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BPH and prostate disease
Original article

Angiogenesis in prostate cancer and benign prostatic hyperplasia assessed by VEGF and CD-34 IHC: A comparative clinico-pathological study

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Angiogenesis;
Vascular endothelial growth factor;
VEGF;
CD34;
Microvessel density

Abstract

Introduction: Prostate carcinoma is still a dreaded disease wanting more effective treatment and definitive early detection for a better prognosis and cure of life.

Objective: The present study was planned to investigate the correlation of vascular endothelial growth factor (VEGF) expression level and microvessel density (MVD) between the BPH and prostate cancer subjects to analyze their diagnostic and prognostic value.

Subjects and methods: Freshly diagnosed histopathologically confirmed 50 cases of prostate cancer and 50 cases of BPH were included. Expression level of VEGF was measured using Immunohistochemistry (IHC), while MVD was determined via CD34 endothelium-specific antibodies. In the case group, we have also recorded the Gleason's score of prostate cancer and investigated its correlation with angiogenic factor VEGF and MVD CD34.

Results: The study showed a statistically significant difference value of VEGF expression level between the prostate cancer and BPH group ($p < 0.001$). The mean MVD CD34 in the prostate cancer and BPH groups were 29.66 ± 0.21 and 9.96 ± 0.25, respectively. The difference of MVD CD34 expression between the groups was also found significant ($p < 0.001$). VEGF scoring was significantly correlated with Gleason's scores of prostate cancer ($p = 0.005$).

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Conclusions: The present findings may support the assumption that VEGF and CD34 expression level might have an important role in the prediction of prostate cancer as it was significantly differed with BPH. In addition, VEGF expression level showed intense staining in the tissue samples with higher grading of prostate cancer which reveals its importance as prognostic marker.

Introduction

Prostate cancer is the most prevalent cancer worldwide. In the USA, 233,000 new cases of prostate cancer were diagnosed and 29,490 deaths occurred in 2014 alone [1]. According to Indian Council of Medical Research (ICMR) report, 2009, the estimated age-adjusted incidence rates of prostate cancer in India was 3.7/100,000 person during the year 2008. Expected cases of prostate cancer all over India for the periods 2010, 2015 and 2020 were estimated as 26,120, 28,079 and 30,185, respectively. According to United Nations Development Program, 2013, India has been grouped in the category of medium human development index (HDI). Usually, prostate cancer is the most commonly diagnosed cancer of men in very high HDI countries, but as India is also leading toward westernization with respect to life style, as a result the same pattern of prostate cancer is likely to follow that seen in high HDI countries [2].

Prostate specific antigen (PSA) test is contemporary and preliminary screening test for the early detection of prostate cancer, which is released by normal epithelial cells as well as neoplastic epithelial cells [3]. Since, serum PSA level may also be raised in benign prostatic hyperplasia (BPH), therefore PSA is not specific only in the case of prostate cancer [4,5]. PSA levels between 4.1 and 9.9 ng/ml are more challenging for differentiating BPH and prostate cancer patients. The patients of this group are sent for confirming the abnormal PSA levels with repeat test and if confirmed prostate biopsy is performed for histopathological analysis. In addition, prostate biopsy screening results in pain, fever, bleeding, infection, transient urinary difficulties as well as psychological harm of false-positive test results, and over diagnosis.

The U.S. Preventive Services Task Force (USPSTF, 2012) does not recommend PSA based screening test for prostate cancer in grade D patient [6]. Therefore, due to unequivocal results of PSA in prediction of prostatic neoplasia and its behavior, there is an urgent need of other diagnostic markers for the early screening of progressive prostate cancer. The essential characteristic of any tumor to develop and progress is the formation of new blood vessel from the preexisting vasculature known as angiogenesis. Vascular endothelial growth factor (VEGF) is one of the angiogenic factors that induces neovascularization and allow tumor to grow beyond 2–3 mm [7], whereas MVD is a quantitative parameter of angiogenesis. Endothelium-specific antibodies are used for the IHC staining of vessels.

Differences of VEGF expression level and MVD between prostate cancer group and BPH group have been reported, however results are inconclusive. Recently published research articles have observed that expression level of VEGF and MVD were higher at initial prostate biopsies in patients who were later diagnosed with prostate cancer [8]. Therefore, by observing prostate cancer at initial stage, survival rate can be increased as well as efforts to stop metastasis of cancer through blood stream in other vital organ like brain, lungs, kidney or liver where cancer cells may grow and disrupt tissue organization and destroy normal cells, eventually leading to organ failure and death, can be done.

The purpose of the present study was to evaluate the diagnostic and prognostic value of VEGF expression level and MVD CD34 for prostate cancer by comparing BPH and prostate cancer groups in freshly diagnosed subjects.

Subjects and methods

Study subjects

Initially, we screened 255 patients on the basis of serum PSA level $\geq$2 ng/ml, abnormalities found in digital rectal examination (DRE) investigations and abnormal prostate ultrasound report. These subjects were recommended for the trans-rectal ultrasound (TRUS) guided prostate biopsy as well as sextant biopsy sample were stored for the study of VEGF expression and MVD. On basis of histopathological examination we further categorized these subjects into two groups (Case group and control group) which were age and ethnicity matched. The prostate cancer patients were considered as case group and BPH served as control group. A total of 100 subjects were included on the basis of diagnosis in time interval of June 2013 to July 2016, out of which 50 were in case group and 50 in control group. Demographical and clinical information of all the subjects were recorded in a systematic questionnaire. Subjects having any immunodeficiency disease like AIDS and any other debilitating disease like hepatitis or any patient receiving treatment of cancer were excluded from the study.

The study was approved by the Institutional Ethics Committee of the King George's Medical University, Lucknow, U.P., India (Ref. Code: XLII ECM/B-P31). The informed consent was obtained from all the participants prior to sample collection.

Immunohistochemistry

We used the monoclonal antibodies VEGF (Monoclonal mouse anti-human VEGF clone VG1, Dako, Denmark) and CD34 (RE7290-K Novolink Polymer Detection Systems Novocastra, Leica Biosystems, UK) and the sections were stained using the streptavidin-biotin-peroxidase method as per standardized protocol for the VEGF expression and MVD assessment. Briefly, 4-μm-wide histological sections were retrieved from formalin-fixed paraffin-

embedded (FFPE) blocks of tissue and were deparaffinized in xylene, rehydrated through a series of bath of graded alcohols (100%, 90%, 70%, 40%, . . .) for 5 min, immersed in 10 mM Tris and 0.5 M EDTA, pH 9.0 and microwaved twice for 5 min each. Subsequently, the slides were incubated with 0.3% hydrogen peroxidase for 30 min to block endogenous peroxidase activity. The sections were then incubated overnight at 4 °C with the primary antibodies (dilutions: VEGF, 1:30 and CD34, 1:50).

Evaluation of VEGF expression and CD34 MVD levels

VEGF expression was mostly observed in the epithelial cells of the prostate. Cytoplasmic/membranous staining was considered as positive immuno-reactivity to VEGF while fibroblast staining occurred in fewer amounts (less than 5%), therefore, we excluded fibroblast staining from evaluation. The intensity of staining was scored as follows: 0 (no staining), 1 (<10% of staining – mild staining), 2 (10–50% of staining – moderate staining), or 3 (>50% of staining – intense staining). The mean VEGF score was calculated by multiplying the percentage of stained cell by the intensity of the VEGF staining.

Vascularity was evaluated by average numbers of CD34 positive stained vessels. Three areas with the highest number of CD34 MVD staining (hot-spots) were examined with the help of light microscope at 400× magnification. For MVD scoring, the intense vascularized areas on each slide were selected at low magnification, and the microvessels were counted in three non-overlapping regions at high-power magnification (400×). The mean value of MVD for each case was calculated by three MVD counts divided by three. Sections, which showed less than three 'hot-spots' were excluded from further analysis.

Statistical analysis

The data are expressed as the mean ± SD. Statistical analyses were performed by using SPSS 16.0 version for the comparison of demographical characteristics, VEGF score and MVD CD34 values between the two groups. The two-sided Chi-square test was used to establish comparative association between categorical data, and the Spearman rank correlation coefficient was used to characterize the correlation between ordinal and continuous variables. Independent *t*-test was used to compare the data between the two groups. The correlations between the biomarkers were observed using Pearson correlation coefficient. Differences with a $p < 0.05$ were considered significant with 95% confidence intervals.

Results

Clinicopathologic findings

The mean age of the BPH (65.90 ± 8.0, range 48–87 year old) and prostate cancer (68.10 ± 6.9, range 50–81 year old) patients

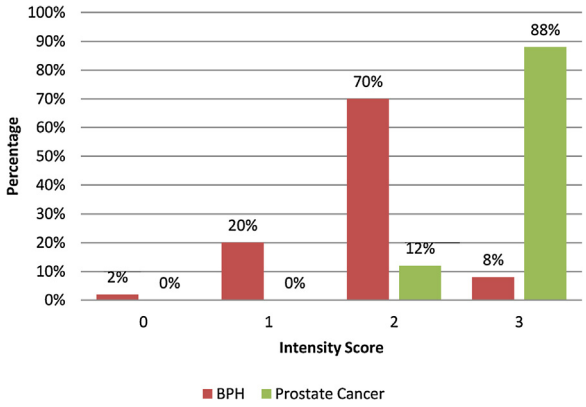

Figure 1 Intensity score of VEGF staining in case (Prostate cancer) and control (BPH) groups. The staining was significantly differed when compared the both groups ($p < 0.001$).

was significantly differed ($p = 0.14$). The mean total serum PSA levels of case group and control group were 48.64 ± 11.68 ng/ml and 7.61 ± 1.27 ng/ml, respectively and their difference was also significantly differed ($p < 0.001$). TNM staging was done at the time of clinical workup of the cases the finding are as under T1N0M0, 4; T2N0M0, 8; T3N0M0, 12; T4N0M0, 16; T4N1M0, 6; and T1N1M1, 4. The Gleason scores of the case group were <7 in 31 (62%) and ≥7 in 19 (38%) patients. The mean Gleason score was 5.98 ± 0.27 ranging from 3 to 10.

Immunohistochemical findings

The percentage of VEGF staining was significantly higher in case group as compared to control group ($p < 0.00001$). In addition, the VEGF score was also significantly differed between the groups ($p < 0.00001$) (Table 1). The staining was observed intense in the case group while control group showed a moderate staining (Fig. 1). In both groups, the subjects are showing either no staining (0) or very rare staining (1) were very few (Fig. 2).

CD34 MVD staining showed a statistically significant difference between the cases and controls ($p < 0.001$) (Table 1). The staining of CD34 positive vessels were relatively abundant in prostate cancer tissue samples while BPH tissue samples were lower in number of positive vessels staining (Fig. 3).

Correlations of VEGF and CD34 with the Gleason's scores of prostate cancer

We did not observe any significant relationship between the CD34 MVD staining and Gleason's scores of prostate cancer ($p = 0.30$). However, VEGF staining scoring was significantly correlated with Gleason's scores of prostate cancer ($p = 0.005$) (Table 2).

Table 1 CD 34 staining (mean ± SE) of two groups.

Staining		BPH ($n = 50$)	Cancer ($n = 50$)	*t*-Test	*p* Value
CD34 staining		9.96 ± 0.25	29.66 ± 0.21	60.238	**<0.001**
VEGF staining	Score %	47.1 ± 26.87	220.9 ± 54.44	16.07	**<0.001**
		21.7 ± 12.11	75.2 ± 16.31	15.83	**<0.001**

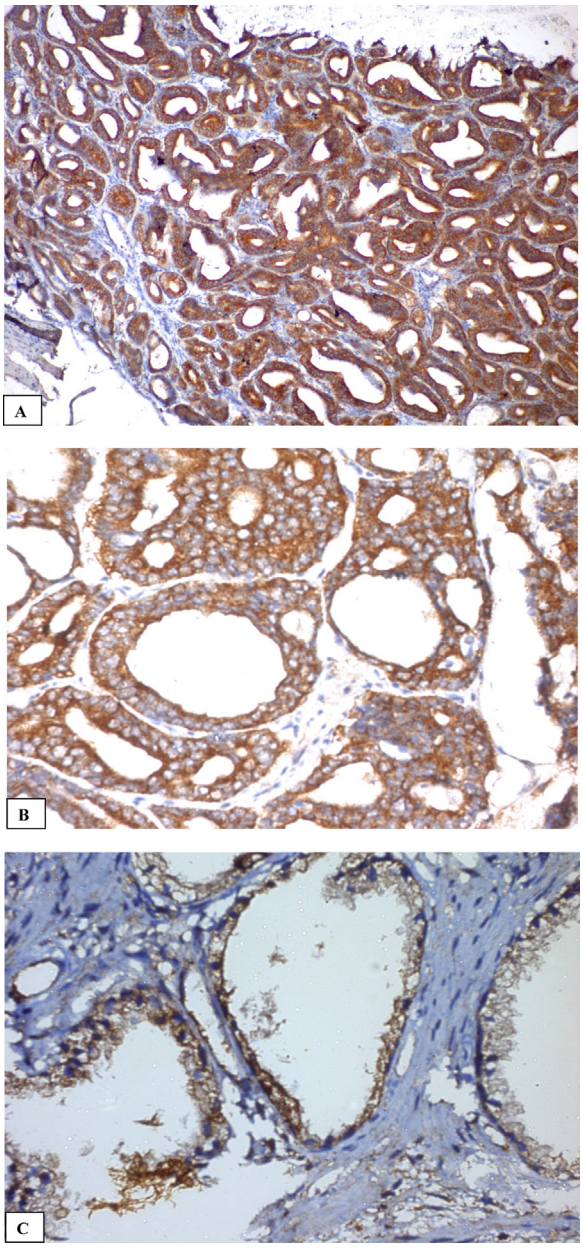

Figure 2 VEGF expression (A) In cytoplasm large number of epithelial cells showing positive VEGF staining in the prostate cancer group (100×); (B) Same microsection at 400×; (C) Showing minimal VEGF staining in cytoplasm of cell lining enlarged glands in the control group. The glands are being looking intervening stroma is fibroblastic and does not show any antibody staining (400×).

Table 2 Distribution of VEGF and CD34 staining results among the Gleason's score and their correlation.

Percentage staining	Gleason's score		*t*-Test	*p* Value
	<7 ($n=31$)	≥7 ($n=19$)		
CD34	1.44 ± 0.26	1.47 ± 0.34	1.05	0.30
VEGF	70.57 ± 14.12	25.98 ± 5.19	3.74	**0.005**

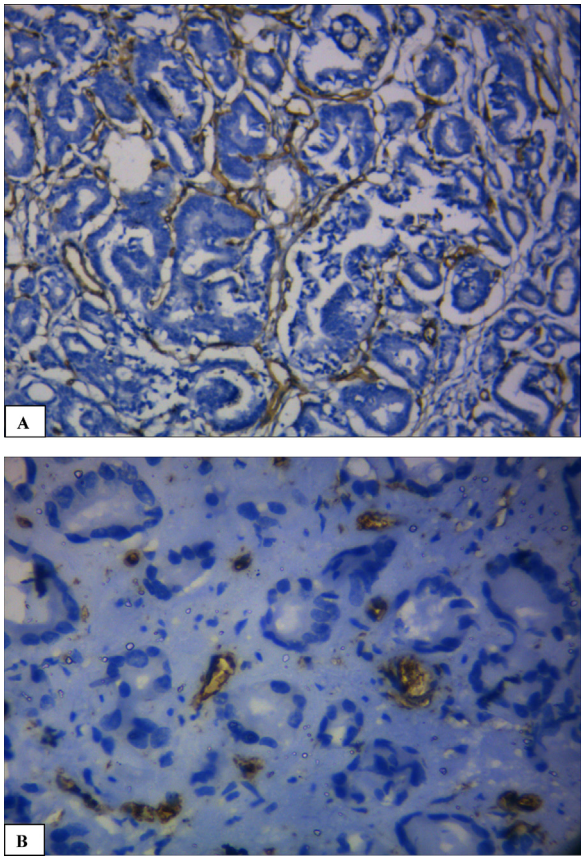

Figure 3 Microvessel density determined by CD34 (A) Networking of MVD in a cancer area of prostate, large number of newly sprouting blood vessel are seen (400×); (B) in benign area of BPH, showing low MVD area (400×).

Correlation of VEGF and CD34 with the PSA levels

We observed the correlation between the examined biomarkers and PSA levels. The correlation between PSA and VEGF has been found to be significant however, the correlation coefficient was very small ($r=0.26$, $p=0.008$). We also observed a correlation between PSA and CD34 MVD ($r=0.33$, $p=0.0008$) but the correlation coefficient was small.

Discussion

PSA-based screening programs helps in the diagnosis of asymptomatic prostate disease which can be benign or malignant but the percentage of men having the asymptomatic prostate cancer is very less moreover such type of cancer either will not progress or will progress so gradually that it would have remained asymptomatic for the man's lifetime [6]. Depending on PSA-based diagnosis, one cannot predict which patient will develop malignant tumor and which will remain with benign tumor [9,10]. On the other hand, prostate cancer develops infrequently and spread quickly to distant site of body, making them potentially life threatening disease. Therefore, we need to search crucial markers whose altered concentration level can give an idea about the invasion and metastasis condition of the tumor. Till date, the search for bonafide marker is inconclusive or with conflicting results.

Angiogenesis is a primary and essential requirement for growth of a tumor. VEGF is a growth factor, which appears to be important for sustaining tumor growth by triggering angiogenesis via binding to specific receptor proteins located on endothelial cells of the prostate and other tissues, thereby, involve in the development of prostatic hyperplasia as well as carcinoma [11,12]. Supporting to previous study, we observed that VEGF expressed in prostate cancer is in significantly higher rates than in BPH [8,13,14]. Furthermore, we observed that quantitative marker of angiogenesis MVD CD34, showed higher counting of microvessels in prostate cancer group as compared to BPH group. Significantly varying levels of MVD in prostate cancer group and BPH tissues have also been observed by several studies [8,13,15].

The Gleason's score is the most significant pathological examination of grading of prostate cancer which provides some general information about the likely behavior of malignancy. When we evaluated staining of angiogenic VEGF marker with Gleason's score <7 and ≥7, a statistically significant result was observed. In accordance to our finding, Kaygusuz (2007) has also observed that VEGF expression level and the Gleason score were closely linked [15]. Prostate cancer patients with high Gleason's score (≥7) showed intense staining of VEGF suggesting higher production of angiogenesis activator (VEGF). In addition, various other studies have also reported that elevated VEGF level was significantly associated with a worse outcome in prostate cancer patients [16]. Contrary to some previous studies, we did not find any significant relationship between expression levels of MVD CD34 and Gleason's score of prostate cancer (<7 and ≥7) [8,17,18].

As India is leading toward western life style, the occurrence of prostate cancer is also increasing due to changes in the life-style as well as in the diet. Further, in India, people do not go for regular check-ups due to very little awareness regarding cancer screening programs. Therefore, there is a very pressing need for the search of markers exclusively based on tumor specific antigen instead of tumor associated antigen, in order to enable the identification of cancer at initial stages because during the late stages, cancer may metastasize in distant parts of the body and form secondary cancer which can progress more aggressively than the primary cancer. Moreover, it is the metastasis tumor rather than primary tumor which is responsible for most of the cancer deaths. Researcher should also closely investigate the role of angiogenesis inhibitors in normal prostate as well as in pathological conditions. It may be possible that imbalance between angiogenesis inhibitors and promoters may lead to cancer formation as well as progression.

The present study included limited number of the cases as well as controls that belonged to north Indian population only therefore; this data cannot be generalized for complete Indian population. This can be attributed as a limitation of the present study. In addition, the present study investigate only angiogenic marker and its effects on prostate cancer, most of studies also included few markers, henceforth, there is a need to study the inter-related action of angiogenesis promoters and inhibitors factors in the tissue and blood samples of normal as well as different pathologic prostate conditions, as these factors can be useful in investigating the actual condition of prostate cancer and recommending suitable treatment accordingly.

Investigated biomarkers VEGF and CD34 may be useful markers as an adjunct in the management of prostate cancer but it has most of the problems associated with PSA. In addition, these are expensive and cumbersome as well as at this stage, these cannot rule out the need of histological diagnosis and prediction for tumor progression. Therefore, more extensive and well designed, large population based studies are needed for establishing their relationship with tumor diagnosis and progression.

Conclusion

The present findings may support the assumption that VEGF and CD34 expression level might have an important role in the prediction of prostate cancer as it was significantly differed with BPH. In addition, VEGF expression level showed intense staining in the tissue samples with higher grading of prostate cancer which reveals its importance as prognostic marker.

Authors' contributions

Conception and design of the study: SNS and AN Srivastav.

Lab work, Acquisition of data, analysis and interpretation of data: KAG and AN Singh.

Drafting the article: KAG.

Revising it critically for important intellectual content: SNS and AN Srivastav.

Ethical committee approval

Institutional Ethics Committee of the King George's Medical University, Lucknow, U.P. India (Ref. Code: XLII ECM/B-P31).

Conflict of interest

The authors indicated no potential conflicts of interest.

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