Serum Vascular Endothelial Growth Factor and Fibronectin Predict Clinical Response to High-Dose Interleukin-2 Therapy

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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

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ABSTRACT

Purpose

High-dose interleukin-2 (IL-2) induces durable therapeutic responses in a small subset of patients with metastatic melanoma and renal cell carcinoma, but simple pretreatment predictors of response have not been identified.

Patients and Methods

To identify predictive biomarkers of clinical response, sera from patients treated with high-dose IL-2 were collected for analysis using a customized, multiplex antibody-targeted protein array platform that surveyed expression of soluble factors associated with tumor immunobiology. Soluble factors associated with clinical responses were analyzed using a multivariate permutation test, and survival outcomes were determined using Kaplan-Meier and log-rank tests.

Results

A training set from 10 patients identified 68 potentially relevant soluble factors that were then tested in an independent validation set of 49 patients. Class comparison revealed a cluster of 11 biomarkers that were associated with therapeutic outcome. Vascular endothelial growth factor (VEGF) and fibronectin were identified as independent predictors of response. In particular, high levels of these proteins were correlated with lack of clinical response and decreased overall survival.

Conclusion

Serum VEGF and fibronectin are easily measured pretreatment biomarkers that could serve to exclude patients unlikely to respond to IL-2 therapy.

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INTRODUCTION

Recombinant interleukin-2 (IL-2) is associated with durable therapeutic activity in a small but consistent cohort of patients with metastatic melanoma and renal cell carcinoma.¹⁻⁶ Systemic administration of IL-2 is also associated with significant toxicity, largely related to vascular leak syndrome, which limits the use of this therapy in many patients. Thus pretreatment parameters that could better predict the likelihood of therapeutic response or toxicity to IL-2 therapy is a high priority and could help improve the risk/benefit ratio associated with high-dose IL-2 administration.^{4,7}

Transcriptional profiling of tumors during IL-2 administration identified changes in genes related to monocyte and lymphoid cell migration, activation, and proliferation.⁷ These studies suggested that the maximum alteration in gene transcription occurred early after initiation of IL-2

treatment and resulted in significant shifts in the levels of cytokines and chemokines within the tumor microenvironment. The impact of transcriptional changes on circulating protein levels, however, has not been previously evaluated. Thus, in this report, we analyzed sera from patients treated with high-dose IL-2 to see whether we could detect changes in specific serum proteins before or after treatment. To identify potential biomarkers of clinical response, we used a customized high-sensitivity, high-throughput, antibody-targeted proteomics approach.⁸ This strategy was selected because of the disproportional concentrations of IL-2-induced cytokines typically observed during therapy compared with resident serum.^{9,10}

Using a gene microarray profiling approach of the tumor microenvironment in patients treated with IL-2, treatment was associated with differential expression of numerous proinflammatory cytokines and chemokines.⁷ This suggested

that IL-2 induced a wide range of shifts in protein expression, although this has not been previously evaluated. In this report, we used a multiplex antibody-targeted proteomics platform to analyze pre- and post-treatment serum factors in a prospective cohort of patients undergoing treatment with a standardized high-dose bolus IL-2 treatment regimen. Although we detected a large number of proteins shifts related to IL-2 administration (Appendix Table A1, online only), the most significant finding was that high pretreatment **vascular endothelial growth factor (VEGF)** and fibronectin levels were highly predictive of a lack of clinical response to IL-2 and were associated with decreased overall survival (OS). The methods to measure serum VEGF and fibronectin levels are simple and could thus impact the selection of appropriate patients for IL-2 therapy.

PATIENTS AND METHODS

Patients and Treatment

Sixty consecutive patients with metastatic melanoma or renal cell carcinoma eligible for high-dose recombinant human IL-2 therapy were enrolled. The protocol was approved by the institutional review board, and all patients provided written informed consent. Recombinant IL-2 (600,000 U/kg; Proleukin, Novartis, Morristown, NJ) was administered by 15-minute bolus infusion every 8 hours to a maximum of 15 total doses or nonresolving grade 4 toxicity. Each course consisted of two cycles of therapy spaced at 3-week intervals. Clinical response status was determined according to Response Evaluation Criteria in Solid Tumors. Subjects were divided into a training set (10 patients) and a **validation** set (49 patients), as discussed in Results. There were no significant differences in demographic information between training and validation sets.

Fig 1. Unsupervised cluster analysis of serum proteins from interleukin-2 (IL-2)-treated patients. Dendrogram shows 59 serum samples (columns) and 65 soluble serum factors (rows). Serum samples obtained before IL-2 treatment are depicted as open circles, and serum samples obtained after IL-2 treatment are shown as closed circles. Patients with clinical responses (R) are shown in green, and nonresponders (NR) are shown in red.

Sample Collection and Protein Platform

All serum samples were prospectively collected, processed, and stored in an identical fashion. Sixty milliliters of peripheral blood was collected in Vacutainer plus (BD, Franklin Lakes, NJ) plastic serum tubes before (pre) and 3 hours after (post) the fourth dose of IL-2. To minimize protein degradation, sera were immediately processed, stored upright for 10 minutes, and centrifuged at room temperature in a horizontal rotor at 3,500 rpm for 8 minutes. Serum aliquots were snap-frozen in dry ice and stored at -80°C until use. Thawed aliquots were used only once.

Serum protein levels were tested with a customized antibody-based platform (Pierce SearchLight Proteome Arrays, Boston, MA) consisting of a multiplex array measuring 16 proteins per well in standard 96-well plates where different monoclonal antibodies were spotted.⁸ A sandwich enzyme-linked immunosorbent assay technique was used to generate signals via chemiluminescent substrate. Light corresponding to each spot in the array was captured by imaging entire plates with a commercially available cooled charge coupled device camera. Data were reduced using image analysis software (Pierce SearchLight Proteome Arrays, Boston, MA) that calculates exact values (pg/mL) based on standard curves. For the training set, a protein array platform containing 111 human soluble factors was used; on identification of those likely to be informative (see Results), the repertoire was restricted to 68 soluble factors for the subsequent analyses.

Statistical Analysis

Data were compared separately for training and validation sets to obtain unbiased estimates of true effects. Paired or unpaired two-tailed *t* tests were used to determine the level of significance in serum concentration changes for the soluble factors before and after treatment (paired) or to discriminate clinically responding from nonresponding subjects (unpaired). The levels of IL-2 were included in the analysis as an internal control. In the training set, differences were considered of potential significance at an exploratory cutoff P_2 value of ≤ 0.1 without adjustment for the number of tests performed to include all potentially informative cytokine information.¹¹

Two sets of cytokines were selected based on the training set for validation: those modulated by IL-2 administration (ie, proteins with or approaching significant differences between pre- and post-treatment levels) and those proteins that exhibited significant differences between clinically responding and nonresponding patients, regardless of whether the changes were seen in pretreatment or post-treatment samples. This preliminary analysis resulted in the selection of 48 soluble factors, and this number was used for Bonferroni's adjustment of *P* values in the validation set. In addition, another 20 soluble factors deemed to be of potential interest based on various criteria (see Results) were added as secondary exploratory parameters totaling the number of factors evaluated in the validation set to 68. This number was used for Bonferroni's adjustment of *P* values related to these secondary factors.

Unsupervised cluster analysis based on 65 proteins of 68 that passed the filter criteria (80% cytokines presence across all experiments and at least one experiment with ratio fold change > 3) clearly separated pre- from post-treatment samples, with only two pretreatment samples clustering together with the post-treatment samples (one sample from a responding and the other from a nonresponding patient) and none of the post-treatment samples clustered with pretreatment samples (Fisher's exact test P_2 value $< .001$; Fig 1). There was not, however, a rigorous separation between samples from clinically responding and nonresponding patients. The mean-centered ratios of protein expression are depicted by a log-transformed pseudocolor scale.

General criteria of significance were followed for the validation and combined sets by accepting an exploratory P_2 value less than .05 as significant without Bonferroni's correction for the number of tests performed. Values remaining significant after Bonferroni's correction were considered robust candidate biomarkers. Further assessment of significance for the combined cluster of 10 potential predictors of response identified by the validation set was based on a multivariate permutation test, as previously described.¹² This test determines whether the number of differentially expressed proteins is higher than expected because of chance by randomly shuffling the phenotype labels (eg, responder *v* nonresponder) and recomputing the two-sample *t* statistics for each protein. This analysis was repeated 10,000 times, and the proportions of the random replications that resulted in as many significant

proteins as seen in the actual data were reported as the significance levels for the number of proteins.^{13,14} Significance of class prediction dependent on unsupervised analysis was based on a Fisher's exact test or χ^2 test, as appropriate. Relatedness in cytokine expression patterns before and after IL-2 administration was displayed by Eisen's hierarchical clustering applied to proteomics as previously described.⁸ Data are presented according to the central method for normalization.¹⁵ Differences in selected cytokine expression between samples from clinically responding and nonresponding subjects are shown as box and whisker graphs.

OS was calculated by the method of Kaplan-Meier. OS was calculated from the first date of IL-2 treatment to date of death or last known date alive.

Table 1. Patient Demographics and Clinical Characteristics

Characteristic	No. of Patients	%
Age, years		
Median		55
Range		22-74
Sex		
Male	38	64
Female	22	37
Melanoma, n = 48		
Stage		
M1a	10	21
M1b	3	6
M1c	35	73
Response		
ORR	18	38
CR	5	10
PR	13	27
PD	30	63
RCC, n = 11		
Stage M1	11	100
Response		
ORR	1	9
CR	0	0
PR	1	9
PD	10	91
Training set, n = 10		
Melanoma, response		
ORR	5	50
CR	2	20
PR	3	30
PD	4	40
RCC, response		
ORR		
CR		
PR		
PD	1	10
Test set, n = 49		
Melanoma, response		
ORR	13	27
CR	3	6
PR	9	18
PD	27	55
RCC, response		
ORR	1	10
CR		
PR	1	10
PD	9	90

Abbreviations: ORR, objective response rate; CR, complete response; PR, partial response; PD, progressive disease; RCC, renal cell carcinoma.

Data were collected from at least one of the following sources: Columbia-Presbyterian Hospital inpatient and outpatient medical records, Social Security Death Index, primary physician's office, and patient or family contact. Permission to contact patients and their families to obtain follow-up information was included in our original consent form.

RESULTS

Clinical Characteristics and IL-2 Responses

Sixty patients were enrolled onto this study (Table 1). One patient did not complete staging scans and could not be included in response determination, leaving 59 patients for complete analysis. The median age of all patients was 55 years (range, 22 to 74 years). All patients completed one cycle of IL-2, and 57 (96.6%) of 60 patients completed one full course of treatment (Appendix Table A1). Nineteen patients exhibited an objective response to IL-2 therapy; 18 of 48 patients with melanoma achieved response (five complete and 13 partial responses), and one of 11 patients with renal cell carcinoma achieved a response (partial response). There were no serious adverse events or treatment-related mortality.

Before proteomic analysis, subjects were divided into a training ($n = 10$ patients) and a validation set ($n = 49$ patients). The training set served three purposes: (1) to define a panel of candidate factors to be validated in a larger and independent population (validation set); (2) to define the basis of a sound statistical strategy a priori to avoid potential data dredging based on multiple statistical approaches and defining a strategy for Bonferroni's adjustment of statistical values for each factor¹¹; and (3) to identify potentially informative biomarkers predictive of clinical effectiveness that could be validated in the subse-

quent larger population. The training set population was biased by selecting samples from five responding and five nonresponding patients to increase the exploratory power of the set.

Training Set

For training, only samples from IL-2 course 1, cycle 1 (C1C1) were used to avoid possible alterations of baseline values owing to previous IL-2 therapy. The training set included 111 proteins, largely composed of cytokines, chemokines, and proinflammatory and angiogenesis-related factors. Forty-eight of the 111 soluble factors tested by the array platform increased significantly (P_2 value $< .1$) after IL-2 administration (Appendix Table A2, online only). Class comparison confirmed previous results obtained in a smaller series and were selected for analysis in the validation set.⁸ In addition, VEGF, the expression of which was not significantly modulated by IL-2, was included for the validation study based on an exploratory P_2 value (< 0.1) trending toward significance when pretreatment values were compared between responding and nonresponding patients. Thus the number of "trained" factors selected for further validation was 49. On the basis of our previous analysis⁸ and other literature-based criteria, a total of 68 factors of potential interest were included in the validation set.

Pretreatment samples segregated into three major clusters, of which two (Fig 1, **a** and **b**) were disproportionally enriched with samples from responding patients (four of seven and 13 of 29 samples, respectively; expected proportion was two of seven for **a** and nine of 29 for **b**) compared with the remaining cluster (Fig 1, **c**, two of 19; expected proportion = seven of 14, $\chi^2 P = .01$; right panel). The post-treatment samples clustered into two separate groups, of which

Table 2. Pretreatment Differences Between Responding and Nonresponding Patients (top) and Pretreatment Combined Test Sets (bottom)

Factor	Nonresponder* (n = 27)		Responder† (n = 9)		t test P_2	BC P_2
	Average (ng/mL)	SEM	Average (ng/mL)	SEM		
Pretreatment C1C1						
IL-2	8.0	2.3	3.1	0.9	.0538	NS
CRP	7,478‡	2,138.6	834‡	452.4	.0047	NS
Fibronectin	8,680‡	1,415.3	2,951‡	670.0	.0007	.0472§
IFN α	8.9	3.4	1.0	0.1	.0270	NS
MMP8	920.7	3,21.5	231.5	82.9	.0433	NS
MPIF1	435.3	43.2	226.0	47.3	.0024	NS
PAI1-total	23,260.6	3,844.0	12,265.6	1,354.5	.0099	NS
P-selectin	44,613.3	3,713.4	30,631.8	2,690.7	.0035	NS
TIMP1	103,125.0	12,535.8	59,712.5	10,536.0	.0100	NS
TNF-RI	1399.4	175.0	935.6	79.1	.0187	NS
VEGF	129.5	23.9	34.1	6.6	.0005	.0266
Pretreatment ALL C1C1						
IL2	7.1	2.0	6.1	3.3	.7893	38.6773
CRP	7,848‡	2,006.2	1,287‡	602.2	.0032	.1547
Fibronectin	9,190‡	1,699.3	2,917‡	443.0	.0010	.0481
TIMP1	106,090.3	11,830.9	61,446.3	9,663.2	.0047	.2313
VEGF	133.5	20.8	43.4	7.4	.0002	.0101

Abbreviations: BC, Bonferroni's correction; C1C1, course 1 and cycle 1; IL-2, interleukin-2; NS, not significant; CRP, C-reactive protein; IFN α , interferon alfa; MMP, matrix metalloproteinase 8; MPIF1, myeloid progenitor inhibitor factor-1; PAI1, plasminogen activator inhibitor-1; TIMP1, tissue inhibitor of metalloproteinase 1; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

*Nonresponders, $n = 27$ for pretreatment C1C1; $n = 32$ for pretreatment combined test sets (ALL C1C1).

†Responders, $n = 9$ for pretreatment C1C1; $n = 14$ for pretreatment combined test sets (ALL C1C1).

‡Bonferroni's correction.

§Fibronectin correction was based on 69 permutations.

Fig 2. Supervised cluster analysis of serum proteins from interleukin-2 (IL-2)-treated patients before treatment. (A) Ten genes with significant variation in expression level among responders (R, green bars) and nonresponders (NR, red bars) from the validation set. (B) Cluster analysis of combined training and validation sets for vascular endothelial growth factor (VEGF) and fibronectin. MMP8, matrix metalloproteinase 8; PAI1, plasminogen activator inhibitor-1; TIMP1, tissue inhibitor of metalloproteinase 1; MPIF1, myeloid progenitor inhibitor factor-1; CRP, C-reactive protein; TNF-R1, tumor necrosis factor receptor 1; IFN α , interferon alpha.

one (cluster *d*) was disproportionately enriched with treatment samples from responding patients (16 of 37; expected proportion = 12 of 37), whereas the other was enriched with those of nonresponding patients (four of 22 in cluster *e*, expected proportion = eight of 22; $\chi^2 P = .05$). Thus unsupervised analysis suggested a baseline biologic difference in patients likely to respond to IL-2 therapy. Differences among pretreatment samples associated with clinical response seemed to reside in the overexpression of serum proteins from nonresponding patients rather than changes in protein levels after exposure to IL-2 (Fig 1, dashed blue bar).

Validation Set

To verify predictive markers identified in the training set, a larger validation set using sera from 49 additional patients was tested. In this cohort, there were 14 patients who achieved response (responders) and 35 patients who did not achieve response to treatment (nonresponders). As shown in Table 2 (top), 10 soluble factors were differentially expressed between responding and nonresponding patients with P_2 values less than .05. The density of factors between responding and nonresponding patients was highly significant based on multivariate permutation test ($P < .001$). The proportional display of protein expression according to experimental class is shown in Figure 2A. The data suggest that although samples from responding subjects tended to have lower protein expression, sera from nonresponding patients could be separated into two categories: samples lacking expression of these factors, similar to those of responding patients, and samples that cocoordinately expressed most of these factors.

After Bonferroni's correction for the number of tests, only fibronectin and VEGF levels maintained significance as individual

predictors of response to IL-2 therapy (Fig 2B). Self-organizing reclustering of pretreatment samples from the validation set based on the proportional expression of these two factors segregated samples from responding patients into a preferential cluster in which six of nine samples belonged to responders, whereas another two independent clusters included two samples from responders and the remaining 21 samples from nonresponders (expected ratios = two of nine and six of 23 respectively, $\chi^2 P < .001$). Both samples from responding patients that clustered with those from responding patients demonstrated low level of VEGF expression. Combining training and validation analyses identified only VEGF as a predictor of response to IL-2 therapy, although fibronectin maintained statistical significance after combining the data sets, suggesting a potential role as an independent predictive biomarker (Table 2, bottom).

Correlation With Clinical Outcome

Serum VEGF and fibronectin levels were independently predictive of IL-2-related clinical responses (Fig 3A and 3B). A receiver operating characteristic curve based on the distribution of VEGF levels is shown in Figure 3C (and Appendix Table A3, online only). The accuracy of discrimination was absolute beyond a cutoff value ≥ 125 pg/mL, with no responding patients in this cohort. Figure 4 shows that patients with VEGF levels more than 125 pg/mL exhibit significantly reduced OS (13 v 23.3 months; $P < .007$; Fig 4A). Similarly, those patients with fibronectin levels greater than 8×10^6 pg/mL showed reduced OS (11 v 23.3 months, $P < .04$; Fig 4B). When both proteins were considered together, there was a significant correlation between reduced survival and elevated VEGF and fibronectin levels (9 v 35.7 months, $P < .01$; Fig 4C).

Fig 3. Serum vascular endothelial growth factor (VEGF) and fibronectin predict clinical response to interleukin-2 (IL-2) therapy. (A) VEGF and fibronectin levels from pretreatment sera are shown for responding (R) and nonresponding (NR) IL-2-treated patients as medians (central line), quartiles (boxes), and ranges (whiskers). (B) Receiver operating characteristic curve analysis for accuracy of VEGF as a biomarker for clinical responsiveness to IL-2 treatment.

DISCUSSION

Serum VEGF and fibronectin were identified as significant predictive biomarkers of IL-2 therapeutic response. Patients with serum VEGF levels greater than 125 pg/mL or fibronectin levels greater than 8×10^6 pg/mL did not have a response to IL-2 therapy, and elevated levels of these proteins were also associated with a significantly worse OS. Fibronectin binds to VEGF and potentiates VEGF signaling, which has been associated with suppression of antigen presentation and T-cell immunity.^{16,17} Thus serum VEGF and fibronectin may serve as easily measurable pretreatment biomarkers that can predict clinical responses to high-dose IL-2 therapy in patients with metastatic melanoma and renal cell carcinoma.

Specifically, we identified serum VEGF and fibronectin as negative predictors of clinical response to IL-2 therapy. The VEGF family plays a critical role in mediating angiogenesis, lymphangiogenesis, and vasculogenesis and has an impact on host innate and adaptive immunity.¹⁸⁻²⁰ The role of excess VEGF on tumor angiogenesis is well documented, and recently, high circulating serum levels of VEGF were associated with poor prognosis in patients with metastatic melanoma and ovarian cancer.^{21,22} VEGF has also been implicated as an important therapeutic target in colorectal, breast, and renal cell carcinoma.²³⁻²⁴ Recently, VEGF has been shown to block maturation of dendritic cells and inhibit effective priming of T-cell responses.^{25,26} These data support an important role for VEGF in the progression of cancer and evasion of antitumor immunity.

In addition to VEGF, serum fibronectin was also found to be predictive of IL-2 response and was associated with a decreased OS.

Fig 4. Serum vascular endothelial growth factor (VEGF) and fibronectin levels predict survival after interleukin-2 (IL-2) treatment. (A) Overall survival (OS) by pretreatment serum VEGF level median cutoff of 125 pg/mL ($P < .007$). (B) OS by fibronectin at cutoff of 8×10^6 pg/mL ($P < .04$). (C) OS by both VEGF (125 pg/mL) and fibronectin (8×10^6 pg/mL) concentrations ($P < .01$).

Fibronectin is a key extracellular matrix protein and binds to the $\alpha_5\beta_1$ receptor, resulting in sustained mitogen-activated protein kinase activation, which can enhance VEGF and integrin signaling.²⁷ Stimulation of the β_1 receptor by fibronectin has also been shown to increase VEGF production by tumor cells.²⁸ Fibronectin can bind directly to VEGF, and such complexes have been shown to be secreted by thrombin-activated platelets and to induce an association between VEGF receptor 2 and $\alpha_5\beta_1$ receptors.¹⁷ These data suggest that a close association between fibronectin and VEGF may be

a common physiologic phenomenon and could explain why we detected the segregation of these two factors in our proteomic profile.

To date, the majority of putative predictors of IL-2 response have been post-treatment related variables, such as the height of rebound lymphocytosis, treatment-induced thrombocytopenia, development of autoimmune thyroiditis and vitiligo, and decreases in absolute number and frequency of peripheral Tregs.²⁹⁻³² In renal cell carcinoma, the level of expression of carbonic anhydrase IX (CAIX) in primary tumors was found to be a potentially useful pretreatment predictor of IL-2 response, but prospective validation and the mechanism through which CAIX might predict clinical response to IL-2 remains to be elucidated.^{6,33} Because primary tumors are not always available for analysis and because CAIX expression is not found in melanomas, the potential usefulness of this biomarker is limited.³³

In summary, we have identified high levels of serum VEGF and fibronectin as potential predictors of poor response to high-dose IL-2 treatment and decreased OS in patients with metastatic melanoma and renal cell carcinoma. These data will need to be further validated in larger, prospective clinical trials. The identification of a pretreatment serum marker will help guide important clinical decisions for patients with metastatic melanoma and renal cell carcinoma contemplating treatment with high-dose IL-2.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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

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Glossary Terms

Biomarker: A functional biochemical or molecular indicator of a biologic or disease process that has predictive, diagnostic, and/or prognostic utility.

Interleukin-2 (IL-2): A cytokine that stimulates proliferation of activated T cells and, at high doses, is used as antitumor therapy in metastatic renal cell carcinoma.

Proteomic studies: With the proteome-indicating proteins expressed by a genome of an individual, protein informatics or proteomics is a growing field that uses technologies like electrophoresis and mass spectrometry to identify and quantify cellular proteins that may be expressed, even in small amounts, and to determine their role in the physiologic and pathophysiologic conditions of an individual.

Vascular endothelial growth factor (VEGF): VEGF is a cytokine that mediates numerous functions of endothelial cells including proliferation, migration, invasion, survival, and permeability. VEGF is also known as vascular permeability factor. VEGF naturally occurs as a glycoprotein and is critical

for angiogenesis. Many tumors overexpress VEGF, which correlates to poor prognosis. VEGF-A, -B, -C, -D, and -E are members of the larger family of VEGF-related proteins.

Validation: The process that tests the performance of a previously defined classifier or prognostic model on a new set of patients. For example, a gene expression signature classifier developed using data from one set of patients might be validated on another, independent set of patients.

Clustering: Organization of data consisting of many variables (multivariate data) into classes with similar patterns. Hierarchical clustering creates a dendrogram based on pairwise similarities in gene expression within a set of samples. Samples within a cluster are more similar to one another than to samples outside the cluster. The vertical length of branches in the tree represents the extent of similarity between the samples. Thus, shorter the branch length, the fewer the differences between the samples.

Cytokines: Cell communication molecules that are secreted in response to external stimuli.