as follows: anti-actin (Sigma Aldrich, Munich, Germany; A5441) and anti-BNIP3 (R&D Systems, Wiesbaden, Germany, AF4147). CoCl2 (60818), etoposide (E1383), and staurosporine (4400) were obtained from Sigma Aldrich. The RCC cell lines A704, ACHN, CAKI-2, 769-P, and 786-0 were purchased from ATCC (Rockville, MD).

Tissue Microarray and Immunohistochemistry

A series of tissue microarrays containing 932 primary tumor and corresponding normal tissue samples was created as described previously [22]. The tissue microarray slides were stained on a semiautomated staining system (Techmate 500; DakoCytomation, Waldbronn, Germany) with a goat anti-BNIP3 polyclonal antiserum (1:2000) for 45 minutes. Visualization was performed as described previously [22]. The arrays were independently scored by two experienced pathologists (S.M.G., W.R.) blinded to tissue annotations and patient outcomes. For the immunohistochemical semi-quantitative assessment of BNIP3 expression, the product of the scores of staining intensity and quantity of immunoreactive tumor cells was calculated based on the following scoring system: the intensity ranged from 0 = negative to 3 = high; the quantity comprised 0 = no expression, 1 = positivity in less than 1%, 2 = positivity in less than 10%, 3 = positivity in less than 50%, and 4 = positivity in more than 50%. The final immunohistochemical score (IHS; ranging from 0 to 12) is obtained by multiplication of the intensity score and the quantity score. Only cases with two properly stained tumor tissue specimens (duplicates) were included in the subsequent analyses. In case of discordance, the average of the two IHSs was used for further analysis. Furthermore, intracellular staining pattern was registered as follows: cytoplasmic, nuclear, and cytoplasmic and nuclear.

Preparation of Cell Pellets

Exponentially growing cells were harvested using trypsin, resuspended and washed with PBS, and fixed in 4% neutral buffered formalin. After a second wash step, cells were transferred in 100% alcohol and precipitated with 30% BSA. Paraffin embedding was done according to standard protocols used for tissue samples.

Immunoblot Analysis

Cells were washed in ice-cold PBS and lysed on ice with 1× cell lysis buffer (Cell Signaling; Danvers, MA; 9803) containing 1× protease/phosphatase inhibitor cocktail (Cell Signaling; 5872). After 15-minute incubation on ice, lysates were centrifuged at 13,000 g at 4°C for 20 minutes. The total protein concentration of the lysates was measured using the Bradford assay (Bio-Rad Protein Assay, Bio-Rad, Munich, Germany). Twenty-five to 50 μg of protein per lane was separated on 6% to 12% polyacrylamide gels and blotted onto nitrocellulose membrane by standard procedures. The membranes were blocked and incubated O/N with primary antibody at 4°C, followed by another extended washing procedure and incubation with the appropriate secondary antibody. Band antibodies were visualized by an enhanced chemiluminescence detection system (Western Lightning Plus-ECL, Perkin Elmer, Hamburg, Germany).

Flow Cytometry

As described previously [25], for measurement of cell death, RCC cells were treated as indicated and stained with propidium iodide (50 μg/ml in Nicoletti buffer). Subsequently, cells were subjected to flow cytometry analysis using a Becton Dickinson FACScalibur cytometer (BD, Heidelberg, Germany) and Cell Quest Software.

Colony-Forming Assay

Cells were treated as indicated and plated in variable density (500, 2000, 5000) per well and allowed to grow to form colonies, which were then stained with crystal violet (5 g/l; ACROS Organics, Geel, Belgium).

siRNA Transfection

RCC cells were transiently transfected with a BNIP3-siRNA (Thermo Fisher Scientific, Waltham, MA; #J-004636-08) using Lipofectamine 2000 (Thermo Fisher Scientific). A nonspecific siRNA served as a control (Thermo Fisher Scientific, #D-001810-01). All siRNAs were used in a concentration of 20 nM.

Stable Transfectants

Cells were selected using complete medium (RPMI 1640 89%, FBS 10%, penicillin/streptomycin 1%) containing G418 (Geneticin) at a final concentration of 1.4 mg/ml. The pcDNA-BNIP3 expression vector was kindly provided by Dr. Nathan Brady.

Statistical Methods

Data were analyzed using the R software package (version 2.5.1, https://cran.r-project.org). For count data, Fisher’s exact test (two-sided) was used. The Kaplan-Meier method was applied to calculate survival probabilities for both progression-free and cancer-specific overall survival. For multivariate analysis, the Cox proportional hazards regression model was used. Univariate survival data were tested for significance using the Mantel-Haenszel log-rank test. P values less than .05 were considered significant.
Results

BNIP3 Expression in RCC Cell Lines and Human RCC Tissue Samples

First, we examined endogenous BNIP3 expression in different RCC cell lines by immunoblot analysis (Figure 1A). High BNIP3 protein levels were detected in CAKI-2 and A704 RCC cell lines. In a next step, we performed BNIP3 immunohistochemistry on cell block sections to compare immunoblot and immunohistochemical analysis. As expected, strong positive BNIP3 staining in A704 and CAKI-2 cells was observed, whereas ACHN cells show only faint staining.
These results demonstrate that the antibody used recognized BNIP3 in formalin-fixed and paraffin-embedded cells.

Next, we investigated BNIP3 expression in human tumor tissue using a tissue microarray containing tumor samples of 932 patients with RCCs. Only cases with 2 evaluable tumor cores were considered; 345 cases were excluded from further analyses due to insufficient tumor tissue, fixation artifacts interfering with BNIP3 immunohistochemistry, or incomplete clinical or pathological information. The remaining 569 cases could be evaluated. Seventy-three tumors were negative for BNIP3 by immunohistochemistry. The remaining 496 cases showed heterogeneous cytoplasmic and/or nuclear positivity (Figure 1C). Of note, an upregulation of BNIP3 next to necrotic tumor areas could be observed when investigating whole slides. However, necrotic or nearby necrotic tumor regions have been excluded during tissue microarray construction.

BNIP3 Expression and Relationship between Clinical and Pathological Characteristics

To study possible relations between BNIP3 expression and tumor-dependent patient survival, we grouped tumors according to BNIP3 expression and performed a univariate survival analysis (median follow-up time 48 months; mean 60 months).

We observed a statistically significant association of high cytoplasmic BNIP3 expression (defined as IHS ≥ 6) with reduced cancer specific survival compared to tumors with low cytoplasmic or nuclear BNIP3 expression (Figure 2A). This was evident not only for the whole collective but also for subset analyses limited to ccRCC. In addition, similar results were obtained for low- and high-stage RCC as well as for nonmetastatic tumors in subsequent subgroup analyses (Figure 2, B-D).

Next, we examined if BNIP3 expression correlated with clinical or pathological characteristics. The proportion of tumors with high cytoplasmic BNIP3 expression increased with tumor grading ($P = .0001$) and lymph node metastasis ($P = .015$), indicating that BNIP3 expression was associated with a more aggressive tumor phenotype. The clinical and pathological features in relation to BNIP3 expression are depicted in Table 1.

In multivariate analysis using the Cox proportional hazards model including grade of malignancy, tumor extent, distant and regional...
lymph node metastasis, sex, and performance status, adjusted BNIP3 hazards for cancer-specific survival did not reach statistical significance based on the whole collective and the subset of ccRCC (n = 481) (Supplementary Table 1 and Table 2). Importantly, multivariate analysis confined on cases without distant metastasis (n = 392) (Table 2) revealed statistically significant association of BNIP3 expression and cancer-specific survival (HR: 2.24; 95% CI: 1.26-3.96; P = .001). These results suggest that high BNIP3 expression is an independent prognostic factor for poor cancer-specific survival in ccRCC patients without distant metastasized disease.

### Table 1. Correlation of BNIP3 Expression in RCC with Clinicopathological Characteristics

| Variable                  | High BNIP3, n (%) | Low BNIP3, n (%) | P     |
|---------------------------|-------------------|------------------|-------|
| Sex                       |                   |                  | .25   |
| Male                      | 55                | 286              |       |
| Female                    | 28                | 200              |       |
| Karnofsky rating, %       |                   |                  | .36   |
| ≥80                       | 74                | 451              |       |
| <80                       | 9                 | 35               |       |
| Tumor extent              |                   |                  | .18   |
| Stage 1                   | 37                | 268              |       |
| Stage 2                   | 10                | 48               |       |
| Stage 3                   | 35                | 160              |       |
| Stage 4                   | 1                 | 10               |       |
| Fuhrman grade             |                   |                  | .0001 |
| G1                        | 12                | 120              |       |
| G2                        | 44                | 294              |       |
| G3                        | 27                | 72               |       |
| Distant metastasis        |                   |                  | .058  |
| No                        | 61                | 403              |       |
| Yes                       | 22                | 83               |       |
| Lymph node metastasis     |                   |                  | .015  |
| No                        | 71                | 456              |       |
| Yes                       | 12                | 30               |       |
| Type of surgery           |                   |                  | .75   |
| Partial nephrectomy       | 14                | 72               |       |
| Radical nephrectomy       | 69                | 414              |       |
| Histopathological subtype |                   |                  | .23   |
| ccRCC                     | 66                | 415              |       |
| Papillary RCC             | 11                | 45               |       |
| Chromophobe RCC           | 2                 | 23               |       |
| Other types               | 4                 | 3                |       |

*pT3/pT4 vs pT1/pT2.  
*b G3 vs G1/G2.

### Table 2. Uni- and Multivariate Analyses of Prognostic Factors Influencing Cancer-Specific Survival (CSS) in ccRCC

| Grade of malignancy       | HR (95% CI) | P     | HR (95% CI) | P     |
|---------------------------|-------------|-------|-------------|-------|
| pT3/pT4 vs pT1/pT2.       | 5.0 (4.10-6.1) | <.001 | 2.0 (1.34-2.96) | <.001 |
| Male vs male.             | 4.97 (4.05-6.6) | <.001 | 2.02 (1.37-2.99) | <.001 |
| Karnofsky performance status | 10.2 (8.34-12.47) | <.001 | 5.7 (3.86-8.42) | <.001 |
| No lymph node metastasis  | 5.36 (4.22-6.796) | <.001 | 1.1 (1.2-2.92) | .19   |
| Sex f                     | 0.66 (0.49-0.73) | <.001 | 0.55 (0.37-0.79) | .0016 |
| Karnofsky performance status | 2.42 (1.83-3.19) | <.001 | 1.81 (1.12-2.39) | .015  |
| Nephropathy expression    | 3.44 (2.3-5.24) | <.001 | 2.09 (0.91-4.82) | .083  |
| BNIP3 expression           | 1.9 (1.26-2.85) | .0021 | 1.33 (0.85-2.08) | .21   |

**Bold P values are significant at <.05.**  
* pT3/pT4 vs pT1/pT2.  
* M1 vs M0.  
* pN1/pN2 vs pN0/pN0.  
* Female vs male.  
* <80% vs >80%.  
* Radical vs partial.  
* BNIP3-IRS 26 vs <6.

### Functional Characterization of BNIP3 in RCC Cell Lines

BNIP3 is implicated in starvation-induced autophagy [2]; consistently, we found elevated protein levels in A704 RCC cell line upon serum depletion (Figure 3A). To examine effects of BNIP3 in RCC cell lines, we silenced BNIP3 expression by siRNA knockdown and studied cell viability under different conditions. Silencing of BNIP3 by siRNA increased cell death induced by serum depletion, staurosporine, etoposide, and CoCl2, a hypoxia-mimetic agent (Figure 3B). Furthermore, reduced colony formation was observed (Figure 3C). In turn, stable overexpression of BNIP3 resulted in increased colony formation under serum starvation (Figure 3D). These results indicate that BNIP3 mediates prosurvival effects in RCC cell lines.

### Discussion

BNIP3 is a direct target of HIF-1 [21]. Although deregulation of BNIP3 expression, including both up- and downregulation, has been described in different malignancies [19], comprehensive analysis of BNIP3 expression and function in ccRCC is not available at present. This is astonishing as ccRCC qualifies as the most frequent malignancy associated with VHL loss and consecutive HIF-1 activation. We here show that upregulation of BNIP3 is frequently observed in ccRCC and tumors with high BNIP3 expression show a more aggressive phenotype with high-grade morphology and more frequent nodal metastasis. Importantly, multivariate analysis revealed that high BNIP3 expression was an independent prognostic factor for cancer-specific survival in ccRCC patients without distant metastasis.

Although initially described to activate apoptosis [4], BNIP3 seems to possess context-depending functions including induction of prosurvival autophagy [8,9]. Our functional analysis showed increased cell death and reduced clonogenicity upon silencing of BNIP3 and vice versa increased clonogenicity upon stable overexpression, indicating protumorigenic effects of BNIP3 in RCC cell lines. Immune evasion is a hallmark of tumor progression [26], and natural killer (NK) cells play an important role in the immune response against cancer [27]. Recently, Messai et al. described a HIF2α/ITPR1 axis regulating RCC cell survival, whereby HIF2α-dependent autophagy prevented NK-mediated lysis [28]. This result is in agreement with a recent report showing that lymphocyte-induced cell-mediated autophagy promotes cancer cell survival [29]. Potentially,
Figure 3. Functional characterization of BNIP3 in RCC cell lines. (A) Immunohistochemistry on cell pellets demonstrating induced BNIP3 expression upon starvation. (a) Corresponding immunoblot analysis demonstrates the 60 kDa dimer of BNIP3. (B) Flow cytometric analysis of A704 cells after BNIP3 silencing by siRNA (20 nM) for 24 hours and consecutive treatment with staurosporine (STS; 100 nM), etoposide (ETO; 50 nM), CoCl2 (400 μM), or serum depletion for 48 hours. Data indicate the percentage of cells showing a sub-G1-DNA content (mean ± SEM). (C) Colony formation assay of A704 after BNIP3 silencing by siRNA (20 nM) for 24 hours. (c) Immunoblot analysis of transfected cells demonstrates the 60-kDa dimer of BNIP3. (D) Colony formation assay of stably transfected 786-O cells. Cells were plated in serum depleted medium for 24 hours and consecutively allowed to grow in rich medium. (d) Immunoblot analysis of transfected cells demonstrates the 60-kDa dimer of BNIP3.
BNIP3 is involved in RCC immune escape attributed to autophagy. RCC is an immunogenic tumor, and therefore, immune therapy has been a historical standard of care in metastatic RCC [30], and new checkpoint inhibitors also show promising results [31,32]. Indeed, sunitinib or pazopanib was approved in both the United States and Europe for the treatment of patients with advanced RCC who have received prior therapy [33]. Potentially, BNIP3 expression indicates resistance to lysis of tumor cells by NK cells and may provide potential as a predictive biomarker for immune therapy.

In conclusion, our findings reveal protumorigenic effects of BNIP3 in RCC cell lines and highlight BNIP3 as a promising new prognostic biomarker in ccRCC.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2017.08.008.

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