Original Research Article

Utility of Immunophenotyping at diagnosis in Multiple Myeloma-

An observational study from a tertiary care center in India

Abstract

Introduction and aims- Flow cytometry (FCM) has been useful in differentiating abnormal plasma cells (APCs) from normal plasma cells (NPCs) based on the different surface antigen expressions as well as the clonality analysis. The enumeration of NPCs and APCs is of prognostic significance. The phenotypic expressions of different antigens have been found prognostically relevant in myeloma. We intended to evaluate the prognostic significance and utility of immunophenotyping of multiple myeloma (MM) cases at diagnosis.

Material and methods- We evaluated 48 newly diagnosed MM cases. Immunophenotyping was done by a 3-tube 6-color FCM panel. NPCs and APCs were defined based on light chain restriction and immunophenotypic aberrancies. **Results-** Cases with >3% NPCs did not show a RISS3 disease (P=0.024). Cases with <3% NPCs at diagnosis were predominantly ISS2 or ISS 3 disease. Cases with >3% NPCs in bone marrow presented with a higher hemoglobin (P=0.004), lower creatinine (P=0.035) and lower beta-2 microglobulin (P=0.003) compared to cases with <3% NPCs at diagnosis.

Conclusion-Cases with increased number of NPCs at diagnosis are associated with good risk factors and may fare well after treatment. A diagnostic immunophenotyping should be encouraged to find out sub-groups of MM patients with higher NPCs.

Keywords- Flow cytometry, plasma cell, immunophenotyping

Introduction

Multiparametric flow cytometry (FCM) has been the forerunner in diagnosis and monitoring in many hematological neoplasms mostly because of the high sensitivity, specificity along with the ability to provide results within a few

hours(1). Multiparametric FCM has been used to diagnose variety of plasma cells disorders (PCDs) especially to distinguish myeloma from other lymphoid malignancies with plasmacytic differentiation.(2) Enumeration of total PCs by FCM is of prognostic significance in terms of survival as well as association of adverse baseline parameters. (3, 4, 5) Lately, prognostic significance of enumerations of NPCs and APCs have been proven in multiple studies.(3, 4, 5, 6, 7) Most of the MM cases have been shown to have a reduced NPCs in total PC compartment in BM.(3, 8, 9) Cases of MM with more NPCs at diagnosis have a MGUS like signature (5) The percentage of NPCs at diagnosis is a powerful prognostic factor at all stages of MM, from diagnosis to follow-up post therapy.(3,5, 7, 10) Though a prognostic significance of immunophenotypic aberrancies in MM is proven in studies but discrepancies exist.(3,11)

We studied the immunophenotype of newly diagnosed MM cases and enumerated the total PCs, NPCs and APCs. We tried to correlate the total PCs, NPCs and immunophenotypic expression with baseline cytogenetics, laboratory parameters and stage of the disease.

Patients and Methods

BM aspirate samples of 48 newly diagnosed MM cases were evaluated for diagnostic FCM. The diagnosis of MM was done in these cases according to IMWG criteria(12). A 3-tube 6 color multiparameter FCM panel was used for plasma cell immunophenotyping (table-1).

TABLE-1 (Panel for plasma cell immunophenotyping)

Tube	FITC	PERCPCy5.5	PE	PECy7	APC	APCH7
1	**CD81**	**CD45**	**CD138**	**CD19**	**CD56**	**CD38**
COMP./CLONE	**BL/JS81**	**BL/30-F11**	**BD/MI15**	**BL/HIB19**	**BD/B159**	**BD/HB7**
2	**CD27**	**CD45**	**CD138**	**CD19**	**CD117**	**CD38**

COMP./CLONE	**BD/M-T271**	**BL/30-F11**	**BD/MI15**	**BL/HIB19**	**BD/104D2**	**BD/HB7**
3	**cLAMBDA**	**CD45**	**CD138**	**CD19**	**cKAPPA**	**CD38**
COMP./CLONE	**BL/MHL38**	**BL/30-F11**	**BD/MI15**	**BL/HIB19**	**BL/MHK49**	**BD/HB7**

COMP- Company, BL- Biolegend, San Diego, CA, BD- BD biosciences, San Jose, CA

BM aspirate samples were received in EDTA anticoagulant and processed within 12 hours. An ammonium chloride based bulk lysis/ pre-lysis protocol (RBC lysis buffer, Biolegend, San Diego, CA) was used for all the samples. Antibodies against surface antigens and intracytoplasmic light chain antigens were stained according to previously described protocols(13). For cytoplasmic light chain staining permeabilizing solution (BD Perm/wash, BD biosciences, San Jose, CA) was used after surface staining. In all washing steps aspiration of supernatant was done in place of simple decantation of tube to minimize cell loss. A minimum of 0.5 million events were acquired on BD FACSCanto II 3-laser flow cytometer (BD biosciences, San Jose, CA) immediately after sample processing in all the cases.

NPCs were defined based on polyclonal cytoplasmic kappa and lambda light chain expression. APCs were based on antigen expression profile and monoclonality on cytoplasmic light chain staining. An aberrant antigen expression profile was assigned when at least two surface antigen expressions were abnormal. Antigen expression intensity were characterized as negative (N), dim (D), partial positive (PP), subpopulation positive (SPP), subpopulation negative (SPN) or moderate/ strong positive (P).

Bone marrow aspirate smear and peripheral smears were stained with Jenner-Giemsa stain and evaluated for PCs. The baseline characteristics including serum protein electrophoresis (SPEP), immunofixation electrophoresis (IFE), free light chain assay (sFLC) and radiological features were retrieved from patients' medical records.

A sequential gating strategy was used for immunnophenotyping of PCs.

Gating strategy

a. A CD38 vs time dot plot was used to assess the quality of data acquisition.
b. FSC-H vs FSC-A dot plot to exclude doublets.
c. FSC-A vs SSC-A to exclude debris.
d. A broad gating (PC gate) on CD38 vs CD138 dot plot to include all CD38 and CD138 positive events.
e. A refined PC gate (CD38 vs CD45 dot plot) on cells gated in PC gate to include only CD38+ bright events.
f. A CD19 vs CD45 plot was used to characterize refined PCs with expression of CD19 and CD45.
g. Further characterization of each subset of PCs was done with CD56, CD81, CD117, and CD27.
h. Each subset on a CD19/CD45 dot plot was assessed for cytoplasmic kappa or lambda restriction.
i. Mast cells, hematogones and NK cells were also evaluated to assess sample dilution.

STATISTICAL ANALYSIS

The presentation of the Categorical variables was done in the form of number and percentage (%). On the other hand, the quantitative data were presented as the means ± SD and as median with 25th and 75th percentiles (interquartile range). The data normality was checked by using Kolmogorov-Smirnov test. The cases in which the data was not normal, we used non parametric tests. The following statistical tests were applied for the results:

1. The comparison of the variables which were quantitative and not normally distributed in nature were analyzed using Mann-Whitney Test (for two groups) and Kruskal Wallis test (for more than two groups) and independent t test was used for comparison of normally distributed data between two groups.
2. The comparison of the variables which were qualitative in nature were analyzed using Chi-Square test. If any cell had an expected value of less than 5 then Fisher's exact test was used.
3. Spearman rank correlation coefficient was used for correlation of BM PC (%) and FCM PC(%) and for correlation of FCM PC(%) with Haemoglobin (g/dL), Total leucocyte count(cells/cubic mm), Platelet count(cells/cubic mm), Serum creatinine(mg/dL), ß2M(mg/L), Albumin(g/dL) and LDH(U/L).

   The data entry was done in the Microsoft EXCEL spreadsheet and the final analysis was done with the use of Statistical Package for Social Sciences (SPSS) software, IBM manufacturer, Chicago, USA, ver. 21.0.

For statistical significance, p value of less than 0.05 was considered statistically significant.

Results

We studied 48 MM cases with male a male predominance. Mean age in our cohort was 58.4 years. The baseline characteristics are shown in table-2.

Table-2 Baseline characteristics of MM cases

Age in years (n=48) mean/median/range	58.4/60.5/40-79
Sex (n=48)	Male(M)=33, Female(F)=15 M:F=2.2:1
Lytic Lesions on radiology (n=48)	68.75% (33/48)
Hb (g/dL) (n=48) mean, median, range	8.3/8.2/4-15.1
TLC($\times 10^9$/L) (n=48) mean/median/range	7.0/6.0/1.39-17.6
Platelets($\times 10^9$/L) (n=48) mean/median/range	158.29/144.0/20.0-503
Calcium(mg/dL) (n=48) Mean/median/range	9.34/9.1/7.9-13.8
Creatinine(mg/dL) (n=48) Mean/median/range	2.01/1.4/0.5-7.2

M Band (g/dL) (n=44) Mean/median/range	3.58/3.8/0.3-8.63
No M bands	4 of 48 cases
Albumin (g/dL) (n=48)	3.37/3.45/1.5-5.6
LDH (U/L) (n=48) Normal range (120-240) Mean/median/range	241/184/56-940
sFLC ratio (n=48) Normal sFLC ratio (0.26-1.65) Mean/median/range	67.8/6.25/0.001-657.3
Beta2M (mg/L) (n=48) Mean/median/range	8.02/6.25/3.04-20
Type of immunoglobulin (n=48)	IgG Kappa- 20 IgG Lambda-09 IgA Kappa-07 IgA lambda-06 Kappa Light chain-01 Lambda light Chain-04 Not available-01
FISH (fluorescent in situ hybridization) abnormalities (n=41)	Del 13q- 17 Del17p-05 t(4;14)-04 t(11;14)-03 No abnormalities-17 Other anomalies-08

Comparison between BM morphology and FCM PC

There was a significant reduction in PCs in FCM processing in comparison to morphology (BMPC). Total PCs by morphology and FCM ranged from 4-96% (mean/median, 44.5%/43%) and 0.5-75.9% (mean/median, 16.07% and 9.5%). Only two cases had FCMPC% more than BMPC and both the cases had marrow fibrosis of grade1-2 (WHO scoring system grade 0-3). A mean of 59.77% reduction was seen in PCs enumeration by FCM in comparison to BM. However, a moderate correlation was present between PCs in both the methods (R^2=0.458, P=0.001).

Revised International Staging System (RISS) categories of MM cases (n=41)

Most of the patients in our study were with a RISS2 (62%) or RISS3 (31%) disease. Only 3 patients (7%) had a RISS1 diseasetable. When categorized with the international staging system (ISS) criteria most patients (65%) had ISS3 stage followed by ISS2 (31%) and ISS1 (4%)

Patterns of antigen expression in MM cases

CD19 showed abnormalities in 100% of cases followed by CD81 (93.6%) and CD27 (89.3%) cases. 14.6% cases (n=7) showed a moderate to strong positivity for CD45. CD56 showed a dim, partial or strong positivity in 79.1% cases (n=38). CD117 had the lowest frequency of abnormalities with positivity in only 31.9% of cases (n=15).

None of the cases showed a moderate to strong positivity for CD19. CD45 was moderate to strong positive in 7 cases (14.6%). CD56 positivity (D/PP/P) was seen in 38 cases (79.1%) cases. CD81 and CD27 were abnormal (N/D/PP) in 44 (93.6%) and 42 (89.3%) cases respectively. CD117 expression was abnormal in 15 (31.9%) cases only.

Clinical significance of FCMPC enumeration

We tried to correlate FCMPC% with high-risk and standard-risk cytogenetics along with baseline laboratory parameters. High-risk cytogenetics were associated with a higher number of PC% by FCM (mean, 22.21%) versus

standard risk (mean FCMPC%, 14.84) (P=0.197). With increasing FCMPC% there was an association of reduced haemoglobin (Hb) (P=0.415), increased total leukocyte count (TLC) (P=0.596), decreased platelet count (PLT) (P=0.803) , raised serum creatinine (P=0.040) increased beta-2 M (P=0.149) and raised LDH (P=0.158) however, only correlation with serum creatinine was statistically significant.

Significance of NPCs in diagnostic bone marrows in MM cases

Only 5 (10.41%) MM cases with NPCs >3% were evaluated for association with FISH and baseline laboratory parameters. Cases with >3% Pcs did not show any high-risk cytogenetics (P=0.568). Patients with >3% NPCs had a significant correlation with higher Hb (P=0.004), lower serum creatinine (P=0.035), and lower beta-2 microglobulin (P=0.003).

Correlation of FCMPC% and NPC% with RISS (n=41) and ISS (n=48)

R-ISS 3 was associated with a higher FCMPC% (mean-20.78%, median-14.9%) in comparison to R-ISS 2 (mean-15.3%, median-8.9%) and R-ISS 1 (mean-7.4%, median-7.3%) (P=0.334). Similarly, a higher FCM PC% (mean-18.98%) was associated with ISS3 compared to ISS2 (mean FCMPC%-11.02%) and ISS-1 (mean FCMPC%-8.9%) (P=0.3). Cases with >3% NPCs did not show a RISS3 disease (P=0.024). Similarly, cases with <3% NPCs at diagnosis were predominantly ISS2 or ISS 3 disease (P=0.006).

Correlation of immunophenotypic expression with cytogenetics and laboratory parameters

The expressions of CD19, CD45, CD81, CD56, CD117 and CD27 were correlated with cytogenetics, stage and baseline laboratory parameters. However none of the antigens showed a significant correlation (supplementary file).

Discussion

Most of our MM cases had NPCs <3% at diagnosis. Only 5 (10.41%) patients had >3% NPCs and 3 (6.25%) patients had >5% NPCs. Only a single patient had NPC of >20% at diagnosis. In our study, though there was an association of higher FCM PC with high risk CTG, higher RISS/ISS, low Hb, high LDH, high beta-2M, lower PLT count, and high creatinine, only a higher creatinine (P<0.05) was statically significant. Cases with >3% NPCs at

diagnosis had a significant (P<0.05) correlation with high Hb, low beta-2M, lower creatinine, lower incidence of high-risk CTG, and lower ISS/RISS in our study cohort.

The number of residual polyclonal PCs is a useful discriminating marker between MGUS and MM.(7, 11) In a series of 595 MM cases, Paiva et al. found only 14% MM cases with >5% NPCs at diagnosis. Cases with >5% NPCs had higher Hb(P<0/001%), lower beta-2M (P=0.009), higher platelet (P<0.001), higher albumin(P=0.02), higher percentage of ISS1 (P=0.03) and lower incidence of high-risk cytogenetics (P<0.001)(5). In another study in 76 MGUS and 65 MM patients, Ocqueteau et al. found only 1.8% MM cases with >3% NPCs in comparison to 98% cases of MGUS(9). A study conducted by Tian et al. found higher FCMPC to be significantly (P=0.05) correlated with low Hb, high LDH, higher beta-2M, higher RISS, and ISS.(14)

The number of residual polyclonal PCs (NPCs) is a useful discriminating marker between MGUS and MM at diagnosis(4, 9). MGUS usually has more than 5% NPCs within total bone marrow PCs(11). Apart from diagnosis an abnormal to total PC ratio has prognostic significance too. MGUS and SMM with abnormal to total PC ratio more than 95% have a higher risk of progression to symptomatic MM with time to progression at 5 years of 25% vs. 5% and 64% vs. 8% respectively(4).In a series of 594 MM patients, cases with less than 95% abnormal to total PC ratio (14%) had an MGUS like signature and low incidence of high-risk cytogenetic anomalies(5). These patients had a better response to therapy, longer progression free survival (PFS) and overall survival (OS) compared to the rest of the patients. MM patients with more than 5% NPCs in BM have a lower frequency of immune paresis (42% vs. 83%, p=0.003) and a greater response to autologous stem cell transplantation (ASCT) (complete remission (CR) rate after ASCT of 64% vs. 33%, p=<0.001)(15).In series of 765 newly diagnosed MM patients, patients with less than 15% PCs detected by FCM had significantly longer PFS and OS than cases with more than 15% PCs(6). BM PCs have been shown to have a significant correlation with beta-2 microglobulin (ß2M), LDH, creatinine and hemoglobin(14). These findings suggest that FCM is very useful in defining subgroups of patients with better prognoses irrespective of achievement of CR or not.

We also tried to correlate abnormal expression of CD19, CD45, CD27, CD81, CD117 and CD56 with stage of MM, baseline cytogenetics and laboratory parameters. We did not get any significant correlation of adverse/high-risk parameters with immunophenotypic aberrancies.

Different studies have shown prognostic significance of antigenic expression in MM. For example, absence of CD117 is associated with high-risk cytogenetics like t(4;14) and del (17p). In one study CD56 negative patients had higher ß2M levels, a higher incidence of extramedullary disease, Bence Jones protein, renal insufficiency, and thrombocytopenia and were more likely to have a plasmablastic morphology compared to CD56 positive patients(16) CD56 positivity and absence of CD45 have been associated with advanced ISS stage.(17) CD19, CD45 absence and over-expression of CD56 have been found to be associated with low hemoglobin, higher beta-2 microglobulin and higher ISS stage.(18) Gupta et al found CD27 aberrancy association with normal albumin and association of CD117 expression with normal hemoglobin.(19) However, none of the findings have been proven in large multi-center trials.

FCM immunophenotyping though may not be relevant to diagnose MM, it can provide prognostic information. The presence of higher number of NPCs at diagnosis are associated with good prognostic factors and hence worth enumerating. Most cases of MM have a very minor population of NPCs at diagnosis. Immunophenotype of PCs in MM does not provide any additional prognostic information but can help differentiating APCs from NPCs. The sample size in our cohort is small, hence further study on large sample size may help in establishing the findings.

Bibilography

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