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Original Article

Pathology Section

Cyclin D1: A Prognostic Marker in Multiple Myeloma and its Association with CRP and β2-Microglobulin Level

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ABSTRACT

Introduction: Cyclin D1 is a protein encoded by the *CCND1 (BCL-1)* gene on chromosome 11q13 and it is an important regulator of G1 to S phase progression. Over expression of cyclin D1 protein releases cells from their normal controls when they need to exit from the cell cycle. This obstructs their maturation, and promotes transformation into a malignant phenotype.

Aim: To study the role of cyclin D1 expression in trephine biopsies of multiple myeloma patients and its association with C-Reactive Protein (CRP), β2-microglobulin level and treatment response rate.

Materials and Methods: This prospective observational study was conducted at Department of Pathology in collaboration with Department of Clinical Haematology, King George's Medical University, Lucknow, India, from September 2018 to August 2019. Total 40 cases of multiple myeloma fulfilling inclusion and exclusion criteria were enrolled. Bone marrow aspiration and biopsy was done in all the cases. Immunohistochemical (IHC) expression of cyclin D1 on trephine biopsy was associated with CRP levels and β2-microglobulin expression. All investigations were repeated at six months follow-up and response was compared with expression of cyclin D1. The statistical tests applied were Chi-square test, Student t-test and paired t-test.

Results: The age of cases ranged between 44 to 78 years and the mean age of the study subjects was 64.40±7.13 years. Total 67.5% of patients were males. On IHC, cyclin D1 expression was not observed in majority of cases (n=23), weak cyclin D1 expression was observed in 8 cases, while strong cyclin D1 expression was observed in 9 cases. Out of eight cases with weak cyclin D1 expression, five cases achieved partial response and two cases achieved complete response. One case was lost to follow-up. Among nine patients with strong cyclin D1 expression, six patients expired on six months follow-up and three patients achieved partial response. On comparison of two groups cyclin D1 positive and cyclin D1 negative cases it was found that cyclin D1 positive cases had an early age of onset, more than 50% plasma cells on marrow aspirate and were associated with plasmablastic morphology. Cyclin D1 positive cases also had increased CRP level as compared to cyclin D1 negative cases. Similarly, serum calcium, serum creatinine and β2-microglobulin levels were more in cyclin D1 positive group.

Conclusion: Cases who have strong cyclin D1 expression at time of diagnosis showed poor response to treatment. This was also associated with increased serum CRP and β2-microglobulin levels. Hence, cyclin D1 can be used as a prognostic marker in multiple myeloma.

Keywords: Bone marrow, C-reactive protein, Immunohistochemical expression, Trephine biopsy

INTRODUCTION

Cyclin D1 is a product of *CCND1 (BCL-1)* gene. *CCND1* gene in association with cyclin dependent kinase causes phosphorylation of retinoblastoma gene causing release of transcription factor E2F which is an important regulator of G1 to S phase progression. This inturn causes increased cell proliferation and release of cells from their normal controls causing malignant transformation. It is commonly seen in multiple myeloma and other malignancies like mantle cell lymphoma, breast cancer and hepatocellular carcinoma. Studies have shown that *CCND1* overexpression represents an unfavorable prognostic factor in multiple myeloma [1,2]. This overexpression of the cyclin D1 protein t (11;14) (q13; q32) can be easily demonstrated by immunohistochemical stain [3,4]. Patients who expressed cyclin D1 require bone marrow transplant to achieve complete response [5-7].

C-Reactive Protein (CRP) is secreted in increased amounts by hepatocytes in response to myeloma-derived cytokines and it activates myeloma cells to promote osteoclastogenesis and bone destruction. There is highly positive correlation between the level of serum CRP and number of osteolytic bone lesions. High level of circulating CRP protects myeloma cells from chemotherapy induced apoptosis. It binds activating Fcgamma receptors; activates PI3K/Akt, ERK, and NF-kappaB pathways; and inhibits caspase cascade activation induced by chemotherapy drugs. It also enhances myeloma cell secretion of Interleukin-6 (IL-6) and synergise with IL-6 to protect myeloma cells from chemotherapy drug-induced apoptosis. Thus, CRP is implicated as a potential target for cancer treatment [8,9].

β2-microglobulin is the light chain of the Human Leukocyte Antigen (HLA) histocompatibility complex. It is a low molecular weight protein found on the surface of all nucleated cells. The increased levels of serum β2-microglobulin in patients with multiple myeloma have been associated with a poor prognosis and correlates with the tumour load [10,11].

Hence, this study was conducted to study the role of cyclin D1 expression as a prognostic marker in multiple myeloma and its association with CRP and β2-microglobulin levels.

MATERIALS AND METHODS

This prospective observational study was conducted at Department of Pathology in collaboration with Department of Clinical Haematology, King George's Medical University, Lucknow, Uttar Pradesh, tertiary care center in North India, from September 2018 to August 2019. Ethical clearance from the Institutional Ethics Committee was obtained (95th ECM II B-Thesis/P23).

Inclusion criteria: All cases of suspected myeloma were enrolled. The diagnosis of multiple myeloma was based on various parameters, including age, sex, presenting complaints, radiological findings, Complete Blood Count (CBC) parameters (haemoglobin, red blood

count count, total leucocyte count, differential leucocyte count, platelet count), biochemical parameters including total serum protein, albumin, Albumin to Globulin (A:G) ratio, serum creatinine, calcium, lactate dehydrogenase, serum CRP levels, β2-microglobulin, bone marrow aspiration with trephine biopsy, serum protein electrophoresis and immunofixation.

Exclusion criteria: Patients with the reactive cause of marrow plasmacytosis, patients with asymptomatic (smoldering) myeloma and subjects who were already on myeloma treatment were excluded. Patients with Immunoglobulin M (IgM) related disorders or with primary amyloidosis were also excluded from the study.

After these investigations, 40 cases of multiple myeloma confirmed by bone marrow aspiration, trephine biopsy, serum protein electrophoresis and immunofixation were enrolled in this study. International Staging System (ISS) was used for staging [12]. Nine patients were in stage I, 23 patients in stage II and 8 patients in stage III.

Bartl's grading system: Number and morphology of plasma cells were assessed on aspirate smears using modified Bartl's grading system [13]:

- Grade I is assigned to cases with more than 70% plasma cells showing mature morphology i.e., small medium size eccentric nucleus, coarsely clumped chromatin and inconspicuous nucleoli.
- Grade II has less than 50% plasma cells with immature morphology like nuclear atypia, immature chromatin and prominent nucleoli but lack of significant pleomorphism.
- Grade III shows greater than 50% of plasma cells with marked cytologic atypia showing open chromatin, pleomorphism, and prominent nucleoli.

The infiltration pattern of plasma cells was studied on trephine sections [1].

Procedure

Immunohistochemistry was performed with antibodies against cyclin D1. Cyclin D1 manufactured by Dako (Prediluted ready-to-use Rabbit monoclonal antibody, Clone: EP12). Dako EnVision ™M FLEX detection system was used as the secondary antibody.

On biopsy sections, only nuclear positivity in atleast 10% of myeloma cells was considered as positive. Positive reaction was further graded as 1+ (10-19% nuclei positive), 2+ (20-50% nuclei positive) and 3+ (>50% nuclei positive) [1]. Negative control sections were processed by omitting primary antibodies. Immunohistochemical expression of cyclin D1 was also associated with CRP levels and β2-microglobulin expression.

Patients with symptomatic myeloma (patients with Hypercalcaemia, Renal failure, Anaemia and Bone lesions-CRAB features) received VTD (Bortezomib/Thalidomide/Dexamethasone) protocol. Bortezomib was given subcutaneously @ dose of 1.3 mg/m^2 in a weekly schedule (days 1, 8, 15, 22). Thalidomide was given orally at fixed doses of 100 mg once a day, and dexamethasone was given 40 mg/day orally in a weekly schedule (Days 1, 8, 15, 22). Patients also received acyclovir for herpes zoster prophylaxis, acetylsalicylic acid 75 mg daily for venous thrombosis prophylaxis, and monthly zolendronic acid 4 mg infusion to prevent bone-related events. All patients received uniform treatment.

All the patients were followed-up every month in haematology Outpatient Department (OPD). All investigations were repeated at six months follow-up, and response was compared with the expression of cyclin D1. Responses were assessed using the International Myeloma Working Group (IMWG) uniform response criteria for multiple myeloma. Patients were broadly categorised into two groups, those having Complete Response (CR) and those with Partial Response (PR) as per IMWG uniform response criteria [14].

The CR was defined as less than 5% clonal plasma cells in bone marrow, the disappearance of any soft tissue plasmacytomas, and negative immunofixation on serum and urine samples. PR was defined as >50% reduction of serum M-protein and reduction in 24 hours urinary M-protein by >90% or to < 200 mg/24 hour [14].

If the serum and urine M-protein are unmeasurable, >50% decrease in the difference between involved and uninvolved Free Light Chain (FLC) levels is required in place of the M-protein criteria [14]. If serum and urine M-protein is not measurable, and the serum-free light assay is also not measurable, a >50% reduction in plasma cells is required in place of M-protein, provided baseline bone marrow plasma cell percentage is >30% [14].

In addition to the above-listed criteria, if present at baseline, >50% reduction in the size of soft tissue plasmacytomas was also required [14].

STATISTICAL ANALYSIS

Authors analysed the data using Statistical Package for Social Sciences (SPSS) version 21.0 statistical analysis software. The values were represented in number, percentage and mean±SD. The statistical tests applied were Chi-square test, Student's t-test and paired t-test.

RESULTS

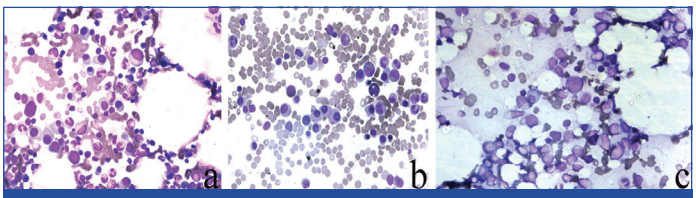
This study was conducted to evaluate the prognostic impact of the immunohistochemical expression of cyclin D1 in the plasma cells of multiple myeloma and its association in myeloma prognosis (short term/long-term responses). Total 40 cases of recently diagnosed myeloma were included. [Table/Fig-1] shows the clinicopathological parameters of enrolled cases.

Parameters	Results
Age (years) Mean±SD	64.40±7.13
Gender (Male/Female)	27/13
Lytic lesions	19
Splenomegaly	4
Associated plasmacytoma	1
Mean Haemoglobin (gm/dL)	9.0
Serum creatinine (mean±SD)	2.12±0.80
Albumin to Globulin (A:G) ratio (less than 1)	32
M band detected	33
M Band IgA and Kappa positive	2
M Band IgA and Lambda positive	2
M Band IgG and Kappa positive	24
M Band IgG and Lambda positive	5
Kappa/Lambda ratio (Mean±SD)	18.81±28.86 (0.03-78.10; n=7)
Bartl's histological grade	
Grade I (Mature)	13
Grade II (Immature)	17
Grade III (Plasmablastic)	10
Plasma cells	
<25%	4
25-50%	32
>50%	4
Pattern of marrow infiltration	
Diffuse	13
Interstitial	14
Nodular	7
Nodular and paratrabecular	6

[Table/Fig-1]: Clinicopathological parameters of 40 enrolled cases.

The age of cases ranged between 44 to 78 years, the mean age being 64.40±7.13 years. Total 67.5% of patients were males. The most common X-ray findings were lytic lesions (47.5%) and osteopenia (32.5%). Only four patients had splenomegaly with lytic lesions.

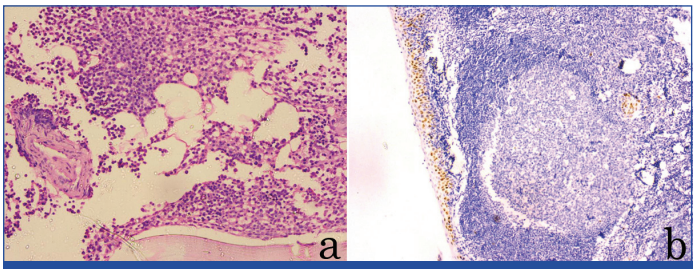
Bone marrow aspirate smears showed an increased number of plasma cells (more than 10%) in these cases. In most cases (80%), plasma cells were in the range of 25-50%. A 10% of cases had more than 50% plasma cells. Cases were classified as modified Bartl's grading system into grade I (13 cases) [Table/Fig-2a], grade II (17 cases) [Table/Fig-2b], grade III (10 cases) [Table/Fig-2c].



[Table/Fig-2]: a) Bone marrow smears showing mature grade I plasma cells, with mature and lymphoplasmacytic morphology (Leishman stain; 400X); b) Bone marrow smears showing immature grade II plasma cells, with high nuclear cytoplasmic ratio (Leishman stain; 400X); c) Bone marrow smears showing plasmablastic grade III plasma cells, with pleomorphic appearance and high nuclear cytoplasmic ratio (Leishman stain; 400X).

An even distribution between diffuse (32.5%), interstitial (35.0%) and nodular patterns (including nodular and paratrabecular; 32.5%) was observed on trephine biopsy sections [Table/Fig-3a]. M band was detected in 33 cases on serum protein electrophoresis, and 7 cases showed no M band. Total 26 cases showed kappa light chain restriction on immunofixation, and 7 had lambda light chain restriction [Table/Fig-1].

Tonsillar tissue was used as the positive control for cyclin D1 staining. Immunohistochemical analysis of tonsillar tissue shows strong nuclear positivity [Table/Fig-3b].



[Table/Fig-3]: a) Trephine biopsy section showing interstitial pattern of infiltration by plasma cells (Haematoxylin and Eosin, 100X); b) Cyclin D1 nuclear positivity in stratified squamous epithelium of tonsillar tissue- Positive control for Cyclin D1 (10X).

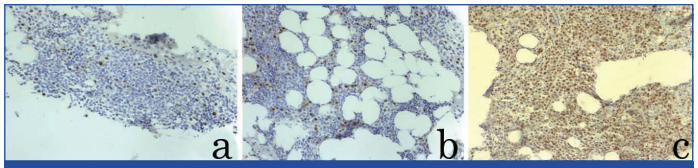
On immunohistochemistry, cyclin D1 expression was not observed in majority cases (n=23; 57.5%), weak cyclin D1 expression was observed in 8 (22.5%) cases, while strong cyclin D1 expression was observed in 9 (20.0%) cases.

The expression level was divided into three categories based on the number of cells expressing cyclin D1 [Table/Fig-4,5a-c].

IHC findings	Number of cases	Percentage
Cyclin D1 characteristics on IHC (n=40)		
Negative	23	57.5
Weak	8	22.5
Strong	9	20.0
Level of expression in plasma cells (n=17)		
10-19% (1+)	5	29.4
20-50% (2+)	5	29.4
>51% (3+)	7	41.2

[Table/Fig-4]: Pretreatment IHC findings of study population.

Out of 40 cases enrolled in the study, 6 (15.0%) were lost to follow-up, and 10 (25.0%) expired during the study. Only 24 (60.0%) patients were followed-up till six months and responded to the treatment. Nine (22.5%) patients achieved CR, while 15 (37.5%) had PR per IMWG protocol.



[Table/Fig-5]: a) Strong Cyclin D1 nuclear positivity in 10-19% of myeloma cells (1+); b) Strong Cyclin D1 nuclear positivity in 20-50% of myeloma cells (2+); c) Strong Cyclin D1 nuclear positivity in more than 50% of myeloma cells (3+).

Out of 40 cases, eight cases were weakly positive at pretreatment. On six month follow-up, 2 (25%) cases showed weak cyclin D1 positivity and had a PR, 5 (62.5%) cases turned out to be negative, and the remaining 1 (12.5%) was lost to follow-up. Out of these five patients who turned out to be negative at follow-up, 3 (60%) had PR, and 2 (40%) had CR. Similarly, Out of nine cases that were strongly positive for cyclin D1 at pre-treatment, 6 (66.6%) cases expired, and the remaining 3 (33.3%) cases showed weak positivity and PR [Table/Fig-6]. Out of 10 patients who expired during the study, 6 (60.0%) cases were strongly positive for cyclin D1 and the remaining 4 (40%) showed negative expression at the time of diagnosis.

Pretreatment	At six months follow-up	
Weak cyclin D1 expression-8/40	Weak cyclin D1 expression-2/8	2/8-PR
	Negative expression-5/8	3/8-PR; 2/8-CR
	Lost to follow-up-1/8	
Strong cyclin D1 expression-9/40	Expired-6/9	3/9-PR
	Weak cyclin D1 expression-3/9	

[Table/Fig-6]: Comparison of cyclin D1 expression at pretreatment and at six months follow-up and correlation with treatment response.
*PR: Partial response; CR: Complete response

Biochemical parameters: Pretreatment mean serum CRP, calcium, creatinine, and β2-microglobulin levels were found to be 28.80±12.19 mg/L, 7.98±1.46 mg/dL, 2.12±0.80 mg/dL and 10155±12836 µg/mL. At six months follow-up on treatment, an increment of 7.65±16.21 mg/L in CRP levels was observed. This increment (26.56% of pretreatment) was found to be statistically significant. In contrast, a decline in serum calcium, serum creatinine and β2-microglobulin levels were observed [Table/Fig-7].

When these biochemical parameters were compared among PR and CR groups (total 24), increment in CRP was more in the PR group, i.e., 8.35±17.58 vs. 6.47±14.55 in the CR group.

On comparing the two groups, cyclin D1 positive and cyclin D1 negative cases, it was found that cyclin D1 positive cases had an early age of onset; more than 50% plasma cells on marrow aspirate and associated with plasmablastic morphology. Cyclin D1 positive cases also had increased CRP level as compared to cyclin D1 negative cases. Similarly, serum calcium, serum creatinine and β2-microglobulin levels were more in cyclin D1 positive group. It was also observed that serum calcium and serum creatinine levels were significantly more in patients who showed strong cyclin D1 expression than those with weak cyclin D1 expression [Table/Fig-8].

DISCUSSION

Cyclin D1 belongs to the family of protein kinase that is involved in normal cell cycle regulation. Its dysregulations are associated with early oncogenic changes in myeloma like monosomy 13, deletion of 13q14, t (4;14), t(14;16) [7]. Since none of present cases were subjected to molecular analysis, study was done to associate the overexpression of cyclin D1 on IHC with prognosis. Advantage of cyclin D1 on IHC is its ready availability and less cost than molecular analysis.

Variables	Pretreatment	Post-treatment	Change in pretreatment		Paired 't' test	
	Mean±SD	Mean±SD	Mean±SD	% change	t-value	p-value
Serum C-Reactive Protein (CRP) (mg/L)	28.80±12.19	36.45±14.88	7.65±16.21	26.56	2.312	0.030
S. Calcium (mg/dL)	7.98±1.46	7.33±1.57	-0.65±2.63	-8.12	-1.207	0.240
S. Creatinine (mg/dL)	2.12±0.80	1.74±1.22	-0.38±1.40	-17.94	-1.330	0.197
β2-microglobulin (µg/mL)	10155±12836	6012±2010	-4143±13189	-40.80	-1.539	0.137

[Table/Fig-7]: Changes in pre-treatment and post treatment biochemical parameters (Paired 't' test).

Parameters	Cyclin D1 Negative (n=23)	Cyclin D1 Positive (n=17)	p-valaue[1]	Cyclin D1 weak positive (n=8)	p-value[2]	Cyclin D1 strong positive (n=9)	p-value[3]
Age* (in years)	65.04±4.91	63.53±9.44	0.513	62.63±10.62	0.390	64.33±8.83	0.733
Male	60.9	76.5%	0.298	62.5%	0.935	88.9%	0.124
Plasma cells >50%	8.7	11.8%	0.749	12.5%	0.754	11.9%	0.833
Lytic lesions	37.8	47.1	0.962	62.5	0.474	33.3	0.457
Morphological pattern							
Mature	21.7%	17.6%	0.231	37.5%	0.518	55.6	0.168
Immature	47.8%	35.3%		50.0%		22.2	
Plasmablastic	30.4%	47.1%		12.5%		22.2	
Biopsy pattern							
Diffuse	34.8%	29.4%	0.355	25.0%	0.289	33.3%	0.523
Interstitial	43.5%	23.5%		25.0%		22.2%	
Nodular	13.0%	23.5%		12.5%		33.3%	
Nodular and paratrabecular	8.7%	23.5%		37.5%		11.1%	
CRP levels*(mg/L)	27.78±12.41	30.44±11.31	0.485	27.86±13.29	0.599	27.71±12.38	0.555
Serum calcium* (mg/dL)	7.49±1.12	8.21±1.60	0.103	7.65±0.64	0.714	8.71±2.04	0.038
Serum creatinine* (mg/dL)	1.96±0.65	2.36±1.04	0.151	2.00±0.96	0.896	2.67±1.07	0.029
β2-microglobulin* (µg/mL)	6978±2984	14053±19213	0.089	14708±21639	0.096	13471±18103	0.099
Complete response	7/14 (50.0%)	2/10 (20.0%)	0.134	2/7 (28.6%)	0.350	0/3 (0)	0.110

[Table/Fig-8]: Clinicopathologic Characteristics of Subsets of Myeloma cases Positive and Negative for Cyclin D1 expression.
*Student 't' test (1 Negative vs. Positive; 2 Negative vs. Weak positive; 3 Negative vs. Strong positive)

Previous studies have described a robust positive association between cyclin D1 expression with advanced clinical stage, higher histologic stage and grade, increased proliferative activity (plasma cell labeling index), impaired renal functions, and higher β2-microglobulin levels [3]. Hoyer JD et al., analysed 24 cases of multiple myeloma, of which 10 had a lymphoplasmacytic morphology and four were in the leukaemic phase. Strong cyclin D1 positivity was noted in 19/24, and all 19 patients succumbed to the disease [3].

Tasidou A et al., in their study, found 35 out of 115 myeloma cases showing strong cyclin D1 expression and found an unfavorable response to conventional chemotherapy without allogeneic stem cell transplant [8]. Hoechtlen-Vollmar W et al., Athanasiou E et al., and Pruneri G et al., found a positive correlation of cyclin D1 expression with higher histological grade and stage and thus with adverse outcome [15-17].

Other studies in this context have shown conflicting results. Cook JR et al., found that strong cyclin D1 expression was associated with increased numbers of plasma cells, better survival and strong correlation with t (11;14) (q13; q32). Improved survival was seen in the strongly positive and weakly positive groups compared with cyclin D1 negative cases [18]. Kelley TW et al., recruited 94 myeloma patients and found no difference in the pattern of cyclin D1 expression in a newly diagnosed (n=49) and relapsed/refractory (n=45) myeloma [19]. Moreover, no significant association was found with the degree of expression and clinicopathologic features in that study. Similarly, in another series by Markovic O et al., of 59 newly diagnosed myeloma patients, cyclin D1 positivity or negativity did not convey any significant correlation with clinicopathological features or survival [20].

In the present study, it was observed that majority of the patients with PR had positive expression (53.3%) of cyclin D1 while the majority of the patients with CR had negative expression (77.8%), but this association was not found to be statistically significant, which could be attributed to small sample size.

Bataille R et al., carried out a survival analysis in 162 multiple myeloma patients at diagnosis and showed that serum CRP level is a highly significant prognostic factor. Moreover, serum CRP was independent of serum β2-microglobulin. This feature allowed stratification of multiple myeloma patients into three groups according to CRP and β2-microglobulin serum levels: (1) low-risk group, CRP and β2-microglobulin less than 6 mg/L (50% of patients); (2) intermediate-risk group, CRP or β2-microglobulin greater than or equal to 6 mg/L (35% of patients); (3) high-risk group, CRP and β2-microglobulin greater than or equal to 6 mg/L (15% of patients). Survival was 54, 27, and 6 months, respectively (p-value <0.0001) [21].

Tienhaara A et al., studied the prognostic significance of serum Interleukin-6 (IL-6) and serum CRP in multiple myeloma. They found a linear association between concentrations of CRP and IL-6. CRP was a slightly more substantial prognostic factor than IL-6 and was one of independent prognostic significance [22]. CRP binds activating Fc gamma receptors and thus activates PI3K/Akt, ERK, and NF-kappa-B pathways. This event inhibits caspase cascade activation induced by chemotherapy drugs. It is also found that CRP enhances myeloma cell secretion of IL-6 protects myeloma cells from chemotherapy drug-induced apoptosis [9].

In this study, mean serum CRP, serum calcium, serum creatinine, and β2-microglobulin levels of the study population were found to be 28.80±12.19 mg/L, 7.98±1.46 mg/dL, 2.12±0.80 mg/dL and 10155±12836 µg/mL at the time of diagnosis, while at 6 month follow-up, mean serum CRP, serum calcium, serum creatinine and β2-microglobulin levels of the study population were measured

as 36.45±14.88 mg/L, 7.33±1.57 mg/dL, 1.74±1.22 mg/dL and 6012±2010 µg/mL. An increment of 7.65±16.21 mg/L in CRP levels was observed on treatment. This increment (26.56% of pretreatment) was found to be statistically significant. While a decline in serum calcium, creatinine, and β2-microglobulin levels was observed on treatment. Though percentage change in pretreatment serum calcium, creatinine, and β2-microglobulin levels were 8.12%, 17.94% and 40.80%, respectively, these changes were not statistically significant. Serum CRP, β2-microglobulin, serum calcium, and serum creatinine levels were more in cyclin D1 positive group and were associated with a bad prognosis.

Among patients with PR, an increment in CRP levels was observed while the decline in serum calcium, serum creatinine, and β2-microglobulin levels occurred. This increase in CRP levels was lesser in patients with CR. Serum CRP did not show any association with the response status of patients.

Limitation(s)

The sample size was small in this study. There is need to conduct study on larger number of cases to find out the significant association between cyclin D1 expression, serum CRP and prognosis in myeloma patients.

CONCLUSION(S)

Cases that have strong cyclin D1 expression at the time of diagnosis show poor response to treatment. While on treatment, increasing serum CRP and β2-microglobulin levels is associated with a bad prognosis and incomplete response.

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