Gene Expression Signature in MGUS and Multiple Myeloma

Christoph J. Heuck, Sarah K. Johnson, Qing Zhang, and John D. Shaughnessy Jr.

Abstract Despite improvement in therapeutic efficacy, multiple myeloma (MM) remains incurable with a median survival of approximately 10 years. Gene expression profiling (GEP) can be used to elucidate the molecular basis for resistance to chemotherapy through global assessment of molecular alterations that exist at diagnosis, after therapeutic treatment and that evolve during tumor progression. Unique GEP signatures associated with recurrent chromosomal translocations and ploidy changes have defined molecular classes with differing clinical features and outcomes. When compared to other stratification systems, the GEP70 test remained a significant predictor of outcome, reduced the number of patients classified with a poor prognosis, and identified patients at increased risk of relapse despite their standard clinicopathologic and genetic findings. GEP studies of serial samples showed that risk increases over time, with relapsed disease showing GEP shifts toward a signature of poor outcomes. GEP signatures of myeloma cells after therapy were prognostic for event-free and overall survival and thus may be used to identify novel strategies for overcoming drug resistance. This chapter will focus on the use of GEP of MM to define high-risk myeloma and elucidate underlying mechanisms that are beginning to change clinical decision-making and inform drug design.

C.J. Heuck, M.D. • Q. Zhang, Ph.D.
Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR, USA

S.K. Johnson, Ph.D.
Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

J.D. Shaughnessy Jr., Ph.D. (✉)
Signal Genetics, LLC, 667 Madison Ave., New York 10065, USA

Myeloma Health, LLC, 667 Madison Ave., New York 10065, USA
e-mail: jdsjr@signalgenetics.com; ShaughnessyJohn@uams.edu

S. Lentzsch (ed.), *Genetic and Molecular Epidemiology of Multiple Myeloma*,
DOI 10.1007/978-1-4614-4660-6_2, © Springer Science+Business Media New York 2013

Keywords Gene expression profiling • fluorescence in-situ hybridization • cytogenetics • multiple myeloma • classification • prognosis • high risk • therapeutic management • microenvironment • chromosomal abnormalities • standard-risk • low bone

1 Introduction

Multiple myeloma (MM) is a malignancy of antibody secreting plasma cells that accumulate within bone marrow. Its sequelae include renal failure, osteolytic bone destruction and osteoporosis, anemia, and immunosuppression (Palumbo and Anderson 2011). Prior to 1999, the primary treatment approach for the MM patient was mainly palliative in nature despite the use of potent chemotherapeutic drugs such as melphalan, prednisone, dexamethasone, vincristine, and Adriamycin, as well as stem cell transplantation (SCT) procedures that were introduced in the early 1990s (Palumbo and Anderson 2011; Barlogie et al. 2006a; Anderson 2011), but new drugs have become a promising new feature of the therapeutic landscape. The clinical outcome has dramatically improved with the successive introduction of increasingly efficacious drugs such as thalidomide (in 1999), bortezomib (in 2003), and lenalidomide (in 2004). A large proportion of MM patients can now look forward to substantial increases in progression-free survival (PFS) and overall survival (OS) (Palumbo and Anderson 2011; Lonial et al. 2011) resulting in a median survival of approximately 10 years (Hoering et al. 2009; Barlogie et al. 2006b). Despite this dramatic improvement, cure in MM remains a rare event, and a significant portion of newly diagnosed patients (about 15%) do not derive significant benefit from these novel agents and are thus at high risk for early relapse and death (Mitsiades et al. 2011; Barlogie et al. 2008). These sobering facts underscore the reality that a number of critical aspects of the biology and underlying genetics of MM in general and treatment-refractory MM in particular remain elusive. Consequently, a major research focus is to elucidate the molecular and genetic basis for the observed resistance to current chemotherapeutic regimens as well as novel targeted approaches to overcome it.

The advent of powerful technologies, such as interphase fluorescence in situ hybridization (FISH), spectral karyotyping, comparative genomic hybridization, single nucleotide polymorphism (SNP) genotyping, and microarray-based gene expression profiling (GEP), has provided the necessary tools to study MM in exceptional detail. We have used GEP to elucidate both the biology and clinical behavior of MM by globally assessing molecular alterations that exist in individual patients at diagnosis and that evolve during tumor progression and after therapeutic treatment. We have shown, for example, that unique gene expression patterns associated with recurrent chromosomal translocations and ploidy changes have defined molecular classes with differing clinical features and outcome (Zhan et al. 2006). Furthermore, GEP studies of serial samples showed that risk increases over time, with relapsed disease showing dramatic GEP shifts toward a signature of poor outcomes (Nair et al. 2009). This latter observation suggests a common mechanism of

disease evolution, driven by a small cohort of genes, and potentially reflects preferential expansion of therapy-resistant cells.

2 Pathogenesis at the Molecular Level

Long-term survival in patients with MM can vary considerably, and it is difficult to predict outcome based on current laboratory tests. Several large clinical trials have shown that before any treatment, a high-risk signature is present in approximately 15% of new cases of MM and is associated with shorter durations of complete remission, event-free survival, and overall survival (Barlogie et al. 2008). Among the 85% of MM patients presenting with low-risk disease and who respond to initial treatment, a high percentage of these patients will develop resistance over time and relapse with high-risk disease. Present consensus among MM practitioners is that the observed heterogeneity in outcomes for MM patients with similar clinical parameters (e.g., age, clinical presentation, functional status, and plasma cell burden) is the result of a differential evolution of complex genetic abnormalities that involve the malignant myeloma cell as well as the bone marrow microenvironment that lead to disease progression. A range of genetic and molecular tests have been incorporated as part of the standard of care for MM patients in order to refine diagnosis and to provide more rational clinical guidance regarding the selection of the most effective therapy.

MM can be characterized as either hyperdiploid (containing trisomies) or nonhyperdiploid (containing a number of translocations and other structural rearrangements) (Fonseca et al. 2004). Interphase fluorescence in situ hybridization (FISH) and metaphase cytogenetics have identified specific recurrent genetic abnormalities that are associated with inferior outcomes in MM patients and are used as a basis to risk-stratify patients (Chapman et al. 2011). Historically, poor long-term survival has been associated with the presence of several chromosomal abnormalities, including translocations of the immunoglobulin heavy chain locus on chromosome 14 and chromosomes 4, 16, and 20; deletion of 17p; deletions of all or parts of chromosome 13; and amplification of chromosome 1q. Conversely, patients who have extra chromosomes ("hyperdiploidy"), translocation t(11;14), or t(6;14) appear to have longer duration of response to therapy and better survival (i.e., standard-risk myeloma). However, these methods remain insufficient in capturing the broad spectrum of genetic abnormalities that cause such a heterogenous clinical course. We have utilized GEP as a highly sensitive method to both stratify MM patients in terms of outcome and to more fully characterize an individual's tumor at the molecular level using RNA extracted from a highly purified population of CD138+ myeloma plasma cells retrieved from the bone marrow (Zhan et al. 2002). Combining the microarray data and multivariate analysis of several large cohorts of patients with over a 10-year continuous follow-up and complete clinical and pathological outcomes analysis (Barlogie et al. 2008; Ribatti et al. 2006; Alizadeh et al. 2000), we identified a distinctive 70-gene molecular signature (GEP70) that identified a

patient population with myeloma that was at high risk of early recurrence and shorter overall survival compared to patients with low-risk MM who had a much greater probability of maintaining a longer-term remission (Shaughnessy et al. 2007a, b). We found, comparing this model to other stratification systems that are used clinically, including the International Staging System (ISS) for multiple myeloma, the GEP70 test remained a significant predictor of outcome (Fig. 1a) (Waheed et al. 2011). These 70 genes affect overlapping functions known to be important for relapse including cell-cycle regulation, angiogenesis, cell adhesion, cell migration, and proliferation. Compared to standard risk assessment factors or the presence of abnormal metaphase or interphase genetic abnormalities (Fig. 1b), the GEP70 gene signature significantly reduces the number of patients traditionally classified with a poor prognosis. In other words, it identifies the "false positive" high-risk patients, thus offering the clinician the opportunity to hold aggressive treatments and spare the patient unnecessary side effects. At the same time, it can distinguish those patients who may be at increased risk of relapse despite their standard clinicopathologic and genetic findings.

The ability of the GEP70 signature to distinguish between high- and low-risk myeloma patients regardless of where or how the MM patient was treated has been validated in publicly available datasets (Table 1), in other medical centers, and in clinical trials using differing clinical treatment protocols from those utilized at the UAMS as shown by the Phase II CREST and SUMMIT trials (Mulligan et al. 2007) as well as the Phase III APEX trials using bortezomib in a relapsed/refractory setting (Zhan et al. 2008). In addition, the GEP70 signature score has been further validated in a cohort of high-risk patients with specific chromosomal translocations at the Mayo Clinic (Chng et al. 2008). Our group was not the only one to develop a GEP-based prediction model for MM. Decaux and colleagues recently used a custom cDNA microarray to define a 15-gene model of high risk related to cell proliferation, with a hyperdiploid signature being related to better survival (Decaux et al. 2008). Multivariate analysis comparing the GEP70-gene model with the 15-gene model revealed that the GEP70-gene model was significant in all datasets tested, but the 15-gene model was significant in bortezomib trials only. These data, together with more of our unpublished data, suggest that the GEP70-gene model captures more outcome variability than other models or indices of cell proliferation.

3 The Relationship Between GEP and Recurrent Chromosomal Abnormalities

MM is currently classified according to recurrent chromosomal abnormalities (CAs). It is crucial to define the relationship of these abnormalities to clinical outcome and to identify groups that benefit from treatment with new therapeutic approaches. We performed GEP of a large number of MM cases that has allowed us to identify a pattern that divides MM into at least seven definable and reproducible disease classes (Fig. 2) (Zhan et al. 2006). Four (MF, MS, CD-1, and CD-2) of the

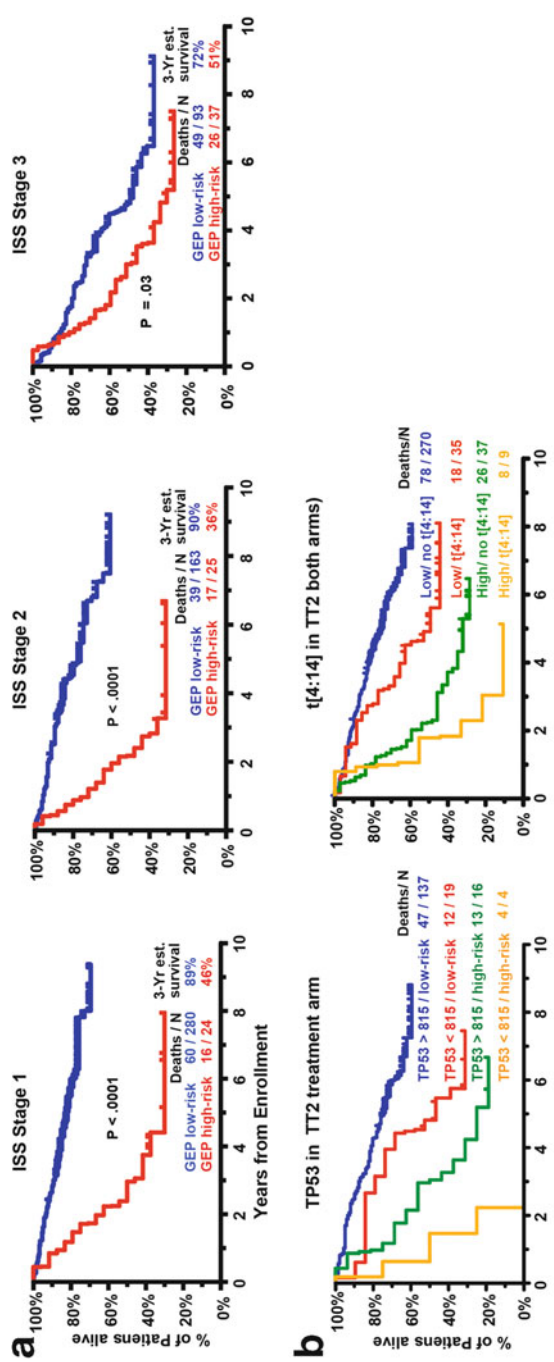


Fig. 1 Impact of gene expression profiling (GEP)-defined risk across total therapy (TT) regimens in newly diagnosed patients (**a**) within International Staging System (ISS) stage subsets on overall survival in ISS-defined low risk (Stage I, *left*), intermediate risk (Stage II, *center*), and high risk (Stage III, *right*) or (**b**) with low *TP53* (indicative of deletion, Xiong et al. 2008, *left*) or that harbor t(4;14) (*right*) (Adapted from Waheed et al. 2011)

Table 1 Studies incorporating multiple myeloma GEP70 risk model in outcome prediction

Study	Type of treatment	Patient (*n*)	GEP70 Independence in MVA	Tests competed	% population high risk	Conclusions	Ref
Comparison of ISS, CAs, and GEP risk stratification	TT2: induction, Mel-based ASCT, consolidation, maintenance TT3: added Bzb	Newly diagnosed (TT2, 351) (TT3, 275)	Yes	ISS3 CA GEP	21 33 14	After accounting for GEP-derived risk and delTP53 status, ISS stage and CAs still retain independent prognostic implications. GEP high risk is identified as having the highest HR for death and EFS. The GEP70 model identifies high-risk patients with shorter OS in each of the ISS stages	Waheed et al. 2011
GEP of relapsed myeloma	TT2	Relapsed (120)	Yes	CA GEP	68 38	GEP-defined high-risk status at relapse predicts shorter OS, while GEP-derived HY subgroup at relapse implied a decreased risk for death in MVA	Nair et al. 2009
GEP-defined proliferation for prognosis	HM1 and HM2: Mel, ASCT	Newly diagnosed (298)	ND	GEP-PI ISS3 GEP15 GEP70	7.7 17 12.4 18.7	Proliferation of malignant plasma cells, assessable by GEP-defined proliferation indices, is a central biological and prognostic factor in MM, independent of B2M, ISS stage, high-risk-associated CAs, and gene expression-based risk scores	Raje et al. 2006

GEP-defined PI and risk, t(4;14), low TP53	Mayo Clinic: high-dose Mel, ASCT	Newly diagnosed (71)	ND	GEP17[a]	13	GEP 17 identifies a high-risk subgroup with shorter OS in the Mayo Clinic dataset	Chng et al. 2008
GEP-defined risk in MM patients receiving Len and Dex	E4A03: Len plus Dex or Dex	Newly diagnosed (45)	ND	CA ISS3 GEP15 GEP70	23 21 22 16	GEP70 and GEP15 identify a high-risk subgroup with shorter OS in the non-auto-transplant population thus highlighting the importance of GEP scoring in the setting of initial therapy with Len and Dex	Sukhdeo et al. 2007
Identify broadly applicable prognostic markers	IFM 99: double ASCT, then no treatment or Pam or Thal w/Pam	Newly diagnosed (250)	Yes	ISS3 GEP15 GEP17	22.5 25 25	GEP 17 independently identifies high-risk myeloma in 5 independent datasets. GEP17 outperforms GEP15 (IFM-model) when tested on the Dex only arm of the APEX trial. GEP17 identifies high-risk myeloma in the IFM dataset. GEP15 does not remain an independent significant variable in the UAMS dataset	Decaux et al. 2008
Phase III APEX trial	Bzb or Dex, Dex pts w/progressive disease receive Bzb	Relapsed, 1–3 or >3 lines of therapy (156)	ND	GEP17	13.5	GEP17 identifies a high-risk subgroup in the APEX dataset that has a significantly shorter OS	Zhan et al. 2008

(continued)

Table 1 (continued)

Study	Type of treatment	Patient (*n*)	GEP70 Independence in MVA	Tests competed	% population high risk	Conclusions	Ref
Homozygous deletion mapping identifies genes and an associated GEP in MM	MRC Myeloma IX: Younger: ASCT, Cyc, Thal, and Dex or Cyc, Vin, Adr, and Dex. Older: Cyc, Thal, and Dex or Mel and prednisolone	Newly diagnosed (258)	Yes	GEP97 GEP15 GEP70	34 25 35	The cell death-97 GEP identifies 89 cases with poor prognosis, and the IFM 15-GEP identifies 64 cases. The GEP97 is almost equally as sensitive to identifying poor prognosis cases as the GEP70, which identifies 90 cases	Neri et al. 2007

Gene expression profiling (GEP), multivariate analysis (MVA), proliferation index (PI), autologous stem cell transplant (ASCT), cytogenetic abnormalities (CA), International Staging System (ISS), beta-2-microglobulin (B2M)

Bzb Bortezomib®, *Thal* thalidomide, *Dex* dexamethasone, *Mel* melphalan, *Cyc* cyclophosphamide, *Vin* vincristine, *Adr* Adriamycin, *Pam* pamidronate, *Len* lenalidomide

[a]GEP17 was derived from, and can substitute for, GEP70 (Shaughnessy et al. 2007a)

seven subclasses are characterized by elevated expression that results from recurrent chromosomal translocations present in approximately 40% of MM, which occur as a result of errors in switch recombination and/or somatic hypermutation (Kuehl and Bergsagel 2002). These translocations cause normally silent genes to become juxtaposed with powerful immunoglobulin enhancer elements, resulting in expression "spikes" readily detectable in microarray studies (Fig. 2). The HY subtype is characterized by low-level ectopic expression of *CCND1* and overexpression of genes mapping to the odd-numbered chromosomes that typically exhibit trisomy in MM. The LB class, characterized by a low incidence of magnetic resonance imaging (MRI)-defined bone lesions, expresses high levels of *CCND2* and a unique constellation of genes, including *endothelin-1/EDN1*. The seventh class of MM, PR, is not related to a primary genetic lesion but to high expression levels of proliferation-associated genes. This class likely constitutes an advanced stage of the other classes with the underlying hallmark features of the respective subclass masked by expression of proliferation genes. The 700 most differentially expressed genes allow segregation of these seven molecular MM subclasses (Zhan et al. 2006).

The MS class of MM is characterized by the t(4;14)(p16;q32) translocation. It is a high-risk subtype that predicts poor prognosis (Keats et al. 2003). The t(4;14)(p16;q32) reciprocal translocation results in hyperactivation of both *FGFR3* and *MMSET/WHSC1* genes (Chesi et al. 1998). All t(4;14)-positive diseases express elevated levels of *MMSET*, but in about 30% of these cases, expression of *FGFR3* is lost (Keats et al. 2003; Santra et al. 2003). It thus appears that *MMSET* plays a central role in driving downstream transcriptional events in the MS class. It is important to note that 25% of MM cases in other classes also exhibit upregulation of *MMSET*, supporting its importance in MM pathogenesis (Dring et al. 2004). Functional studies show that suppression of *MMSET* decreases cell viability and cell-cycle progression which indicates a role in cell proliferation (Brito et al. 2009).

Accounting for approximately 6% of cases, the MF class of MM is characterized by the t(14;16)(q32;q23) and t(14;20)(q32;q11) translocations, which result in activation of *c-MAF* and *MAFB* proto-oncogenes, respectively. A small portion of cases lacking characteristic *c-MAF* or *MAFB* spikes can also be classified as MF, suggesting that other genes of the MAF family may be activated in these cases or that low ectopic expression of either gene is sufficient to drive this classification. Although translocations involving *c-MAF* are seen in less than 5% of MM cases, *c-MAF* expression is elevated in myeloma cell lines lacking the translocations and in up to 50% of primary samples (Hurt et al. 2004). These data strongly suggest that *c-MAF* expression may be activated by other mechanisms and attest to the importance of this family of transcription factors in MM pathogenesis. Clinically, the MF class has relatively low incidence of bone lesions and, consistent with this, has low expression of *DKK1*, a Wnt antagonist produced by myeloma cells and associated with bone disease (Tian et al. 2003). Although mutually exclusive, *MAF*- and *MAFB*-activating translocations induce a common gene expression signature, suggesting that ectopic expression of the MAF family of transcription factors results in dysregulation of common downstream targets.

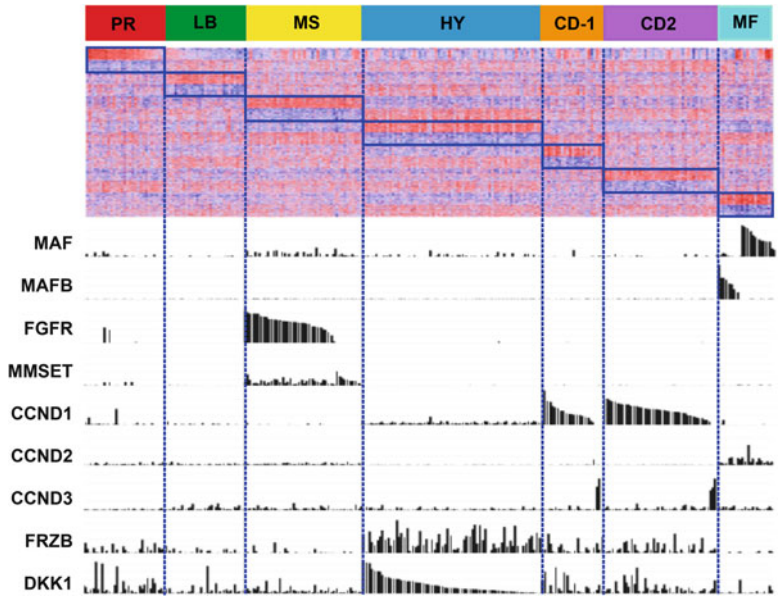


Fig. 2 Classes are characterized by unique GEP patterns. *Upper panel:* A supervised clustergram of the expression of 700 genes (50 SAM-defined overexpressed and 50 underexpressed genes from each of the 7 classes) across 256 newly diagnosed cases. Genes are indicated along the vertical axis and samples on the horizontal axis. The normalized expression value for each gene is indicated by a color, with red representing high expression and blue representing low expression. *Lower panel*: The Affymetrix gene expression signal (expression level: vertical axis) for the mRNA of *MAF, MAFB, FGFR3, MMSET, CCND1, CCND2, CCND3, FRZB,* and *DKK1*, within classes presented in the upper panel, is indicated. The normalized expression level for each gene across the samples is given by the height of each bar (Adapted from Zhan et al. 2006)

The t(11;14)(q13;q32) and t(6;14)(p21;q32) translocations, characteristic of the CD-1 and CD-2 classes, directly activate expression of *CCND1* and *CCND3*, respectively. Tumors with *CCND1* and *CCND3* spikes have gene expression profiles that cluster together tightly, suggesting that activation of the two cyclin D orthologs results in dysregulation of common downstream transcriptional programs. Nevertheless, *CCND1* and *CCND3* spikes are associated with two distinct, nonoverlapping gene expression signatures that were used to distinguish the CD-1 and CD-2 classes. CD-2 is characterized by elevated expression of *CD20/MS4A1, VPREB3, and PAX5*—genes expressed in B cells but normally extinguished in terminally differentiated plasma cells. Of note, *CD20* mRNA and protein levels are correlated (Zhan et al. 2002), but cells expressing elevated *PAX5* mRNA do not express the protein (Lin et al. 2004). Unlike CD-2, CD-1 lacks expression of *CD59* (potent inhibitor of complement membrane attack complex), Notch-like protein *NOTCH2NL*, and Notch target gene *HES1*. CD-1 is characterized by overexpression of *KLHL4* (a transcription factor), *INHBE, FYN* proto-oncogene, *CEBPB*/*NF-IL6*, and *EVER1* and *EVER2*, two cytoplasmic proteins that colocalize

with calnexin, an integral membrane protein of the endoplasmic reticulum (Zhan et al. 2006).

Hyperdiploid MM is characterized by trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19, and 21. The trisomies are one of two central genetic pathways in development of MM, and this type of disease was previously shown to have a distinct gene expression signature (Bergsagel and Kuehl 2005). The hyperdiploid signature is present in nearly 60% of cases and is associated with hyperdiploid karyotypes in more than 90% of these; however, the signature is also observed in cases that are not identified as hyperdiploid by flow cytometry analyses. Genes overexpressed in the HY subtype include Wnt signaling antagonists, *FRZB* and *DKK1*, which have been associated with bone disease in myeloma (Zhan et al. 2006).

A novel class of MM, LB (low bone disease), is characterized by low incidence of MRI-defined focal bone lesions and lacks evidence of translocation spikes or HY gene expression features. Consistent with the absence of MRI-defined focal lesions in the LB class, recent studies integrating positron emission tomography (PET) imaging with GEP also revealed that the LB class is uniquely inversely correlated with F18-fluorodeoxyglucose PET-defined focal lesion number and intensity (Bartel et al. 2009). LB disease is distinguished by overexpression of *endothelin 1/EDN1*, a soluble factor that is secreted by prostate cancers and that causes osteoblastic metastases of prostate cancer. Interestingly, purified EDN1 induces osteoblast differentiation via suppression of Wnt/β-catenin signaling antagonist *DKK1* (Yin et al. 2003; Clines et al. 2007), and the LB class is characterized by significantly lower expression of *DKK1*, suggesting that EDN1 may downregulate *DKK1* in myeloma cells.

The PR (proliferation) class is characterized by overexpression of numerous genes related to cell-cycle progression and cell proliferation, including *CCNB2*, *CCNB1*, *MCM2*, *CDCA2*, *BUB1*, *CDC2*, and *TYMS*, and also cancer-testis antigen genes, including *MAGEA6*, *MAGEA3*, *GAGE1*, and *GAGE4*. Plasma cells from all MM classes have a higher gene-expression-defined proliferation index than plasma cells from healthy donors but that of the PR class is similar to myeloma cell lines and is significantly higher than non-PR classes. Metaphases CAs, a surrogate for cell proliferation, are present in an extraordinarily high percentage of cases in the PR class. The PR class is associated with poorer survival than other classes, and both hyperdiploid and nonhyperdiploid cases are equally common, with and without concomitant translocation spikes. This suggests that hyperdiploid versus nonhyperdiploid status is not sufficient as a sole parameter for risk assessment. Indeed, those cases with both PR signature and hyperdiploidy are at higher risk than those with the HY signature. Several factors—overexpression of proliferation-associated genes in all non-PR classes, the presence of expression spikes in the PR class, and a shift to the PR class upon disease progression—suggest that the PR class is driven by a transformation event secondary to underlying primary genetic lesions.

These molecular classes are largely consistent with the TC classification schema of Bergsagel et al. who used correlated gene expression profiles with specific recurrent translocations, trisomies, and expression of cyclin D genes (Bergsagel and

Kuehl 2005) to identify eight translocation/cyclin D (TC) groups. These were termed the 11q13/TC1, 6p21/TC2, 4p16/TC3, maf/TC4, D1/TC5, D1+D2/TC6, D2/TC7, and none/TC8 classes (Bergsagel et al. 2005). The authors proposed that these genetic entities are defined by early, perhaps initiating, oncogenic events. The classes exhibited significant, uniform differences in global gene expression profiles and clinical features, such as prevalence of bone disease, frequency distribution at relapse, and progression to extramedullary tumor growth (Bergsagel et al. 2005). Genomic profiling of a large number of MM cases revealed that dysregulated expression of cyclin D might be a universal event in myelomagenesis. Relative to plasma cells from the bone marrow of healthy donors, myeloma plasma cells exhibit increased and/or dysregulated expression of either *CCND1*, *CCND2*, or *CCND3* (Bergsagel et al. 2005). *IgH*-mediated translocations can directly activate *CCND1* (11q13) (Chesi et al. 1996) or *CCND3* (6p21) (Shaughnessy et al. 2001); MAF- (16q23) or MAFB- (20q11) activating translocations lead to their transactivation of adhesion molecules and *CCND2*, which is also elevated in t(4;14)-positive tumors (Hurt et al. 2004). Biallelic dysregulation of *CCND1* occurs in nearly 40% of tumors, most of which are hyperdiploid (Bergsagel et al. 2005). Elevated levels of *CCND2* and the absence of IgH translocation spikes characterize a novel form of MM discovered through GEP of primary disease (termed "low bone disease," discussed above) (Zhan et al. 2006).

Of significant note is that the GEP70 high-risk signature is evident in a subset of all MM molecular classes defined to date. Moreover, the presence of this high-risk signature even in MMs defined as low risk by conventional cytogenetic and FISH analysis negatively influences clinical outcome in these patients, for example, low-risk MS subclass disease fares much better than high-risk MS disease.

In addition to its ability to predict the outcome of newly diagnosed MM patients, the GEP70-gene model is also an independent and significant prognostic factor in predicting post-relapse survival in relapsing MM (Nair et al. 2009). We assessed 120 MM patients previously enrolled in tandem transplantation trial total therapy 2 and observed that the 3-year post-relapse survival (PRS) was 71% in patients with GEP70 low-risk disease present both at baseline and relapse (Fig. 3a) (Nair et al. 2009). This contrasted with only 17% of patients with GEP70 high-risk disease at relapse. On multivariate analysis of relapse parameters, we showed that a GEP70 high-risk signature conferred short PRS. Thus, the conversion from GEP70 low to high risk severely impacted post-relapse survival and was unrelated to the salvage therapies used. Recognizing that GEP risk status increases in a sizeable proportion of patients, GEP analysis can be repeated on treatment failure so that salvage strategies can be selected according to this powerful risk discriminator. Improving on risk stratification provided by the ISS, Mulligan and colleagues used U133A microarray data to develop response and survival classifiers for relapsed disease treated with single-agent bortezomib or high-dose dexamethasone that were significantly associated with outcome (Mulligan et al. 2007); a modified version of the GEP70-gene model also predicted poor outcome in relapsed disease (Zhan et al. 2008).

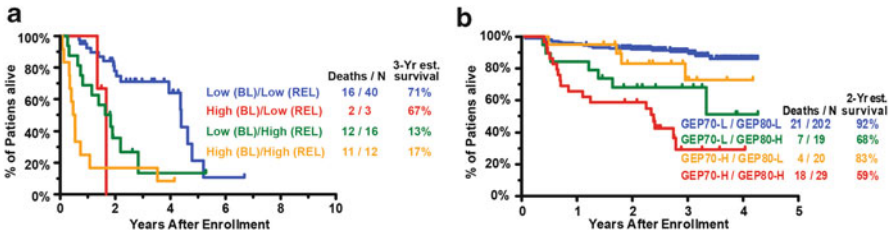


Fig. 3 Impact of the GEP70 risk model. (**a**) Post-relapse survival (PRS) by GEP-defined risk in the context of GEP data at both baseline before therapy and at relapse revealed that PRS was superior in the absence of high risk at both observation times. (**b**) Applying the GEP80 model to GEP70-defined low- and high-risk disease revealed significant segregation in survival outcomes. OS in both GEP70 low- and high-risk settings could be further discriminated by the GEP80 model (Adapted from Nair et al. 2009 and Shaughnessy et al. 2011)

The marked increase in the frequency of a GEP70 high-risk designation from 13% at diagnosis to 76% at relapse provides strong molecular evidence of disease evolution that influences post-relapse outcome, indicating that the underlying molecular mechanisms that drive disease progression can be identified. We observed that this quantifiable increase in the high-risk score over time was concordant with gains of chromosome 1q (Shaughnessy et al. 2007a), suggesting that expansion of a dominant clone with survival and/or proliferation advantages may involve altered regulation of genes on this chromosome. Abnormalities of chromosome 1 are among the most frequent chromosomal alterations in MM being observed in >45% of patients (Hanamura et al. 2006) with the short arm being often associated with deletions and the long arm with amplifications (Carrasco et al. 2006). Gains/amplification of 1q21 increases the risk of MM progression, and incidence of the amplification is higher in relapsed than in newly diagnosed MM (Hanamura et al. 2006; Rosiñol et al. 2005). GEP studies comparing MM with and without 1q gains (Fabris et al. 2007) identified 61 genes that distinguished the two groups. In cases with 1q gains, 41 of the 43 upregulated genes mapped to 1q12–q44, whereas most of the 18 downregulated genes were localized to chromosomes 13q (7/18) and 11 (6/18). These data suggest that cases with 1q gains typically also harbor del13 and lack trisomy of chromosome 11. Integrating GEP and DNA copy number variation data, several independent studies revealed that numerous 1q genes are copy number sensitive in MM (Carrasco et al. 2006; Agnelli et al. 2009). Taken together, these data show that there is a correlation between cytogenetic abnormalities identified by fluorescence in situ hybridization analysis (FISH) and GEP data in MM (Table 2).

Gains of 1q are associated with upregulation of genes involved in intracellular protein transport, including *COPA* and *ARF1*, which play roles in vesicle-mediated transport from the endoplasmic reticulum (ER) to the Golgi, and *RABIF* and *RAB3GAP2*, which are related to the Rab GTPases that regulate membrane-vesicle transport. These findings may also partially account for increased expression of genes encoding proteins involved in energy production pathways. In cases with 1q

Table 2 Studies correlating data obtained from FISH with data obtained from GEP

Cytogenetic abnormality	Conclusion	Ref
t[6:14]	By metaphase chromosome analysis, the t(6;14) (p21;q32) translocation was identified in 6 of 150 (4%) primary MM tumors. Overexpression of cyclin D3 messenger RNA (mRNA) was identified by microarray RNA expression analysis in 3 of 53 additional primary MM tumors, each of which was found to have a t(6;14) translocation breakpoint by interphase fluorescence in situ hybridization analysis (FISH)	Chesi et al. 1996 Heller et al. 2008 Duus et al. 2006
t[4:14]	t[4;14] is detected by FISH in 18% of newly diagnosed MM cases and is strongly associated with overexpression of MMSET. 32% of these cases lack expression of FGFR3	Santra et al. 2003 Heller et al. 2008 Duus et al. 2006
t[11:14]	11 of 11 MM[s] samples with spiked expression of CCND1 by GEP had a detectable t[11:14] by TRI-FISH	Zhan et al. 2002 Heller et al. 2008 Duus et al. 2006
t[14:16]	Gene expression profiling analysis revealed strong peaks of MAF expression in the cases with t[14;16] detected by FISH	Heller et al 2008
t[14:20]	*c-MAF*, which is deregulated by t(14;16), and *MAFB*, deregulated by t(14;20), were observed only in a subset of patients, which clustered separately within this MF cluster. The remaining samples in the MF cluster clustered with these samples on the basis of overexpression of downstream targets of *MAFB* and/or *c-MAF*: *RND3*, *CCND2*, and *ITGB7*	Duus et al. 2006
del 13	Ten genes, including RB1, were identified as correctly discriminating 41 (94%) of 48 cases from validation group in which FISH 13 status was known	Ocio et al. 2009
del 17p13	TP53 deletion was highly correlated with low TP53 expression. Comparison of TP53 expression level on the basis of deletion status revealed that TP53 expression was lower in 36 monoallelic deletion cases and even lower in 4 biallelic deletion cases than in 154 cases lacking deletion	Avet-Loiseau et al. 2007 Duus et al. 2006
amp 1q21	Interphase fluorescence in situ hybridization revealed that CKS1B expression was strongly correlated with DNA copy number in a subset of 197 cases with both measurements by GEP and FISH for 1q21	Rosiñol et al. 2005 Burington et al. 2008

gains, three genes involved in protein translation (*RPLP2*, *RPL21*, and *FAU*) are downregulated, which is potentially significant because recent studies suggest that survival of B-cell malignancies (including MM) may be highly dependent on ER–Golgi protein transport, thus targeting this process may be a novel therapeutic strategy (Carew et al. 2006). Cases with 1q gains also showed significantly modulated expression of genes involved in ER stress-induced responses, including upregulation of *CLN3* (chaperone), *UBAP2L* and *UBE2Q1* (ubiquitin cycle), *PSMD4* (proteasome degradation), and *CASP4* (initiates apoptosis in response to ER stress) (Fabris et al. 2007). The importance of the unfolded protein response

(UPR) in maintaining tissue health is evidenced by the fact that a chronic increase in defective protein structures plays a pathogenic role in diabetes, cardiovascular disease, Alzheimer's and Parkinson's syndromes, and cancer. Since the UPR promotes cell survival by reducing the accumulation of aberrantly folded proteins through translation arrest, production of chaperone proteins, and increased degradation and because ER stress-induced apoptosis can play an important role in malignant cells' sensitivity to certain drugs, including bortezomib, a better understanding of ER stress-induced responses may contribute to important new treatment strategies (Fabris et al. 2007).

Based on the GEP data, we also developed a virtual karyotype model, or vCA, that predicts cytogenetic abnormalities (CAs) in MM. We determined genome-wide gene expression profiles and DNA copy numbers (CNs) in purified plasma cell samples obtained from 92 newly diagnosed MM patients, using the Affymetrix GeneChip and the Agilent aCGH platforms, respectively. Applying the criterion of a Pearson's correlation coefficient >0.35, which kept the false discovery rate to ≤5%, we identified 1,114 CN-sensitive genes that were used to develop a vCA model for predicting CAs in MM patients by means of GEP. We validated the model with a FISH dataset compiled from 262 independent MM samples for which both FISH records and GEP data were available as well as a set of cytogenetic data generated by conventional karyotyping that included 533 independent MM samples for which both karyotype records and GEP data were available (Zhou et al. (2012). Thus, the vCA model can be used to identify CAs in patients for whom FISH and cytogenetic data are not available.

4 Risk Factors in the Era of Novel Agents Based on GEP Treatment Options

In the new era of targeted therapies and new drugs, the focus has rapidly shifted from treating MM using a one-size-fits-all therapy to one where the ultimate goal is to predict which drug regimen will provide the optimal outcome for an individual patient. The ability to assess the genetic response of a particular MM to an administered drug will be of enormous advantage in selecting the most efficacious approach. However, since MM is not a single genetically distinct disease but one with many molecular subtypes that manifest significant differences in clinical presentation and outcome, sensitive techniques like GEP that identify specific defects which accurately predict treatment options for newly diagnosed and relapsed/refractory MM patients are critical. This is of particular importance in the era of new therapies since the introduction of agents like bortezomib and lenalidomide has been shown to overcome high-risk chromosomal abnormalities identified by FISH and cytogenetics such as t(14;16) and t(4;14). For example, although many studies predict a poor prognosis for patients who manifest t(4;14) translocations (Chng et al. 2008; Avet-Loiseau et al. 2007; Fonseca et al. 2003), recent studies at the University of Arkansas have shown a large proportion of these high-risk

patients (by FISH standard) who undergo long-term treatment with bortezomib can on average expect almost similar outcomes as patients with standard-risk myeloma if they have low-risk disease by the GEP70 model (Pineda-Roman et al. 2008). Other studies have confirmed these data (San Miguel et al. 2008; Avet-Loiseau et al. 2010). However, despite intensive use of the current arsenal of drugs as well as incorporation of stem cell support, there continues to be a subset of ultrahigh-risk MM patients that have inferior outcomes indicating the impact of other genetic abnormalities that continue to exert a dominant role in tumor progression and which are unaffected by current therapeutic approaches. For example, the presence of 17p13 deletions still predicts an inferior outcome even with novel drug therapy (Munshi and Avet-Loiseau 2011). This high-risk feature presumably leads to loss of heterozygosity of *TP53*, a tumor suppressor gene that transcriptionally regulates cell-cycle progression and apoptosis to modulate cellular responses to DNA damage. Xiong et al. (2008) found that low expression of *TP53*, seen in approximately 10% of newly diagnosed patients, is highly correlated with FISH-defined *TP53* deletion and inferior clinical outcome and is an independent risk factor. Only a few of the more than 122 known p53 target genes were highly correlated with *TP53* expression in primary myeloma cells. GEP following ectopic expression of *TP53* in four *TP53*-null cell lines identified 85 significantly differentially expressed genes (50 upregulated and 35 downregulated). Using these 85 putative target genes, unsupervised hierarchical clustering of myeloma cell samples from 351 newly diagnosed and 90 relapsed patients revealed two major subgroups that strongly correlated with *TP53* expression and survival. These data suggest that loss of *TP53* expression in MM confers high risk and probably results in deregulation of a novel set of p53 target genes specific to MM and perhaps unique to different cell lineages (Xiong et al. 2008). We had identified del *TP53* as a poor risk feature in patients treated on total therapy 2, regardless of GEP risk designation. However, when examined for its prognostic consequences in TT3, we found *TP53* haploinsufficiency did not compromise overall or event-free survival, in the 83% with genomically defined low-risk myeloma. Thus, the prognostic implications of del *TP53* were protocol, genome-defined risk, and molecular subgroup dependent (Shaughnessy et al. 2009).

MicroRNAs (miRNAs) are noncoding RNAs that regulate global gene expression. miRNAs often act synergistically to repress target genes, and their dysregulation can contribute to the initiation and progression of a variety of cancers. To investigate the potential involvement of miRNA in MM, we used whole-genome microarray analyses of CD138-enriched plasma cells from 52 newly diagnosed cases of MM to correlate miRNA expression profiles with the GEP70 risk score, proliferation index, and predefined gene sets (Zhou et al. 2010). In stark contrast to mRNAs, we discovered that all tested miRNAs were significantly upregulated in high-risk disease as defined by GEP70 risk score and proliferation index. Increased expression of *EIF2C2/AGO2*, a master regulator of the maturation and function of miRNAs and a component of the GEP70 risk model, is driven by DNA copy number gains in MM (Zhou et al. 2010). Genome-wide elevated expression of miRNAs in high-risk MM may be secondary to deregulation of AGO2 and the enzyme complexes

that regulate miRNA maturation and function. Thus, additional detailed molecular studies will no doubt further clarify which genetic/chromosomal abnormalities are pivotal to the various dysregulated biological attributes of MM and which are susceptible to modulation by current drug regimens.

5 Using GEP to Predict Therapeutic Response

The ability to generate detailed knowledge of the molecular defects in a cancer holds significant potential to identify which drug or combination of drugs will impact that specific cancer and provide long-term benefit to an individual patient. Mechanisms of cancer cell resistance to chemotherapy are poorly understood, and efficacy measures typically rely on clinical outcome data. In order to determine which genes may confer drug resistance, we used GEP to identify the set of genes impacted in MM cells after exposure to two clinically relevant drugs, dexamethasone and bortezomib in vitro (Chauhan et al. 2002; Mitsiades et al. 2002, 2003). Similar studies have used GEP to identify the transcriptional signatures of a number of potentially active drugs in MM cells, such as 2-methoxyestradiol (2ME2), an estrogen derivative (Chauhan et al. 2003); two histone deacetylase inhibitors, SAHA and VPA (Mitsiades et al. 2004a; Neri et al. 2008); insulin-like growth factor receptor (IGFR) inhibitors (Mitsiades et al. 2004b), an IL-6 receptor inhibitor, Sant7 (Tassone et al. 2005); an inhibitor of Wnt signaling pathway, PKF115-584 (Sukhdeo et al. 2007); ribonucleotide reductase inhibitor, didox (Raje et al. 2006), and atiprimod, an oral agent with anti-inflammatory properties (Neri et al. 2007). Using GEP, the transcriptional signatures of cellular responses to 5-aza-2'-deoxycytidine and trichostatin (Heller et al. 2008); an HSP90 inhibitor, 17-AAG (Duus et al. 2006); and a marine-derived compound, Zalypsis (Ocio et al. 2009) have also recently been described.

The power of GEP to study drug responses of MM patients in vivo was provided by Burington et al. who compared GEP signatures of myeloma cells before and 48 h after single-agent therapy with dexamethasone, thalidomide, or lenalidomide. The results showed that genes differentially expressed after therapy were prognostic for event-free survival (EFS) and overall survival (OS) (Burington et al. 2008). Remarkably, gene expression altered by thalidomide in newly diagnosed disease and associated with subsequent survival was also altered by lenalidomide, a thalidomide analogue, and the changes were associated with EFS in a salvage trial of patients with relapsed disease (Burington et al. 2008). This finding strongly suggests that these genes are powerful biomarkers, and the similar acute gene expression responses to two related chemotherapeutic agents may provide important insights into the drugs' potential mechanism(s) of action. These results also highlight the similar acute molecular responses to chemotherapies in both primary and refractory disease.

A second study used GEP signature differences following therapy with the proteasome inhibitor bortezomib in 142 newly diagnosed symptomatic MM cases

to identify 80 highly survival-discriminatory genes (Shaughnessy et al. 2011). Analysis of the 80 genes revealed two major gene clusters with the upper gene cluster comprising many genes coding for subunits of the proteasome. The 26 patients in the high-risk groups demonstrated a concerted upregulation of 42 "bad" genes (including genes coding for the proteasome) and downregulation of 38 "good" genes. In the context of the 70-gene risk model, the GEP80 model identified 9% of patients with a poor prognosis among those with GEP70-defined low-risk disease and 41% of patients with a favorable prognosis among those with GEP70-defined high-risk disease (Fig. 3b) (Shaughnessy et al. 2011). *PSMD4*, which is common to both models, resides on chromosome 1q21 and is associated with progressively shortened PFS and OS with an increase in copy number (Shaughnessy et al. 2011). GEP80 baseline-defined high risk, high LDH, and low albumin were the only independent adverse variables surviving the multivariate survival model. These data imply that the activation status of proteasome genes in tumor cells after short-term proteasome inhibition is associated with significant outcome differences in patients with MM receiving polychemotherapy that includes bortezomib (Shaughnessy et al. 2011).

Finally, a recent study by Kumar and colleagues (Kumar et al. 2011) examined the utility of GEP-based risk stratifications systems in newly diagnosed patients undergoing therapy with lenalidomide and dexamethasone in the context of a Phase III trial. Among the major findings from this study were that the high-risk GEP70 signature had excellent prognostic value in predicting inferior OS in the context of primary treatment with novel agents alone. And that a GEP70 high-risk signature portends a poorer outcome in patients treated primarily with novel agents like lenalidomide.

6 Molecular Diagnostic Models for MGUS

Monoclonal gammopathy of undetermined significance (MGUS) is a benign disease identified by the presence of a serum monoclonal protein (M protein) in the absence of clinical parameters attributable to monoclonal gammopathy such as MM. In a recent survey, the prevalence of MGUS was 3.2% and 5.7% in persons older than 50 and 70 years, respectively (Rosiñol et al. 2007). Patients with MGUS are characterized by a detectable PC population in the marrow (<10%) and secretion of M protein in the serum (<30 g/L), but they lack clinical features of end organ damage, such as hypercalcemia, renal failure, anemia, or bone lesions. Patients with overt MM have increased marrow plasmacytosis (>10%), exhibit serum levels of M protein >30 g/L, and generally present with anemia, lytic bone disease, hypercalcemia, or renal insufficiency.

Especially in early phases of MM, the differential diagnosis may be associated with a degree of uncertainty. This has important implications, since the majority of MGUS patients will never require therapy, whereas MM can have a very heterogenous disease course and in the vast majority of cases, is a fatal malignancy. As the premalignant

precursor, MGUS progresses to MM at the rate of about 1–2% a year; thus, identifying predictors of MGUS transformation into overt MM has become an important area of research (Rosiñol et al. 2007).

We are just beginning to understand the complex molecular mechanisms underlying the progression of MGUS to MM. Microarray analysis of NPCs, MGUS, and MM by Davies et al. has revealed differences between these PDs. Although the GEP between NPC and MGUS or NPC and MM were drastically different, only 74 genes were differentially expressed between MGUS and MM samples, indicating that the differences between MGUS and MM are smaller than those between NPC and MM or NPC and MGUS but due to their partially overlapping oncogenic features, still prevent definitive diagnosis (Davies et al. 2003; Zingone and Kuehl 2011). Evaluation of common MM CAs (immunoglobulin heavy chain (IgH) translocations, 13q14 and 17p13 deletions, and 1q21 gains) by FISH in MGUS, SMM, and MM demonstrated an increase in the number of clonal PCs with these CAs during progression from MGUS to MM (López-Corral et al. 2011). Thus, current models propose a multistep process initiated by IgH translocations and an altered bone marrow microenvironment followed by alterations of the MYC (Chng et al. 2011), Ras, and other oncogenic pathways. Despite this increased understanding, we are still not able to predict which MGUS patients will ultimately progress to MM.

This need for more robust methods of classification and stratification led us to compare six different statistical and data mining algorithms (logistic regression, decision trees, ensemble of voters, naïve Bayes, nearest shrunken centroid, support vector machines) for their ability to discriminate groups of highly purified PCs from patients with newly diagnosed MM (n = 218), patients with MGUS (n = 21), and healthy donors (NPCs, n = 45) on the basis of the expression of approximately 12,000 genes. The models were highly accurate in distinguishing NPCs from abnormal cells; however, they displayed a modest failure in the discrimination between the hyperplastic and malignant cells (Hardin et al. 2004). A more recent study analyzing 877 GEPs compiled from six publicly available datasets found an increase of the MYC, RAS, and E2F pathway activation with progression of the disease from normal plasma cell to ultimately relapsed MM. Although the authors proposed a high-risk signature derived from MGUS samples clustering with relapsed MM, this model could not be validated due to limited follow-up. Unsupervised hierarchical clustering could differentiate between NPCs and MGUS or NPCs and relapsed MM, yet the authors were not able to differentiate plasma cell dyscrasias (PCDs) (Anguiano et al. 2009). These results reflect our observation using GEP data generated on the Affymetrix U133Plus 2 microarray. In unsupervised clustering, we are not able to differentiate MGUS from MM samples, while NPCs are easily separated (Shaughnessy, unpublished data). In the past, we have shown that 52 genes, involved in important pathways related to cancer, were differentially expressed between plasma cells from healthy subjects (n = 22) and patients with stringently defined MGUS/smoldering MM (n = 24) and symptomatic MM (n = 351) (P < .001). Among the patients with PCD, we could further separate a non-MGUS-like MM cluster from a cluster that contains both MGUS and MGUS-like MM (Fig. 4);

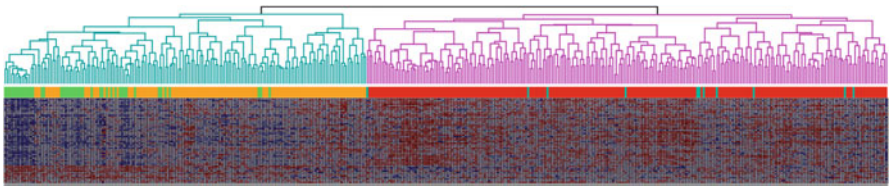


Fig. 4 Hierarchical clustering of GEP MGUS and MM cannot separate between MGUS and MM. Expression pattern of 52 genes that were identified as differentially expressed between PCs of healthy donors and MGUS and samples from patients with PCDs segregates MGUS and MGUS-L MM from non-MGUS-L MM. Two-dimensional unsupervised hierarchical cluster analysis of 52 MGUS genes (*rows*) in CD138-enriched plasma cells of patients with MGUS ($n = 56$) and newly diagnosed MM ($n = 351$) (*columns*). The left branch consists of MGUS (samples marked in green below the clustering tree in *green*) and MGUS-like MM samples (*orange*), and the right branch contains the non-MGUS-L MM (*red*). MM-like MGUS patients (MM-L MGUS, teal) (Adapted from Zhan et al. 2007)

however, we could not clearly segregate MGUS and MM. It is interesting to note that MGUS-like MM has a significantly better survival than their non-MGUS-like counterparts (Zhan et al. 2007). To date, a gene signature that differentiates between MGUS and MM is not available.

DNA methylation profiles appear to be very promising in distinguishing MGUS from more advanced PCDs. A study analyzing the DNA methylation fingerprints of CD138-sorted plasma cells of 8 healthy donors, 8 MGUS, 13 newly diagnosed MM, and 18 relapsed MM generated using the high-throughput microarray-based HELP (HpaII tiny fragment enrichment by ligation-mediated PCR) assay found predominant hypomethylation in MGUS and MM. Unsupervised clustering segregated cohorts into two major clusters with newly diagnosed and relapsed MM on one side and NPCs and MGUS cases on the other, demonstrating great epigenetic dissimilarity between them. Each of these clusters was separated into two subgroups resulting in a total of four clades each one representing the majority of NPCs, MGUS, and newly diagnosed and relapsed MM, respectively (Heuck 2011, unpublished data).

MGUS has all the features of overt malignancy; however, only a small portion of MGUS cases actually convert to MM. The explanation for this apparent paradox might lie in the microenvironment. It has been shown that the tumor environment supports the growth of MM (Anderson 2007) and probably all cancers (Whiteside 2008). In order to shed some light on the genomic changes of the bone marrow microenvironment, we have begun analyzing the GEP of whole bone marrow biopsies. We expect to report data in the near future.

Ongoing longitudinal studies such as the SWOG 0120 observational study of MGUS and SMM will prove valuable in elucidating the pathophysiology involved in the progression of MGUS to MM.

7 Conclusions

Utilizing high-throughput genomic analyses and data mining techniques, a complete landscape of MM molecular pathogenesis is emerging, and powerful validated prognostic models have been developed. Unsupervised clustering of GEP data of large patient cohorts also has revealed that MM heterogeneity can be accurately cataloged and disease classes defined. Importantly, the improved survival observed in specific classes through the use of new treatments, such as thalidomide and bortezomib, buttresses the concept of personalized treatment approaches.

Large-scale gene expression data and large cohorts of uniformly treated patients with long follow-up times have provided more precise and independent prognostic models for stratifying patients with MM. Investigating GEP changes between baseline and relapse has shed light on the mechanisms underlying MM progression and the nearly universal development of multidrug-resistant MM. Pharmacogenomics studies comparing gene expression profiles at diagnosis and following short-term single-agent therapy have identified genes associated with drug responses, contributing to mechanistic understanding. This understanding is critical for improving existing therapies with personalized treatments and for informing research and discovery of new therapeutics. We have shown that risk stratification using standard methods is too rigid to account for the heterogeneity of MM. In the future, risk will have to be evaluated in light of novel therapies, and we can expect paradigms to shift with the emergence of new treatments.

While the majority of MM patients can anticipate long-term disease control via a variety of treatment approaches, patients with molecularly defined high-risk disease do not benefit from current approaches. To address this, clinical trials designed to reduce toxicities in low-risk disease and to test new treatment strategies in high-risk disease are underway. When routinely available, molecular-based classification and risk stratification will meet their potential to shift strategies for MM treatment and cure (Stewart 2010).

Combining these approaches with increasingly sensitive esoteric technologies, such as high-throughput proteomics, microRNA profiling, whole-genome sequencing, and advances in sophisticated bioinformatic data mining algorithms, will broaden the defined spectrum of molecular variables and provide clinicians with relevant therapeutic targets to improve clinical management of the MM patient.

Acknowledgements This work was supported by the Lebow Fund to Cure Myeloma.

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http://www.springer.com/978-1-4614-4659-0

Genetic and Molecular Epidemiology of Multiple Myeloma

Lentzsch, S. (Ed.)
2013, VII, 125 p., Hardcover
ISBN: 978-1-4614-4659-0