Bad expression influences time to androgen escape in prostate cancer

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OBJECTIVE

To assess the role of selected downstream Bcl-2 family members (Bad, Bax, Bcl-2 and Bcl-xL) in the development of androgen-independent prostate cancer (AIPC), as androgen-deprivation therapy is the treatment of choice in advanced prostate cancer, yet patients generally relapse and progress to an AI state within 18–24 months.

PATIENTS, MATERIALS AND METHODS

The patient cohort was established by retrospectively selecting patients with prostate cancer who had an initial response to androgen-deprivation therapy, but subsequently relapsed with AIPC. In all, 58 patients with prostate cancer were included with matched androgen-dependent (AD) and AI prostate tumours available for immunohistochemical analysis; two independent observers using a weighted-histoscore method scored the staining. Changes in Bad, Bax, Bcl-2 and Bcl-xL expression during transition to AIPC were evaluated and then correlated to known clinical variables.

RESULTS

High Bad expression in AD tumours was associated with an increased time to biochemical relapse ($P = 0.007$) and a trend towards improved overall survival ($P = 0.053$). There were also trends towards a decrease in Bad ($P = 0.068$) and Bax ($P = 0.055$) expression with progression to AIPC. There were no significant results for Bcl-2 or Bcl-xL.

CONCLUSION

There is evidence to suggest that Bad expression levels at diagnosis influence time to biochemical relapse and overall survival, and that levels of pro-apoptotic proteins Bad and Bax fall during AIPC development. Bad might therefore represent a possible positive prognostic marker and potential therapeutic target for AIPC in the future.

KEYWORDS

Bad, Bax, Bcl-2, Bcl-xL, prostate cancer

INTRODUCTION

Prostate cancer is the most common malignancy among men in the UK and remains the second leading cause of male cancer-specific mortality after lung cancer, being responsible for nearly 10 000 deaths in the UK each year [1]. Androgen-deprivation therapy has remained the treatment of choice in advanced prostate cancer since the observations made by Huggins and Hodges in the 1940s [2]. While initial response rates to androgen-deprivation therapy are high, patients generally relapse within 18–24 months [3] with rising PSA levels indicative of progression to hormone-refractory or androgen-independent prostate cancer (AIPC) [3]. There are few therapeutic options available to patients after transition to the AI state; second-line androgen-deprivation therapy, chemotherapy and radiotherapy can be beneficial in symptom control; however, approach to management is generally of a palliative intent [4] and the median survival of patients after progression to AI is just 12 months [3]. It is therefore important that we further our knowledge of the molecular mechanisms driving the development of AIPC, in doing so new therapeutic targets might be identified, leading to the development of effective treatments for this patient group.

There is evidence to suggest that receptor tyrosine kinases, such as epidermal growth factor receptor and HER2, in the plasma membrane contribute to hormone escape in prostate cancer [5,6]. The PI3K/Akt pathway is activated via receptor tyrosine kinases in response to extracellular growth and survival factors and represents one of several independent routes to androgen escape in prostate cancer [7]. As we previously reported activation of the cascade in this laboratory [7], it was appropriate to examine the activity of downstream members. Akt is a protein capable of influencing cellular proliferation and survival in several ways, including inhibition of apoptosis via phosphorylation of the pro-apoptotic protein Bad [8]. Bad is a member of the Bcl-2 family, a group comprising proteins that are important regulators of apoptosis. The proteins are divided into three groups according to their relative actions and the number of Bcl-homologuey domains present [8]. The prosurvival family members include Bcl-2 and Bcl-xL, and are located in the outer mitochondrial membrane where they have the capacity to inhibit specific apoptotic stimuli [8,9]. There are two groups of pro-apoptotic proteins; Bax/Bak-like proteins and BH3-only proteins. Bax and Bak can disrupt the outer mitochondrial membrane, resulting in the release of apoptogenic molecules, such as cytochrome c, from the mitochondria into the cytoplasm, leading to cell death [8]. Bad, Bim and Bid act upstream of Bax and Bak and are examples of BH3-only proteins that exert their pro-apoptotic influence by binding to and antagonizing pro-survival members and/or by activating the pro-apoptotic Bax/Bak-like members [8].

The BH3-only protein Bad acts principally by heterodimerizing with either of pro-survival proteins Bcl-xL and Bcl-2; however, it appears to bind more strongly to Bcl-xL in mammalian cells [9]. Bad associates with Bcl-xL or Bcl-2 and modulates its survival functions by preventing Bcl-xL or Bcl-2 from binding to...
and thereby hindering the death-promoting actions of Bax and Bak [10]. Therefore when Bad binds to Bcl-xL or Bcl-2, Bax and Bak can continue to exert their pro-apoptotic influence, causing the release of apoptogenic molecules from the mitochondria into the cytosol that culminates in caspase activation and cell death [10]. Bad undergoes translocation from the cytosol to the outer mitochondrial membrane during the apoptotic process [11].

Bad comprises 23 serines and 10 threonines within 204 amino acids, and of these, serines 112, 136 and 155 have been identified as key phosphorylation sites [12]. Phosphorylation by Akt at Ser136 prevents the association of Bad with Bcl-xL or Bcl-2 on the outer membrane of mitochondria, and causes alteration in Bad subcellular distribution from mitochondria to bind to 14–3–3 proteins present in the cytosol [12,13]. Bad is sequestered in the cytoplasm by 14–3–3 proteins and therefore unable to perform pro-apoptotic activities [12,13]. As a result, Bcl-xL and Bcl-2 are free to support cell survival and thus bind to and antagonize the actions of Bax and Bak.

Analysis of these events at a molecular level has not been a straightforward task, as most prostate tumours available for study only represent the disease at time of diagnosis, because it is not usual practice to take biopsies of recurrent disease [14]. However, the present study measures the expression of selected Bcl-2 family members, namely Bad, Bax, Bcl-xL and Bcl-2, in paired androgen-dependent (AD) and AI tumour specimens, to further characterize the role of this protein family in the development of AIPC.

PATIENTS, MATERIALS AND METHODS

The patient cohort was established by retrospectively selecting patients with prostate cancer who had an initial response to androgen-deprivation therapy, but subsequently relapsed with AIPC. This provided the following numbers of matched AD and AI tumour pairs for analysis; 58 (Bad and Bcl-xL), 53 (Bax) and 51 (Bcl-2), it was originally planned to do 58 pairs for all proteins but some pairs were lost due to insufficient tissue. Tumours classed as AD were obtained from patients diagnosed with locally advanced or metastatic prostate cancer who had had surgery, followed by conventional androgen-deprivation therapy. Inclusion criteria were applied according to Djavan et al. 2003 [16]. To meet the inclusion criteria there had to be a response to androgen-deprivation therapy; defined as a decrease in PSA levels by at least half, with a nadir being reached of <0.1 ng/mL. The AD samples were obtained from either a TURP or a TRUS-guided biopsy. Patients were also required to relapse with AIPC to meet the inclusion criteria; relapse corresponded to failure of androgen-deprivation therapy and was defined clinically as two consecutive rises in PSA concentration >10%, PSA levels had to rise to >0.4 ng/mL to have clinical relevance and disease recurrence had to be within 6–49 months after surgery. After identification of these patients, AI tumour specimens were only available for analysis if additional surgery was required to treat the clinical symptoms of BOO, and therefore AI tumour samples were obtained only by TURP. To further confirm progression to AIPC, the proliferation index using MIB-1 Ki67 staining was calculated, the proliferation index significantly increased from 2.9 (1.2–6.1) to 7.8 (2.7–15.8), P < 0.001. Androgen receptor and PSA expression was observed in all AI tumours.

All tumours had patient identification removed, including block number and hospital number, and were coded to make the database anonymous. Ethics approval was obtained from the Multicentre Research Ethics Committee for Scotland and relevant Local Research and Ethical Committees.

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The specificity of antibodies used in this study was confirmed by Western blotting. All immunohistochemistry was done on 5µm, archival formalin-fixed, paraffin-embedded prostate tumour sections, therefore overcoming the problem of heterogeneity. The sections were dewaxed in xylene and then rehydrated through graded alcohols. Antigen retrieval was done by incubating sections in antigen unmasking solution at 96°C for 10 min (Bax) and 20 min (Bad), or using heat treatment under pressure in a Tris-EDTA buffer (Bcl-xL and Bcl-2). Endogenous peptidase was destroyed by incubating sections in 10% peroxidase for 20 min The following antibodies and dilutions were selected; Bad (CST#9292) at 1: 25, Bcl2 (Dako M0887) at 1: 50, Bax (Dako A3533) and Bcl-xL (CST #2762) both at 1: 1000. The sections were incubated with primary antibody overnight at 4°C, except those stained for Bcl-2, which were incubated for 1 h at room temperature. Staining was developed using the EnVision kit (DakoCytomation, Glostrup, Denmark), and chromagen was detected using diaminobenzidine (Vector Laboratories, UK). A positive and negative control slide was included in each run. Slides were counterstained in haematoxylin and Scotts tap water substitute, dehydrated through graded alcohols and xylene, and lastly mounted in DPX.

Tissue staining intensity was scored ‘blind’ by two independent observers using a weighted-histogram method. Histoscores were calculated from the sum of (1 × percentage of cells staining weakly positive) + (2 × percentage of cells staining moderately positive) + (3 × percentage of cells staining strongly positive), with a maximum of 300. Inter-class correlation coefficients (ICCC) were calculated to confirm consistency between observers and ICCC >0.7 was acceptable. The mean of the two observers scores was used for analysis.

Wilcoxon signed-rank tests were used to compare expression between AD and AI tumours. The Kaplan–Meier method was used for survival analysis and curves were compared using the log-rank test. Cox regression analysis was used for multivariate analyses and hazard ratios (HRs); P < 0.05 was considered to indicate statistical significance.

RESULTS

In all, 58 patients with prostate cancer (diagnosed 1984–2002) were included in this study with matched AD and AI prostate tumours available for analysis (116 tumours in total). Patients were diagnosed with locally advanced (48) or metastatic prostate cancer (10) and subsequently had surgery and androgen-deprivation therapy (18 orchidectomy, 36 GnRH analogues, three had both and one received antihormone therapy alone). In all, 38 of the 58 patients received antihormone therapy. During the follow-up 48 patients died and 10 were alive.
At the last follow-up; 33 deaths (57%) were cancer-specific. Table 1 summarizes the main patient characteristics.

As expected, the proteins were expressed within the cytoplasm. The ICCC for each protein was >0.7 and was therefore classed as excellent. The median (interquartile range) histoscores for each protein in both AD and AI tissues are given in Table 2. While there was no significant change in median histoscore with transition to AI for Bcl-xL and Bcl-2, there was a trend towards a decrease in the expression of Bad (100–75 units, \( P = 0.068 \)) (Fig. 1) and Bax (70–55 units, \( P = 0.055 \)).

**TABLE 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, years</td>
<td>70.5 (66–74.25)</td>
</tr>
<tr>
<td>Gleason score (complete range 4–10) at diagnosis</td>
<td>8 (7–9)</td>
</tr>
<tr>
<td>at relapse†</td>
<td>9 (8–9)</td>
</tr>
<tr>
<td>PSA level, ng/mL</td>
<td></td>
</tr>
<tr>
<td>at diagnosis</td>
<td>46.50 (22.43–129.25)</td>
</tr>
<tr>
<td>at relapse</td>
<td>16.30 (6.33–39.60)</td>
</tr>
<tr>
<td>Time, years</td>
<td></td>
</tr>
<tr>
<td>to biochemical relapse</td>
<td>2.32 (1.47–4.67)</td>
</tr>
<tr>
<td>to death from relapse</td>
<td>1.39 (0.75–2.46)</td>
</tr>
<tr>
<td>Overall survival, years</td>
<td>4.39 (2.81–7.04)</td>
</tr>
<tr>
<td>Metastasis*, n (%)</td>
<td></td>
</tr>
<tr>
<td>at diagnosis</td>
<td>10 (17.24)</td>
</tr>
<tr>
<td>at relapse</td>
<td>30 (51.72)</td>
</tr>
</tbody>
</table>

*Wilcoxon signed-rank test; †trends.

**TABLE 2**

<table>
<thead>
<tr>
<th>Bcl-2 family members</th>
<th>AD</th>
<th>AI</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bad</td>
<td>100 (62–131)</td>
<td>75 (37–120)</td>
<td>0.068†</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>95 (69–155)</td>
<td>105 (63–125)</td>
<td>0.705</td>
</tr>
<tr>
<td>Bax</td>
<td>70 (24–140)</td>
<td>55 (28–93)</td>
<td>0.055†</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>120 (60–190)</td>
<td>120 (60–190)</td>
<td>0.842</td>
</tr>
</tbody>
</table>

*Wilcoxon signed-rank test; †trends.

There was a trend to a decrease in Bad expression when AD (a) progresses to AI disease (b) (\( P = 0.068 \)).

The influence of Bad expression in AD tumours upon time to relapse and overall survival appears to be a delayed effect. This was confirmed after repeated analysis using patients relapsing or surviving for >24 months only, with \( P < 0.001 \) and \( P = 0.031 \) for time to relapse and overall survival, respectively. The degree of Bad expression in AI tumours was not related to either time to death from relapse or overall survival (\( P = 0.695 \) and 0.638, respectively). A change in protein expression with progression to hormone-refractory disease was defined as the mean difference between the independent observers scores + 2 SD and on this basis, a change in Bad expression was determined to be 57 histoscore units. However, a change in Bad expression was not associated with time to biochemical relapse (\( P = 0.431 \)), time to death from biochemical relapse (\( P = 0.359 \)) or overall survival (\( P = 0.361 \)). There were no significant associations between expression of Bax, Bcl-2 and Bcl-xL and time to relapse, time to death from relapse or overall survival (data not given).

**DISCUSSION**

The association between Bad expression in AD disease and time to biochemical relapse was the most notable finding in this study, with patients expressing high levels of Bad at diagnosis relapsing after a period significantly longer than patients with low Bad expression (\( P = 0.007 \)). Furthermore, there is evidence that Bad expression at diagnosis is an independent determinant of time to relapse, as shown by the multivariate analysis (\( P = 0.033 \)). There was a trend towards improved overall survival in patients with
high Bad expression at diagnosis ($P = 0.054$), the lack of statistical significance in this instance might be partially explained by a lack of power owing to the few patients. Power calculations estimate that $n=104$ patients would be required to achieve a significant result in this instance. The patient cohort continues to expand and new samples can be stained for Bad in an attempt to strengthen existing results.

Although not statistically significant, high Bad expression in AD disease did confer a greater than median 2 years extended survival relative to low Bad expression. As the degree of Bad expression at diagnosis appears to influence clinical outcome, this protein might represent a possible positive prognostic indicator in prostate cancer. The present results confirm that Bad serves a protective role in AD disease, which would be expected as Bad is a pro-apoptotic protein capable of binding to and inhibiting anti-apoptotic Bcl-2 family members Bcl-xL and Bcl-2, thus enabling the release of cytochrome c from the mitochondria and subsequent activation of the apoptosis cascade [8–13]. High levels of Bad might increase apoptosis and slow down tumour growth in AD prostate tumours, thereby delaying time to biochemical relapse and death. Interestingly, these results are akin to those reported in a similar study of breast cancer tissue where patients with high Bad expression had a significantly improved disease-free survival compared with patients expressing low levels of Bad ($P = 0.049$) [16].

The present data suggests that the influence of Bad expression at diagnosis upon both time to relapse and overall survival is a delayed effect occurring after $\approx 24$–months. This observation might be explained by the effect of androgen-deprivation therapy, which achieves responses for up to 24 months as measured by decreasing PSA level. After this time the protective role of Bad might become more apparent as its effects are no longer masked by those of androgen-deprivation therapy subsequent to hormone escape. This observation is in agreement with that made in breast cancer, Cannings et al. [16] reported that their correlation with overexpression of Bad and disease-free survival was most significant after 3 years of tamoxifen treatment. They suggested that this is of current interest as it might influence decisions about switching from tamoxifen to aromatase inhibitors. This might also reflect the biological effect that androgen deprivation has on prostate cancer cells; it has been shown that long-term treatment with androgen-deprivation therapy alters expression of members of apoptotic pathways [17].

Molecular markers have the potential not only to act as prognostic markers, but also to contribute to new therapeutic strategies thereby providing possible targets for molecular-based intervention. BH3 mimetics are a new class of anticancer drugs, and one approach involves the use of compounds designed to mimic pro-apoptotic proteins and shaped to ‘fit into the groove’ of pro-survival proteins [18]. ABT-737 is a recently described BH3 mimetic that behaves like Bad and targets pro-survival Bcl-2, Bcl-xL and Bcl-w with high affinity, thus hindering their anti-apoptotic activity [19]. In prostate cancer specifically, adenoviral technology has been used to induce overexpression of Bad and Bax [20]. Given their pro-apoptotic role, levels of protective proteins Bad and Bax might be expected to decrease with disease progression, as in the present study, which reinforces the logic in replenishing diminished levels of these proteins in prostate cancer.

That Bcl-2 was not up-regulated with progression to AI was an unexpected finding, given the evidence supporting an increased expression in AIPC [21–24]. This has most probably resulted from a difference in sample size, with some previous studies using more tissue sections, e.g. Zellweger et al. [24] stained 181 localized prostate cancer and 120 AI sections reporting significant overexpression in the latter group. Some studies highlight Bcl-xL as the optimum target in prostate cancer cells [25] and it has remained uncertain as to the extent each of the anti-apoptotic proteins Bcl-xL and Bcl-2 serve functional roles in this disease, and with no significant results for either protein, that study was unable to clarify the matter. Both pro-survival proteins are located in the outer mitochondria, where they can hinder specific apoptotic stimuli, unless bound by Bad which heterodimerizes with either of Bcl-xL and Bcl-2 [8,9] and it is likely that the proteins act synergistically. There has been recent interest in use of antisense oligonucleotides to block the action of Bcl-2 or Bcl-xL [26–28].

Bad is phosphorylated by other proteins in addition to Akt, and it would therefore be of interest to investigate 90-kDa serine/threonine ribosomal S6 kinase (p90RSK or MAPK activated protein kinase 1) for example, which is a downstream effector in the MAPK-signalling cascade that maintains Bad in an inactive state by phosphorylation at Ser112, thus promoting cell survival [29,30]. Additionally, recent cell line work identified the Pac/PAK1 cascade as a parallel anti-apoptotic signalling pathway, mediating Bad phosphorylation at Ser136 [31].

Apoptotic and proliferative index studies are underway and it would be useful to assess whether expression of the Bcl-2 family members examined in the present study correlates with the degree of apoptosis or proliferation occurring within the tumour samples.

In summary, there is evidence to indicate a role for Bad in AD prostate cancer, with the degree of Bad expression at diagnosis
influencing time to biochemical relapse. There was a trend towards improved overall survival in patients with high Bad expression at diagnosis. In addition, there were trends towards a decrease in Bad and Bax expression with disease progression. It thus follows that Bad might represent a possible positive prognostic marker and useful therapeutic target in AIPC management in the future.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

None declared.

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Abbreviations: AI, AD, androgen-independent, -dependent; AIPC, AI prostate cancer; ICCC, inter-class correlation coefficients.