Original Article

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Diagnostic Utility of Proliferative Cell Markers in Prostatic Lesions: An Institutional Experience

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ABSTRACT

Background: Prostatic cancer is a complex and biologically heterogenous disease. Diagnosis of prostatic lesions with immunohistochemistry still faces challenges because of difference in reactivity of monoclonal antibodies in benign, equivocal, and malignant lesions. Proliferative markers Ki-67 and Proliferating Cell Nuclear Antigen (PCNA) can be used for diagnosis and prognostic stratification of prostatic carcinoma.

Methods: A total of hundred prostate biopsies with 50 cases each of benign prostatic hyperplasia and prostate carcinoma received in the Department of Pathology, PGIMS, Rohtak, India were included in the study. Ki-67 and PCNA expression was studied immunohistochemically in each case.

Result: Ki-67 expression was significantly upregulated in malignant cases and increased with increasing Gleason grade. PCNA expression was also found to be increased in increasing Gleason grades in carcinoma cases, however, the results were ambiguous as it was found to be positive in all benign cases also.

Conclusion: Ki-67 is useful in predicting biological behavior in prostate carcinoma cases, however PCNA expression needs to be studied further.

Keywords: *Benign Prostatic Hyperplasia, Ki-67, Proliferating Cell Nuclear Antigen, Prostate Carcinoma*

Introduction

Prostate cancer (PCa) is the second most prevalent cancer in men. Thus, screening, and early detection becomes important to help in detection of curable disease. Early detection of cancer in the younger age group results in a better cure of the disease as these patients have less comorbidities and can readily opt for surgical treatment options. [1]

Implementation of various histopathological and molecular markers is required for better stratification of prostate cancer cases. Molecular markers reflecting tumor characteristics, progression and biology could therefore, act as a novel threshold in active surveillance and watchful waiting. A major problem with prostate biopsies is that they only sample about 0.05% to 0.5% of the total prostatic volume, which results in under-sampling of the most significant areas of cancer tissue. Therefore, along with improved image-guided biopsy procedures, implementing these novel molecular markers might help in predicting the presence of unsampled significant areas in case molecular aberrations are preceded by pathologically recognizable patterns. [2] Despite the available grading systems, diagnosis of prostate cancer remains a challenge on hematoxylin and eosin staining in cases of pre-malignant lesions like prostatic intra-epithelial neoplasia and atypical adenomatous hyperplasia. To combat this diagnostic dilemma, several immunohistochemistry markers have been used to differentiate benign and malignant prostatic lesions. Loss of basal cell layer in malignant cases and its presence in the benign lesions has been exploited as the basis for use in immunohistochemistry. Basal cell markers like p63 and high molecular weight cytokeratin are used. The proliferative activity in the cells also signify the nature of the lesion. Proliferative markers like Ki-67 and proliferating cell nuclear antigen (PCNA) are of great significance in this regard. [3]

Ki-67 is a nuclear antigen associated with proliferation of cells and is expressed in G1, S, G2 and M phases of the cell cycle but not in the resting cells of the G0 phase. Ki-67 has been shown to have a role in early and accurate detection of prostate cancer and is an independent prognostic factor in prostate cancer. [4,5]

Proliferating cell nuclear antigen (PCNA), also called cyclin, is an acidic nuclear protein and is a marker of G1/S phase of the cell cycle. Expression of PCNA depends on the phase of the cell cycle. As compared to G0 levels, PCNA increases in late G1 phase, and it increases even further in S phase, and declines back in G2/M phase. PCNA expression is thus associated with the proliferative state of the cell. [4]

Several studies have been conducted which concluded that Ki-67 is a useful prognostic biomarker and had shown

statistically significant correlation between Ki-67 and the increasing Gleason grade. [6,7,8] Also, it was observed that cases with higher Gleason grade had a higher PCNA value. [3,8,9]

Various studies done in the past signify the role of Ki-67 and PCNA as diagnostic and prognostic markers, making the basis of our study to analyze their role in benign and malignant lesions of prostate and to correlate their expression with various clinicopathological parameters.

Materials and Methods

A total of hundred prostatic biopsies, with fifty cases each of BPH and prostate carcinoma were included in the study, conducted in Department of Pathology, Pt. B.D. Sharma, PGIMS, Rohtak. Tissue was fixed in 10% formalin and processed for histopathological examination. Histopathological diagnosis as benign prostatic hyperplasia (BPH), equivocal or prostatic carcinoma was established on routine hematoxylin and eosin staining. Special histochemical stains were applied wherever necessary. Carcinoma cases were further scored as per Gleason scores by two independent observers. [10]

Based on the Gleason scores, histopathological grading was done as per **the new 5 grade group system** which is as follows: [11]

Grade Group 1 (Gleason score ≤6)	Only individual discrete well-formed glands
Grade Group 2 (Gleason score 3+4=7)	Predominantly well-formed glands with a lesser component of poorly formed/fused/cribriform glands
Grade Group 3 (Gleason score 4+3=7)	Predominantly poorly-formed/fused/cribriform glands with a lesser component of well-formed glands
Grade Group 4 (Gleason score 8)	Only poorly formed/fused/cribriform glands or predominantly well-formed glands with a lesser component lacking glands or predominantly lacking glands with a lesser component of well-formed glands
Grade Group 5 (Gleason scores 9-10)	Lacks gland formation (or with necrosis) with or without poorly formed/fused/cribriform glands

Number of cores received per biopsy and percentage of core involved by the tumor were noted in carcinoma cases. Areas of perineural invasion as well as presence of prostatic intraepithelial neoplasia (PIN), were also reported.

Representative sections from each case were subjected to immunohistochemistry for Ki-67 using Rabbit monoclonal antibody by Bio SB inc and PCNA using Mouse monoclonal antibody by Dako. Antigen retrieval was done using fully automated system- Dako PT Link. Specimens from tonsil served as positive control for Ki-67 and human lymph node with germinal centers as positive control for PCNA. Negative control was obtained by substituting the primary antibody with antibody of non-specific relevance. [12,13]

Both for Ki-67 and PCNA, the slides were scanned at 40x to look for yellow or brown color staining which was interpreted as positive nuclear staining. A magnification of 100x was used to confirm that the positive staining was in the area of interest (benign glands in BPH and malignant cells in PCa). Magnification of 400x was used to count the percentage of positively stained cells among the cells of interest. Counting was done in the highest density area of the positive staining.

Ki-67 Score was derived by counting the total number of positively stained nuclei and expressing it as a percentage of total cells counted.[12] **Ki-67 score** was then grouped into four categories: Negative: No nuclear positivity seen; <5% : Less than 5% of the total cells counted showed nuclear positivity; 5-10% : 5-10% of the total cells counted showed nuclear positivity; >10% : More than 10% of the total cells counted showed positive nuclear staining. For PCNA, similar method of counting the number of positively stained nuclei and expressing it as a percentage of the total cells counted was used. **PCNA scores** were grouped as follows:[14] <33%: less than 33% of the total cells counted show positive staining; 33-66%: Out of the total cells counted, 33-66% show positive nuclear staining; >66%: More than 66% cells show positive nuclear staining. PCNA was further categorized as per the intensity of staining as weak, weak-moderate, moderate, moderate-strong, and strong.

Both the observers independently scored the cases for Ki-67 score and PCNA scoring, while being blinded to the clinicopathologic data of the cases.

Ki-67 and PCNA immunohistochemical expression was assessed in BPH and in distinct categories of carcinoma cases. The expression of these markers was also correlated with various clinico-pathological parameters like age, PSA levels, percentage of cores involved and Gleason grade groups in carcinoma cases.

The data was compiled, tabulated, and analyzed using SPSS 20.0 software. The statistical tests applied for analysis were percentages, proportions, and chi square test.

All procedures performed in the current study were

approved by Institutional Ethics Committee (No: IEC/Th/17/Patho/06, Dated 30.11.17) in accordance with the 1964 Helsinki Declaration and its later amendments.

Informed consent was obtained from all individual participants included in the study.

Result

In the present study, maximum number of cancer cases were seen in the older **age groups**, i.e., 61-90 years (78%), whereas majority of BPH cases were in the younger age group of 41-70 years (76%). This observation was found to be statistically significant. Low serum **PSA levels** of <10 ng/mL were found in around 80% of the BPH cases, whereas only 5% prostate cancer cases had a serum PSA value of less than10 ng/mL. In cancer patients, 33% cases had serum PSA levels of >100 ng/ mL while only 5% of BPH cases had PSA levels of >100 ng/mL. The difference in serum PSA levels between benign and malignant cases was found to be statistically significant. 78% of BPH cases were TURP specimens, while 72% of the carcinoma cases were reported in prostate needle biopsies.

Gleason Score: Maximum number (60%) of cases of prostate cancer were scored with Gleason score 4+3=7 and 8. The variability between the Gleason scoring by the 2 observers was seen between the Gleason score 4+3=7, 8 and 9. No variability was seen in scores 6 and 10. The interobserver agreement of both observers was excellent (Kappa= 0.910).

Gleason Grade: The interobserver agreement was noted to be excellent with a kappa value of 0.866. Maximum number of (60%) prostate cancer cases were reported as Gleason grade III and IV. Variability in results of the 2 observers was noticed in grade groups III, IV and to a lesser extent in grade group V. No interobserver variability was seen in Grade groups I and II. (Figure 1)

Ki-67 Expression: All BPH cases showed negative staining with Ki-67. Foci of PIN also showed negative Ki-67 staining. All the carcinoma cases revealed positive Ki-67 expression. There was no interobserver variation (kappa=1.0). (Figure 2,3, Table 1)

Ki-67 Expression and correlation with clinicopathological parameters: Ki-67 expression was seen in variable intensity in different age groups. No statistically significant correlation of Ki-67 was observed with age, PSA levels and percentage of core involved.

Ki-67 expression was correlated with **Gleason grade groups in our study**. Ki-67 expression was observed to be increased with increasing grade by both observers and also found to be statistically significant by observer 1. (Table 2)

PCNA expression: All benign and carcinoma cases revealed positive PCNA expression. (Figure 2,3, Table 3)

PCNA Expression and correlation with clinicopathological parameters: PCNA expression was seen in variable intensity in different age groups. No statistically significant correlation of PCNA was observed with age, serum PSA levels and percentage of core involved.

The PCNA expression as well as intensity increased with advancing Gleason grade groups as per findings of both observers and were found to be statistically significant with grade by observer 1 and with intensity by both observers. (Table 4)

Table 1: Comparison of Ki-67 expression in BPH and PCa cases (n=100).

Ki-67 Score	Observer 1		Observer 2	
	BPH (%)	PCa (%)	BPH (%)	PCa (%)
Negative	50 (100)	0 (0)	50 (100)	0 (0)
<5%	0 (0)	32 (64)	0 (0)	32 (64)
5-10%	0 (0)	8 (16)	0 (0)	9 (18)
>10%	0 (0)	10 (20)	0 (0)	9 (18)
Total	**50**	**50**	**50**	**50**

Table 2: Correlation of Ki-67 expression with clinicopathological parameters of prostate cancer.

Parameter <5%		Ki-67 score Observer 1			P Value	Ki-67 score Observer 2			P Value
		5-10%	>10%		<5%	5-10%	>10%		
Age	<50 yrs	2	0	1	0.461	2	0	1	0.331
	51-60 yrs	5	2	1		4	3	1	
	61-70 yrs	8	2	3		8	2	3	

Parameter <5%		Ki-67 score Observer 1			P Value	Ki-67 score Observer 2			P Value
		5-10%	>10%		<5%	5-10%	>10%		
	71-80 yrs	11	2	3	0.461	11	2	3	0.331
	81-90 yrs	5	2	2		6	2	1	
	>90	1	0	0		1	0	0	
PSA	<10	0	0	2	0.223	1	0	1	0.345
	11-100	20	3	3		20	3	3	
	>100	9	3	2		8	4	2	
% Core	<20%	0	0	1	0.177	0	0	1	0.421
	21-40%	3	3	1		3	3	1	
	41-60%	15	2	5		15	2	5	
	61-80%	14	3	3		14	4	2	
	81-100%	0	0	0		0	0	0	
GG	I	1	0	0	0.045	1	0	0	0.204
	II	8	0	0		7	1	0	
	III	12	0	4		11	0	2	
	IV	7	5	2		8	5	3	
	V	4	3	4		5	3	4	

Table 3: Comparison of PCNA expression in BPH and PCa cases (n=100).

PCNA Score	Observer 1		Observer 2	
	BPH (%)	PCa (%)	BPH (%)	PCa (%)
<33%	0 (0)	7 (14)	0 (0)	9 (18)
33-66%	0 (0)	12 (24)	0 (0)	10 (20)
>66%	50 (100)	31 (62)	50 (100)	31 (62)
Total	**50**	**50**	**50**	**50**

Table 4: Correlation of PCNA expression with clinicopathological parameters of prostate cancer.

Parameters <33%		PCNA score Observer 1			P value	PCNA score Observer 2			P Value
		33-66%	>66%		<33%	33-66%	>66%		
Age	<50 yrs.	1	0	2	0.123	1	0	2	0.066
	51-60 yrs.	2	1	5		3	0	5	
	61-70 yrs.	0	4	9		1	3	9	
	71-80 yrs.	4	3	9		4	3	9	
	81-90 yrs.	0	4	5		0	4	5	
	>90	0	0	1		0	0	1	
PSA	<10	0	1	1	0.464	0	1	1	0.219
	11-100	3	7	16		4	6	16	
	>100	3	1	10		4	0	10	

Parameters <33%		PCNA score Observer 1			P value	PCNA score Observer 2			P Value
		33-66%	>66%		<33%	33-66%	>66%		
% Core	<20%	0	0	1		0	0	1	
	21-40%	0	2	5		2	1	4	
	41-60%	3	8	11	0.421	3	7	12	0.622
	61-80%	4	2	14		4	2	14	
	81-100%	0	0	0		0	0	0	
GG	I	1	0	0		1	0	0	
	II	3	2	3		1	3	4	
	III	2	4	10	0.05	3	4	6	0.107
	IV	1	5	8		4	2	10	
	V	0	1	10		0	1	11	

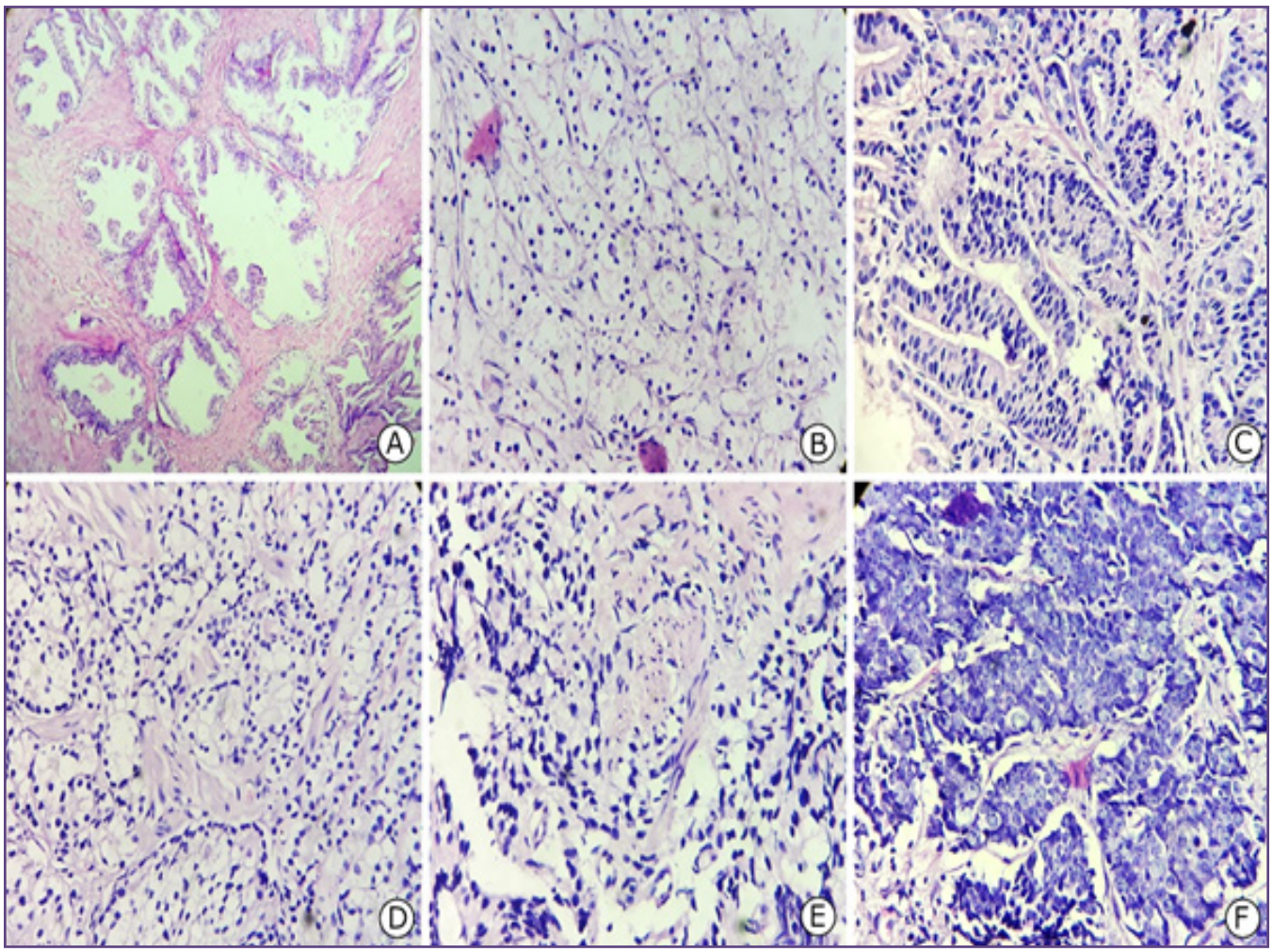

Fig. 1: Hematoxylin and Eosin (H&E) sections at 400x; A- BPH, B- Prostate carcinoma Gleason grade group 1, C- Prostate carcinoma Gleason grade group 2, D- Prostate carcinoma Gleason grade group 3, E- Prostate carcinoma Gleason grade group 4, F- Prostate carcinoma Gleason grade group 5.

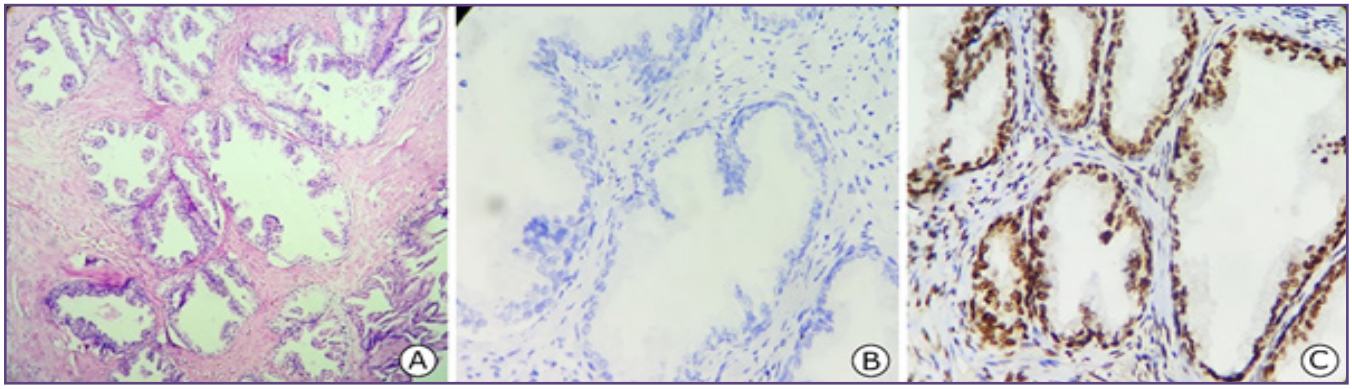

Fig. 2: A- H&E section of BPH at 400x, B- Negative Ki-67 expression in BPH (400x), C- PCNA expression >66% in BPH at 400x.

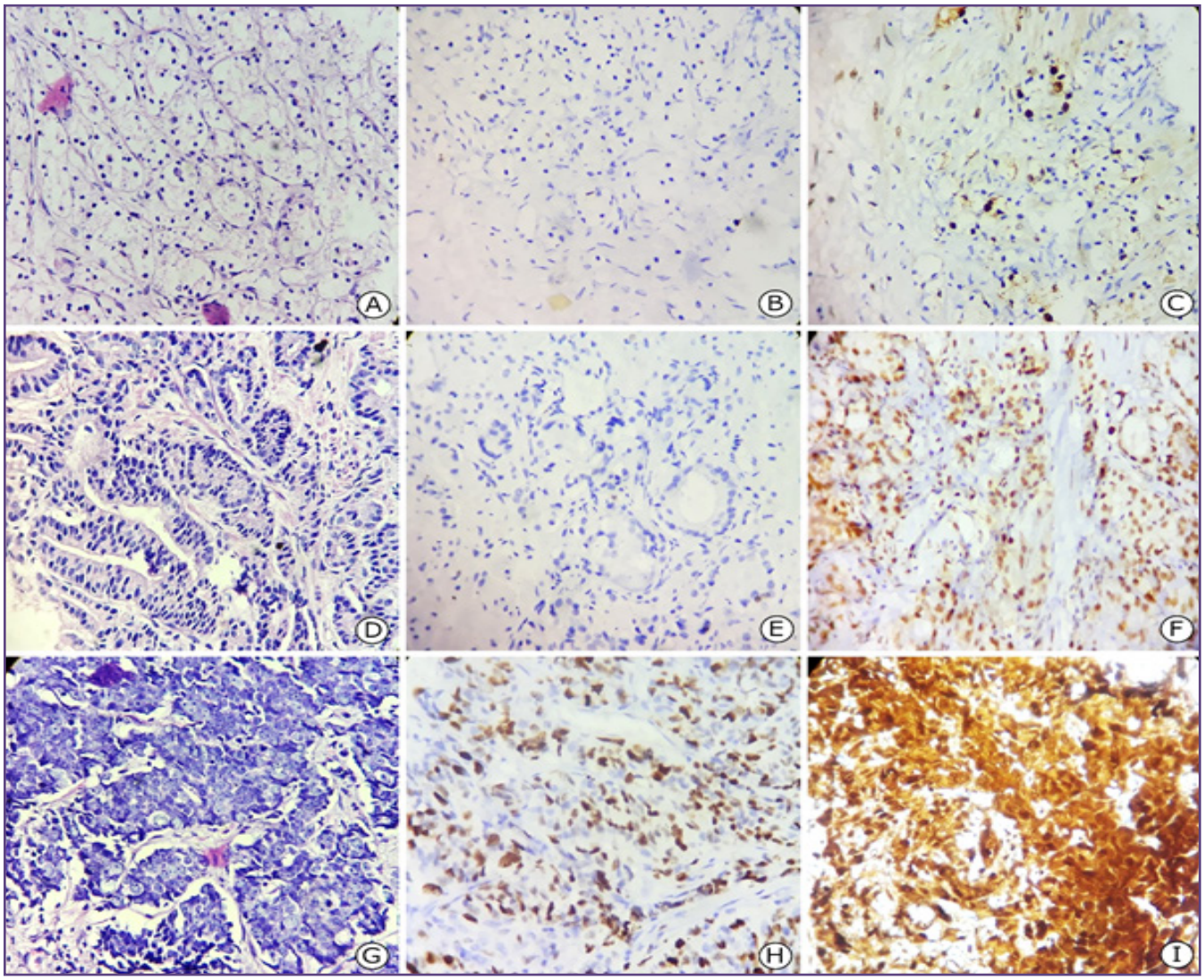

Fig. 3: A- H & E section of Gleason grade group 1 at 400x, B- <5% Ki-67 in Gleason grade group 1 (400x), C- <33% PCNA in Gleason grade group 1, D- H&E section of Gleason grade 2 (400x), E- <5% Ki-67 in Gleason grade group 2 (400x), F- 33-66% PCNA in Gleason grade group 2, G- H&E section of Gleason grade group 5 (400x), H- >10% Ki-67 in Gleason grade group 5 (400x), I- >66% PCNA in Gleason grade group 5 (400x).

Discussion

In the present study, prostate carcinoma cases were graded on the basis of Gleason scoring and Gleason grade groups by 2 independent pathologists. Mild interobserver variability was seen in Gleason score 7 (4+3), 8 and 9. Uemura H et al reported that the reason behind the interobserver variability is because needle biopsies have an inherent sampling error and prostate cancer is a multifocal disease with satellite tumors. Also, the objective evaluation becomes difficult due to the heterogeneity within the different histologic patterns of prostate. [15]

Further, grading the cases based on Gleason grade groups, interobserver variation was seen in grade groups III, IV and V, which was in concordance with study by Montironi et al who stated that assessing Gleason pattern 4 is difficult, due to intimate admixture of patterns 3 and 4. Under-grading of cancer results due to failure of recognition of small areas of gland fusion. [10]

Ki-67 expression was negative in all cases of BPH and positive in 100% of carcinoma cases with no interobserver variability. Two cases showed foci of PIN, with Ki-67 score <5%, however, further studies involving a greater number of cases of PIN are required to clarify its expression. Our findings are almost similar to the studies done by Mohamed et al and Verma et al who observed Ki-67 to be expressed only in 19% and 10% cases of BPH respectively, whereas in malignant cases, it was expressed in 81% and 64% of cases respectively. [6,8]

Ki-67 expression was observed to be increased with increasing grade by both observers and also found to be statistically significant by observer 1.

The results of our study are in agreement to the previous studies which observed an increasing trend in Ki-67 expression with advancing tumor grade, although statistically significant difference could not be reached in these studies. [5,7,12]

Munoz et al did not find any significant difference between Ki-67 expression and Gleason score. The reason for variations in the results could be because of variable number of patients included in the numerous studies. Also, there may be interobserver variations in the estimation of Ki-67 score, different ways of data categorization, variability in the cut off points used to assess Ki-67 expression, variation in the fixation time of specimens and difference in the monoclonal antibody used. [16,17]

PCNA was found to be expressed in all cases of BPH and prostate cancer. 100% cases revealed a score of >66% in BPH, and 62% cases revealed >66% score in carcinoma, with no interobserver variability. 2 cases with foci of PIN also showed >66% PCNA expression.

Our results are in concordance with the study done by Zhong et al who showed that PCNA expression was high (grade 2+ and 3+) in the BPH specimens and was significantly inhibited by treatment with Qianliening capsule (QC) which is an herbal medicinal treatment of BPH used in China. The PCNA scores after administration of QC was dropped down to negative or grade 1 PCNA staining. [18]

This observation is in discordance with previous studies done by Manna AK et al and Wang et al who reported no/ low PCNA staining in BPH and significantly higher PCNA levels in prostate carcinoma. They concluded that expression of caPCNA isoforms contribute to the carcinogenesis of the prostatic cancer. The discrepancy in results can be explained by the facts that multiple isoforms of PCNA reside within cancer cells and tissues, reflecting the apparent acetylation states of the protein. Also, the difference in antibody used, fixation procedure and influence of tissue handling, and sampling error may interfere with the results. [3,9,19,20]

The PCNA expression as well as intensity increased with advancing Gleason grade groups as per findings of both observers and were found to be statistically significant with grade by observer 1 and with intensity by both observers. The above findings are in concordance with the studies done by Miyamoto S, Manna AK, and Wang et al. Wang et al reported that high Gleason grades showed higher PCNA expression and stronger intensity of staining. Shiraishi et al stated that PCNA index showed a higher trend in moderately and poorly differentiated malignancies, but difference was not found to be statistically significant. [3,9,19,20]

Proliferating cell nuclear antigen (PCNA), a proliferative marker although found to be increased with advancing Gleason grade groups in the present study, the results were however ambiguous as it was found to be positive in all benign cases also. So, further studies are needed to clarify its expression in benign, equivocal, and malignant cases.

Ki-67, a reliable indicator of proliferative activity, is significantly upregulated in malignant cases and is useful in differentiating it from benign lesions. Ki 67 expression score also increases with increasing Gleason score and grade group and thus may serve as an indicator of more aggressive tumor.

Conclusion

Diagnosis of prostatic diseases with immunohistochemistry still faces challenges because of the difference in reactivity of monoclonal antibodies in benign, equivocal, and malignant lesions.

Further studies are needed to clarify the expression of PCNA in benign, equivocal, and malignant cases as the results were ambiguous in the present study also. The study of Ki 67 expression should be routinely for the diagnosis of prostate carcinoma and to predict its biological behavior. Also, its role in identifying premalignant lesions needs to be studied further.

We would also like to extend the study involving cocktail use of biomarkers in prostatic lesions falling in the grey zone.

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Competing Interests
The authors declare that they have no potential conflicts of interest to disclose.

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