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Assessment of proliferative index and its association with Ki-67 antigen molecule expression in nodular hyperplasia of prostate

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Abstract: The cytoplasmic expression of Ki-67, a nuclear protein that appears primarily during the proliferative phases of the cell cycle was studied in benign tumours of the prostate gland. Archival prostatic tissue from 39 patients with nodular hyperplasia and no prior or subsequent prostatic carcinoma that have been obtained through transurethral prostatectomy (TURP) procedure, were used in this study. The proliferative index was assessed by calculating the number of actively proliferating cells in the H&E sections in varied histologic patterns like hyperplastic epithelium, proliferating stroma, normal glands and normal stroma. The nuclear protein Ki-67 was analyzed by immunohistochemistry for determining the cytoplasmic positivity of the tumour cells. The proliferative index in the hyperplastic tissues was higher, indicating an increased activity of cellular proliferation, compared with the normal tissues, which was highly significant (p<0.01). Out of 39 cases of prostatic tissue, 25 (64 %) showed positivity for Ki-67 expression. Pearson's correlation test was applied to and showed significant association (p<0.05) between the intensity of Ki-67 expression with proliferative index. Comparisons of proliferative indices between the normal cells and tumour cells showed significant correlation, strongly suggesting the higher cell proliferation in the benign lesions. Enhanced expression of Ki-67 by the tumour cells suggests a growth imbalance in favour of cell proliferation that might ultimately promote prostatic hyperplasia.

Keywords: prostate, mitosis, Ki-67, nodular hyperplasia.

Introduction

Benign prostatic hyperplasia (BPH), a relatively common disease, is caused by the excess proliferation of prostatic stroma and glands. The resultant prostate gland enlargement is a cause of many lower urinary tract symptoms which decrease the quality of life, particulary for ageing males (>40 years) (Robbins & Cotran, 2005). It is believed that worldwide 30 million men have symptoms due to this benign enlargement (Barry & O'Leary,1995). The aetiology of this disease still remains unclear, though there is evidence pointing to the relationship between the levels of male hormones and their metabolites and the onset of prostatic hyperplasia (Trachtenberg *et al*., 1980). Like any other organ in the human body, the size of prostate is maintained by a delicate balance between apoptosis and mitosis. Therefore any imbalance of mitosis over apoptosis would lead to an increase in organ size (Kyprianou *et al*., 1996).

Ki-67 is a nuclear protein that is related to the proliferative phase of the cell cycle (Key *et al*., 1994). This protein is detected exclusively within the nucleus during the interphase (G0). However during mitosis it is relocated to the surface of chromosomes. It is present during all the active phases of the cell cycle (G1, S, G2 and M phases) but absent during G0. This makes it an excellent marker for the detection of cell proliferation (Schlüter *et al*., 1993). The over-expression of Ki-67 would therefore suggest an increase in mitotic activity of the prostatic cells.

The objectives of this study are to observe the cytoplasmic expression of the mitotic regulatory protein, Ki-67 in benign nodular hyperplasia of prostate in histopathological sections by immunohistochemical staining, and to demonstrate that the increase of cell proliferation activity is an important regulatory mechanism in benign tumours of prostate, by calculating the proliferative index in the histopathological sections. Subsequently, to compare Ki-67 index of hyperplastic tissues with that of the normal tissues to assess for the proliferative potential of the tumour.

Materials and methods

Archival paraffin-embedded prostatic tissue from 39 patients with nodular hyperplasia were obtained from Hospital Seremban after proper informed consent from the patients, and due approval from the IMU Joint Research and Ethical committee after prior submission of research protocol (Research grant I-01-2007/07). The tissue was obtained through transurethral prostatectomy (TURP) procedure that was done on the patients. All patients were of Malaysian origin, and aged between 55 and 73 years old (64.67 $\pm$ 5.24). The inclusion criteria were nodular hyperplasia *per se*, 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 the present 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, more peripheral zone were also removed and then embedded in the same blocks as the nodular areas. These tissues 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.

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For Haematoxylin & Eosin staining, the slides were dewaxed in a hot air oven at 59.5°C for 30 minutes followed by two changes of xylene for 1 minute each and 3 changes of alcohol at 30 seconds 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 for 45 minutes in a hot air oven at 59.5°C, and passed through xylene and decreasing concentrations of alcohol (abosolute alcohol, 90%, 80% 70%) to distilled water at 4 min intervals each. Target retrieval was done by placing the sections in target retrieval solution at 95°C for about 45 minutes in a hot water bath, and then cooled to room temperature for another 20 minutes. The slides were rinsed with Tris Buffer Saline solution and soaked in it for 10 minutes. Dual endogenous enzyme block was applied on the specimens for 20 minutes. The slides were again rinsed with TBS and excess buffer was carefully wiped off.

Monoclonal mouse anti-human ki-67 (primary antibody) (M724001, Clone MIB-1, 1 mL) purchased from DAKO Cytomation, Denmark (IHC Kit from Bita Lifescience, Malaysia), diluted in 1:50 ratio (as recommended), was incubated for 90 min at room temperature. Dako kit Antibody diluent with background reducing components solution (provided with primary antibody) was used as the diluent. Labelled polymer HRP was applied on the slides for 40 min before soaking them in TBS for 5 minutes. This was then rinsed with TBS and buffered for another 5 min. Substrate-chromogen, diluted in 1:50 ratio, (10µL of DAB+ chromogen was added to 500µL of DAB+ substrate buffer and was shaken thoroughly) was added at room temperature for 20 min. The slides were then carefully rinsed with distilled water, and counterstained with haematoxylin (2 minutes), and then rinsed 5 seconds with blueing agent, following that rinsed with water and mounted with coverslip with vecta mounting media after being air dryed.

Table 1. Proliferative index of normal and hyperplastic tissues and their relation to Ki-67 positivity

No.	Normal Tissue (1000 cells)		Proliferative Index (PI)	Hyperplastic tissue (1000cells)		Proliferative Index (PI)	Ki-67 positive
	Gland (500)	Stroma (500)		Gland (500)	Stroma (500)		
1	6	10	0.016	11	14	0.025	-ve
2	2	3	0.005	7	12	0.029	+ve
3	3	3	0.006	8	10	0.018	+ve
4	1	3	0.004	5	6	0.011	+ve
5	4	6	0.010	9	11	0.020	+ve
6	7	8	0.015	8	12	0.020	+ve
7	6	10	0.016	5	11	0.016	-ve
8	1	3	0.004	5	6	0.011	-ve
9	5	8	0.013	5	6	0.011	-ve
10.	4	5	0.009	6	5	0.011	+ve
11.	0	3	0.003	9	10	0.019	+ve
12.	1	2	0.003	4	7	0.011	-ve
13.	4	5	0.009	5	5	0.010	+ve
14.	6	5	0.011	5	4	0.009	-ve
15.	4	6	0.010	5	5	0.010	-ve
16.	2	1	0.003	1	3	0.004	-ve
17.	5	5	0.010	9	6	0.015	-ve
18.	4	6	0.010	7	17	0.023	+ve
19.	4	7	0.011	14	18	0.032	+ve
20.	2	6	0.008	16	5	0.021	+ve
21.	30	45	0.075	47	42	0.089	+ve
22.	9	12	0.021	14	18	0.032	+ve
23.	2	8	0.010	6	12	0.018	+ve
24.	4	14	0.018	8	18	0.026	+ve
25.	20	22	0.044	26	38	0.064	+ve
26.	11	20	0.031	15	20	0.035	+ve
27.	11	26	0.037	11	30	0.041	+ve
28.	4	4	0.008	2	6	0.008	+ve
29.	3	3	0.006	2	9	0.011	+ve
30.	4	3	0.007	3	1	0.004	-ve
31.	3	5	0.008	8	14	0.022	+ve
32.	7	9	0.016	12	15	0.027	+ve
33.	2	6	0.008	5	12	0.017	+ve
34.	0	1	0.001	2	4	0.006	-ve
35.	0	4	0.004	3	6	0.009	-ve
36.	3	7	0.010	4	9	0.013	-ve
37.	3	3	0.006	7	12	0.019	+ve
38.	6	5	0.011	8	10	0.018	+ve
39.	1	3	0.004	2	3	0.005	-ve

The mean PI for normal tissues is 0.013, and for hyperplastic tissues is 0.02

For the tissues that were stained with Haeamatoxylin and eosin, the proliferative index was calculated by observing the number of mitotic cells in the glands and stroma of tissues out of 1000 cells. The mitotic cells were recognized by of each of the various morphological features that the cell nuclei undergo during the various phases of mitosis. The counting of the mitotic cells was done for 500 cells for each group of glandular tissue and stromal tissue in the normal and hyperplastic tissue. The proliferative index (PI) was calculated as the number of mitotic activity in a total of 1000 cells.

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Statistical analysis

For the correlation between PI of normal tissue to Ki-67 positivity and PI of hyperplastic tissues to KI-67 positivity, Pearson's test and Wilcoxon rank test were employed. For analysing normal distribution of data Spearman's test and Kolgomorov Smirnov test were used. A $p > 0.05$ was taken as normal distribution of data. In this study, the following were compared:

I. Normal gland versus Hyperplastic gland
II. Normal stroma versus Hyperplastic stroma
III. Normal proliferative index versus Hyperplastic proliferative index

While correlation was determined between the following:

IV. The correlation between Hyperplastic proliferative index and Ki-67 positivity
V. The correlation between Normal proliferative index and Ki-67 positivity
VI. The correlation between Ki-67 positivity for various age groups

Table 3. PI Correlation of tissues to Ki-67 positivity

Correlation	R^2	P value	Significance
PI hyperplastic tissue to Ki-67 positivity	0.619	0.000	YES (p<0.05)
PI normal tissue to KI-67 positivity	0.253	0.119	NO (p>0.05)

For statistical analysis, the data was explored using Kolgomorov Smirnov test for distribution of the data. A $p > 0.05$ was taken as normal distribution of data. If data is normally distributed, pairseel test will be employed to compare studies I, II and III. Otherwise, Wilcoxon test will be used. A p value less than 0.05 was taken as significant.

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

Study	Z Value	P value Kolgomorov Simirnov Test of normality
I	-4.719	0.000
II	-4.520	0.000
III	-4.960	0.000

Results

The Proliferative index (PI) of normal and hyperplastic tissues and their relation to Ki-67 positivity is provided in Table 1. The mean PI for normal tissues is 0.013, and for hyperplastic tissues is 0.020. Results of the Kolgomorov Smirnov test on distribution of data (Table 2) showed that the distribution was skewed and not normally distributed. Thus, Wilcoxon rank test was used (Table 4) for comparison.

The Study I of PI between normal gland (0.010) versus that of the hyperplastic gland (0.017) was highly significant p=0.00 (p<0.01) (Table 3).

Study II, the difference of proliferative index between normal stroma (0.012) versus that of hyperplastic stroma (0.023) was highly significant p=0.00 (P<0.01), and significantly raised in older age groups, p=0.00 (p<0.05).

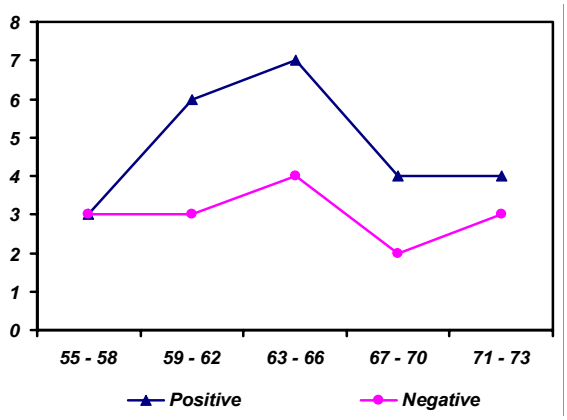
Study III, the difference between PI of hyperplastic tissue (0.020) and the PI of normal tissue (0.013) was highly significant, p=0.00 (p<0.05) and significantly raised in older age groups, p=0.01 (p<0.05).

Table 4. Results of Wilcoxon rank test

Study	P Value Wilcoxon Rank Test	Z Value
I	0.000*	-4.719
II	0.000*	-4.520
III	0.000*	-49.60

**Significant difference at p < 0.01*

Fig.1. Ki-67 positivity for various age groups



Correlation of Proliferative index to Ki-67 positivity

For the correlation between PI of normal tissue to Ki-67 positivity and PI of hyperplastic tissues to KI-67 positivity, Pearson's or Spearman's test was employed, respectivley depending upon whether data in distributed normally or not (Fig.1; Table 4). A p value less than 0.05 was taken as significant.

In this study, the data was not normally distributed; therefore Spearman's test was employed.

Spearman's correlation test indicated that PI of hyperplastic tissues (0.020) is significantly correlated to Ki-67 positivity while PI for normal tissue is not correlated.

Discussion

Proliferative index (Mitosis) and benign prostatic hyperplasia

Benign prostatic hyperplasia is due to excessive cellular growth of both the glandular and the stromal elements of the gland. 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). The present study has focused on mitosis and the factor related to mitosis (the nuclear protein Ki-67).

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).

The study examined the incidence of mitosis cells in human BPH, establishing its significance in regulating the dynamics of prostate growth. Also it examined the relationship between ki-67 and incidence of mitosis as proliferative index (PI). The present finding shows that the PI of the tumour was significantly higher than that of the normal tissues. Low PI in normal tissues results in the maintenance of the normal size of the prostate, which is consistent with previous studies (Kyprianou *et al*.,1996). However, in BPH tissues,

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the reverse was observed, with a significantly higher proliferative index, thus proving the resistance to apoptosis and increase in mitosis (Jefferson *et al*., 2000) that resulted in benign prostatic hyperplasia.

Ki-67, and its association with mitosis and benign prostatic hyperplasia

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 Ki-67 was observed (Fig. 2-4). Slides that were positive for Ki-67 expression were then marked as + and those were negative for Ki-67 was marked as 0. Interestingly, Tamboli *et al.* (1996) and Kyprianou *et al.* (1996) arrived with similar findings that the mean Ki-67 antigen expression significantly increases in hyperplastic glands when compared to normal.

We found that out of 39 BPH tissue stained by immunohistochemistry, 25 (64%) showed positivity for Ki-67 expression. There was significant correlation between the intensity of Ki-67 expression with mitosis. This proved our theory that Ki-67 regulates cell mitosis up to certain extent. Hence, the result present here supports the theory that with an increase of ki-67 level, there was an increase in mitosis, which eventually leads to enlargement of the organ.

Fig.2. Photomicrograph of benign prostatic hyperplasia tissue stained with immunohistochemistry for Ki-67, showing positive glandular staining at 400X (IHC with Ki-67, original magnification x 1000)

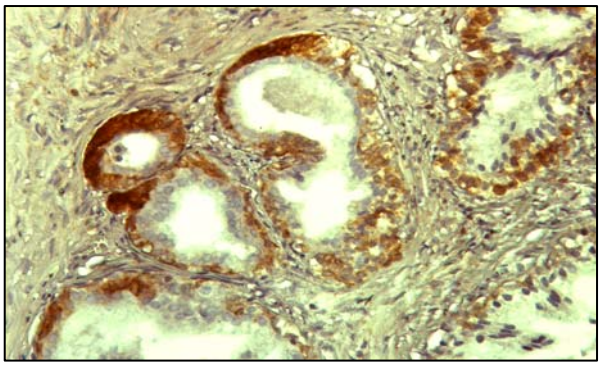


Fig. 3. Photomicrograph of diffuse glandular expression of Ki-67 (IHC with Ki-67, original magnification x 1000)

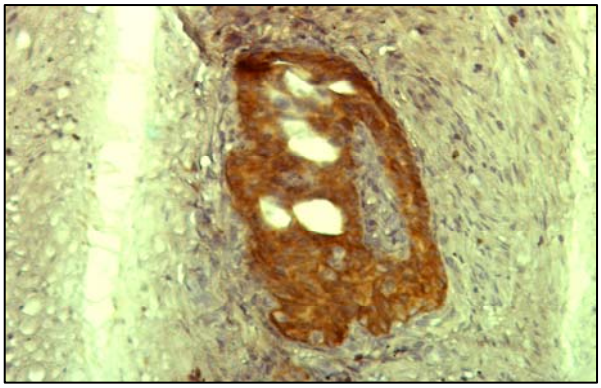
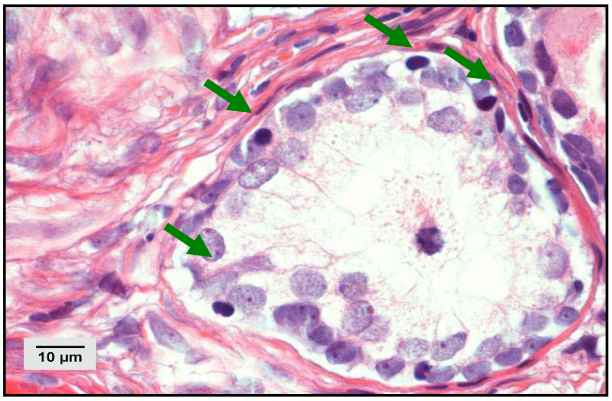


Fig.4. Photomicrograph of benign prostatic hyperplasia, showing cells undergoing mitosis (arrows) in the gland and stroma. (H&E, original magnification x 1000)



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