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BAX-INTERACTING FACTOR-1 (BIF-1) EXPRESSION IN PROSTATE CANCER

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Abstract

Background—Bif-1 protein is a member of the endophilin B family that binds to and activates the pro-apoptotic Bax protein in response to apoptotic signals. Loss of Bif-1 suppresses the intrinsic pathway of apoptosis and promotes tumorigenesis. We examined the expression levels of Bif-1 protein in human prostate cancer.

Methods—Thirty-nine archival tissues specimens of human prostate cancer, and a human prostate cancer tissue microarray containing 19 samples of normal prostate (NR), 26 samples of benign prostatic hyperplasias (BPH), 30 samples of high grade prostatic intraepithelial neoplasia (PIN), and 153 samples of prostate cancer (CA), were selected for immuno-histochemical staining with Bif-1 antibody. The slides were scored by two independent observers.

Results—Non TMA samples: moderate to strong Bif-1 staining was identified in 38 of 39 CA. In 32 cases foci of PIN were identified adjacent to CA. Of these, twenty-nine (91%) showed strong and diffuse Bif-1 staining. BPH, identified in 27 cases, was weakly Bif-1 positive in 89% of cases. TMA samples: 38.6% (59/153) of CA showed moderate-strong Bif-1 expression, and 21.5% (33/153) were Bif-1 negative. Bif-1 expression was moderate-strong in 76.6% (23/30) of PIN. Bif-1 was weak-moderate in 53.8% (14/26) of BPH and negative in 46.1% (12/26) of them. Low-moderate Bif-1 was seen in 89.5% of NR.

Conclusions—The loss of Bif-1 expression in a subset of CAs is in agreement with the proapoptotic function of Bif-1. The significance of the increased Bif-1 in a subgroup of CA and in PIN remains to be determined. It seems that Bif-1 has a role in prostate cancer, providing the rationale for using Bif-1 as a target for prostate anticancer therapy.

Keywords

Bif-1; prostate adenocarcinoma; PIN; immunohistochemistry

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1. Introduction

Prostate adenocarcinoma is the most common non cutaneous malignancy in men (1), and the second leading cause of cancer death among men in the United States (1,2).

High grade prostatic intraepithelial neoplasia (PIN) is the putative premalignant lesion of prostatic adenocarcinoma, and it is characterized by the proliferation of high grade dysplastic cell within the prostatic acini and duct (3). PIN has been reported to be a risk factor for subsequent detection of adenocarcinoma (4). Several studies have shown that high grade PIN is usually seen adjacent to or intermingled with prostatic adenocarcinoma in up to 75% of cases (5), and that large areas of high grade PIN may be associated with microinvasive carcinoma (6). In addition, it seems that, similarly to what is observed with adenocarcinoma, the incidence of high grade PIN increases with age (7).

Recently, it has been reported that inhibition of apoptosis is critical in prostate cancer (8). It has also been proposed that overexpression of Bcl-XL in prostate cancer may suppress the activity of the proapoptotic molecules Bax and Bak and may contribute to androgen resistance and progression of prostate cancer (9).

It seems that overexpression of Bax and a lower Bcl 2/Bax ratio in human prostatic cancer tissues may have a proapoptotic stimulus, and a high Bcl 2 level may represent a tentative of counterbalancing the tendency to cell death (10).

Bif-1 (Bax-Interacting Factor-1) has been shown to interact with Bax and to induce its conformational change in mammalian cells during apoptosis. We have shown that knockout of Bif-1 suppresses Bax/Bak conformational change, cytochrome c release, caspase activation and cell death (11), suggesting that Bif-1 may represent a new type of Bax activator controlling the mitochondrial pathway of apoptosis. Along this line, a recent study has reported the decrease expression of Bif-1 in malignant gastric epithelial cells as compared to the normal gastric mucosal cells (12). To date the expression of Bif-1 protein in prostate cancer has not been reported.

In this study we focused on the evaluation of Bif-1 expression and significance in prostatic hyperplasia, high grade PIN and prostatic adenocarcinoma. To determine the level of Bif-1 expression we used qualitative immunohistochemistry in archival specimens of prostate cancer resections containing prostatic hyperplasia and/or high grade PIN adjacent to the prostatic adenocarcinoma. Our data demonstrated for the first time that while Bif-1 is highly expressed in high grade PIN, a significant portion of carcinomas are Bif-1 negative.

2. Materials and methods

2.1 Selection of cases

Following institutional review board ethics approval, archival pathology specimens (paraffin embedded tissue) of 39 prostatic adenocarcinomas, were identified from the H. Lee Moffitt Cancer Center Anatomic Pathology Division's database, CoPath®, for surgical specimens obtained between 2000 and 2006. The patients selected for this study did not undergo pre-operative neoadjuvant therapy as part of their treatment. The selected blocks included adjacent areas of prostatic hyperplasia in 27 of the cases, and high grade PIN in 32 of the cases.

In addition, human prostate cancer tissue microarray (TMA), prepared in the Histology Laboratory of the Moffitt Cancer Center Tissue Core Facility, were also tested for Bif-1 expression. The prostate TMA included 19 samples of normal prostate (NR), 26 samples of benign prostatic hyperplasias (BPH), 30 samples of prostatic intraepithelial neoplasia (PIN),

and 153 samples of prostate carcinoma. When considered together, TMA and resection specimens accounted for 19 cases of NR, 53 cases of BPH, 62 cases of PIN, and 192 prostatic cancer samples.

All of the specimens were preserved in 10% buffered formalin prior to embedding in paraffin. The tissue sections were stained to assess the variations in Bif-1 expression during the progression from normal prostate to hyperplasia, to prostatic intraepithelial neoplasia, to carcinoma.

2.2. Immunohistochemistry

Unstained tissue sections were cut from representative blocks of the formalin-fixed, paraffin embedded tissues of thirty nine resection specimens, as well as from the prostate TMA block. These sections were mounted on slides and stained for Bif-1, using a mouse monoclonal antibody (Imgenex, San Diego, CA).

The slides were dewaxed by heating at 55° C for 30 minutes and by three washes, five minutes each, with xylene. Tissues were rehydrated by a series of five-minute washes in 100%, 95%, and 80% ethanol, and distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 minutes. After blocking with universal blocking serum (Ventana Medical Systems, Inc., Tucson, Arizona OMNIMAP) for 30 minutes, the samples were incubated with a Bif-1 mouse anti-human monoclonal antibody (Imgenex, dilution 1:2500; final concentration of 1 µg/ml) at 4° C overnight. The sections were then incubated with biotin-labeled secondary antibody and streptavidin-horseradish peroxidase for 30 minutes each (Ventana Medical Systems). The samples were developed with 3,3'-diaminobenzidine tetrahydrochloride substrate (Ventana Medical Systems Inc. Tucson, Arizona) and counterstained with hematoxylin (Ventana Medical Systems Inc. Tucson, Arizona, product #760-2021). The slides were dehydrated and coverslipped. Standard cell conditioning (following the Ventana proprietary recommendations) was used for antigen retrieval. Negative controls were included by omitting Bif-1 antibody during the primary antibody incubation. The positive controls consisted of a cytopsin preparation of cells constitutively expressing Bif-1 protein (Dr. Wang's laboratory).

2.3. Data analysis

The Bif-1 stained slides were examined by two independent observers (CO, DC) simultaneously; and a consensus score was reached for each specimen. The positive reaction of Bif-1 was scored into four grades, according to the intensity of the staining: 0, 1+, 2+, and 3+. The percentages of Bif-1 positive cells were also scored into four categories: 0 (0%), 1 (1–33%), 2 (34–66%), and 3 (67–100%). The product of the intensity by percentage scores was used as the final score. The final scores were classified as: 0, negative; 1–3, weak; 4–6, moderate; and 7–9, strong. The specimens were also classified by the types of tissue staining positive: normal prostate, prostatic hyperplasia, high grade prostatic intraepithelial neoplasia, and prostatic adenocarcinoma.

Descriptive statistics for the scores obtained from the non-TMA samples were generated and reported for each group. The statistical method used to compare the scores by groups was the Wilcoxon Signed Rank test. Three tests were performed and a Bonferroni-Holm adjustment for multiple testing was performed to correct for multiple testing, using SAS software (SAS Institute Inc., Cary, North Carolina). Comparisons were done for 1) CA versus PIN, 2) CA versus BPH and 3) PIN versus BPH. Spearman's correlation coefficient was used to analyze the relationship between Bif-1 expression and Gleason's grading score.

The analysis on the TMA data was included as a follow-up, confirmatory analysis to the original BIF analysis. The method used to compare Bif-1 expression in CA, PIN, BPH and NR is the Wilcoxon Rank sum test since the data in this TMA portion of the study was not paired data. Six tests were performed and a Bonferroni-Holm adjustment for multiple testing was used to correct for multiple testing.

3. Results

3.1 Clinical Pathologic Findings

When considering the non-TMA specimens, the patients had an average age of 60 years (range between 42 and 85 years old). Six of the cases were metastatic resections (4 cases of lymph nodes excision, 1 local excision of chest wall tumor, and 1 excisional biopsy of a tumor of the posterior bladder wall). Thirty-three cases were primary tumor resection. These tumors ranged in size between 0.4 cm. and 2.5 cm, mostly multifocal and bilateral. The most common Gleason score was 7 (16 cases), followed by 6 (8 cases), 9 (5 cases), and 8 (4 cases). Twenty-three patients had stage II, 10 had stage III, and 6 had stage IV disease. All the patients were treated with surgery: excision or resection for the metastases, and radical retropubic prostatectomy for the primary tumors. Twenty-seven patients were treated with surgery alone; twelve patients received additional therapy: radiation (3), chemotherapy (2), or both (2), hormonal therapy (1), radiation, chemotherapy and hormonal therapy (2), hormonal plus radiation (1) and hormonal plus chemotherapy (1). All additional therapy was administered postoperatively (adjuvant therapy). The patients were followed up between 8 to 98 months. The mean follow-up time of all patients was 30.5 months. At the end of the study 32 patients were alive with no evidence of disease (mean follow-up of 31 months) 1 patient was dead of disease (follow-up of 27 months), two were alive with disease (mean follow-up of 44 mo.), and 4 patients were lost to follow up. Since there was only one event, a survival analysis with respect to Bif-1 expression was not informative.

No clinical-pathological information was available for the cases included in the prostate TMA.

3.2 Immunohistochemical results and data analysis

All of the positively stained cases had cytoplasmic staining. Nuclear staining by Bif-1 was not seen. The cytoplasmic staining was diffusely granular with variation in intensity seen within the same lesion in some cases. Cases with variable staining were graded based on the predominant staining intensity and the percentage of positive stain was determined based on the amount of the lesion demonstrating the predominant intensity.

Of the non-TMA cases, approximately 72% (28/39) of the prostatic adenocarcinomas exhibited strong Bif-1 staining, and 26% (10/39) of patients demonstrated moderate Bif-1 staining. The mean score was 6.7 (2.0 s.d.) for adenocarcinoma. Similarly, 91% (29/32) cases of high-grade prostatic intraepithelial neoplasia demonstrated strong Bif-1 staining. The mean score was 7.2 (2.1 s.d.) for high grade PIN. Only 1 of the adenocarcinoma and none of the high grade PIN had weak Bif-1 expression. There was no discernable difference in Bif-1 expression in adenocarcinomas with different Gleason's grading score (Spearman's correlation coefficient: -0.054 ; p value = 0.76). When comparing the difference in the staining scores between high grade PIN and adenocarcinoma, a p -value of 0.0171 was calculated.

No cases of carcinomas and/or high grade PIN were Bif-1 negative. Most of the prostatic hyperplasia (88.9%; 24/27) exhibited weak Bif-1 staining. The mean score was 2.0 (1.2 s.d.) for prostatic hyperplasia. Moderate Bif-1 stain was only observed in 3 of 27 cases (11%). When comparing the differences in the staining scores between adenocarcinomas and hyperplasia, a

p-value of <0.0001 was calculated. A similar value was calculated when comparing high grade PIN to prostatic hyperplasia.

When considering the TMA cases, 38.6% (59/153) of CA showed moderate to strong Bif-1 expression, 39.8% (61/153) had weak Bif-1 staining, and 21.5% (33/153) were Bif-1 negative. The CA mean score was 3.5 (2.9 s.d.). Bif-1 expression in PIN was strong in 16.6% (5/30), moderate in 60% (18/30), and weak in 20% (6/30) of cases. Only one PIN case was Bif-1 negative. The PIN mean score was 5.3 (2.4 s.d.). When considering BPH, weak to moderate Bif-1 expression was observed in 53.8% (14/26) of cases and it was absent in 46.1% (12/26) of them. The BPH mean score was 1.6 (1.9 s.d.). Normal prostate tissues expressed low to moderate levels of Bif-1 in 89.5% of cases. Only 2 NR were Bif-1 negative. The NR mean score was 1.9 (1.4 s.d.). Strong Bif-1 expression was not noted in NR and BPH cases.

When comparing the differences in the staining scores between the TMA CA and TMA BPH, an adjusted p-value of 0.0060 was calculated. The difference in IHC score between TMA PIN and TMA BPH ($p = <0.0001$), TMA NR and TMA PIN ($p = <0.0001$), TMA PIN and TMA CA ($p = 0.003$), TMA NR and TMA CA ($p = 0.049$) were all statistically significant.

These results demonstrate a statistically significant difference in staining between prostatic hyperplasia, high grade PIN, and prostatic adenocarcinoma.

3.3 Antibody specificity

The specificity of the anti-Bif-1 monoclonal antibody was confirmed by immunoblot analysis of Bif-1-positive and -negative cells. As shown in Fig.2, an approximately 41-kDa band corresponding to Bif-1 was detected in wild type cells. In contrast, this monoclonal antibody failed to react with any of the proteins in cell lysate prepared from Bif-1 knockout cells. The specificity of the anti-Bif-1 antibody was further confirmed by its reactivity with a single 41-kDa protein in Bif-1 knockout cells transfected with Bif-1 expression plasmid.

4. Discussion

In recent years it has become evident that dysregulation of apoptosis contributes to the pathogenesis of prostate cancer (8-10).

Bif-1, also known as endophilin B1 and SH3GLB1 (SH3 domain GRB2-like endophilin B1), was originally identified as a Bax-binding protein by yeast two-hybrid screens using Bax as the bait (13,14). The interaction between Bif-1 and Bax is enhanced in mammalian cells during apoptosis, which is accompanied by a conformational change in the Bax protein (11,13,15).

Overexpression of Bif-1 in hematopoietic cells promotes Bax activation and apoptosis following IL-3 withdrawal (13). Conversely, knockout of Bif-1 suppresses Bax/Bak conformational change, cytochrome c release, caspase activation and cell death (11). This suggests that Bif-1 may represent a new type of Bax activator that controls the mitochondrial pathway of apoptosis.

Consistent with its pro-apoptotic function, suppression of Bif-1 expression by RNA interference in HeLa cells promotes tumor growth in nude mice (11) and Bif-1 knockout mice have a higher frequency of tumor incidence (16). It has been shown that the Bif-1 mRNA levels are downregulated in lung carcinomas (17), and that 60% gastric carcinomas express undetectable Bif-1 protein by using a tissue microarray technology (12). Interestingly, loss of heterozygosity (LOH) on 1p22, where the *bif-1* gene is localized, is frequently observed in many types of tumor (18-26). Taken together, these results suggest that Bif-1 is a candidate tumor suppressor.

Here we report for the first time that Bif-1 protein was absent in 17.2% (33/192) of the total number of prostate cancer examined. These findings support the pro-apoptotic and tumor suppressor functions of Bif-1.

However, our results also demonstrate increased Bif-1 protein expression in a subset of CAs. The biological meaning of this finding remains to be determined. In particular, we found the highest Bif-1 expression in PIN, a precursor of prostatic adenocarcinoma. In prostatic cancer, the expression of Bif-1 persisted but at a lower level compared to that in PIN, and the difference in Bif-1 score between PIN and CA was statistically significant in both the non TMA samples ($p=0.0171$) and in the TMA samples ($p = 0.003$). One possibility is that Bif-1 may play an important role in the early stages of prostate tumor development. For example, transformed prostate acinar cells are upregulating Bif-1 expression in a tentative to induce apoptosis of these abnormal cells. In addition to its proapoptotic function, we have recently discovered that Bif-1 plays a critical role in autophagy as a positive regulator of autophagosome formation (16). Autophagy is a self-eating process connected to cell survival by supplying the cell with recycled nutrients under starvation conditions or removal of malfunctioning proteins and organelles (27). Therefore, Bif-1 overexpression in PIN may be a mechanism to induce autophagy by which the cell is trying to survive the environmental stresses associated with limited angiogenesis and nutrition. Once the tumor is established the function of Bif-1 as an activator of autophagy is no longer required, but its expression may persist as a mechanism to counteract tumor growth.

5. Conclusions

This study describes, for the first time, the expression of Bif-1 protein in prostate cancer. We found that Bif-1 expression is decreased in a subset of CA as compared to NR. This findings support the candidacy of Bif-1 as tumor suppressor. In addition, we found increasing levels of Bif-1 protein expression in high grade PIN and in a subset of prostate cancer. The significance of this finding remains to be determined. Future studies are also warrant to evaluate the value of Bif-1 as an early diagnostic biomarker for prostate cancer. The results of this study may help in allowing evolution of improved therapies for prostate cancer based on the better understanding of the underlying biology of this disease process.

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B

Non-TMA Samples

Diagnosis	<i>n</i>	L	M	H	Score (mean $\pm$ SD)	<i>p</i>
Carcinomas	39	1	10	28	6.7 $\pm$ 2	0.0171 (CA vs PIN)
PIN	32	0	3	29	7.2 $\pm$ 2.1	<0.0001 (CA vs BPH)
Hyperplasia	27	24	3	0	2.0 $\pm$ 1.2	<0.0001 (PIN vs BPH)

IHC score: L = 1-3; M = 4-6; H = 7-9

Figure 1. Bif-1 Immunohistochemistry

Strong immunoreactivity for Bif-1 is seen in PIN and CA. In contrast, a case of BPH shows weak Bif-1 staining (A). The statistical data for the non-TMA samples are presented in B.

Figure 2. Immunoblot analysis of specificity of anti-Bif-1 antibody

Cell lysates were prepared from Bif-1 knockout (lane 1), knockout transfected with Bif-1 (lane 2), and wild type (lane 3) mouse embryonic fibroblasts and subjected to SDS-PAGE/immunoblot analysis with monoclonal antibodies specific for Bif-1 (top) or β -actin (bottom).