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Original Article

Pathology Section

Angiogenic Index as a Measure of Angiogenesis in Prostate Cancer and Its Correlation with Gleason Grade and Score

SHIKHA PRAKASH[1], PRASHANT SINGH[2], POOJA NAGAYACH[3], KALPANA SINGH[4], RAJNI BHARTI[5], GUNJAN PRAKASH[6]

ABSTRACT

Introduction: Microvessel density, as a measure of angiogenesis, predicts prognosis in prostate cancer. Angiogenic Index (AI, numerical value of angiogenesis) minimises the possible variation concerning the width of the microscopic field, stromal epithelial relations and cellular tumour size.

Aim: To study AI in prostate cancer and its correlation with Gleason Grade (GG) and Gleason Score (GS).

Materials and Methods: The present cross-sectional, study was done at Postgraduate Department of Pathology, Sarojini Naidu Medical College, Agra, India, from September 2019 to December 2020. Twenty five histopathologically confirmed prostate adenocarcinoma specimens from radical prostatectomy, Transurethral Resection of Prostate (TURP), and needle biopsy were included in the study. These cases were categorised according to Gleason Grade (GG); Gleason Score (GS) was assigned to each case. The GSs were simplified into three groups: low (GS 2-6), intermediate (GS 7) and high-grade (GS 8-10). Immunohistochemical {Cluster Differentiation (CD) 31} blood vessel staining was done to calculate AI. Statistical significance was determined by Unpaired t-test.

Results: All the cases were males with age range from 55-76 years (mean age was 65.48±5.62 years). Mean AI was 13.74, 83.76, 163.27, 299.12 for the GG1, GG3, GG4, GG5, respectively. Mean AI was 29.72, 82.67, 129.15, 190.31, 206.71, 307.34 for GS 2, 6, 7, 8, 9, 10, respectively. Comparing GG among themselves, statistically significant difference in AI was found between GG3 vs GG4 (p-value=0.0056, r-value=0.5269). Difference was also statistically significant between GG3 vs GG5 (p-value=0.000011, r-value=0.8030) and GG4 vs GG5 (p-value=0.0036, r-value=0.5806). In all scores combined, the mean AI was 56.20 for low-grade (GS 2-6), 129.15 for intermediate-grade (GS 7), 247.35 for high-grade (GS 8-10). Statistically significant difference was found in between AI (p-value <0.05) in all Gleason scores.

Conclusion: Positive correlation was observed between AI, GG and GS in prostatic adenocarcinoma. AI may be of immense value to predict prognosis of prostatic adenocarcinoma.

Keywords: Microvessel density, Prognosis, Prostatectomy, Prostate adenocarcinoma

INTRODUCTION

Worldwide, clinically detected prostate cancer is the second most common malignancy, with an estimated 1.41 million new cases in 2020 [1]. Incidence is low in Asian and North African countries but on the rise in India [2,3]. Most cases of prostate carcinoma are diagnosed after the age of 50 years, and frequency increases with age [4].

Angiogenesis is implicated as part of the process of tumour metastasis. Since the clinical significance of Microvessel Density (MVD) in human prostate cancer tissues was first reported by Weidner N et al., [5], several reports have shown that MVD is increased in carcinogenesis [6-10]. MVD is significantly associated with pathological features and outcomes in patients with prostate cancer [11]. Antiangiogenic therapy might be a novel treatment option in advanced or metastatic prostatic carcinoma [12].

Earlier methods to quantify the microvessels in a tumour were microvessel count, MVD and maximal MVD. A different method of quantification of vascularisation is the Angiogenic Index (AI) that has been found to be associated with greater tumour size and grade, lymph node metastases and early death in cancer of breast [13].

Laforga JB and Aranda FI determined the number of microvessels by 1,000 tumour cells (AI) to perform a more accurate method for counting microvessels in breast cancer cases [13]. This method minimised the possible variations concerning the width of the microscopic fields, stroma/epithelium relations and cellular tumour size. Results showed that the AI correlated more significantly than the classic MVD determination with other prognostic factors in breast cancer. Therefore, they recommended determining AI because of its reliable calculation and significant correlation with other prognostic factors [13].

Assessing angiogenesis with a numerical value that can be evaluated in small biopsy specimen will have more reproducibility than just counting MVD. No study is reported regarding AI measurement in prostate cancer. As blood microvessels are essential for tumour growth, the present study was performed to calculate the angiogenic index (using CD31 marker) in prostate cancer.

Cluster Differentiation 31 is more specific for endothelial cells than CD34 [14] and has been described on human blood platelets, monocytes, neutrophils, as well as on large and small vessels' endothelial cells and naïve T-lymphocytes. Silberman MA et al., also used CD31 for staining microvessels [15]. Hence, the present study was aimed to evaluate the angiogenic index in prostate cancer and its correlation with Gleason Grade (GG) and Gleason Score (GS) using Gleason System (histological prognostic factor).

MATERIALS AND METHODS

This cross-sectional study was conducted in Postgraduate Department of Pathology, Sarojini Naidu Medical College, Agra, India, from September 2019 to December 2020. Ethical clearance was obtained from Institutional Ethics Committee (SNMC/IEC/2022/130). Written consent was taken from the patients and their confidentiality was maintained.

Inclusion criteria: All the prostatectomy, Transurethral Resection of Prostate (TURP), needle biopsy specimens with definite histopathological diagnosis of prostate cancer were included in the study.

Exclusion criteria: Very small needle biopsy specimens (≤1 mm) and samples with extensive necrosis were excluded from the study.

Sample size calculation: Total 25 cases were included, of which 14 cases were fresh (during the study period) and 11 cases were archival (December 2018 to August 2019) formalin-fixed and paraffin-embedded diagnosed adenocarcinoma samples of needle biopsy (four cases), TURP (12 cases) and radical prostatectomy (nine cases).

Study Procedure

On Haematoxylin and Eosin (H&E) sections, the cases of prostate adenocarcinoma were categorised according to Gleason Grading (GG) system [16]. It is based on the degree of glandular differentiation and growth pattern of the tumour in relation to the stroma as evaluated on low power (4X, 10X objective eye piece) examination. The predominant (primary) tumour pattern was graded according to Gleason pattern from GG1 to GG5; non dominant (secondary) tumour pattern was similarly graded according to Gleason Pattern [16]. Gleason Score is obtained by adding these two values together.

Further, the scores were simplified in three groups as described by Humphrey PA [17]:

a) Low-grade (GS2-6)

b) Intermediate-grade (GS7)

c) High-grade (GS8-10)

Immunohistochemical staining for Cluster Differentiation (CD) 31 was performed by means of a modified labelled avidin-biotin technique in which a biotinylated secondary antibody forms a complex with peroxidase-conjugated streptavidin molecules using monoclonal antibody and staining kit (Universal DAKO Labelled Streptavidin-Biotin®2 System, Horseradish Peroxidase, Denmark; DAKO LSAB®2 System, HRP) [14].

Calculation of Angiogenic Index

Counting procedure: Counting of vessels was done in three consecutive high-power fields (x400, Olympus CH20i). The total number of microvessels was recorded along with the number of nuclei of tumour cells located across the central straight line drawn across the maximum diameter of the eyepiece. The cell count was calculated as equal to $\pi(^{n}/_{2})^{2}$, where 'π' is equal to 3.14 and 'n' is the number of nuclei that intercept the line [13]. Angiogenic index was calculated for each field and mean was derived.

In cases with primary and secondary Gleason grades, AI was calculated in individual grades in the similar way and the mean of the two grade was considered for those cases. To avoid bias, counting was done by two different observers and average AI was noted.

STATISTICAL ANALYSIS

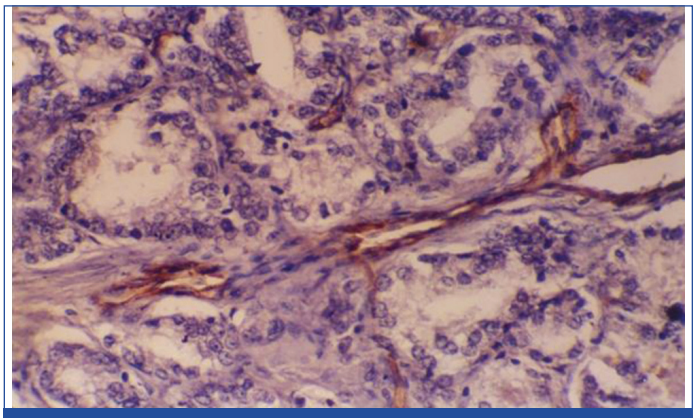
Data was entered in Microsoft excel software and analysis was done using Statistical Package for the Social Science (SPSS) software version SPSS-12. The data was analysed to determine statistical significance using unpaired t-test and p-value ≤0.05 was considered significant.

RESULTS

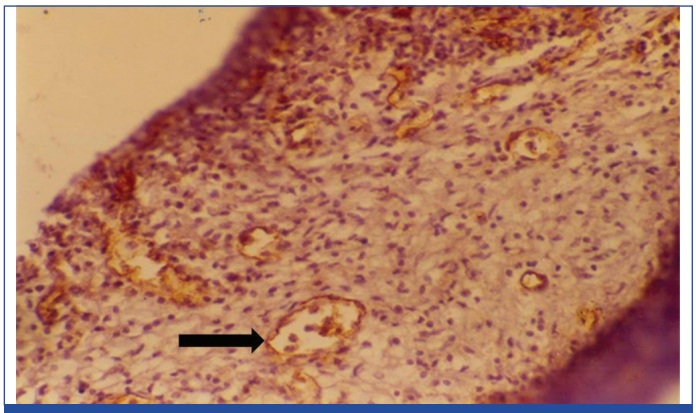
All the 25 specimens were males and the age of cases ranged from 55-76 years with a mean age of 65.48±5.62 years. Fourteen showed single GG and 11 showed variable (primary grade+secondary grade) GG making a total of 36 variables to be studied [Table/Fig-1]. GG1 adenocarcinoma was identified in one (2.78%) of 36 variables studied. GG3 [Table/Fig-2] was identified in 12 (33.33%) of 36 variables, GG4 was identified in 14 (38.89%) of 36 variables and GG5 [Table/Fig-3] was detected in nine of 36 (25%) variables studied. On Gleason Scoring, there was overlapping between various grades [Table/Fig-4]. Seven study cases were of Intermediate Grade [Table/Fig-5] and 12 were High-Grade.

Histological diagnosis (adenocarcinoma)	Number	Percentage
Grade 1	1	2.78
Grade 3	12	33.33
Grade 4	14	38.89
Grade 5	9	25
Total	36	100

[Table/Fig-1]: Distribution of cases in various Gleason grades.



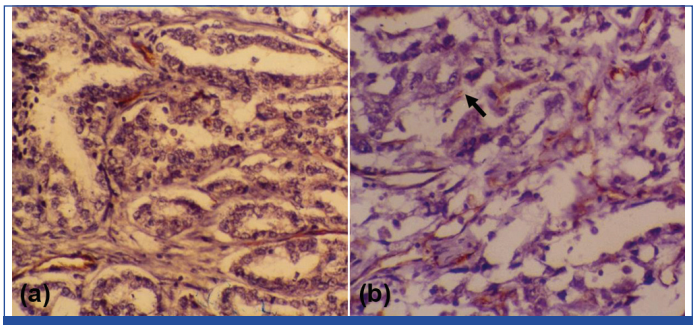
[Table/Fig-2]: Gleason grade 3 prostate adenocarcinoma (CD31 stain, 40X).



[Table/Fig-3]: Gleason grade 5 showing highlighting blood vessels (black arrow) (CD31 stain, 40X).

Histological diagnosis (adenocarcinoma)	Grade	n (%)
Grade 1+1, score 2	Low	1 (4)
Grade 3+3, score 6	Low	5 (20)
Grade 3+4/4+3, score 7	Intermediate	7 (28)
Grade 4+4, score 8	High	3 (12)
Grade 4+5/5+4, score 9	High	4 (16)
Grade 5+5, score 10	High	5 (20)

[Table/Fig-4]: Distribution of cases (N=25) in various Gleason scores.



[Table/Fig-5]: Gleason score 7: a) Intermediate-grade adenocarcinoma; b) depicting single endothelial cell (black arrow) (CD31 stain, 40X).

In Gleason grade 1 adenocarcinoma, the angiogenic index ranged from 15.64-40.28. Mean AI was 29.72. The range and mean values

of angiogenic index for other Gleason grades [Table/Fig-6] are tabulated.

Gleason grade	Range	Angiogenic index (Mean±SD)
1	15.64-40.28	29.72±10.36
3	26-437.90	83.76±40.11
4	14.13-596.18	163.27±78.82
5	7.54-876.43	299.12±111.50

[Table/Fig-6]: Angiogenic index in various Gleason grades.

On comparing the mean AI in GG3 (83.76) and GG4 (163.27) adenocarcinoma, statistically significant difference (p-value=0.0056) was found [Table/Fig-7]. On comparing the mean AI in GG3 (83.76) and GG5 (299.12) adenocarcinoma, a statistically significant difference (p-value=0.000011) was observed. On comparing the mean AI in GG4 (163.27) and GG5 (299.12) adenocarcinoma, the difference was statistically significant (p-value=0.0036). Only single case of GG1 was there, so no correlation could be carried out for this grade.

AI correlation	p-value	r-value
GG 3 vs GG 4	0.0056	0.5269
GG 3 vs GG 5	0.000011	0.8030
GG 4 vs GG 5	0.0036	0.5806

[Table/Fig-7]: Correlation of angiogenic index among various Gleason grades.

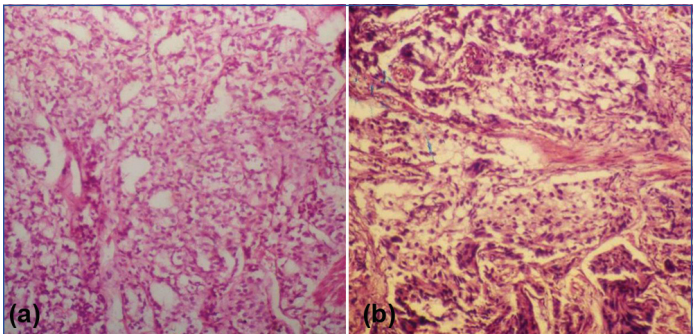
The range and mean values of AI for different Gleason scores [Table/Fig-8] are tabulated. Statistically significant difference (p-value <0.05) was found amongst all Gleason scores [Table/Fig-9]. Mean AI increased from low-grade to intermediate-grade to high-grade. There was statistically significant difference in AI in cases with intermediate-grade and high-grade prostatic adenocarcinoma (p-value <0.02) [Table/Fig-10].

Gleason score	Grade	AI range	Mean AI	Average mean AI
Score 2	Low	15.91-41.40	29.72	56.20
Score 6	Low	28.01-437.90	82.67	
Score 7	Intermediate	22.41-596.18	129.15	129.15
Score 8	High	63.69-398.09	190.31	247.35
Score 9	High	7.54-876.43	206.71	
Score 10	High	127.26-844.66	307.34	

[Table/Fig-8]: Angiogenic Index (AI) in various Gleason score.

AI correlation	p-value	r-value
Low-grade (GS 6) vs Intermediate-grade (GS 7)	<0.05	0.37
Low-grade (GS 6) vs High-grade (GS 8, 9, 10)	0.002371	0.69
Intermediate-grade (GS 7) vs High-grade (GS 8, 9, 10)	0.01554	0.55

[Table/Fig-9]: Correlation among various Gleason scores.



[Table/Fig-10]: Gleason Score 9: High-grade adenocarcinoma; a) Grade 4; b) Grade 5 (H&E, 10X).

No statistically significant difference (p-value >0.05) was seen on comparing the AI between GG (mean AI=143.97) and GS (mean AI=157.65).

DISCUSSION

Angiogenic index has been found to be a reliable quantitative measure of angiogenesis in immunohistochemistry-stained sections [13]. In this study, IHC marker CD31 was used to highlight blood vessels. The degree of tumour angiogenesis was determined by measuring AI. An increase in microvessel count was observed from low-grade to high-grade cancers.

Silberman MA et al., examined the relationship of MVD in intermediate grade prostate carcinomas with stage at radical prostatectomy (RP) and progression after RP [15]. They observed MVD (using CD31) and Gleason score were independent statistically significant predictors of progression. Similar findings were reported by Strohmeyer D et al., [7]. Brawer MK et al., demonstrated that MVD in prostatic carcinoma is an independent predictor of pathologic stage and, presumably, malignant potential [18]. Contrarily, Arakawa A et al., observed that Gleason Score, but not MVC, was the best prognostic indicator in their study [19]. CD-31 can detect pre-existing and newly formed vessels in cancer tissues [20]. Parums DV et al., reported CD31 (antibody JC70) to be more specific, more sensitive and more reproducible in staining blood microvessels. JC70 does not stain lymphatic endothelium in benign lesions [21].

Comparing AI of intermediate grade (score 7) with high-grade (score 8-10), we found that the difference in Angiogenic index was statistically significant (p-value <0.02) [Table/Fig-9]. The increase in microvessels from low to high grade, as observed in various studies, is tabulated [Table/Fig-11] [8-10,15,18,19,22-24]. The present study results were in accordance with the findings of Upadhyay P et al., Gautam KA et al., Bigler A et al., [9,10,22]. Strohmeyer D et al., revealed that tumour angiogenesis was associated with a negative clinical prognosis in prostate cancer after radical prostatectomy [23]. Bostwick DG et al., showed a statistically significant contribution of MVD (along with Gleason score) in the prediction of pathologic stage [8]. In the present study, the cases were divided according to different Gleason grades of adenocarcinoma. On comparison of GG among themselves, statistically significant difference in AI was found between GG3 vs GG4 (p-value=0.003). The difference was also statistically significant between GG3 vs GG5 (p-value=0.0003), GG4 vs GG5 (p-value=0.007). Also, statistically significant difference was found in between AI (p-value <0.05) in all Gleason scores. The observations of this study were in agreement with Upadhyay P et al., and Bettencourt MC et al., [9,24]. They found that the MVD in the tumour area significantly increased with increasing Gleason score and nuclear grade. In the present study, comparing the angiogenic index between grade (mean AI: 143.97) and score (mean AI: 157.65), no statistically significant difference (p-value >0.05) was found.

Gleason grading system is simplified (in low sample size studies) by compressing the scores into three groups: low-grade (score 2-6), intermediate-grade (score 7), high-grade (score 8-10) [17]. In the present study, AI was calculated in various Gleason scores. No studies have been reported to find out AI in prostate cancer so a comparison cannot be done. Other authors have reported MVC values, but there was variation in the values as different methods were used [Table/Fig-12] [10,25]. Microvessel density has not always been an independent prognostic parameter for prostate cancer [26]. Such disparate findings may be due to the use of different antibodies and immunohistochemistry techniques, tumour heterogeneity or the practice of analysing the most vascular portion of the tumour, which introduces a subjective assessment into the analysis. It was also noted, in almost all the sections there was an increased background staining owing to the cytoplasmic staining of tumour cells. This might be due to the presence of angiogenic factors in the cytoplasm of tumour cells.

Author and year of the study	Place of the study	Sample size	Objective of the study	Conclusion
Bigler SA et al., 1993 [22]	University of Washington, USA	15 RP	Develop a method to quantify the relative density of microscopic vessels in PC compared with benign prostatic glandular tissue.	Increased density of capillaries in PC as compared to benign prostatic tissue.
Brawer MK et al., 1994 [18]	University of Washington, USA	32 RP	Study angiogenesis to predict pathologic stage in PC.	MVD‖ is an independent predictor of pathologic stage. Quantification of tumour angiogenesis may help in planning of management strategies.
Bostwick DG et al., 1996 [8]	Mayo Clinic, USA	186 needle biopsy PC	Evaluate the predictive value of MVD‖ in combination with biopsy Gleason Score and preoperative serum PSA concentration for extraprostatic extension.	MVD‖ addition significantly enhance the power of Gleason Score and serum PSA to predict pathologic stage and extraprostatic extension.
Arakawa A et al., 1997 [19]	Methodist Hospital, Texas, USA	101 RP	Assess the importance of MVC as an independent predictor for pathologic stage and progression.	MVC in PC was predictive of pathologic stage and Gleason grade, but not an independent prognostic predictor.
Bettencourt MC et al., 1998 [24]	Bethesda, Maryland	149 RP	Assess the neovascularity of clinically localised PC in an attempt to identify association between angiogenesis and disease progression following RP*.	Significant association between the MVC and nuclear grade, Gleason Score and pathological stage. MVC in the tumour area significantly increased with increasing Gleason Score and nuclear grade.
Silberman MA et al., 2000 [15]	John Hopkins Medical Institutions, Maryland	196 RP	Determine the relationship of MVD‖ with stage at RP (109 cases). Study progression after RP in PC with Gleason score 5 to 7 (87 cases).	MVD‖ was found to be a statistically significant predictor of tumour progression.
Strohmeyer D et al., 2000 [23]	Austria and Germany	98 RP	Correlation of MVD‖ with clinical outcome in patients with prostate cancer of different tumour stages, grades and preoperative PSA values.	Neoangiogenesis correlates with tumour stage, grade and clinical outcome in pT2/pT3 prostate cancer after radical prostatectomy. MVD‖ is the most predictive single independent prognostic factor in PC and therefore of potentially high clinical relevance.
Upadhyay P et al., 2016 [9]	Nepal	65 prostatic biopsies	Assess if there was a significant difference in MVD‖ in benign and malignant lesions of prostate and correlate the vascularity with increasing grade of PC.	Increased MVD‖ was significantly associated with high-grade carcinoma. Therefore, vascular density can be added as one of the indicators for predicting the disease outcome.
Gautam KA et al., 2017 [10]	Lucknow, India	50 benign prostatic hyperplasia, 50 prostate cancer	The diagnostic and prognostic value of VEGF expression level and MVD‖ for PC by comparing benign prostatic hyperplasia and PC groups in freshly diagnosed subjects.	MVD‖ showed higher counting of microvessels in prostate cancer as compared to BPH group. No significant relationship between CD34 MVD staining while VEGF staining score was significantly correlated with Gleason score.
Present Study. 2020	Agra, India	25 (needle biopsy 4 cases, TURP 12 cases, RP 9 cases).	Calculate angiogenic index in PC. Find correlation of AI with Gleason grade and score.	Cases with high Gleason grade and score have a high AI as compared to low Gleason grade and scores.

[Table/Fig-11]: Enumeration of angiogenesis In Prostate Cancer (PC) in various studies [8-10,15,18,19,22-24].
RP: Radical prostatectomy; MVD: Micro vessel density; MVC: Micro vessel count; PSA: Prostate-specific antigen; T: Tumour; BPH: Benign prostatic hyperplasia; CD: Cluster differentiation; VEGF: Vascular endothelial growth factor; AI: Angiogenic index; TURP: Trans urethral resection of prostate

Author and year of the study	Well to moderately differentiated	Intermediate-grade	High-grade	p-value
Pallares J et al., 2006 [25]	Gleason Score 4,5,6 Mean MVC 7 (3-13)		Gleason Score 7, 8, 9, 10 Mean MVC 9 (4-16)	0.006
Gautam KA et al., 2017 [10]	Gleason Score <7 Mean MVD 1.44±0.26		Gleason Score ≥7 Mean MVD 1.47±0.34	0.30
Present study, 2020	Gleason score <7 Mean AI 56.20	Gleason score=7 Mean AI 129.15	Gleason Score 8,9,10 Mean AI 247.35	<0.02

[Table/Fig-12]: Comparison of microvessel count in different studies [10,25].

Limitation(s)

The limitation of the present study was smaller sample size. Also, only a single IHC marker was used due to cost constraints. Follow-up of patients would have been useful to correlate angiogenic index with prognosis.

CONCLUSION(S)

Angiogenic index seems to be more reliable method in quantifying angiogenesis as it minimises the possible variations concerning the width of the microscopic fields, stroma/epithelium relations and cellular tumour size. It can be used especially in TURP and needle biopsy specimens where grading and scoring may not be proper due to small tissue size. Future large-scale studies similar studies should be done to come to a consensus for the prognostic cut-off value of the Angiogenic Index. Further studies should also include the correlation of AI with the clinical outcome of the patients, so that the prediction of the prognosis may be supported by the AI values in prostatic adenocarcinoma cases. The subsequent compilation of similar multi-centric studies may result in the overall better management of prostatic adenocarcinoma patients.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Pathology, Sarojini Naidu Medical College, Agra, Uttar Pradesh, India.
2. Assistant Professor, Department of Pathology, Sarojini Naidu Medical College, Agra, Uttar Pradesh, India.
3. Assistant Professor, Department of Pathology, Sarojini Naidu Medical College, Agra, Uttar Pradesh, India.
4. Professor, Department of Pathology, Sarojini Naidu Medical College, Agra, Uttar Pradesh, India.
5. Professor, Department of Pathology, Sarojini Naidu Medical College, Agra, Uttar Pradesh, India.
6. Associate Professor, Department of Ophthalmology, FH Medical College, Firozabad, Uttar Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Gunjan Prakash,
B-2/22, Kamla Nagar, Agra-282005, Uttar Pradesh, India.
E-mail: gunjanprakash@gmail.com

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