

Gene Expression and Pathway Analysis of Quiescent CD8+ T Cells from Liver Cancer, Liver Sinusoid and Peripheral Blood

Study on toxicogenomics and prevention targeting

Zhaohui Wang[#]

Department of Cell Biology and Genetics
University of North Texas Health Science Center
Fort Worth, TX 76107, U.S.A.
E-mail: vincent_wang@hotmail.com

Hongliang Hu[#]

Renji Hospital
Shanghai Jiaotong University
Shanghai 200001, China
E-mail: hongliang_hu1115@hotmail.com

Jie Zheng*

School of Computer Engineering
Nanyang Technological University
Singapore 639798
E-mail: zhengjie@ntu.edu.sg

Biaoru Li*

Department of Pathology and Biochemistry
School of Medicine, Case Western Reserve University
Cleveland, OH 44106, U.S.A.
E-mail: brli1@juno.com

[#] Co-first authors, *Co-corresponding authors

Abstract—The quiescent status of T cell immunology is associated with the development of tumor in liver cancer. However, the cause of T cell quiescence in the tumor microenvironment is still unclear. Recently, it has been proposed that toxic substances could inhibit T-cell immune response to tumor cells. Since sinusoidal structure is primarily involved in anti-toxic function and toxic retaining mechanism, in this paper we study the role of liver sinusoid in liver tumor by comparing gene expression levels of quiescent genes in CD8+ T cells isolated from three sources: peripheral blood, sinusoidal structure, and tumor tissue. We observed that most quiescent genes are upregulated in CD8+ T cells isolated from sinusoidal structure as compared with those from liver tumor and peripheral blood. The genes and pathways predicted in this project will be candidates for drug targeting.

Keywords- gene expression, pathway analysis, quiescent CD8+ T cells, toxicogenomics, prevention targeting

I. INTRODUCTION

CD8+ T cells are a group of specialized lymphocytes that exert cytotoxicity once activated. As other lymphocytes, CD8+ T cells consist of naïve, effector, and memory phenotypes [1]. CD8+ T cells also display characteristics of immune tolerance in the periphery [2]. It was reported that after experimental immunization, CD8+ T cells displayed quiescent status even with features of tumor-antigen peptide. These cells had no cytotoxicity and low proliferation potential; whereas they could be fully activated when exposed to IL-2 *in vitro* [3]. In our previous work [7], we employed single-cell genomics analysis to elucidate the quiescent status of CD8+ T cells in liver tumors. By studying the quiescent CD8+ T cells obtained from liver tumor infiltrating lymphocytes (TIL), we identified a group of down-regulated genes including T cell receptor (TCR), TNF-

alpha receptor, TRAIL, and Perforin1. Additionally, we have observed a group of up-regulated genes including Tob [4], TGF- β , LKLF [5, 6], SnoA, Ski, Myc, ERF and REST/NRSF complex. The results were further confirmed by quantitative gene expression analysis using real-time PCR [7]. Our observation of actively maintained quiescent CD8+ T cells is consistent with findings from other laboratories using microarray, rtPCR or animal studies [4, 5, 8, 9]. Since ethanol or other toxic factors have been found to inhibit immune responses in tumor environment [8, 10], it was proposed that the quiescent status of CD8+ T cells is actually induced by toxic factors [11].

The liver is a place with enriched lymphocytes under both physiological and pathological conditions [12]. The liver sinusoid is of interest since it receives oxygen from the liver artery while at the same time receiving nutrients/metabolites from the GI tract. It serves as both a molecular sieve and a scavenger, which results in the accumulation of toxic substances in the liver sinusoid [13, 14]. The mechanism that the accumulation of toxic factors in the liver sinusoid affects the immune response of CD8+ T cells, however, remains unknown. In this paper, we attempt to shed light on this question by analyzing CD8+ T cells acquired from two liver cancer patients. The cells come from three sources - peripheral blood, liver sinusoid and liver tumor. We identified 10 genes upregulated in liver sinusoid compared with the other two gene sets. Moreover, using pathway analysis, we discovered 156 nodes from the network profiles of quiescent CD8+ cells. From these nodes, several proteins with high betweenness and low connectivity could be candidates for targeting of toxic prevention.

This study will improve our understanding of the immunity tolerance in the liver tumor and provide a foundation for effective prognosis and treatment in the future.

II. MATERIALS AND METHODS

A. CD8+ T Cells Isolation

CD8+ T cells were isolated from two liver tumors as described before [15]. Briefly, freshly procured tumor tissues were washed in phosphate buffered saline, cut into small pieces and digested with 0.25mg/ml of collagenase IV at 4°C for 24 hours. CD8+ T cells from sinusoids of the same patients were perfused by PBS from liver sinusoid as described in our previous work [16]. Lymphocytes from tumor tissues, sinusoid structures, and peripheral blood mononuclear (PBMN) cells were centrifuged in Ficoll-Hypaque solution at 500g for 15 minutes and were recovered from the interface of cell suspension. CD8+ T cells were isolated from the recovered lymphocytes using magnetic anti-CD8+ microbeads (MACS technology, Miltenyi Biotech, Foster City, CA, USA) following the manufacturer's recommendations. The purities of isolated CD8+ T cells were confirmed by fluorescence-activated cell sorting (FACS) using FITC-labelled anti-CD8+ mAb. The proliferation potential and cytotoxicity of activated CD8+ cells were measured as previously described [7].

B. RNA Extraction

Purified CD8+ T cells were lysed in Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted using RNeasy column (Qiagen, Valencia, CA). Briefly, CD8+ T cells were homogenized in Trizol before the phenol-extracted aqueous layer was mixed with chloroform. The aqueous layer from chloroform extraction was precipitated in 70% ethanol and further purified by passing through RNeasy column. After sequential washing, total RNA was eluted in RNase-free water. Isolated total RNA was quantified and its integrity was confirmed on a RNA denaturing gel.

C. cRNA Synthesis

1ug of total RNA was used to prepare biotinylated antisense RNA (cRNA) using Ambion's MessageAmpII-Biotin Enhanced kit (Ambion, Austin, TX) and Affymetrix's Genechip sample module kit (Affymetrix, CA). Briefly, total RNA was converted to first-strand cDNA using T7 oligo dT primer. The RNA template was removed from the DNA:RNA hybrid using RNase H before double-stranded cDNA was synthesized. Double-stranded cDNA was purified and used as template to synthesize biotin-labelled cRNA by in vitro transcription. Purified biotinylated cRNA was fragmented at 94°C and used for gene chip hybridization.

D. Chip Hybridization

15ug of fragmented biotinylated cRNA was hybridized to Genechip Human Genome U133 Plus 2.0 Array

following standard conditions described in Affymetrix protocol (Expression Analysis Technical Manual, Affymetrix, Santa Clarita, CA). Briefly, fragmented biotinylated cRNA was incubated with GeneChip 133 plus 2.0 for 16 hours at 45 °C before the hybridized chip was washed and stained in an Affymetrix fluidics station. The processed array was scanned and the QC was performed using GeneChip Operating Software (GCOS, Affymetrix, CA). Each hybridized chip was examined against following QC standards (i) scaling factors are below 3, (ii) percentage of present is in the range of 30%-65%, and (iii) for housekeeping gene, such as GAPDH and beta-actin, the ratio of 3'/5' expression is below 3.

E. Analysis of Gene Expression

After QC was performed, the expression profile of each sample was generated by Genechip Expression Console (Affymetrix, CA) and further processed and analyzed in dChip per the instructions [17]. Briefly, dChip algorithm was used to summarize gene expression profile on each sample, where each chip was compared to a reference chip for normalization in overall chip hybridization intensity so as to allow for comparison between different chips. Hierarchical clustering was performed using the same dChip software to examine the similarity of gene expression between different samples. The gene expression profile of each sample was filtered according to the default criteria of dChip. The expression values of genes of interest were listed and compiled. The filtered gene expression profile of each sample was imported into GSEA software [18] for gene enrichment analysis in order to identify statistically significant gene sets that might be involved in the development of immune tolerance in live tumors.

F. Pathway Analysis

Once differential gene expression profiles of CD8+ T cells from peripheral blood, sinusoid structure, and tumor tissue were analyzed, a couple of key genes were utilized for toxicogenomics analysis by database and pathway. Briefly, interaction data was gathered from a number of different datasets from our work described above. The pathway network was generated by using Pathway Studio® 5.0 software (Ariadne Genomics, Inc. Rockville, MD, USA). All datasets with the regulatory connectivity and calculated betweenness of each protein node within these networks were listed and compiled.

To calculate node betweenness within networks, an improved version of the algorithm developed by Newman and Girvan was utilized. (1) Initialize the betweenness of every vertex v in the network $B_v = 0$. (2) Starting from a vertex i , a breadth-first tree is built with i on the top and those that are farthest from i at the bottom. Each node is put at a certain level of the tree based on its shortest distance

from i . (3) A variable $p_i = 1$ is assigned to i . As we are building the tree, for every vertex j ,

$$C_B(v) = \sum_{s \neq v, t \in V} \frac{\sigma_{st}(v)}{\sigma_{st}},$$

where σ_{st} is the number of shortest geodesic paths from s to t and $\sigma_{st}(v)$ the number of shortest geodesic paths from s to t that pass through the vertex v . This value was normalized by dividing by $(n-1) \times (n-2)$, where n is the number of vertices.

An additional analysis was carried out to evaluate the potential importance of individual nodes in protein interaction networks for providing connectivity among quiescent CD8+ T cells.

To identify such “topologically significant” proteins, the set of quiescent CD8+ genes was used to construct the regulatory path network that connected corresponding nodes in the global database of protein interactions (Pathway Studio software). Next, the number of paths that each node in this regulatory path network that was specific for genes differentially expressed in quiescent CD8+ was computed. A detailed description of the regulatory connectivity analysis is available as *Pathway Studio* software 5.0 manual.

Topology analysis was done to identify “topologically significant” proteins relative to their toxicity. This set of differentially expressed genes was then used to construct a path network that connected corresponding nodes in the global database of toxic substance. Briefly, several candidate genes were input into *GeneGo* software. A detailed description of the regulatory connectivity analysis is available as *GeneGo* software manual.

III. RESULTS

A. Gene Expression Analysis

As described in the method above, QC was performed using the GeneChip expression console. Each gene chip was examined according to QC standards including scaling factors, range of percentage of presence, and ratio of 3’/5’ expression of housekeeping genes. All three specimens from the first patient passed QC test, while the liver tumor specimen from the second patient showed significant RNA degradation.

Hierarchical clustering was performed using dChip software to examine the similarity and/or difference of gene expression profiles among the 5 samples (Figure 1). The liver sinusoids from two liver cancer patients presented the most similarity in terms of gene expression, while peripheral blood of first patient had the least similarity versus the liver tumors and liver sinusoids of both patients.

The normalized expression values of quiescent-related genes are summarized as in Table I. Statistically significant changes, i.e. at least 1.5 fold, were present as either upregulation or downregulation. C-Myc, Tob1, Tob2, ERF, KLF2, TGFbeta2, Trail, Granzyme B, TNFalpha, Perforin 1

were upregulated in CD8+ T cells isolated from both liver sinusoid samples as compared with those of peripheral blood, while TGF beta1 and REST were downregulated in liver sinusoid samples. However, C-myc, Tob1, Tob2, ERF, KLF2, TGF beta2 and Trail were upregulated in CD8+ T cells isolated from liver tumor as compared with those of liver sinusoid. By contrast, REST were downregulated in liver tumor, while Perforin1 and TGF1 beta1 presented no statistically significant changes between CD8+ T cells isolated from liver tumor and liver sinusoid.

Furthermore, the expression profiles of each chip were analyzed in GSEA software to investigate potential enrichment of gene sets. As shown in Table II, in comparison with those of peripheral blood, 10 out of 1009 genes were upregulated in CD8+ T cells of liver sinusoids, with 999 gene sets downregulated. However, 80 out of 1009 genes were upregulated in liver tumor, with 929 gene sets upregulated in liver sinusoids. This implies that most of the genes are still in active state in liver sinusoids compared with tumor.

B. Pathway Analysis

The genes were input into *Pathway Studio* software and a total of 156 nodes were discovered in the network profiles of quiescent CD8 cells, including 6 seed-proteins and 150 neighbours (which are derived) out of the 156 nodes. All networks are displayed in Figure 2, in which one seed-protein, Sno-A, which had no links (i.e. there was no *in silicon* evidence of interactions), is not involved in the network analysis. The results of topology regarding regulatory connectivity are that higher regulatory connectivity means the more direct interaction with other proteins in protein-protein reaction, such as c-myc and TGF beta1. The lower regulatory connectivity is a few direct interactions with other proteins in protein-protein reaction, such as Tob1, KLF2, ERF, REST and Ski as shown in Figure 2. The topology regarding higher betweenness means the protein dominating the much higher percentage interaction with other proteins in all proteins in a pathway, such as, ERF, Ski and REST. Lower betweenness means the much lower percentage interaction with other proteins in dominating protein-protein reaction, such as c-myc. As routine process, higher betweenness and lower connectivity are used as targeting proteins so that results of topology from pathway can be subject to select a measurement to target proteins.

C. Prediction of Toxic Substance and Toxic Prevention

Recently, therapeutic targeting including drug targeting, small molecule targeting, and toxicogenomics related with toxic substances has focused on bioinformatics analysis and topology analysis. After the topology was analyzed, some key proteins based on higher betweenness and lower regulatory connectivity are also defined as toxicogenomics

related with toxic substances as shown in Figure 3. As figure demonstration, REST and ERF related for toxic ethanol are discovered to connect several drugs' targeting from GeneGo platform, which can be selected as candidates of targeting drugs for *in vitro*, *ex vivo* and *in vivo* assay to search drugs prevention and study prevention mechanism.

IV. DISCUSSION

Sinusoids are found in the liver, lymphoid organs, bone marrow and spleen. Liver sinusoids consist of a discontinuous endothelium and an underlying complete basal lamina. Liver sinusoids are highly permeable, hence allowing small and medium-sized protein or other substances to freely enter the blood stream. As a result, some toxic substances are highly retained in liver sinusoids. Previously, we reported the quiescent status of CD8⁺ T cells in tumors by mining the differentially regulated genes of CD8⁺ T cells obtained from liver tumor TIL [7]. Nevertheless, several findings suggested that the inactivation of CD8⁺ cell immune response is caused by toxic substances. In order to address this issue, we used CD8⁺ cells from sinusoids to investigate the potential changes of gene expression profiles, which may be related to quiescent CD8⁺ T cells.

In this pioneering study, we selected only a pair of specimens. The number of specimens has been limited by various conditions such as the mass size required to isolate T-cell from liver sinusoid, informed consent from patients and the difficulty of harvesting T-cells from liver sinusoids. Nevertheless, we still got some promising results in spite of limited specimens. As shown in our results, some quiescent genes were produced in the liver sinusoid after we analyzed CD8⁺ T-cell genomes in the two pairs of specimens coming from three sources, peripheral blood, sinusoid, and tumor tissue. The research also addresses the pathway in quiescent status of CD8⁺ T cells from sinusoid structure, in which we have mined some quiescent genes related with ethanol, so that the toxicogenomics analysis will produce a new research model in the future. In future work, we plan to use the recently developed method of network topology analysis as described in [19] and dynamic simulation using SimBoolNet [20] for pathway analysis of our data. Last but not least, our results indicate that toxicogenomics analysis can be extended to the targeting identification (TI) and analysis which will give us a new model for preventing toxic substances in genome level by targeting identification.

Toxicogenomics is a field that deals with toxic substances related with genomics within particular cell or tissue of an organism. It can elucidate molecular mechanisms being evolved in the expression of toxicity, and derive molecular expression patterns (i.e., molecular biomarkers) that predict toxicity or the genetic susceptibility to it. The current data and the methods presented in this paper can shed light on the observed relationship between T-cell quiescence and toxic substances.

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TABLE I. GENE EXPRESSION OF KEY QUIESCENT GENES IN CD 8+ T CELLS FROM PERIPHERAL BLOOD (PB), LIVER SINUSOID AND LIVER TUMOR.

Gene Symbol	Probe Set	1_PB	1_Liver Sinusoid	1_Liver Tumor	2_Liver Sinusoid	2_Liver Tumor ^a	1- Liver Sinusoid/1-PB	1-Liver tumor/1-Liver Sinusoid	2- Liver Sinusoid/1-PB
C-Myc	202431_s_at	73.79	547.66	249.48	283.75	71.66	up	down	up
TOB1	202704_at	179.52	1346.79	732.36	913.25	397.78	up	down	up
	228834_at	33.76	185.1	104.9	116.44	44.39			
TOB2	222243_s_at	24.57	705.75	313.62	458.46	302.47	up	down	up
ERF	203643_at	102.59	430.09	273.62	291.8	101.49	up	down	up
KLF2	219371_s_at	91.99	2647.71	429.02	1310.72	121.59	up	down	up
TGFB2	209909_s_at	20.25	40.71	27.78	42.22	4.02	up	down	up
TRAIL	202688_at	54.84	1408.42	508.78	2460.63	114.55	up	down	up
	202687_s_at	39.97	850.97	454.45	1087.64	201.23			
	214329_x_at	9.37	1138.32	347.61	743.01	165.75			
Granzyme B	210164_at	68.72	179.93	551.26	102.23	24.45	up	up	up
ADAM10/TNFA	202604_x_at	14.45	99.27	147.01	96.48	26.37	up	up	up
	214895_s_at	14.43	157.9	172.17	119.8	27.23		no change	
Perforin 1	214617_at	63.57	328.05	393.67	269.71	45.9	up	no change	up
TGFB1	203085_s_at	3656.89	278.2	207.01	282.64	13.64	down	no change	down
REST	204535_s_at	68.73	3.61	1.99	16.4	22.02	down	down	down

a 2-liver tumor displayed features of mRNA degradation by QC analysis.

TABLE II. GENE SET ENRICHMENT ANALYSIS (GSEA) OF CD 8+ T CELLS ISOLATED FROM PERIPHERAL BLOOD, LIVER SINUSOID AND LIVER TUMOR.

Phenotype	Gene sets Upregulated in Phenotype
Peripheral Blood (1 samples)	999 / 1009
Liver Sinusoid (2 samples)	10 / 1009

Phenotype	Gene Sets Upregulated in Phenotype
Liver Sinusoid (2 samples)	929 / 1009
Liver Tumor (1 sample)	80 / 1009

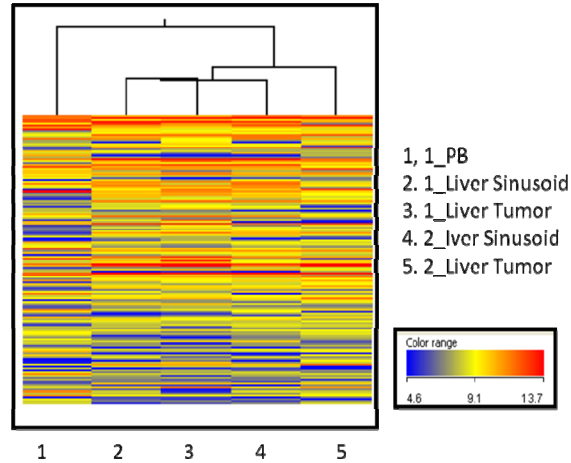


Figure 1. Hierarchical clustering of CD8+ T cells isolated from peripheral blood, liver sinusoids and liver tumors. Hierarchical clustering indicates five columns (i.e. genomic expression in different groups): 1. CD8+genomic expression of peripheral blood from patient one, 2. CD8+genomic expression of liver tumor from patient two, 3. CD8+genomic expression of liver tumor from patient one, 4. genomic expression of liver sinusoid from patient one and 5. genomic expression of liver sinusoid from the patient No. 2 (because of mRNA degrade from peripheral blood of patient No. 2, we missed CD8+genomic expression of peripheral blood from patient No. 2). Color “red” means high expression and “green” means low expression.

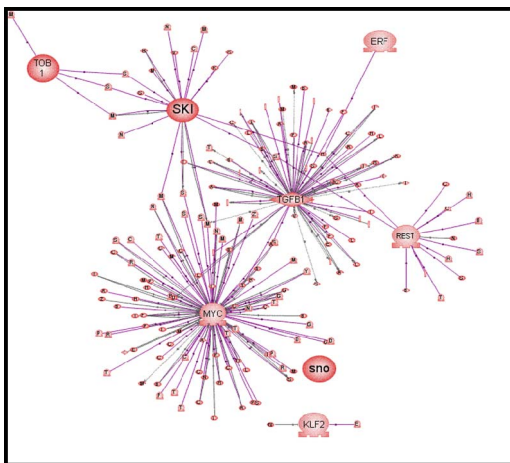


Figure 2. Pathway study for toxicogenomics targeting. These pathway (based on topology processed by *Pathway Studio* software) included large nodes (seed proteins) and small nodes (non-seed proteins).

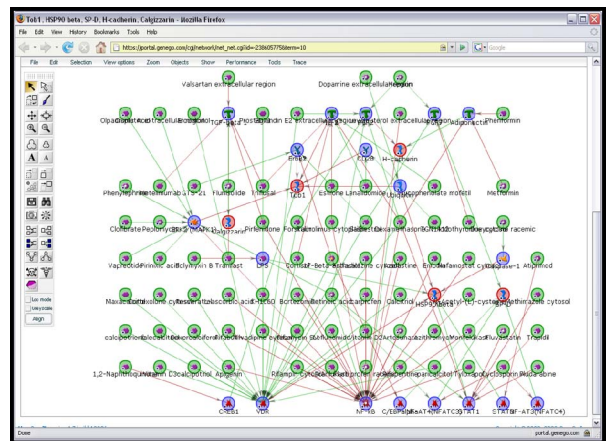


Figure 3. Toxicogenomics and toxic prevention. These pathways (processed by *GeneGo* software) included large nodes from pathway and network based on topology analysis and toxic substances such as ethanol and toxic prevention drugs.