EVALUATION OF p300 AND VEGF EXPRESSION AND MICROVESSEL DENSITY IN PLASMA CELL MYELOMA

MYELOMDA p300 VE VEGF EKSPRESYONU İLE MİKRODAMAR YOĞUNLUĞUNUN ARAŞTIRILMASI

ANGIOGENESIS RELATED PARAMETERS IN MYELOMA

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Öz

Amaç: Çalışmanın amacı plazma hücreli myelom (PHM)'da anjiogenez ile ilişkili parametrelerden vaskuler endotelyal büyüme faktörü (VEGF) ve p300 ekspresyonu ile mikrodamar yoğunluğunu (MDY) araştırmak idi. Gereç ve Yöntem: PHM tanılı 45 hastaya ait parafine gömülü kemik iliği biyopsilerine immünhistokimyasal olarak p300, VEGF ve Faktör VIII ilişkili antijen (MDY'yi hesaplamak için) uygulandı. Histomorfolojik faktörler, klinik ve laboratuvar bulgular ve toplam sağ kalım (TSK), p300 ekspresyonu, VEGF ekspresyonu ve MDY ile korele edildi. Bulgular: Yüksek (≥%50) VEGF ekspresyonu ile diffüz infiltrasyon patterni ve yüksek plazma hücre infiltrasyon oranı arasında anlamlı ilişki saptandı (p=0.014, p=0.002, sırası ile). Yüksek MDY, diffüz infiltrasyon patterni ve artmış CRP düzeyi ile direkt korelasyon göstermekteydi (p=0.026, p=0.015, sırası ile). p300, VEGF ve MDY'ye göre sağ kalanların yüzdesi ve TSK açısından anlamlı bir fark saptanmadı. p300, VEGF ve MDY arasında anlamlı ilişki gözlenmedi. Tartışma: Bulgularımız, kemik iliği infiltrasyon paterni ve neoplastik hücrelerin miktarı ile MDY ve VEGF ekspresyonu arasında anlamlı ilişki olduğunu ortaya koymuştur. p300'ün histomorfolojik prognostik parametreler, MDY, VEGF ekspresyonu ve TSK ile ilişkisi olmadığı saptanmakla birlikte, bildiğimiz kadarıyla bu çalışma, PHM'li hastalarda p300 ekspresyonunun potansiyel prognostik rolünü araştıran ilk çalışma olma özelliği taşımaktadır. Sonuç olarak, anjiyogenez ile myelom hücreleri arasındaki etkileşimin yanı sıra PHM'de artmış kemik iliği anjiyogenezisinin prognostik rolünün daha kapsamlı çalışmalarla aydınlatılması gerekmektedir.

Anahtar Kelimeler

Plazma Hücreli Myelom; p300; CREB; Anjiyogenez; VEGF; İmmünohistokimya

Abstract

Aim: The aim of the study was to evaluate angiogenesis-related parameters including vascular endothelial growth factor (VEGF), p300 expression, and microvessel density (MVD) in plasma cell myeloma (PCM). Material and Method: p300, VEGF, and Factor VIII related antigen (for measurement of MVD) were applied to the paraffin-embedded bone marrow sections of 45 patients with PCM, immunohistochemically. Histomorphologic factors, clinical and laboratory findings, and overall survival (OS) were correlated with p300 and VEGF expression, and MVD. Results: VEGF overexpression (≥50%) was significantly associated with diffuse infiltration pattern and high percentage of plasma cell infiltration (p=0.014, p=0.002, respectively). Higher MVD was directly correlated with diffuse infiltration pattern and increased CRP levels (p=0.026, p=0.015, respectively). No significant difference was noted in percentage of survivors and OS with respect to p300, VEGF, and MVD. No significant correlation was noted between p300 expression, VEGF expression, and MVD. Discussion: Our findings revealed significant association of bone marrow infiltration pattern and quantity of neoplastic cells with MVD and VEGF expression. Although no association of p300 was shown with histomorphologic prognostic parameters, MVD, VEGF expression, or OS, this is the first study investigating the potential prognostic role of p300 expression in PCM, to the best of our knowledge. In conclusion, more-comprehensive studies are needed to further elucidate the interaction between angiogenesis and myeloma cells as well as the prognostic role of increased bone marrow angiogenesis in PCM.

Keywords

Plasma Cell Myeloma; p300; CREB; Angiogenesis; VEGF; Immunohistochemistry

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Introduction

Plasma cell myeloma (PCM) is a haematological malignancy characterized by accumulation of clonal malignant plasma cells predominantly within the bone marrow (BM) [1]. The role of proliferation, apoptosis, and angiogenesis-related defects has been considered in the pathogenesis of PCM, while a very heterogeneous prognosis of the disease necessitates assessment of prognostic factors and prognostic stratification for implementation of individualized targeted therapy [2].

Progressive increase in the bone marrow microvessel density (MVD) is a prominent feature of active PCM, while along with increased angiogenic cytokine expression it has been associated with pathogenesis and progression of PCM as well as survival [1].

Although the exact mechanisms regulating the progressive increase in angiogenesis in PCM remain unclear, expression of proangiogenic molecules such as vascular endothelial growth factor (VEGF) with stimulatory effects on endothelial cell growth and microvascular permeability is considered to drive tumor related angiogenesis and to influence critical steps of myeloma pathogenesis and progression [3-8].

The hypoxic microenvironment of bone marrow in PCM induces tumor angiogenesis via expression of hypoxia-inducible transcription factor 1 alpha (HIF-1 α) that leads to the upregulation of proangiogenetic VEGF and to subsequent increase in the bone marrow angiogenesis and thus growth of myeloma cells [9-11].

In hypoxic conditions, HIF-1 α can also interact with its transcriptional coactivators including p300 and cyclic AMP response element-binding (CREB) protein (CBP), highly homologous proteins that are required for the optimal function of HIF-1α transcription machinery and subsequent angiogenesis. They have also been shown to play a role in tumorigenesis [11-12]. To our knowledge no data exist investigating the potential prognostic role of p300 expression in patients with PCM.

The present study was designed to evaluate angiogenesis-related immunohistochemical parameters including VEGF, MVD, and p300 in newly-diagnosed PCM patients in terms of their potential association with clinicopathological and laboratory parameters, histomorphologic prognostic factors, and survival.

Material and Method

Study population

After obtaining ethics committee approval from Gazi University School of Medicine and informed consents, 76 consecutive patients with newly-diagnosed PCM between 2001 and 2010 at Gazi University School of Medicine were enrolled. 45 of 76 patients [mean (SD) age: 58.6(10.8) years; 62.2% were males] who had available clinical data and paraffin-embedded tissues for the immunohistochemical analysis of p300 expression, VEGF expression, and MVD were included in this study.

Assessments

Data on patient demographics, disease characteristics including clinical stage (I-III), risk group (low, moderate, high), and laboratory findings [serum M protein (IgG, IgA, IgD, kappa, lambda, non-secretory), light chain (kappa, lambda), hemoglobin (g/dL), WBC (e3/IU), platelet count (e3/IU), serum calcium (mg/dL), serum creatinine (mg/dL), β2-microglobulin (g/dL), serum albumin (g/dL), C-reactive protein (CRP; mg/L), lactate dehydrogenase (LDH; U/L), erythrocyte sedimentation rate (ESR; mm/h), proteinuria (g/24 h) and presence of urinary M protein] were retrieved from medical records at initial diagnosis. The clinical stage of the patients was classified according to the Durie and Salmon's criteria [13]. Risk groups were examined according to the classification of Bataille et al. depending on serum CRP and beta-2 microglobulin (β2M) levels [14].

Formalin-fixed paraffin-embedded bone marrow specimens were re-evaluated in terms of histomorphologic factors and immunohistochemical analysis for p300 expression, VEGF expression, and MVD. Demographic, clinical and laboratory parameters, as well as overall survival were evaluated with respect to level of p300 expression, VEGF expression, and MVD.

Histopathological examination

Hematoxylin and eosin- and CD138-stained sections of bone marrow prepared at the time of initial diagnosis were obtained from the archive of the Department of Pathology and re-evaluated for identification of plasmacytic differentiation and immunohistological assessment of plasma cell morphology, the percentage of plasma cell infiltration (<20%; 20-49% and ≥50%), and the histological infiltration pattern.

Plasma cell morphology was cytologically sub-graded according to level of differentiation into low-grade (characterized by predominantly small cells), intermediate-grade (with predominantly cleaved, polymorphous, asynchronous cells), or high-grade (where most tumor cells are plasmablastic) cells as defined by Bartl [15].

The histological infiltration pattern was classified as interstitial (interstitial or interstitial and sheet-like), nodular (interstitial nodular or nodular), or diffuse (diffuse or sarcomatoid) infiltration pattern [15].

Immunohistochemical analysis

For immunohistochemical staining, 3- to 4-μm thick tissue sections were cut on slides from formalin-fixed, paraffin-embedded bone marrow biopsies of the patients. The slides were deparaffinized for 2 hours in a 60°C oven, and stained for Factor VIII related antigen (vonWillebrand factor Ab-2; 1:100 dilution, clone F8/86, mouse monoclonal, Neomarkers, USA), VEGF (1:50 dilution, clone VG1, mouse monoclonal, IgG1 Kappa, Thermo Scientific, USA), p300 (1:50 dilution, clone Sc-585, rabbit polyclonal, IgG, Santa Cruz, USA), and CD138 antibody (1:40 dilution, rabbit polyclonal, IgG, ThermoFisher Scientific, Rockford, USA) in an automatised stainer (Ventana automated immunostainer Discovery XT, Ventana Medical Systems Inc., Tucson, USA) by using established protocols. Immunostaining was completed with the diaminobenzidine (DAB) detection kit (Ventana), which uses a streptavidin-biotin technique and hematoxylin as a counterstain. Sections of breast invasive ductal carcinoma for p300, angiosarcoma for VEGF, placenta for Factor VIII, and tonsil for CD138 were used as positive controls. For negative controls, the primary antibodies were omitted. Cytoplasmic staining was considered as positive for Factor VIII and VEGF; nuclear staining was considered as positive for p300; and membranous staining was considered as positive for CD138. Each slide was evaluated

under a light microscope (BX53, Olympus, Tokyo, Japan) by two referral pathologists (SS, NA) blinded to the cases.

The measurement of MVD was determined immunohistochemically with Factor VIII. Any positively stained endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered as a single, countable microvessel. Slides stained with antibodies to Factor VIII antigen/von Willebrand complex were first scanned with light microscopy under low power (×10 objective) to identify areas with the greatest number of microvessels (hot spots). The microvessels (capillaries and small venules) at hot spots were counted at one high power field (HPF) (x40 objective with eyepiece diameter of 0.65 mm) of the microscope. Then, MVD was counted as the average number of vessels in the three hot spots. Microvessels were identified as endothelial cells either single or clustered in nests or tubes, clearly separated from one another, with or without lumen not exceeding 10 micrometers in transverse diameter. Larger vessels and vessels in the periosteum were excluded. MVD was categorized into two groups based on the number of microvessels including low (<20 microvessels/HPF) and high (≥20 microvessels/HPF) MVD.

The p300 (low expression: <30%; high expression: ≥30%) and VEGF (low expression: <10%; moderate expression: 10-49%; high expression: ≥50%) expressions were evaluated based on the percentage of positive plasma cells.

Statistical analysis

Statistical analysis was conducted using SPSS software, version 15.0. Chi-square (x^2) and Fisher's exact tests for the comparison of categorical data, while numerical data were analyzed using Student t-test and ANOVA. Spearman correlation analysis was used to analyze correlation between immunohistochemical parameters. Survival analysis was made via Kaplan-Meier analysis and comparisons were made via Log-Rank test. Data were expressed as "mean (standard deviation, SD)", minimum-maximum, and percent (%) where appropriate. p<0.05 was considered statistically significant.

Results

Demographic and clinical characteristics

Most of the patients had clinical stage II-III disease (82.2%) with intermediate-grade cytology (62.2%), diffuse type of infiltration pattern (44.4%), and plasma cell infiltration of ≥50% (68.9%) and were categorized into the moderate risk group (50.0%) (Table 1).

Overall, mean (SD) MVD score was 23.7(13.2, ranged 3 to 54) microvessels/HPF, while MVD score was <20 microvessels/HPF in 19(42.2%) patients, and ≥20 microvessels/HPF in 26(57.8%) patients (Fig 1).

Mean (SD) extent of VEGF expression was 64.2 (29.2, ranged 5 to 100)%, while VEGF expression was <10%, 10-49% and ≥50% in 3(6.7%), 11(24.4%) and 31(68.9%) patients, respectively (Fig 2).

Mean (SD) p300 expression was 38.7 (31.8, ranged 0 to 90)%; while p300 expression was <30% in 17(37.8%) and ≥30% in 28(62.2%) patients (Fig 3).

No significant difference was noted in patient demographics, clinical stage, histomorphologic prognostic factors, or risk group in terms of p300 expression (Table 1).

Higher (≥50%) than lower (<50%) VEGF expression (85.0% vs. 15.0%, p=0.014) and higher (≥20 microvessels/HPF) than lower (<20 microvessels/HPF) MVD scores (80% vs. 20%, p=0.026) were more likely seen in patients with diffuse type plasma cell infiltration pattern (Table 1).

Higher (≥50%) than lower (<50%) VEGF expression (83.9% vs. 16.1%, p=0.002) was more commonly noted in patients with high (≥50%) percentage of plasma cell infiltration. No significant difference was noted in patient demographics, clinical stage, pattern of plasma cell infiltration, risk group in terms of level of VEGF, and MVD score (Table 1).

Survival

The survival analysis was performed for 42 patients. After median (min-max) 17 months (ranged 2 to 94 months) of follow-up, duration of overall survival was median 65.0 months (ranged 25.9 to 104.1 months) and 29 of 42 (69.0%) patients were still alive at the time of study. (Table 1, Fig 4).

No significant difference was noted in percentage of survivors and median duration of OS with respect to p300, VEGF, and MVD (Table 1, Fig 4).

Laboratory parameters

Higher (≥20 microvessels) than lower (<20 microvessels) MVD scores (78.3% vs. 21.7%, p=0.015) were more likely seen in patients with CRP levels of ≥6 mg/L (Table 2).

None of the laboratory parameters showed a significant change with respect to p300 or VEGF. Apart from CRP, no significant change was observed in laboratory parameters also with respect to MVD (Table 2).

Correlation between p300 expression, VEGF expression, and MVD score

No significant correlation was noted between p300 expression, VEGF expression, and MVD score (Table 3).

Discussion

Our findings revealed association of diffuse plasma cell infiltration pattern with increased VEGF expression and higher MVD scores in patients with PCM. Also, higher percentage of plasma cell infiltration and higher CRP levels were associated with increased VEGF expression and higher MVD scores, respectively. p300 expression was not associated with any of the prognostic factors related to plasma cell infiltration, while none of the angiogenesis-related immunohistochemical parameters was associated with cytological grade or survival.

PCM is the first haematological malignancy in which a significant correlation of angiogenesis with prognosis and survival has been identified [16]. Bone marrow MVD was shown to be significantly increased in PCM compared to monoclonal gammopathy of undetermined significance (MGUS) and also in active versus non-active myeloma [1,17] as well as in stage II–III versus stage I myeloma [3].

The type of infiltration pattern is considered to reflect the stage of disease with identification of interstitial and nodular patterns when hematopoiesis is still preserved, unlike occurence of diffuse inflitration with disease progression that results in the supression of hematopoiesis [18].

Table 1. Demographic and clinical characteristics with respect to p300 expression, VEGF expression, and MVD score categories (n=45)

	p300 expression			VEGF expression				MVD score (microvessel)			Total (n=45)
	<%30 (n=17)	≥%30 (n=28)	p value	<%10 (n=3)	10-49% (n=11)	≥%50 (n=31)	p value	<20 (n=19)	≥20 (n=26)	p value	
Age, mean(SD)	59.8(11.9)	57.9(10.2)	0.593a	51.0(23.3)	64.5(10.8)	57.2(10.1)	0.066b	58.6(11.7)	58.6(0.3)	0.991a	58.6(10.8)
Age group, n(%)											
<50	2(22.2)	7(77.8)	0.447	2(22.2)	1(11.1)	6(66.7)	0.142	3(33.3)	6(66.7)	0.712	9(20.0)
≥50	15(41.7)	21(58.3)		1(2.8)	10(27.8)	25(69.4)		16(44.4)	20(55.6)		36(80.0)
Gender, n(%)											
Female	7(41.2)	10(58.8)	0.961	2(11.8)	4(23.5)	11(64.7)	0.674	9(52.9)	8(47.1)	0.410	17(37.8)
Male	10(35.7)	18(64.3)		1(3.6)	7(25.0)	20(71.4)		10(35.7)	18(64.3)		28(62.2)
Clinical stage, n(%)											
Stage I	5(62.5)	3(37.5)	0.204	1(12.5)	1(12.5)	6(75.0)	0.455	5(62.5)	3(37.5)	0.236	8(17.8)
Stage II-III	12(32.4)	25(67.6)		2(5.4)	10(27.0)	25(67.6)		14(37.8)	23(62.2)		37(82.2)
Plasma cell differentiation, n(%)											
Low grade	6(40.0)	9(60.0)	0.764	1(6.7)	5(33.3)	9(60.0)	0.841	5(33.3)	10(66.7)	0.762	15(33.3)
Intermediate grade	11(39.3)	17(60.7)		2(7.1)	6(21.4)	20(71.4)		13(46.4)	15(33.3)		28(62.2)
High grade	0(0.0)	2(100.0)		0(0.0)	0(0.0)	2(100.0)		1(50.0)	1(50.0)		2(4.4)
Pattern of plasma cell infiltration, n(%)											
Interstitial	5(35.7)	9(64.3)	0.132	3(21.4)	6(42.9)	5(35.7)	0.014	8(57.1)	6(42.9)	0.026	14(31.1)
Nodular	7(63.6)	4(36.4)		0(0.0)	2(18.2)	9(81.8)		7(63.6)	4(36.4)		11(24.4)
Diffuse	5(25.0)	15(75.0)		0(0.0)	3(15.0)	17(85.0)		4(20.0)	16(80.0)		20(44.4)
Percentage of plasma cell infiltration, n(%)											
<20%	2(50.0)	2(50.0)	0.796	1(25.0)	1(25.0)	2(50.0)	0.002	2(50.0)	2(50.0)	0.082	4(8.9)
20-49%	3(30.0)	7(70.0)		2(20.0)	5(50.0)	3(30.0)		7(70.0)	3(30.0)		10(22.2)
≥50%	12(38.7)	19(61.3)		0(0.0)	5(16.1)	26(83.9)		10(32.3)	21(67.7)		31(68.9)
Risk group, n(%)											
Low	3(42.9)	4(57.1)	0.373	1(14.3)	1(14.3)	5(71.4)	0.663	3(42.9)	4(57.1)	0.659	7(25.0)
Moderate	5(35.7)	9(64.3)		1(7.1)	5(35.7)	8(57.1)		5(35.7)	9(64.3)		14(50.0)
High	5(71.4)	2(28.6)		0(0.0)	1(14.3)	6(85.7)		1(14.3)	6(85.7)		7(25.0)
Survival, n(%)											
Survivor	13(44.8)	16(55.2)	0.305	3(10.3)	78(24.1)	19(65.5)	0.153	14(48.3)	15(51.7)	0.236	29(69.0)
Non-survivor	4(30.8)	9(69.2)		0(0.0)	2(15.4)	11(84.6)		4(30.8)	9(69.2)		13(31.0)
Overall survival (month), median (95 %CI)	NA	43(40-43)	0.4626c	NA	NA	65(40-65)	0.6314c	NA	65(40-65)	0.1708c	65(25.9-104.1)

MVD: microvessel density; VEGF: Vascular endothelial growth factor
x2 test, aStudent t-test, b ANOVA, cLog-Rank Test,

High percentage of plasma cells infiltration in bone marrow has been suggested to be a reliable predictor of relapse in PCM patients [19], while advanced grade plasma cell infiltration pattern, reflecting a high tumor burden, has been associated with high plasma cell count and poor prognosis [20].

VEGF activity was shown to be associated with tumor grade and prognosis in PCM, while in vivo inhibition of VEGF-induced angiogenesis by antibodies against VEGF was reported to result in suppression of tumor growth [8,21]. Also, high tumor burden and diffuse pattern of infiltration were reported to be associated with higher MVD along with a highly significant correlation between MVD and histologic grade of tumor, extent of bone marrow infiltration, proliferative activity, and treatment response in patients with PCM [22-24].

Hence, association of diffuse plasma cell infiltration pattern both with increased VEGF expression and higher MVD score and association of the high percentage of plasma infiltration with increased VEGF expression in our cohort seem to indicate the role of angiogenesis in promoting disease progression in PCM and supporting the association of increased angiogenesis with advanced PCM [21].

A high clinical stage/cytological grade in PCM has been associated with higher rate of proliferation, higher intratumoral vascularity, and increased VEFG in the neoplastic cells as well as increased MVD [2,8]. However, unlike other plasma cell related prognostic factors including type of bone marrow infiltration and quantity of neoplastic cells, the morphology of the cells was not associated with any of the angiogenesis-related immunohistochemical parameters in our cohort. This seems to be associated with inability to perform appropriate analysis due to observation of high cytological grade only in 2 patients in the overall cohort.

Table 2. Laboratory findings with respect to p300 expression, VEGF expression, and MVD score categories (n=45)

	p300 expression			VEGF expression				MVD score (microvessel)			Total (n=45)
	<%30 (n=17)	≥%30 (n=28)	P value	<%10 (n=3)	10-49% (n=11)	≥%50 (n=31)	p value	<20 (n=19)	≥20 (n=26)	p value	
Serum M protein											
IgG	10(35.7)	18(64.3)	0.0981	3(10.7)	8(28.6)	17(60.7)	0.6971	11(39.3)	17(60.7)	0.2751	28(62.2)
IgA	2(25.0)	6(75.0)		0(0.0)	2(25.0)	6(75.0)		4(50.0)	4(50.0)		8(17.8)
IgD	0(0.0)	1(100.0)		0(0.0)	1(100.0)	0(0.0)		1(100.0)	0(0.0)		1(2.2)
Kappa	4(100.0)	0(0.0)		0(0.0)	0(0.0)	4(100.0)		3(75.0)	1(25.0)		4(8.9)
Lambda	1(33.3)	2(66.7)		0(0.0)	0(0.0)	3(100.0)		0(0.0)	3(100.0)		3(6.7)
Non-secretory	0(0.0)	1(100.0)		0(0.0)	0(0.0)	1(100.0)		0(0.0)	1(100.0)		1(2.2)
Light chain											
Kappa	12(50.0)	12(50.0)	0.1662	0(0.0)	8(33.3)	16(66.7)	0.0931	12(50.0)	12(50.0)	0.4871	24(54.5)
Lambda	5(25.0)	15(75.0)		3(15.0)	3(15.0)	14(70.0)		7(35.0)	13(65.0)		20(45.5)
Hemoglobin											
≥10.0 g/dL	7(43.8)	9(56.3)	0.8061	1(6.3)	2(12.5)	13(81.3)	0.7461	8(50.0)	8(50.0)	0.5621	16(35.6)
8.5-10.0 g/dL	5(38.5)	8(61.5)		1(7.7)	4(30.8)	8(61.5)		6(46.2)	7(53.8)		13(28.9)
<8.5 g/dL	5(31.3)	11(68.8)		1(6.3)	5(31.3)	10(62.5)		5(31.3)	11(68.8)		16(35.6)
WBC											
<4500 e3/IU	3(37.5)	5(62.5)	1.001	0(0.0)	2(25.0)	6(75.0)	1.001	4(50.0)	4(50.0)	0.6931	8(17.8)
4500-11.000 e3/IU	13(39.4)	20(60.6)		3(9.1)	8(24.2)	22(66.7)		14(42.4)	19(57.6)		33(73.3)
>11.000 e3/IU	1(25.0)	3(75.0)		0(0.0)	1(25.0)	3(75.0)		1(25.0)	3(75.0)		4(8.9)
Platelet count											
<150.000 e3/IU	6(46.2)	7(53.8)	0.5111	1(7.7)	3(23.1)	9(69.2)	1.001	4(30.8)	9(69.2)	0.5102	13(28.9)
150.000-400.000 e3/IU	11(34.4)	21(65.6)		2(6.3)	8(25.0)	22(68.8)		15(46.9)	17(53.1)		32(71.1)
Serum calcium											
<12 mg/dL	15(38.5)	24(61.5)	1.001	3(7.7)	10(25.6)	26(66.7)	0.6711	16(41.0)	23(59.0)	1.001	39(90.7)
≥12 mg/dL	2(50.0)	2(50.0)		0(0.0)	0(0.0)	4(100.0)		2(50.0)	2(50.0)		4(9.3)
Serum creatinine											
<2 mg/dL	15(39.5)	23(60.5)	0.6931	3(7.9)	10(26.3)	25(65.8)	0.7941	17(44.7)	21(55.3)	0.6811	38(84.4)
≥2 mg/dL	2(28.6)	5(71.4)		0(0.0)	1(14.3)	6(85.7)		2(28.6)	5(71.4)		7(15.6)
Beta2 microglobulin											
<3.5 g/dL	9(50.0)	9(50.0)	0.3341	2(11.1)	5(27.8)	11(61.1)	0.7031	11(61.1)	7(38.9)	0.1861	18(62.1)
3.5-5.5 g/dL	2(40.0)	3(60.0)		0(0.0)	0(0.0)	5(100.0)		2(40.0)	3(60.0)		5(17.2)
>5.5 g/dL	5(83.3)	1(16.7)		0(0.0)	1(16.7)	5(83.3)		1(16.7)	5(83.3)		6(20.7)
Serum albumin											
≥3.5 g/dL	11(40.7)	16(59.3)	0.7571	2(7.4)	6(22.2)	19(70.4)	0.8811	12(44.4)	15(55.6)	0.7661	27(60.0)
<3.5 g/dL	6(33.3)	12(66.7)		1(5.6)	5(27.8)	12(66.7)		7(38.9)	11(61.1)		18(40.0)
CRP											
<6 mg/L	5(35.7)	9(64.3)	1.001	1(7.1)	4(28.6)	9(64.3)	1.001	9(64.3)	5(35.7)	0.0151	14(37.8)
≥6 mg/L	9(39.1)	14(60.9)		1(4.3)	6(26.1)	16(69.6)		5(21.7)	18(78.3)		23(62.2)
LDH											
<243 U/L	12(40.0)	18(60.0)	0.7161	3(10.0)	9(30.0)	18(60.0)	0.4431	15(50.0)	15(50.0)	0.0851	30(73.2)
≥243 U/L	3(27.3)	8(72.7)		0(0.0)	2(18.2)	9(81.8)		2(18.2)	9(81.8)		11(26.8)
ESR											
≤20 mm/h	0(0.0)	1(100.0)	1.001	0(0.0)	0(0.0)	1(100.0)	1.001	0(0.0)	1(100.0)	1.001	1(3.3)
20 mm/h	12(41.4)	17(58.6)		2(6.9)	7(24.1)	20(69.0)		13(44.8)	16(55.2)		29(96.7)
Bence Jones proteinuria											
<1 g/24 h	9(37.5)	15(62.5)	1.001	2(8.3)	6(25.0)	16(66.7)	1.001	10(41.7)	14 (58.3)	0.7241	24(64.9)
≥1 g/24 h	5(38.5)	8(61.5)		1(7.7)	3(23.1)	9(69.2)		4(30.8)	9(69.2)		13(35.1)
Urinary M protein											
Negative	4(26.7)	11(73.3)	0.4721	2(13.3)	2(13.3)	11(73.3)	0.3841	5(33.3)	10(66.7)	1.001	15(46.9)
Positive	7(41.2)	10(58.8)		0(0.0)	3(17.6)	14(82.4)		6(35.3)	11(64.7)		17(53.1)

Data are shown as n(%). CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; LDH: Lactate dehydrogenase; MVD: microvessel density; VEGF: Vascular endothelial growth factor; WBC: White blood cell
1Fisher Exact test, 2 Chi-Square Yates Continuity Correction

Table 3. Correlation between p300 expression, VEGF expression, and MVD score.

		p300 expression	VEGF expression	MVD score
p300 expression	r	1,000	-,128	,280
	p	.	,403	,063
	n	45	45	45
VEGF expression	r	-,128	1,000	,208
	p	,403	.	,171
	n	45	45	45
MVD score	r	,280	,208	1,000
	p	,063	,171	.
	n	45	45	45

MVD: microvessel density; r: rho correlation coefficient; VEGF: Vascular endothelial growth factor
Spearman correlation analysis

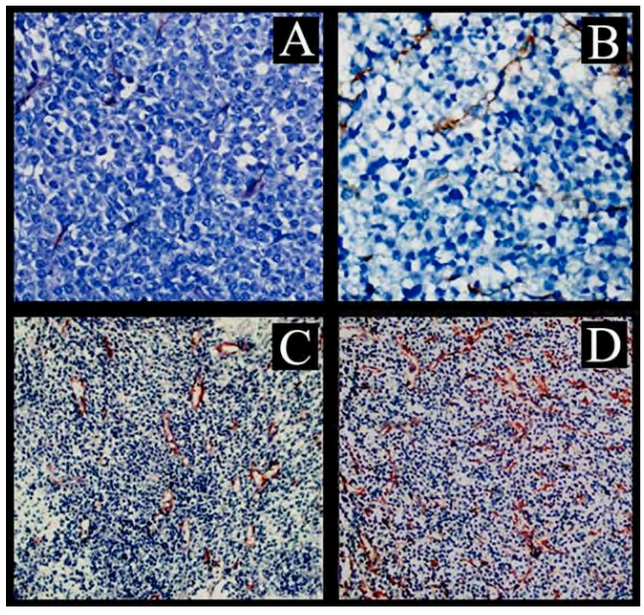

Fig 1. Bone marrow microvessel density (MVD) in PCM cases A-B) <20 microvessel/HPF (Factor VIII related antigen, original magnification x400), C-D) ≥20 microvessel/HPF (Factor VIII related antigen, original magnification x200).

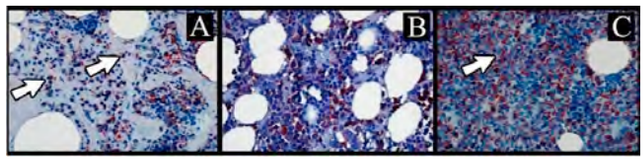

Fig 2. VEGF expression in PCM cases A) <10%, arrows: perinuclear halos in plasma cells (original magnification x400), B) 10-49% (original magnification x400), C) ≥50% (original magnification x400).

Association of higher MVD scores with higher CRP levels in our cohort seems notable given the consideration of both MVD and serum CRP levels as the best variables that predict event-free and overall survival among patients with PCM [22]. This also emphasizes the likelihood of CRP release in response to angiogenic cytokines, given the positive feedback between VEGF and angiogenic cytokines such as IL-6 to promote angiogenesis in proliferating myeloma cells [8].

Providing data on p300 expression for the first time in the literature, our findings revealed high (≥30%) amount of p300 expression in more than half of the patients with PCM, whereas there was no association of p300 expression with clinical or prognostic parameters. Hence, the potential prognostic role

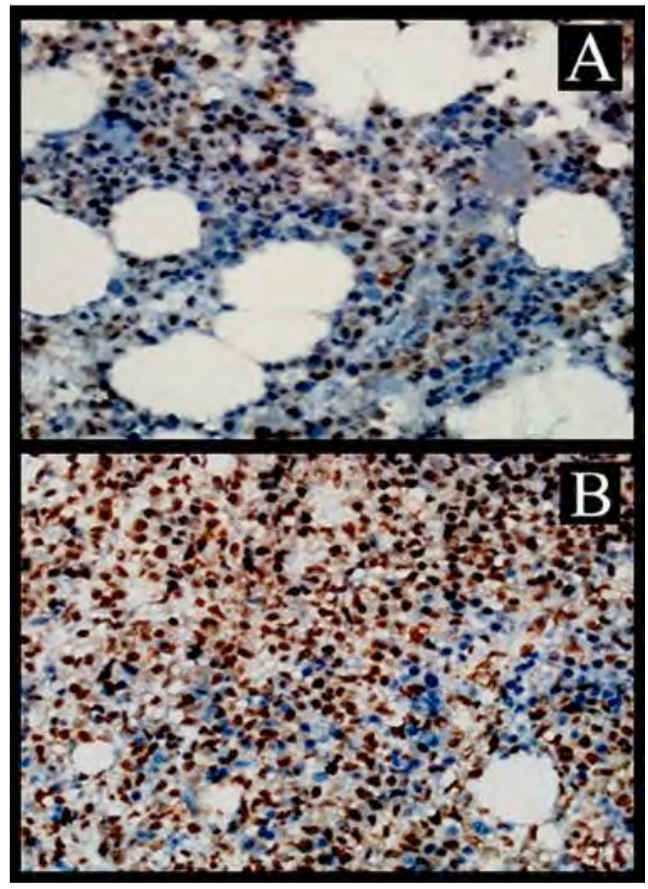

Fig 3. p300 expression in PCM cases A) <30% (original magnification x400). B) ≥30% (original magnification x400).

of increased p300 expression in patients with PCM seems to be justified in larger scale studies. Our findings also revealed no correlation between VEGF expression, p300 expression, and

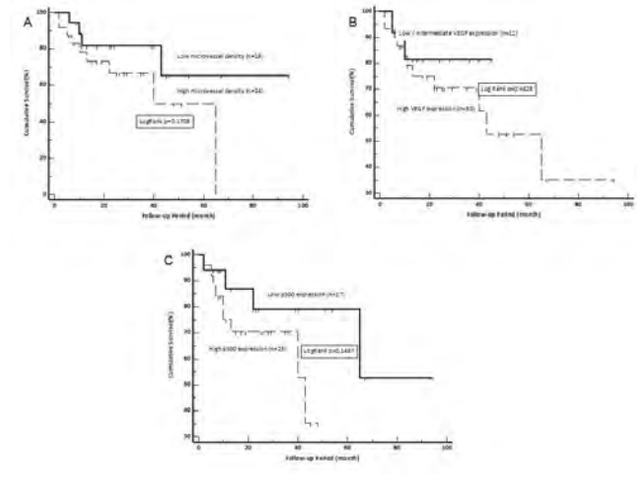

Fig 4. The Kaplan-Meier overall survival curves according to A) MVD B) VEGF expression and C) p300 expression.

MVD. Similarly to our findings, no correlation between the level of bone marrow MVD and plasma levels of VEGF was shown in another study [25]. Besides, high MVD scores were demonstrated in patients both with high and low VEGF expression, indicating the potential role of other angio-regulatory factors in the neo-vascular growth process [8]. Nonetheless, there is inconsistency regarding the correlation of bone marrow angio-

genesis and regulatory factors, and a significant correlation between higher VEGF expression and an increase in the MVD of tumor tissues was also indicated in malignant plasma cell neoplasms [25].

The extent of bone marrow angiogenesis has been reported as a strong indicator of biological potency of malignant clone and a predictor of survival in newly-diagnosed patients with PCM [22-23]. Increased bone marrow MVD was shown to be a significant poor-prognosis factor for survival in patients with newly-diagnosed PCM [6,24].

However, despite a statistically significant relationship between VEGF expression and the OS, expression of VEGF was not considered to be an independent prognostic factor in a past study among PCM patients [8]. Also no significant difference was reported between VEGF-positive and VEGF-negative patients with PCM in terms of overall survival in another study [24]. Bone marrow MVD was not found to be significantly correlated with OS both at diagnosis and prior to transplant [3] and no correlation was noted between baseline bone marrow MVD and PFS and OS in PCM patients [25]. Lack of any association between increased angiogenesis and poorer survival was also reported in another study among patients with advanced PCM [8].

Patients with well differentiated plasma cells and low tumor burden with <50% of bone marrow tumor infiltrates were estimated to have favorable prognosis [18]. However, despite their association with type of bone marrow infiltration and quantity of neoplastic cells, VEGF or MVD had no association with morphology of the plasma cells and had no impact on survival in our cohort. The lack of a statistically significant association of overall survival with VEGF expression and MVD scores in our cohort might be attributed to the potential contribution of angiogenic factors other than VEGF, the small number of cases in study groups as well as in high-grade cytology group along with the influence of different treatment methods and co-morbid disorders.

Conclusions

In conclusion, our findings revealed significant association of bone marrow infiltration pattern and quantity of neoplastic cells but not the morphology of the cells with the angiogenesis-related immunohistochemical parameters. Increased VEGF expression was associated with diffuse type of bone marrow infiltration pattern and high percentage of plasma cell infiltration, while higher MVD score was associated with diffuse type infiltration pattern and increased CRP levels. To the best of our knowledge, this is the first study to date investigating the potential prognostic role of p300 expression in patients with PCM. No association of p300 was shown with histomorphologic prognostic parameters and the angiogenesis-related immunohistochemical parameters and overall survival. Nevertheless, the present study provides the first evaluation and evidence of p300 expression in PCM. Eventually, the interaction between angiogenesis and myeloma cells as well as the prognostic role of increased bone marrow angiogenesis in PCM need further elucidation with larger study groups and longer follow-up.

Competing interests

The authors declare that they have no competing interests.

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