EVALUATION OF IMMUNOPHENOTYPIC EXPRESSIONS OF PLASMA CELLS IN PLASMA CELL MYELOMA PATIENTS AND ITS ASSOCIATION WITH PROGNOSTIC FACTORS AND CLINICAL STAGES

DR LING PEI CHI

DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PATHOLOGY (HAEMATOLOGY)

SCHOOL OF MEDICAL SCIENCES

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

Symbols / Abbreviations	Meaning
%	Percentage
+	Positive or addition
-	Negative
±	Plus-minus
≤	Less than or equal to
≥	Greater than or equal to
κ	Kappa
λ	Lambda
g/L	Gram per litre
AL	Immunoglobulin light chain amyloidosis
ASCT	Autologous stem cell transplant
BMA	Bone marrow aspirate
B2M	Beta-2-microglobulin
bFGF.	Basic Fibroblast Growth Factors,
CAM	Cell adhesion molecule
CD	Cluster of Differentiation
CT	Computer Tomography
CRP	C-reactive Protein
DSS	Durie Salmon Staging
del	Deletion
dl	decilitre
EDTA	Ethylenediaminetetraacetic acid
et al	et alia (and others)
FBC	Full blood count
FBP	Full blood picture
FISH	Fluorescence in situ hybridization
FLC	Free Light Chain

fl	Femtolitre
FSC	Forward scatter
Hb	Haemoglobin
Hct	Haematocrit
HKL	Hospital Kuala Lumpur
IGF	Insulin-like Growth Factors
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IL	Interleukin
ISS	International Staging System
IMWG	International Myeloma Working Group
LDH	Lactate Dehydrogenase
MGUS	Monoclonal Gammopathy of Uncertain Significance
MRD	Monitoring residual disease
MRI	Magnetic Resonance Imaging
OS	Overall Survival
PB	Peripheral Blood
PBS	Phosphate- buffered saline
PCM	Plasma Cell Myeloma/ Multiple myeloma
PET	Positron emission tomography
PFS	Progression Free Survival
R-ISS	Revised International Staging System
sCR	Stringent complete response
SPE	Serum Protein Electrophoresis
SPSS	Statistical Package for Social Sciences
SMM	Smouldering multiple myeloma
TNF-α	Tumour Necrosis Factor Alpha
t	Translocation
USM	UniversitiSains Malaysia
VEGE	Vascular Endothelial Growth Factors

WBC	White Blood Cell
WHO	World Health Organization
WBLD	Whole body low dose

PENILAIAN EKSPRESI IMUNOFENOTIPIK SEL PLASMA DI DALAM PESAKIT MYELOMA SEL PLASMA DAN BERKAITAN DENGAN FAKTOR PROGNOSTIK DAN TAHAP PERINGKAT KLINIKAL

ABSTRAK

Pengenalan: Sel plasma neoplastikmempunyaipenandaimunofenotipik yang berbezadaripadasel plasma yang biasa dan penandaimunofenotipikinimempunyaikepentinganprognostik. **Objektif:** Kajian inidilakukanbertujuanuntukmengkajijenis dan kekerapanekspresipenandaimunofenotipikpesakit myeloma sel plasma semasa diagnosis dan mengkajihubunganantarapenandaimunofenotipikinidengan parameter klinikal dan makmalsertatahapklinikalnya di kalanganpesakit. **Metodologi:** Ujiankajianretrospektifinimelibatkanpengumpulan data penandaimunofenotipikmenggunakansitometrialiran(CD38/CD138/CD19/CD45/CD 56/CD 117 dan sitoplasmarantairingan kappa dan lambda) sampelkespesakit yang barudidiagnosisdengan myeloma sel plasma antara Jun 2016 hingga Jun 2019 di Hospital Kuala Lumpur dan Hospital UniversitiSains Malaysia. Data klinikal dan keputusanmakmaldiambildarirekodperubatan dan dianalisissecarastatistikmenggunakan SPSS26.0. **Keputusan**: Kesemua 78 kessitometrialiran pada myeloma sel plasma yang barudidiagnosismempunyailebihdaripadasatuekspresi antigen yang aberandengankadarekspresi 100% untukkedua-dua CD38 dan CD138 sementara CD19 / CD45 / CD56 / CD117 masing-masing 28.2%, 23.1%, 83.3% dan 25.6%.

Majoritipembatasanrantaianringan kappaterekspresi, 60.3%.DidapatihubunganyangsignifikanantarapenandaCD19dengankreatininserum (p = 0.036). Walaubagaimanapun, tidakterdapathubungan yang signifikanantarapenandaimunofenotipik lain denganfaktor yang berkaitan. **Kesimpulan**: Imunofenotip oleh sitometrialiranmultiparametrikadalahalat yang bergunauntukmembezakansel-sel plasma neoplastikdarisel-sel plasma normal di mana antigen yangaberranterdapat di sebahagianbesarsel plasma neoplastik dan profilimunofenotipheterogendidapati pada populasikitaberbandingdengan yang lain. Selainitu, terdapathubungansignifikanditunjukkanantara CD19 dengankreatinin serum. Namun, keputusaniniperludisahkandengankajian yang mempunyaisaizsampel yang lebihbesar.

(263 perkataan)

EVALUATION OF IMMUNOPHENOTYPIC EXPRESSIONS OF PLASMA CELLS IN PLASMA CELL MYELOMA PATIENTS AND ITS ASSOCIATION WITH PROGNOSTIC FACTORS AND CLINICAL STAGES

ABSTRACT

Introduction: Neoplastic plasma cell expresses aberrant markers which differ from normal plasma cell was postulated to carry prognostic significance. This study aimed to determine the proportion of immunophenotypic expression of plasma cells in plasma cell myeloma patients at diagnosis and to study the association between these markers with clinical and laboratory parameters. **Methods**: A retrospective study was carried out from June 2016 till June 2019 by collecting the flow cytometry results (CD38/CD138/CD19/CD45/CD56/CD117 and cytoplasmic kappa and light chains expression) from newly diagnosed plasma cell myeloma, PCM cases in both Hospital Kuala Lumpur and Hospital USM. Clinical data and laboratory results retrieved from medical record were analyzed statistically using SPSS26.0. **Results**: All 78 cases of flow cytometry results in newly diagnosed PCM had more than one aberrant antigen expression with 100% expression rate for both CD38 and CD138 while CD19/CD45/CD56/CD117 in 28.2%, 23.1%, 83.3% and 25.6% respectively. The majority were expressed kappa light chain restriction, 60.3%. A significant association was demonstrated between CD19 markers with serum creatinine (p=0.036). However, there was no significant association between expression of other immunophenotypic markers with its associated factors. **Conclusion:** Immunophenotyping by multiparametric flow cytometryisausefultoolfordistinguishingneoplasticplasmacellsfromnormalplasma cell

where aberrant antigens were present in most of thePCM withaheterogenous immunophenotypic profile of PCM were defined in our population as compared to others. Moreover, there was a significant association demonstrated between CD19 with serum creatinine. However, this result should be confirmed with a bigger sample size.

(256words)

CHAPTER 1

INTRODUCTION

INTRODUCTION

Plasma cell myeloma, (PCM) is the second most common malignancy after non-Hodgkin lymphoma. It is an incurable disease with increased age incidence affecting male mainly in their sixty. Though it is an incurable disease and inevitably will relapse later despite achieving progressively higher complete remission rates, but with new emergence of novel treatment protocols it might be able to increase remission rates by achieving deeper treatment response with curative potential. This can then be translated into better clinicaloutcomes.

Multiparameter flow cytometry (MFC) is one of the most common available techniques used to monitor treatment response in PCM patients. It can discriminate neoplastic plasma cells from benign by utilizing a panel of combination antigens which include minimally CD38, CD138, CD19, CD45, CD56, CD117 as well as cytoplasmic lambda and kappa in a single tube. Differentiation between neoplastic and benign are based on aberrant immunophenotypic markers expression and light chain clonality. Plenty of studies have emerged to define immunophenotypic profile of neoplastic plasma cells with few looking into prognostic role of immunophenotypic markers in PCM. However, results were heterogenous with frequent discrepancies reported regarding prognostic role of immunophenotypic markers expression in PCM. For instance, absence of CD56 expression was found to be associated with poor prognosis as reported by Pan *et al.,* 2016 but Kraj*et al.,* 2008 did not find such correlation.

Despite the usefulness of these aberrant markers expression on plasma cells in term of disease prognostification and clinical outcome as described by several studies, however, none of the parameters concerning aberrant antigen expression are recruited

or involved in the risk stratification system neither International Staging System (ISS) nor Revised International Staging System (R-ISS). Hence, both clinical and prognostic value of immunophenotypic markers expression in PCM remains questionable (Rawstron*et al.*, 2008).

The aim of the study was to observe the prevalence of expression of immunophenotypic markers in newly diagnosed PCM patients in Malaysia by recruiting PCM from two study centers: Hospital Kuala Lumpur and Hospital USM. To date, there is no local data on immunophenotypic marker expression in newly diagnosed PCM in Malaysia.

The immunophenotypic markers expression were then correlate with associated factors which include both clinical and laboratory parameters that are considered to have prognostic value. The associated factors include demographic characteristic, hematological parameters, biochemical parameters, cytogenetic and molecular abnormalities and its clinical stages according to International Staging System, ISS forPCM.

CHAPTER2

LITERATURE REVIEW

LITERATURE REVIEW

Epidemiology of Plasma CellMyeloma

Plasma cell myeloma (PCM) is a debilitating hematological malignancy resulting from multifocal neoplastic proliferation of plasma cells in the marrow (Siegel *et al.*, 2016). It accounts for 13% of hematological malignancies and 1% of neoplastic cases (Rajkumar, 2018). It is second most common after non-Hodgkin lymphoma with estimated 26000 cases were diagnosed, and more than 11,000 patients died of PCM in 2015 (Siegel *et al*.,2016).

There is geographic and ethnic variation in PCM. The incidence of PCM is higher in African American compared with Caucasians. It is almost twice as frequent in African Americans population as Caucasians population (Landgren *et al.*, 2009).

PCM is commonly seen in elderly population and almost never found in children. It is very infrequently occurred in adults aged less than 30 years old (Crusoe *et al.*, 2015) and about 2% of patients are younger than 40 years old (Rajkumar *et al.*, 2016). The incidence increases progressively with age thereafter with about 90 % cases occur in patients aged more than 50 years old. It has slightly male predominance. The median age at diagnosis for male occurs at 69 years old and 72 years old for female (Ciolli*et al*., 2012).

According to Malaysia National Cancer Registry as reported by Ab *et al*., 2007 PCM is predominantly seen in men with a 5 years incidence of 396 cases from year 2007 till 2011. It accounts for approximately 0.38%. Majority of the cases were presented in advancedstage.

Etiology and clinicalfeatures

Etiology

Causes of PCM is unknown and it has shown radiation may play a role in some cases. There is an increased risk seen in those who use herbicides and insecticides particularly farmer or people who exposed to benzene and other organic solvent (Baris *et al.,* 2013). Familial clustering with high incidence of PCM in African heritage had suggested a genetic component may underlies the disease, with a possible autosomal dominant inheritance pattern (Koura & Langston, 2013). Virtually all PCM is preceded by premalignant lesion called monoclonal gammopathy of unknown significance (MGUS) (Kyle *et al.*,2003).

Clinicalfeatures

Commonly, patients with PCM will present with one or more PCM related end-organ damage symptoms which include hypercalcemia, renal insufficiency, anemia and bone lesions collectively term CRAB (C= hypercalcemia, serum calcium >11mg/dl, R= renal insufficiency, creatinine ≥177 μmol/L, A= anemia, hemoglobin <10g/dl or B= one or more osteolytic lesions (each lesion ≥ 5mm in size on X-ray, CT or PET-CT) attributable to plasma cell proliferation. Among these, the two most common presenting symptoms are anemia and bone pain (Kyle *et al.*, 2003). Anemia is mainly resulted from marrow replacement by unregulated proliferation of neoplastic plasma cells and renal damage. It occurs in about 73% of patients (Kyle *et al.*, 2003) and often contributes to fatigue symptoms. While 80% of patients will have detectable osteolytic bone lesions at diagnosis (Melton *et al.*, 2005). This occurs secondary to PCM-induced osteolytic lesions and resulted in bone pain or pathological fractures with or without symptoms indicative of spinal cord or nerve root compression as well as hypercalcemia. It was found that 20-40% (Knudsen *et al.*, 1994) of patients atdiagnosis and up to 50% (Kyle *et al.*, 2003) of patients during disease course can

present with renal failure. Multifactorial causes have contributed to renal failure and among these, myeloma kidney and hypercalcemia account for the most predominant etiologies (Mateu*et al.*, 2011). The underlying pathophysiology of myeloma kidney contributes to renal failure is by light chain deposition in the renal tubules secreted by neoplastic plasma cells which will results in nephron loss with progressive renal impairment. The renal failure can sometimes sufficiently severe to warrant hemodialysis in 10% cases (Knudsen *et al*., 2000). While hypercalcemia caused by osteolytic bone resorption will lead to hypercalciuria and dehydration and so contributes to renal failure. Besides that, calcium deposits may also cause inflammatory insult to the kidneys result in interstitial nephritis. Hypercalcemia commonly presents at diagnosis in 15-30% patients and 30-40% patients will have it later during disease course. Both renal failure and hypercalcemia would signify poor prognostic factors as patients with hypercalcemia are associated with features of advanced disease (Kastritis*et al.*, 2011) while renal impairment remains therapeutic challenges (Katagiri*et al*., 2013).

Other non-CRAB presenting features include increased susceptibility to infection secondary to leucopenia and depressed normal immunoglobulin production due to immunoparesis, bleeding secondary to thrombocytopenia or hyperviscosity syndrome secondary to high levels circulating monoclonal M protein production. Less commonly, organomegaly due to extramedullary involvement or amyloidosis symptoms may be present (Kyle *et al.*, 2003). Amyloidosis in PCM occurs secondary to accumulation of unstable monoclonal immunoglobulin light chain forming amyloid aggregates in the tissue which will ultimately leads to widespread organ dysfunction and failure. It is called immunoglobulin light chain amyloidosis, AL amyloidosis.

Patients usually will present with non-specific features for example fatigue symptoms, fluid retention and loss of weight. However, the 2 most common organs involved are cardiac and renal.

Pathogenesis of PCM

PCM is a B cell malignancy arising from accumulation of terminally differentiated clonal plasma cells in the bone marrow. The pathogenesis involved in the development of PCM include both cytogenetic and molecular changes occur in the neoplastic clone as well as its complex interaction with bone marrow microenvironment. Although PCM is an incurable disease, however, by understanding of its underlying pathogenesis provides an insight toward novel therapeutic approaches by targeting the bone marrow milieu other than only the neoplastic plasma cells clone thus, prevents PCM disease progression (Manier*et al.*, 2012). Goal of treatment in PCM is to eradicate all clones including subclones population with distinct biological characterized by improving treatment strategies that reflect genomic landscape of the disease (Furukawa *et al.*,2015).

Molecularpathogenesis

As like others, it has been confirmed that the nature of disease biology of all malignancies is resulted from stepwise accumulated mutations that lead to dysregulated cell growth and survival, the defining feature of malignancy. PCM is known to be genetically diverse disease with accumulated mutations found to sequentially drive the abnormal plasma cell towards full malignant proliferation.

Both MGUS and PCM share some genetic abnormalities which provide an apparent early, unifying event in pathogenesis. There is a universal dysregulation and/or

increased expression of cyclin D1,D2, D3 among them (Chesi*et al*., 2013).

There are two genomic events found to occur in PCM which can be divided into primary and secondary. Primary genomic events composed of two oncogenic pathways which have been established during course of MGUS. These primary cytogenetic abnormalities can be detected by fluorescent in situ hybridization studies, FISH and classified into hyperdiploid and non-hyperdiploid pathway (Debes-Marun*et al.*, 2003). Hyperdiploid pathway is associated with trisomies of odd chromosome (chromosome 3, 5, 7, 9, 11, 15, 19 and 21) which lacks recurrent immunoglobulin gene translocation. While non-hyperdiploid pathway involves translocation of immunoglobulin heavy gene, (IgH). With disease progression, secondary genomic events occur involving rearrangement of MYC protooncogene, gain 1q, del (17p), del (13), or RAS mutation. The importance of knowing both primary and secondary cytogenetic abnormalities is important as it will influence disease course, treatment response and prognosis. Few cytogenetic abnormalities are known to be poor genetic prognostic factors and these include t(4;14), t(14;16), t(14;20), del(17p) and gain of 1q (Morgan *et al.*,2012).

Bone marrowmicroenvironment

In addition to genetic abnormalities, complex interaction with altered bone marrow microenvironment also takes part in PCM pathogenesis and progression. It was found that neoplastic plasma cells actively modulate their bone marrow microenvironment in several different ways with bone marrow stromal cells via adhesion molecules to support their growth and survival and promote disease progression. There are multiple adhesion molecules expresses on both neoplastic plasma cells and bone marrow stromal cells. Adhesion molecules which expressed by neoplastic plasma cells includescelladhesionmolecule-1(ICAM-1),fibronectinreceptorVLA-4(α4β1integrin), lymphocyte homing receptor CD44 and neutral adhesion molecule N-CAM while

VCAM-1 is expressed on stromal cell. Interaction among these adhesion molecules will eventually stimulates a storm of cytokine synthesis and secretion which includes interleukin 6 (IL-6), CD138, osteoclast-activating factors for example interleukin 1(IL-1) and IL6, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factors (VEGE) as well as increased apoptotic expression of bcl-2. These in turn eventually increase signaling pathways that mediate growth, survival, drug resistance, migration of neoplastic plasma cells and disease progression (Manier*et al.*, 2012). IL-6 is a potent growth and survival factor which secreted by both stromal and neoplastic plasma cells. It plays an important role to mediate growth and survival of neoplastic plasma cells and stimulates angiogenesis. Besides that, osteoclast-activating factors also promote differentiation and maturation of osteoclast which contributes to bone disease characterized in PCM. CD-138 also play a role by stimulating osteoclastic activity with release of several cytokines from bone matrix which includes TGF-β, IL6, insulin-like growth factors, IGF and basic fibroblast growth factors, bFGF. These in turn will act either directly or indirectly to stimulate neoplastic plasma cells growth with parathyroid hormone related protein creating a vicious cycle whereby neoplastic plasma cells stimulating bone resorption, and bone resorption leading to an increased neoplastic plasma cells growth. VEGF, bFGF and interleukin 8 (IL-8) which acts as proangiogenic factors are increasingly expressed by stromal cells will stimulate neovascularization. This is an important determinant for disease progression. Increased expression of anti-apoptotic proteins is responsible for drug resistance and promote genetic accumulation which important for PCM progression. The interaction among neoplastic plasma cells with bone marrow microenvironmentareillustrateddiagrammaticallyinthefollowingasshownin Figure 2.1.

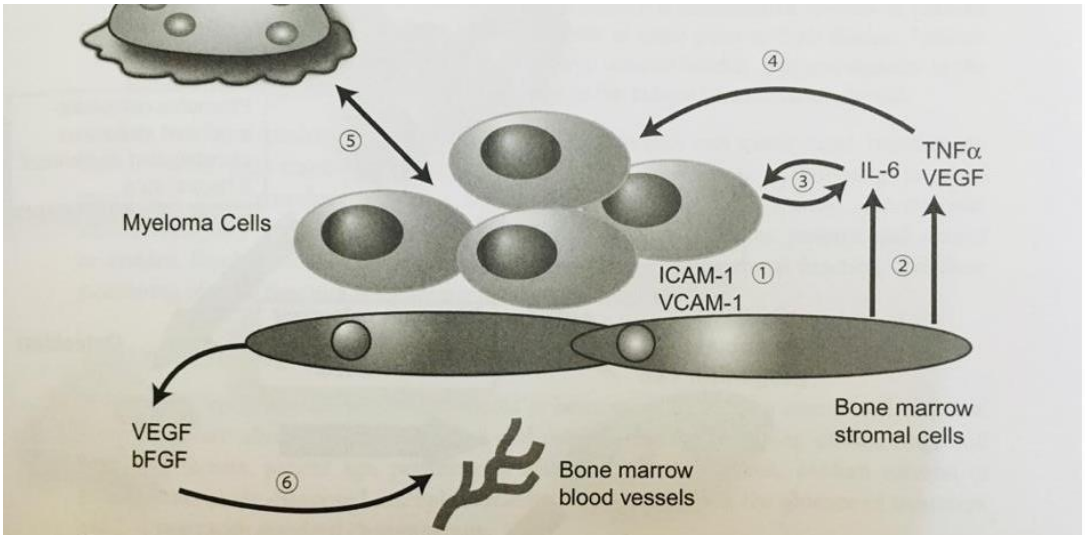

Figure 2.1: Interaction among plasma cells with bone marrow microenvironment (Adapted from Pallister & Watson, 2011).

Diseaseprogression

As reported by Kyle *et al.*, 2003, virtually all PCM is preceded by premalignant lesion called MGUS. MGUS is the most common plasma cell disorder with the presence of clonal marrow plasma cell less than 10% associated with monoclonal M protein less than 30g/L in the absence of CRAB symptoms or end organ damage (Rajkumar *et al*., 2016). Majority of the patients with MGUS are asymptomatic with more than 50% of them have had the condition 10 years prior to diagnosis (Therneau*et al.*, 2012). The M protein can remain stable for years in MGUS. Incidence of MGUS is found increasingly with age where 3% of cases are identified in population older than 50 years old and up to 10% in those who aged more than 70 years old (Kyle *et al.*, 2006). It commonly affects male than female in 1.5:1 ratio. Although MGUS patients do not require treatment but they should be monitored periodically as about 20-25% of them can progress into PCM in an interval between 10 to 20 years (Van Camp *et al.*, 1990). The annual risk of progression is about 1% per year from MGUS to PCM or related disorder (Ola Landgren *et al.*, 2009). Risk for MGUS disease progressionis increased

when M protein is equal or greater than 15g/L with abnormal free light chain ratio and monoclonal protein is of subtype IgA or IgM. These parameters are incorporated into prediction model constructed by Mayo Clinic MM (multiple myeloma) group as predicting risk in MGUS patients for disease progression. Patients with 3 risk factors are high risk MGUS with 58% risk progression compared to those with none of the risk factors have 5% risk progression at 20 years (Rajkumar *et al.*, 2005).

Smoldering PCM is an intermediate between MGUS and PCM. It is characterized by monoclonal M protein more than 30g/L with presence of clonal marrow plasma cells between 10% to 60% in the absence of end organ damage or CRAB symptoms (Rajkumar *et al*., 2015).SmolderingPCM resembles MGUS with absence of end organ damage but is clinically far more likely to progress into PCM or amyloidosis than MGUS (Kyle *et al.*, 2007). The risk of progression to PCM is heterogenous ranging from low risk of about 5% per year to high risk of about 50% within 2 years of diagnosis (Landgren, 2017). The common attributable risk factors for disease progression to PCM includes tumor mass which can be surrogated by level of M-protein, plasma cell infiltration, abnormal serum free light chain ratio (FLC) or presence of immunoparesis (Cesana*et al.*, 2002). Besides that, high risk cytogenetic abnormalities which involving t(4;14), del (17p) or gain 1q also contributes to higher disease progression rate independent of tumor mass (Neben*et al.*, 2013). Two commonly used models for risk stratification in smoldering PCM includes Mayo Clinic and Spanish PETHEMA models (Cherry *et al.*, 2013). Each model identifies unique patients as high risk with some overlap. Mayo risk model is based on bone marrow plasma cells infiltration, serum M protein level and serum FLC ratio (Dispenzieri*et al.*, 2008).WhileSpanish PETHEMA model uses aberrant plasma cells byimmunophenotypingandimmunoparesis(Pérez-Persona*etal.*,2007).However,there is a revised risk stratification by Mayo clinic based on the 20/20/20 criteria where bone

marrow plasma cells more than 20%, M spike more than 20g/L and FLC ratio >20. It is a simple routinely used risk stratification at diagnosis. Patients without any risk factors are stratified as low risk, while those with one factor present are intermediate risk or high risk if two or more factors are present (Lakshman *et al.*, 2018). High risk patient has higher risk of progression with 84% in 10 years; while those with 1 or 2 risk factors are 50% and 65% respectively (Dispenzieri*et al.*,2008).

Diagnosis of plasma cellmyeloma

Diagnosis of PCM usually started from clinical suspicion when they present with clinical features suggestive of PCM. Of this the most common complaints are fatigue due to anemia and bone pain. Based on this suspicion, baseline diagnostic workup for PCM is required. The investigations needed in patients suspected for PCM include complete blood cell count, measurement of serum calcium and creatinine, serum and urinary protein electrophoresis and immunofixation, serum free light chain assay and bone marrow examination. Other than this, imaging to detect osteolytic bone lesion is also needed. The standard diagnosis of lytic disease imaging modalities used is whole-body low-dose computed tomography (WBLD-CT). If this is not available, at minimum, plain radiography of entire skeleton is used to detect osteolytic bone lesion (Regelink*et al.*, 2013). There is no new bone formation in PCM (Roodman*et al.*, 2010)

The diagnostic criteria for PCM required in the WHO classification based on the work of several consensus group are shown in Table 2.1. In addition to WHO criteria, which mainly reflect plasma cell mass, other factors which are prognostically importantrelatestotumorbiologyarealsoimportanttoincludeininvestigationsofPCM patients. These include reduced serum albumin with increased lactate dehydrogenase (LDH), serum beta -2-microglobulin (B2M), C-reactive protein (CRP) and IL-6.

International Myeloma Working Group (IMWG) 2014 has updated criteria diagnosis for PCM which requires presence of 10% or more clonal plasma cells by bone marrow examination or a biopsy-proven plasmacytoma and presence of 1 or more myeloma defining events with presence monoclonal M protein in serum or urine (Rajkumar *et al.*, 2014).

Myeloma defining events are defined as CRAB symptoms with addition of 3 highly specific biomarkers which comprised of clonal bone marrow plasma cells equal or more than 60%, serum free light chain (FLC)ratio ≥ 100 (provided involved FLC level is ≥ 100mg/L and more than one focal lesion of at least 5mm or more on magnetic resonance imaging MRI/CT/PET-CT (Rajkumar *et.al*., 2018). These biomarkers can define patient as PCM even with the absence of end organ damage and allow for early therapy initiation to prevent end organ damage. It has been thought that these patients are associated with higher risk of progression to organ dysfunction at about 80% within 2 years of disease (Rajkumar *et al.*, 2014).

Table 2.1: Diagnostic criteria for PCM (Adapted from Rajkumar et al., 2014).

PCM

Clonal bone marrow plasma cell percentage ≥ 10% or biopsy-proven plasmacytoma and
≥ 1 of the following myeloma-defining events:

End-organ damage attributable to the plasma cell proliferative disorder:

- Hypercalcaemia: serum calcium > 0.25 mmol/L (> 1 mg/dL) higher than the upper limit of normal or > 2.75 mmol/L (> 11 mg/dL)
- Renal insufficiency:
  creatinine clearance < 40 mL/minute or serum creatinine > 177 µmol/L (> 2 mg/dL)
- Anaemia: a haemoglobin value of > 20 g/L below the lower limit of normal or a haemoglobin value < 100 g/L
- Bone lesions: ≥ 1 osteolytic lesion on skeletal radiography, CT, or PET/CT

≥ 1 of the following biomarkers of malignancy:

- Clonal bone marrow plasma cell percentage ≥ 60%
- An involved-to-uninvolved serum free light chain ratio ≥100
- > 1 focal lesion on MRI

Complete blood count(CBC)

It is a baseline investigation for PCM work up. CBC will commonly show normochromic normocytic anemia with hemoglobin ranging between 7-10g/dl and a low reticulocyte count (Kyle *et al.,* 2003). Severity of anemia and reticulocytopenia correlate with extent of bone marrow infiltration and are accentuated by dilution caused by increased plasma volume.

Platelet count often normal or slightly reduced at presentation. Platelet lifespan can be shortened due to intravascular activation by abnormal protein. Besides that, its normal function can also be compromised by increased plasma viscosity, which interferes with coagulation factors activation of factor V, VII and VIII as well as forming complexes with prothrombin and fibrinogen (Gogia*et al.*,2018)

Leucocytecount is usually normal but sometimes can have mild leucopenia. In half cases, differential count will show neutropenia with relative lymphocytosis. Rarely there is marked neutrophilia. This can be due to production of granulocyte colonystimulating factor (G-CSF) and interleukin 6 (IL6) by neoplastic plasma cells (Kohmura*et al.*, 2004).

Sometimes pancytopenia present due to disease progression with extensive bone marrow infiltration resulting in suppression of other hematopoietic cells.

Morphology

Hematological examination of the patients can be assessed through blood and bone marrow smear using May-Grunwald- Giemsa or Wright stain. Blood film of PCM patients with Rouleaux formation reflects an increased immunoglobulin production and when it is present in a very high concentration, it will lead to an increased bluish background staining because thin film of amorphous material that is diffusely weakly basophilic. This phenomenon may compromise appreciation of subtle morphological abnormalities. High monoclonal protein production will also cause a notable increase in ESR as well as interfere with cell counts on impedance instruments, particularly causing a factitious thrombocytosis(Mayer *et al.*, 1980). Recognition of circulating plasma cells is rare, excepts cases of plasma cell leukemia. Minority of the patients can present with leucoerythroblastic picture with atypical plasma cell and this strongly suggestive of thediagnosis.

Bone marrow aspirate and biopsy are standard option to evaluate for plasma cells morphology and percentage within the marrow. This remains ancillary criteria for PCM diagnosis and has prognostic significance. The number of plasma cells on bone marrow aspirate or biopsy should be at least 10% or more to fulfill WHO criteria. However, there are cases clinically suggestive of PCM have bone marrow plasma cells less than 10%. The reasons underlie could be due to suboptimal bone marrow aspirateorfrequentfocaldistributionofPCMinthemarrow.Thisaccountsfor5%incidence of cases (Jalaeikhoo, 2018). Involvement of more than 50% plasma cells in PCM patients at presentation have significantly shorter survival than others (Subramanian *et al.*, 2009).

There is variable of plasma cells morphology seen in PCM patients at diagnosis and it

can be classified according to morphological classification proposed in 1980s into mature, intermediate, immature, or plasmablastic as shown in Table 2.2 based on the predominant type of neoplastic plasms cells in the bone marrowaspirate.

Table 2.2: Morphological classification of plasma cell myeloma (Adapted from Greipp*et al.*,1985)

Mature myeloma
>10% mature plasma cells
<2% plasmablastic myeloma cells
<13% immature myeloma cells
Intermediate myeloma
Not fulfilling criteria for other types
Immature myeloma
>12% immature myeloma cells
<2% plasmablastic myeloma cells
<10% mature myeloma cells
Plasmablastic myeloma
≥2% plasmablastic myeloma cells

The prognostic significance of plasma cell morphology in PCM has been emphasized by study Greipp*et al.*, 1985. Of this, plasmablastic myeloma which defined as presence of plasmablast more than 2% in the marrow is considered as an independent prognostic factor. It is associated with adverse clinical risk (Møller*et al.*, 2015).

Histological examination is also crucial for PCM as it can provides information on the extent and pattern of neoplastic plasma cells infiltration as well as of prognostic value. The two main histological criteria to differentiate among neoplastic plasma cells from reactive plasmacytosis is the distribution and location of plasma cells. Neoplastic plasma cells tend to present early in irregular distribution either in interstitial ornodular distribution or later diffusely in advanced stage. Diffuse involvement is associated with the poorer outcome with reduced survival compared to other (Sukpanichnant*et al.*, 1994). Besides that, neoplastic plasma cells also tend to form aggregates in the periosteal position as compared to normal localization of plasma cells in proximity to bone marrow sinusoids and arterioles. Immunohistochemistry for

CD138 permits an optimal estimate of plasma cells percentage while staining with cytoplasmic κ and λ light chains to demonstrates its clonality. There are cases where discrepancy appears among estimates of plasma cell percentage in the aspirate and trephine biopsy. The higher of two is being selected for diagnostic and prognostic purposes (Rajkumar *et al.*,2001).

Immunophenotyping inPCM

Other than immunofluorescent stain to confirm clonality of neoplastic plasma cells, immunophenotyping by multiparametric flow cytometry using a panel of myeloma markers are also useful to differentiate among neoplastic and normal marrow plasma cells.

Study by Jelinek *et al*., 2018 has reviewed the applications of multiparameter flow cytometry in plasma cell disorders, it has shown its usefulness in differential diagnostic work up, prediction of disease prognostic outcomes, as well as for monitoring of minimal residual disease. Flow cytometry measures the optical and fluorescence characteristic of single cell. It allows cells identification based on expression of surface or cytoplasmic antigen, side scatter (SSC) and forward light scatter (FSC). Forward angle light scatter determines the size while right-angle scatter determine the internal complexity. The advantages of this technique include its high sensitivity and specificity with detection of one tumor cell over 10,000 bone marrow cells, intra-assay quality of whole cell sample via simultaneous detectionofhematopoietic population with wide availability at acceptable cost with fast results turnaround time (Flores-Montero *et al.*, 2016). However, it is not without its limitation. The need for extensive expertise to analyze flow results together with lack of well standardized flow MRD methods are the major drawbacks of flow cytometry (Paiva *et al*.,2015).

Immunophenotyping of neoplastic plasma cells have been studied recently to better

define its immunophenotypic profile with its clinical impact on prognostic and therapeutic significance. No single marker permits definite lineage assessment. A panel of antibodies are commonly used to determine immunophenotypic profile of plasma cells. Myeloma markers that commonly used to discriminate among neoplastic plasma cells from benign plasma cells are CD38/138/19/45/56/117 as well as cytoplasmic kappa and lambda light chains (Raja *et al.*,2010).

Normal marrow plasma cells are characterized by bright positive expression of CD38, positive CD138, CD45 and CD19 but negative for CD56, CD117, CD28, CD33 and CD20 (San Miguel *et al.*, 2002) with heterogenous expression of higher κ to λ ratio expression in benign plasma cells. The ratio of κ to λ is about 0.26 to 1.65 (Katzmann*et al.*, 2002).

(a) Immunophenotypic characteristic of neoplastic plasma cells

Neoplastic plasma cells always characterized by abnormal immunophenotyping. Although both normal and neoplastic plasma cell are positive for CD38 and CD138, but 90% of neoplastic plasma cells are negative for CD19 with 99% negative for CD45 marker or expressed in low intensity. 70% are positive for CD56 (Raja *et al*., 2010). Besides that, neoplastic plasma cells also express aberrant antigens which may confer prognostic significance. For instances, CD117 expression which usuallyabsentin normal plasma cells are commonly detected in about 30% of neoplastic plasma cells (Chang *et al*, 2006). It was reported that patients with CD117 expression are associated with good prognosis as it could serve as anchor molecule to reduce spread of plasma cells(Bataille*et al.*, 2008).Besides that, both CD56 and CD117 expression reduced as clinical stage advancing suggesting an association between antigenic expression of neoplastic plasma cells with cytogenetic abnormality and stage. Other aberrant markers like CD28 and CD33 can also found in 36% and

18% of neoplastic plasma cells respectively with expression of both markers are associated with poorer prognosis (Mateo *et al.,* 2008). CD45/CD56/CD117 and CD28 are also considered prognostic markers for plasma cell myeloma (Rawstron*et al.*, 2008). The comparison of immunophenotypic markers among normal and neoplastic plasma cells are shown in Table2.3.

Table 2.3: Summary of immunophenotypic characteristic of normal and neoplastic plasma cells (Adapted from (Leach *et al*., 2013).

Antigen	Normal plasma cells	Neoplastic plasma cells
CD45	Dim	Neg
CD38	Bright	Pos
CD138	Pos	Pos
CD19	Pos	Neg
CD56	Neg	Pos/neg

(b) Phenotypicmarkers

(i) CD45

CD45, also called leucocyte common antigen. It is a complex family of high molecular weight glycoprotein expressed on majority of hematopoietic cells and their progenitors (Gorczyca, 2017). Neoplastic plasma cells are heterogenous inCD45expression. It has important clinical and biological implication in neoplastic plasma cells. CD45 expression is correlates with proliferation rate of neoplastic plasma cells. Study done by Kumar *et al*., 2004 has shown that CD45 positive neoplastic plasma cells are commonly seen in early disease and associated with low grade angiogenesis. CD45 will eventually lost when disease advanced. It has also shown that CD45 negative PCM often shows aberrant expression of other markers eg CD138, CD54 and CD56 (Kumar *et al.*, 2004).

(ii) CD138

CD138, also called syndecan-1 is hallmark marker typically expressed by both benign plasma cells and neoplastic plasma cells but not T or B cells (Sanderson &Børset, 2002). It is a transmembrane heparan sulfate proteoglycan that plays a role in development and proliferation of plasma cells (Wijdenes*et al.*, 1996). Its inclusion in flow cytometry analysis for PCM is considered the most specific marker for plasma cells (Gorczyca, 2017). CD138 expression in PCM serves a main role in term of treatment purpose as well as for disease monitoring. Majority of PCM patients will show a high prevalence rate of CD138 expression in about 60-100% and this marker has becoming therapeutic interest by clinical researchers. As described before CD138 takes part in PCM disease pathogenesis by stimulating osteoclastic activity with release of several cytokines from bone matrix which in turn will act either directly or indirectly to stimulate myeloma cell growth with parathyroid hormone related protein creating a vicious cycle whereby neoplastic plasma cells stimulating bone resorption, and bone resorption leading to an increased neoplastic plasma cells growth. Hence by using anti-CD138 inhibitor it can downregulate the vicious cycle and hence reduce neoplastic plasma cells proliferation.

Anti-138 immunotherapy is used as an alternative therapeutic option particularly for those who are refractory to chemotherapy. Few clinical trials had reported the efficacy of CD138- directed cytotoxicity therapy and Guo *et al.*, 2016 had demonstrates promising result of adoptive immunotherapy with CART-138. In contrast, there is a new clinical entity observed by study Kawano *et al.*, 2012 where low CD138 expression PCM was associated with poorer prognosis, immature phenotype and refractory to lenalidomide. This implicates an establishment of different therapeutic strategy for low CD138 expression PCM patients.

(iii) CD38

CD38 is a transmembrane glycoprotein with widespread cellular expression and

functional activity. It is expressed by both hematopoietic and non-hematopoietic cell lineages but B-cell precursor and terminally differentiated plasma cells are highly expressing it (Gorczyca, 2017). CD38 has a role in tumor escape pathway to evade immune response. Hence targeted immunotherapy based on monoclonal antibody (mAbs) has become increasingly feasible and highly promising approach in hematological malignancies, particularly in combination with conventional treatments to further increase the potency of anti-tumor effects. One of the anti-CD38 mAbs used in our current practice are daratumumab which kill neoplastic plasma cells via antibody-dependent cell-mediated cytotoxicity and by complement-dependent cytotoxicity in vitro (de Weers*et al.*, 2011).

(iv) CD56

CD56 is expressed by natural killer cells and subset of T cells. It is a neural cell adhesion molecule (N-CAM) not expressed by benign plasma cells (Rawstron*et al.*, 2008). Majority of benign plasma cells are CD19 positive and CD56 negative while neoplastic plasma cells are mainly CD19 negative and CD56 positive.

Immunophenotypic analysis using paired markers has permitted differentiation among benign plasma cells and neoplastic plasma cells. Prevalence of CD56 expression in neoplastic plasma cells are found at 75% by Shin *et al.*, 2015. Its role as prognostication marker is debatable. Absence of CD56 expression was `associated with poor prognosis and might be associated with more aggressive disease and extramedullary dissemination. Sahara *et al.*, 2002a found CD56 negative patients had association with higher serum B2M levels, Bence Jones protein, renal insufficiency, thrombocytopenia and plasmablastic morphology compared to CD56 positive patients. However, Kraj*et al.*, 2008 did not found any association among CD56 with adverse prognosis.

CD56 has a role in as therapeutic target in the current era of targeted immunotherapy.

There are emerging mAbs against CD56 other than CD38 and CD138 as described before. Lorvotuzumabmertansine, is an unique antibody-drug conjugate targeting CD56 has completed phase 1 trial as reported by Ailawadhi*et al.*, 2019 with promising result both in its safety as well as clinical usefulness particularly for refractory or relapse PCMpatients.

(v) CD117

CD117 is a c-kit gene product not expressed by normal plasma cells but can be found in neoplastic plasma cells. About 32% of PCM expresses CD117 (Pan *et al.*, 2016). It is associated with good prognosis by serving as anchor molecule to prevent malignant spread of neoplastic plasma cells (Pan *et al.*, 2016). It has been recommended by European Myeloma Network as prognostic marker (Rawstron*et al.*, 2008). Both Shim *et al.*, 2014 and Pan *et al.*, 2016 had found a strong association between CD117 negativity with higher serum creatinine. However, its underlying mechanism is unclear. Other than its role as prognostication, it can also be used as an indicator fortherapeutic efficacy evaluation in newly diagnosed PCM treated with chemotherapy. It was found by Tang *et al.*, 2015 therapeutic efficacy decreased in CD117 positive patients compared with CD117 negative patients. Mateo *et al.*, 2008 also reported that PCM patients with CD117 positive treated with conventional therapy followed by autologous stem cell transplantation had decreased overall survival and progression free survival with CD117 negative PCMpatients.

(vi) CD19

It is a marker acquired early during B cell differentiation and expressed during B-cell maturation. It is present mainly in normal plasma cells with less than 5% in neoplastic plasma cells (Mateo *et al.*, 2008). Few literatures had reported the role of CD19 in PCM is of diagnostic purpose rather than of prognostic value (Cannizzo*et al.*, 2012).

(vii) CD20

It is a B-cell specific transmembrane protein expressed in committed B-cells throughout their development but is lost upon differentiation to normal plasma cells. However, it can presentin abnormal plasma cells in about 30% (Thakral *et al*., 2015)**.** There is an strong association found between t(11;14) and CD20 expression by Robillard *et al.*, 2003 where 83% patients with CD20 expression had t(11;14).

(viii) CD33

CD33 is a myeloid lineage marker with unknown pathway of mechanism in neoplastic plasma cells. It is not or slightly expressed on normal plasma cells (Robillard *et al.*, 2005) but is expressed by 12% of neoplastic plasma cells (Pan *et al.*, 2016). Expression of CD33 is correlated with poor prognosis and high incidence of t(4;14) (Robillard *et al.*, 2005), increase anemia or thrombocytopenia and elevatedserumB2M and LDH (Sahara *et al.*, 2006). Shim *et al.*, 2014 also report similar findings where expression of myeloid antigen is associated with poor prognosis in PCM.

Cytogenetic and MolecularAbnormalities

Almost 30% of PCM patients have abnormal karyotype at diagnosis (Dewald *et al.*, 1985). However, in view of low proliferative activity of neoplastic plasma cells only a third of patients have demonstrable cytogenetic abnormality at diagnosis. The frequency of detection increases with repeated analysis and with illness evolution, which appears to be associated with a very unstable genome (Sawyer *et al.*, 1995). The most frequent anomalies seen in PCM as shown by study from Kumar *et al.*, 2012 in Table 2.4 are trisomies which accounts for 42% and followed by recurrent chromosomal translocation involving 14q32 locus of immunoglobulin heavy chain, IgH gene, 30%. Among IgH translocations, the most frequent is t(11;14)(q13;q32), found in 15% of all PCM. It is associated with dysregulation of CCND1 encoding cyclin D1 byt(11;14).

These cytogenetic abnormalities can be detected by FISH study using bone marrow sample. FISH is a molecular cytogenetic technique used to detect specific targeted regions of the genetic abnormalities by specific probes. It has higher sensitivity than conventional G-banding karyotype as it can analyses 200 cells in interphase rather than in metaphase hence results are not influenced by infrequency of cells in metaphase. It is particularly useful for low mitotic or non-mitotic cells. The presence and type of cytogenetic abnormalities does provide prognostic significance and determines behavior of neoplastic plasma cells. Hyperdiploidy is associated with better clinical outcome (Bergsagel*et al.*, 2013) whereas IgH translocations especially thosewitht(4;14),t(14;16)andt(14;20)areconsideredhighriskcytogeneticabnormalities and associated with poor prognostic outcome. 5 years survival rate is only 10% (Palumbo *et al.*, 2015).

Table 2.4: Primary Molecular Cytogenetic Classification of Plasma Cell Myeloma (Adapted from Kumar *et al.*, 2012).

Primary Molecular Cytogenetic Classification of Multiple Myeloma

Subtype	Gene(s)/chromosomes affected [a]	Percentage of myeloma patients
Trisomic multiple myeloma	Trisomies of one or more odd-numbered chromosomes	42
IgH translocated multiple myeloma		30
t(11;14) (q13;q32)	*CCND1* (cyclin D1)	15
t(4;14) (p16;q32)	*FGFR3* and MMSET	6
t(14;16) (q32;q23)	*C-MAF*	4
t(14;20) (q32;q11)	*MAFB*	<1
Other IgH translocations[a]	*CCND3* (cyclin D3) in t(6;14) multiple myeloma	5
Combined IgH translocated/trisomic multiple myeloma	Trisomies plus any one IgH translocation	15
Isolated monosomy 14		4.5
Other cytogenetic abnormalities in absence of IgH translocations or trisomy or monosomy 14		5.5
Normal		3