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The Impact of Cyclooxygenase-2 and Proliferating Cell Nuclear Antigen Over-Expressions in Multiple Myeloma Patients' Bone Marrow Biopsies and their Correlations with other Prognostic Parameters

HOMAM M.A. SHARSHIRA, M.D.; SHEREEN M. EL-MAGHRABY, M.D. and AHMED M.L. BEDEWY, M.D.

The Department of Hematology, Medical Research Institute, Alexandria University

ABSTRACT

Background: The survival of patients with multiple myeloma ranges from few years to more than 10 years from the time of diagnosis. The prognosis depends on several clinical, laboratory and histological/cytological parameters. Proliferating cell nuclear antigen (PCNA) correlates with cellular proliferation in benign and malignant neoplasms, including hematological malignancies. Markers of angiogenesis correlate with clinical characteristics in hematologic malignancies, including multiple myeloma (MM). Also Cyclooxygenases (COX)-2 over expression is claimed to be associated with reduced estimated survival in MM.

Objectives: Our aim is to find if COX-2 and PCNA over expressions in MM patients affect their prognosis and survival and/or correlate with other prognostic parameters.

Material and Methods: The study population included 44 newly diagnosed MM patients. Pre-treatment bone marrow (BM) aspiration and biopsy samples were available from all the patients. In addition myeloma work up was done for all patients. BM biopsy samples from 15 patients who had no evidence of BM involvement upon work-up for lymphoma or metastatic tumor were used as normal controls. The biopsies of the myeloma cases and controls were immuno-stained with COX-2 antibodies and antibodies to PCNA and CD 138.

Results: We studied 44 cases, the age of the patients ranged from 37 to 73 with a mean of 56.36±9.84 and a median of 57 years. They included 28 males and 16 females. Patients were followed for 24 months; 8 patients died during the follow-up period. Seven patients (15.9%) were in stage I, 26 (59.1%) in stage II and 11 (25.0%) in Stage III. The trephine biopsy showed >50% plasma cells in 35 cases (59.3%), while 21 cases (35.6%) showed a plasma cell percentage of >50% in the bone marrow aspirate (p<0.001). Positive COX-2 immuno-staining was found in 70.5% with 36.4% having weak to moderate positivity and 34.1% strongly positive. Poor prognostic factors as advanced stage, elevated beta-2 microglobulin (B2M) and reduced albumin were correlated with COX-2 expression. The overall survival estimate of those patients with negative or weak-moderate COX-2 immunoreactivity in myeloma cells was significantly better than that of patients with strong COX-2 immunoreactivity (p=0.001). We also found that PCNA expression increased with advancing disease stage and correlated significantly with prognostic factors such as elevated B2M, reduced albumin. Moreover, there was strong correlation with COX-2 over expression, response to treatment and survival. Conclusion: Bone marrow biopsy is better than aspirate in estimating the plasma cell burden in the marrow. COX-2 and PCNA expressions by immunohistochemistry are associated with shorter survival and poor prognostic factors such as advanced stage, elevated B2M and reduced albumin. Moreover, PCNA expression is associated with plasmablastic morphology and poor response to treatment.

Key Words: *MM – Bone marrow biopsy – COX-2 – PCNA.*

INTRODUCTION

Multiple myeloma is a malignant disorder of monoclonal plasma cells. Besides the serum or urinary M protein, patients also have increased plasma cells in bone marrow, lytic areas in bone and various other clinical and laboratory abnormalities characteristics of this disease. It has been mentioned that approximately 2% of patients with MM are younger than 40 years and it is still rarer in patients younger than 30 years **[1]**.

Multiple myeloma is a well-established clinical and immunological entity with considerable variability in biological behavior and survival **[2]**. Bone marrow examination continues to be the cornerstone for establishing a diagnosis in association with other clinical and laboratory parameters **[3]**.

Several clinical, laboratory and histological/cytological variables help us in determining the prognosis of the disease **[4]**. The first histological classification and staging of multiple myeloma, based on the bone marrow trephine biopsy, was put forward by Bartl et al., in 1987 **[5]**. The Durie and Salmon clinical staging system, proposed in 1977 **[6]**, is still being used today, though it has been replaced by the International staging system (ISS) at many places **[7]**.

Assessment of bone marrow involvement by malignant plasma cells is an important element in the diagnosis and follow-up of patients with multiple myeloma and other plasma cell dyscrasias. However, multiple myeloma is often a focal process, a fact that impacts the accuracy and reliability of the results of bone marrow plasma cell percentages obtained by differential counts of bone marrow aspirate smears. CD138 allows excellent assessment of plasma cell numbers and distribution in bone marrow biopsies. CD138 is a highly specific and sensitive marker of normal and neoplastic plasma cells **[8]**.

Determining those patients with myeloma who will develop progressive disease is an important clinical issue. The ß2 microglobulin and plasma cell labelling index remain important independent laboratory markers of prognosis **[9]**. Immunohistological assessment of plasma cell differentiation, the volume of plasma cell infiltration, and the pattern of infiltration all have prognostic value **[4]**. An increased volume of myeloma in the bone marrow trephine is associated with shorter survival **[10]**.

Proliferating cell nuclear antigen (PCNA) is a 36-KD auxiliary protein of DNA polymerase-delta, that has been found to be a useful marker in immunocytochemical studies of cell proliferation because its expression correlates with the proliferative state of the cell. Its expression increases from the late G1 phase through the S phase of the cell cycle **[11]**.

Exposure of exponentially growing cells to antisense oligdeoxynucleotides to PCNA resulted in complete suppression of DNA synthesis and mitosis, indicating an important role for this protein in cell proliferation **[12]**.

Cyclooxygenases (COX) are enzymes that are involved in the synthesis of prostaglandins (PGs) from arachidonic acid. They catalyze the insertion of molecular oxygen into arachidonic acid to form the unstable intermediate PG-G2 being rapidly converted to PGH2.

PGH2 is the source of several biological active PGs, thromboxanes, and prostacyclins, which contribute to many physiological and pathological processes like hemostasis, kidney and gastric functions, pain, inflammation and tumor defense, and also tumorigenesis **[13]**.

Angiogenesis is defined as the formation of new capillaries from existing blood vessels and plays an important role in the progression of many cancer types. Markers of angiogenesis also correlate with clinical characteristics in hematologic malignancies, including multiple myeloma (MM), serving as predictors of poor prognosis as well as in solid tumors **[14]**.

Forced over-expression of COX-2 stimulates angiogenesis in animal models **[15]**. Pharmacological inhibition of COX-2, but not COX-1, inhibits corneal neovascularization and experimental colon and lung tumor growth **[16]**. Giles et al. **[17]** showed that elevated bone marrow COX-2 levels are associated with reduced survival in chronic phase chronic myeloid leukemia (CML).

In this work, we evaluated cyclooxygenase-2 and PCNA expressions in MM patients' bone marrow biopsies by immunohistochemistry and investigated the relationship of their expression with other myeloma parameters.

MATERIAL AND METHODS

In the current study, we investigated 44 patients (28 males and 16 females) with newly diagnosed multiple myeloma during the period (2008-2011), presented to the Hematology Department, Medical Research Institute. The diagnosis of MM was established using WHO classification 2008 **[18]**. The patients were clinically staged according to Durie-Salmon staging system **[6]**. Treatment regimens were either VAD (vincristine, doxorubicin and dexamethasone) infusion chemotherapy or Thal-dex (thalidomide and dexamethasone) **[19]**. Patients taking medications that could affect the COX-2 metabolism, such as aspirin, and chemotherapeutic agents were excluded from the study. BM biopsy samples from 15 patients who had no evidence of

BM involvement upon investigational work-up for lymphoma or metastatic tumor were used as normal controls for COX2 and PCNA expressions after exclusion of those with significant drug history.

Our patients were followed-up for 24 months or more from diagnosis. Pre-treatment bone marrow aspiration and biopsy samples were available from all the patients. Biopsies were taken from the posterior iliac crests. All patients were subjected to complete blood count, serum beta-2 microglobulin (B2M), serum albumin, serum lactate dehydrogenase (LDH), serum calcium, serum creatinine and serum protein electrophoresis. A radiological skeletal bone survey, including spine, pelvis, skull, humeri and femurs was carried out for all patients.

Bone marrow aspirate smears were stained with Leishman stain and examined using a 100x oil immersion objective. Percentages of plasma cells in the aspirates were estimated by a 500-cell count. The bone marrow aspirates were typed for plasma cell morphology as plasmacytic (well differentiated or intermediately differentiated) or plasmablastic (poorly differentiated plasma cells).

Trephine biopsies were decalcified, processed, paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E) **[20]**. In bone marrow trephine biopsy sections, we estimated the percentages of cellular marrow occupied by plasma cells to the nearest 5% based on the examination of conventional H&E sections and CD138 stained sections. In addition, the pattern of infiltration in the trephine biopsies whether interstitial, mixed (nodular and interstitial) or diffuse was determined. All cases were independently reviewed by 2 hematologists without prior knowledge of the clinical data.

Immunohistochemistry:

Sections of bone marrow trephine biopsies of the myeloma cases and controls were immune-stained according to the manufacturer instructions with CD 138 Ab-2 (Catalog number MS-1793-S0, Thermo Scientific, UK), COX-2 antibody (Catalog number RM-9121-S0) (Thermo Scientific, UK) and antibody to PCNA (Catalog MS-106-R7, Thermo Scientific, UK), using the AEX080-IFU system (Econo Tek HRP Anti-Polyvalent (DAB) (Scy Tek, USA).

We utilized an immune-histochemical score (IHS) for COX-2 based on the German Immunoreactive score. The IHS is calculated by multiplying the quantity and staining intensity scores. The scores could range from 0 to 12 for three groups. An IHS score of (7-12) was considered strong immunoreactivity, (1-6) weak or moderate, and 0 was scored as negative. COX-2 [21].

Two observers, blinded to the clinical outcome of the patients, independently scored the myeloma cell staining for COX-2 and PCNA.

Statistical analysis:

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18). Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Monte Carlo correction. Quantitative data were described using median, minimum and maximum as well as mean and standard deviation.

The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test. D'Agstino test was used if there was a conflict between the two previous tests. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non–parametric tests were used. For normally distributed data, comparison between more than two population were analyzed by F-test (ANOVA). Correlations between two quantitative variables were assessed using Pearson coefficient. For abnormally distributed data, Mann-Whitney Test (for data distribution that was significantly deviated from normal) were used to analyze two independent population. If more than two population were analyzed, Kruskal Wallis test was used. Kaplan-Meier for Survival curve was used. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

RESULTS

During the period (2008-2011) we studied 44 cases, the age of the patients ranged from 37 to 73 years with a mean of 56.36±9.84 and a median of 57 years. They included 28 males and 16 females with a male to female ratio of

1.75:1. Bone pain and easy fatigability were the most common symptoms seen in 37/44 (84.1%) and 33/44 (75%) respectively. Patients were followed during the study period for a minimum of 24 months. Eight patients died during the study period. Serum creatinine more than 2mg/dl was detected in 20.4% and serum calcium more than 11.5mg/dl in 29.5% of the cases. LDH was elevated in 21/44 (47.7%) of the patients, B2M in 13/44 (29.5%) and albumin was reduced in 36/44 (81.8%).

Seven patients (15.9%) were in stage I, 26 (59.1%) in stage II and 11 (25.0%) in Stage III. The biopsy showed >50% plasma cells in 35 cases (59.3%), while only 21 (35.6%) showed a plasma cell percentage of >50% in the bone marrow aspirate (p<0.001) (Table 1).

Twenty-six cases (59.1%) had plasmablastic morphology, while 18 cases (40.9%) had plasmacytic morphology. Nearly 71% of the cases (31/44) had a diffuse pattern of infiltration in the bone marrow trephine biopsy, while interstitial and mixed (nodular + interstitial) patterns were found in 18.2% (8/44) and 11.4% (5/44) of patients respectively.

According to the international uniform response criteria for multiple myeloma, 28 cases (63.6%) responded to therapy in the form of partial or complete remission (Responders) and 16 (36.4%) showed no response (Non–responders).

On comparing the clinical stage of the disease with the response to treatment, it was found that 8/11(72.7%) of patients in stage III were non-responders versus 8/26 (30.7%) of patients in stage II, whereas all patients in stage I were responders (p=0.005). As regards the bone marrow histological features, 8/11 (72.7%) of patients in stage III showed plasmablastic morphology versus 15/26 (57.7%) of patients in stage II and 3/7 (42.9%) of patients in stage I (p=0.426), while 9/11 (81.8%) of patients with stage III had diffuse pattern of infiltration versus 18/26 (69.2%) of stage II patients and 4/6 (57.1%) of stage I patients; the differences are statistically insignificant (p=0.846).

Fig. (1) Bone marrow infiltration by plasma cells highlighted by CD138 stain showing membranous pattern.

COX-2 and PCNA expressions by immunohistochemistry was significantly higher in patients as compared to controls (Table 2). Thirty four percent (15/44) of the cases were strongly positive for COX-2, 36.4% (16/44) showed moderate positivity whereas none of the controls had strong positivity and only 13.3% (2/15) were moderately positive.

COX-2 results were correlated with different prognostic parameters. Thirteen bone marrow biopsy specimens were negative, while 16 and 15 specimens were moderately positive and strongly positive for COX-2 immunostaining respectively. Fig. (2) shows bone marrow biopsy section from a patient with multiple myeloma immunostained for Cox-2 that demonstrates membranous and cytoplasmic staining.

Strong COX-2 expression was associated with higher bone marrow plasma cell burden and diffuse pattern of infiltration but the results were statistically insignificant, whereas advanced stage and non-responders were associated with strong Cox-2 expression with p-value of <0.001 for both. B2M levels were higher while albumin levels were lower in those with strong COX-2 expression with p-value of 0.016 and 0.007 respectively (Table 3). Kaplan-Meier survival estimate of those patients with negative or moderate COX-2 immunoreactivity in myeloma cells was significantly better than that of patients with strong COX-2 immunoreactivity (p=0.001) (Fig. 4).

PCNA positivity ranged from 8% to 80% in myeloma cells. Fig. (3) illustrates bone marrow biopsy section from a patient with multiple myeloma immunostained with PCNA antibody that show nuclear staining. PCNA expression was highly correlated to COX-2 expression (p<0.001). Similarly PCNA expression correlated significantly with elevated ß2M and reduced albumin (r=0.385, p=0.010; r=0.350, p=0.020, respectively). PCNA expression was associated with advanced stage (p<0.001). Higher PCNA expression was associated with plasmablastic morphology (p=0.006). Also higher PCNA expression was detected in non- responders (p<0.001) and in patients with short overall survival (p<0.001) (Table 4).

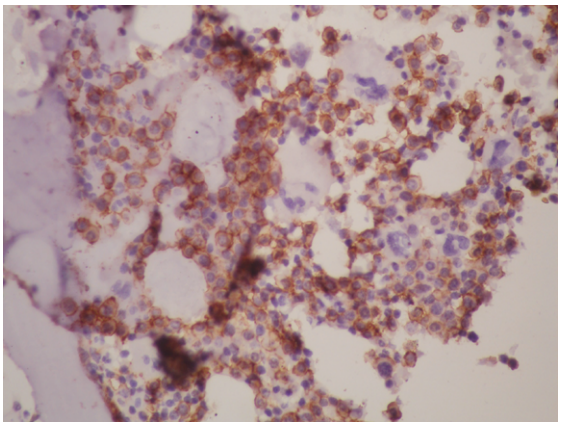

Fig. (1): Bone marrow biopsy section showing infiltration by plasma cells highlighted by CD138 stain that show the membranous pattern of CD138.

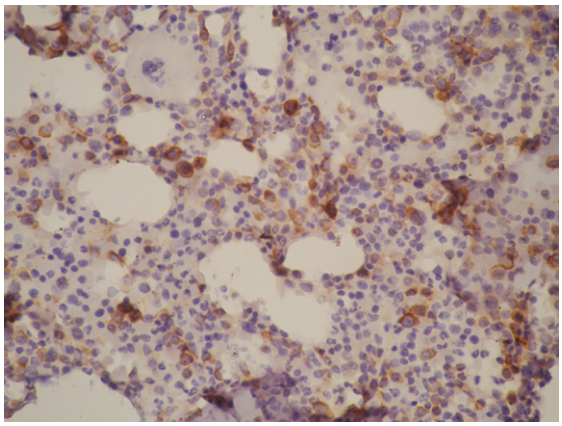

Fig. (2): Bone marrow biopsy section from a patient with multiple myeloma showing immunostaining with antibody with Cox-2 that demonstrates membranous and cytoplasmic staining.

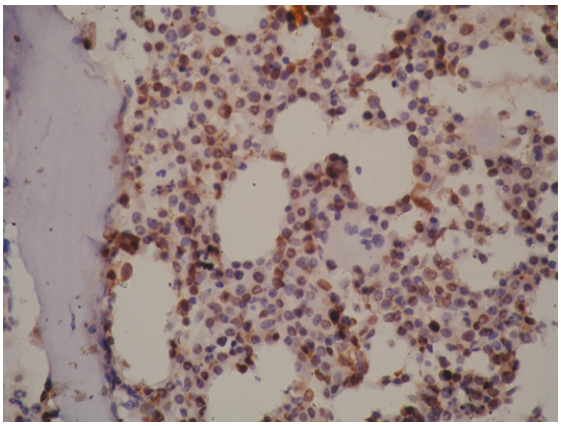

Fig. (3): Bone marrow biopsy section from a patient with multiple myeloma immunostained with PCNA antibody shows nuclear staining.

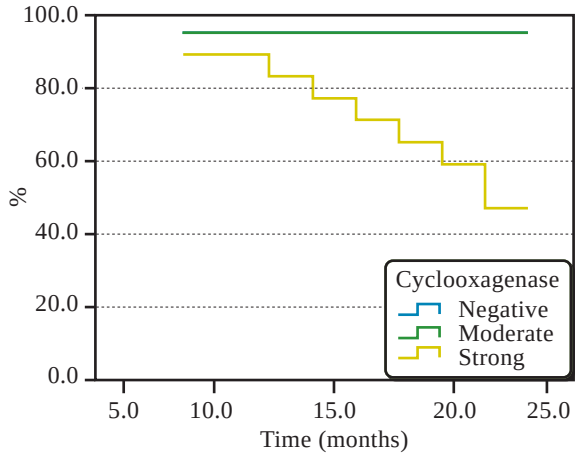

Fig. (4): Kaplan Meyer curve of survival in patients with negative, weak-moderate and strong COX-2 immunoreactivity in myeloma cells.

Table (1): Comparison between plasma cell percent in bone biopsy and aspirate.

	<20		20- 50		>50		MCp
	No.	%	No.	%	No.	%	
Bone marrow biopsy	0	0.0	24	40.7	35	59.3	<0.001*
Bone marrow aspirate	2	3.4	36	61.0	21	35.6	

MCp: *p*-value for Monte Carlo test. *: Statistically significant at $p \leq 0.05$.

Table (2): Comparison between patients and controls according to Cyclooxagenase-2 expression and mean of PCNA expressions.

	Cases (n = 44)		Controls (n = 15)		MCp
	No.	%	No.	%	
Cyclooxagenase-2:					
Negative	13	29.5	13	86.7	0.001*
Moderate	16	36.4	2	13.3	
Strong	15	34.1	0	0.0	
PCNA %:					
Range	8.0-80.0		0.0-3.0		<0.001*
Mean ± SD	32.80±16.81		1.60±0.99		
Median	31.0		2.0		

MCp: *p*-value for Monte Carlo test. *p*: *p*-value for Mann Whitney test. *: Statistically significant at $p \leq 0.05$.

Table (3): Cyclooxagenase 2 immunostaining in relation to the different studied parameters.

		Age (mean±SD)	Serum LDH (median)	Serum B2M (median)	Serum Albumin (mean±SD)	Plasma cell percentage in trephine biopsy			Pattern of infiltration in trephine biopsy			Stage			PCNA immune-staining (median)	Response to treatment	
						<20% Number (%)	20-50% Number (%)	>50% Number (%)	Diffuse Number (%)	Interstitial Number (%)	Mixed Number (%)	I Number (%)	II Number (%)	III Number (%)		Responder	Non-responder
Cyclo-oxygenase 2 lmmune-staining	Negative	53±9.8	218	2.3	3.31±0.31	0 (0)	9 (69.2)	4 (30.8)	8 (61.5)	4 (30.8)	1 (7.7)	6 (46.2)	7 (53.8)	0 (0)	20	13 (100)	0 (0)
	Moderately-positive	58.1±9.2	267.5	3.2	2.95±0.37	0 (0)	8 (50)	8 (50)	10 (62.5)	3 (18.8)	3 (18.8)	1 (6.3)	14 (86.5)	1 (6.3)	30.5	13 (81.3)	3 (18.8)
	Positive	57.4±10.4	250	2.9	2.88±0.38	0 (0)	7 (46.7)	8 (53.3)	13 (86.7)	1 (6.7)	1 (6.7)	0 (0)	5 (33.3)	10 (66.7)	41	2 (13.3)	13 (86.7)
p		0.341#	0.079‡	0.016‡*	0.007#*	0.525#			0.402#			<0.001#*			<0.001‡*	<0.001#*	

*: Significant. ‡: Kruskal wallis test *p*-value.
#: Monte Carlo test p-value. B2M: beta 2-microglobulin.

Table (4): Relation between PCNA and different prognostic parameters.

	Range of PCNA	Mean of PCNA± SD	Median	Test of sig.
Plasma cells % in biopsy:				
<20	0.0-0.0	0.0±0.0	0.0	KW_p=0.272
20-50	10.0-80.0	30.79±16.75	28.0	
>50	8.0-77.0	35.20±17.0	34.50	
Pattern of infiltration in biopsy:				
Diffuse	8.0-80.0	36.06±18.13	34.0	KW_p=0.225
Interstitial	11.0-36.0	24.25±9.29	25.0	
Mixed	12.0-40.0	26.20±11.76	31.0	
Plasma cell morphology:				
Plasmablastic	8.0-80.0	38.62±18.32	35.0	MW_p=0.006*
Plasmacytic	10.0-40.0	24.39±9.72	24.50	
Stage:				
I	8.0-20.0	11.86±3.85	11.0	KW_p<0.001*
II	16.0-60.0	32.48±9.51	32.0	
III	30.0-80.0	58.83±21.02	65.0	
Response:				
Responder (28 cases)	8.0-50.0	25.04±10.34	25.0	MW_p<0.001*
Non responder (16 cases)	25.0-80.0	46.38±17.56	38.50	
Outcome:				
Alive (36 cases)	8.0-64.0	28.17±12.29	29.50	MW_p<0.001*
Dead (8 cases)	35.0-80.0	53.63±19.37	50.0	

KW_p: *p*-value for Kruskal Wallis test. MW_p: *p*-value for Mann Whitney test. *: Statistically significant at $p \leq 0.05$.

DISCUSSION

This study analyzed 44 cases of myeloma, with respect to COX-2 and PCNA expressions by immunohistochemistry on bone marrow biopsies. The correlation of COX-2 and PCNA expressions with other myeloma parameters (clinical stage, laboratory parameters, bone marrow plasma cell infiltration, clinical outcome and survival) were investigated.

In the studied cohort, bone pain and easy fatigability were the most common symptoms seen in 84% and 75% respectively as was reported by others **[22]**. Most of our patients were in stage II (59.1%). When the morphology of the plasma cells and pattern of infiltration were compared with the clinical stage of the disease it was seen that although 72.7% of patients in stage III had plasmablastic morphology and 81.8% had diffuse pattern of infiltration, the results were statistically insignificant (p=0.426 and p=0.846 respectively). This may be due to the small number of cases in stage III (11 patients).

In our study, we observed that 59.3% of the patients had >50% plasma cell infiltrate in the biopsy, compared to only 35.6% in the aspirate (p<0.001), this is in agreement with Subramanian et al **[24]** who found that 71% of the patients had >50% plasma cell infiltrate in the biopsy, compared to only 40% in the aspirate (p<0.001). Also Pich et al **[23]** reported a higher mean percentage of plasma cell infiltrate in the biopsy (50.3%) as compared to the aspirate (32.89%).

In our study, positive COX-2 immunostaining was found in 70.5% of our patients; 36.4% were weak to moderate and 34.1% were strongly positive. Poor prognostic factors as clinical stage, B2M and albumin were correlated with COX-2 expression. Kaplan-Meier overall survival estimate of those patients with negative or weak-moderate COX-2 immunoreactivity in myeloma cells was significantly better than that of patients with strong COX-2 immunoreactivity. Cetin et al **[24]** found that COX 2 overexpression was associated with reduced estimated survival. Poor prognostic factors such as LDH, age and b2-microglobulin were also correlated with COX-2 expression.

We also found that PCNA expression increased with advancing disease stage and correlated significantly with prognostic factors such as B2M and albumin. Also there was strong correlation with COX-2 over expression, response to treatment and survival. Tsirakis et al **[25]**. found that PCNA value increased with advancing disease stage and correlated significantly with prognostic factors, such as IL-6, ß2 microglobulin and LDH. Pretreatment PCNA expression correlated significantly with bone marrow MVD (p<0.05) plasma cell infiltration (p<0.01) and IL-6 (p<0.01) as reported by Alexandrakis et al **[26]**.

In agreement with our results Usnarska-Zubkiewicz et al **[27]** found that PCNA positive cells ranged from 2% to 100%, higher PCNA expression was observed in patients with immature type of MM (mean 29.5%, SD=5.5), the highest expression was seen in plasmablastic type MM (mean 60.5%, SD=22.6) and correlated positively with the cytomorphology of plasma cells and clinical outcome.

In conclusion, Bone marrow biopsy is better than aspirate in estimating the plasma cell burden in the marrow. Also COX-2 over-expression by immunohistochemistry is associated with reduced survival and poor prognostic factors such as clinical stage, B2M and albumin. PCNA expression in myeloma indicates the myeloma's proliferative activity and correlates positively with the different parameters as advanced stage, plasmablastic cell morphology, elevated B2M, reduced albumin, poor response to treatment and shorter survival.

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