



## Study of Immunohistochemical Marker PsmA and Ki 67 Expression and Its Relation with Grading in Prostate Carcinoma

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Cite this article: Varma VA, Jindal S, Sharda B, Malukani K, Dosi S, Kesharwani P: Study of Immunohistochemical Marker PsmA and Ki 67 Expression and Its Relation with Grading in Carcinoma Prostate. Ann Urol Oncol 2022; 5(1): 20-33. <https://doi.org/10.32948/auo.2022.08.29>

### Abstract

**Background** Prostate cancer (PCa) is the second most frequent malignancy (after lung cancer) in men worldwide. In prostate cancer, immunohistochemistry (IHC) has an important role in the diagnostic confirmation. Gleason score, tumour volume, surgical margins and Ki-67 index are the most significant prognostic factors. The value of different biomarkers like p53, Ki-67, PSMA, androgen receptor mutations, IGF, E-cadherin remains to be applied in clinical practice. In the present study we studied the expression of PSMA and Ki 67 IHC marker in prostatic carcinoma cases and its relation with Gleason score and Gleason grade group of tumour.

**Method** A total of 52 cases of prostate carcinoma diagnosed on histopathology as adenocarcinoma in the Department of surgical pathology within 2.5 years duration were further studied immunohistochemically by PSMA and Ki 67 antibodies.

**Results** At the time of presentation most of the cases have serum PSA level 11-50 ng/ml and with increasing Gleason score it can be  $\geq 100$  ng/ml. Majority of cases have Gleason score 7 and the most common Gleason grade group is 2. PSMA and Ki 67 IHC marker is significantly correlated with Gleason score and Gleason grade group. PSMA expression is significantly correlated with perineural invasion and Ki 67 with bone metastasis.

**Conclusion** PSMA and Ki 67 IHC marker can be used in conjunction with or as a substitute to Gleason scoring system for proper risk.

**Key words** Prostate cancer, Ki-67, PSMA, Gleason scoring system

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## Introduction

Prostate cancer (PCa) is the sixth leading cause of cancer death among men worldwide. The worldwide prostate cancer burden is expected to grow to 1.7 million new cases and 499000 new deaths by 2030 because of growth and aging of the global population [1]. Worldwide, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020. Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers [2]. Prostate cancer incidence and mortality rates are strongly related to the age with the highest incidence seen in elderly men (> 65 years of age) [3]. Prostate cancer is thought to arise after a sequence of at least eight genetic mutational events. Early events appear to be the loss of tumour suppressive genes such as p53 which is mutated in approximately 64% of tumours and p21 in 55% of tumor [4]. Prostatic adenocarcinoma being the most common histological type [5]. Despite the controversy, prostate-specific antigen (PSA) serum level screening routinely accompanies digital rectal examination. Diagnostic confirmation is accomplished by prostate biopsy guided by transrectal ultrasonography with or without magnetic resonance imaging (MRI). In addition to elevated PSA levels, clinicoradiological, signs of local extension or metastasis, detailed histopathological characterization of prostate cancer at needle-biopsies predict clinical tumor behavior and helps therapeutic decision-making [6]. The heterogeneity of PCa histology was first described by Donald Gleason in the 1960s and has improved over the years [7]. Incorporating modifications in the Gleason grading system, the methodology used nowadays is according to the International Society of Urological Pathology (ISUP). Several new biomarkers appear promising in individuals with elevated PSA levels or those with diagnosed prostate cancer, these are likely to guide in separating individuals who can be spared of aggressive treatment from those required aggressive treatment [8]. Another reason of implementing novel markers in prostate cancer diagnosis and clinical decision-making is because of considerable inter-observer variability in Gleason grading among pathologists [9]. This inter-observer variability influences decision making and therapeutic approaches [10]. Thus, it is important to improve the reproducibility of Gleason grading by more objective parameters. In particular, molecular markers reflecting tumor biology can act as novel threshold in active surveillance or watchful waiting [11]. In prostate cancer, immunohistochemistry (IHC) has an important role in the diagnostic confirmation of borderline cases due to the presence (or absence) of basal cells, detected by specific antibodies against it combined with racemase expression in luminal epithelial cells [12-14]. Gleason score, tumour volume, surgical margins and Ki-67 index are the most significant prognostic factors. Additionally, the volume of the tumour, lymphovascular invasion, perineural invasion (PNI), extension of the tumour through the prostate capsule, and invasion to the seminal vesicle and bone metastasis are also valid prognostic factors for disease progression and survival [15]. The value of different biomarkers like p53, ki-67, PSMA, androgen receptor mutations, IGF, E-cadherin remains to be applied in clinical practice [16]. In the present study we studied the expression of Ki 67 and PSMA IHC markers in prostatic carcinoma cases and their relation with Gleason score and Gleason grade group of tumour.

## Materials and Methods

This was an observational study conducted in the Department of Surgical Pathology, Shri Aurobindo Medical College and PG Institute, Indore [M.P.] A total of 52 cases of prostate carcinoma

diagnosed on histopathology within 2.5 years (Retrospectively Jan to Dec 2018 and prospectively Jan 2019 to June 2020) were further studied immunohistochemically. All new cases of carcinoma prostate whose Biopsy or TURP specimen received during study period for histopathology were included in the study while prostate cancer histology other than adenocarcinomas, very small specimen with inadequate tissue for further processing to apply IHC and proven case of carcinoma prostate who had received prior treatment of chemotherapy, radiotherapy or hormonal treatment were excluded. Scoring of PSMA marker – Focal ( $\leq 10\%$  of stained PCa tumor cells ), Regional (11-50% of stained PCa tumor cells ) and Diffuse ( $\geq 50\%$  of stained PCa tumor cells ). Scoring of Ki 67 marker - 1+ ( $\leq 25\%$  of stained PCa tumor cells ), 2+ (26-50% of stained PCa tumor cells ), 3+ (51-75% of stained PCa tumor cells ) and 4+ (76-100% of stained PCa tumor cells.) [17-18].

### *Haematoxylin Solution (Harris's) and Eosin Solution*

In this study the histopathological sections were evaluated after staining by Haematoxylin and Eosin technique.

Haematoxylin 2.5gm, Absolute alcohol 25 ml, Potash alum 50 gms, Mercuric oxide 1.25 gms, Distilled water 500 ml, Glacial acetic acid 20 ml.

Haematoxylin was dissolved in alcohol and added to alum dissolved in warm distilled water. This mixture was brought rapidly to boil and mercuric iodide was added slowly and carefully. The solution was cooled rapidly and glacial acetic acid was added and kept in dark brown bottle.

Eosin 1 gm, Distilled water 100 ml.

### *Staining Procedure for Histological Section*

Glass slide with tissue section was put over a hot plate for few seconds. Xylene 15 mins, Xylene 15 mins, Hydrate through 95% alcohol – 2 min; 80% alcohol – 10 dips; 70% alcohol – 10 dips; 50% alcohol – 10 dips; Bring to water – 10 dips; Stain in alum haematoxylin – 15 minutes Wash in running tap water, Differentiate 1% acid alcohol – 3-4 dips, Wash in running tap water till sections blue, Ammonia – 1 min, Stain in 1% Eosin solution – 8-10 minutes, Wash in running tap water – 1-5 minute, Dehydrate through 50% alcohol – 10 dips, 70% alcohol 10 dips, 90% alcohol 10 dips, 95% alcohol 10 dips, Absolute alcohol 10 dips. Sections were dried and mounted using DPX mountant. Sections were studied under the microscope and histopathological diagnosis was made as per WHO classification for prostate cancers. Histological grading of prostate adenocarcinoma was done and categorized as Grade-I, II, III, IV, & V. The cases that were reported as Adenocarcinoma were further studied for Immunohistochemical staining by PSMA and Ki 67 antibodies.

### *Immunostaining protocol*

Representative formalin fixed Paraffin embedded blocks were selected. Three um thick sections were cut from each block and sections were taken on poly-L-lysine coated slides. Immunostaining was done by Streptavidin- biotin immunoperoxidase technique (LSAB) using readymade antibody to PSMA and Ki 67 (Diagnostic Biosystem, USA). Serial 3um thick sections were cut from representative paraffin embedded tissue blocks and taken on poly-L-lysine coated slides. Deparaffinization was done as per standard protocol (three changes of xylene for 15 minutes each and two change of ethyl alcohol for 1 minute each). Then the slides were rehydrated in decreasing concentration of alcohol (95% alcohol for 3 minutes, 70% alcohol for 3 minutes, distilled water for 1 minute). Antigen retrieval of all slides was done by heating the sections immersed in appropriate buffer in a

**Table 1. Clinical characteristics of patients (N = 52).**

Parameters	Data	
Age (years) (mean /min–max) (SD)	67.9 (48–87) 9	
Symptoms - Acute urinary retention (most common) (n)	26	
PSA (ng/mL) (mean /min–max) (SD)	50.10 (4.44 – 115) 33.8	
BMI (kg/m <sup>2</sup> ) (mean /min–max) (SD)	25.9 ( 17.9 – 42.2 ) 2.28	
PV (mL) (mean /min–max) (SD)	46.0 (19 – 107) 17.6	
Perineural invasion (n)	29	
Bone metastasis (n)	11	
PSMA scoring	Focal	2
	Regional	14
	Diffuse	36
Ki 67 scoring	1+	32
	2+	17
	3+	2
	4+	1
Gleason score	6	8
	7	8
	8	12
	9	4
	1	8
Gleason grade group	2	26
	3	2
	4	12
	5	4
	High	16
Histopathological grade of tumor	Intermediate	27
	Low	9

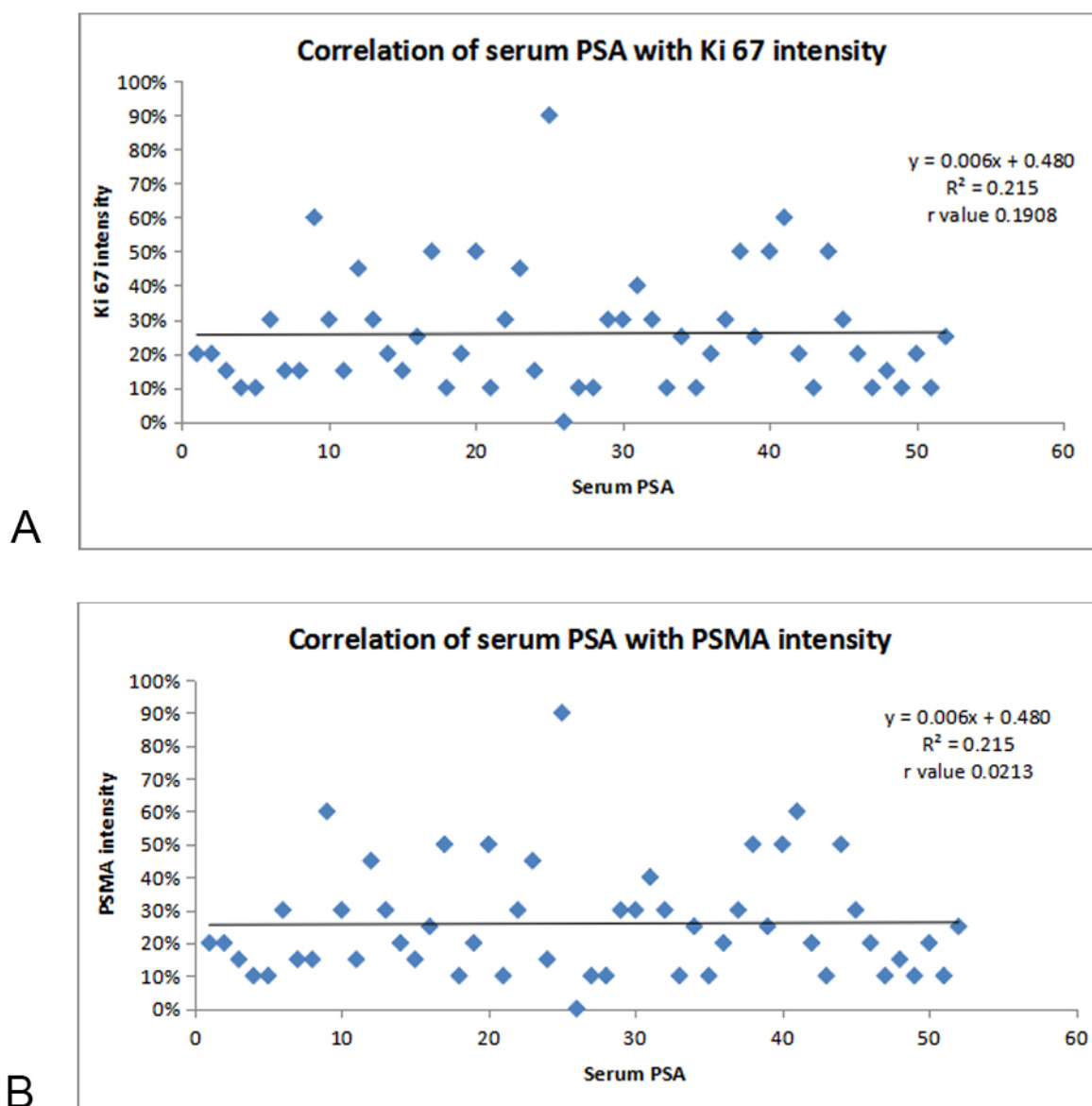
**PV: prostate volume; BMI: body mass index; PSA: prostate-specific antigen; SD: standard deviation.**

pressure cooker till first whistle and then continues to heat till 7 minutes.

Buffer used- Citrate buffer (10mMol/L) was used in antigen retrieval, Anhydrous citric acid crystals 2.4 gram, Distilled water: 1litre (Dissolved, made solution of 1litre), pH: 6.4. All slides were brought to room temperature and then treated with 0.05M Tris-HCL buffer pH 7.4.

#### *Protocol of Staining*

To minimize the non-specific staining (due to action of endogenous peroxidase) each slide was treated with methanol containing 4% hydrogen peroxidase for 30 minutes. After rinsing, the slides were placed in 0.05M-Tris – HCL buffer pH 7.4 for 10 minutes. Excess buffer was removed by wiping of the slides. Sections were covered with adequate amount of primary antibody



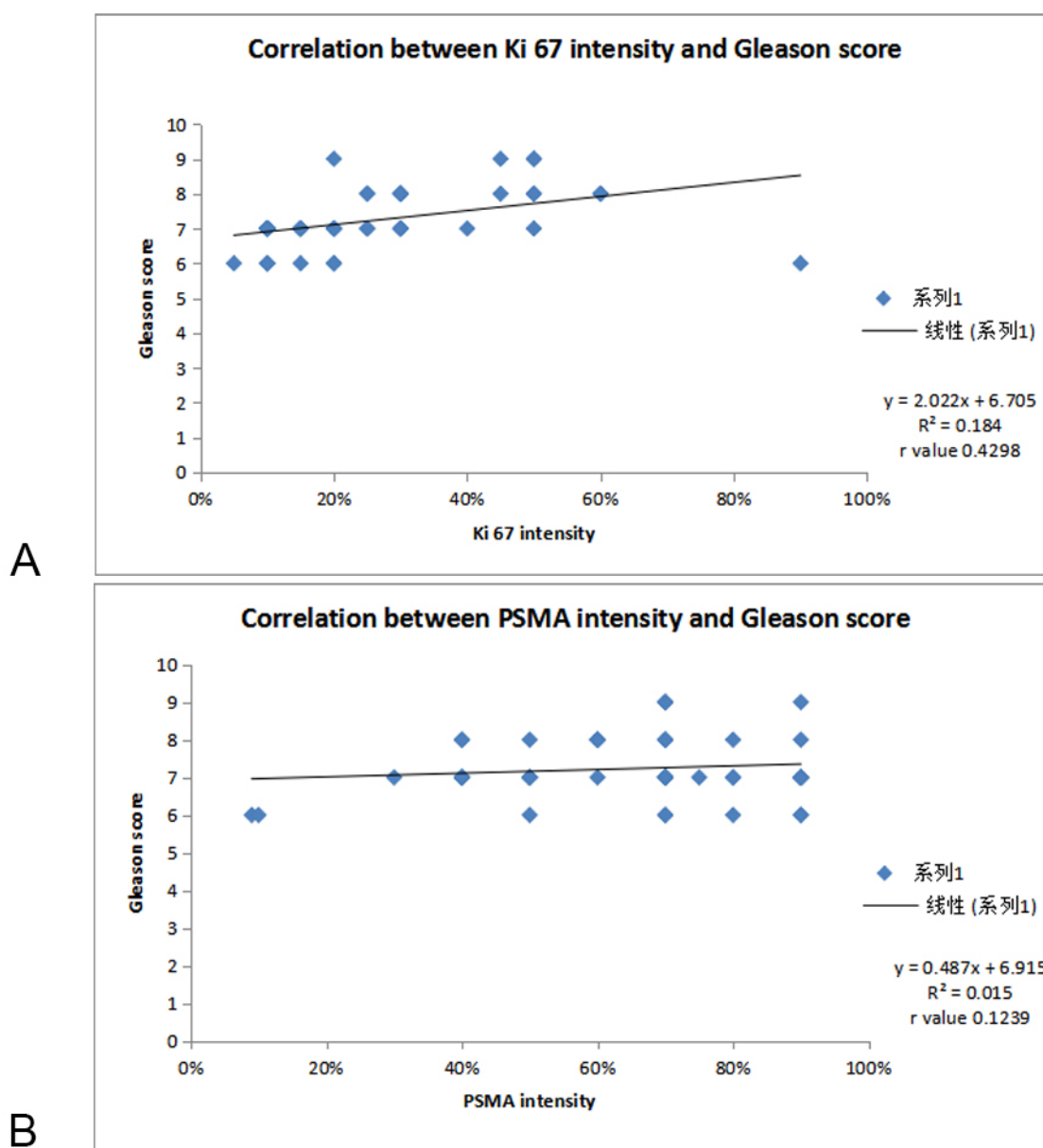
**Figure 1A: Correlation of SERUM PSA with Ki 67 intensity; Figure 1B: Correlation of SERUM PSA with PSMA intensity.**

in the specified dilutions and incubated for 1 hr 20 minutes in a humid chamber at room temperature for PSMA and at 22°C for Ki 67. Primary antibody (purified rabbit for IgG for Ki 67 and mouse monoclonal for PSMA) was obtained from (Diagnostic Biosystem, USA) in dilution 1:50 for Ki 67, and PSMA). The slides were washed three times in 0.05M Tris-HCL buffer pH 7.4 followed by incubation at room temperature for 25 minutes for PSMA and at 22°C for ki 67 with biotinylated secondary antibody of anti-mouse antiglobulins in phosphate buffer saline (PBS) containing carrier protein and Sodium Azide (15mMOL/l) large volume (universal Biogenex kit). After 3 washings (5 minute each) in Tris-HCL buffer, Horse Radish peroxidase (HRP) conjugated Streptavidin was used to cover the slides at room temperature for PSMA and at 22°C for ki 67 and incubated for 30 minutes. After finishing of the above steps, slides were again rinsed thrice in 0.05M Tris-HCL buffer PH 7.4 for 5 minutes each. Slides were then covered with substrate chromogen solution freshly prepared by dissolving 1mg of 3,3'-diaminobenzidine tetra hydrochloride (DAB) in 1ml

of 0.05M Tris- HCL buffer PH 7.4 containing 1µl of H<sub>2</sub>O<sub>2</sub>. Then the slides were incubated at 22°C for 10 minutes for both till development of optimum brown colour peroxidase product. After rinsing in distilled water, sections were counterstained with Harris Haematoxylin and then mounted with coverslip using DPX as mounting media. Precautions were taken to avoid drying of tissue at any step of processing. Each batch of slides was immunostained with appropriate positive controls of sections for PSMA and Ki 67 from Prostate and breast tissue only.

#### *The IHC staining Criteria*

The pathology biopsies and immunohistochemistry slides were reviewed. The pathological diagnosis was considered definite for Ki 67 when tan or brown particles in the nucleus were seen whereas for PSMA tan brown particles were seen in the cytoplasm or along the membrane. Accounting to the percentage of positive cells, Ki 67 and PSMA expression was considered positive or



**Figure 2A: Correlation of Ki 67 scoring with Gleason score; Figure 2B: Correlation of PSMA scoring with Gleason score.**

negative. At high magnification 10 different views were selected; for each view, 100 tumor cells were counted. The staining was considered as negative for both Ki 67 and PSMA when less than 10% cells were stained.

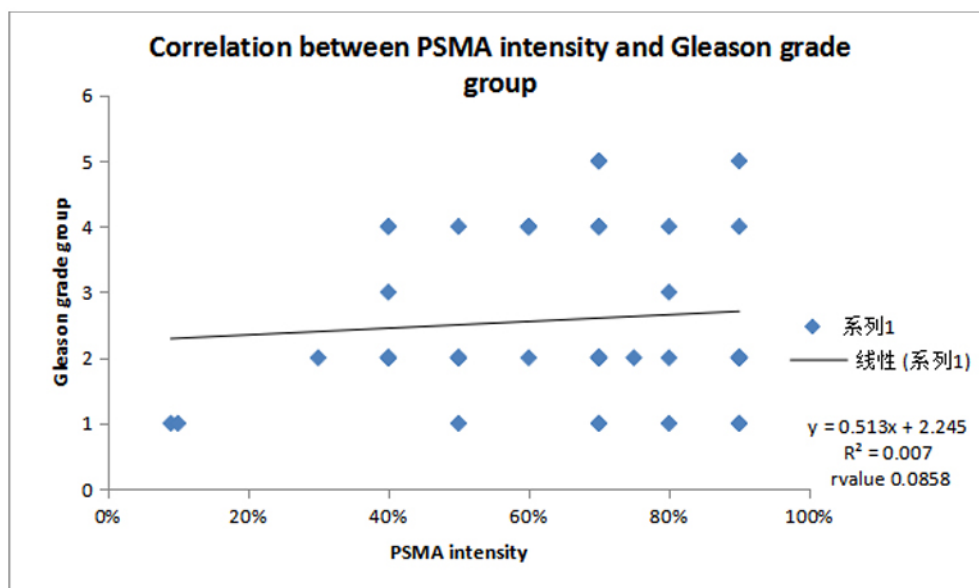
#### Statistical tests applied

Descriptive and inferential, both statistics were used. Mean and Percentage proportion was calculated of cases as per age, PSA level, histopathology typing, grading, IHC staining pattern. For categorical data, Chi square test was applied. T-test for assessing correlation. Pearson correlation coefficient (r value) was employed to test relationships between serum PSA, Gleason score, Gleason grade, PSMA intensity, Ki 67 intensity, bone metastasis and perineural invasion. P value of <0.05 was considered as significant.

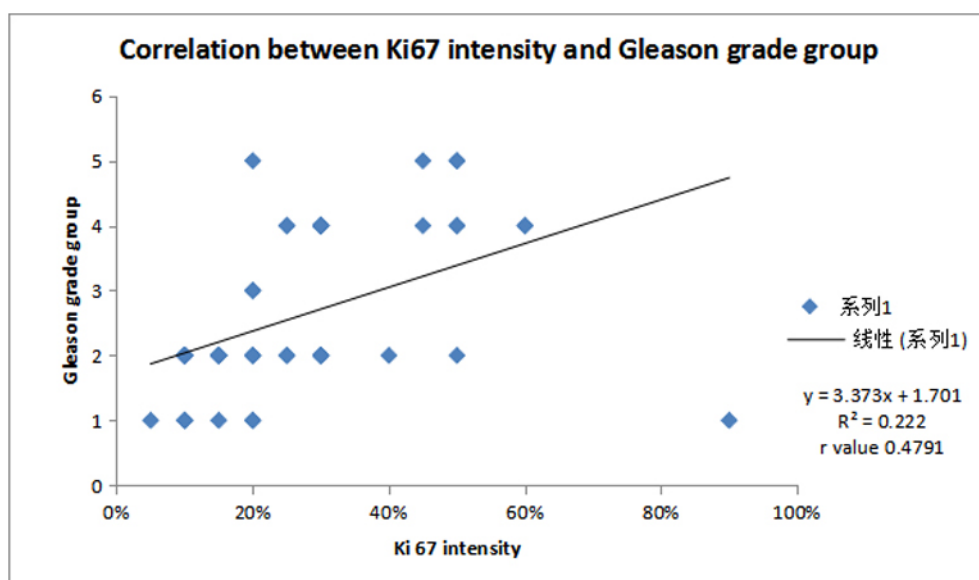
#### Results

In our study, mean age of the patients was  $67.9 \pm 9$  years (Table 1) with majority of patients presented with acute urinary retention (50%) followed by obstructive LUTS in (19.23%) and storage LUTS (11.53%) cases. The prostate volume PV before biopsy was calculated on TRUS according to the ellipsoid formula (height x width x length x 0.52). In our study, Prostate volume mean  $\pm$  SD was  $46.0 \pm 17.6$  ml and median  $\pm$  IQR was 40.5 (19 – 107) ml. The weight of the patients was recorded in kilograms and height in meters. The body mass index (BMI) was also calculated (kg/m<sup>2</sup>) (BMI; 25 to 29.9, 30 to 34.9, > 35.0, < 25 kg/m<sup>2</sup>). Patients were divided according to the Serum PSA level. 46.1% cases had PSA level between 11-50 ng/ml, 25% with PSA level of 51-100 ng/ml, 17.3% had serum PSA level >100 ng/ml and 11.5% had serum PSA level between 4-10 ng/ml. Gleason score in our study ranged from 6 to 9. None of the patient have Gleason score 10. According to the ISUP Gleason Grade Grouping system, the cases were divided into Gleason grade group (GGG) 1-5. Majority of cases (26) belonged to the GGG2, 12 cases belong to GGG4, 8 cases belong to GGG1,

A



B



**Figure 3A: Correlation of PSMA scoring with Gleason grade group; Figure3B: Correlation of Ki 67 scoring with Gleason grade group.**

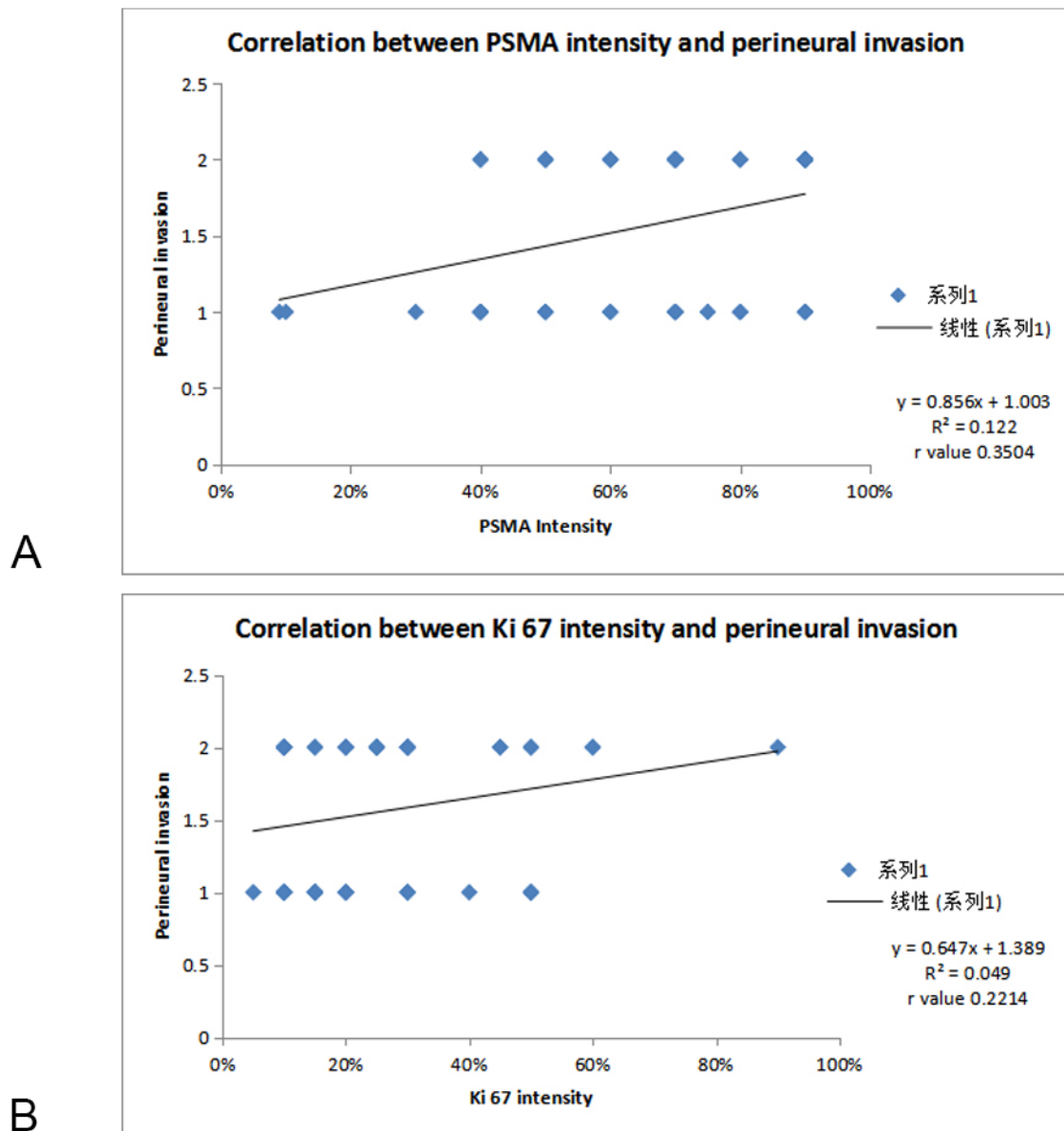
4 cases belong to GGG5 whereas only 2 cases have GGG3. **Figure 1 & 2** shows positive correlation between Serum PSA and percentage of Ki 67 and PSMA intensity (Pearson correlation coefficient r value 0.1908, 0.0213 respectively).

We distribute the cases in various Gleason grade groups as per serum PSA levels. Out of 8 cases of GGG 1, 4 cases had serum PSA level in between 4-10ng/ml. In GGG 2 majority of cases i.e. 20 cases had serum PSA level in between 10-50 ng/ml. GGG 3 had only 2 cases, in which 1case had serum PSA level 10-50ng/ml and another was in 50-100ng/ml. Out of 12 cases in GGG 4, maximum cases i.e. 7 had serum PSA level in between 50-100 ng/ml and 4 cases have serum PSA level > 100 ng/ml. Maximum cases of GGG 5 (3) had serum PSA level > 100 ng/ml and another one had serum PSA level between 50-100 ng/ml. On applying Chi square test statistically highly significant correlation was seen between Gleason Grade group and serum PSA level ( $p < 0.001$ ). On applying statistics, the correlation between Gleason grade group and perineural invasion (PNI) was statistically non-significant

( $p = 0.133$ ). In present study, there is no difference in presence of bone metastasis between GGG3, 4 and 5, as all have approx 50 % bone metastasis. But as the GGG increases from 1,2 to 3,4,5, presence of metastasis increases. On applying statistics between low GGG (1 & 2) and high GGG (3, 4, and 5) in relation with bone metastasis a significant correlation ( $p = 0.01$ ) was seen.

Out of 52 cases, maximum number of cases 32 (61.53%) showed 1+ Ki 67 score followed by 17 cases (32.69%) had 2+ Ki 67 score. 2 cases (3.84%) had 3+ ki 67 score while only one patient had 4+ Ki 67 score. Out of 52 cases, 36 cases (69.2%) showed diffuse PSMA score followed by 14 cases (26.9%) had Regional PSMA score, while only 2 cases (3.8%) showed Focal PSMA score. There was no significant correlation found between age of the patients and Ki 67 and PSMA scoring. In Gleason score 6 category all cases (8) showed Ki 67 1+ expression. 28 cases belong to Gleason score 7 in which 22 cases (78.5%) show 1+ ki 67 scoring and 6 cases (21.4%) show 2+ ki 67 scoring. But in Gleason score 8 maximum cases 8 out of 12 i.e. (66.6%) show 2+ ki 67 scoring and 2 (16.6%)



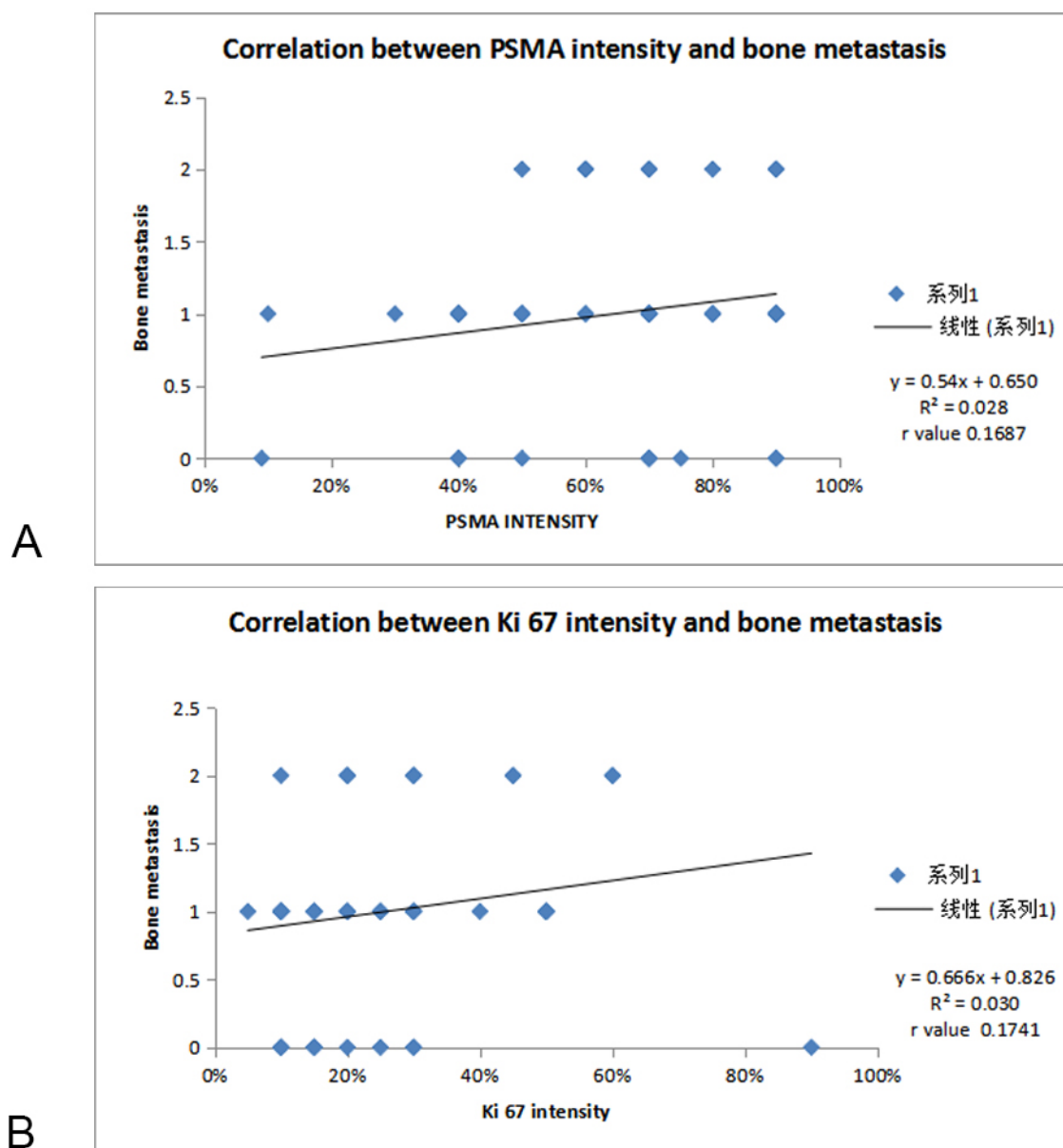


**Figure 4A: Correlation of PSMA scoring with PNI; Figure 4B: Correlation of Ki 67 scoring with PNI.**

show 3+ ki67 scoring. In Gleason score 9, 3(75%) cases show 2+ ki 67 scoring and 1(25%) case show 4+ ki 67 scoring. As per **Figure 3**, higher the Gleason score, the more was the expression of Ki 67 which showed high tumor aggressiveness. On applying chi-square test the relation of Gleason score to Ki 67 scoring was found to be statistically highly significant ( $P < 0.0001$ ). 8 cases showed Gleason score 6, out of which 05 cases (62.5%) showed diffuse PSMA expression, whereas 01 case (12.5%) and 2 cases (25%) show regional and focal PSMA expression respectively (**Figure 4**). The higher the Gleason score, the more was the expression of PSMA which shows high tumor aggressiveness. The P value obtained was 0.02 which was statistically significant. There was a statistically significant positive correlation between Gleason score and PSMA Ki67 intensity on applying Pearson correlation coefficient (r value 0.1239, 0.4298 respectively).

It is evident from the **Figure 5 & 6** that with increasing GGG, Ki 67 proliferation and PSMA expression also increased. On distribution of cases as per GGG and Ki 67 expression, we

observed that all 8 (100%) cases of GGG 1 show 1+ Ki- 67 expression. In Gleason grade group 2, 20 cases (76.9%) show 1+ ki 67 proliferation index and 6 cases (23.07%) show 2+ proliferation index. GGG 3 has only 2 cases, both shows 1+ proliferation index. In GGG 4 majority of cases i.e., 8 (66.66%) cases shows 2+ proliferation index and 2 cases (16.66%) showed 3+ ki 67 proliferation. In Gleason grade group 5 majority of cases 3 (75%) show 2+ proliferation and 1 (25%) case show 4+ Ki67 proliferation index. When Pearson chi square test was applied between Ki 67- GGG and PSMA- GGG, p value was highly significant ( $p = 0.0001$  and 0.04 respectively). On applying T-test in between GGG and Ki 67 expression p value in between GG1 and GG3 ( $p = 0.04$ ), GG2 and GG3 ( $p = 0.004$ ), GG2 and GG5 ( $p = 0.01$ ) was significant ( $P < 0.05$ ) and comparison of other grade groups were insignificant. On applying T-test in between Gleason grade groups and PSMA expression p value in between GGG2 and GGG3 ( $p = 0.04$ ), GGG3 and GGG4 ( $p = 0.03$ ) is significant i.e.,  $p < 0.05$ . On applying Chi Square test statistically no significant relation was found



**Figure 5A: Correlation of PSMA scoring with bone metastasis; Figure 5B: Correlation of Ki 67 scoring with bone metastasis.**

between serum PSA levels & corresponding Ki 67 proliferative index ( $p = 0.08$ ) and PSMA expression ( $p = 0.9$ ). But there was a positive correlation between Gleason grade group and PSMA Ki67 intensity on applying Pearson correlation coefficient (r value 0.0858, 0.4791 respectively).

Distribution of cases of Perineural invasion (PNI) in different PSMA and Ki 67 score as per **Figure 7 & 8**, out of 2 cases in focal grade, none of the case showed PNI. While in Diffuse grade, out of 36 cases, PNI was seen in 24 cases (66.66%). When Pearson correlation test was applied, statistically significant positive correlation was seen between PSMA expression & PNI ( $p = 0.03$ ) while correlation between PNI and Ki 67 labelling index was not significant ( $p = 0.41$ ). On applying Pearson correlation coefficient, there was a strong positive correlation between PNI and PSMA Ki67 intensity (r value 0.3504, 0.2214 respectively).

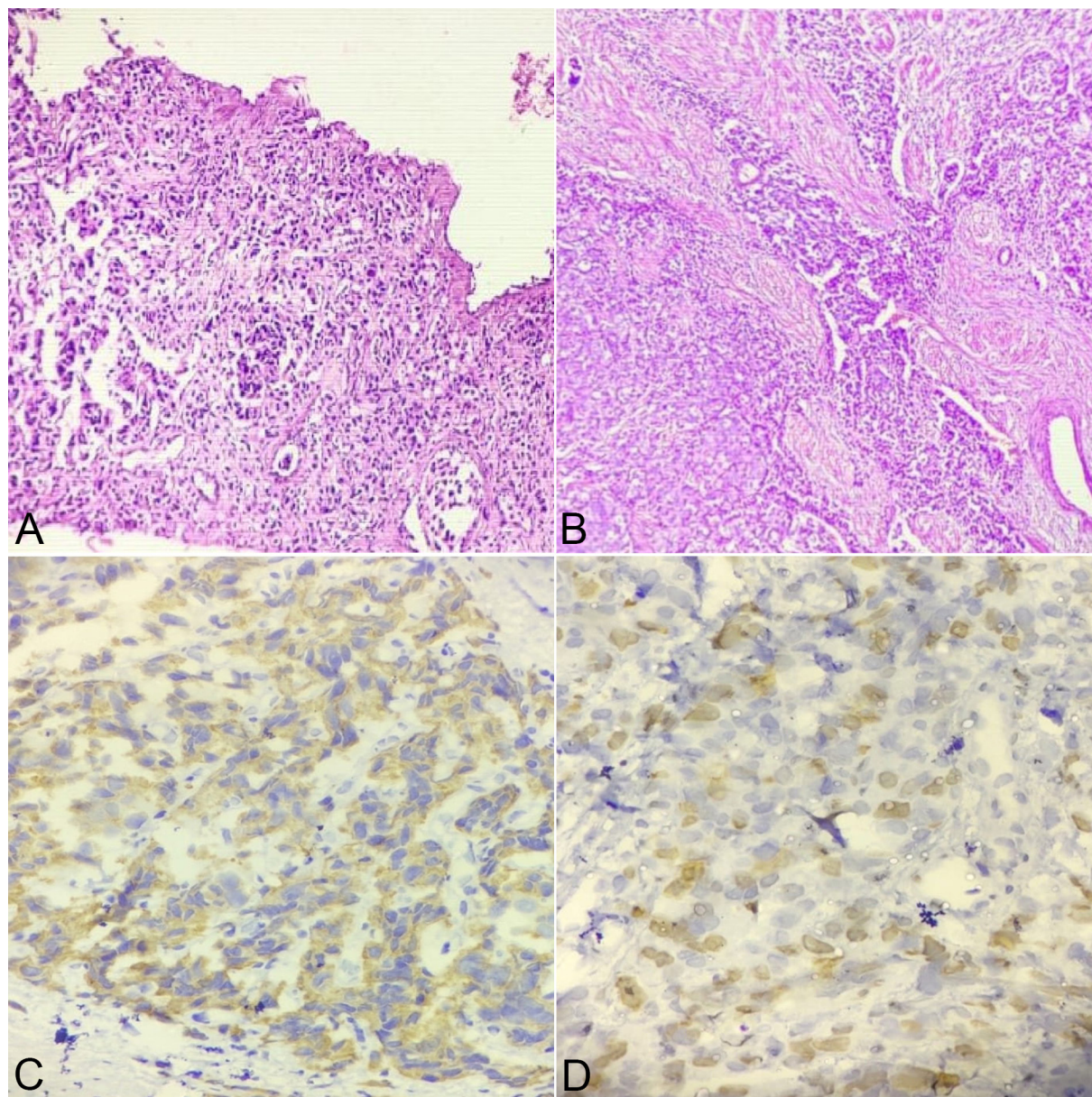
In our study, out of 52 cases, only 41 cases metastatic workup data was available. Out of 41 cases bone metastasis was present in only 11 cases. In 23 cases of Ki 67 1+ category, only 3(13%) cases showed bone metastasis. In 3+ and 4+ category, bone metastasis

was present in all 100% cases (**Figure 9 & 10**). On applying statistics between bone metastasis and Ki 67 score a significant positive correlation was observed ( $p = 0.01$ ) while no significant correlation was seen between PSMA and bone metastasis ( $P = 0.41$ ). In our study, there was a positive correlation between bone metastasis and PSMA Ki67 intensity on applying Pearson correlation coefficient (r value 0.1687, 0.1741 respectively).

## Discussion

The age of the patients in our study varied from 45 years to 87 years with a mean age of 67.9 yrs, this was similar to a study done by Deepak P et al (mean age 68.8 year & range 50-90 year), Gurumurthy et al (68.4 year), Anderson Jackson et al (68.5 year). This wide age range in various studies could be due to, the longer life expectancy and increased screening of serum PSA, lead to increasing the number of elderly men diagnosed with prostate cancer [19-21]. The most common presentation was acute urinary retention (50%) followed by obstructive LUTS in 19.23% and





**Figure 6A:** Microphotograph showing sheets and acini (Gleason score 5+3) arrangement in Prostatic Adenocarcinoma at 10X view; **Figure 6B:** Microphotograph showing sheets and cribriform pattern (Gleason score 5+4) in Prostatic Adenocarcinoma at 10X view; **Figure 6C:** Microphotograph showing Diffuse PSMA expression in high Gleason grade group at 40X view; **Figure 6D:** Microphotograph showing High (4+) Ki 67 expression in High Gleason grade group at 40x view.

Storage LUTS in 11.53%. Hematuria and burning micturition were the least common presenting symptoms seen in 9.61% cases each. Hamilton et al and Siddharth Gangwar et al also reported acute urinary retention (67%) as the most common symptom [22-23]. In our study, serum PSA levels ranged from 4.4 to >100 ng/ml. C Marchal et al, Sladana Zivkovic et al, Goswami AP et al found serum PSA range from 1.0 ng/ml to 523.0 ng/ml. In the present study mean serum PSA level was 43.59ng/ml which was similar to study done by Gurumurthy et al and Zhigun Cao et al (55.1ng/ml and 32.91ng/ml respectively). Contrary to this Albasri A et al found very high mean serum PSA (303.4 ng/ml) in his study. The variations in the PSA level may be attributed due to factors that alter the serum PSA level other than malignancy [24-29]. In the present study, majority of cases (89.5%) had PSA level > 10 ng/ml, least no. of cases i.e. 6 (11.5%) had serum PSA level between 4-10 ng/ml. Our findings are in concordance with the findings of

Deepak P et al, G Fischer et al and I Putugde Sanjaya et al (92.5%, 80.2% and 88.3% respectively). Contrary to our observation, Karnes R J et al and Marcos F et al in their study found maximum no. of cases (55.9%, 60.3% respectively) in PSA range 4-10 ng/ml, followed by serum PSA >10 ng/dl (23.5%, 33% respectively). The variations in the PSA level may be attributed to the other factors that alter the serum PSA level other than malignancy like, associated urinary tract infection or bacterial prostatitis, recent catheterization or urological intervention like prostatic biopsy or TURP [21] [30-33]. We have classified the cases according to the Gleason score into 3 groups. We found that most of the cases, i.e 53.84% were present in the category of Gleason score 7 whereas 30.76% cases belonged to higher Gleason score group (8-10) and least no. of cases (15.38%) had Gleason score 6. This observation is closer to that of Anderson Jackson et al (52.5%) and Kumari K et al (39.2%). Our findings here did not match with that of Marchal

**Table 2. Comparison of PSMA expression in the present study with various other Studies.**

Study	Year	PSMA positive cases	Focal	Regional	Diffuse
Jeffrey S. Ross et al <sup>[47]</sup>	2003	100%	52%	48%	0%
Tomomi Kusumi et al <sup>[46]</sup>	2008	100%	0%	11.90%	88%
Kurt D Bernacki et al <sup>[48]</sup>	2013	87.25%	6.25%	0%	81.25%
Tsouralaki Marie et al <sup>[49]</sup>	2015	97.60%	22.20%	29.50%	45.90%
Sara Bravaccini et al <sup>[44]</sup>	2018	89.80%	11.25%	45.79%	32.70%
Marie CHupe et al <sup>[45]</sup>	2019	87%	41.90%	33.70%	11.40%
Present Study 2020	2020	100%	3.80%	26.90%	69.20%

C et al, ShaneMesko et al, Gong-Wei Wang et al and Verma R et al, they observed most of the cases in Gleason score 6 group (49%, 44%, 37.7% & 36% respectively). I Putugde Sanjaya et al reported the reverse findings and had maximum cases (53.6%) in higher Gleason score group (8-10) [19] [34-37] [24] [31]. In the present study, we have classified the cases on the basis of Gleason grade group. We found that most of the cases (50%) were present in the GGG 2 whereas only 3.84% cases belonged to GGG3 group similar to that of Elin Richardson et al (41%), Solene Florence et al (33%) and Julie et al (37.1%) [38-40].

Israeli R.S. 1994, Cunha AC 2006, Bostwick et al proposed that PSMA is a type II transmembrane glycoprotein marker in many cancers, including Prostate cancer. Colombatti M et al have shown that high PSMA expression activates signaling pathways that promote tumor cell survival and proliferation. Chang S.S.et al and Sweat SD et al have concluded that increased PSMA expression in prostate cancer is associated with higher tumor grade and a high risk of disease progression. Sara Bravaccini and Marie Christine Hupe et al in 2019 indicated that, detection of PSMA expression can serve as a powerful tool for the diagnosis of prostatic cancer and is an independent prognostic marker on biopsies at time of initial diagnosis and can predict disease recurrence following curative therapy for Prostate cancer [41-45]. In the present study, the percentage of PSMA expression positive cases was 100%

(52/52), which was similar to studies by Tomomi Kusumi et al, Jeffrey S. Ross et al. Different frequencies of PSMA expression prostatic carcinomas reported in various studies could be due to geographic variation and various PSMA marker used for IHC staining [46-47]. In the present study, diffuse PSMA expression was more common (69.2%) similar to the studies done by Tomomi Kusumi et al, Kurt D Bernacki et al, Tsouralaki Marie et al whereas, it did not match with the findings of Sara Bravaccini et al, Marie Cristine Hupe and Jeffrey S. Ross et al [47-49] [Table 2]. In our study, the PSMA expression in Prostatic adenocarcinoma cases was compared with clinicopathologic parameters like age, serum PSA level, Gleason score and Gleason Grade Group (ISUP) and histological features like perineural invasion and presence of metastasis. There was no statistically significant relationship between the expression of PSMA and age. This was in concordant with the study of Sara Bravaccini et al and Alberto et al. In contrary to this, Julie L. et al. in his study found positive correlation between PSMA expression and age. Furthermore, the correlation of PSMA expression was significant (p value 0.02) with the Gleason score of the tumor, which was at par with the finding of Sara Bravaccini et al, George L Wright et al, Jeffery S Ross et al, Sven Perner et al, Julie L. et al. With increasing Gleason score, the PSMA expression increased significantly with highest PSMA expression in high Gleason score. The correlation was also

**Table 3. Comparison of Ki 67 expression in the present study with various other Studies.**

Study	Year	Ki 67 Positive Cases	1+	2+	3+	4+
R Urs AN et al <sup>[52]</sup>	2008	72%	54%	12.00%	4%	2%
Madani SH et al <sup>[18]</sup>	2011	71%	48.90%	12.20%	4.08%	6.12%
G Fischer et al <sup>[32]</sup>	2013	81.90%	-	-	-	-
Verma R et al <sup>[37]</sup>	2015	64.00%	30.00%	26.00%	8.00%	0%
K S Mahadev et al <sup>[53]</sup>	2018	100.00%	12%	85.00%	0%	3%
SidharthGangwar et al <sup>[23]</sup>	2020	66.65%	29.62%	25.92%	0%	11.11%
Present Study 2020	2020	100%	61.53%	32.69%	3.84%	1.92%



**Table 4. Conclusion of various studies related to correlation between Ki67 expression and Gleason score.**

Study	No. of cases	Conclusion
M.Tsuji et al 1998 <sup>[54]</sup>	79	Ki67 labelling index increased with increasing grade. Mean Ki67 LI for Gleason score 2-6 was 11.6% and patients with score 8-10 had LI of 24.7
Munoz E et al 2003 <sup>[55]</sup>	35	No statistically significant differences between the immunolabeling for Ki-67 and Gleason's score
Aaltomaa et al 2006 <sup>[56]</sup>	211	Positive correlation between Ki67, pT and differentiation of tumours
Mesko et al 2013 <sup>[36]</sup>	77	Ki67 labelling index was significantly different for Gleason scores of 6,7, and $\geq 8$ , ( $P = 0.01$ )
Verma et al 2015 <sup>[37]</sup>	60	Statistically significant correlation between Ki67 positivity and increased Gleason's grade ( $P=0.002$ )
Present study 2020	52	Highly significant correlation between Ki 67 expression and Gleason score (p value $< 0.0001$ )

significant (p value 0.04) with the Gleason Grade Group. With increasing Gleason Grade Group of tumor from 1 to 5 the PSMA expression also increased significantly with 100 % cases in GGG5 showed diffuse PSMA expression. The correlation between PSMA expression and serum PSA level (p value 0.08) was statistically insignificant. In this study, diffuse PSMA expression was equally more common in all categories of cases as per serum PSA level. This finding is similar to the finding of Jia-Qiang Ren et al, and Alberto et al. The difference in the results can be because of the difference of the staining procedure of PSMA as well as the difference in sample size and specimen type of the study [47] [51] [44] [45] [38] [50]. In the recent few years, the focus has been on the assessment of the tumor cell kinetics, since as it can reflect the degree of tumor aggression. High Ki 67 index has recently been correlated with poor clinical outcome in many malignancies. In the present study, Ki 67 was positive in 100% (52/52) cases similar to K S Mahadev et al. Our finding were discordant with study of Verma R et al and R Urs AN et al (64%, 72%,) [Table 3]. Different frequencies of Ki 67 positive cases reported in various studies could be due to geographic variation and various Ki 67 antibodies used for staining. In the present study 1+ Ki 67 expression was more common (61.53%) , this observation was similar to the studies done by Madani SH et al (48.9%), R Urs AN et al (54%) and Verma R et al (30%) [18] [32] [37] [52] [53].

We correlated Ki 67 expression in Prostatic adenocarcinoma cases with clinicopathologic parameters like age, serum PSA level, Gleason score and Gleason Grade Group (ISUP) and histological features like perineural invasion and presence of metastasis. There was no statistically significant relationship observed between the expression of Ki 67 expression and age. This was in concordant with the study of Solène-Florence Kammerer-Jacquet [40]. Furthermore, the correlation of Ki 67 expression was highly significant (p value  $<0.0001$ ) with the Gleason score of the tumor, which was at par with the finding of studies shown in Table 4. With increasing Gleason score the Ki 67 expression increased significantly. Whereas Munoz E et al, did not find any significant correlation with Ki67 and Gleason score [18] [36-37] [54-56]. The correlation was also significant (p value 0.0001) with the Gleason Grade Group and insignificant with serum PSA level.

Pretreatment serum PSA levels are a prognostic marker and stratify patients into differing prognostic categories. In present

study, the correlation between Ki 67 expression and serum PSA level (p value 0.9) was statistically insignificant. This finding is in concordance with the finding of Rugwizangoga B, Cowen D, Sulik M. On contrary to this Luczynska E et al and Mahadev K S et al, observed positive correlation between serum PSA and Ki 67 expression. The difference in the results can be because of the difference of the staining procedure of Ki 67 as well as the difference in sample size and specimen type of the study [53] [57-59]. Prostate cancer has a propensity to invade and grow along nerves, a phenomenon called perineural invasion (PNI). Recent studies suggest that the presence of PNI in prostate cancer has been associated with cancer aggressiveness. In the present study, PNI was present in 29 (55.7%) cases and was absent in 23 (44.2%) cases similar to the study of Lorenzo Masieri et al, (65.7%). Contrary to this Elin Richardson et al (25%). The difference in the occurrence of PNI can be attributed to the fact that cases having more number of well differentiated tumors have low percentages of evidence of PNI while studies with more number of high grade tumors have higher percentages of evidence of PNI. Our study has significant number of cases with higher Gleason grade and high serum PSA level [39] [53]. We also correlate PNI with Ki67 labeling index and PSMA expression. In our study, we did not observe significant correlation between Ki67 and PNI whereas statistically significant correlation was present between PSMA and PNI ( $p=0.03$ ). We did not find any literature related to such correlation between PNI with Ki 67 and PSMA so that comparison with other study was not possible here. In our study, we found PNI was more commonly present in cases of higher GGG but there was no statistically significant ( $p=0.133$ ) correlation. Our observation did not match with studies done by Hwang Gyun Jeon et al, F Ozcan, Jun Taik Lee et al.

Furthermore, out of 52 cases, data related to metastatic workup was available in only 41 cases. Out of 41 cases bone metastasis was present in only 11 (26.8%) cases and absent in 30 (73.1%) cases. This finding is discordant with those of previous studies by Ito et al and Atausetalin in which bone metastasis was present in 36.1% and 24% cases respectively. On contrary to this Oesterling et al, Zaman et al and Wolff et al reported very less incidence of bone metastasis (0.8%, 12.6% and 11.2% respectively). This high number of bone metastasis could be due to several reason like most of patients come with advance stages, majority of the patients

came with high PSA and intermediate or high Gleason score at diagnosis [60-64] [41] [29]. In our study, we observed statistically significant correlation between Ki67 and bone metastasis ( $p=0.01$ ) and it is evident that higher the Ki 67 labelling index, the more was the presence of bone metastasis. Whereas there was no significant correlation present between bone metastasis and PSMA expression ( $p=0.41$ ). We did not find any literature related to such correlation between bone metastasis with Ki 67 and PSMA so that comparison with others studies was not possible here.

There was positive correlation between Serum PSA, Gleason score, Gleason grade group, Bone metastasis, PNI and PSMA – Ki 67 intensity on applying Pearson correlation. Our findings are in agreement with results from studies carried out by other researchers [65-66].

Limitation of our study is retrospective and prospective data, not able to follow up the patients and the relatively small sample size due to time constraints. Therefore large randomised trials should be conducted to support our data. In 1970s, PSA was discovered and today it remains the most widely used biomarker in prostate carcinoma. The search for a more rapid, specific marker for detection of prostate carcinoma has led to numerous laboratories examining biomarkers. Although a number of markers have been acknowledged, there is yet to be one that is widely accepted and used. Researches are now more concentrated on genomic markers which are beyond our study scope.

## Conclusion

Ki-67 immuno-labeling index and PSMA IHC marker can be used in conjunction with or as a substitute to Gleason scoring system for proper risk stratification to aid in therapeutic intervention and proper prognostication of prostatic carcinomas.

## Acknowledgements

We extend our sincere thanks to the patient who participated in the study.

## Ethical policy

Approval from institutional ethical committee was taken.

## Author contributions

AVV & KM: They supervise the whole study, and helps in Critical Review; SJ & PK: Conception, Design, Materials, Data collection; SD & BS: Writing, Analysis and Interpretation, Literature Review, Drafting of manuscript. All authors read and approved the final manuscript.

## Competing interests

All authors declare no competing interests.

## Funding

This study was financially not supported by any institution.

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