Abstract. Background: To determine whether an imbalanced interaction between proapototic and antiapoptotic signals may account for the loss of the normal cell growth control in benign prostatic hyperplasia (BPH), the expression of some apoptosis-regulating genes (bcl-2, bax, c-myc, fas) was investigated. Patients and Methods: BPH specimens were obtained from 20 patients who underwent trans-urethral resection of the prostate (TURP) or adenomectomy. Gene expression was studied by reverse transcriptase-polymerase chain reaction (RT-PCR) and its correlation with age and serum PSA level was also investigated. Results: Genes were found to be differentially expressed in BPH tissues. In particular, the antiapoptotic gene bcl-2, which was found in 18/20 samples, gave the weakest signal (p<0.05-p<0.001, Wilcoxon’s signed rank test), whereas the cell cycle regulator c-myc was detected in all the specimens and was the most highly expressed (p<0.001). A positive relationship between the expression of bcl-2 and that of the two proapoptotic genes bax and fas was observed (p<0.05, Spearman’s rank correlation test), as well as between c-myc and fas levels (p<0.005). Moreover, bax expression positively correlated with age and serum PSA level (p<0.02), which have also been shown to directly correlate (p<0.01). Conclusion: The higher expression of the oncogene c-myc suggests the activation of mitogenic signals within hyperplastic prostate tissue which a relatively high expression of the proapoptotic genes bax and fas fails to counterbalance.

Benign prostatic hyperplasia (BPH) is the most frequently occurring non-malignant urological disease among aging men, afflicting more than 40% of individuals over the age of 60 (1). In the pathogenesis of BPH, the unregulated proliferation of both the epithelial and stromal cell populations of the transition zone of the prostate is involved. An impairment of the delicate equilibrium between mitogenic and cell death-inducing stimuli is believed to be important in prostate tumorigenesis, but its role in the development of BPH is somewhat unclear (2, 3). Apoptosis, responsible for the programmed elimination of cells, is involved in physiological processes such as embryonic development and homeostatic maintenance of tissue and organs (4-6), being genetically controlled by a number of distinct death- or survival-related genes (2, 7). Since resistance to apoptosis can occur due to the abnormal expression of such genes (2, 7, 8), the characterisation of the apoptotic pattern of prostate hyperplastic cells may also yield useful information concerning the possible malignant transformation and may provide novel therapeutic targets.

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 (9). In the normal prostate, bcl-2 expression is limited to the basal cells of the glandular epithelium, which are resistant to the effects of androgen deprivation (10, 11). Bcl-2 protein is a potent repressor of apoptosis, contributing to the selection and maintenance of long-living cells (12). The proapoptotic bax protein resides in the cytosol or is loosely attached to cell membranes. In response to cytotoxic signals, it translocates into the mitochondria where it triggers cytochrome c release, which in turn activates the caspase-dependent DNA fragmentation (13). While not dependent on each other for their individual functions, bcl-2 and bax share homology and may heterodimerize to antagonize the effects of each other (14, 15).

The bcl-2/bax ratio has been considered to be an apoptotic index and it seems to be a main determinant of relative resistance to cell death-inducing stimuli (16).
The fas antigen (CD95) is a cell-surface receptor protein belonging to the tumour necrosis factor receptor superfamily (17, 18). Binding of the fas ligand (fasL) to the receptor extracellular domain leads to the activation of the apoptotic signalling pathway in a variety of human tissues. Some authors have reported that the expression of the soluble form of fas receptor, which down-regulates apoptosis by binding to fasL (19), might be considered a useful discriminator between BPH and prostate cancer (CaP). In fact, significantly lower levels of soluble fas were detected in BPH patient serum compared to CaP patient serum (20).

Molecules involved in cell-cycle regulation have also been intimately linked with apoptosis regulation. Among these, the c-myc protooncogene is a strong positive regulator of cell growth and its mutations represent common genetic alterations found in a wide variety of human cancers (21). c-myc expression has not been found in normal prostate, but it has been demonstrated in both epithelial and stromal cells of BPH, and is believed to be involved in the development of this pathology (22).

The study of the expression of apoptosis regulators in benign prostate disease would be of great interest both for a better understanding of the hyperplastic process and to establish whether the gene expression in BPH is different from that reported in the literature concerning CaP. For this reason, in the present study, the expressions of the apoptosis-regulating genes bcl-2, bax, fas and c-myc were investigated by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) in tissue samples obtained from human BPH and the correlation of these biomarkers with age and serum PSA level was evaluated.

### Patients and Methods

Twenty patients affected by BPH were enrolled in this study at the "Divisione di Urologia, Ospedale di Circolo e Fondazione Macchi, Varese, Italy". The criteria for patient selection were: obstructive prostate hyperplasia necessitating surgery; absence of clinical history of urinary retention; absence of positive urine-culture; absence of suspicion of CaP on digital rectal exam (DRE) and trans-rectal ultrasonography (TRUS); absence of previous hormonal treatments. The patient characteristics are reported in Table I. BPH samples were obtained from 20 patients who underwent trans-urethral resection of the prostate (TURP) or adenomectomy. Following surgical removal, the BPH specimens underwent trans-rectal resection (HGPIN/ASAP) or cancer, while the other was excluded high-grade prostate intraepithelial neoplasia/atypical small acinar proliferation (HGPIN/ASAP) or cancer, while the other was.

### RT-PCR analysis

Total RNA was prepared using TRIzol reagent (Life Technologies, Paisley, UK), according to the manufacturer’s protocol.

First-strand cDNA was then synthesized from 1 µg of total RNA using 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies), in a 20 µl volume reaction containing 10 mM dithiothreitol, 10 U RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), 1 mM dNTPs and 2.5 µl random hexamers. The reaction mixture also contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂. 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 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 coamplification with bcl-2 (500 bp) and bax (323 bp), Ald1 and Ald2 primers were used while Ald1 and Ald3 primers (300 bp) were utilized in the coamplification with c-myc (217 bp) and c-Fas (194 bp), to distinguish between 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.

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

### Table I. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (range)</th>
<th>PSA (ng/ml) (range)</th>
<th>Free/total PSA (median) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>69 (44-86)</td>
<td>1.8 (0.4-11.4)</td>
<td>0.19 (0.12-0.33)</td>
</tr>
</tbody>
</table>

### Table II. Oligonucleotide primer sequences.

<table>
<thead>
<tr>
<th>MYC F</th>
<th>5'-CAAGAGCGAACACAGACACGT-C3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYC R</td>
<td>5'-CTGTCTCCTGTTCCGCAAC-C3'</td>
</tr>
<tr>
<td>FAS-TM F</td>
<td>5'-GCACACTCAGCAGCAACCC-3'</td>
</tr>
<tr>
<td>FAS-TM R</td>
<td>5'-GTTTCTCCTTGCTGTTTC-3'</td>
</tr>
<tr>
<td>BAX F</td>
<td>5'-ATGGACGTCGGAGGCAGACG-3'</td>
</tr>
<tr>
<td>BAX R</td>
<td>5'-CCCAGTTGAAGTTGGCCGTACG-3'</td>
</tr>
<tr>
<td>BCL-2 F</td>
<td>5'-GGTGGCAACCTGTTGTCACCCTG-3'</td>
</tr>
<tr>
<td>BCL-2 R</td>
<td>5'-CTTACCTGTTGGCCCCAATAGGG-3'</td>
</tr>
<tr>
<td>Ald F</td>
<td>5'-GCAGAAGGGTGCTGTTGA-3'</td>
</tr>
<tr>
<td>Ald R₁</td>
<td>5'-CAAGCTTCTTCTCTGTCTCCGAGG-3'</td>
</tr>
<tr>
<td>Ald R₃</td>
<td>5'-GTGTCTCCTGTTGTTTCG-3'</td>
</tr>
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TM (fragment of mRNA encoding the transmembrane region of FAS), F (forward), R (reverse).
Aliquots of the RT-PCR products were visualized after electrophoresis migration in a 1.8% ethidium bromide-stained agarose gel. The bands were viewed under UV light and analyzed by densitometric analysis using the ImageMaster W DS gel analyzer (Amersham Biosciences Europe, Freiburg, Germany).

**Statistical analysis.** Differences in expression of the apoptosis-related gene mRNAs within BPH samples were assessed using Wilcoxon’s signed rank test. 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 age or serum PSA.

**Results**

The expression of the apoptosis-related genes bcl-2, bax, fas and c-myc was investigated by RT-PCR analysis in BPH specimens obtained from 20 patients who underwent TURP or adenomectomy. All the BPH samples examined were found to express bax, fas and c-myc, while the bcl-2 transcript was detected in 18/20 patients. Remarkable differences were observed among the four gene signals. The bcl-2 and c-myc transcript levels were found to be significantly different from those of the other genes (\( p < 0.05 \) to \( p < 0.001 \), Wilcoxon’s signed rank test). In particular, bcl-2 resulted the least expressed gene (\( p < 0.05 \) to \( p < 0.001 \), Figures 1 and 2, Table III), whereas c-myc was the most highly expressed (\( p < 0.001 \), Figures 1 and 2, Table III). No statistically significant differences were observed between bax and fas expression (Figures 1 and 2, Table III). The ratio between bcl-2 and bax was 0.56.

The Spearman’s rank correlation test demonstrated a significant positive relationship between the expression of bcl-2 and that of the two proapoptotic genes, bax and fas (\( p < 0.05 \)). C-myc and fas mRNA levels were also found to be positively correlated (\( p < 0.005 \)). No significant relationship was observed between c-myc and bcl-2 or bax gene expressions as well as between bax and fas levels. Moreover, a statistically significant positive association was observed between bax and age or PSA (\( p < 0.02 \)). These latter two parameters were positively associated (\( p < 0.01 \)).

**Discussion**

Apoptotic pathways have received much attention in the management of CaP, but little information exists about the key events that trigger cell death in BPH. The logical premise in developing new therapeutic strategies for this...
disease is the disclosure of the molecular mechanisms underlying prostate cell growth, so that proliferative markers can be identified.

This study aimed at gaining a better understanding of the behaviour of apoptosis-regulating genes in BPH, which might render them usable, not only as prognostic indicators, but also as potential therapeutic targets. The expression of bcl-2, bax, fas and c-myc in BPH samples was investigated by RT-PCR. To our knowledge, this is the first study in which this technique has been utilized to evaluate the expression of all the four genes in prostate tissue samples, since the great majority of inherent data derives from immunohistochemical analysis (23-27).

Our results indicated that bcl-2, bax, fas and c-myc were expressed in all the BPH samples. Nevertheless, significant differences were observed in their respective values. In particular, bcl-2 resulted the least expressed gene, whereas c-myc was the most highly expressed. This latter finding suggests the presence of a strong proliferative signal within hyperplastic prostate tissue, since little or no detectable c-myc transcript has been described in normal prostatic tissues (28).

It is interesting to point out that a similar trend of gene expression was observed by our group in another series of BPH samples obtained from 51 patients enrolled in a study in which apoptosis-regulating gene expression was also investigated in CaP samples (manuscript in preparation). Moreover, the same tendency was found by our group in the androgen-sensitive prostate cancer cells LNCaP, in which bcl-2, bax and c-myc expressions were investigated at both the mRNA and protein levels (29).

The bcl-2/bax ratio is a significant prognostic factor for many tumours and non-malignant proliferative diseases, and it is often considered a more reliable indicator of prognosis than the level of any one protein alone (30-34). Given the relatively low bcl-2 levels detected, the observed bcl-2/bax ratio value of 0.56 could be regarded as a positive feature of the disease. If we take into account CaP, a bcl-2/bax ratio higher than 1 is associated with significantly poorer response to radiotherapy and therapy failure (35). Similarly, Lohmann et al. (36) reported that a ratio less than 1 correlated with survival advantage in ovarian cancer patients.

The Spearman’s rank correlation test demonstrated a significant positive relationship between the expression of bcl-2 and that of the two proapoptotic genes bax and fas. Analogously, the c-myc mRNA levels were positively correlated with fas values. In accordance, Martin et al. (37) reported that, in human gliomas, the expression of the two opponent members of the bcl-2 gene family, bcl-2 and bax, rose in parallel in low-grade to high-grade tumours. This seems to suggest that apoptosis-promoting and -inhibiting genes are both deeply involved in the progression of such proliferative diseases, each counteracting the other. In the same context, the positive relationship observed between the death-inducer gene bax and PSA, a marker for increased crowding and growth of prostate cells, may take its place. Along with the correlation found between the above gene expression and age, it may represent an intriguing question that will need to be addressed in future investigations.

Serum PSA levels have also been found to be positively correlated with age, as previously reported by other authors in BPH as well as in early CaP (38, 39).

In conclusion, the current study suggests the existence of a strong mitogenic signal within hyperplastic prostate tissue, supported by the high expression of the c-myc gene. Nevertheless, apoptotic stimuli are also likely to be present in BPH, as testified by the expression of the bax and fas genes, by the low bcl-2/bax ratio and by the significant positive correlation found between growth-promoting and cell death-inducing genes. Whether the imbalanced expression of the above genes with opposing activity could play a leading role in the development of prostate hyperplasia remains to be established. In fact, it should be taken into account that, in this disease, the apoptotic/proliferative stimuli derive from heterogeneous sources such as oncogenes, cytokines and growth factors. Studies that attempt to correlate the expression of the genes investigated with the actual presence of apoptosis and/or with the expression of consolidate markers of proliferation are needed.

Acknowledgements

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References