Apoptosis-related Gene Expression in Benign Prostatic Hyperplasia and Prostate Carcinoma

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Abstract. Background: The aim of this study was to examine the expressions of the bcl-2, bax, fas and c-myc apoptosis-related genes in benign prostatic hyperplasia (BPH) and prostate carcinoma (CaP) to determine whether significant differences exist within each disease and between the two groups of patients. The correlation between gene expression and tumour diameter, stage, Gleason score and serum PSA was also investigated. Patients and Methods: Tissue specimens from 51 cases of BPH and 27 cases of CaP were examined for bcl-2, bax, fas and c-myc expression by reverse transcriptase-PCR (RT-PCR). Results: In BPH, bcl-2 and bax gave the weakest signals (p<0.001). In CaP, bcl-2 was the least expressed gene (p<0.001). In both patient groups, fas and c-myc were the most highly expressed genes (p<0.05). Both bcl-2 and bax were expressed at higher levels in CaP than in BPH (p<0.02). The bcl-2/bax ratio was lower in CaP than in BPH (p<0.001). Bcl-2 was more highly expressed in high Gleason grade (>7) tumours (p<0.05). In the BPH group, bax showed a positive relationship with fas (p<0.01), while the bcl-2 level inversely correlated with that of c-myc (p<0.05). Conclusion: Our data showed that all the apoptosis-related genes were expressed in both BPH and CaP. The stronger expression of bax and the lower bcl-2/bax ratio observed in CaP may suggest a pro-apoptotic stimulus, while the higher bcl-2 levels appear to counterbalance the tendency to cell death.

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Apoptosis is a genetically-regulated cellular suicide mechanism that plays a crucial role in development and in cellular homeostasis (1). The characterisation of the apoptotic patterns of prostate hyperplastic and cancer cells by analysing the gene expression which regulates programmed cell death may yield important prognostic information and provide new therapeutic targets, since the abnormal expression of such molecules can result in resistance to apoptosis (2, 3).

The bcl-2 gene family encodes a group of homologous proteins forming two functionally antagonistic groups that regulate distal and crucial commitment steps of the apoptotic pathway, often through protein-protein interaction. The bcl-2 gene encodes a 26 kDa protein, a potent blocker of apoptosis, expressed in several epithelial tissues (4). In the normal prostate, bcl-2 expression is limited to basal epithelial cells, which are resistant to the effects of androgen deprivation (5). In prostate tumours, overexpression of the bcl-2 protein has been associated with the development of hormone-refractory advanced disease, increased tumour stage and poor outcome (5, 6).

The pro-apoptotic bax protein also belongs to the bcl-2 family and resides in the cytosol or is loosely attached to cell membranes. In response to cytotoxic signals, bax translocates into the mitochondria, where it triggers cytochrome c release which can be blocked by bcl-2. Released cytochrome c then activates the pathway of caspases which eventually cause DNA fragmentation (7). While not dependent on each other for their individual functions, bcl-2 and bax do share homology and each may heterodimerise to antagonise their effect on each other (8). A discriminant role of the bcl-2/bax ratio as a predictive marker for response to chemotherapy has been suggested in
various human neoplasms (9, 10). In prostate cancer a reduced expression of \textit{bax} and a high \textit{bcl-2}/\textit{bax} ratio correlated with radiation therapy failure (11).

The \textit{fas} antigen (CD95) is a cell-surface receptor protein belonging to the tumour necrosis factor receptor superfamily (12). When the \textit{fas} ligand binds to the extracellular receptor domain it leads to the activation of the apoptotic signalling pathway in a variety of human tissues. Activation of the \textit{fas-fas} ligand pathway has been demonstrated to sensitise androgen-independent prostate cancer cells to chemotherapeutic drugs (13).

Molecules involved in cell-cycle regulation have also been intimately linked with apoptosis regulation. Among these the \textit{c-myc} proto-oncogene, a strong positive regulator of cell growth, has been found to be amplified in up to 72% of androgen-independent prostate cancers (14) and a role for its deregulated expression has recently been reported in the development of the androgen-independent state (15).

Apoptotic pathways have received much attention in the management of CaP, but there are limited data on the apoptosis-related processes that may govern benign prostatic hyperplasia (BPH). In a recent study, the expression pattern of \textit{bcl-2}, \textit{bax}, \textit{fas} and \textit{c-myc} genes was determined in tissue samples from 20 patients with BPH (16). Thus, in an effort to gain a deeper insight into the molecular mechanisms which influence the onset and progression of the two prostate proliferative diseases, the expressions of the above apoptosis-regulating genes were investigated by RT-PCR in both BPH and CaP specimens in order to determine whether significant differences exist between benign and malignant lesions. Moreover, the correlation of these biomarkers with prognostic variables, such as cancer diameter, stage, Gleason score and serum prostate-specific antigen (PSA) level, was evaluated.

### Patients and Methods

A total of 51 cases of BPH and 27 cases of CaP were examined. The specimens were obtained from the Department of Surgical Sciences, Division of Urology, Catholic University of the Sacred Heart, Rome; the Department of Surgical and Anesthesiological Sciences, Division of Urology, S. Orsola-Malpighi Hospital, University of Bologna; and from the Section of Urology, Department of Internal Medicine, Cardiovascular and Nephro-Urological Diseases, University of Palermo, Italy. The patient characteristics are reported in Table I.

The patients’ ages ranged from 52 to 89 years (average age 67.9) in the BPH group and from 57 to 82 years (average age 67.6) in the CaP group. BPH specimens were obtained from 34 patients undergoing open prostatectomy and from 17 patients undergoing transurethral resection of the prostate (TURP). CaP samples were obtained by radical prostatectomy from 23 patients, biopsy from three and TURP from one. The CaP patients had received no previous hormonal treatment or chemotherapy. All the prostate specimens were immediately frozen in liquid nitrogen following surgical removal and were stored at –80°C until use. The histopathology of the samples was determined and the prostate cancers were graded according to the Gleason system. The grades varied from 3 to 9, with a median of 7.

Reverse transcriptase-PCR (RT-PCR). Total RNA was prepared using TRIzol reagent (Life Technologies, Paisley, UK), according to the manufacturer’s protocol. First-strand cDNA was then synthesised from 1 μg of total RNA using 200 U Moloney murine leukaemia virus reverse transcriptase (Life Technologies), in a 20 μl reaction volume containing 10 mM dithiothreitol, 10 U RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), 1 mM dNTPs, 2.5 μM random hexamers. The reaction mixture also contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂.

<table>
<thead>
<tr>
<th>TM (fragment of mRNA encoding the trans-membrane region of FAS), F (forward), R (reverse).</th>
<th>\textit{Myc} F</th>
<th>\textit{Myc} R</th>
<th>\textit{Fas-TM} F</th>
<th>\textit{Fas-TM} R</th>
<th>\textit{Bax} F</th>
<th>\textit{Bax} R</th>
<th>\textit{Bcl-2} F</th>
<th>\textit{Bcl-2} R</th>
<th>\textit{Ald} F</th>
<th>\textit{Ald} R₁</th>
<th>\textit{Ald} R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Fas-TM} (170 bp)</td>
<td>5’-CAAGAGGGCAACACACACACGTGTCGACAC-3’</td>
<td>5’-CTGTTCCTCTCGTCGTTTCGACAC-3’</td>
<td>5’-GCACACTACCAAGACACACC-3’</td>
<td>5’-GGTTTTCCCTTTCTGTGCTGTC-3’</td>
<td>5’-ATGGAGGGTGCCGGGAGCACG-3’</td>
<td>5’-CCCAGGTGAAGTGGCCGTCAG-3’</td>
<td>5’-GGTGTCACCTGGTGTCACCTG-3’</td>
<td>5’-CTTCAGCTTGGTGCCGAGAAGATAG-3’</td>
<td>5’-CGCAGAAGGGGTGGTTCTGGTGA-3’</td>
<td>5’-CAGCTCTTCTTCTCTCGCTGGGG-3’</td>
<td>5’-GGTTTCCCTCGGTGTTCTCG-3’</td>
</tr>
</tbody>
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| Table I. Clinical characteristics of patients and histopathological details of samples used in the study. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BPH | CaP |
| Total patient no. | 51 | 27 |
| Mean age (years, range) | 67.9 (52-89) | 67.6 (57-82) |
| Biopsy | 3 |
| Open prostatectomy | 34 |
| Radical prostatectomy | 23 |
| TURP | 17 |
| Mean pre-operative PSA (ng/ml, range) | 5.81 (0.6-23.3) | 17.14 (0.13-78.62) |
| Tumour diameter (cm) | | |
| ≤1.5 | 7 |
| >1.5 | 18 |
| Tumour volume (cm³) | | |
| ≤1.76 | 8 |
| >1.76 | 19 |
| Stage: | | |
| T₁N₀M₀-T₂bNₓMₓ | 13 |
| T₃aN₀M₀-T₃bN₁Mₓ | 14 |
| Gleason score | | |
| (≤7) | 21 |
| (>7) | 5 |

BPM, benign prostatic hyperplasia; CaP, prostate carcinoma; TURP, transurethral resection of the prostate.
Each sample was incubated for 45 min at 45°C, followed by 10 min at 72°C. For the PCR reaction, 2 μl of each cDNA template solution was amplified using 10 pmol of each specific primer of the apoptosis-related genes combined with 1 pmol of aldolase (Ald) primers in a 25 μl reaction mixture. The primer sets are listed in Table II. In the co-amplification with bcl-2 (459 bp) and bax (323 bp), Ald1 and Ald2 primers were used while Ald1 and Ald3 primers (300 bp) were utilised in the co-amplification with c-myc (217 bp) and fas (149 bp) to distinguish the different products.

cDNA was amplified in a thermal cycle (Applied Biosystems, Foster City, USA) in a solution containing 1x PCR buffer (15 mM Tris-HCl, pH 8.0 and 50 mM KCl), 1.5 mM MgCl2, 100 μM dNTPs, the above-mentioned primers and 1.5 U of Taq polymerase (AmpliTaq Gold, Applied Biosystems). The PCR amplification was performed as follows: hot-start at 95°C for 7 min, followed by 28 cycles of amplification (denaturation: 45 sec at 94°C, annealing: 45 sec at 60°C, elongation: 45 sec at 72°C). A final extension was performed for 7 min at 72°C.

It is important to specify that preliminary experiments had been performed to determine the exponential phase of amplification for each marker and the optimal primer ratio. The optimal primer ratio between the different primer couples, used in amplifying Ald and the various specific markers, was 1:10.

The apoptosis-gene expression level was compared with the PCR product of the Ald gene co-amplified in the same reaction tube by using the primers listed in Table II.

Aliquots of the RT-PCR products were visualised after electrophoresis migration in a 1.8% ethidium bromide-stained agarose gel. The bands were viewed under UV light and analysed by densitometric analysis using the ImageMaster W DS gel analyser (Amersham Biosciences Europe, Freiburg, Germany).

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Semi-quantitative analysis of bcl-2, bax, fas and c-myc mRNA expression in tissue specimens from patients with BPH and CaP. The values represent the mean densitometric units of each gene normalised to the co-amplified Ald cDNA fragment ± SE.

\( p < 0.02 \) vs. CaP, \( p < 0.001 \) vs. CaP, Mann-Whitney U-test.

**Table III. Apoptosis-related gene expression in BPH and CaP.**

<table>
<thead>
<tr>
<th></th>
<th>bcl-2</th>
<th>bax</th>
<th>fas</th>
<th>c-myc</th>
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<tbody>
<tr>
<td>BPH</td>
<td>0.39 ± 0.04</td>
<td>0.38 ± 0.03</td>
<td>2.20 ± 0.14</td>
<td>2.38 ± 0.11</td>
</tr>
<tr>
<td>(range)</td>
<td>(range)</td>
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<td>(range)</td>
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</tr>
<tr>
<td>CaP</td>
<td>0.61 ± 0.11</td>
<td>1.37 ± 0.07</td>
<td>2.09 ± 0.26</td>
<td>2.19 ± 0.15</td>
</tr>
<tr>
<td>(range)</td>
<td>(range)</td>
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</table>

Statistical analysis. Differences in the mRNA expression of the apoptosis-related genes were assessed between the two patient groups as well as within the BPH or CaP populations using the Mann-Whitney U-test and Wilcoxon’s signed rank test, respectively. Probability values of \( p < 0.05 \) were considered statistically significant. Spearman’s non-parametric correlation test was applied to investigate the degree of correlation among the apoptosis-related gene mRNA levels, as well as those existing between the above genes and other prognostic variables such as the Gleason score, stage, tumour diameter and PSA.

**Results**

A total of 51 cases of BPH and 27 cases of CaP were entered into the study. The patients of the two groups were comparable with regard to age and, as expected, they differed for PSA levels, which were higher in the CaP than in the BPH group (\( p < 0.01 \), Table I).

The expressions of the bcl-2, bax, fas and c-myc apoptosis-related genes was studied in tissue specimens from CaP and BPH patients by RT-PCR. All four genes investigated were found to be expressed in BPH and CaP tissues, although at different levels. In the BPH group, bcl-2 and bax gave the weakest signals (\( p < 0.001 \)) and were expressed in 98% (50/51) and 92% (47/51) of the samples, respectively; fas

![Figure 1. Distribution of the apoptosis-related gene mRNA levels in patients with BPH and CaP analysed by RT-PCR. The levels of each apoptosis-related gene mRNA (in densitometric units) was normalised to Ald mRNA to yield relative densitometric units (R.D.U.). The horizontal bars represent the medians.](image-url)
and c-myc were expressed in 96% (49/51) and 98% (50/51) of BPH cases, respectively. In the CaP group, bcl-2 was the least expressed gene ($p<0.001$). In the latter group the four genes were expressed in 100% of the samples. In both patient groups, fas and c-myc were the most highly-expressed genes ($p<0.05$) (Figure 1, Table III).

Whether there were any statistically significant differences in the transcript levels of the above genes between the two clinically distinct groups was subsequently investigated. Bcl-2 was expressed at a higher level in the CaP than in the BPH group ($p<0.02$) and the same was true for the bax gene ($p<0.001$, Figure 2, Table III). The bcl-2/bax ratio was significantly lower in the CaP than in the BPH samples ($p<0.001$).

Bcl-2 was expressed to a lesser extent ($p<0.05$) in low- to medium-grade tumours (Gleason grade: ≤7) than in high-grade ones (Gleason grade: >7).

In the BPH group, when each biomarker expression was compared with that of the others and with serum PSA, bax showed a significant positive relationship with fas ($p<0.01$), while bcl-2 expression inversely correlated with that of c-myc ($p<0.05$). In CaP samples, no significant correlation was found when the expression of one gene was compared with that of the others or with the various clinicopathological parameters studied (tumour diameter, stage, Gleason score, serum PSA).

**Discussion**

Alterations in the expression of apoptosis-related genes can contribute to the origin of cancer and influence the response to therapeutic treatments.

Using RT-PCR, the expressions of the bcl-2, bax, fas and c-myc apoptosis-related genes in tissue samples obtained from BPH and CaP. To our knowledge, this is the first report in which the expressions of the above genes have been simultaneously investigated at the mRNA level by RT-PCR and one of the very few studies in which this methodology has been applied to prostate tissue samples. In the great majority of studies, the gene expression was evaluated by immunohistochemistry (5, 6, 9, 11).

Our findings clearly indicate that bcl-2, bax, fas and c-myc were expressed in all the BPH and CaP specimens analysed. Nevertheless, differences in gene transcript levels were observed within each patient population, as well as between the two groups. Bcl-2 and bax mRNAs generally gave the weakest signals, whereas in both patient groups, fas and c-myc were the most highly-expressed genes. In another series of BPH specimens obtained from 20 patients enrolled in a study performed in collaboration with Prof. A.V. Bono at the Division of Urology, Ospedale di Circolo e Fondazione Macchi, Varese, Italy, the bax expression did not significantly differ from that of fas and bcl-2 was the least expressed gene, while c-myc was the most abundant transcript (16). It is interesting to point out that a similar trend in gene expression has previously been demonstrated by our group in LNCaP androgen-sensitive prostate cancer cells, in which bcl-2, bax and c-myc expressions were investigated at both the mRNA and protein level (17); this finding suggests that, in hyperplastic or neoplastic lesions, apoptosis-related gene expression may generally be ascribed to the epithelial compartment of the gland.

The balance between promoting and inhibiting cell-death signals characterises normal cell growth. In this context, the interaction of the bcl-2 and bax genes was univocally described as the most important regulator of this relative equilibrium. It has commonly been reported that cells overexpressing bax show a higher susceptibility to apoptosis, while overexpression of the bcl-2 protein is mainly associated with tumorigenesis by enhancing the survival of otherwise doomed cells (8). Activation of the fas-fas ligand pathway also sensitises cells to apoptotic stimuli (13), while amplification of the c-myc proto-oncogene, a strong positive
regulator of cell growth, was found in the majority of androgen-independent CaP (15). Thus, the above pattern of gene expression observed within each patient group may reflect an attempt by the cells to counteract the mitogenic stimuli coming from anti-apoptotic genes such as bcl-2 and c-myc, with corresponding pro-apoptotic incentives coming from the bax and fas genes.

When the expression of each of the four genes was compared between the BPH and CaP samples, no statistically significant differences were observed in the transcript levels of fas or c-myc. This result was not completely unexpected as it has previously been reported by other authors for the same human pathologies, but using different techniques (18, 19).

In the present study, both bcl-2 and bax were more expressed in the CaP than in the BPH group, although bax showed a stronger signal than bcl-2 in the CaP samples.

Our data are in general agreement with those of other authors. Royuela et al. (20) hypothesised that an increased expression of both bcl-2 and bax in CaP compared to BPH, as determined by immunohistochemistry, might be involved in the higher apoptosis index reported in CaP samples. Moreover, a study by Zhou et al. (21) on an animal model demonstrated that both the bcl-2 and bax proteins were expressed more in androgen-insensitive CaP than in androgen-sensitive tumours, suggesting that both genes could play an antagonistic but important role in the progression of this pathology.

The stronger expression of bax compared to bcl-2 in CaP specimens may represent a driving stimulus toward apoptosis, which has also been described in other tumours (22). This hypothesis seems to be borne out by the lower bcl-2/bax ratio observed in the CaP group when compared to that of the BPH group. It is interesting to note that a low bcl-2/bax ratio assumes a positive predictive significance for the therapeutic response in CaP as well as in other malignancies (11, 23).

Although no statistically significant correlation was found between the gene expression and the various clinical variables studied (tumour diameter, stage, Gleason score, serum PSA) in the CaP group, bcl-2 showed a trend for a stronger expression in high-grade tumours than in low-grade ones. This finding is in complete agreement with the literature which reports an increase in the expression of anti-apoptotic members of the bcl-2 family during progression of CaP (5). Of interest were data coming from Spearman’s non-parametric analysis, which demonstrated a significant positive correlation between the two bax and fas pro-apoptotic genes in the BPH group, where an inverse relationship was found between bcl-2 and c-myc expression. This latter finding deserves further investigation in view of the intrinsic apoptotic properties that the c-myc oncogene has been shown to have, at least when cells expressing high c-myc levels are grown in a serum-poor environment or are arrested at various points in the cell cycle (24).

It is fully recognised that our study would have been more significant with the addition of a control arm. There is, however, no easy way to obtain normal prostate tissue. Even the prostates obtained through radical cystectomy for bladder cancer are likely to be affected by BPH or even CaP. It is also difficult to circumvent other possible causes of bias, such as lack of homogeneity of the tissues obtained by different procedures, namely needle biopsy, TURP or open surgery. Small specimens may not be representative of neighbouring tissues, particularly with regard to the Gleason grade. When the study was implemented, more accurate methods to obtain more homogeneous cell populations, such as laser-microdissection, were not available. We believe, however, that our results are valid to the extent that they show a statistically significant difference between certain groups or subgroups.

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