# Primer on Medical Genomics
# Part IX: Scientific and Clinical Applications of DNA Microarrays—
# Multiple Myeloma as a Disease Model

JOHN SHAUGHNESSY, JR, PHD

**Multiple myeloma (MM) is a poorly understood and uniformly fatal malignancy of antibody-secreting plasma cells. Although several key molecular events in disease initiation or progression have been confirmed (eg, 14q32 translocations) or implicated (eg, chromosome 13 deletion), a unifying mechanism of myelomagenesis has eluded investigators. Furthermore, although MM is generally indistinguishable morphologically, it exhibits a tremendous degree of variability clinically with some patients surviving only months and others many years, suggesting that MM is composed of distinct clinical entities. Given that abnormal gene expression lies at the heart of most, if not all, cancers, high-throughput global gene expression profiling has become a powerful tool for investigating the molecular biology and clinical behavior of cancer. DNA microarray technology has facilitated the simultaneous quantification of thousands of cellular messenger RNAs (ie, gene expression). This review discusses progress made in the development of molecular-based diagnostics and prognostics for MM through the dissection of the transcriptome of plasma cells from healthy individuals and patients with MM and other plasma cell dyscrasias.**

***Mayo Clin Proc*. 2003;78:1098-1109**

CA = cytogenetic abnormalities; CA 13 = CA of chromosome 13; CCND = D-type cyclin gene (cell cycle regulatory gene); CDK = cyclin-dependent kinase; EDG = early-stage differentiation genes; FGFR = fibroblast growth factor receptor; FISH = fluorescence in situ hybridization; FISH 13 = FISH evidence of chromosome 13 deletion; GEP = gene expression profiling; GTF2F2 = general transcription factor IIF-polypeptide 2; IGF1R = insulin-like growth factor 1 receptor; IGH = immunoglobulin heavy chain; LDG = late-stage differentiation genes; MAF = musculoaponeurotic fibrosarcoma oncogene homologue; MGUS = monoclonal gammopathy of undetermined significance; MM = multiple myeloma; MMSET = MM set domain (a novel gene); MSDA = multivariate step-wise discriminant analysis; NO CA = without CA; NO FISH 13 = without FISH 13; PC = plasma cell; RB1 = retinoblastoma tumor suppressor gene; TSC22 = transforming growth factor β-stimulated protein; UBE2C = ubiquitin-conjugating enzyme E2C; vEDG = variable EDG

The recent completion of the Human Genome Project and the resulting advent of technologies allowing a global interrogation of the entire expressed human genome from very small initial tissue samples promise to revolutionize medical diagnosis, prognosis, and treatment. The diagnosis and management of multiple myeloma (MM) is no exception and will be changed irrevocably by the application of such technologies. Although the technology is new, dramatic progress is already being made.

## GENOMIC TECHNOLOGIES

Inasmuch as all cancer mutations appear to affect gene transcription directly or indirectly, the ability to follow these changes represents a powerful way in which to classify and study the molecular biology of malignancies. An important spin-off from the Human Genome Project has been the capacity to rapidly acquire large datasets of DNA sequence (high-throughput sequencing, express sequence tags, serial analysis of gene expression libraries) at relatively low cost from target cells and subsequently to mine these libraries using gene expression technology. Changes in gene expression can be quantitatively monitored on the basis of complementary base pairing of nucleic acids. At its simplest level, gene expression profiling (GEP) uses microarrays of complementary DNAs detected on nylon membranes or on glass slides. The advantage of such microarrays is that they are relatively inexpensive and may be rapidly customized to contain only genes of interest. Alternatively, more sophisticated high-density oligonucleotide microarrays were developed by exploiting technologies adapted from the semiconductor industry using photolithography and solid-phase chemistry to produce arrays containing hundreds of thousands of oligonucleotide probes packed at extremely high densities.[1] The probes are designed to maximize sensitivity, specificity, and repro

ducibility, allowing consistent discrimination between specific and background signals and between closely related target sequences.[2] Microarray technology was first used to study cancer in 1996[3] and now has been used (1) to identify disease subtypes in morphologically indistinguishable cancers[4-7] and (2) to develop molecular predictors of response to therapeutic interventions.[8,9] Microarrays can simultaneously monitor the expression of nearly all the estimated 35,000 human genes on a 2-array platform.

## MULTIPLE MYELOMA

Multiple myeloma is a plasma cell (PC) dyscrasia that accounts for 10% of all hematologic malignancies and is the second most frequently occurring hematologic cancer in the United States after non-Hodgkin lymphoma.[10,11] At any given time, the prevalence of MM is nearly 1 in 50,000 persons, and almost 15,000 new patients are currently diagnosed as having MM; 11,000 patients die of the disease each year. Although MM is uniformly fatal, long remissions can be achieved with high-dose chemotherapy and stem cell support. The clinical spectrum of PC dyscrasias is wide, ranging from the presumptive precursor condition termed *monoclonal gammopathy of undetermined significance* (MGUS) with an annual progression to overt MM of 1%[12] to solitary plasmacytoma of bone and soft tissue, smoldering MM (representing more advanced MGUS), and truly overt and symptomatic MM. Symptoms of advanced disease consist of anemia, discrete lytic or diffuse bone lesions with hypercalcemia, renal failure, and recurrent infection due to profound immunosuppression. The presence of somatic hypermutation of immunoglobulin variable region genes in MM PCs suggests that malignant transformation occurs in a B cell that has traversed the germinal centers of lymph nodes. Multiple myeloma cells originate in, survive, and expand exclusively in the bone marrow until late stages of the disease, when growth can occur independent of the bone microenvironment.

Despite recent advances in our understanding of the biology of MM,[13] the genetic complexity of the disease has hindered the identification of a suspected unifying molecular mechanism. Karyotype analyses in leukemias and lymphomas have proved invaluable for identifying nonrandom, recurrent chromosomal abnormalities that have provided the framework for molecular characterization of the genetic lesions responsible for the cellular transformation in these cancers. However, obtaining information from MM karyotypes has proved difficult because of low mitotic indexes and a so-called genomic chaos, with karyotypes revealing an average of 7 different chromosomal abnormalities. Although much of this karyotypic heterogeneity in MM is thought to result from stochastic events associated with tumor progression, undefined dis-

crete genetic abnormalities suspected to explain the variable clinical course of MM may exist beneath this cloud of genomic chaos. Thus, more powerful techniques of genomic analyses, such as microarray profiling, are envisioned to provide a deeper understanding of the molecular biology and clinical behavior of the disease, which is likely to reveal that MM constitutes several distinct clinical entities, each with unique mechanisms of transformation and response to therapeutic interventions.

## NORMAL AND MALIGNANT PCs CAN BE DISTINGUISHED BY MOLECULAR SIGNATURES

Typically, PCs make up less than 5% of the cells in normal human bone marrow and can even represent a minor population in the bone marrow of patients with MM. Thus, one of the most important considerations in the development of microarray profiling in MM is sample preparation. Unlike the leukemias and lymphomas in which relatively pure populations of highly homogeneous tumor cells can be obtained from peripheral blood or biopsies, respectively, the anatomical location of MM combined with the fact that tumor cells are mixed with a multitude of nontumor cells, each with unique gene expression signatures, indicated that PC purification would be required to obtain a robust PC-specific gene expression signature. Two different and complementary techniques have been used to accomplish this objective. My colleagues and I have used automated immunomagnetic bead sorting of PCs from large-volume bone marrow aspirates by using a monoclonal antibody, BB4, raised against syndecan-1/CD138.[5] This technique has been used to rapidly isolate (<3 hours) highly homogeneous populations of normal PCs from both bone marrow and tonsil.[5] Isolation of sufficient numbers of PCs from normal human marrow for large-scale GEP experiments makes our approach an impractical endeavor for most laboratories. Thus, to create a source of polyclonal PCs from normal donors, Tarte et al[14] developed a method for in vitro differentiation of B cells from peripheral blood. Global expression profiling of these polyclonal PCs and normal bone marrow PCs derived from immunomagnetic sorting has revealed not only strong similarities but also importantly distinct differences between the 2 populations and MM.[14,15]

To develop a comprehensive picture of the gene expression changes associated with the normal development and neoplastic transformation of human PCs, we have been profiling CD138-enriched PCs from the bone marrow and tonsils of normal donors, patients with newly diagnosed and end-stage MM, and patients with other PC dyscrasias, including MGUS, smoldering MM, and Waldenström macroglobulinemia, since January 2000. As of this writing, we have placed more than 800 cases on high-density oligonucleotide microarrays investigating the expression of ap

proximately 10,000 named and annotated genes. Recently, we began putting most of these samples on the third-generation Affymetrix GeneChip platform, allowing the surveillance of nearly all the estimated 35,000 human genes. In an initial study investigating the expression of 6800 genes in 74 newly diagnosed cases of MM, 7 of MGUS, and 31 with normal bone marrow PCs,[5] we showed that (1) short-term serial GEP reveals little intrasample variability, suggesting that changes between GEP at diagnosis and GEP at fulminate relapse may reveal clues to the mechanisms of resistance; (2) microarray-derived gene expression levels and protein levels as determined by fluorescence-activated cell sorter analysis are tightly correlated; (3) "spiked" expression of *CCND1*, *CCND3*, *MAF*, and *FGFR3/MMSET* reflects the presence of 14q32 translocations in these loci; (4) MM can be significantly differentiated from normal bone marrow PCs by approximately 120 of 6800 genes (*P*<.001), possibly reflecting fundamental genetic changes involved in, or a reflection of, neoplastic transformation; (5) GEP can accurately distinguish normal PCs from MM, but MGUS is indistinguishable from MM; and (6) 4 distinct molecular subgroups of MM (MM1, MM2, MM3, and MM4) can be identified using unsupervised hierarchical cluster analysis, with the MM4 subgroup having features of highly proliferative human MM cell lines and the MM1 subgroup being MGUS-like (Figure 1). As expected, the most important expression differences between MM1 and MM4 were found to be of genes related to cell cycle and DNA metabolism that are highly expressed in MM4. The MM4 group also exhibits a significant increase in the incidence of cytogenetic abnormalities (CA) and elevated serum $\beta_2$-microglobulin compared with the other 3 subgroups. From these data we concluded that the MM4 subgroup represents a high-risk entity. However, long-term follow-up of these patients will be required to realize these predictions. An important observation of these studies is that a subgroup of patients with newly diagnosed MM has GEP features of cell lines and as such suggests that MM cell lines represent an appropriate model system for studying the biology and evaluation of preclinical drug efficacy for at least the human myeloma cell line–like subgroup of the disease.

## MM SUBGROUPS CAN BE LINKED TO DISTINCT STAGES OF LATE-STAGE B-CELL DEVELOPMENT

Based on morphology, PCs have been thought to represent a homogeneous end-stage B cell. Recent phenotypic analysis and GEP have shown that PCs isolated from distinct organs can be recognized as belonging to distinct stages of development.[15,16] Multiple myeloma PCs are derived from the bone marrow and are thought to represent a transformed counterpart of normal terminally differentiated bone mar-

row PCs. However, the low labeling indexes of these PCs have prompted many experts to hypothesize that MM PCs actually represent terminally differentiated progeny of a transformed B cell. Thus, it is possible that MM, although generally morphologically indistinguishable, represents a spectrum of disease entities harboring molecular fingerprints that might point to their derivation at different stages of late-stage B-cell development. Precedence for such a hypothesis comes from work by Alizadeh et al,[6] who showed that diffuse large B-cell lymphoma, although representing a single disease diagnosis based on morphologic criteria, can be differentiated into 2 molecular subtypes: 1 having an activated B cell-like and 1 a germinal center B cell-like gene expression signature, each with a distinctly different clinical course.

Using microarray profiling, we previously showed that MM can be classified into 4 distinct gene expression–based subgroups.[5] Recently, we also showed that GEP can be identified that distinguish the late stages of B-cell differentiation (Figure 2, A).[15] By comparing and contrasting the gene expression patterns of highly purified populations of normal cells, we first showed that all the genes coding for cell surface markers and transcription factors known to shift during late-stage B-cell development followed the expected patterns. We then identified a panel of highly significant *early-stage differentiation genes* (EDG), marking the transition between tonsillar B cells and tonsillar PCs, and another group of genes termed *late-stage differentiation genes* (LDG), which mark the transition between tonsillar PCs to bone marrow PCs.[15] Furthermore, we recognized that MM exhibited highly variable expression of many of these EDG and LDG. An example of a so-called variable EDG (vEDG) is the gene for cyclin-dependent kinase 8 (*CDK8*). *CDK8* is not expressed in tonsillar B cells but is up-regulated to extremely high levels in tonsillar PCs and bone marrow PCs; however, *CDK8* is expressed at low or undetectable levels in all MMs. Thus, with respect to this gene, MM would be considered more tonsillar B cell-like than bone marrow PC-like as would be expected. We used 1-way analysis of variance to identify the most vEDG and LDG (v1LDG and v2LDG) and used hierarchical cluster analysis with these genes and revealed that previously defined MM gene expression subgroups (MM1-MM4) could be linked to 1 of 3 normal cell types (Figure 2, B).[15] Importantly, these studies showed that the MM4 subtype is a tonsillar B cell-like MM. Thus, the same subgroup with aggressive clinical features, eg, high incidence of CA, high $\beta_2$-microglobulin level, and being related to MM cell lines, also has the most dedifferentiated transcriptome. The MM3 subgroup was strongly linked to tonsillar PC, and the MM2 subgroup was more related to bone marrow PC. Therefore, these data support the notion
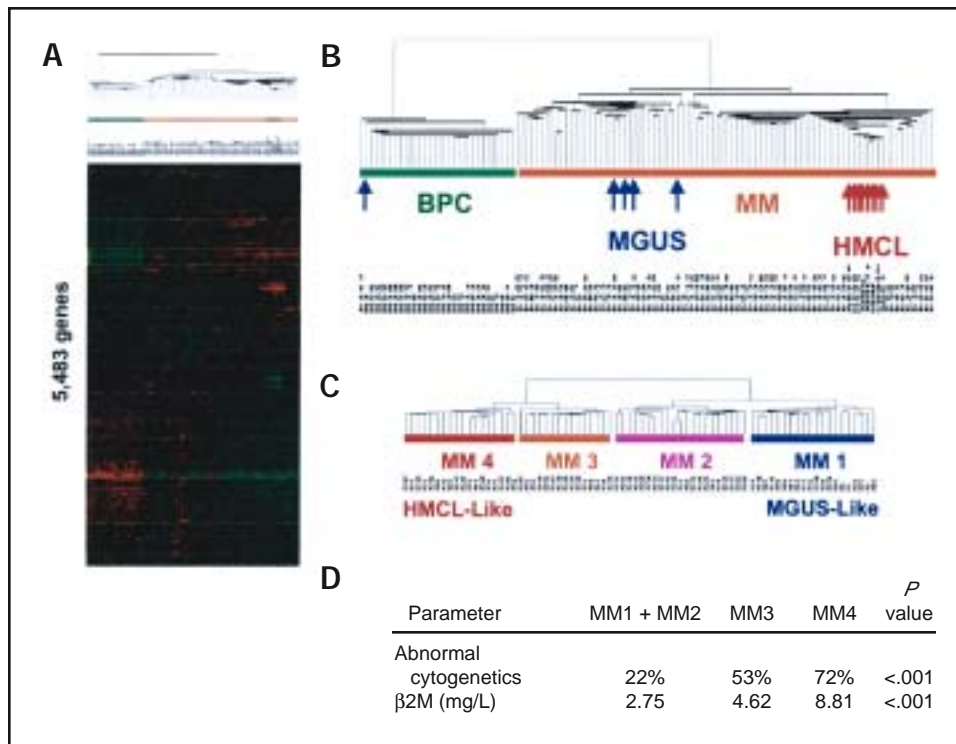
Figure 1. Global gene expression profiling distinguishes plasma cell (PC) subgroups. A, Two-dimensional hierarchical cluster analysis of 74 cases of newly diagnosed multiple myeloma (MM), 31 cases of normal bone marrow PCs (BPC), 5 cases of monoclonal gammopathy of undetermined significance (MGUS), and 6 human MM cell lines (HMCL) samples clustered on the basis of the correlation of 5483 genes (probe sets). The clustering is presented graphically as a colored image. Along the vertical axis, the analyzed genes are arranged as ordered by the clustering algorithm. The genes with the most similar patterns of expression are placed adjacent to each other. Likewise, along the horizontal axis, experimental samples are arranged; those with the most similar patterns of expression across all genes are placed adjacent to each other. Both sample and gene groupings can be further described by following the solid lines (branches) that connect the individual components with the larger groups. The color of each cell in the tabular image represents the expression level of each gene, with red representing an expression greater than the mean, green representing an expression less than the mean, and the deeper color intensity representing a greater magnitude of deviation from the mean. B, Enlarged view of patient sample cluster in A. Colored bars under samples indicate normal bone marrow PC (green) and MM (orange) clusters. Cases of MGUS are indicated by blue arrows and MM cell lines by red arrows. Note that major branches in the MM samples contain MGUS or cell line samples. C, Dendrogram of a hierarchical cluster analysis of 74 cases of newly diagnosed, untreated MM (clustergram note shown). Two major branches contained 2 distinct cluster groups. The subgroups under the right branch, designated MM1 (blue) and MM2 (magenta), were more related to the MGUS cases in B. The 2 subgroups under the left branch, designated MM3 (orange) and MM4 (red), represent samples that were more related to the human MM cell lines in B. D, Correlation between gene expression subgroups and clinical parameters indicates that there is a significant increase in incidence of abnormal cytogenetics and elevated serum $\beta_2$-microglobulin ($\beta$2M) from the MM1 to the MM4 subgroup.

that MM may be derived from cells immortalized or transformed at distinct stages of development. More elaborate studies are needed to confirm this controversial theory. Nevertheless, the strong correlations between the developmental stage–based system and our GEP-based system represent a critical affirmation of the potential validity of the 2 different classification schemes.

Most of the *vEDG* able to classify MM4 as being tonsillar B cell-like belonged to a vast array of functional classes,

eg, adhesion, transcription, signaling, and metabolism, with very few of the genes being associated with cell proliferation. Because we previously showed that MM4 has proliferation characteristics,[5] we investigated the MM4 tonsillar B-cell relationship further by correlating the expression of a panel of proliferation markers across normal samples and the gene expression–defined subgroups. These results showed a clear and strong correlation between MM4 and the tonsillar B cells, with both

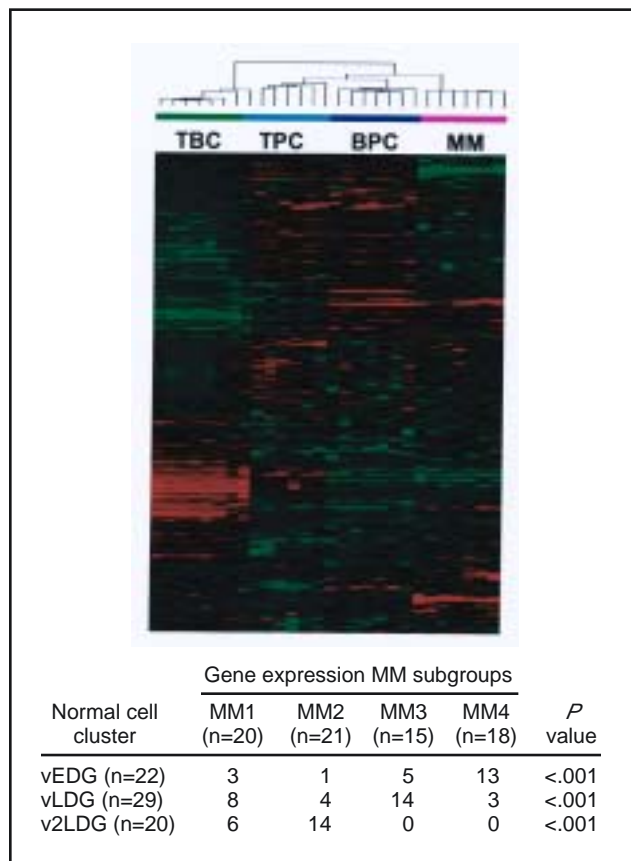| Normal cell cluster | Gene expression MM subgroups | | | | |
|---|---|---|---|---|---|
| | MM1 (n=20) | MM2 (n=21) | MM3 (n=15) | MM4 (n=18) | *P* value |
| vEDG (n=22) | 3 | 1 | 5 | 13 | <.001 |
| vLDG (n=29) | 8 | 4 | 14 | 3 | <.001 |
| v2LDG (n=20) | 6 | 14 | 0 | 0 | <.001 |

Figure 2. Gene expression patterns mark late-stage B-cell differentiation and reveal links of multiple myeloma (MM) to different stages of B-cell development. A, Two-dimensional hierarchical cluster analysis of 7 tonsillar B cells (TBC), 7 tonsillar plasma cells (TPC), 7 bone marrow PCs (BPC), and 7 MM PC samples based on the correlation of experimental expression profiles of 3288 probe sets. Image is as described in Figure 1, A. Note a major branch in the dendrogram between the CD19-enriched tonsillar B-cell samples and all the CD138-enriched PC samples, with the exception of 1 tonsillar PC sample being clustered with tonsillar B cells. The CD138-enriched PC branch was further subdivided into 2 distinct subbranches: 1 containing the tonsillar and bone marrow PC and 1 containing the MM PC. The tonsillar and bone marrow PC were further separated on a separate subbranch. B, Correlation analysis between gene expression profiling–defined subgroups (MM1, MM2, MM3, and MM4) and developmental stage–defined clusters. vEDG = variable early-stage differentiation genes; vLDG = variable late-stage differentiation genes.

groups having high levels of expression of these genes.[15] Of interest, the essential transcription factor required for PC differentiation, XBP1, showed a progressive reduction in expression across all gene expression–based subgroups, with MM1 having the highest expression and being the most similar to normal bone marrow PC and MM4 having the lowest level. One future issue is whether the reduction in expression of XBP1 is cause or effect in

the apparent dedifferentiated state of the MM3 and MM4 subtypes. The answer(s) will be aided through an understanding of the transcriptional regulation of *XBP1* expression. Of note, other transcription factors important in regulating PC development, eg, *IRF4*, *BCL6*, *CIITA*, *STAT6*, and *PAX5*, did not show the down-regulation seen with *XBP1*.

## NUMERIC CHROMOSOME ABNORMALITIES IN MM REFLECTED IN GENE EXPRESSION CHANGES

Cytogenetic abnormalities are seen in only 33% of MM cases, but abnormal DNA content is found in virtually 100% of cases by fluorescence in situ hybridization (FISH) and flow cytometry.[17-20] When observed, there are an average of 6 karyotypic abnormalities per karyotype that can affect all chromosomes.[21] Given the genomic chaos in MM, it is important to identify which chromosomal abnormalities represent key transforming or progression lesions and which are stochastic events associated with genomic instability and tumor progression. Toward this end, we determined the ploidy status of all autosomes and the X chromosome from abnormal G-banding karyotypes in 231 patients with newly diagnosed MM treated with tandem transplants.[15] In at least 10% of patients, trisomies involved chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, whereas monosomies and deletions usually of the q arm affected predominantly chromosomes 6 (6%), 13 (13%), 16 (10%), and 22 (6%). To determine whether gene expression patterns in a new and different cohort of patients could be linked to the changes in ploidy observed in the historical group, we compared the GEP of PCs from 200 patients with newly diagnosed MM and 45 normal healthy donors.[22] A total of 2476 of approximately 12,000 genes were found to be significantly down- or up-regulated in MM (*P*<.001). The chromosome map position of these genes and the number of abnormally expressed genes from individual chromosomes were determined. A greater number of altered genes from chromosomes 3, 5, 7, 9, 15, and 19 were up-regulated compared to down-regulated, with *P* values ranging from .04 for chromosome 7 to <.001 for chromosome 9. This was in contrast to genes from chromosomes 10, 13, 14, 16, and 22 in which a greater number of down-regulated genes were noted, with *P* values ranging from .03 for chromosome 10 to <.001 for chromosome 13, which was the most significant (Figure 3). No significant differences were observed for other chromosomes. One of the most remarkable observations of this study was that virtually all the genes from chromosome 13 that were altered in MM were down-regulated. This phenomenon may have profound implications given the powerful influence of chromosome 13 loss in MM outcome. Patterns of gene expression changes appear to be strongly correlated with ploidy for many chro
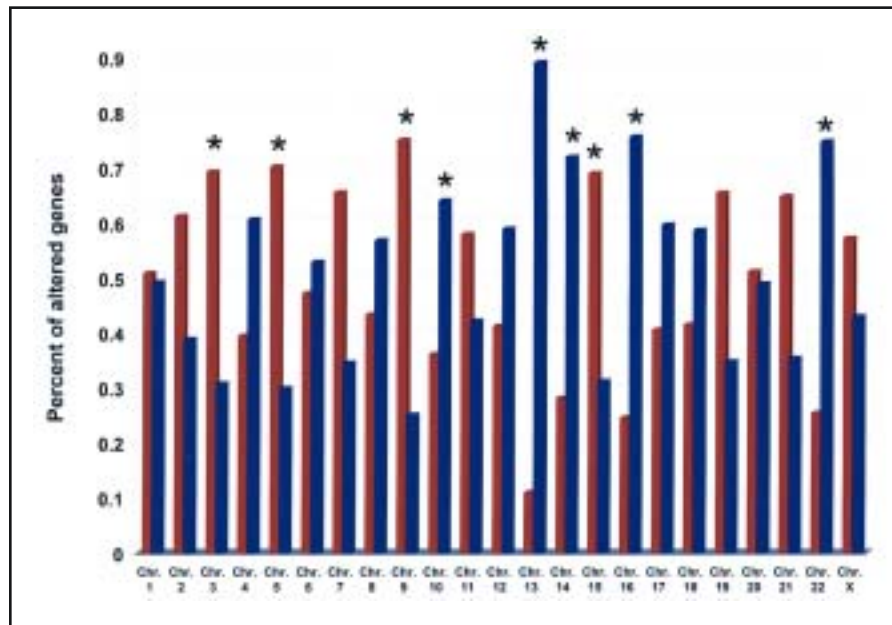
Figure 3. Gene expression reflects ploidy changes in multiple myeloma (MM) karyotypes. A, A total of 2476 genes were identified as being significantly differentially expressed between normal bone marrow plasma cells (PCs) and MM PCs. The chromosome locations of the genes were determined and broken down based on whether the gene was up-regulated or down-regulated relative to normal PCs. The percentage of up-regulated (red) and down-regulated (purple) genes from each chromosome is indicated on the y-axis. Chromosomes 1 to 22 and X are ordered from left to right on the x-axis. Asterisks indicate significant difference between up- and down-regulated genes for given chromosome. Note that chromosomes containing genes showing significant up-regulation or down-regulation also tend to be the same chromosomes exhibiting trisomy and monosomy, respectively, in karyotype analysis (see text for further details).

mosomes in MM, supporting the notion that chromosome copy number translates into altered gene dosage for select genes, which is reflected in reduced or enhanced transcript levels. One glaring exception to this trend in the present study involved the lack of a significant increase compared with a decrease of gene expression for genes mapping to chromosome 11, a frequently duplicated chromosome in MM. Comparative studies from different cancers will be informative to determine whether (1) chromosome ploidy-gene expression correlations exist in other cancers and (2) whether the same genes correlating with ploidy of a chromosome in one disease, eg, chromosome 13 in MM, are the same genes correlating with ploidy of the same chromosome in another disease, eg, chromosome 13 in chronic lymphocytic leukemia.

### IDENTIFICATION OF KNOWN AND SUSPECTED MM-ASSOCIATED 14Q32 TRANSLOCATIONS BY GEP

Like many tumors of the B-cell lineage, MM shows recurrent rearrangements of the immunoglobulin heavy chain (*IGH*) locus at 14q32. Because of the unique transcriptional activating nature of immunoglobulin-associated translocations, microarray profiling represents a powerful

means to both monitor the expression levels of known partner genes and identify new novel recurrent translocations. Recently, we showed that GEP can be used to identify 14q32 translocations in MM by the use of so-called spike gene profiles. Briefly, spiked genes are those that are not expressed in normal bone marrow PCs and most MMs but are expressed at high levels in a subset of MM cases. Spikes of *FGFR3*, *CCND1*, and *CCND3* show a 100% correlation with the presence of t(11;14)(q13;q32), t(4;14)(p16;q32), or t(6;14)(p21;q32), respectively.[5,23] In an analysis of 145 newly diagnosed cases of MM, *CCND1* and *FGFR3* spikes were seen in 13%, *MAF* in 7.5%, and *CCND3* in 4.1%. Although *CCND1* and *FGFR3* spikes never overlapped, 3 *MAF* spikes overlapped with an *FGFR3* spike and 1 with a *CCND1* spike, and 1 *CCND3* spike overlapped with a *CCND1* spike. Taken together, these data show that 34% of primary MM exhibits spikes of 1 of the 4 recurrent *IGH* translocation partners.[24] The data also point out that for the most part spikes identify unique disease entities but that cases with dual spikes indicate the presence of biallelic 14q32 translocations within 1 cell or a biclonal tumor or that spikes can be induced through a non–translocation-associated mechanism. A recent evalua
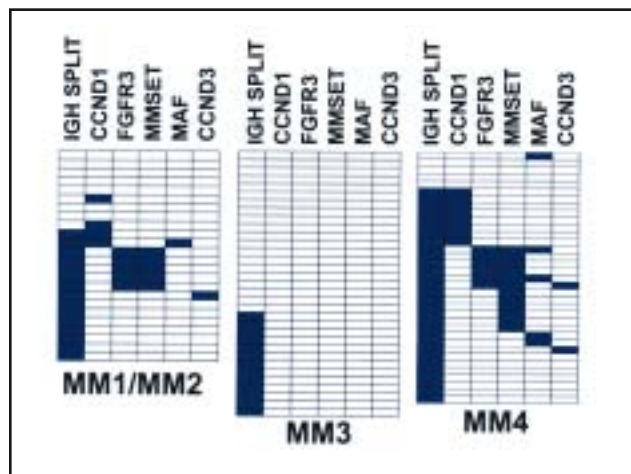
Figure 4. Visual representation of 14q32 translocations and gene expression spikes in patients with newly diagnosed multiple myeloma (MM). The gene expression subgroups MM1, MM2, MM3, and MM4 are separated (MM1 and MM2 are combined) with each row representing a single patient sample. A filled rectangle in the IGH SPLIT column indicates that more than 20% of the clonotypic plasma cells from the patient sample harbor a split signal of the IGH *CH* and *VH* fluorescence in situ hybridization probes, indicating that a 14q32 translocation has occurred. Split signals were seen in 60 of 100 (60%) of these patients (J.S., unpublished data, 2003). A filled box in the *CCND1*, *MMSET*, *FGFR3*, *MAF*, or *CCND3* column indicates that a spike (Affymetrix signal >10,000) exists in the sample. Note that spikes are highly correlated with the presence of 14q32 translocations; however, not all spikes are correlated with translocations. We are currently attempting to identify novel translocation partners in these cases. Note that the MM3 subgroup lacks spikes of any of the common partner loci. Also note that the so-called variant t(4;14)(p16;q32), in which *MMSET* spikes exist in the absence of *FGFR3* spikes, is found only in the MM4 subgroup. CCND = D-type cyclin gene; FGFR = fibroblast growth factor receptor; IGH = immunoglobulin heavy chain; MAF = musculoaponeurotic fibrosarcoma oncogene homologue; MMSET = MM set domain (a novel gene).

tion of the distribution of *CCND1*, *MMSET*, *FGFR3*, and *MAF* spikes in the 4 GEP-defined subgroups revealed that the MM3 subgroup completely lacks translocations involving these genes (J.S., unpublished data, 2003) (Figure 4). Interestingly, we also found that whereas *FGFR3* and *MMSET* spikes were together in the MM1/MM2 subgroups, *MMSET* spikes in the absence of concomitant *FGFR3* spikes were observed only in the MM4 subgroup. The exclusive presence of this unique type of translocation in the MM4 subgroup is currently under investigation.

As *MMSET* and *FGFR3* are concomitantly activated by t(4;14)(p16;q32), a surprising discovery of spike profiling was that a number of patients with elevated expression of *MMSET* lacked spikes for *FGFR3*. As of this writing, of 180 newly diagnosed cases of MM, 10 were found to have

spikes for *MMSET* but not for *FGFR3*, and 23 had spikes for both genes.[25] All 33 cases had evidence of the *MMSET-IGH* fusion transcript, a marker of the t(4;14)(p16;q32) translocation. Thus, the t(4;14)(p16;q32) translocation appears to be present in 19% of patients with newly diagnosed MM, with 32% lacking expression of *FGFR3*. Importantly, *FGFR3* was deleted in more than 90% of clonotypic PCs in 7 of the 10 patient samples, suggesting that loss of *FGFR3* occurred early in the disease evolution. Thus, these data indicate that the activation of *MMSET* may be a critical transforming event of t(4;14)(p16;q32). It is not yet clear whether *FGFR3* is activated and crucial in the initiation of MM but not essential for and thus lost during progression. Nevertheless, the consistent retention of the der(4) chromosome suggests that *MMSET* may be indispensable for both and that this chromosome should be used in routine FISH analysis for this translocation.

In our GEP spike analysis, 21 genes, including the 4 common translocation partners, were spiked in 109 (75%) of 145 cases of MM. We suspect that many of the 17 unclassified spike genes represent candidates for unknown 14q32 translocation partner genes.[23] The identification of novel translocation partners is currently being pursued by correlating FISH-detected 14q32 rearrangements (*IGH* CH/VH break-apart strategy) with GEP spike profiles using the whole-genome GeneChip analysis on a large population of patients with newly diagnosed MM.

## CHROMOSOME 13 DELETION REFLECTED IN GENE EXPRESSION PATTERNS

Previous studies from our group and others have revealed that chromosome 13 deletion represents one of the most powerful prognostic variables predicting a brief survival in patients with MM. In a comprehensive interphase FISH analysis of chromosome 13 ploidy in more than 150 cases of newly diagnosed MM, we showed that monosomy is present in about 50% to 60%, 13q14 is a deletion hot spot, and FISH deletion is linked to inferior overall survival of patients with MM.[5,25-27] To determine whether gene expression changes reflect deletion of single chromosomes and thus aid in risk stratification and identification of a putative MM tumor-suppressor gene on the chromosome, we combined FISH deletion analysis with GEP. From a training set of 47 samples with FISH evidence of chromosome 13 deletion (FISH 13) (deletion being defined as >50% of clonotypic PCs harboring 1 signal for all 4 probes spaced along the q arm of chromosome 13) and 51 without FISH 13 (NO FISH 13), 36 genes were identified at the intersection of datasets from $\chi^2$, Wilcoxon rank sum, and significance analysis of microarray[28] statistical analyses (*P*<.001).[21] Thirty-five genes, 32 mapping to chromosome 13, including several at 13q14—*GTF2F2*, *TSC22*, and

*RB1*—were underexpressed, and only 1 gene, *IGF1R* (insulin-like growth factor 1 receptor) was overexpressed in the FISH 13 group. A multivariate step-wise discriminant analysis (MSDA) was used to find a subset of the 36 differentially expressed genes that could accurately predict FISH 13 in a set of new patient samples. Ten genes, including *RB1*, were identified as correctly discriminating 41 (85%) of 48 cases in a held-out validation group in which FISH 13 status was known.[21] Thus, GEP can act as a surrogate for FISH and points to possible candidate tumor-suppressor genes. Importantly, the data indicate that one of the possible consequences of chromosome 13 deletions is the creation of a haploinsufficiency of the tumor-suppressor gene *RB1*. That a simple reduction in dosage of tumor-suppressor genes can influence cancer is supported by recent data linking an increased risk of human colon cancer and murine lymphoma to haploinsufficiency of the caretaker gene *BLM* (the gene mutated in the Bloom syndrome).[29,30] Importantly, a haploinsufficiency model can account for the inability, even after exhaustive searches, to identify a novel tumor-suppressor gene mapping to the 13q14 region in chronic lymphocytic leukemia.[31-34] An additional compelling finding of the correlation analysis was that *IGF1R*, a powerful growth-signaling receptor in MM,[35-37] represented the only gene significantly up-regulated in FISH 13 MM. Recently, we reported that both normal bone marrow and MM PCs express *IGF1*, whereas tonsil-derived PCs and tonsillar B cells do not.[15] Thus, we speculate that activation of *IGF1R* on FISH 13 MM cells could create an autocrine growth signal loop in FISH 13 MM. This model is especially relevant given that recent studies have shown that serum IGF1*R* levels are linked to survival in patients with MM.[38] Future studies will be aimed at determining whether *IGF1R* expression is indirectly or directly negatively regulated by a gene that maps to chromosome 13.

The analysis of *RB1* expression in cases of FISH 13 and NO FISH 13 revealed that a subset of patients exhibiting NO FISH 13 deletion expressed *RB1* at levels lower than the lowest level seen in 45 cases of normal bone marrow PCs (range of signal, 991-3000) and thus more closely resembled FISH 13 cases. This was initially thought to indicate that microinterstitial deletions had occurred at or near the *RB1* locus and went unrecognized by the FISH probes used in the study. However, preliminary FISH analysis with an *RB1*-specific probe failed to confirm this hypothesis (J.S., unpublished data, 2003). More recent analyses showed that, of the 32 genes mapping to chromosome 13 that are significantly down-regulated as a result of chromosome 13 deletion, only *RB1* exhibits a highly significant down-regulation in cases with low *RB1* and NO FISH 13 deletion (J.S., unpublished data, 2003). We are currently investigating whether there is a loss of heterozygosity near the *RB1* locus in these cases, which may explain the reduced *RB1* expression.

We have analyzed the relationship of FISH 13 deletion status, *RB1* expression levels, and GEP-defined subgroup designation. These analyses have revealed that nearly all the cases of NO FISH 13 and low *RB1* belonged to the MM3 subgroup (J.S., unpublished data, 2003). This was deemed to be an important finding given that (1) *RB1* is an *LSDG*[15] expressed at significantly lower levels in tonsillar PCs than in bone marrow PCs and that (2) MM3 is a tonsillar PC-like form of MM.[15] Thus, one possible explanation for the observation of low *RB1* expression in the absence of chromosome 13 deletion in MM cases could be that these forms of MM are derived from a lymph node or tonsillar PC in which immortalization and/or transformation occurred at a stage of development in which *RB1* expression is physiologically low, thus eliminating the need for chromosome 13 deletions. Of importance, the MM3 subgroup is also unusual in that it lacks chromosomal translocations of *CCND1*, *MMSET*, *FGFR3*, and *MAF* (Figure 4).

If a reduction in *RB1* gene expression is in fact the ultimate consequence of chromosome 13 deletion, then it stands to reason that patients with the MM3 subtype with no evidence of chromosome 13 deletion, yet exhibiting low *RB1*, should also have as poor a prognosis as those with FISH 13 deletion and low *RB1*. We are waiting for sufficient follow-up to test this hypothesis. Nevertheless, given the extremely strong link between FISH 13 and gene expression, we are confident that, after sufficient follow-up, gene expression patterns, possibly of a single gene, will be linked to poor survival and thus may point to the culpable gene(s) linked to this dismal prognostic subgroup.

## INTEGRATION OF GENE EXPRESSION WITH CYTOGENETICS, CHROMOSOME 13 FISH, AND PATIENT OUTCOME

With a median follow-up of almost 9 years, we recently showed that metaphase CA, especially of chromosome 13 (CA 13), confer a grave prognosis in MM treated with high-dose chemotherapy and tandem autotransplants as applied in Total Therapy I.[21] Prospective concurrent evaluation of CA, FISH 13, and GEP is being conducted as part of the successor protocol, Total Therapy II. Among approximately 50% of patients with FISH 13, only those that also harbor CA 13 had inferior survival, whereas those with FISH 13 but not CA 13 fared as well as patients lacking FISH 13.[26] Therefore, we reasoned that in vitro MM cell division, required for metaphase CA to be detected, was a critical biological feature of stroma cell–independent growth shared by CA 13 and other CA.[17] Availability of
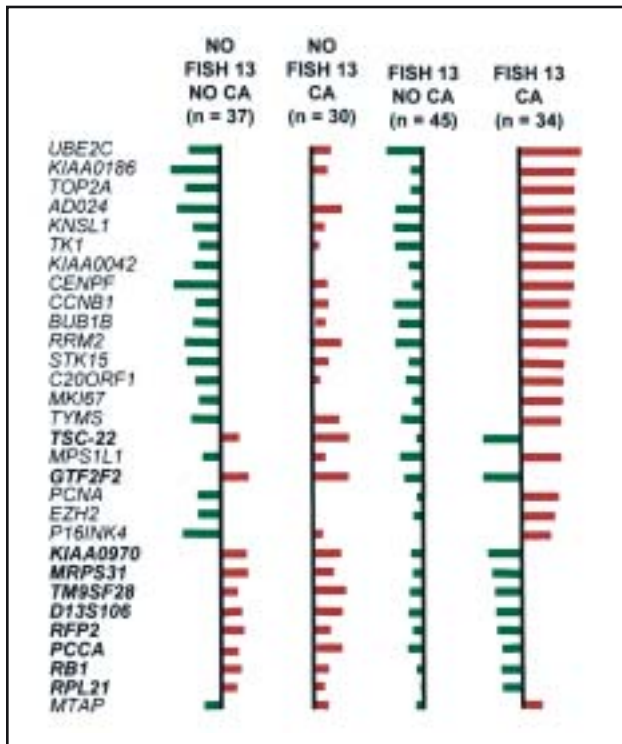
Figure 5. Cell cycle and chromosome 13–related genes distinguish fluorescence in situ hybridization evidence of chromosome 13 (FISH 13)/cytogenetic abnormalities (CA) subgroups. The so-called shrunken centroids method was applied to reveal significant differences across 4 FISH 13/CA subgroups in the expression of 30 genes (20 genes being up-regulated in the presence of CA [17 related to cell cycle/DNA metabolism and 3 having unknown function] and 10 showing significant reduction as a result of chromosome 13 deletion in multiple myeloma [indicated in bold]). The genes with non-zero components in each class are mutually exclusive. Left-sided bars indicate the degree of low "signal" relative to overall centroid; right-sided bars represent the degree of high "signal" relative to overall centroid. Differences in expression across comparisons were significant for all genes. FISH 13/NO CA (45 patients) is characterized by uniformly green, left-sided bars (underexpression of both cell cycle/DNA metabolism and chromosome 13–related genes). NO FISH 13/CA (30 patients) show mainly red, right-sided bars, indicative of expression of both cell cycle/DNA metabolism and chromosome 13–related genes. NO FISH 13/NO CA (37 patients) is characterized by overexpression of chromosome 13 genes (right-sided bars) and underexpression of all 17 cell cycle/DNA metabolism–related and 3 unknown function genes (left-sided bars). FISH 13/CA (34 patients) show the mirror image of NO FISH 13/NO CA in that all cell cycle/DNA metabolism genes are hyperactivated (more so than in the NO FISH 13/CA group) and chromosome 13 genes are inactivated (more so than in the FISH 13/NO CA group).

GEP data from 146 patients in Total Therapy II who had CA and FISH 13 status provided the opportunity to determine the molecular basis for the uniquely grave prognosis associated with FISH 13/CA (CA 13) compared with all other cytogenetic/FISH–defined groups, especially the NO FISH 13/CA group. Thus, sequential statistical analyses were performed on 37 samples with NO FISH 13 and NO CA (without CA) and 34 cases with FISH 13 and CA. A total of 157 genes were identified with significantly altered messenger RNA expression levels ($P<.001$) between the 2 groups. Most of the genes (91%) were overexpressed in the FISH 13/CA group; only 14 genes (8 mapping to chromosome 13) were underexpressed in the FISH 13/CA group. The largest class of significant genes coded for proteins involved in different checkpoints of cell cycle progression and DNA replication. Genes included those related to proliferation, *PCNA*, *MKI67*, and *MCM2*; *G1/S* transition, *TK1* and *CDI1*; *G2/M* checkpoint, *CCNB1*, *CDC2*, *UBE2C*, and *BUB1B*; chromosome segregation, *CENPA*, *CENPE*, *CENPF*, *KNSL1*, *MAD2L1*, *STK12*, *STK15*, and *STK18*; and DNA replication, *TOP2A*, *PRKDC*, *TK1*, and *TYMS*.

To show that expression levels of these genes could discriminate 4 cytogenetic subgroups (NO FISH 13/NO CA, NO FISH 13/CA, FISH 13/NO CA, and FISH 13/CA), the so-called shrunken centroids method was used with 30 genes: 20 differentially expressed genes in a comparison between FISH 13/CA and NO FISH 13/NO CA and 10 genes discriminating FISH 13 from NO FISH 13[21] (Figure 5). Forty-five patients with FISH 13/NO CA were characterized by uniform underexpression of both cell cycle and chromosome 13–related genes. By contrast, 30 patients in the NO FISH 13/CA group showed relative overexpression of both cell cycle– and chromosome 13–related genes. The third group of 37 patients with NO FISH 13/NO CA was characterized by expression of chromosome 13 genes and underexpression of all cell cycle genes. Finally, 34 patients with FISH 13/CA represented the mirror image of the previous group in that all cell cycle genes were particularly strongly expressed (more so than in the NO FISH 13/CA group) and chromosome 13 genes were profoundly underexpressed, again more so than in the FISH 13/NO CA group. Thus, collectively, it appears that FISH 13/CA is characterized by a net overexpression of all cell cycle genes and underexpression of chromosome 13 genes. The mirror image of this pattern is seen in the NO FISH 13/NO CA group. However, the underexpression of cell cycle–related genes does not match their overexpression in the FISH 13/CA group. In either comparison, FISH 13 appears to be a modulator of cell cycle gene expression. We suggested previously that *RB1* represents a strong candidate for a putative chromosome 13 tumor-suppressor gene in MM. Given the critical role that *RB1* plays in cell cycle control, the massive up-regulation of cell cycle genes in the FISH 13/CA group again implicates *RB1* loss as the essential event associated with chromosome 13 loss in MM.

Elevated expression of cyclin B1 (*CCNB1*) and the mitotic cyclin-specific ubiquitin-conjugating enzyme E2C (*UBE2C*) in MM with CA and especially in FISH 13/CA is notable. Since mutant *UBE2C* results in stabilization of both cyclin A and B, arrests cells in M phase, and inhibits the onset of anaphase, destruction of mitotic cyclins by ubiquitin-dependent proteolysis seems to be required for cells to complete mitosis and enter interphase of the next cell cycle.[39] Also, *UBE2C*-mediated ubiquination is involved in inactivation of *CDC2* (also hyperactivated in FISH 13/CA-type MM) and in sister chromatid separation, 2 processes normally coordinated during a cell's exit from mitosis. We speculate that a therapeutic strategy of treating patients with FISH 13/CA might be to inhibit *UBE2C* function. A candidate drug for such a function may be XK469; it was recently shown to induce mitotic arrest, which was correlated with the inhibition of cyclin B1 ubiquination.[40] Overexpression of topoisomerase II (*TOP 2A*) in the FISH 13/CA MM subgroup also suggests that inhibitors of this gene product, such as VP-16, may be especially effective in these patients.

## GEP IN RESPONSE PREDICTION

The identification of multiple gene expression–based subgroups is consistent with the variable clinical course of MM, with survival ranging from as little as 2 months to more than 80 months after diagnosis. An important hope for GEP is that the outcome variability, which cannot be accurately accounted for with current clinical parameters, might be better anticipated. Currently, we are prospectively attempting to correlate GEP with outcome in patients with newly diagnosed MM entered into our National Cancer Institute–sponsored clinical trial Total Therapy II, which is testing the efficacy of thalidomide combined with high-dose chemotherapy and tandem stem cell autotransplants. To date, 233 of an estimated 400 such patients have now joined this GEP study. In addition, we are performing serial GEP to catalog changes that occur in samples compared at diagnosis and relapse. This database will be used to identify common events among patients that may point to common mechanisms of resistance. Currently, there is insufficient follow-up time to establish correlations between GEP and outcome in this population; however, we have demonstrated links between GEP and initial response to VAD (vincristine, doxorubicin [Adriamycin], and dexamethasone) chemotherapy. We identified 4 subgroups based on expression of 10 cell cycle and DNA metabolism genes (Figure 6, A) (J.S., unpublished data, 2003). There is a significant correlation between elevated expression of the cell cycle genes and the presence of CA (Figure 6, B) and response to VAD chemotherapy (Figure 6, C). The data showed that, whereas 70% of patients with higher than
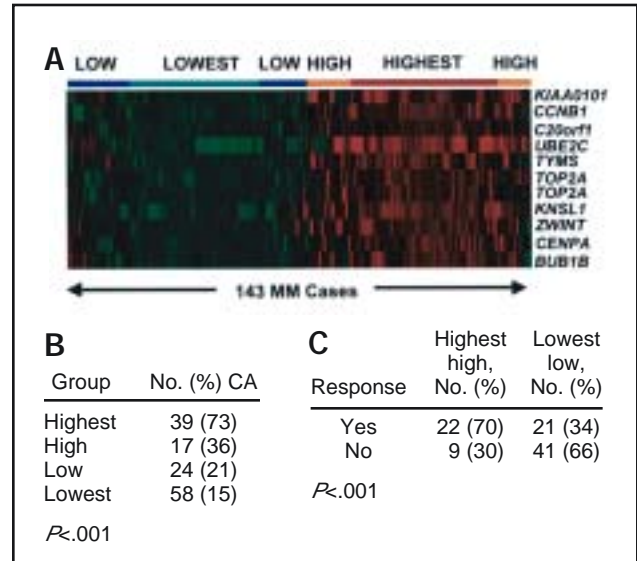


Figure 6. Expression of cell cycle and DNA metabolism genes distinguishes subgroups of newly diagnosed multiple myeloma (MM). A, Hierarchical clustering of 143 patients with newly diagnosed MM using 10 genes associated with 4 gene expression subgroups. Each row represents the expression level for a given gene, and each column represents a patient. Note that these genes are highly correlated, ie, when 1 gene is elevated all genes tend to be elevated and vice versa. Note that the clear demarcation between the highest and lowest gene expression subgroups is highly correlated among the 10 genes. B, Gene expression subgroups are correlated with presence of cytogenetic abnormalities (CA) as determined by G-banded karyotype analysis. C, Gene expression subgroups are also correlated with initial response of MM to VAD (vincristine, doxorubicin [Adriamycin], and dexamethasone) chemotherapy.

median expression of at least 8 of the 11 cell cycle genes showed a partial or complete response, only 34% of the patients with lower than median expression of 8 of 11 of these genes responded (J.S., unpublished data, 2003). Although initial response to VAD is not necessarily an accurate predictor of overall response, this study bodes well for the use of GEP in MM patient risk stratification.

To evaluate the ability of GEP to predict response to single-agent drugs, we performed GEP in 30 patients before initiation of treatment with the proteasome inhibitor PS-341. After sufficient follow-up, responders (n=15) and nonresponders (n=15) were identified, and gene expression differences in baseline samples were examined. Of the 12,000 genes surveyed, Wilcoxon rank sum test identified 44 genes that distinguished response from no response, with *P* values ranging from .009 to <.001 (Figure 7, A). An MSDA revealed that 5 of the 44 genes could be used in a response predictor model. A leave-one-out cross-validation analysis performed on a training group revealed that the
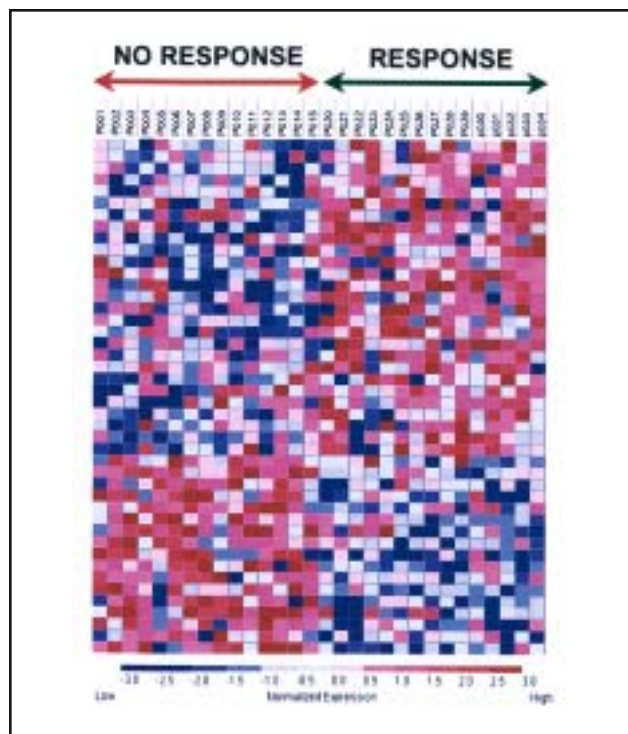
Figure 7. Gene expression patterns can be used to predict response to chemotherapy. Gene cluster of normalized expression values of a panel of genes demonstrating significant expression differences between multiple myeloma (MM) cases exhibiting a response (>50% reduction in serum M protein) or no response to proteasome inhibitor (PS-341) therapy. Plasma cells were purified from bone marrow aspirates from 15 responders and 15 nonresponders and analyzed using the Affymetrix U95Av2 GeneChip investigating approximately 12,000 genes. Using a combination of $\chi^2$, Wilcoxon rank sum test, and significance analysis of microarray, a total of 44 genes, 17 higher in the nonresponders and 27 higher in the responders, significantly differentiated the 2 groups. Genes are in rows and patient samples in columns.

model was 96.7% accurate. A held-out validation group was then tested in which 10 (71%) of 14 cases were correctly classified. Importantly, 5 of 6 responders were accurately predicted (Figure 7, B).[41] A more recent analysis of a cohort of 60 patients allowed the development of a new 10-gene MSDA that is capable of complete discrimination of a validation test group of 15 responders and 15 nonresponders. Importantly, running the same model with use of 8 of the 10 genes resulted in 3 of the 30 patients being misclassified (J.S., unpublished data, 2003). Since these data are based on an initial clinical response, at least a 50% reduction in serum M protein, it will be important to determine whether these predictive models hold up on a long-term response analysis. Nevertheless, these data indicate that correlating clinical response with GEP of purified

tumor cells before the initiation of single-agent chemotherapy may prove to be a useful tool in future clinical trial design.

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Primer on Medical Genomics Part X will appear in the November issue.