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Machine-learning Modeling for Personalized Immunotherapy- An Evaluation Module

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ABSTRACT

Immune-cell therapy and targeting therapy are in rapid development to treat tumor diseases. However, current immune-cell therapy and targeting immunotherapy often face three challenges (three Ss): safety challenges such as cytokine releasing syndrome (C.R.S.); specificity targeting problems such as low efficacy caused by off-targeting tumor cells; unsatisfying payment are confounded to clinical patients and physicians. We have been studying immunotherapy for more than thirty years, and recently, personalized immunotherapy to treat tumor disease has been proposed. After we discovered quiescent genes from immune cells within the tumor microenvironment, we set up single-cell genomics analysis, studying heterogeneous immune responses from multiple tumor antigens (neo-antigen); here, we further introduce a new generation of immunotherapy module by using a machine-learning model to assess optimal immunotherapy. The machine-learning model combined with single-cell genomic analysis can predict optimal immune-cell (such as T-cells) and other optimal targeting drugs such as PD1 and CTLA4 inhibitors for the patient to use.

Keywords: Machine-Learning; Gene Expression; Pathway Analysis; Tumor-Infiltrating Lymphocytes; Targeting Therapy; Single-Cell Genomic Analysis; Personalized Immunotherapy

Abbreviations: CRS: Cytokine Releasing Syndrome; TCR: T Cell Receptor; CSC: Cancer Stem Cell; TAA: Tumor-Associated Antigens; TSA: Tumor-Specific Antigens; CT: (I) Cancer-testis; AI: Artificial Intelligences; TIME: Tumor Immune Microenvironment; PBMN: Peripheral Blood Mononuclear; CC: Clustering Coefficient; CD: Connectivity Degree

Introduction

TILs (tumor-infiltrating lymphocytes) are a group of lymphocytes that engage and kill tumor cells in tumor tissues. As with other lymphocytes, the TIL consists of CD^{3+}cell (CD8+T-cell, CD^{4+}T-cell), CD19 (B-cell infiltrating lymphocyte, BIL), $CD1^{6+}/CD5^{6+}$cell (NK cells) and other immune cells (macrophage and neutrophil) [1]. T.I.L.s, including CD^{8+}T-cells, demonstrate characteristics of main immune surveillance in the tumor location, which has been specifically recognizing tumor antigens [2]. Moreover, we have also reported that CD^{8+}T-cells from TIL displayed quiescent status and even lower response to tumor-antigen, which are obtained from pre-immunization induction. These cells, during quiescent periods, 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 [4], we employed single-cell genomics analysis

to elucidate the quiescent status of CD^{3+} (such as CD^{8+}T-cells) in tumor microenvironments. By studying the quiescent TIL obtained from a solid tumor, we have identified a group of down-regulated genes, including T cell receptor (TCR), TNF-alpha receptor, TRAIL, and Perforin. More interestingly, we have observed a group of upregulated quiescent genes, including Tob [5], TGF-β, LKLF [6,7], SnoA, Ski, ERF, and REST/NRSF complex. The quiescent genes are discovered in the tumor microenvironment by Western blot and quantitative rtPCR analysis [4]. Furthermore, the observation of actively quiescent T-cells, including CD^{8+}T-cells, is consistent with findings from other laboratories using genomic analysis and animal studies [5,6,8,9].

Since different inhibitors have been found to block immune responses to tumor cells in the tumor environment [8,10], it was further proposed that factors from the quiescent T-cells, such as PD-1 or CTLA-4, are actually one of the cores of immunotherapy [11]. Additionally, the tumor location is a place enriched with specifically activated lymphocytes, such as from an early monoclonal cancer stem cell (C.S.C.) into multiple differentiating tumor antigens within tumor cells, such as tumor-associated antigens (T.A.A.) and tumor-specific antigens (T.S.A.). T.A.A. consists of

(I) Cancer-testis (C.T.) antigens, overexpressed antigens,

(II) Differentiation antigens, and

(III) Oncofetal antigen. T.S.A. contains neo-antigens and onco-viral antigens such as HBV, EBV, CMV, and human papillomavirus (HPV) E6/E7 protein. In addition, neo-antigens have individual expressions for each patient, although shared neo-antigens have been increasingly discovered [12-14]. This background has guided us to set up personalized immunotherapy by single-cell genomics analysis with the machine-learning model.

This manual will use single-cell genomic data to address these questions combined with a machine-learning model. Machine learning is the study of computer algorithms, which can improve automatically through experiments with the use of data. As one of the artificial intelligences (A.I.) algorithms, here, our machine learning algorithms can build a model based on single-cell genomic data from quiescent genes of individual patients. It is called "personal trained data" from the tumor immune microenvironment (TIME) of each patient. The program can make predictions or decisions and then be used in personalized immunotherapy. The treatment module combined three sources:

(I) Immune cell therapy such as T-cell immunotherapy,

(II) Targeting immunotherapy, and

(III) Other different immune therapy methods such as IL2 and TNF and so on. Using the machine-learning analysis, we can address three challenge issues (three Ss) of immunotherapy: Safety: avoiding cytokine releasing syndrome (C.R.S.); Specificity: targeting free off-targeting tumor cell resulting in low efficacy; Satisfying payment avoiding confounded to clinical scientists and physicians. This study will improve our understanding of artificial intelligence, the personal machine-learning application for personalized immunotherapy, and provide a foundation for effective immunotherapy for tumor disease.

Materials and Methods

Immune Cell Isolation

Immune cells were isolated from tumors as described before [15-16]. 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. Lymphocytes from tumor tissues and peripheral blood mononuclear (PBMN) cells were centrifuged in Ficoll-Hypaque solution at 500g for 30 minutes and were recovered from the interface of cell suspension. CD^{3+}cell (including CD^{8+}T-cells), non-CD^{3+}cells were isolated from the lymphocytes using magnetic anti-CD^{3+} microbeads (MACS technology, Miltenyi Biotech, Foster City, CA, U.S.A.) following the manufacturer's recommendations. The purities of isolated CD^{3+}T-cells were confirmed by fowl cytometry using FITC-labelled CD^{3+}mAb/anti-CD^{8+}mAb. The proliferation potential and cytotoxicity of activated TIL were measured as previously described [4].

R.N.A. Extraction

Purified immune cells were lysed in Trizol reagent (Invitrogen, Carlsbad, CA), and total R.N.A. was extracted using the RNA easy column (Qiagen, Valencia, CA). Briefly, CD^{8+}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 the RNA easy column. After sequential washing, total R.N.A. was eluted in RNase-free water. Isolated total R.N.A. was quantified, and its integrity was confirmed on an R.N.A. denaturing gel.

cRNA Synthesis

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

Microarray Hybridization

15ug of fragmented biotinylated cRNA was hybridized to Gene Chip 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 Gene Chip 133 plus 2.0 for 16 hours at 45oC before the hybridized chip was washed and stained in an Affymetrix fluidics station. The processed array was scanned, and the Q.C. was performed using Gene Chip Operating Software (GCOS, Affymetrix, CA). Same specimens performed by RNA-seq will be reported separately (although all data from single-cell RNA-seq will be presented by other manuals).

Analysis of Gene Expression

After QC was performed, the expression profile of each sample was generated by Gene Chip Expression Console (Affymetrix, CA) and further processed and analyzed in dChip per the instructions [17]. Briefly, the dChip algorithm was used to summarize the 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 BRB Array Tool [18] for gene enrichment analysis in order to identify statistically significant gene sets that might be involved in the development of immune quiescence in tumors.

Machine-Learning Analysis

Once differential gene expression profiles of TIL cells from tumor tissue were analyzed, a couple of key genes were utilized for quiescent and active analysis by database list. Briefly, interaction data was gathered from a number of different datasets from our work described above. Machine learning was generated by using algorithms that we have published. All datasets with the Regulatory Connectivity and calculated Betweenness of each protein node within these networks were listed and compiled in the Cytoscape platform.

Three indices were used for machine-learning analysis: Betweenness Centrality (B.C.), Connectivity Degree (CD), and Clustering coefficient (CC). In detail,

1) Input a pair of clinical genome profiles from genome diagnosis (including genome profiles CD^{3+}cell (such as CD8+cell), non-CD^{3+} cells) into Cytoscape platform (plugin Reactome F.I. platform or plugin Mimi platform).

2) Collect a series of genes with a higher degree of BC., lower CD and lower CC, as genes with higher inducing to TIL cells and lower toxicity to T-cells.

3) Enter this genome into the T-cell therapy database (such as CD^{8+} for TIL, $CD16/5^{6+}$ for N.K., $CD1^{9+}$ for B.I.L., CD11b for TAM1/TAN1 and so on) and the Drug Genome Response Database (DGIdb) for immune targeting therapy or combined chemotherapy.

The personal machine-learning design, performance and clinical information will be reported by another manual from single-cell RNA-seq based Machine learning.

Results

Immune Cells Isolation and Gene Expression Analysis

Table 1.

Biomarker expression related to prediction for immune cell therapy						
Source	**Immune cell therapy prediction**	**Marker-1**	**Marker-2**	**Marker-3**	**Marker-4**	
Tumor Source	TIL	CD8	CD4	CD3		
	BIL	CD19	CD20			
	Functional TIL	CD8	CD4	CD3	IL10	TGF-beta
	TCR T- cell	CD8	CD4	CD3		
	Macrophage (TAM1)	CD11b	CD68		IL4	IL13
	Leukocyte (TAN1)	CD11b				
Source	Cell Type	Marker-1	Marker-2	Marker-3	Marker-4	
Blood	CAR T-cell	CD8	CD4		CD3	
	TCR T- cell	CD8	CD4	CD3		

	DCCIK	CD11b	CD68	CD8		
	LAK	CD8	CD4	CD3		
	CIK	CD8	CD4	CD3		
	NK	CD16	CD56			
	NKT	CD16	CD56	CD3		
	Macrophage (TAM1)	CD11b	CD68			
	Leukocyte (TAN1)	CD11b				
	DN	CD8	CD4			

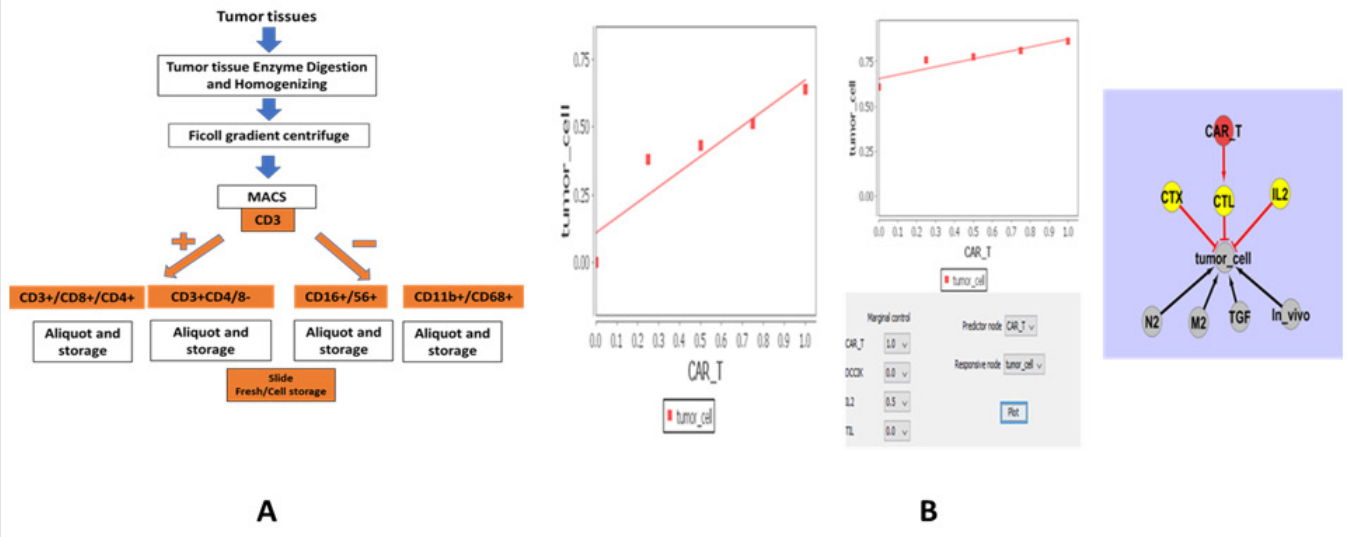

Figure 1.

Results and performance of immune cells and R.N.A. were isolated from tumors as our previous report. After total R.N.A. extract and microarray performance, Q.C. was performed using the Gene Chip expression console. Each gene chip was examined according to Q.C. standards, including scaling factors and range of percentage of presence. All three pairs of specimens from each patient passed the Q.C. test, while CD^{3+}cell and CD^{3-}cell from TIL with paring T-cell from PBMN as shown in (Figure A). As (Table 1) shows, further immune cells related to biomarker expression to discover the quiescence or activity according to gene expression profiles among the three pairs of samples. In a table list, the CD^{3+}cell from TIL or CD^{3-}cell or CD16/CD56, or CD11b presented quiescence or activity of gene expression. At the same time, peripheral blood of similar patients had quiescence or activity from PBMN of each patient as Table demonstrated that blue color is quiescence and red color is an activity for each immune cell.

The expression values of quiescent-related and activity genes are summarized as a database list if significant changes of quiescent genes were upregulated in CD^{8+}T-cells or $CD16/5^{6+}$ N.K. cell or CD11b (macrophage or neutrophil) isolated from patient tumor samples as compared with those of peripheral blood. For example, T.G.F. beta and REST were upregulated in CD^{8+}cell higher expression section isolated from the tumor as compared with those of PBMN so that the quiescent status was upregulated in CD^{3+}/CD^{8+}cell which we can culture TIL as major cell therapy for the patient because the CD8 quiescent changes can play an important role to treat patient, individually.

Furthermore, the expression profiles of each chip were analyzed in Cluster analysis to investigate the potential enrichment of gene sets. In comparison with those of peripheral blood, quiescent gene profiles were upregulated in CD^{8+}T-cells from TIL; This implies that most of the genes are still in active state tumor while active gene profiles were down-regulated in CD^{8+}T-cells from TIL so that we can further confirm TIL (CD8) as major cell therapy for the patient because inducing the active genes can be significant changes and more function for CD^{8+}T-cells to treat patient.

Machine-Learning Analysis

The genes were entered into the Cytoscape platform for discovering in the network profiles of quiescent CD^{8+}cells, or quiescent $CD16/5^{6+}$cell, or quiescent CD11b, including their seed-proteins and their neighbors (which are derived) for a network.

All networks are displayed in a configuration in which one seed protein is involved in the network analysis. Three results include Betweenness Centrality (B.C.), Connectivity Degree (CD), and Clustering coefficient (CC). The clinical genome profiles from genome diagnosis (including genome profiles from PBMC and genome profiles from TIL including CD^{3+} cell such as CD^{8+}cell, non-CD3 cells indicate a higher degree of B.C., lower CD and lower CC, as genes with higher inducing to the immune cells for cell therapy and lower toxicity to the immune cells. The results also can cover other cell therapy such as N.K. cell (CD16/56), and macrophage therapy (CD11b).

Prediction of the Combination of Immune Therapy

Recently, two immune therapies are emerging:

(A) Immune targeting inhibiting therapy such as PD1 or CTLA4 inhibitors and

(B) CAR-T or TCR-T therapy. If we want to find the PD1 or CTLA-4 efficacy, we can put it into Drug Genome Response Database (DGIdb) to discover the immune targeting therapy.

If we want to discover a new generation of cell immune therapy, such as CAR-T or TCR-T therapy, we can use the machine-learning model to discover which cells are optimal. If we want to find a combination, as shown in (Figure-B), therapeutic targeting, including drug targeting, and small molecule targeting, was used for the machine-learning analysis. After the machine-learning was analyzed, (Figure B) finds CAR-T and targeted therapeutic drugs and targeted molecules and links the genome expression profiles with targeted therapeutic drugs and targeted molecules. As the figure demonstrates, CAR-T therapy related to chemotherapy and targeting therapy are discovered to connect as a prediction for the patient individually.

Discussion

In this manual, after we set up single-cell genomic data to address the quiescent and active status of immune response for each patient, here we combined a machine-learning model to discover an optimal therapy for each patient. Machine learning can improve automatically through experiment data from individual genes so that the machine learning algorithms can be used in personalized immunotherapy. The treatment model also combined three sources:

1) Immune cell therapy such as CD^{8+}T-cells or $CD16/5^{6+}$ N.K. cell or CD11b (macrophage or neutrophil), with totally current thirteen immune cell therapy.

2) Targeting immunotherapy with all clinical trial and F.D.A. approved drugs.

3) All other different treatment such as HDACI or DNMA inhibitors so that we can use the machine-learning analysis to resolve three challenge issues from immunotherapy: safe treatment voiding cytokine releasing syndrome (C.R.S.); specific targeting treatment free off-targeting tumor cell; satisfying payment avoiding confounded to clinical patients for a higher payment. In the near future, we will report using single-cell R.N.A. seq data to mimic machine-learning with the results from clinical patients. Hopefully, it also will support machine-learning to support personalized immunotherapy.

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