Gene Expression Profiles of Tumor Biology Provide a Novel Approach to Prognosis and May Guide the Selection of Therapeutic Targets in Multiple Myeloma

Ariel Anguiano, Sascha A. Tuchman, Chaitanya Acharya, Kelly Salter, Cristina Gasparetto, Fenghuang Zhan, Madhav Dhodapkar, Joseph Nevins, Bart Barlogie, John D. Shaughnessy Jr, and Anil Potti

From the Institute for Genome Sciences and Policy, Duke University; Department of Medicine, Duke University Medical Center, Durham; Regional Cancer Care, US Oncology Network, Chapel Hill, NC; Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR; and Section of Hematology, Yale University, New Haven, CT.

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A.A. and S.A.T. contributed equally to the work.

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Corresponding author: Sascha A. Tuchman, MD, Division of Medical Oncology, Duke University Medical Center, Morris Building 25155, Box 3872, Durham, NC 27710; e-mail: sascha.tuchman@duke.edu.

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose

Monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) comprise heterogeneous disorders with incompletely understood molecular defects and variable clinical features. We performed gene expression profiling (GEP) with microarray data to better dissect the molecular phenotypes and prognoses of these diseases.

Methods

Using gene expression and clinical data from 877 patients ranging from normal plasma cells (NPC) to relapsed MM (RMM), we applied gene expression signatures reflecting deregulation of oncogenic pathways and tumor microenvironment to highlight molecular changes that occur as NPCs transition to MM, create a high-risk MGUS gene signature, and subgroup International Staging System (ISS) stages into more prognostically accurate clusters of patients.

Results

Myc upregulation and increasing chromosomal instability (CIN) characterized the evolution from NPC to RMM ($P < .0001$ for both). Studies of MGUS revealed that some samples shared biologic features with RMM, which comprised the basis for a high-risk MGUS signature. Regarding MM, we subclassified ISS stages into clusters based on shared features of tumor biology. These clusters differentiated themselves based on predictions for prognosis (eg, in ISS stage I, one cluster was characterized by increased CIN and a poor prognosis).

Conclusion

GEP provides insight into the molecular defects underlying plasma cell dyscrasias that may explain their clinical heterogeneity. GEP also may refine current prognostic and therapeutic models for MGUS and MM.

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INTRODUCTION

Monoclonal gammopathy of undetermined significance (MGUS) likely progresses to multiple myeloma (MM) in a multistep process.^{1,2} Initially, intracellular events such as immunoglobulin heavy chain translocations occur, and deleterious influences arise from an altered bone marrow microenvironment.³ Later, secondary immunoglobulin heavy chain translocations⁴⁻⁶ and deregulation of *Myc*, *Ras*, or other oncogenic pathways^{7,8} ensue, as do further microenvironmental perturbations.⁹⁻¹² Our understanding of this pathogenic process has advanced significantly, yet we remain unable to reliably anticipate which 1% of MGUS patients will annually experience progression to MM.¹ Furthermore, MM is a complex disease,¹³ and although work defining molecular markers and the Interna-

tional Staging System (ISS) has somewhat improved our ability to predict outcomes in MM patients,^{14,15} treatment approaches remain controversial, and no gold standard exists.¹⁶

Gene expression profiling (GEP) has been used to define prognosis in human malignancies, and multiple genomic approaches have been developed to dissect the biology of human cancers¹⁷⁻²⁰ and tailor therapy.²¹⁻²³ Indeed, relevant phenotypes have recently been identified using GEP in MM,²⁴⁻³² but less has been done to illuminate the complexity of MM progression^{33,34} or to predict responses to specific chemotherapies. Our hypothesis for this study was that applying gene expression signatures of oncogenic pathway deregulation¹⁸ and altered tumor microenvironments³⁵⁻³⁸ to certain plasma cell dyscrasias (MGUS, smoldering MM [SMM], MM, and relapsed MM [RMM]) would

shed light on the biology of myeloma, such that we could refine current prognostic models and provide a rational basis to explore individualized therapeutic strategies in future studies.

METHODS

Patient and Tumor Samples

We compiled Affymetrix (Santa Clara, CA) mRNA gene expression data from 877 CD138-purified plasma cell samples, including normal plasma cells (NPCs), MGUS, SMM, MM, and RMM, from patients within the following six publicly available data sets: GSE2658, GSE5900, GSE6205, GSE2113, GSE6980, and GSE6477. The largest data set, GSE2658, originated from the University of Arkansas Myeloma Institute for Research and Therapy and included 560 newly diagnosed MM patients enrolled onto two clinical trials. Total Therapy 2 trial tested a multiagent treatment regimen comprising induction with serial cycles of vincristine, doxorubicin, and dexamethasone; dexamethasone, cyclophosphamide, etoposide, and cisplatin; and cyclophosphamide, doxorubicin, and dexamethasone; random assignment to thalidomide or placebo; stem-cell collection with tandem autologous hematopoietic stem-cell transplantation; and consolidation with dexamethasone, cisplatin, doxorubicin, cyclophosphamide, and etoposide. Total Therapy 3 trial combined Total Therapy 2 agents, including thalidomide and autologous transplantation, with random assignment to bortezomib or placebo.³⁹ Some MGUS and SMM patients came from a Southwest Oncology Group prospective clinical trial. Table 1 lists MM patient demographic data.

Cross-Platform Comparison, Filemerger and ComBat Methods

Gene expression data came from several generations of Affymetrix microarray chips with comparable probesets. We used an in-house program, Chip Comparer (<http://tenorio.duhs.duke.edu/genearray/perl/chip/chipcomparer.pl>), to convert probesets to a common format, the HG-U133A chip. Another in-house program, Filemerger (<http://tenorio.duhs.duke.edu/genearray/perl/filemerger>), enabled us to combine data sets. We then normalized data sets using the program ComBat (<http://statistics.byu.edu/johnson/ComBat/Abstract.html>), which attenuates batch effects⁴⁰ (ie, differences between data sets that arise as a result of dissimilarities in RNA and microarray chip processing that occur in different settings).

Hierarchical Clustering Analysis

We performed hierarchical clustering, meaning the grouping of individual samples according to similarities in patterns of gene expression, using the R/Bioconductor statistical package (version 2.4.0).^{41,42} Individual samples were clustered by probability values reflecting the likelihood of deregulation of

specific aspects of tumor biology described later. Complete linkage clustering was accomplished using the Euclidean distance metric, which brings together samples whose absolute expressions are similar.⁴³ We generated heatmaps with R/Bioconductor and GenePattern (version 2.0).⁴⁴

Development of Signatures

This process has been described previously.^{18,19,45} Briefly, we performed initial unsupervised hierarchical clustering (grouping based on gene expression patterns and without clinical data) and observed that certain MGUS samples clustered with the RMM cohort, indicating potentially high-risk biologic features. These RMM-like MGUS samples thus provided the basis for a high-risk MGUS gene signature. We constructed the signature by applying class labels to individual samples: high-risk MGUS samples, referring to those clustering among RMM samples, were termed 1, whereas MGUS samples clustering separately from RMM were termed 0, indicating low-risk comparators. Within the MATLAB environment (version 7.1), we then performed Bayesian probit binary regression analysis on gene expression data from all samples in the new signature. In this technique, each sample is compared with all other samples, and a resultant probability value is generated that reflects that sample's similarity to the high-risk phenotype. A probability value of 0 indicates complete dissimilarity from the phenotype, whereas 1 indicates complete similarity to the high-risk phenotype. Most samples fall somewhere between those extremes. Samples with high probability values and hence molecular resemblance to the high-risk MGUS samples could be expected to carry a poor prognosis, whereas the converse would stand for samples with low values. We then performed leave-one-out cross-validation, in which each sample is removed from the signature serially and the model is repeatedly regenerated without that sample. Robust, stable signatures do not change markedly with removal of single samples.

Application of Oncogenic Pathway and Tumor Biology Signatures

Using R/Bioconductor and MATLAB, we combined, standardized, and applied Bayesian analysis to validated tumor biology gene signatures and experimental samples to generate probability values regarding the likelihood in experimental samples of activation of the β -catenin (B-CAT), *E2F*, *Myc*, phosphatidylinositol 3-kinase (PI3K), *Ras*, and *Src* oncogenic pathways, as well as angiogenesis (the wound healing phenotype [WH]), chromosomal instability (CIN), hypoxia, and tumor necrosis factor α . We then synthesized heatmaps and assigned clusters, which reflect subphenotypes. Finally, we investigated clinical correlates by plotting Kaplan-Meier survival curves for individual clusters in GraphPad Prism (version 4.03; GraphPad, La Jolla, CA). Statistical significance was calculated with unpaired *t* tests when assaying continuous variables between two groups, analysis of variance when assaying continuous variables between more than two groups, and the log-rank method when comparing survival data.

RESULTS

Signatures of Oncogenic Pathway Activation and Tumor Microenvironment Characterize Phenotypes of Plasma Cell Dyscrasias

We hypothesized that by applying genomic predictors of tumor biology to gene expression data from normal patients (NPC controls; *n* = 37) and patients with plasma cell dyscrasias (MGUS, *n* = 66; SMM, *n* = 36; MM, *n* = 74; and RMM, *n* = 27), we could further dissect the biology of MM progression. Clear patterns characterized the evolution from NPC to RMM, with increasing CIN and *Myc* and *E2F* overexpression most notably predominating (Figs 1A and 1B; *P* < .0001 for CIN and *Myc*, *P* = .01 for *E2F*). Relative activation of each pathway for each plasma cell dyscrasia phenotype is detailed in Appendix Figure A3 (online only).

Table 1. Patient Demographics and Clinical Characteristics by International Staging System Stage

Patient Demographics and Clinical Characteristics	Stage I (n = 300)	Stage II (n = 145)	Stage III (n = 115)
Age, years			
Average	56.0	57.7	59.2
Range	24.8-75.9	35.8-74	36-76.5
SD	3.1	12.7	21.3
Sex, %			
Male	59	66	53
Female	41	34	47
Laboratory data			
Mean β_2 -microglobulin, mg/mL	2.2	4.1	12.4
Mean serum albumin, g/dL	4.2	3.9	3.8
Median survival time, months			
Event-free survival	72.9	54.1	34.7
Overall survival	Not yet reached	Not yet reached	56.2

Fig 1. Genomic expression signatures of tumor biology characterize phenotypes of plasma cell dyscrasias. (A) Mean predicted probabilities of oncogenic pathway deregulation in normal plasma cells (NPCs; $n = 37$) and certain plasma cell dyscrasias: monoclonal gammopathy of undetermined significance (MGUS; $n = 66$), smoldering multiple myeloma (SMM; $n = 36$), multiple myeloma (MM; $n = 74$), and relapsed multiple myeloma (RMM; $n = 27$). (B) Application of signatures of an altered tumor microenvironment to the same samples as in A. B-CAT, beta-catenin; HYP, hypoxia; CIN, chromosomal instability; TNF, tumor necrosis factor; WH, wound healing.

High-Risk MGUS Signature

The clear molecular transformation that occurred with progression from NPC to RMM led us to believe that when applied to MGUS, GEP directed at identifying MGUS samples with an RMM-like phenotype may pinpoint higher risk MGUS patients who are likely to fall within the 1% of MGUS patients who experience progression to MM annually.¹ We turned to our MGUS samples ($n = 66$) to investigate this question. Using predictions of tumor biology, we were able to delineate four clusters characterized by unique molecular features (Appendix Fig A1A, online only). Most significantly, *Myc* pathway activation was increased in cluster 1 compared with cluster 2 ($P = .0003$), as was activation of *Ras* ($P < .0001$) and *Src* ($P = .004$). We hypothesized that these molecular differences would correlate to differences in prognosis as well and that we could thus use GEP to subclassify MGUS into clinically relevant subphenotypes. Consequently, we proceeded to develop a high-risk MGUS gene signature.

As an initial proof of concept, we applied unsupervised hierarchical clustering analysis to 240 plasma cell dyscrasia samples (GSE5900

and GSE6477), and discrete gene sets emerged that could differentiate NPCs from RMM and MGUS (Appendix Fig A1B, top left panel). When we combined samples from all three phenotypes, NPCs still reliably separated from the RMM samples, but MGUS samples were interspersed, with some samples clustering with the NPC cohort and others with RMM (Fig A1B, top row, third panel). Direct comparison of MGUS to RMM again demonstrated a collection of MGUS samples clustering with RMM samples (Fig A1B, top right panel). These samples became the high-risk MGUS signature (Fig A1B, bottom left panel). The novel signature was validated in leave-one-out cross-validation with an accuracy of 0.96 (Fig A1B, bottom right panel), yet clinical validation of this signature was constrained by the limited duration of clinical follow-up in MGUS cohorts. This accordingly remains a work in progress.

Prognostic Substratification Based on Oncogenic Pathway Deregulation and Tumor Microenvironment Status

In addition to characterizing the biology of plasma cell dyscrasias, we further evaluated the likelihood that gene signatures representative of tumor biology could refine the broad prognoses offered by ISS stage. Beginning with ISS stage I patients, using GEP, we identified five distinct clusters based on biologic differences (Fig 2, top row). On applying survival data by cluster, clusters 1 and 3 represented the extremes of survival (Fig 2, bottom left panel; $P = .016$ for cluster 1 ν 3; data for other clusters not shown). Cluster 1, which survived the longest, was characterized by higher activation of the B-CAT and *Myc* pathways, as well as increased angiogenesis (WH; $P < .0001$ for all), as opposed to the shortest surviving cohort, cluster 3, which demonstrated markedly increased levels of CIN (Fig 2, bottom right panel; $P < .0001$).

Within ISS stage II patients ($n = 145$), survival differences between clusters did not reach statistical significance (Fig 3, top and bottom left panel; survival data for the two clusters with most extreme survival shown; $P = .059$; survival data for other clusters not shown). Compared with the good prognosis cluster (cluster 3), the poor prognosis cluster (cluster 2) showed increased activation of *Myc* ($P < .0001$) and B-CAT ($P < .0001$). The CIN and WH phenotypes, which distinguished clusters among stage I patients, were not expressed at different levels by the subphenotypes delineated here.

Lastly, among ISS stage III patients ($n = 115$), clusters were again discernable (Fig 4, top panel) with markedly different survival trends, although statistical significance was not met as a result of the small numbers of patients (Fig 4, lower left panel; $P = .09$). Both B-CAT and *Myc* were upregulated in the poor prognosis compared with the good prognosis subphenotype ($P < .0001$ for both), and interestingly, both groups had markedly increased CIN and angiogenesis (Fig 4, lower panel). This is thought provoking because both features likely represent key contributors to the aggressiveness of MM in certain patients.

DISCUSSION

Our ability to understand biologic complexity in a particular disease process is frequently limited by our ability to define pertinent phenotypes. This is most relevant for MM, in which the complex oncogenic process involves the somatic acquisition of myriad mutations coupled

Fig 2. Dissection of International Staging System (ISS) stage I (low risk) into subphenotypes based on tumor biology. Subgroups based on molecular differences within ISS stage I carry prognostic implications. CIN, chromosomal instability; WH, wound healing; TNF, tumor necrosis factor; HYP, hypoxia; B-CAT, beta-catenin.

Fig 3. Dissection of International Staging System (ISS) stage II (intermediate risk) into subphenotypes based on tumor biology. Subgroups based on molecular differences within ISS stage II carry prognostic implications. CIN, chromosomal instability; WH, wound healing; TNF, tumor necrosis factor; HYP, hypoxia; B-CAT, beta-catenin.

Fig 4. Dissection of International Staging System (ISS) stage III (high risk) into subphenotypes based on tumor biology. Subgroups based on molecular differences, within ISS stage III carry prognostic implications. CIN, chromosomal instability; WH, wound healing; TNF, tumor necrosis factor; HYP, hypoxia; B-CAT, beta-catenin.

with genetic variability within the host. To develop effective therapeutic strategies, an understanding of the unique characteristics of cancer in individual patients is important. Gene expression profiles, which represent biologic states in the form of patterns of gene expression, offer the opportunity to characterize and treat tumors in an individualized fashion. In this study, we sought to apply gene expression analysis to plasma cell dyscrasias.

MGUS is a predecessor of MM, and MGUS can be conceptualized as a complex, molecular first hit that is followed by an equally complex second hit that provokes transformation to MM.² The *Myc* pathway, for instance, was recently identified as central in the evolution of MGUS into MM³² and could represent such a second hit. Our work supports these concepts. In our results (Fig 1), *Myc* pathway activation increases as NPCs progress toward MM, and similar increases in *Ras* and *E2F* activation, CIN, and angiogenesis also ensue. Importantly, GEP also shows that *Myc* activation coincides with B-CAT activation, which has been associated with extensive bone involvement and more aggressive forms of MM.⁷

We used these patterns to generate a high-risk MGUS gene signature based on the molecular similarities between certain MGUS patients and RMM. Our goal was to exploit these similarities to isolate subgroups of MGUS patients who, as a result of the presence of a biologic phenotype similar to that of high-risk (relapsed) MM, would be more likely to belong to the 1% of MGUS patients who experiences progression to MM annually. Subphenotypes are indeed identifiable (Appendix Fig A1B), but adequate follow-up is unavailable to draw further conclusions about the prognostic capacity of this model. Therefore, work continues on collecting more clinical data.

In terms of MM, GEP differentiated ISS stages into good and poor prognosis subphenotypes based on unique molecular patterns of biologic deregulation (eg, CIN, *Ras*, or *Myc* activation), some of which have been previously associated with poor outcomes in MM.¹⁴ In addition to prognostic information, another benefit of using gene signatures of pathway deregulation is that they also provide an opportunity to develop and validate novel, individualized treatment strategies in subgroups of MM patients because deregulation of particular pathways has been shown to be associated with sensitivity to inhibitors specific to those pathways.¹⁷ However, this concept will take years to validate in prospective clinical trials.

Limitations to this work are important to highlight. First, our data derive from CD138-selected plasma cells, which inevitably comprise a blend of normal and malignant tissue. Therefore, gene expression data constitute a hybrid reading of both cell populations. In our prior work, this has not presented significant problems, yet we recognize it as an important potential confounder. Regarding the gene signature, its stability in leave-one-out cross-validation offers promising preliminary validation, but the demonstration of reproducibility in independent data sets with extensive clinical follow-up is needed, and ultimately, evaluation in prospective clinical trials remains the gold standard. In MGUS in particular, such extensive follow-up is only now being collected. Much of the published work regarding GEP of both MGUS and MM has met this limitation^{5,14,24-31,33,34}; our MGUS data embody no exception because they contain less than 5 years of follow-up and relatively few transformations to MM. Therefore, longer follow-up is planned.

In terms of subtyping ISS stages of MM, the data described also represent a proof of concept, and our ability to draw definitive conclusions about subphenotypes with ever smaller numbers of patients, such as within ISS stage 3 (Fig 4), is limited by the numbers of patients available for analysis. Collaborative, multicenter trials that enroll large numbers of patients are crucial for further progress.

Lastly, the external validity of this data is also limited, in that our work does not incorporate some of the latest developments in MM research and clinical practice. Molecular aberrations with prognostic significance have been recently described but are not addressed here, such as chromosomal hyperploidy versus nonhyperploidy.⁴⁷

We view the work described here as a platform for the future development of highly refined genomic prognostic models. We believe the broader aim should be to merge genomic models with established clinical markers, such as ISS, into comprehensive systems that accurately predict prognosis and tailor treatment regimens for individual patients. Again, only collaborative research efforts that maximize patient numbers and available data will make possible the creation of such models that not only work on paper, but that are also truly relevant in today's rapidly evolving clinical protocols and therapeutic paradigms. Ultimately, we believe that prospective trials incorporating these models (Appendix Fig A2,

online only), such as those underway in non-small-cell lung cancer²² and breast cancer,⁵⁶ will eventually demonstrate that combined clinical-genomic approaches are far superior to either approach alone in optimizing the care we provide for MM patients.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Ariel Anguiano, Cristina Gasparetto, Joseph Nevins, Anil Potti

Provision of study materials or patients: Fenghuang Zhan, Madhav Dhodapkar, Bart Barlogie, John D. Shaughnessy Jr

Collection and assembly of data: Ariel Anguiano, Fenghuang Zhan, Madhav Dhodapkar, Bart Barlogie, John D. Shaughnessy Jr

Data analysis and interpretation: Ariel Anguiano, Sascha A. Tuchman, Chaitanya Acharya, Kelly Salter, Joseph Nevins, Anil Potti

Manuscript writing: Ariel Anguiano, Sascha A. Tuchman, Anil Potti

Final approval of manuscript: Ariel Anguiano, Sascha A. Tuchman, Chaitanya Acharya, Kelly Salter, Anil Potti

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