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A Study of the Significance of Apoptosis and its Association with Abnormalities in Expression of BCL-2 Proto-Oncogene in Benign Nodular Hyperplasia of Prostate

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Abstract: Benign Prostatic Hyperplasia (BPH) is an enlargement of the prostate gland caused by an increase in the number of glandular units. Apoptosis is a programmed cell death necessary for the regulation of the size of organs in adult life. Disruption of apoptotic pathways has been suggested as an important regulatory mechanism in BPH. A high level of the BCL-2 protein suppresses apoptosis by preventing the activation of the enzymes that carry out the process. In this study, an attempt was made to observe the abnormal expression of BCL-2 protein in BPH tissues in paraffin sections and to demonstrate the disruption of apoptotic pathways in BPH. Prostatic tissue from 30 patients with BPH and no prior prostatic carcinoma were obtained by transurethral resection of prostate procedure. Apoptotic index was compared in the H and E sections. Expression of BCL-2 was analyzed by immunohistochemistry and evaluated. Apoptotic index in BPH tissues was found to be twice lower than that of normal tissues. Wilcoxon signed rank test was employed and the p-value proved that the results were highly significant ($p<0.01$). This data supported the research hypothesis that apoptotic index is decreased in benign prostatic hyperplasia. Out of 30 tissue samples, 20 (67%) shown positivity for BCL-2 expression. Kendall's Tau-B test was applied and the result showed negative correlation between the intensity of BCL-2 expression and apoptosis, however not significantly. This proves, the theory that BCL-2 regulates individual cell death up to a certain extent.

Key words: Apoptosis, mitosis, immunohistochemistry, BCL-2, benign prostatic hyperplasia

INTRODUCTION

Benign nodular hyperplasia of prostate is a slowly progressive abnormal enlargement of the prostate with heterogeneous morphology. It involves proliferation of glands and stroma. There is an increase in the size of the prostate gland that is so common as to be normal with advancing age and there is no malignancy present. It is also, referred to as Benign Prostatic Hyperplasia (BPH) (Berry *et al*., 1984).

The aetiology of this extremely common disease remains unknown. Much of the research on the origins of BPH have focused on defining relationship between the levels of male hormones or their metabolites and the onset of abnormal prostate growth (Trachtenberg *et al*., 1980).

The size of the prostate, like most organs in human body is maintained in delicate balance by apoptosis and proliferative activity. Therefore, disruption of the molecular mechanism that regulates these two processes may underline the abnormal growth of the gland leading to BPH (Kyprianou *et al*., 1996).

Apoptosis is a form of cell death designed to eliminate unwanted host cells through activation of coordinated, internally programmed series of events affected by a dedicated set of gene products (Cotran *et al*., 2004). It is controlled by a proto-oncogene, BCL-2. Expression of this gene inhibits apoptosis of the prostate cells.

The objectives of this study are to observe the abnormal expression of apoptosis regulatory protein BCL-2 in benign nodular hyperplasia of prostate in paraffin sections by immunohistochemistry and to demonstrate that the disruption of apoptotic pathways is an important regulatory mechanism in BPH by calculating the apoptotic index in the histopathological sections. The research hypothesis for this study is: apoptotic index is decreased in benign prostatic hyperplasia and over expression of BCL-2 is an indicator of suppressed apoptosis.

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MATERIALS AND METHODS

Chips of prostate tissue from 30 cases were obtained by the Transurethral Resection of Prostate (TURP) procedure, after prior informed consent. All patients were of Malaysian Origin and aged between 55 and 73 years old (64.67±5.24). The inclusion criteria were nodular hyperplasia per sec, which have been confirmed by high PSA levels and per rectal digital examination. Hyperplasia associated with inflammation, suggesting prostatitis and/or associated urinary tract infections and carcinoma prostate were excluded, so as to avoid deviation in the hypothesis of our study. The normal healthy tissues were obtained as comparison from the same patients. During the TURP, few chips of the healthy prostate tissue from the deeper zone were also removed. These chips of tissue, embedded in the same blocks as the abnormal ones, were used as the control in this project.

The bits of prostate tissue were fixed in formalin and processed through alcohol, acetone and xylene. Blocks of tissue were subsequently prepared using paraffin wax and sections of 4 μm on silanized slides were taken from the blocks using a rotary microtome.

For Haematoxylin and Eosin staining, the slides were dewaxed in a hot air oven at 56°C for 30 min followed by two changes of xylene for 1 min each and 3 changes of alcohol at 30 sec each. The slides were then immersed in tap water to render it aqueous. The slides were then stained with haematoxylin and eosin and mounted with coverslips using vectamount mounting media.

Immunohistochemistry staining: The slides were dewaxed and passed through xylene and decreasing concentrations of alcohol to water. Target retrieval was done by placing the sections in target retrieval solution at 90°C for about 40 min and then cooled to room temperature for another 10 min. The slides were rinsed with Tris Buffer Saline solution and soaked in it for 15 min. Dual endogenous enzyme block was applied on the specimens for 10 min. The slides were again rinsed with TBS and excess buffer was carefully wiped away.

Monoclonal mouse anti-human BCL-2 oncoprotein (primary antibody, DAKO, Bita Lifescience) was diluted in 1:25 ratio, as recommended in the immunohistochemistry staining guide. Antibody diluent with background reducing components solution was used as the diluent.

Substrate-chromogen used in this research was diluted in 1:40 ratio. Twenty microliter of DAB + chromogen was added to 1000 μL of DAB + substrate buffer and was shaken thoroughly.

The primary antibody, which was diluted earlier was applied into the sections and left for 2 h in room temperature. The slides were then rinsed again in TBS and excess buffer was carefully wiped dry. Labelled polymer HRP was applied on the slides for 30 min before soaking them in TBS for 5 min. Substrate chromogen which was prepared earlier was then applied into the slides and left in room temperature for 15 min. The slides were then carefully rinsed with distilled water and counterstained with haematoxylin and mounted with coverslip.

Analysis of studies: For the tissues stained in haematoxylin and eosin, apoptotic index was observed by counting the number of apoptotic and mitotic cells in glands and stroma of the tissues out of 1000 cells. The apoptotic cells were counted based on the morphology, such as cell shrinkage, chromatin condensation, formation of cytoplasmic blebs and phagocytosis of apoptotic bodies by adjacent macrophages or other neighbouring phagocytic cells. The mitotic cells were counted by observing the different phases-prophase, metaphase and apaphase. In the slides stained by immunohistochemistry, cells that expressed BCL-2 were stained brown. Grading of the staining was done to determine the strength of the BCL-2 expression. Grade 0 indicated negative staining (0% of the cells expressed BCL-2), grade 1 indicated <30% of the cells expressed BCL-2, grade 2 indicated 31-60% of the cells expressed BCL-2 and grade 3 indicated >60% of the cells expressed BCL-2.

Statistical analysis

Apoptotic index evaluation: The samples were divided into 4 groups. Group I was apoptotic cells in hyperplastic tissues, group II was apoptotic cells in normal tissues, group III was mitotic cells in hyperplastic tissues and group IV was mitotic cells in normal tissues.

Appropriate statistical tests were employed on the data using SPSS. A ($p<0.05$) was taken as significant. The parametric or non-parametric statistics were selected based on the distribution of the data.

Kolmogorov Smirnov test was employed to test the distribution of data. Between two groups (I vs. II, III vs. IV, I vs. III and II vs. IV), Wilcoxon rank test was employed on the ratio of number of observed cells to the total number of cells taken as 1000. Before statistical analysis, the missing values were treated by putting the lowest values in the ratios (Bolton *et al*., 2004).

BCL-2 evaluation: Kendall's Tau correlation was employed to test the association between the BCL-2 levels and the apoptosis in abnormal tissues.

RESULTS

Apoptotic index analysis: After haematoxylin and eosin staining was done, the cells were observed under microscope for apoptotic and mitotic cells (Fig. 1 and 2). The apoptotic and mitotic index were counted and the ratio of each cell was calculated (Table 1).

Kolgomorov Smirnov test was then applied to the results to test on the distribution of data (Table 2). The distribution of data based on p-value obtained from Kolgomorov Smirnov test indicated that all the data sets were not distributed normally except for the data for group II (apoptotic cells in normal tissues) (Table 2). Therefore, the comparison was noted by using Wilcoxon signed rank test (Table 3). As shown in Table 3, the apoptotic index in hyperplastic tissues was lower than that in the normal tissues. Apoptotic index in the normal tissues was 2 times higher than that in the hyperplastic tissues, which was highly significant ($p<0.01$).

The mitotic index in the hyperplastic tissues was three times higher, indicating an increased activity of mitosis compared with the normal tissues (0.0066 vs. 0.0013, which was highly significant as well ($p<0.01$).

In comparison between the apoptotic and mitotic indices in both hyperplastic tissues and the normal tissues, the results shown that there was a highly significant decrease in the apoptotic activity ($p<0.01$) and increase in mitotic activity in hyperplastic tissues ($p<0.01$). Whereas, the apoptotic index in normal tissues was noted to be significantly higher than the mitotic index.

The highly significant difference between group I vs. II and group II vs. IV indicates that there was a disruption in the normal regulation between the cell proliferation and cell death in benign prostatic hyperplasia.

After immunohistochemistry staining, the slides were analysed under microscope and the BCL-2 expression was graded (Janku *et al*., 2005) and were compared with the apoptotic indices (Table 4).

Out of 30 slides that were stained, 20 (67%) of them shown positivity (Fig. 3 and 4). Kendall Tau's B test was employed to test the association between the BCL-2 levels and the apoptosis in abnormal tissues. The result shows a negative correlation between the apoptotic indices and BCL-2 (-2.66). However, the relationship was not significant ($p = 0.083$).

Table 1: Result of apoptotic and mitotic index ratio

	Hyperplastic tissues (1000 cells)		Normal tissues (1000 cells)	
Slides No.	Apoptosis	Mitosis	Apoptosis	Mitosis
519	0.01	0.006	0.012	0.003
2953	0.002	0.005	0.007	0.002
266	0.001	0.004	0.006	0.001
2644	0	0.006	0.01	0
2677	0.002	0.003	0.001	0.001
267	0.001	0.006	0.00267	0.00267
601	0.002	0.005	0.006	0.001
409	0.002	0	0.003	0
355	0.001	0.003	0.002	0.001
209	0	0.006	0.003	0.001
245	0.002	0.008	0.001	0.002
522	0.001	0.009	0.002	0
2923	0	0.006	0.022	0.002
2919	0.001	0.004	0.003	0.001
2912	0.001	0.005	0.001	0.001
510	0	0.002	0	0.001
2941	0	0.007	0.0053	0
2910	0.002	0.015	0.004	0.001
101	0.002	0.007	0.005	0.001
2814	0.001	0.007	0.0075	0.0025
3122	0.003	0.007	0.007	0.002
3368	0.002	0.008	0.008	0.003
3942	0.005	0.015	0.008	0.005
4011	0.005	0.008	0.01	0.004
4137	0.006	0.011	0.007	0.001
4688	0	0.004	0.004	0.002
4595	0.002	0.005	0.0075	0
5103	0.002	0.007	0.0075	0
5168	0.003	0.008	0.005	0
5376	0.003	0.012	0.007	0.002

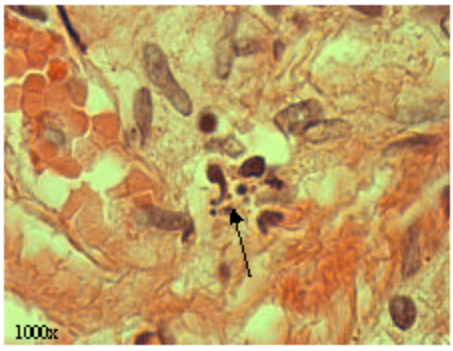

Fig. 1: Photomicrograph of benign prostatic hyperplasia, haematoxylin and eosin stained, showing apoptotic bodies (arrow) viewed under microscope at 1000× magnification

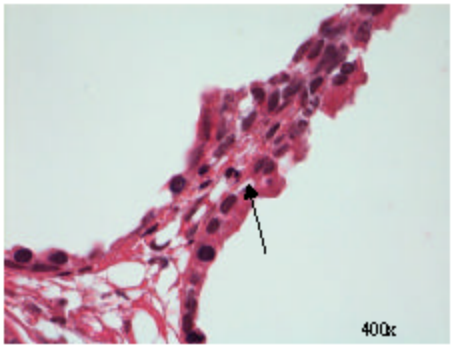

Fig. 2: Photomicrograph of benign prostatic hyperplasia gland, haematoxylin and eosin stained, showing apoptotic cells (arrow) viewed under microscope at 400× magnification

Table 2: Results of the Kolgomorov Smirnov test on distribution of data

Groups	p-value
I	0.00
II	0.20
III	0.02
IV	0.01

Table 3: Results of Wilcoxon rank test

Comparison between	Results	p-value
I vs. II	0.0020±0.0020↔0.0049±0.0030	0.000*
III vs. IV	0.0066±0.0033↔0.0013±0.0013	0.000*
I vs. III	0.0020±0.0021↔0.0066±0.0033	0.000*
II vs. IV	0.0040±0.0032↔0.0013±0.0130	0.000*

*Significant difference at p<0.01

Table 4: Comparison between the apoptotic indices with the level of BCL-2

Slides No.	Apoptotic index ratio in hyperplastic tissues	BCL-2 grade
519	0.010	0
2953	0.002	2
266	0.001	3
2644	0	3
2677	0.002	1
267	0.001	3
601	0.002	2
409	0.002	0
355	0.001	2
209	0	0
245	0.002	2
522	0.001	3
2923	0	3
2919	0.001	2
2912	0.001	3
510	0	0
2941	0	1
2910	0.002	1
101	0.002	2
2814	0.001	0
3122	0.003	0
3368	0.002	2
3942	0.005	2
4011	0.005	1
4137	0.006	1
4688	0	0
4595	0.002	0
5103	0.002	0
5168	0.003	0
5376	0.003	3

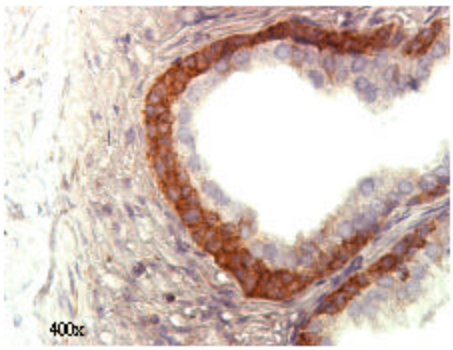

Fig. 3: Photomicrograph of benign prostatic hyperplasia, immunohistochemistry stained, BCL-2 positive, viewed at 400× magnification

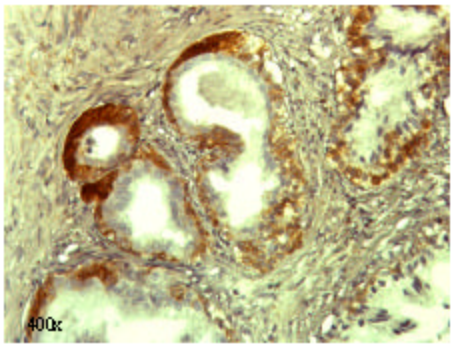

Fig. 4: Photomicrograph of benign prostatic hyperplasia, immunohistochemistry stained. Grade 3, BCL-2 positive, at 400× magnification

DISCUSSION

Apoptosis and benign prostatic hyperplasia: Benign prostatic hyperplasia is a non-malignant enlargement of the prostate that is due to excessive cellular growth of both the glandular and the stromal elements of the gland. The condition is a normal phenomenon and considered associated with ageing. Despite the disease being common, the aetiology of this disease is unknown. There are theories suggesting that ageing and hormonal factors are central to the development of BPH. Androgens, especially, Dihydrotestosterone (DHT), stimulate cell proliferation activity and inhibit cell death (Wright *et al*., 1996). In this research, we have focused on apoptosis and the factors affecting apoptosis.

Apoptosis is the molecular mechanism of physiologically relevant cell death in eukaryotic cells and is primarily responsible for the programmed elimination of cells and hence, the regulation of the size of the organ in adult life (Kyprianou *et al*., 1996). In every human being, about a hundred thousand cells are produced every second by mitosis and a similar number die by apoptosis. It is therefore, crucial that the balance between cell death and proliferation is tightly regulated (Jacobson *et al*., 1997).

In this study, we examined the incidence of apoptotic cells in human BPH, establishing its significance in regulating the dynamics of prostate growth and to examine the relationship between BCL-2 and its importance in suppressing apoptosis. Apoptotic index was counted by counting the number of apoptotic and mitotic cells in glands and stroma of the tissues out of 1000 cells. The present finding shows that apoptotic index in normal tissues of prostate was >2 times higher than that of the BPH tissues and the mitotic index of normal tissues was 3 times lower than that of the BPH tissues. This data supported the research hypothesis (apoptotic index is decreased in benign prostatic hyperplasia).

In normal prostatic tissues, apoptotic index (0.004) was found to be higher than mitotic index (0.0013). This low mitotic index results in the maintenance of the normal size of the prostate, which is consistent with the previous studies that documented low levels of cell proliferation in normal prostatic tissues (Kyprianou *et al*., 1996). However, in BPH tissues, the reverse was observed. Mitotic index (0.002) was found to be 3 times higher than the apoptotic index (0.0066), again confirming the research hypothesis.

The findings confirmed the theories that in benign prostatic hyperplasia, there was a resistance to apoptosis and that there was a decrease in apoptosis (Jefferson *et al*., 2000). Based on this, now focus can be done on the treatment for this disease using 5α-reductase, an enzyme that converts testosterone to its metabolite, DHT, which is an inhibitor for apoptosis (Thomas *et al*., 2000). In other studies Chon *et al*. (1999), pro-apoptotic agents like doxazosin and terazosin were found to increase the apoptotic index in both prostatic epithelial and stromal cells within the first month of the therapy.

BCL-2, apoptosis and benign prostatic hyperplasia: BCL-2 is a member of a large family of proteins that promotes apoptosis (bax, bad) and inhibits apoptosis (such as BCL-2 itself and BCL-xl). It acts by regulating the activation of casapaces.

Proapoptotic member of the BCL-2 family (bax) is strategically located on the outer mitochondrial membranes. It is believed to regulate the exit of cytochrome c from the mitochondrion to the cytoplasm by forming a channel in the mitochondrial membrane. BCL-2 blocks this channel-forming activity of bax. By blocking this channel, release of cytochrome c, which is the critical step in the chain of events that lead to apoptosis is prevented.

The proapoptotic and antiapoptotic members of BCL-2 family act as a rheostat in regulating programmed cell death. The ratio of death antagonist to agonist determines whether a cell will respond to apoptotic stimulus.

BPH seems to stem from the imbalance between the cell proliferation and cell death. In this study, slides of BPH tissues were stained using immunohistochemistry staining and the expression of BCL-2 was observed. Slides that were positive for BCL-2 expression were then graded (1 indicated that the glands shown <30% of BCL-2 expression, 2 indicated that the glands expressed 30-60% BCL-2 and 3 indicated that the glands shown >60% of BCL-2 expression) and those were negative for BCL-2 was marked as 0.

Studies have shown that 77.6% of the 30 samples was stained positive for BCL-2 expression. There was an increase in BCL-2 expression, which accounted for the decrease in the apoptotic index in a research done by (Colombel *et al*., 1998; Royuela *et al*., 2000) and in the present study, we found that out of 30 BPH tissue slides that we stained, 20 (67%) of them showed positivity for BCL-2 expression.

CONCLUSION

This study proves the theory that BCL-2 regulates cell death up to certain extent and also seemed to correlate well with the two other recent comparable studies. Kendall Tau-B test was applied to find any association between the intensity of BCL-2 expression with apoptosis and the result shown a significant correlation. This finding supported the theory that with an increase of BCL-2 level, there was a decrease in apoptosis, which eventually leads to enlargement of the organ.

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