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Bad expression influences time to androgen escape in prostate cancer

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SUMMARY

Objective:
Androgen deprivation therapy is the treatment of choice in advanced prostate cancer, yet patients generally relapse and progress to an androgen independent state within 18-24 months. The PI3K/Akt pathway is known to represent one of a number of routes to hormone resistance and this study assessed 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).

Materials and Methods:
Immunohistochemistry was performed on matched hormone sensitive and hormone refractory tumours. Staining was scored by 2 independent observers using a weighted histoscore method. Change in Bad, Bax and Bcl-xL expression during transition to AIPC was evaluated and then correlated to known clinical parameters.

Results:
High Bad expression in androgen sensitive tumours was associated with increased time to biochemical relapse (p=0.0072) and a trend towards improved overall survival (p=0.0532) was observed. There were also trends towards a fall in Bad (p=0.068) and Bax (0.055) expression with progression to AIPC. No significant results were yielded 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 may therefore represent a possible positive prognostic marker and potential therapeutic target for AIPC in the future.
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 (ADT) 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 ADT 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 androgen independent state; second-line ADT, 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 androgen independence 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 may be identified, leading to the development of effective treatments for this patient group.

There is evidence to suggest that receptor tyrosine kinases (RTKs), such as EGFR and HER2, in the plasma membrane contribute to hormone escape in prostate cancer [5, 6]. The PI3K/Akt pathway is activated via RTKs in response to extracellular growth and survival factors and represents one of a number of independent routes to androgen escape.
in prostate cancer [7]. Having previously observed 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 which are important regulators of apoptosis. The proteins are divided into three groups according to their relative actions and the number of Bcl-homology (BH) domains present [8]. The pro-survival 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 have the ability to 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 which 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, which 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 sub-cellular 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, since most prostate tumours available for study only represent the disease at time of diagnosis, as it is not usual practice to take biopsies of recurrent disease [14]. However, this study measures the expression of selected Bcl-2 family members, namely Bad, Bax, Bcl-xL and Bcl-2, in paired androgen dependent and androgen independent tumour specimens, in order to further characterise the role of this protein family in the development of AIPC.
MATERIALS AND METHODS

Patient cohort

The patient cohort was established by retrospectively selecting prostate cancer patients who demonstrated an initial response to ADT, but subsequently relapsed with AIPC. This provided the following numbers of matched androgen dependent and androgen independent 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, however some pairs were lost due to insufficient tissue. Tumours classed as androgen dependent were obtained from patients diagnosed with locally advanced or metastatic prostate cancer who had received surgery, followed by conventional ADT. Inclusion criteria were applied according to Djavan et al. 2003 [15]. In order to meet the inclusion criteria a response to ADT had to be observed; defined as a fall in PSA levels by at least 50%, with a nadir being reached of less than 0.1 ng/ml. The androgen dependent 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 ADT and was defined clinically as 2 consecutive rises in PSA concentration greater than 10%, PSA levels had to rise to more than 0.4ng/ml to have clinically relevance and disease recurrence had to be within 6-49 months following surgery. Following identification of these patients, androgen independent tumour specimens were only available for analysis if additional surgery was required to treat the clinical symptoms of bladder outflow obstruction, and therefore androgen independent tumour samples were obtained only by TURP. In order to further confirm progression to hormone refractory disease proliferation index using MIB-1 Ki67
staining was calculated in the cohort, proliferation index significantly increased from 2.9 (1.2-6.1-) to 7.8 (2.7-15.8), p= 0.0001. Androgen receptor and PSA expression was observed in all hormone refractory 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 (MREC) for Scotland and relevant Local Research and Ethical Committees (LREC).

**Immunohistochemistry**

The specificity of antibodies used in this study was confirmed by western blotting. All immunohistochemistry (IHC) was performed on 5 µm, 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 performed by incubating sections in antigen unmasking solution at 96ºC for 25 minutes (Bad) or 40 minutes (Bax), or using heat treatment under pressure in a Tris EDTA Buffer (Bcl-xL and Bcl-2). Endogenous peptidase was destroyed by incubating sections in H₂O₂ at the following concentrations; 0.3% for 20 minutes (Bad), 1% for 10 minutes (Bax and Bcl-2), 3% for 10 minutes (Bcl-xL). Non-specific background staining was blocked using horse serum in TBS for 20 minutes. The following antibodies and dilutions were selected; Bad (CST#9292) at 1:25, Bcl-2 (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 4ºC, except those stained for
Bcl-2 which were incubated for 1 hour at room temperature. Staining was developed using the EnVision kit (DakoCytomation, Glostrup, Denmark), and chromagen was detected using DAB (Vector Labs, UK). A positive and negative control slide was included in each IHC run. Slides were counterstained in haematoxylin and Scotts tap water substitute, dehydrated through graded alcohols and xylene, and lastly mounted in DPX.

Scoring

Tissue staining intensity was scored blind by 2 independent observers using a weighted histoscore method. Histoscores were calculated from the sum of (1 x % cells staining weakly positive) + (2 x % cells staining moderately positive) + (3 x % 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.

Statistics

All statistical analyses were performed using SPSS version 11 for windows. Wilcoxon signed rank tests were used to compare expression between androgen dependent and androgen independent tumours. Survival analysis was conducted using the Kaplan Meier method and curves were compared using the log rank test. Multivariate analysis and hazard ratios were performed using Cox Regression analysis. A value of p<0.05 was considered statistically significant.
RESULTS

Patient characteristics

A total of 58 prostate cancer patients (diagnosed between 1984 and 2002) were included in this study with matched hormone sensitive and hormone refractory prostate tumours available for analysis (116 tumours in total). Patients in this cohort were diagnosed with locally advanced (48) or metastatic prostate cancer (10) and subsequently received surgery and ADT (18 orchidectomy, 36 GnRH analogues, 3 had both and 1 received anti-androgen therapy alone). In total 38 of the 58 patients received anti-androgen therapy. Forty-eight patients died during the course of follow-up, and 10 patients were alive at last follow-up. Thirty-three deaths (57%) were cancer-specific. Table 1 summarises the main patient characteristics.

Protein expression

Protein expression was observed within the cytoplasm as expected. The interclass correlation coefficient (ICCC) for each protein was greater than 0.7 and therefore classed as excellent. The median histoscores with inter-quartile range (IQR) for each protein in both androgen dependent and androgen independent tissue are given in table 2. While Bcl-xL and Bcl-2 demonstrated no significant change in median histoscore with transition to androgen independence, there was a trend towards a fall in expression of Bad (100 to 75 units, p=0.068) (figure 1) and Bax (70 to 55 units, p=0.055).
Protein expression and clinical outcome

When patients were divided into those with high or low levels of Bad expression (above or below the median histoscore), a high level of Bad expression at diagnosis (androgen dependent tissue) was associated with increased time to biochemical relapse ($p=0.0072$) (figure 2a). The median time to biochemical relapse for patients whose tumours demonstrated low levels of Bad expression was 2.17 (1.83-2.51) years, compared to 3.26 (0.90-5.62) years for patients whose tumours expressed high levels. On multivariate analysis when combined with Gleason grade at diagnosis, Bad expression was independently significant for time to relapse ($p=0.0325$ and hazard ratio 0.50 (0.26-0.94)). There was a trend towards improved overall survival associated with high Bad expression in hormone sensitive tissue ($p=0.0539$); the median survival of patients whose tumours showed a high degree of Bad expression was 6.57 (3.61-9.53) years, in comparison to 4.15 (2.18-6.12) years in patients with low levels of expression (figure 2b). The influence of Bad expression in androgen dependent tumours upon time to relapse and overall survival appears to be a delayed effect. This was confirmed following repeated analysis using patients relapsing or surviving beyond 24 months only, with $p$-values of 0.0001 and 0.0309 obtained for time to relapse and overall survival respectively.

The degree of Bad expression in androgen independent tumours was not related to either time to death from relapse or overall survival ($p=0.6946$ and 0.6376 respectively). A change in protein expression with progression to hormone refractory disease was defined as the mean difference between the independent observers scores + 2 standard deviations and on this basis, change in Bad expression was determined to be 57 histoscore units.
However, 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 androgen dependent disease and time to biochemical relapse was the most noteworthy finding in this study, with patients expressing high levels of Bad at diagnosis relapsing after a time period significantly longer than patients with low Bad expression (p=0.0072). Furthermore, there is evidence that Bad expression at diagnosis is an independent determinant of time to relapse, as demonstrated by multi-variant analysis (p=0.0325). A trend towards improved overall survival was observed in patients with high Bad at diagnosis (p=0.0537), the lack of statistical significance in this instance may be partially explained by a lack of power owing to the size of this patient cohort. Power calculations estimate that approximately 104 patients would be required to achieve a significant result in this instance. The patient cohort continues to be expanded and new samples can be stained for BAD in attempt to strengthen existing results.

Although not statistically significant, high Bad expression in androgen sensitive disease did confer a greater than median 2 year extended survival relative to low Bad expression. As the degree of Bad expression at diagnosis appears to influence clinical outcome, this protein may represent a possible positive prognostic indicator in this malignancy. The results confirm that Bad serves a protective role in hormone sensitive disease, which follows 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.
High levels of Bad may increase apoptosis and slow down tumour growth in hormone sensitive prostate tumours, thereby delaying time to biochemical relapse and death. Interestingly, these findings are akin to those found in a similar study of breast cancer tissue where patients with high Bad expression had a significantly improved disease-free survival compared to patients expressing low levels of Bad (p=0.049) [16].

The data here suggests that influence of Bad expression at diagnosis upon both time to relapse and overall survival is a delayed effect occurring after an approximate 24-month period. This observation may be explained by the effect of ADT therapy, which achieves response for up to 24 months as measured by falling PSA level. After this time the protective role of BAD may become more apparent as its effects are no longer masked by those of ADT subsequent to hormone escape. This observations is in agreement with that made in breast cancer, Cannings et al. observed that their correlation with over-expression of Bad and disease free survival was most significant after 3 years of tamoxifen treatment [16]. 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 demonstrated that long term treatment with ADT alters expression of members of apoptotic pathways [17].

Molecular markers have the potential not only to act as prognostic markers, but to contribute to new therapeutic strategies thereby providing possible targets for molecular based intervention. BH3 mimetics are a new class of anti-cancer 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 which 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 utilised to induce overexpression of Bad and Bax [20]. Given their pro-apoptotic role, levels of protective proteins Bad and Bax may be expected fall with disease progression, as demonstrated here, which reinforces the logic in replenishing diminished levels of these proteins in prostate cancer.

That Bcl-2 was not upregulated with progression to androgen independence was an unexpected finding, given the body of evidence supporting an increased expression in AIPC [21-24]. This has most likely resulted from a difference in sample size, with some previous studies utilising a larger number of tissue sections, for example Zellweger et al [24], stained 181 localised prostate cancer and 120 hormone refractory sections noting 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, this study was unable to clarify this 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 anti-sense 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 which 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 in the laboratory and it would be useful to assess whether expression of the Bcl-2 family members examined here 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 hormone sensitive 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, trends towards a fall in Bad and Bax expression with disease progression were noted. It thus follows that Bad may represent a possible positive prognostic marker and useful therapeutic target in AIPC management in the future.
Acknowledgements

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References


11. Chao OS, Clement MV. Epidermal growth factor and serum activate distinct pathways to inhibit the BH3 only protein in prostate carcinoma LNCaP cells. Oncogene 2006: 25: 4458-69


Figure Legends

Figure 1
Figures 1a and 1b visually demonstrate the trend to fall in Bad expression when androgen sensitive (1a) progresses to androgen insensitive disease (1b) observed in this study (p=0.068). Magnification x400.

Figure 2
Kaplan-Meier plots demonstrating increased time to biochemical relapse (2a) and trend towards improved overall survival (2b) in patients with high levels of Bad expression at diagnosis (androgen dependent tumour), compared to patients with low levels of expression (p=0.0539).
Table 1

Patient characteristics: age (years), Gleason score (complete range 4-10), PSA level (ng/ml), time to biochemical relapse, time to death from relapse and overall survival are shown as median and interquartile range. Metastasis is the number and percentage of patients with metastasis at diagnosis and relapse. Those with metastasis at diagnosis are included in the number of patients with metastasis at relapse. Note that as Gleason score is directly affected by ADT, the score at relapse may not necessarily reflect tumour biology.

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<tbody>
<tr>
<td>Age (years) at diagnosis</td>
<td>70.5 (66-74.25)</td>
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<tr>
<td>Gleason score at diagnosis</td>
<td>8 (7-9)</td>
</tr>
<tr>
<td>Gleason score at relapse</td>
<td>9 (8-9)</td>
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<tr>
<td>PSA level at diagnosis (ng/ml)</td>
<td>46.50 (22.43-129.25)</td>
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<tr>
<td>PSA level at relapse (ng/ml)</td>
<td>16.30 (6.33-39.60)</td>
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<tr>
<td>Time to biochemical relapse (years)</td>
<td>2.32 (1.47-4.67)</td>
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<tr>
<td>Time to death from relapse (years)</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 at diagnosis</td>
<td>10 (17.24%)</td>
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<tr>
<td>Metastasis at relapse</td>
<td>30 (51.72%)</td>
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</table>
Table 2

The median histoscore and inter-quartile range (IQR) for Bad and Bcl-xL expression in both androgen dependent and androgen independent tumour samples are shown. P-values derived from Wilcoxon signed rank tests are given and trends observed are marked with an asterisk*.

<table>
<thead>
<tr>
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<th>Androgen Dependent</th>
<th>Androgen Independent</th>
<th>P-value</th>
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<tr>
<td>Bad</td>
<td>100 (61.88-130.63)</td>
<td>75 (36.88-120)</td>
<td>0.068*</td>
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<tr>
<td>Bcl-xL</td>
<td>95 (68.75-155)</td>
<td>105 (62.50-125)</td>
<td>0.705</td>
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<tr>
<td>Bax</td>
<td>70 (23.75-140)</td>
<td>55 (27.50-92.5)</td>
<td>0.055*</td>
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<tr>
<td>Bcl-2</td>
<td>120 (60-190)</td>
<td>125 (50-180)</td>
<td>0.842</td>
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