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Novel approach to gene expression profiling in Sezary Syndrome

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Abstract

Background—Microarray hybridization studies in Sezary Syndrome (SS) have compared T lymphocytes from cutaneous T-cell lymphoma patients to those of normal controls; a major limitation of this design is that significant inherent genetic variability of lymphocyte populations between individuals may produce differences in gene expression unrelated to disease state.

Objective—The objective of this study was to minimize the heterogeneity of information derived from whole-genome expression analysis and to identify specific genetic differences between highly purified malignant and nonmalignant (control) T cells from the same SS patient.

Patients/Methods—Peripheral blood mononuclear cells were obtained from a SS patient, stained with anti-T cell receptor-V β (TCR-V β) antibodies, and sorted by multiparameter flow cytometry. Malignant cells expressed the dominant TCR-V β ; control T cells lacked the dominant TCR-V β but were otherwise phenotypically identical (CD3+ CD4+ CD45RO+). These cell populations were compared using the Illumina Inc. Sentrix Human-6 expression BeadChip system.

Results—Transcriptome analysis using the J5 test, which was selected for data analysis based on an efficiency analysis of competing statistical methods, showed differential expression of 44 genes between the malignant and nonmalignant cell subsets. Promyelocytic leukemia zinc finger protein (ZBTB16) was the most profoundly upregulated gene in the malignant cell population, while interferon regulatory factor 3 (IRF3) and interferon-induced protein 35 (IFI35), which are important elements of the cellular response to viral infection, were significantly downregulated.

Conclusions—The results of this study suggest the feasibility of this novel comparative approach to genomic profiling in SS. Using this approach, we identified several differentially expressed genes and pathways not previously described in SS. While these findings require validation in larger studies, they may be important in SS pathogenesis.

Keywords

Cutaneous T-Cell Lymphoma; Sezary Syndrome; Gene Expression Microarray Analysis; genomics; T-Cell Receptor beta-Chain

What's already known about this topic?

- A number of microarray hybridization studies have compared Sezary cells to peripheral blood mononuclear cells from individuals without CTCL, and have identified differentially expressed genes.

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Conflict of interest disclosures: None

What does this study add?

- This study introduces a novel approach to genomic profiling in Sezary syndrome, comparing leukemic to non-tumour CD4+ cells from the same Sezary syndrome patient by genomic hybridization. This new method minimizes the confounding effects of genetic variability unrelated to tumour status, which has likely contributed to inconsistent results in previous Sezary syndrome microarray studies.

Introduction

The pathogenesis of Sezary syndrome (SS), a rare leukemic form of cutaneous T cell lymphoma (CTCL), is largely unknown, and the discovery of disease-specific biomarkers is important for the advancement of diagnostic and therapeutic paradigms for the disorder. Several studies have compared CTCL gene expression profiles to those of various controls, yielding highly inconsistent results¹⁻⁴. We sought to minimize this variation through the novel approach of comparing malignant and nonmalignant CD4+ T cells in a patient with SS, with the patient's own nonmalignant CD4+ cells serving as an internal control.

Methods

Blood was drawn from a patient with histologically confirmed Sezary Syndrome (SS), seen at the Cutaneous Lymphoma Clinic at the University of Pittsburgh, in accordance with an Institutional Review Board-approved protocol.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare Life Sciences, Piscataway, NJ) density centrifugation and stained with eight TCR-V β cocktails (Beckman Coulter, Fullerton, CA), each containing three TCR-V β antibodies conjugated to FITC, PE, or both FITC and PE (representing a total of 24 V β families). Stained cells were incubated for 20 minutes on ice, washed, resuspended in fixative, and analyzed by multiparameter flow cytometry. In this patient, malignant CD3+/CD4+ cells were identified as possessing an expansion of V β 5.1. For malignant cell isolation, PBMCs were stained with anti-CD3-ECD, anti-CD4-PC7, anti-CD45RO-APC, and anti-V β 5.1-PE antibodies (Beckman Coulter). CD3+ CD4+ CD45RO+ T cells were sorted into V β 5.1+ and V β 5.1- subsets using a Dako-Cytomation MoFlo high speed cell sorter. A Giemsa stained blood smear confirmed Sezary cell morphology.

Total RNA was isolated using the Ambion RNAqueous®-Micro RNA isolation kit (Applied Biosystems, Austin, TX). RNA Amplification and conjugation were performed using the BD Biosciences Super SMART RNA amplification and labeling system (Clontech Laboratories, Inc. Mountain View, CA), per manufacturer instructions. Biotinylated cRNA was hybridized to an Illumina Inc. Sentrix Human-6 Expression BeadChip (Illumina, Inc., San Diego, CA). The BeadChip was processed and scanned per manufacturer instructions.

Based on an efficiency analysis, performed to determine the analysis method and normalization/feature selection combination leading to the most internally consistent gene set⁵, data was normalized with log2 and z-transformation, and the J5 test was used to identify differentially expressed genes⁶.

A pathway level impact analysis was implemented, which was designed to provide both statistical and biological significance in indicating the pathways affected by observed gene expression changes⁷. The results are summarized as impact scores and p-values.

To compare relative frequencies of gene ontology categories represented in the gene list, we used the Webgestalt module of the Gene Ontology Tree Machine⁸.

Results and Discussion

Several microarray studies have compared the PBMCs of CTCL patients to those of normal controls¹⁻⁴. Genomic differences were demonstrated, but with widely inconsistent results. Major limitations in comparing SS PBMCs to normal controls are (1) significant inherent genetic variability of lymphocyte populations between individuals, which could produce differences in gene expression unrelated to disease state, and (2) heterogeneity of SS patients' circulating T lymphocytes, which do not consist exclusively of the malignant cell population.

To minimize the heterogeneity of information derived from SS patients' PBMCs and improve the purity of controls, we compared malignant to control T cells from the same SS patient that were phenotypically identical except for TCR- $\nu\beta$ expression. The majority of malignant cells from CTCL/SS patients possess a mature memory phenotype and express markers (CD3+CD4+CD45RO+) which can be used to non-selectively separate cells containing the malignant population⁹. Expression of dominant TCR- $\nu\beta$ was then used to distinguish between the malignant (expressing the dominant TCR- $\nu\beta$) and nonmalignant (all other TCR- $\nu\beta$ families) mature memory CD4+ populations. The patient's circulating CD4+ T cell population showed expansion of the TCR- $\nu\beta$ 5.1 family, with the $\nu\beta$ 5.1+ subset constituting 36% of the patient's CD3+CD4+CD45RO+ cells. Blood smear of isolated $\nu\beta$ 5.1+ cells demonstrated cells with convoluted nuclei, consistent with Sezary cell morphology. Thus, highly purified and selected cell populations were identified and isolated from an SS patient's blood based on the central memory phenotype of CD4+ cells and TCR- $\nu\beta$ identity.

To further improve assay specificity and reduce the signal/noise ratio, we used BeadChip microarray technology (Illumina Inc.), which had not previously been used in CTCL. The J5 test⁶, chosen to analyze the microarray data based on an efficiency analysis analysis⁵, demonstrated 44 genes significantly over- or under-expressed in malignant $\nu\beta$ 5.1+ circulating CD4+ T cells relative to nonmalignant $\nu\beta$ 5.1- circulating CD4+ T cells (Table 1). Importantly, many of these genes are known to be associated with carcinogenesis and immunologic dysfunction.

Previous gene array studies have identified a number of genes, including T-Plastin, STAT4, and integrin β 1, that may be differentially expressed in CTCL¹⁻⁴. Despite some overlap among these studies' findings, no single gene differentially expressed in all CTCL tumor studies has been identified. It may be the case, for instance, that elucidated genetic abnormalities represent the "downstream" dysregulation of various genes, while the inciting "upstream" events may still be unknown. At the very least, the published data suggest that CTCL pathogenesis and maintenance are multifactorial and complex. In the context of this intricate milieu, the analysis of gene classes is of utmost importance, providing important insights into a multifaceted disease process.

To better classify and contextualize our differentially expressed genes, we next performed gene ontology analysis⁸. This process mines input data for statistically significant over- or under-expression of gene classes, providing insight into domains of cellular function that may be of great import in a particular disease. Our analysis showed two affected ontological classes, regulation of gene expression and response to virus (Fig. 1), indicating that perturbations in these areas of cellular operation may be important to CTCL pathogenesis.

Using our differentially expressed gene list, we also performed a pathway analysis to evaluate clusters of functionally related genes (Table 2). We found two pathways to be significantly affected ($p < 0.05$) in malignant cells, leukocyte transendothelial migration and cellular adhesion pathways, both well known to be important in cancer pathogenesis.

Confirmatory studies are necessary to determine the role of these differentially expressed genes in CTCL pathogenesis. Nevertheless, this pilot study suggests the feasibility of this novel approach to genomic profiling in SS, in which malignant T cells are compared to control T cells from the same SS patient. This study will hopefully stimulate further work applying this experimental design to genomic profiling in SS.

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Fig 1.

Gene ontology category analysis. Two affected ontological classes are shown in black. Overexpressed (gray) and underexpressed (white) genes belonging to each class are shown.

Overexpressed (positive J5) and underexpressed (negative J5) genes in malignant circulating CD4+ T cells relative to non-malignant circulating CD4+ cells. Shown in order of decreasing J5 score

Table 1

Gene symbol	Gene title	J5	Accession
ZBTB16	Zinc finger and BTB domain containing 16 (promyelocytic leukemia zinc finger protein)	15.893	NM_006006.3
CD6	CD6 antigen	15.797	NM_006725.1
OK/SW-cl.56	Beta 5-tubulin	13.379	XM_114617.3
UBE4B	Ubiquitination factor E4B	12.16	NM_006048.2
ZNF331	Zinc finger protein 331	12.132	NM_018555.3
SFXN1	Sideroflexin 1	12.021	NM_022754.3
HNRPU	Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A), transcript variant 2	11.904	NM_004501.2
BIK	BCL2-interacting killer (apoptosis-inducing)	11.903	NM_001197.3
LOC286379	LOC286379	11.321	XM_212297.1
KIAA1030	Immunoglobulin superfamily, member 9B	11.297	XM_290502.1
DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	11.275	NM_016222.2
DKFZP43410714	Hypothetical protein DKFZP43410714	11.188	XM_098247.1
KCNK4	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	11.148	NM_002250.2
LOC351347	LOC351347	11.146	XM_304482.1
RNF19A	Ring finger protein 19A, transcript variant 1	11.077	NM_183419.1
TMEM175	Transmembrane protein 175	10.652	NM_032326.2
PTOV1	Prostate tumor overexpressed gene 1	10.57	NM_017432.2
ITGB1	Integrin, beta 1, transcript variant 1A	10.409	NM_002211.2
LOC157562	Hypothetical protein LOC157562	10.374	XM_098779.1
LOC283745	LOC283745	10.371	XM_211188.1
CNIH4	Cornichon homolog 4 (Drosophila)	10.324	NM_014184.1
BAIAP2L2	BAI1-associated protein 2-like 2	10.184	NM_025045.3
MED27	Mediator complex subunit 27	10.155	NM_004269.2
CHIC2	Cysteine-rich hydrophobic domain 2	10.063	NM_012110.2
CD52	CD52 antigen (CAMPATH-1 antigen)	10.024	NM_001803.1
PEBP1	Phosphatidylethanolamine binding protein 1	10.012	NM_002567.2

Gene symbol	Gene title	J5	Accession
CAPN3	Calpain 3, (p94), transcript variant 5	9.949	NM_173089.1
PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein	9.936	NM_005608.1
SP140	SP140 nuclear body protein	-9.898	NM_007237.2
KIAA1171	KIAA1171 protein	-9.983	XM_370928.2
LOC388299	Similar to coenzyme A diphosphatase	-9.998	XM_370993.1
RPS2	Ribosomal protein S2	-10.302	NM_002952.2
SLC27A3	Solute carrier family 27 (fatty acid transporter), member 3	-10.341	NM_024330.1
LOC388011	Hypothetical gene supported by AK091668	-10.442	XM_373606.2
RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3	-10.593	NM_006105.3
VAMP8	Vesicle-associated membrane protein 8 (endobrevin)	-10.792	NM_003761.2
IFI35	Interferon-induced protein 35	-11.481	NM_005553.2
LOC167454	LOC167454	-11.98	XM_094496.3
ARID1A	AT rich interactive domain 1A (SWI-like)	-12.227	NM_006015
PWWP2	PWWP domain containing 2	-12.412	NM_138499.1
LOC285535	Hypothetical protein LOC285535	-14.14	XM_211930.1
PAG	Phosphoprotein associated with glycosphingolipid-enriched microdomains	-14.344	NM_018440.2
IRF3	Interferon regulatory factor 3	-15.17	NM_001571.1
LOC144571	Hypothetical protein LOC144571	-16.063	XM_096630.1

Table 2
Impacted pathways, shown in order of decreasing impact factor and increasing p-value

Pathway	Impact Factor	p-value	Differentially expressed gene(s) in pathway	Gene symbol (s)
Leukocyte transendothelial migration	3.889	0.020	Integrin, beta 1; Rap guanine nucleotide exchange factor 3	ITGB1; RAPGEF3
Cell adhesion molecules (CAMs)	3.716	0.024	CD6 antigen; Integrin, beta 1	CD6; ITGB1
SNARE interactions in vesicular transport	2.687	0.068	Vesicle-associated membrane protein 8	VAMP8
Pathogenic Escherichia coli infection	2.43	0.088	Integrin, beta 1	ITGB1
Acute promyelocytic leukemia (M3)	2.247	0.106	Zinc finger and BTB domain containing 16 (promyelocytic leukemia zinc finger protein)	ZBTB16 (PLZF)
Ribosome	2.095	0.123	Ribosomal protein S2	RPS2
Long-term potentiation	2.067	0.127	Rap guanine nucleotide exchange factor 3	RAPGEF3