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# Therapeutic Targets and Signaling Pathways for Diagnosis of Myeloma

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**Abstract**

Multiple myeloma (MM) is a malignancy of plasma cells that not only shows different clinical behavior but also depicts heterogeneous groups at molecular level. The prognosis of the disease has been dramatically changed with the arrival of new drugs in the past few years. In this context of better therapeutic agents, there are important challenges for accurate evaluation of patients by better prognostic and predictive tools. Transcriptomic studies have largely added to decipher MM heterogeneity, dividing MM patients into different subgroups according to prognosis. Micro-arrays and more recently RNA sequencing have helped in evaluating coding and non-coding genes, mutations, unique transcriptome convertors and different splicing events giving new information concerning biology, outcome and treatment options. Initial data from gene expression profiling studies have also pointed out genes that predict prognosis, i.e., CSK1-B, and can deliver pharmacogenomics and biologic vision into the pathophysiology, targeted treatment, and future direction. Importantly, we suggest that all prospective studies and clinical trials now accept genetic testing and risk stratification of MM patients. In this review, we discuss the part and effect of gene expression profiling in myeloma.

**Keywords:** multiple myeloma, monoclonal gammopathy of undetermined significance, smoldering multiple myeloma, gene expression profile

## 1. Background

In literature, multiple myeloma accounts 1% of all malignancies and almost 10% of all hematologic malignancies [1, 2]. Every year more than 20,000 new patients are diagnosed in the

United States [3]. The age-adjusted annual incidence in the United States has lingered similar for years at almost 4 per 100,000 [4]. Multiple myeloma is marginally more commonly reported in men than in women, and is twofold as common in African-Americans as compared with Caucasians [5]. At time of diagnosis of this disease, the median age is about 65 years [6].

## 2. Approach for diagnosis

The diagnosis of multiple myeloma requires the presence of one or more myeloma defining events (MDE) in addition to evidence of either 10% or more clonal plasma cells on bone marrow examination or a biopsy-proven plasmacytoma [7–12]. MDE consists of established CRAB (hypercalcemia, renal failure, anemia, or lytic bone lesions) features as well as three specific biomarkers: clonal bone marrow plasma cells ≥60%, serum free light chain (FLC) ratio ≥100 (provided involved FLC level is ≥100 mg/L), and more than one focal lesion on magnetic resonance imaging (MRI). Each of the new biomarkers is associated with an approximately 80% risk of progression to symptomatic end-organ damage in two or more independent studies. The updated criteria represent a paradigm shift since they allow early diagnosis and initiation of therapy before end-organ damage [13–16]. The rate of progression is influenced by the underlying cytogenetic type of disease; patients with t(4;14) translocation, del(17p), and gain(1q) are at a higher risk of progression from SMM to multiple myeloma [17–19].

When multiple myeloma is suspected clinically, patients should be tested for the presence of M proteins using a combination of tests that should include a serum protein electrophoresis (SPEP), serum immunofixation (SIFE), and the serum free light chain (FLC) assay [20]. Approximately 2% of patients with multiple myeloma have true non-secretory disease and have no evidence of an M protein on any of the above studies [6]. Bone marrow studies at the time of initial diagnosis should include fluorescent in situ hybridization (FISH) probes designed to detect t(11;14), t(4;14), t(14;16), t(6;14), t(14;20), trisomies, and del(17p) [21]. Conventional karyotyping to detect hypodiploidy and deletion 13 has value, but if FISH studies are done, additional value in initial risk-stratification is limited. Gene expression profiling (GEP) if available can provide additional prognostic value [22].

## 3. Molecular classification

Although multiple myeloma is still thought to be a single disease, it is in reality comprises of collection of variable cytogenetically distinct plasma cell malignancies [23–30]. On fluorescent in situ hybridization (FISH) studies of the bone marrow, approximately 40% of multiple myeloma cells have trisomies (trisomic multiple myeloma), while remaining have translocation involving the immunoglobulin heavy chain (IgH) locus present on chromosome 14q32 (IgH translocated multiple myeloma) [31–34]. In small subset of patients both trisomies and IgH translocations are found simultaneously. Trisomies and IgH translocations are primary cytogenetic abnormalities and observed at the time of establishment of MGUS. In addition,

secondary cytogenetic abnormalities developed during the disease course of multiple myeloma, including gain(1q), del(1p), del(17p), del [13], RAS mutations, and secondary translocations of MYC. Both primary and secondary cytogenetic abnormalities can influence disease progression, response to treatment, and overall prognosis [30].

# 4. Prognostication

The median survival of this disease is approximately 6–7 years; especially ASCT (Autologous stem cell transplant) eligible patients 4 year survival rates exceed 80%. However, behavior ofmalignancies is unpredictable, prognosis depends on patient characteristics such as age, co-morbids as well as disease characteristics such as disease stage, biology (cytogenetic abnormalities), and response to therapy [35, 36] Stage, i.e., tumor burden in multiple myeloma, is being evaluated by using the Durie-Salmon Staging (DSS) and the International Staging System (ISS) [37–39]. Disease biology best assessed by molecular abnormalities of multiple myeloma and the presence or absence of secondary cytogenetic abnormalities such as del(17p), gain(1q), or del(1p) [21, 29]. In literature, it is emphasized that the interpretation and impact of cytogenetic abnormalities are different according to the disease phase [30]. The recent staging system, Revised International Staging System (RISS) combines stage and disease biology (presence of high risk cytogenetic abnormalities or elevated lactate dehydrogenase level) to better define not only prognosis but guide treatment options [40].

It is important to note that in order to ensure constant availability, only three widely available cytogenetic markers are used in the RISS. Patients with standard risk multiple myeloma have a median overall survival (OS) of >7 years while those with high risk disease have a median OS of approximately 3 years despite tandem autologous stem cell transplantation (ASCT) [41, 42]. In addition to cytogenetic risk factors, two other markers that are related with rapid disease progression are elevated serum lactate dehydrogenase and plasma leukemic cells in circulation [43].

# 5. Pathways involved in multiple myeloma

## 5.1. PI3K/MEK/ERK pathways in myeloma

The phosphatidylinositol 3-kinases (PI3Ks) are a group of intracellular enzymes that phosphorylate the 3-OH group at the inositol ring of phosphatidylinositol leads to activation of PI3K/AKT signaling pathway that is responsible for chemoresistance [44]. PI3K signaling is inhibited by Phosphatase and tensin homolog (PTEN) and activated by insulin like growth factor 1 (IGF-1) and interleukin-6 (IL-6) But there is no FDA approved PI3K inhibitors for MM [44]. Inhibition of this pathway alone is not showing meaningful clinical responses in studies. MEK/ERK pathway is co-functioning with the PI3K/AKT [45]. Both pathways decrease apoptosis [45]. Resistance to treatment develops secondary to cross talk between pathways [45]. Therefore, targeting both pathways together may be an effective therapeutic strategy and has been proved in certain cancers, i.e., in melanoma and renal cell carcinoma [45].

### 5.2. Ras/MAPK pathway in myeloma

Ras protein family (H, K and N-Ras) send downstream signals that attracts growth-factor-receptor bound protein 2 (Grp2) and sons of Seven less (SOS) [46]. The grp2/SOS combination then converts Ras to active form by changing GTP to GdP [46]. Activated Ras recruits Raf to the cell membrane by phosphorylation [46]. This process is antagonized by GTPase-activating proteins, which promote GTP hydrolysis and the formation of inactive Ras-GDP complexes [46].

Mitogen-activated protein kinases (MAPKs) are a family of expressed kinases that convey cell surface signals into the cell. MAPK pathways are activated via a phosphorylation cascade [47]. The most proximal kinase in these pathways, the MAPK kinase kinase (MAPKKK or MAP3k), engaged by extracellular signals, phosphorylates a dual specificity MAPK kinase (MAPKK or MAP2K), which in turn phosphorylates and actives the distil effector MAPK [47].

The Ras/MAPK pathway consists of the Ras proteins, a family of small G-coupled molecules, the Raf kinases (MAP3K), the MAP2K kinases (MEK1 and MEK2) and ERK1 and ERK2 [47]. The Ras/MAPK network is frequently deregulated in malignancy and causes uncontrolled cellular proliferation and resistance to drug [47]. MEK is present at a junction of the Ras/MAPK pathway [47]. Amplification of Ras/MAPK pathway leads to the aggressive tumor characteristics [45]. In MM, certain translocation points the overall prognosis. The t(4;14) translocation overexpresses fibroblast growth factor receptor 3 overexpression that activates the Ras/MAPK pathway that subsequently leads to decreased apoptosis [45]. Incidence of activating Ras mutations is between 32 and 50% in MM (K-Ras and N-Ras), are also deregulate this pathway [46]. Novel agent RO5126766 showed activity in *RAS*- and *RAF*-mutated malignancies (lung and gynecological cancers) [48]. It also showed partial response in myeloma patient in Maxime Chenard-Poirier study [48].

### 5.3. Bruton's tyrosine kinase (BTK)

Bruton's tyrosine kinase (BTK) belongs to Tec family of tyrosine kinases [49]. The Tec family comprises of BTK, BMX, ITK, TEC, and RLK. BTK is the most commonly studied member of the Tec family and is present in different stages of B cells [49]. But this protein is absent in T lymphocytes and normal plasma cells [50]. On B cells and myeloma cells BTK controls signal pathways including PI3K, PLCγ, and PKC in multiple myeloma [49]. These pathways play important functions in cell propagation, expansion, delineation and survival [49]. BTK attract MM cells toward stromal cell-derived factor-1 (SDF-1) which is present at high levels in the BM [49]. BTK expression is correlated with SDF-1 receptor CXCR4 in myeloma cells [51]. BTK inhibition leads to the inhibition of anti-apoptotic proteins Bcl-xL, survivin and FLIPL and stimulates caspase-controlled apoptotic death within the myeloma cells [52]. One of the BTK inhibitor, ibrutinib, inhibits MM cell growth, osteoclasts or mesenchymal stem cells growth in vitro [53]. As a single agent, BTK inhibitor CC-292 did not show anti-myeloma activity in vitro but reveals negative impact on osteoclasts function. Interestingly, high levels of BTK have been reported as a poor prognostic marker in MM patients [52]. Therefore, we need targeting agents against this protein (BTK) to not only control microenvironment but also malignant plasma cells [54].

### 5.4. HSP70

Pathways, like HSP70, ubiquitin-proteasome and unfolded protein response (UPR) and auto-phagy pathways help neoplastic cells to adjust according to stress that is produced by immu-noglobulin overload in the endoplasmic reticulum (ER) [55]. Heat shock protein 70 is one of the pathways that increase survival of myeloma cells by inhibiting gh APAF-1 and caspase 9 [55]. HSP expression is activated by heat and other stressors, i.e., radiation and chemotherapy exposure [56]. HSP70 family comprises of 13 proteins. Proteins of this family are: HSPA1A and HSPA1B (called together as HSP70 or HSP72), HSPA5 (BIP), HSPA8 (HSC70), and HSPA9 [55]. Hsp proteins consist of an N-terminal ATPase domain, a C-terminal domain, and a middle portion. After binding of ATP, Hsp undergoes a conformational change [57]. The middle segment is binding site for protein kinase PKB/Akt and is implicated as the main site for client protein interactions [58].

Recent studies reveal that tumor cells with high levels of HSP70 have beneficial effect of proteasome inhibitors [55]. This protein represents a possible target to establish a new approach for multiple myeloma treatment [55]. HSF1 knockdown sensitizes myeloma cells to bortezomib treatment [55]. Bustany et al. study strongly suggests that HSF1(HSP) inhibitors might be promising agents in combination with bortezomib-based therapeutic protocols to treat MM patients with adverse prognosis or in relapse [59]. Bustany et al. study, strongly suggest that HSF1(HSP) inhibitors might be promising agents in combination with bortezomib-based thera-peutic protocols to treat MM patients with adverse prognosis or in relapse [60].

### 5.5. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a group of 18–24 nucleotides, non-coding RNA molecules. Mature one attaches to 3″UTR non translated site and control gene expression by translation modifi-cation or mRNA degradation [61]. They have substantial impact on post-transcriptional nega-tive regulation of oncogenes (e.g. MYC, MDM2) and tumor suppressor genes (e.g. TP53, PTEN) [62]. miR-145 is tumor suppressor miRNA in MM, miR-145 mimics inhibited p-AKT and p-PI3K, impairing proliferation and survival of MM cells [63]. Until now, around 700 miRNAs have been revealed in humans. Each miRNA can target at least 200 genes [64]. Anderson et al. identified a MM-specific miRNAs print that is evident by degradation of miRNAs -15a/-16 and over expression of miRNAs -222/-221/-382/-181a/-181b [64]. They also reported that these miRNAs control proliferation and growth of MM cells by inhibiting AKT serine/threonine protein-kinase (AKT3), ribosomal-protein-S6, MAP-kinases, and NF-_B-acti-vator MAP3KIP3. Furthermore these miRNAs exerted their activity even on bone marrow microenvironment [64]. One of the poor prognostic cytogenetics in myeloma is deletion of chromosome 13 that has been associated with overexpression, of miRNA-17_92 cluster (located on chromosome 13) in these patients [80]. In another study, miRNA-15 and -16, were down-regulated in MM patients having ch13 deletion [63].

The miRNA analysis showed contrary relationship between five assumed target genes (RAD54L, CCNA2, CYSLTR2, RASGRF2 and HKDC1) [61]. Anti-MM effects are also linked with miR-137 and miR-197. Studies showed that miR-34 and miR-125a inhibitors upregulates

p53 related miR-192 and -194 and inhibits oncogenesis and migration while enhance apoptosis [63]. miR-202 is down-regulated in bone marrow microenvironment and treatment with miR-202 mimics to inhibit growth by decreasing BCL-2 and BAFF levels [63].

## 5.6. Histone

Histone acetyl transferase (HAT) and histone deacetylase (HDAC) are enzymes that regulate expression of genes by moving acetyl from acetyl-CoA to the lysine residue of histones [65]. Subsequently, hyper acetylated histones aggravate transcription [65]. HDACs are enzymes that catalyze the removal of acetyl groups from amino lysines in histones, resulting in relaxation of the DNA around the histones and suppression of transcription [66]. HDACs are divided into five groups: class I (HDAC1, HDAC2, HDAC3, and HDAC8), class IIa (HDAC4, HDAC5, HDAC7, and HDAC9), class IIb (HDAC6 and HDAC10), class III (SIRT family), and class IV (HDAC11) [66]. Inhibiting HDAC converts histones in hyperacetylation form and leads to alter gene expression [67]. In malignant cells, many HDAC inhibitors (HDACi) have shown good anti-tumor activities with anti-proliferative, pro-apoptotic and anti-angiogenic properties [67]. SAHA (suberoylanilide hydroxamic acid) is one of the HDACi, showed antimyeloma activity by inhibiting proteasome and expression of its subunits, and increases myeloma cell sensitivity to Bortezomib [68]. Extrinsic and intrinsic apoptotic pathways, non-apoptotic cell death, i.e., autophagy pathways and cytokines and proteins implicated in multiple myeloma survival, progression and immune escape have been documented in myeloma cells treated with an HDACi [67]. The cellular pathways controlled by SAHA include IGFIGF-1RAkt, IL-6gp130 and proliferative/antiapoptotic factors (e.g., NF-B, XBP-1, and E2F-1) [68].

Myeloma cells have overexpression of antiapoptotic proteins Bcl2 and Mcl1 and down regulation of pro-apoptotic protein Bax [67]. These findings depict resistance to chemotherapeutic agents [67]. Treatment with depsipeptide in myeloma cell line resulted in a decrease of the anti-apoptotic proteins Mcl1, Bcl2, BclxL and an increase in Bax [67]. 5-azacitidine is a DNA methyltransferase inhibitor shows activity against myeloma [67]. Azacitidine and analogs such decitabine are interesting agents to investigate hypermethylation in tumorigenesis and the clinical efficacy is under investigation in phase II trials [67]. In S. B. Khan s study, depsipeptide (HDACi) induces apoptosis in MM cells and shows an additive effect with melphalan [65].

## 5.7. Microenvironment

Microenvironment is defined as surrounding cells and tissues can impact the growth of specific cells by changing the pH, oxygen levels, nutrients, and antiapoptotic factors [69]. when this microenvironment is dysfunctional leads to disease progression, particularly in cancer. Like in other parts of body, the bone marrow has own microenvironment [69]. That is classically defined as to have two niches: the endosteal (osteoblastic) niche and the vascular (sinusoidal) architecture [69]. The osteoblastic niche comprises of reticular cells, fibroblasts, and adipocytes [70]. They provide supportive matrix for stem cells [70]. The vascular niche has important functions in bone marrow: transfer oxygen, nutrients and growth factors to hematopoietic cells for proliferation and differentiation of cells; support of homing and recruitment through chemokines [70].

The BM milieu of MM consists of extracellular matrix, hematopoietic and nonhematopoietic cells along with cytokines, growth factors, and adhesion molecules [70]. The increased osteo-clastic activity is secondary to increase cytokines, i.e., IL-6, IL-1b, tumor necrosis factor (TNF)-$\alpha$, and parathyroid hormone-related protein [70]. Other causes of osteoclast activation are: Mye-loma cells express RANKL, TNF-$\alpha$, and inactivation of RANKL decoy receptor and OPG. The destroyed bone environment stimulates platelets to release TGF-$\beta$ and IGF-I that will cause myeloma genesis [70]. Not only osteoclast is activated, osteoblasts are also inhibited in myeloma. Factors responsible for inhibiting osteoblast are TGF-$\beta$ and IL-3 [70]. Extracellular matrix of the myeloma show increased expression of angiogenic factors and their receptors, i.e., vascular endothelial growth factor (VEGF) and VEGF receptor-2, fibroblast growth factor-2 (FGF-2) and FGF-2 receptor-2, platelet-derived growth factor receptor beta (PDGFR-$\beta$) and ECs-released VEGF and IL-8 [70]. In the bone microenvironment, myeloma cell are surrounded by immune competent cells [68]. Because of certain growth factors rapid expansion of immature myeloid cells which fail to differentiate and, impede immune system and leads to oncogenesis [68]. Specific CD8+ T cells has been recognized in microenvironment, inhibiting CD4 + −cell growth by releasing interferon gamma causing immunosuppression [68]. The T-cell activity is also suppressed by the activation of PD-1 receptor with its ligand [69].

The PI3K-Akt signaling has been demonstrated to phosphorylate HKII Hexokinase II to acti-vate Glycolytic pathway in MM cells [69].

A number of intracellular signaling pathways, i.e., NF-κB, Akt, p38MAPK, protein p62, Pim-2 are over-in both MM cells and their BM microenvironment [69]. Pim kinases are also involved in drug resistance by activating drug efflux transporters [69]. Pim-1 phosphorylates the ATP-binding cassette (ABC) transporter ABCG2 that subsequently causes drug resistance [69]. The side population (SP) phenotype is a feature of stem cells in tissues. The SP cells are associated with the expression of genes involved in the glycolytic pathway including GLUT1, GLUT3, and PDK1 and the glycolysis appears to be highly accelerated in SP cells [69]. The inhibition of glycolysis via targeting these SP cells can disrupt the drug resistance [69].

Immune microenvironment consists of T Cells, NK and NKT Cells, dendritic cells, myeloid derived suppressor cells and adipocytes [70]. Reciprocal increase in IL-17, IL-17 induces mye-loma tumor cell growth and inhibits immune function in myeloma patients [70]. Impaired differentiation and function of NK and NKT cells have been recognized in MM. A major contributing factor to this immune dysfunction is believed to be IL-6 mediated [70]. Myeloid-derived suppressor cells (MDSCs) expands during cancer, inflammation and infection and have ability to suppress T-cell responses (**Table 1**) [71]. Recently, it has been proposed that a 5-fold increase in MDSCs in newly diagnosed MM [70] **Tables 1–3**.

### 5.8. Marrow-infiltrating lymphocytes

Lymphocytes residing in the bone marrow are called marrow infiltrating lymphocytes [72]. These MILs need to be activated and expanded in vitro to destroy malignant cells. Difference between peripherally derived T lymphocytes and marrow derived lymphocytes is: MILs have a ability to recognize a wide variety of proteins on the surface of the tumor cells than do cells

| Growth factors/cytokines | Possible mechanism of actions |
|---|---|
| PTH, VIT D3, IL-1, IL-11 | Activates osteoblast and stromal cells |
| PD-1 on T cells | PD-L1 on myeloma cells |
| VEGF, IL-6 on stromal cells | Raf/MEK/ERK activation on myeloma cells |
| VEGF, TGF-β, FGF from stromal cells | Angiogenesis |
| G-CSF and IL-6 induced a higher level of phospho-STAT3 in neutrophils | Angiogenesis |
| IL-10 | Plasma cell proliferation and angiogenesis |
| Wnt, Dickkopf Wnt signaling pathway 1 (DKK1), fibroblast growth factor (FGF) | Decreased increased osteoblast number Decreased bone mineral density |
| Downregulating expression of the RANK-L decoy receptor (OPG) Elevated levels of IL-6 induce RANK-L expression and decrease INFγ production | Osteoclastogenesis Bone resorption |

**Abbreviations**: PTH, parathyroid hormone; VIT D3, Vitamin D3; IL-1, IL-11:Interleukin1/11; VEGF, vascular endothelial growth factor receptor; TGF-β, transforming growth factor beta; FGF, fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; RANK-L, receptor activator of nuclear factor kappa beta.Source: Mondello et al. [71].

**Table 1.** Bone marrow micro-environment.

| Pathways | Consequences of activation of pathways |
|---|---|
| Raf/MEK/P42/44 MAPK[*] | Proliferation |
| β-catenin[*] | Proliferation |
| PI-3 K/Akt[**] | Proliferation Anti-apoptosis Drug resistance |
| JAK/STAT3[*] | Proliferation Anti-apoptosis Drug resistance |
| NF-κB[*] | Proliferation Anti-apoptosis Drug resistance |
| Notch-1[*] | Anti-apoptosis Drug resistance |
| MEK/ERK/P27[**] | Proliferation Anti-apoptosis Drug resistance (Cytokine mediated) |

[*]van de Donk et al. [80].
[**]Kizaki and Tabayashi [81].

**Table 2.** Intracellular signaling pathways in the pathogenesis of multiple myeloma.

that obtained from the blood [73]. So on relapse after receiving CAR T cells therapy, new type of antigen or protein are developed on tumor cell surface (similar to the antibiotic resistance) [73]. While MILs can identify a huge variety of proteins on tumor cells, problem of resistance is significantly lower [73].

| | | |
|---|---|---|
| Protein BMI-1 | substance PTC-209 | Preclinical studies |
| Inhibitor of microRNA genes | EZH2 inhibitor | Preclinical studies |
| Irreversibly inhibition of 20S proteasome, pan-proteasome inhibitor | Marizomib | Phase 1 clinical trials, relapsed/refractory. Trials ongoing for CNS involvement in myeloma |
| Oral 26S proteasome inhibitor | Oprozomib | Phase 1 studies relapsed/refractory |
| Anti-CD138 monoclonal antibody conjugated to DM4, inhibitor of the microtubule assembly | Indatuximab | Phase 1/2 clinical trials, relapsed/refractory |
| Monoclonal antibody to CD38 | SAR (SAR650984) | Phase 1 clinical trials, relapsed/refractory |
| Histone deacetylase (HDAC) 6 inhibitor | Panobinostat | Phase 3 clinical trials |
| HDAC6-specific histone deacetylase inhibitor | Ricolinostat | Preclinical studies |
| Non-specific histone deacetylase inhibitor | Vorinostat | Phase 3 clinical trials |
| Alkylating agent | Bendamustine | Phase 1/2 trial PR rate of 52%, with very good PR achieved in 24% |
| AKT kinase inhibitor | Afuresertib (PKB115125) | Phase 1 studies ORR—50% in relapsed/refractory |
| Bcl-2 inhibitors | ABT 199 | Preclinical studies |
| BTK inhibitors | Ibrutinib | Phase 2 dose escalation study, relapsed or refractory |
| Inhibitor of cyclin-dependent kinases (CDKs) | Dinaciclib | Phase 2 dose escalation study |
| IL-6 inhibitors | Siltuximab | Phase 2, newly diagnosed MM, VGPR rate was significantly improved but not CR rate |
| Kinesin spindle protein (KSP) | Filanesib (ARRY-520) | Phase 2 clinical trials, ORR was 58%, relapsed/refractory |
| Phosphoinositide 3-kinase (PI3K) | Idelalisib BAY80-6946 GDC-0941 | Relapsed/refractory, preclinical investigation |
| Heat-shock protein 90 inhibitor | Tanespimycin | Phase 1 dose-escalation study |

Source: Refs. [139, 140].

**Table 3.** Potential Target for Multiple Myeloma.

Adoptive T-cell therapy (ACT) has been assessed in trials, in which activated tumor-specific T cells has been used to activate antitumor immunity after myeloablative chemotherapy in patients with multiple myeloma (MM) [73]. But efficacy of this approach is limited by the tumor-non specific T cells [73]. In phase I study, Noonan and colleagues assess the safety, and efficacy of this approach in 25 patients in multiple myeloma patients [74]. MILs infused after autologous stem cell transplant in 22 patients and found complete remission/partial response/stable disease in six/seven/five patients [74]. Progression-free survival was correlated with greater than 90% reduction in tumor burden (25.1 vs. 11.8 months) [74]. Borrello and colleagues also showed that marrow-infiltrating T lymphocytes (MILs) can led to clinical

antitumor immunity [73]. Results from small studies are encouraging but need confirmation in a larger trials [73].

### 5.9. PD-1/PD-L1

The PD-1 receptor is present on T, B cells, monocytes, and natural killer (NK) T cells when activated to certain antigen stimulus [75]. PD-L1 and PD-L2 are present on antigen presenting cells, i.e., dendritic cells and macrophages [75]. After contact of PD-1 to PD-L1 or PD-L2, this complex reduces secretion of Th1 cytokines, inhibits T-cell proliferation and inhibits CTL-mediated killing [75]. In the physiologic state, this pathway maintains immunologic equilibrium. While, in pathologic settings, e.g., in malignancy, over expression of this pathway leads to to activation and function of cancer related T-cell populations, which supports for immune escape and tumor proliferation [75]. PD-L1 expression is also documented in cells of the tumor microenvironment, i.e., myeloid-derived suppressor cells that helps in escape to natural body defense system [75]. To improve already decrease immunity in myeloma patients, strategies have been explored at molecular and cellular levels [76]. These are: passive immunotherapy with monoclonal antibodies that hit myeloma specific antigens; cancer vaccines; T-cell therapy and change immunosuppressive microenvironment of the bone marrow via immunomodulatory medicines or by inhibiting immune checkpoints [76]. There are studies under process for PD-1 receptor/PD-L1 and PD-L2 inhibitors in myeloma, i.e., Pembrolizumab in combination with IMiDs [77]. Preliminary results of a phase II trial with pembrolizumab with pomalidomide showed 50% objective response with near complete and very good partial responses in refractory patients [77].

### 5.10. Monoclonal antibodies

In 2015, two monoclonal antibodies were approved for the treatment of relapsed or refractory multiple myeloma (RRMM), elotuzumab and daratumumab [78]. CD38 is a type II cell membrane glycoprotein. It has multiple functions in cell to cell adhesion, enzymatic (cellular nucleic acid metabolism) activity [77]. It is present on a multiple hematopoietic and non-hematopoietic cell types. Cell that harbors this receptor are: medullary thymocytes, activated B and T lymphocytes, NK cells and dendritic cells [77].

Daratumumab is a fully humanized monoclonal IgG-κ antibody directed that works against CD38 of myeloma cells [77]. It exerts its effects like other monoclonal antibodies, i.e., antibody dependent cytotoxicity, complement mediated cytotoxicity and antibody dependent phagocytosis (ADCP), induction of autophagy/apoptosis [77].

Antibodies targeting CD38 are easily tolerated and showed partial response or better in approximately 30% of relapsed/refractory MM patients as single agent [79]. In future, deep responses and better progression-free survival can be obtained by combining them with immunomodulatory agent or proteasome inhibitors [79].

In phase I/II study recently published by Lokhorst et al., impressive clinical responses were seen in heavily pretreated patient population with 64% double refractory to PIs and IMiDs and

had undergone ASCT in 76% [77]. Daratumumab as a single agent yielded 36% overall response rate in 16 mg/kg arm and remarkably, in the responder group, 65% remained progression free in 12 months [77].

Elotuzumab is a monoclonal IgG-κ antibody works against signaling lymphocytic activation molecule F7 (l surface receptor helps in activation of natural killer cell) [78]. This antibody induces cell death via antibody dependent cytotoxicity (ADCC) and inhibits CS1-mediated MM cell adhesion to bone marrow stem cells [79].

In phase III ELOQUENT-2 study, different regimens with this agent were tried in relapsed/ refractory setting. It was found that 1-year PFS rate was higher in the ELO-LEN-DEX (- Elotuzumab-Lenalidomide-Dexamethasone) arm (68 vs. 57%), and this difference was slightly greater at 2 years (41 vs. 27%). Other targeted antigens on which trials are being conducted are: CD74, CD138, B-cell activating factor, interleukin-6 [79].

### 5.11. CART cells

CART cells, is made by fusing the variable fragment (scFv) of a monoclonal antibody (mAb) with intracellular signaling domain related to CD-19 related antigen [77]. The MHC-independency, in vivo expansion and memory cell growth make these cells more beneficial the antibodies [77]. Plasma cells do not have a strong CD-19 expression but Garfall et al. have observed a relatively more frequent expression than previously reported [77]. In 43 years old patients after nine lines of treatment this approach showed remission. This generates a hypothesis that there may be a role of this strategy even in minimally/weakly expressed antigens Currently, it remains unclear whether concurrent targeting of multiple antigens (such as CD38, CS1, BCMA, CD138, etc.) is helpful for achieving eradication of myeloma clone [77]. For CART cells, costimulatory molecules are required to prevent the immune system from eradication of these cells, but best costimulatory antigen is not known yet. Few costimulators are under study, these are: CD19, CD138, CD38, CD56, Lewis Y, CD44v6, CS1, and BCMA.

In new data from a Garfall pilot study, after anti-CD19 CAR and a salvage SCT, progression-free survival (PFS) was reported after first-line SCT in 3 of 10 study participants. In 2017, studies with chimeric antigen receptor (CAR)-T cells targeting B-cell maturation antigen (BCMA) have shown good response in relapsed/refractory myeloma patients. But this option is impeded by short half lived effector cells, acute toxicity, and host immune responses against CARs.

# 6. Pathways involved in multiple myeloma

## 6.1. Gene expression profile (GEP) and molecular variability in myeloma

The MM transcriptome has been evaluated in different groups [81–84]. Different genes have been explored between MM and normal plasma cells and also during different phases of disease. Impaired control of certain genes of the Cyclin D family (CCND1, CCND2 and CCND3) appeared to be a universal characteristic of MM cells, especially early MGUS

(monoclonal gammopathy of undetermined significance) stage [43]. The mechanisms elaborate in Cyclin D mutation are multiple and comprised of 1—cyclin D amplifications, 2—translocation of CCND1 or CCND3 with the IgH gene in the t(11;14) and the t(6;14), 3—trisomies and other cytogenetics events that incidentally contribute to over-expression of CCND genes. In particular, CCND2 is overexpressed in certain group of patients that carry t(4;14) and t(14;16) MM [81, 82]. These observations allowed classification of MM in eight subgroups in the translocation cyclin D (TC) classification [43]. Additional studies have observed other molecular subgroups independent of Cyclin D involvement and linked with other clinical and phenotypical characteristics. For example, a Low-Bone subgroup, that includes MM patients with minimal or few bony lesions and minimal expression of Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1) or the proliferative subgroup which shows over expression of specific cell cycle- and proliferation-associated genes [83]. Overall, GEP emphasis an important molecular heterogeneity in this disease. Over 500 genes have a substantial difference between the different clinical subgroups [43]. Cytogenetic changes, mainly hyperdiploidy and translocations involving IgH are highly connected with certain molecular subcategories clusters. For example, t(4;14) which primes to the over-expression of the histone methyl transferase Multiple Myeloma SET Domain (MMSET) is linked with a specific gene expression profile secondary to MMSET activity [85]. More globally, HDMM and NHDMM can be observed by using GEP [86].

## 6.2. Definition of myeloma pathogenesis by using GEP

In order to explore the molecular basis of myeloma cell development, several studies have observed GEP at the different stages of the disease [87]. In these studies, normal plasma cell was compared with cells during different stages of MM i.e. MGUS cell, Myeloma, smoldering MM, newly-diagnosed symptomatic MM, relapsed MM and cells from patients with plasma cell leukemia (PCL) by using GEP [87]. In one study of 877 patients, authors concluded that MGUS plasma cells share similar features with MM and relapse MM but have different genes and pathways that are expressed lately during MM progression [87]. These activated pathways comprises of E2F activation, cMYC and chromosomal instability genes and these demonstrates a possibility of progression to MM if exist at MGUS or SMM stage [88]. Other groups have examined other different genes, i.e., antiapoptotic DNA repair, NF-kB and cytokines-signaling pathway related genes in established MM cells in comparison with premalignant MGUS cells [88]. Interestingly, influence of microenvironment on gene profile of the MM cells has been assessed that confirmed activation of crucial critical pathways such as Notch and Ras, NF-kB, and genes affecting proliferation, survival, cell cycle regulators/activation in MM cells [89].

## 6.3. Link of prognosis with GEP

Ability to explore complete transcriptomic expression profile of MM cell provided an unique opportunity to confirm predictive role of GEP on disease behavior. Clinical trials and long term follow-up of MM patients revealed the ability of GEP to predict prognosis in different cohorts. Many studies have identified gene expression signatures capable of predicting EFS and OS in MM by using different approaches. Most of these studies have shown GEP profile as

an independent prognostic factor. Some studies have used a biological approach with respect to specific features of MM cells. Chromosome instability signature [90], centrosome index signature, and cell death profile [91] were explained based on instability of genomes, whereas a 7-gene prognostic expression profile was developed from MM cell lines study [92, 93]. Other prognostic signatures like the 15-gene prognostic signature or the proliferation signature have also been published in literature [94]. Other groups evaluated GEP signature correlation between GEP with overall survival of MM patients in separate cohorts. The HOVON-65/GMMG-HD4 clinical trial researchers [94], the Intergroup Francophone du Myeloma 99 clinical trial [84], and UAMS researchers [95] published reports on 92, 15 and 70 genes signature respectively [95]. Importantly, only minimal or no genes overlay between these signatures signifying that each signature does not encompass all high risk patients and also highpoints the dismissal in the system. In an attempt to streamline GEP use in clinical practice and to define a distinctive tool, amalgamation of existing prognostic signatures have been recently reported. That combination will define a single reliable signature that might be able to predict outcome in MM at diagnosis and relapse [96].

Interestingly, GEP signature has also shown significance in early stages of MM or in plasma cell leukemia patients. Investigators from UAMS have reported that 70-genes signature and its derivative are able to predict outcome in context of MGUS and SMM [97]. In the context of PCL, in a cohort of 21 patients, a 27 gene expression signature was identified as an independent prognostic factor [24].

## 6.4. Transcriptome modifiers profiling

The RNA-sequencing have been created and will be incorporated into GEP to enhance estimation of the outcome in the future [98]. Of these modifiers, non-coding RNA are mainly researched in MM since reports have already proved that micro-RNA contribute to myeloma formation and can be used to predict prognosis or response to auto-transplant [98]. MiR17 and miR886-5p have been observed as a strong prognostic indicator in a study of 163 newly diagnosed patients from the MRC Myeloma IX l trial [99]. Recent literature is now signifying importance of microRNAs MM and separate MM subgroups [100]. For example, miR-126 stimulates cMYC overexpression in t(4;14) MM [101], and miRs-192, -194, and -215 leads to impaired control of p53 and MDM2 in a subgroup of MM, causing poorer outcome [102, 103]. Very interestingly, overexpression of circulating microRNAs, which are easily access for investigations has been researched and may represent a decent prognostic biomarkers in MM [104]. Furthermore, management options that can reestablish miRNAs (Tumor suppressor miRNA) or impede miRNAs (Oncogene miRNA) are in process to be used as major therapeutic option in the future [105, 106]. Long noncoding RNAs (lnc RNA) are also being sensibly studied inMM. Samur et al. with others is currently identifying important changes in deregulation of lncRNAs over- or underexpression and its impact on clinical outcome [108].

In post-transcriptional event, alternate splicing is an important event that extremely increases the transcript collection affecting number of cell development process including cell growth and survival. It has been documented as important marker of malignant phenotype and the knowing the alternate splicing events will help in future to better predict prognosis in MM.

There is evidence that splicing events affect specific genes as hyaluronan synthase 1 (HAS1) [109] or deleted in colorectal carcinoma gene (DCC) occur repeatedly in MM [110], or that a targeted therapy that control the splicing of X-box binding protein 1 (XBP1) increases sensitivity of MM cells to proteasome inhibitor. Pilot investigations by Nagoshi et al. as well as and by some other researchers have identified important number of spliced isoforms in myeloma in comparison to normal plasma cells with regards to both functional concern as well as prognostic importance. Interestingly, the ability to depict mutations at the RNA level is becoming well recognized entity. DNA-based studies in MM, including mainly whole exome sequencing, have emphasized the mutational background of the disease, which includes few repeated mutations (NRAS, KRAS, TP53, DIS3, and FAM46C). NFkB and ERK trails are the most involved pathways, with mutations in 43 and 17% of MM cases respectively [111–114]. Although only specific mutations have a clear impact on prognosis (TP53, ATR, and ATM) until now. The capability to diagnose these mutations at the RNA level [114] can now be used to predict outcome that can be integrated in the future models expecting prognosis in MM. Finally, next generation sequencing helps us to perform single cell studies. This method, exemplified by the drop-seq technology [115], allows documentation of the variable clones as well as identification of the transcriptome in the reference of the microenvironment. The initial data regarding single cell transcriptome assessment depicts exciting applications [116] including amalgamation into a new GEP signature [117].

## 6.5. GEP and variability in clones

Intraclonal variability is an important characteristic of cancer that has been shown in MM [118, 119]. It refers to malignant cells having same genomic changes but with subtle differences in mutations, copy number abnormalities and chromosome changes including translocations among different clones. In MM cells, the evaluation of Ig gene rearrangement by next-generation sequencing is particularly helpful. Munshi NC et al. did deep sequencing of the IgH gene at time of diagnosis and relapse in a large series of patients emphasizing the complexity of the clonal and sub-clonal architecture of the disease [120]. However, only few reports have been published the evolution of in MM. Four patterns of this clonal changes have been observed [112, 121]. The modification in sub clonal copiousness will be correlated with changes in GEP. For example a linear development may not meaningfully influence on overall GEP, on the other hand branching evolution may reveal decrease in expression of genes representing clones The ability to assess transcriptome at a single cell level might be essential in order to define the true influence of intraclonal heterogeneity on GEP and to recognize potential marker of sensitivity or resistance to specific therapeutic drugs [116, 122].

## 6.6. Significance of GEP in combination with ISS

A recent study reported GEP in combination with clinical prognostic marker in MM comprising cytogenetic alterations and ISS score. This study used different GEP signature and revealed that the combination of GEP with ISS is a useful and better prognostic tool that significantly improves risk stratification then alone ISS [123]. Recognizing high risk patients remains an

important task to try and modify treatment in future discussed by Landgren and Rajkumar in this CCR Focus section [124]. Currently, no specific targeted agent therapy is indicated especially for the high-risk patients in upfront setting, there is increasing emphasis on including multi-agent therapy as consolidation followed by transplant and post-transplant maintenance in transplant eligible patients. High dose melphalan followed by autologous stem cell transplant (ASCT) appears to be the best consolidation therapy to date in multiple studies [125].

## 6.7. Response to treatment prediction by using GEP

GEP has also been assessed to forecast complete response (CR) to different treatment as well. CR is an independent factor to not only tell progression free survival but also an indirect marker of overall survival [126]. A precise GEP signature has been identified with reference to upfront three drug combinations (VTD) in newly diagnosed MM, high dose therapy (54 IMiDs/dexamethasone, tandem auto-transplant at relapse and the bortezomib-based regimen [129]. However, a prediction model research that compared different dataset has shown that GEP alone is not well-organized to predict CR in different datasets [127–130].

In this study, various methods have been used to develop a response predictive model; even with the best GEP-based CR predictive model, precision was between 56 and 78% that was found in different datasets. The ability to predict CR was not affected by different methods used measure GEP, or treatment regimens used or in newly-diagnosed or at time of relapsed patients. This study signifies the fact that it may be necessary to combine multiple other genomic parameters in response predicting model in future.

## 6.8. Personalized therapy assortment

Based on GEP, the derangements in certain pathways can be controlled and offer an important information to guide treatment therapy. For example, the presence of high DKK1 level, that shows bone involvement can be explored for the use of anti-DKK1 drug [131, 132] or the assessment of the ratio between BCL2/MCL1 level can point out the sensitivity to BH3 mimetic drugs [133]. On the other side, combining the information of gene expression with mutation expression helps to select treatment options as personalized medicine [134]. The detection of precise mutations such as BRAF V600E can direct for use of BRAF inhibitor such as vemurafenib [135, 136], or mutations triggering the MAPK pathway can give us rationale for the use of MEK inhibitors such as trametinib [137]. Other targetable mutations such as SF3B1, FGFR3, ATM/ATR, IDH1/2, and CCND1 as well as RAS/RAF, NFkB pathway-linked genes have been described in myeloma. These mutations can be controlled by appropriate inhibitors.

Some mutations can also be assessed to predict drug sensitivity. Initial data of one study, revealed that the presence of NRAS mutations in relapsed cases is associated with inferior response to bortezomib [138] or in contrast, that the occurrence of IRF4 mutations is related with higher sensitivity to immuno-modulatory agents [111]. These data needs confirmation in further clinical trials but it is hypothesis generating study.

The documentation of specific micro-RNA expression profile can also be exploited to guide therapy. Several microRNAs are being researched as treatment targets with hopes for development of small molecules that target these micro-RNA function.

Similarly, GEP has been employed to predict resistance to antimyeloma drugs with an interpretation that harmful agents are avoided that are not helpful. With the help of number of B-cell lines including multiple myeloma cell lines, a microarray-based GEP signature was established to predict resistance of melphalan. Although the expression profile was able to predict sensitivity vs. resistance in cell lines, its practical application needs further studies to be done [102, 103]. Interestingly, a pharmacogenomic study of global GEP of myeloma cells recovered from myeloma patients and specific time after administration of different drugs have been assessed [104, 105]. Prognostic information was acquired from GEP of refined plasma cells 2 days after providing thalidomide and dexamethasone or bortezomib to newly-diagnosed myeloma patients. An 80-gene signature was recognized following bortezomib administration that will guide us in future for better patients' risk stratification [105].

From treatment as well as prognostic points, it is also important to consider persistent changes in genome which occurs without stimulus or as well as under the influence of microenvironment, epigenomic changes or therapy. Therefore, assessment by GEP at a single time point may not be meaningful. The advancement of GEP from diagnosis, response and relapse should be interpreted intelligently to have an answer for proper selection of the most appropriate therapy.

## 7. Potential target for multiple myeloma

### 7.1. Constraint of GEP in existing clinical practice

Important impediments still present to prevent application of this important investigation in general clinical practice. Although many specific GEP signatures have been recognized and a recent study has joint some of these signatures to create a unique signature [32] but no consensus have been accepted to date for universal use for every MM patients. GEP remains a research tool and is not yet authenticated by the FDA. For clinician point of view, the GEP data have been created generally in a setting of certain treatments that includes thalidomide, lenalidomide and bortezomib with or without auto-transplant. Since the therapeutic landscape is largely progressing in MM, re-assessments are required for each novel drug and/or combination. In particular the arrival of new therapeutic classes such as antibody drug conjugates, targeted agents (Elotuzumab, Daratumumab) and new IMiDs and proteasome inhibitors [106, 107] markedly improve the prognosis and may need different GEP studies and signatures [107, 114]. GEP has been utilized to date in few myeloma centers and mostly for investigational purposes. The development of investigators friendly and quicker methods should be considered. Simple quantitative PCR has been assessed in a group of 157 newly diagnosed patients proved good acceptable results [115]. However, a final conclusion about this test is still pending. Most importantly

an integrated approach that includes gene signatures, mutational profile and microRNA expression will be requisite to allow a wider application of genomic information to direct for treatment selection as well as prognostication. Taking the current understanding of these landscape genetic assessment to the next level, it will be essential to understand the clinical influence of clonal content and advancement along with identification of sub-clonal variants and molecular alterations on disease outcome [141]. The current information about mutational load that predicts outcome will need to be re-investigated for treatment purpose. These algorithms will be amended with the arrival of immunotherapeutic strategies which may have great achievement in malignancies with high mutational load. Again, as demonstrated by Rashid NU et al. with other colleagues Mutations must be studies further as predictive markers for treatment decisions [97].

## 8. Future trend

There is tremendous progress has reported so far, newer high-throughput technologies are being added with clinical parameters [142]. Array-based methodologies, sequencing-based method, and newer bio-informatics methodologies are in process of development. Furthermore, integrative oncogenomic work are merging new markers such as mutations, splicing events, noncoding RNA, miRs with older ones to help in better prognostication [143]. The personalized medicine depends on the assortment of a targeted therapy guided by the specific mutation or GEP signature is attractive tool treatment option. However, in future, in MM patients, treatment option selection depends on coexistence of sub-clones, dynamic evolution of the disease and triggering mutations in pathway, i.e., KRAS and BRAF for the ERK pathways [144].

To conclude, gene expression profile studies provide important knowledge regarding MM pathogenesis, and establish a powerful tool for prediction of outcome and to direct clinicians for selection of therapy [145]. The grouping of mutational profile, gene expression and splicing events with ISS and cytogenetic may become a standard of care in MM care [97].

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