

Tumor Cell Gene Expression Changes Following Short-term *In vivo* Exposure to Single Agent Chemotherapeutics are Related to Survival in Multiple Myeloma

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Abstract Changes in global gene expression patterns in tumor cells following *in vivo* therapy may vary by treatment and provide added or synergistic prognostic power over pretherapy gene expression profiles (GEP). This molecular readout of drug-cell interaction may also point to mechanisms of action/resistance. In newly diagnosed patients with multiple myeloma (MM), microarray data were obtained on tumor cells prior to and 48 hours after *in vivo* treatment using dexamethasone ($n = 45$) or thalidomide ($n = 42$); in the case of relapsed MM, microarray data were obtained prior to ($n = 36$) and after ($n = 19$) lenalidomide administration. Dexamethasone and thalidomide induced both common and unique GEP changes in tumor cells. Combined baseline and 48-hour changes in GEP in a subset of genes, many related to oxidative stress and cytoskeletal dynamics, were predictive of outcome in newly diagnosed MM patients receiving tandem transplants. Thalidomide-altered genes also changed following lenalidomide exposure and predicted event-free and overall survival in relapsed patients receiving lenalidomide as a single agent. Combined with baseline molecular features, changes in GEP following short-term single-agent exposure may help guide treatment decisions for patients with MM. Genes whose drug-altered expression were found to be related to survival may point to molecular switches related to response and/or resistance to different classes of drugs.

Multiple myeloma (MM) is a malignancy of terminally differentiated, antibody-secreting plasma cells that reside in the bone marrow, where they cause a constellation of clinical symptoms including immunosuppression, anemia, and lytic bone destruction (1). High-dose melphalan treatment with autologous stem cell support has emerged as the standard of care for younger patients with MM (2, 3). Various approaches to remission induction have been applied, including the use of recently identified new agents such as thalidomide, lenalidomide, and bortezomib (4).

Although small-molecule inhibitors, imatinib (ABL) and erlotinib (EGFR), and monoclonal antibodies, trastuzumab (HER2), rituximab (CD20), and alemtuzumab (CD52) target specific protein functions, traditional genotoxic anticancer

agents may affect numerous biochemical targets. While MM is initially sensitive to a variety of compounds, such as standard melphalan and prednisone as well as immunomodulatory agents (thalidomide, lenalidomide) and the first-in-class proteasome inhibitor, bortezomib, first-cycle complete responses are rare and, once achieved, are seldom durable as a consequence of secondary resistance. The mechanisms of both primary and secondary resistance are poorly understood. Genomic signatures of tumor cells have led to the recognition of MM subtypes with markedly different long-term prognoses, although initial complete response rates are similar (5). These differences in event-free survival (EFS) and overall survival (OS) likely reflect differences in the depth of initial tumor cytoreduction and eventual regrowth kinetics, which can be readily studied in some leukemias through the application of PCR-based analysis of unique gene translocations (6).

To elucidate the genomics of cellular responses to cancer treatment, Cheok and colleagues (7) applied a genomics approach to acute lymphoblastic leukemia (ALL) cells before and after *in vivo* treatment with methotrexate and mercaptopurine, alone or in combination. Based on changes in gene expression, they identified 124 genes that accurately discriminated treatments. Only 14% of genes that changed when these medications were given as single agents also changed when they were given together. The authors showed that lymphoid leukemia cells of different molecular subtypes of ALL share common pathways of genomic response to the same treatment, that changes in gene expression are treatment-specific, and that gene expression profiles (GEP) can illuminate differences in cellular response to drug combinations versus single agents (7). ALL can be cured with combination chemotherapy

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in >75% of children, but the cause of treatment failure is unknown. By testing leukemia cells for *in vitro* sensitivity to prednisone, vincristine, asparaginase, and daunorubicin, and by applying genomics to identify genes differentially expressed in drug-sensitive and drug-resistant ALL, groups have identified gene expression patterns of resistance and sensitivity that predict outcome independent of other conventional variables (8–10).

We hypothesized that, similar to observations in acute leukemia (7), drug-induced gene alterations, especially after *in vivo* application, may reveal not only drug-unique mechanisms of action but also contribute to the predictive power provided by the knowledge of MM cell GEP changes. Here, we examine whether changes in GEPs of purified plasma cells (PC) obtained prior to and 48 hours after single-agent therapy with dexamethasone (DEX), thalidomide (THAL), or lenalidomide (LEN) differed by agent and could be used to predict outcome in patients with myeloma.

Materials and Methods

Patients. PCs were obtained from 87 newly diagnosed MM patients enrolled into total therapy 2 (TT2; ref. 11) both prior to and 48 h after single-agent therapy with either DEX (40 mg/d for 2 days; 45 patients randomized to the no-THAL arm) or THAL (400 mg/d for 2 days; 42 patients randomized to the THAL arm). An additional 36 patients with advanced and refractory MM were enrolled into a salvage trial with single-agent LEN, 19 of whom had paired PC samples obtained prior to and after LEN at 25 or 50 mg/d for 48 h. Patient baseline characteristics for both TT2 (11) and LEN (12) trials have been previously reported. The Institutional Review Board of the University of Arkansas for Medical Sciences approved the research studies, and all subjects provided written informed consent approving the use of their samples for research purposes.

Microarray samples and processing. PC purification and GEP, using the Affymetrix U95Av2 microarrays, were done as previously described (13). Microarray data and clinical annotations on the 87 newly treated patients and 36 relapsed patients used in this study have been deposited in the NIH Gene Expression Omnibus under accession number GSE8546.

Statistical methods. Prior to the analysis of changes in gene expression levels, genes were screened to include only those with an average expression level of ≥ 500 . For each remaining gene, likelihood ratio *P* values for changes in expression after 48 h by treatment, adjusted for baseline expression level, were computed with a linear mixed effects regression model for log₂ gene expression. Significant changes were declared by applying a 2.5% false discovery rate (FDR) cutoff to the multiple *P* values. The significant genes were further characterized according to the strength of evidence for differential (by drug) up-regulation or down-regulation, as measured by *P* values for the drug-by-48-h change interaction in the linear mixed-effects models. Due to the large number of selected genes (1,225), we further screened the significantly up-regulated or down-regulated genes for association with EFS. Proportional hazards *P* values for the association of baseline expression and change over 48 h with EFS were computed for each of the 1,225 genes, and the 95 genes with $P \leq 0.025$ (FDR 20%) were retained.

A validation cohort of 36 patients with relapsed disease was used to confirm the association of the 95 genes with EFS and OS. Unsupervised K-means clustering was applied to the 95 identified genes in these 36 patients, and the clusters were used to predict EFS and OS. Nineteen of the 36 relapsed-disease patients had baseline and 48-h (after LEN) samples, and the heat-map for these paired samples was compared, qualitatively, to that for the 87 newly diagnosed patients.

Kaplan-Meier product-limit estimates were used to estimate censored outcome distributions, log-rank tests were used for associated *P* values,

and proportional-hazards regression analysis was used to compute hazard ratio estimates. All statistical analyses were done using the R statistical package, version 2.01.³

Results

We measured gene expression levels in 87 newly diagnosed patients both prior to and 48 hours after single-agent administration of DEX or THAL, preceding the initiation of TT2; these patients compose a subsample of a 668-patient clinical trial whose EFS and OS were similar whether enrolled on the experimental arm with THAL or on the control arm (Fig. 1A and B).

The 12,625 probe sets on the Affymetrix U95Av2 array were first reduced to the 6,603 genes that had a baseline average expression level of ≥ 500 , an arbitrary threshold commonly used with this platform to screen out unexpressed genes. To assess 48-hour GEP changes, we did multivariate mixed-effect regressions separately on the log-scale intensities of each of the 6,603 genes. This standard technique accounts for the correlation between each patient's paired baseline and posttreatment measurements, whereas allowing for the two significance tests of interest to be performed simultaneously poses the questions (a) is the fold change over 48 hours significant; (b) if so, is the fold change over 48 hours different for DEX versus THAL. Thus, a single analysis for each gene provides one *P* value for change over 48 hours and another for differential change by treatment. The former were used to select 1,225 genes, using a FDR (14) of 2.5% ($P \leq 0.01$). The large number of significant genes constitutes strong evidence that transcriptional changes due to the administration of DEX and THAL can be detected after 48 hours. In order to select genes of interest from the 1,225 genes, we did a gene-by-gene Cox regression analysis of the association of baseline and 48-hour changes with EFS, which yielded a final set of 95 genes with $P < 0.025$ (FDR 20%). We used a more liberal FDR of 20% because the genes had already satisfied one differential expression multiple comparisons threshold. Table 1 summarizes this analysis.

In order to assess the degree of differential change by drug, we sorted the 1,225 genes by the significance of the DEX versus THAL difference in 48-hour fold changes. For illustrative purposes, we divide the genes into three arbitrary groups: 279 exhibited changes that were similar for THAL and DEX on average ($P \geq 0.20$), 535 changed significantly but only marginally or mildly differentially ($0.02 < P < 0.20$), and the remaining 411 exhibited changes that were significantly different after DEX versus THAL ($P \leq 0.002$). Figure 2A depicts baseline and 48-hour expression levels in these genes, with pretreatment data on the left (columns) and posttreatment data on the right, divided within by DEX (brown) and THAL (blue). The upper group of gene probe sets (rows, yellow band to the left) depicts genes that change similarly after both DEX and THAL, with two evident subgroups, one of the genes up-regulated by both treatments [expression trending to higher (red) from left to right] and another gene down-regulated by both treatments [trending to lower (green) from left to right]. The orange band indicates gene probe sets with marginally differential changes over 48 hours, many of which seem to trend in the same direction for both treatments, although magnitude and uniformity across patients tended to be higher

³ <http://www.R-project.org>

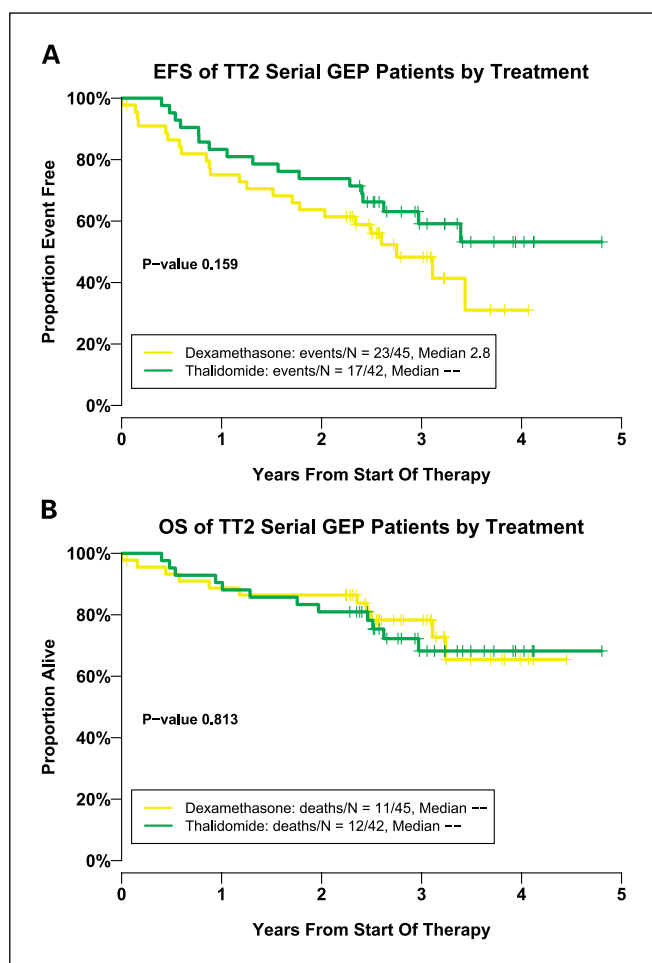


Fig. 1. A, EFS of TT2 patients with pretreatment and 48-h posttreatment paired GEP samples. Patients treated with THAL were drawn from the THAL arm of the TT2 trial, and the EFS advantage for this arm is evident, although not yet significant with the 40 available events in the subsample. B, OS of TT2 patients with pretreatment and 48-h posttreatment paired GEP samples. As in the larger TT2 trial patient sample, no significant difference between the two arms in OS is evident.

for DEX. Gene probe sets in the bottom group (purple band) were significantly differentially regulated after 48 hours of DEX or THAL. Figure 2B depicts expression levels for the 95 genes with changes associated with EFS, 22 of which changed similarly for THAL and DEX, 43 of which changed moderately differentially, and 30 of which changed significantly differentially. The patterns of expression changes are comparable to those for the larger set of 1,225 genes.

Table 2A shows that 23 of the 30 genes that change differentially by treatment are regulated in the opposite direction by THAL and DEX; the remaining 7 genes are regulated in the same direction but with a significantly greater change for one or the other treatment. Table 2B reveals that, of 43 genes with marginal changes associated with treatment, only 10 are regulated in the opposite direction; in Table 2C, all 22 of the 95 genes with $P \geq 0.2$ for differential change are changes in the same direction.

A list of the 95 genes is provided in Supplemental Table S1A-C, along with P values and effect estimates from the expression change over time and EFS analyses. Although the tabulated EFS P value concerns the overall effect of baseline expression and change over 48 hours (i.e., the P value used to select the 95 genes), the tabulated effect estimates are for the change over 48 hours by drug (adjusted for baseline), providing a quantitative assessment of whether up-regulation is associated with better EFS [hazards ratio (HR) < 1] separately for THAL and DEX. For example, *G1P3*, the gene in the 11th row of Supplemental Table S1A, is down-regulated on average by an estimated 0.56 (56% of a 2-fold decrease, or decrease on the log 2 scale) after DEX, although up-regulated on average by an estimated 17% after THAL. For both drugs, up-regulation (i.e., fold increase) is associated with improved EFS (HR, 0.46 for DEX; HR, 0.54 for THAL).

An independent cohort of 36 relapsed-disease patients was used to confirm the overall association of the 95 genes with EFS and OS. Figure 3A and B illustrate the distributions of EFS and OS, respectively, grouped by an unsupervised K-means clustering of the 36 patients based on baseline expression of the 95 genes. Note that no risk classifier was created in the original cohort; the procedure applied here constitutes only a simple, robust confirmation of the overall association of the 95 identified genes with clinical outcome, not the validation of an explicit expression biomarker (i.e., classifier). The unsupervised cluster designation is significantly associated with both EFS and OS in the 36 relapsed-disease patients ($P = 0.033$ and $P < 0.001$, respectively). Nineteen of the 36 patients had paired baseline and 48-hour post-LEN data available for comparison with DEX and THAL results. Figure 4 adds these 19 paired patient samples (columns beneath gray band) to the heat-map of Fig. 2B. Qualitatively, the 48-hour changes induced by LEN are similar to those for THAL, particularly in the bottom group (purple-banded rows), in which the changes mirror those for THAL in contrast to DEX.

Discussion

The potential for the development of molecular-based assays that are predictive of patient outcome following particular

Table 1. Analysis of 48-h treatment-induced expression fold changes

$n = 87$	Average expression screen only
Platform: U95Av2 probe sets	12,625
Prescreen: average expression ≥ 500	6,603
Significance analysis: 48-h fold change and DEX vs. THAL differential fold change (i.e., modification of the 48 h effect by treatment; FDR 2.5% threshold for overall change; $P \leq 0.01$)	1,225
Focus gene postscreen using multivariate proportional hazards: EFS as predicted by baseline log-scale expression plus 48-h fold change, adjusted for treatment (FDR 20%, $P < 0.025$)	95

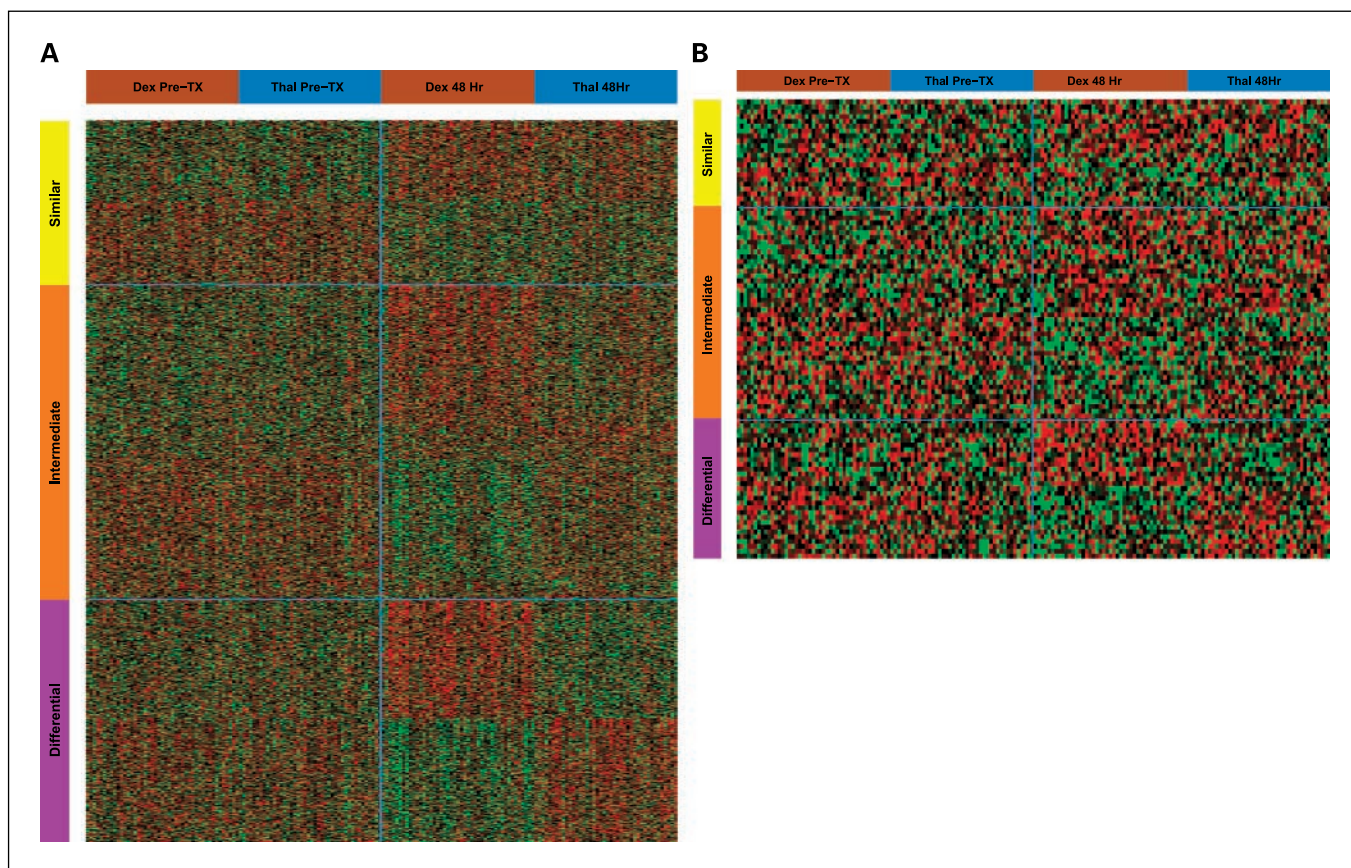


Fig. 2. *A*, heat-map of all 1,225 genes with significant overall fold changes after 48 h of treatment with THAL or DEX (FDR 2.5%, $P \leq 0.01$). The vertical line splits the sample pairs (columns) between pretreatment and 48 h posttreatment. The upper group of genes (rows above upper horizontal line) exhibited changes that were on average unassociated with treatment ($P \geq 0.2$). In the center group, the P values for differential fold change were between 0.002 and 0.2, but the smaller P values did not satisfy the FDR 2.5% threshold; the changes in these genes' expression seemed to be mildly to moderately differential. The bottom group of rows depicts genes with significantly differential fold changes over 48 h by treatment (FDR 2.5%, $P \leq 0.002$). *B*, heat-map of 95 EFS-associated genes obtained by secondary screening of the 1,225 genes with significant overall fold changes. The secondary screening of genes for association with EFS served to select potential focus genes from the large pool of 1,225 declared to be significantly changed over 48 h (FDR 2.5%). The combined baseline expression and 48-h fold change of these genes predicted EFS after adjustment for treatment using a liberal FDR 20% threshold ($P < 0.025$). As in (*A*), the upper horizontal group of genes changed on average similarly across the two treatments. In the center group, the mildly to moderately differential fold changes tended to be in the same direction (see Table 2B) on average for the two treatments, but with an apparent difference in magnitude. The bottom group of rows depicts genes with significantly differential fold changes over 48 h by treatment (FDR 2.5%, $P \leq 0.002$), most of which differed in the average direction of change (see Table 2C).

therapeutic regimens may be aided by the assessment of drug-specific gene expression changes in tumor cells early in the course of therapy. Such changes might also be detectable directly by measuring GEP changes following a so-called "test dose" of a single agent or multiagent combinations early in the course of therapy. Baseline GEP classifiers for treatment outcome prediction, trained using homogeneous samples of newly diagnosed patients receiving high-dose therapy (5, 15), and patients with relapsed disease treated with the single agent bortezomib (16), have been defined. Such classifiers can be expected to be prognostic for EFS and OS in MM patient samples having differing treatment histories and being treated under different regimens, although most likely lacking the accuracy obtained with classifiers customized for specific patient characteristics and treatments. A salient concern is whether such single-treatment outcome classifiers contain any information at all that is specific to the treatment being studied.

DEX and, more recently, THAL are central components to most therapeutic strategies for the treatment of MM. In this report, we have used, for the first time in MM, genomic profiling of CD138-selected PCs prior to and after 48 h of

in vivo single-agent therapy with THAL or DEX to predict EFS. We identified genes that were uniquely influenced by each drug and genes that were commonly altered by both drugs. Similarly, Cheok and colleagues (7) showed that cells of different molecular subtypes of ALL shared common pathways of genomic response to treatment with methotrexate or mercaptopurine. Although we have recently identified seven distinct molecular subtypes of MM (5), the sample size used here prevented an assessment of the relationship between subgroup designation and drug-induced changes, but our data suggest that, as in ALL, changes are consistent across subgroups.

A particularly remarkable finding in the current study was that genes whose expression was altered by THAL in newly diagnosed disease and associated with subsequent survival were also altered by the THAL analogue LEN, and their change was associated with EFS in a salvage trial of patients with relapsed-disease. This finding strongly suggests that these genes are powerful biomarkers and the similarity in acute gene expression responses to two related chemotherapeutic agents may provide important insights into the potential mechanism(s) of action of the drugs. These results also highlight the fact that

refractory disease retains a similar acute molecular response to chemotherapies seen in primary disease.

Reduced or increased expression of genes after 48 hours could be due to a general change in the transcriptional activity or posttranscriptional modification of mRNA of the genes identified. Given intratumor heterogeneity, the 48-hour changes might reflect rapid loss of a cell subpopulation. Thus, one could imagine that the given average expression within a population of tumor cells is derived from cells expressing a certain gene at different levels, including some that do not express the gene at all. DEX or THAL may preferentially eliminate a subset of cells not expressing a certain gene, whereas the remaining cells expressing this gene survive, leading to a net increase in gene expression after therapy. Superior EFS associated with an increase in the expression of genes may therefore reflect the preferential elimination of cells not expressing a given gene. Alternatively, in the absence of tumor cell apoptosis within 48 hours, increased expression of a gene may arise in a cell subpopulation that is destined to die subsequently or recover from sublethal damage. This scenario may be particularly important for genes whose up-regulation is associated with a poorer outcome. Nevertheless, as only a few of the many altered genes were related to EFS, it is likely that these genes represent powerful biomarkers. If this is true, the types of studies described here could be used to dissect

the nature of drug sensitivity in tumor cell populations. In such a scenario, both the direction of the change and the relative degree by which the gene change influences the HR might tell more about the underlying biology of the tumor and its response to therapy. In any event, the data presented provide the platform for a prospective evaluation of a treatment approach that randomizes patients, deemed to have an inferior prognosis based on the knowledge of the 48-hour sample, to continuation of the original therapy versus an investigational drug or combination regimen.

We showed that short-term drug exposure-induced GEP changes in MM tumor cells *in vivo* could be related to subsequent survival. This approach provides a particularly powerful advantage over the evaluation of changes following *in vitro* exposure, in that *in vivo* changes more accurately reflect the actual pharmacokinetics, as well as potentially important interactions of tumor cells with their microenvironment. To obtain a better understanding of cell-intrinsic and stroma-influenced changes, current studies are aimed at (a) comparing GEP in tumor cells following an *in vitro* and *in vivo* exposure and (b) comparing the GEP of both purified PCs and unseparated bone marrow biopsy samples prior to and following treatment. Indeed, as in other cancer types, the microenvironment in MM has been linked to disease progression and drug resistance and can be targeted by agents such as

Table 2. Relational changes in gene expression following short-term *in vivo* drug treatment

(A) DEX- and THAL-associated 48-h differential expression fold changes*

	Up-regulated by THAL	Down-regulated by THAL	
Up-regulated by DEX	5 [†]	10	14 (48%)
Down-regulated by DEX	13	2 [‡]	15 (52%)
	17 (59%)	12 (41%)	30

(B) DEX- and THAL-associated 48-h marginally differential expression fold changes[§]

	Up-regulated by THAL	Down-regulated by THAL	
Up-regulated by DEX	18	5	23 (53%)
Down-regulated by DEX	5	15	20 (47%)
	23 (53%)	20 (47%)	43

(C) DEX and THAL similar 48-h expression fold changes[¶]

	Up-regulated by THAL	Down-regulated by THAL	
Up-regulated by DEX	12	0	12 (55%)
Down-regulated by DEX	0	10	10 (45%)
	12 (55%)	10 (45%)	22

*All of these genes exhibited both significant overall 48-h fold changes (FDR 2.5%, $P \leq 0.01$) and significant differential changes by treatment (FDR 2.5%, $P \leq 0.002$). In this case, all genes that satisfied the differential change FDR threshold independently also satisfied the overall change FDR threshold. The significant genes were postscreened for association with EFS (FDR 20%, $P \leq 0.025$).

[†]All five genes, on average, were differentially less up-regulated after THAL. Three of these exhibited average fold change of <0.04 (little change after THAL), whereas the other two were up-regulated by fold changes of 0.42 and 0.27 after THAL and 1.19 and 0.96 after DEX (*SLC16A3* and *MERTK*, respectively, see Supplemental Table 1A for details).

[‡]Both genes, on average, exhibited a smaller magnitude of fold down-regulation after THAL.

[§]All of these genes exhibited significant overall 48-h fold changes (FDR 2.5%, $P \leq 0.01$), but nonsignificant differential changes by treatment (FDR $> 2.5\%$, $0.002 < P < 0.20$). The significant genes were postscreened for association with EFS (FDR 20%, $P \leq 0.025$).

^{||}Although the FDR multiple-comparisons adjustment allows for genes in this category with P values for differential change <0.005 , note that fold change estimates differing in direction for the two treatments are uncommon. Interpretively, we regard any difference in fold change estimates for the two treatments with P between 0.002 and 0.20 in Table 2B to have weaker evidence for differential change than the genes in Tables 2A and 2C; the evidence for overall change is nonetheless significant, at FDR 2.5%.

[¶]All of these genes exhibited significant overall 48-h fold changes (FDR 2.5%, $P \leq 0.01$), but their changes were weakly associated with treatment ($P \geq 0.2$). The significant genes were postscreened for association with EFS (FDR 20%, $P \leq 0.025$).

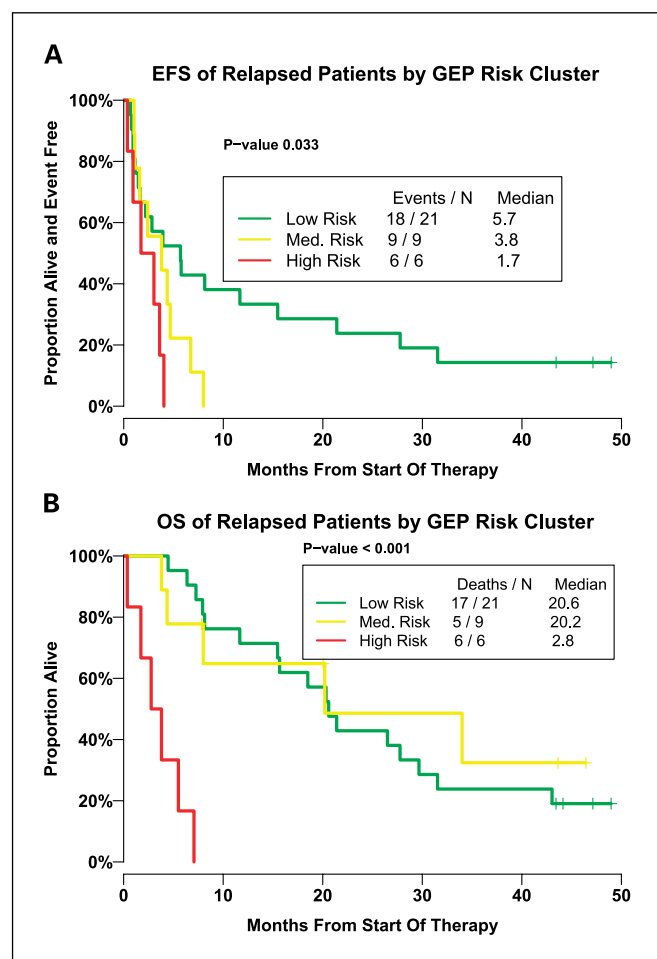


Fig. 3. A, EFS in a validation cohort of 36 relapsed-disease patients by baseline GEP risk group. Risk groups were determined by unsupervised K-means clustering within the validation cohort using the 95 genes from the DEX/THAL cohort. Even though the rate of progression was high in all three groups, with a median time to progression of 5.7 mo in the low-risk group, the 95-gene unsupervised risk group was a significant prognostic factor. B, OS in the validation cohort. Unsupervised high risk based on the 95 genes was strongly associated with poor survival. This shows that the expression profile of genes discovered in a cohort of newly diagnosed patients treated under a different protocol was generally prognostic of EFS and OS in an independent cohort of relapsed-disease patients. These genes are probably not optimal in any sense for prognostic applications in this population, but the confirmation of overall association with clinical outcome is simple and robust.

THAL, LEN, DEX, and bortezomib (17). As many new agents used to treat MM are thought to affect both tumor cells and the microenvironment (18, 19), our current total therapy 3 trial evaluates bortezomib-induced 48-hour GEP changes, in the context of baseline findings, not only in PCs but also in whole bone marrow biopsies in order to investigate the potential contribution to outcome prediction of both baseline and posttherapy alterations of microenvironment-associated genes (20).

In addition to their potential in aiding therapy decisions, the drug-induced changes have the potential to reveal important insights into the molecular mechanisms of action of chemotherapeutics. For example, the discovery that MM PCs up-regulate the Wnt signaling antagonist dickkopf-1 (*DKK1*) following 48-hour drug exposure to THAL *in vivo* (21) indirectly led to the discovery that *DKK1* activation in

embryonic mesenchyme and subsequent cell apoptosis are the root cause of THAL-induced limb deformities (22). Although *DKK1* activation was not discovered to be linked to survival in the current analysis, these data suggest that perhaps the antimyeloma effects of THAL might be traced to negative effects on bone marrow stromal cells through hyperactivation of *DKK1*. This hypothesis requires testing.

We identified a subset of genes whose expression was significantly differentially altered by DEX relative to THAL, i.e., up-regulated by one and down-regulated by the other; another subset exhibiting marginal differential changes; and a third subset of genes whose expression was similarly altered by the two drugs.

Of the genes that were significantly differentially altered by the two drugs, two groups could be identified: one with down-regulation of expression by DEX and up-regulation by THAL and the other vice versa. Importantly, the influence of the change on outcome was not always consistent with the direction in the change in expression. For example, although tending to be down-regulated by DEX and up-regulated by THAL, clinical outcome was superior in the cases in which both drugs up-regulated *GGA2* (a gene coding for the Golgi-associated, γ adaptin ear-containing, ARF binding protein 2), which plays a role in protein sorting and trafficking between the trans-Golgi network and endosomes. Likewise, although the glucocorticoid receptor *NR3C1* tended to be down-regulated by DEX and up-regulated by THAL, up-regulation of the gene by either drug was associated with a better outcome.

A converse situation applied to the putative tumor suppressor gene *ST7*, which tended to be down-regulated by DEX and up-regulated by THAL: up-regulation by DEX was associated with a better outcome, whereas up-regulation by THAL was prognostically unfavorable. Although tending to be down-regulated by both drugs, up-regulation of an estrogen receptor-associated gene, solute carrier family 33 (acetyl-CoA transporter), member

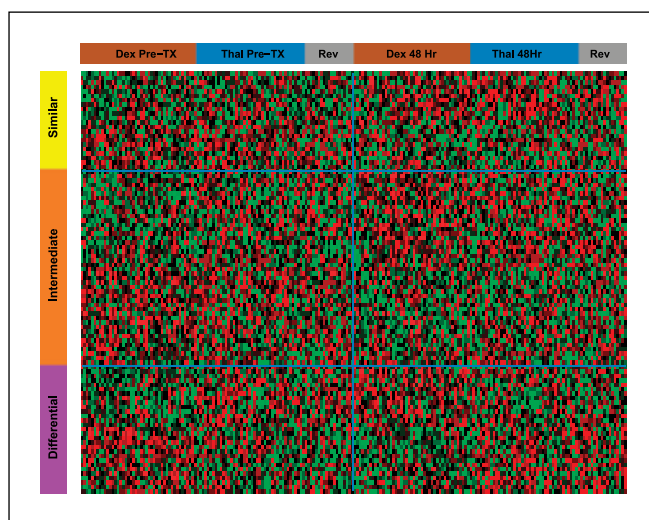


Fig. 4. Heat-map of 95 EFS-associated genes, including an independent cohort of 19 relapsed-disease patients with serial GEP microarrays at baseline and 48 h after treatment with LEN. The expression fold changes of the 19 relapsed-disease patients treated with LEN generally mirrored the 48-h post-THAL fold changes in the 87 newly diagnosed patients. This was particularly evident in the 30 bottom gene rows, in which the LEN fold changes follow the significant contrasts of THAL changes to DEX changes.

1 (*SLC33A1*), by DEX was moderately associated with a better outcome, whereas its down-regulation by THAL was strongly associated with a better outcome.

MACF1, encoding the microtubule-actin cross-linking factor 1 and mapping to 1p34, is a member of the spectraplakins family of genes that are important for controlling microtubule dynamics and reinforcing links between microtubules and polarized filamentous actin, so that cellular polarization and coordinated cell movements can be sustained. Chen and colleagues (23) found that small interfering RNA knockdown of *Macf1* inhibited Wnt signaling in a mouse embryonic carcinoma cell line. The gene coding for transporter 1, ATP-binding cassette, subfamily B (*TAP1/ABCB2*) is a multipass endoplasmic reticulum membrane protein that forms [as shown by Neefjes and colleagues (24)] heterodimers with TAP1 and is involved in assembly of class I molecules and presentation of endogenous peptides derived from nuclear and cytosolic proteins to CD8(+) T cells. *TAP1* up-regulation by both drugs was associated with a poor outcome. The activin A receptor type I gene (*ACVR1*) is part of a complex of two type II and two type I transmembrane serine/threonine kinases that together bind activin. Type II receptors phosphorylate and activate type I receptors, which autophosphorylate and then bind and activate SMAD transcriptional regulators. Up-regulation of this gene by either drug was associated with a good prognosis.

Increased expression of genes mapping to chromosome 1q and loss of expression of genes mapping to 1p are associated with poor survival in patients with myeloma (15). The gene coding for CD53 and mapping to a deletion hotspot at chromosome 1p13 (25) tended to be up-regulated by both DEX and THAL, and up-regulation by either drug was associated with a better outcome. Given that CD53 is a cell surface protein, it is possible that flow cytometric analysis of its expression on PCs following short-term exposure to these drugs could be used as a surrogate predictor for long-term outcome. Another example is syntaxin binding protein 3 (*STXB3*), also mapping to the deletion hotspot at 1p13, which, when up-regulated by both DEX and THAL, was also associated with improved survival.

Of the subset of genes tending to be up-regulated by DEX and down-regulated by THAL, the only gene whose elevated expression after exposure to both drugs was associated with improved survival was the DNA excision repair gene *ERCC1*. On the other hand, up-regulation by either drug of the genes coding for the ABL proto-oncogene interacting protein (*ABI1*), RNase P 40-kDa subunit (*RPP40*), tissue-specific transplantation antigen P35B (*TST3*), phosphatidylserine synthase 1 (*PTDSS1*), phosphormannomutase 1 (*PMM1*), and plectin 1 intermediate filament binding protein 500-kDa (*PLEC1*) was associated with inferior survival, with the HR associated with these changes being >5-fold for some of the genes activated by THAL. In theory, such a high hazard associated with a short-term readout might prove invaluable. Remarkably, whereas up-regulation of the gene coding for postmeiotic segregation increased 2-like 1 (*PMS2L1*) was associated with an improved outcome in DEX-treated cases, its up-regulation following THAL exposure was associated with a pronounced increased risk of early relapse. Although the majority of genes exhibiting similar changes in the direction of expression following treatment with the two drugs showed a similar association with

outcome, there were many exceptions. For example, whereas the gene for phosphoenolpyruvate carboxykinase 2 (mitochondrial; *PCK2*) and GTP-binding protein (*GTP*) tended to be down-regulated by both drugs, this down-regulation was only associated with a better survival in the case of THAL. Conversely, genes changing in the same direction with both drugs could have dramatically different influences on outcome. For example, whereas expression of the gene coding for activating transcription factor 5 (*ATF5*) tended to be down-regulated by both DEX and THAL, the influence on outcome of that change was 5-fold greater for THAL than DEX.

Although many of the gene expression changes linked to outcome did not influence risk by >5-fold, two genes were exceptional. Up-regulation by THAL of the gene coding for seryl-tRNA synthetase (*SARS*) was associated with a HR of 12.40. Surprisingly, up-regulation of the same gene by DEX was associated with a HR of 0.59 and thus a survival benefit. Although the gene for oxidative-stress responsive 1 (*OXSRI*) tended to be up-regulated by both DEX and THAL, the HR associated with up-regulation of the gene by THAL was 3.14, and the HR for its up-regulation by DEX was a remarkable 12.37. It is currently unclear why such disparate effects of the drugs exist, which imply different mechanisms of action.

The dramatic increase in the hazard associated with drug-induced activation of *OXSRI* warrants a discussion of its biological function(s). *OXSRI* is a serine/threonine kinase belonging to the STE20/germinal center kinase subfamily. It binds to and phosphorylates PAK1 and interacts with chloride channel proteins SLC12A6 isoform 2, SLC12A1, and SLC12A2 (NKCC1), but not with SLC12A4 and SLC12A7, through interaction with the R/K-F-X-V/1 motif, possibly establishing sensor/signaling modules that initiate the cellular response to environmental stress. Anselmo and colleagues (26) recently reported that *OXSRI* interacts with lysine-deficient kinase 1 (WNK1) through conserved COOH-terminal motifs. They also showed that *OXSRI* was phosphorylated in a WNK1-dependent manner and that depletion of WNK1 from HeLa cells with small interfering RNA reduced *OXSRI* kinase activity. Depletion of either WNK1 or *OXSRI* also reduced NKCC1 activity, suggesting that WNK1 and *OSR1* are required for NKCC1 function.

In examining the protein kinase components of mitogen-activated protein (MAP) kinase (MAPK) cascades that regulate the *c-Jun* NH₂-terminal kinase (JNK), Chen and colleagues (27) found that Fray, a putative *Drosophila* MAP4K, provided a major contribution to JNK activation by sorbitol. To explore the possible link to JNK in mammalian cells, they isolated *OXSRI* as a human Fray homologue widely expressed in mammalian tissues and cell lines (23). A two-hybrid screen identified p21/CDC42/RAC1-activated kinase 1 (PAK1) as an *OXSRI* target protein (23). PAK1 interacts tightly with GTP-bound but not GDP-bound CDC42 and RAC1. Parrini and colleagues (28) showed that PAK1 formed homodimers *in vivo* and that dimerization was regulated by the intracellular level of GTP-CDC42 or GTP-RAC1. Binding of GTP-bound CDC42 or RAC1 to the autoregulatory region released monomers from the autoinhibited dimer, which enabled phosphorylation of Thr⁴²³ and allowed the kinase domain to adopt an active structure. PAK1 is involved in the dissolution of stress fibers and the reorganization of focal complexes and is recruited to focal adhesions upon activation. *OXSRI* phosphorylates PAK1 at

Thr⁸⁴ within the NH₂-terminal regulatory domain, and replacement of Thr⁸⁴ with Ala reduces the activation of PAK1 by an active form of CDC42, suggesting that phosphorylation by OXSR1 modulates the G protein sensitivity of PAK1 (23). Chen and colleagues concluded that phosphorylation of PAK1 by OXSR1 desensitized PAK1 to activation by RAC1 and CDC42. The authors suggested that this might slow the disassembly of the cytoskeleton under conditions of osmotic stress. We hypothesize that OXSR1 hyperactivation following THAL, or more importantly DEX, exposure may hyperphosphorylate PAK1, rendering it insensitive to CDC42 and RAC1. Thus, it seems that hyperactivation of OXSR1 in myeloma PCs using chemotherapy may be associated with poor outcome through OXSR1's ability to regulate cytoskeletal dynamics. Along these lines, it is interesting to note that up-regulation of RAC1 by both THAL and DEX was associated with improved survival.

ROBO1, 1 of the 51 overexpressed genes in high-risk disease (15), is a receptor for SLIT ligands and acts as a chemorepellent in axon guidance and neuronal migration and as an inhibitor in leukocyte chemotaxis; it also plays a role in tumor angiogenesis. Recombinant Slit2 protein attracts endothelial cells and promotes tube formation in a Robo1-dependent and phosphatidylinositol kinase-dependent manner, and neutralization of Robo1 reduces microvessel density and tumor mass of human malignant melanoma A375 cells *in vivo* (29). Interestingly, in the current study, we found that the gene coding for the SLIT-ROBO Rho GTPase activating protein 2 (*SRGAP2*) tended to be up-regulated by both DEX and THAL, and when up-regulated, was associated with a poorer outcome. Wong and colleagues (30) have proposed a working model in which SLIT-ROBO signaling increases *SRGAP2* activity, which in turn, inactivates CDC42 in its GDP-bound form and thus would be unable to activate PAK1. Taken together, these data suggest a possible important role of OXSR1/PAK1/CDC42 and RAC1 dynamics in controlling the sensitivity of MM cells to chemotherapy.

Global gene expression profiling of purified tumor cells and whole-biopsy samples from normal healthy donors and patients with MM has revealed that although *ROBO* expression is low to undetectable in normal PCs, it is expressed in a majority of myeloma PCs and almost all myeloma cell lines (5, 13). Both normal and malignant MM PCs and their microenvironment express *SLIT2* (5, 13). Interestingly, given the association of ROBO-SLIT signaling with angiogenesis and THAL's antiangiogenic properties (31), syndecan-1, which is expressed at high levels by MM cells and is released by the activity of heparanase into the extracellular matrix (32, 33), where it has been associated with increased angiogenesis (19, 34) and poor survival (33, 35), regulates myotube guidance induced by ROBO-SLIT signaling (36). A question that emerges from these observations is whether drug-induced activation of *SRGAP2* negatively influences survival only in patients whose tumor expresses ROBO1 and SLIT2.

We recently showed that the classic and alternative nuclear factor- κ B signaling pathways were activated in MM through diverse genetic mechanisms, including hyperactivation of NIK (37). Neumann and colleagues (38) showed that the PAK1 autoregulatory domain was required for interaction with NIK in *Helicobacter pylori*-induced nuclear factor- κ B activation, and Frost and colleagues (39) showed that sti-

mulation of nuclear factor- κ B activity by multiple signaling pathways required PAK1. Receptor expressed in lymphoid tissues (RELT) is a recently identified tumor necrosis factor receptor superfamily member that is selectively expressed in hematopoietic tissues and activates nuclear factor- κ B (40). Recently, Cusick and colleagues (41) identified RELL1 and RELL2 as homologues of RELT and showed that these three proteins formed a complex in the plasma membrane and that OXSR1 interacted with the complex and phosphorylated all three members. As pointed out above, OXSR1 interacts with cation chloride cotransporters through interaction with the R/K-F-X-V/1 motif, a motif that is also contained in RELL1, RELL2, and REL. Thus, it is possible that hyperactivation of OXSR1 may negatively affect survival by influencing nuclear factor- κ B signaling, perhaps through regulation of RELT, RELL1, and/or RELL2.

We found that the gene coding for the c-mer proto-oncogene tyrosine kinase (*MERTK*) receptor tyrosine kinase, not normally expressed in normal B and T lymphocytes but expressed in numerous neoplastic B-cell and T-cell lines (42), tended to be up-regulated by both drugs, and its up-regulation was associated with a poor outcome. Similarly, the gene coding for the MAPK kinase 1 (*MAP2K1*) gene also tended to be up-regulated by both drugs, and such up-regulation was associated with an inferior survival. These data, combined with the fact that constitutive activation of *MAP2K1* results in cellular transformation, suggest that this kinase represents a target for pharmacologic intervention. Sebolt-Leopold and colleagues (43) reported the discovery of a highly potent and selective inhibitor of *MAP2K1*, which they called PD184352. PD184352 is orally active, and tumor growth was inhibited as much as 80% in mice with colon carcinomas of both mouse and human origin after treatment with this inhibitor. Efficacy was achieved with a wide range of doses (with an IC₅₀ of 17 nmol) with no signs of toxicity, and correlated with a reduction in levels of MAPK in excised tumors. Thus, PD184352 may prove to be an effective adjuvant to DEX-containing and THAL-containing therapies, especially in tumors that over-express or activate *MAP2K1* following short-term exposure to DEX or THAL.

In conclusion, we have used gene expression profiling of purified tumor cells prior to and following short-term drug exposure to identify genes whose drug treatment-altered expression could be linked to the eventual outcome of patients. This kind of approach might be applied to other drugs and other cancers to better identify disease entities for which a drug will have maximal benefit and for which a given drug whose administration will have limited or possibly even detrimental effects should be withheld. These data suggest that gene expression profiling of tumor cells following a test dose might prove useful in the development and administration of tailored combination chemotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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