Medical Research Archives

European Society of Medicine

OPEN ACCESS

Published: August 31, 2023

Citation: Li B, 2023. Personalized Immunotherapy of Patients: Defining by Single-cell RNA-seq with Artificial Intelligence, Medical Research Archives, [online] 11(8). Vhttps://doi.org/10.18103/mra.v11i8.4293

DOI
https://doi.org/10.18103/mra.v11i8.4293

ISSN: 2375-1924

RESEARCH ARTICLE

Personalized Immunotherapy of Patients: Defining by Single-cell RNA-seq with Artificial Intelligence

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ABSTRACT:
Immunotherapy, including immune cell therapy and targeted therapy, is gradually developed through the ongoing discovery of molecular compounds or immune cells. Choosing the best one or the best combination of target compounds and immune-cell therapy is a challenge for clinical scientists and clinicians. We have found variable efficacy individually after tumor-infiltrating lymphocyte (TIL) therapy, and now TILs have been discovered in a group of heterogeneous immune cells. To select the best immunotherapy for each patient, we started to study TIL genomics, including single-cell mRNA differential display from TIL published in 2007 and single-cell RNA-seq from TIL published in 2013, set up TIL quantitative network in 2015, researched machine-learning model for immune therapy in 2022. These manual reports single-cell RNA-seq data combined with machine learning to evaluate the optimal compounds and immune cells for individual patients. The machine-learning model, one of artificial intelligence, can estimate targeting genomic variance from single-cell RNA-seq so that they can cover thirteen kinds of immune cell therapies and ongoing FDA-approved targeted therapies such as PD1 inhibitors, PDL1 inhibitors, and CTLA4 inhibitors, as well as other different treatments such as HDACI or DNMT1 inhibitors, FDA-approved drugs. Moreover, also cover Phase-1, Phase-2, Phase-3, and Phase-4 of clinical trials, such as TIL, CAR T-cells, TCR T-cells. Single-cell RNA-seq with an Artificial intelligence estimation system is much better than our published models from microarrays or just cell therapy. The medical goal is to address three issues in clinical immunotherapy: the increase of efficacy; the decrease of adverse effects and the decrease of the cost in clinical applications.

Keywords: Artificial intelligence (AI), tumor-infiltrating lymphocytes (TIL), cytokine release syndrome (CRS), chimeric antigen receptor T-cells (CAR T-cell), T-cell receptor T-cell (TCR), natural killer cell (NK), natural killer T-cell (NKT), specific T-cells, PD-1 inhibitor, CTLA4 inhibitor, single-cell genomic analysis, single-cell RNA-seq and personalized immunotherapy.

Introduction

Since 1986, TIL has been discovered through fantastic responses to treat tumor disease[1]. Many immune therapies, including adoptive cellular therapy (ACT), targeting checkpoint blockade, and tumor vaccine, have increasingly become feasible immune therapy for tumor disease. However, how to increase efficacy, decrease adverse effects, and decrease cost for immune therapy puzzle most clinical physicians, so the introduction will focus on the issues in immune therapy, and then, introduce an importance for personalized immunotherapy to resolve the issues:

DIFFERENT EFFICACY TO TREAT DIFFERENT TUMOR PATIENT.

The immune therapeutic efficacy of tumor diseases such as ACT, targeted checkpoint blockers, and tumor vaccines vary from person to person. In an early period, TILs administrated at the National Cancer Institute's Surgical Branch, and objective responses were observed in 11 of 20 patients, with 5 of 29 patients (17%) achieving a complete response (CR) for metastatic melanoma[2]. Now TILs have shown overall response rates (38%) with cyclophosphamide lymph depletion for the treatment of recurrent refractory melanoma presented American Society of Clinical Oncology (ASCO) in the 2021 meeting[3]. In the early periods, we also reported TIL efficacy with 27% responses (both Partial responses, PR and complete response, CR) from clinical analysis of more than 300 patients suffering from solid tumors and 37% responses (PR and CR) by combination treatment of TILs and chemotherapy after selecting compounds by drug sensitivity test (DST) from 83 patient's treatment[4-10]. Now with CAR T-cell clinical trials, complete remission occurred in 6 of 14 patients (43%) with diffuse large B-cell lymphoma (DLBCL) and 10 of 14 patients (71%) with follicular lymphoma[11-12]. The results show different efficacy in treating oncological patients from person to person. Now, it also indicates that individual patients respond differently to targeted immune checkpoint blockers; though targeted immune checkpoint blockers produce successful results, only 15-60% of patients with response[13], so now physicians have focused on tumor signatures such as microsatellite instability, programmed death-ligand 1 (PD-L1) expression and tumor mutational burden, and the occurrence of immune-related adverse events (IRAEs)[14]. Anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) poses similar challenges to efficacy[15]. Moreover, the tumor vaccine will become a promising therapeutic strategy in immunotherapy with strategies for antitumor immunity from tumor antigens, including whole cells, peptides, and nucleic acids[16], however, the clinical efficacy of tumor vaccines is still poorly reported because the results have not been extensively analyzed.

DIFFERENT ADVERSE EFFECTS INDIVIDUALLY

ACTs, targeted checkpoint blockers, and tumor vaccines can produce adverse effects on patients. Current CAR T-cell therapies have experienced varying degrees of severity from different subjects. The most severe symptoms are cytokine release syndrome (CRS) and neurotoxicity. CRS, such as shock and multisystem organ failure, is caused by the strong activation and proliferation of CAR T-cells *in vivo*. At the same time, some side effects can be mild, such as flu-like symptoms[17], Although glucocorticoids or tocilizumab (anti-IL6) and IL-1 blockade are available for the treatment of CRS and encephalopathy syndrome, neurotoxicity. The mechanism of adverse effects is caused by non-specific immune cells from ACT treatment[18]. Additionally, CTLA4 and PD1 targeted therapy occurred in normal autologous tissue with side effects in 15-90% of patients[19]. The common immune feature of toxicity is the accumulation of non-specific memory T-cells, which invade the gastrointestinal tract and lungs, causing inflammatory damage such as itching and mucositis. The toxicities affecting the gastrointestinal tract and brain were more familiar with anti-CTLA4 therapy, while patients receiving PD1 axis-targeted therapy were at higher risk for hypothyroidism, hepatotoxicity, and pneumonia. On the other hand, following the application of tumor vaccines, the adverse effects of tumor vaccines also occur from one person to another person[20].

HIGH COST FOR IMMUNE THERAPY FROM ONE AGENT TO ANOTHER AGENT

A third challenge to consider is the cost of using ACTs, targeted checkpoint blockers, and tumor vaccines[20]. Currently, CAR T-cell therapy costs between $500,000 and $1,000,000 per patient, while the production of a tumor vaccine takes about four months and costs about $100,000 per patient. Moreover, targeted checkpoint blockers come at a cost with pembrolizumab doses. The monthly fee for an average is $9,000 for 2 mg/kg every three weeks; $46,000 for 10 mg/kg every three weeks; $69,000 for 10 mg/kg every two weeks, and finally, $83,500 for the highest dose. At this cost, 26 courses of pembrolizumab for a melanoma patient weighing 75 kg would incur a treatment cost of $1,009,944 for the highest dose administered, so it is difficult to pay a high cost for immune therapy from one agent to another agent if one of the agents cannot produce good efficacy as Fig-1A shown.

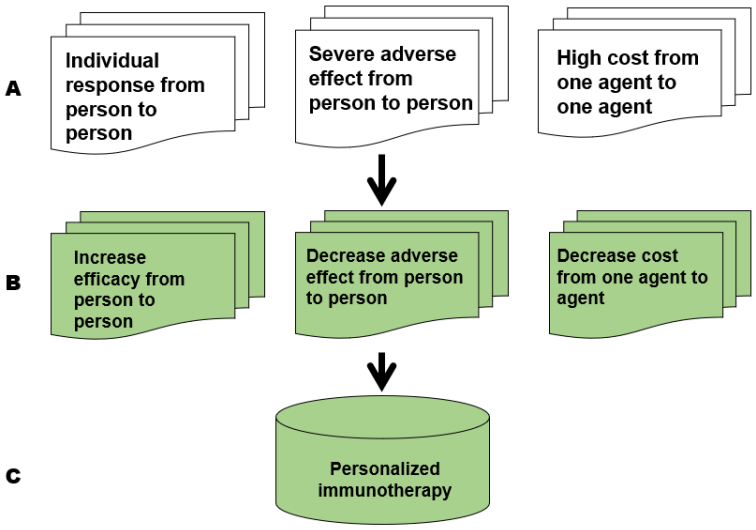

Fig-1. Immune therapy challenge and resolution for efficacy, adverse and cost. A) is shown three challenges, B) shown three resolutions. C). personalized immune therapy is to resolve the three challenges.

According to these three issues, we need to develop an effective immune therapeutic strategy to increase efficacy, reduce adverse effects, and decrease coating, as in shown Fig-1B/1C. After more than twenty-five years effort, now here is first to report a fundamental of personalized immunotherapy by studying three fields, those are, 1) Specific targeted therapy, 2) Safe treatment without off-target non-tumor-cell voiding CRS and 3) Satisfactory treatment, to avoid repeated agents from one agent to another agent, to address the three issues regarding efficacy, side effect and cost.

Fundamental of Personalized Immunotherapy

In the early periods. After we studied TIL characteristics from 83 patients suffering from solid tumors in 1994[4,21], we found TIL response differences in individuals. So, we began to use TIL and primary tumor cells from tumor tissues as subjects to study the immune therapy individually to address the issues as above. Since then, the research experiences TIL genomics (single-cell mRNA differential display from TIL published in 2007 and single-cell RNA-seq from TIL posted in 2013), set up TIL quantitative network presented in 2015, research TIL artificial intelligence (AI) in 2022 [22-25]. We find at least three areas to involve in individual responses and different adverse effects as Fig-2.

These are Immune cell Silencing in tumor tissue which cannot actively kill tumor cells. Heterogeneous immune responses from immune cells, which can be toxic to normal cells, and immune cell Infiltration into tumor sites which immune cells require moving into tumor location to kill tumor cells as Fig-2.

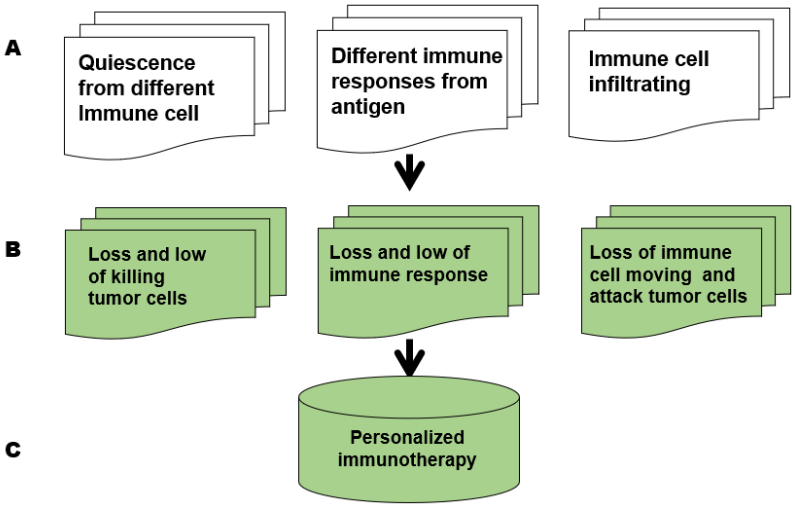

Fig-2. Fundamental of personalized immunotherapy. A) is shown three reasons, B) shows three mechanism. C). personalized immune therapy is to resolve the three mechanisms.

TILs quiescent status: Even if TIL, freshly isolated from solid tumor tissues, has activated T-cell surface markers such as HLA DR and IL-2 receptor, we found the cells did not have potent cytotoxicity against tumor cells published in 1995[26]. After more than twenty years study, we understand that TIL quiescence consists of at least two major factors: internal factors and external factors[27-30]. The internal factors included quiescent gene expressions such as Tob (transducer of ERBB2), LKLF (Lung Krupple-like factor, or KLF2), TGF-beta, ERF (ETS2 Repressor Factor), REST gene (RE1 silencing transcription factor), Bach-2, FOXO1, and STAT3. Tob is a negative regulator of IL-2 transcription and T-cell for T-cell proliferation, which inhibits the Ag-MHCII pathway. LKLF is a zinc-finger-containing transcription factor that plays a negative regulatory role in CTL. Ski, Sno, and TGF-β are involved in the TGF-β pathway to keep T-cells quiescence. ERF negatively regulates TIL infiltration, migration, and migration to tumor sites. The REST gene, TCF-1, Bach-2, FOXO1, and STAT3, can block the CTL pathway.

The external factors also be increasingly studied to involve tumor microenvironment (TME) components, regulatory cells including tumor-associated macrophages (TAM), neutrophils (tumor-related neutrophils-2, TAN-2), cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs); signaling molecules such as TGF-β, ADO. signaling molecules and IDO regulatory molecules[31-35].

Heterogeneous immune response: After 25 years of studying individual TIL responses through single-cell gene expression from solid tumors[36-40], we learned that the TIL immune could produce a response to autologous tumor cells by re-activity of self-tolerance to homologous antigens, heterogeneous response, and heterogeneous network from TIL to autologous tumor cell. Re-activity of self-tolerance against homologous antigens has been largely studied. For example, cancer-testis (CT) antigen, differentiation antigen (DA), and onco-fetal antigen (OFA) are involved in shared tumor-associated antigens (TAAs), while tumor-specific antigens (TSAs) consist of neo-antigens and tumor viral antigens such as HBV, EBV, CMV, and human papillomavirus (HPV) E6/E7 proteins. Not only do neoantigens caused by mutations in coding regions generate antigens that do not exist in normal cells, but also neoantigens can be induced by viral infection, such as alternative splicing and gene rearrangements. These variant antigens can be recognized by immune cells, such as TILs. Current data show neoantigens, including shared neo-antigens and personalized neoantigens. Shared neoantigens refer to mutated antigens common in different cancer patients. In contrast, Individual neoantigens refer to mutated antigens that differ from patient to patient, all of which can be designed for personalized immunotherapy.

TIL Infiltrating into tumor tissue to attack tumor cells: A key to TIL attacking tumor cells includes TIL signal intactness and TIL infiltrating into tumor tissues. In some earlier reports, the infiltration of TIL into tumor tissue to kill tumor cells has been studied in two areas[41-51]: (1) TIL CD8 cells should have intact signaling molecules after harvesting TIL from tumor tissue; (2) *ex vivo* cultured TIL specifically enters tumor tissue from circulating blood after reinfusion. The first question has been largely studied by our colleagues for about 30 years[4, 26]. Earlier, we found changeable results based on Rosenberg's tumor disaggregation (with triple enzymes by collagenase type-IV, hyaluronidase, and DNase) to disaggregate solid tumors. After we found the enzyme digestion damaged TIL intactness, we modified the enzyme condition so that the TILs function could be maintained with optimal proliferation, activity, and cytotoxicity to kill autogenous tumor cells. As in the earlier publication, we isolated and cultured TILs from solid tumors using mild enzymatic digestion (cold enzymatic digestion with lonely collagenase IV), and the results showed that 65% of TILs proliferated more than 1000-fold. The 3TdR incorporation rate peaked at 45-75 days. Cytotoxicity to tumor cells was maintained for 56 days. The phenotypes of TILs after IL-2 induction were CD3 80+21%, CD4 37+21%, CD8 44+18%, and HLA DR 69+24%, respectively. CD3 and CD8 were significantly higher than other clinical laboratories. Recently, TIL mobilization into tumor tissue has become increasingly accepted, such as Dr. Torcellan's *in vivo* TIL-labeling technique in 2017[45] and Bai's on-site antigen presentation for clonal expansion in 2001[46]. Now scientists have found CXCR2 (the receptor for CXCL1 secreted by tumor cells) and CCR4 (CCL17, the ligand for CCR4) involved in T-cells chemotaxis including rolling, adhesive, and extravasate from blood circulation into tumor tissue.

Strategies and Design for Personalized Immunotherapy

In earlier studies, as mentioned above, we found that the efficacy of TILs in treating solid tumors varies from person to person. Then we used several strategies to address the issues shown in Fig-3[4,26, 52-61]. In that period, we have used three ways to resolve these issues: removing inhibitory factors

during TIL culture and reducing T cell quiescence; studying increasing the immune response to tumor cells such as using TNF-α retroviral vectors transduced into TILs; Collagenase IV under mild digestion conditions maintains TIL integrity to increase TIL exposure to tumor cells. Clinically, in order to enhance the efficacy of TIL, we also developed a clinical program that combines TIL with sensitive chemotherapy drugs obtained from sensitive drugs test (SDT) to autologous tumor cells[4].

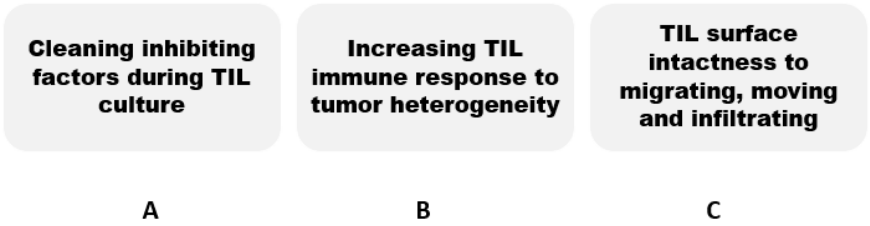

Fig 3. Early strategies for TIL efficacy from experimental level to solid tumor. A) is shown to clean inhibiting factors; B) shows to increase TL functions; C). maintaining CTL intactness.

In order to study increased efficacy individually, we first studied TIL genomics by single cell mRNA differential display system published in 2007 and discovered Tob, LKLF, TGF-beta, ERF, Bach-2, and then now FOXO1 and STAT3 were found to have higher expression in the quiescent status[62-70]. Moreover, TILs are heterogenous cells consisting of $CD3^+$cell ($CD8^+$T-cell, $CD4^+$T-cell), $CD19^+$cell (B-cell infiltrating lymphocyte, BIL), $CD16^+/CD56^+$cell (NK cells), and other immune cells (macrophage and neutrophil so that we can determine different adoptive immunotherapy individually. Furthermost, RNA-seq techniques can discover both RNA expression and RNA-coding SNPs from primary tumor cells and TILs. All the Research and development (R&D) from RNA-seq and RNA-coding SNPs from primary tumor cells and TILs can contribute to analyzing immune cell Silencing in tumor tissue, Heterogeneous immune responses from immune cells to tumor cells, and immune cell Infiltration into tumor sites for new strategies as Fig-4.

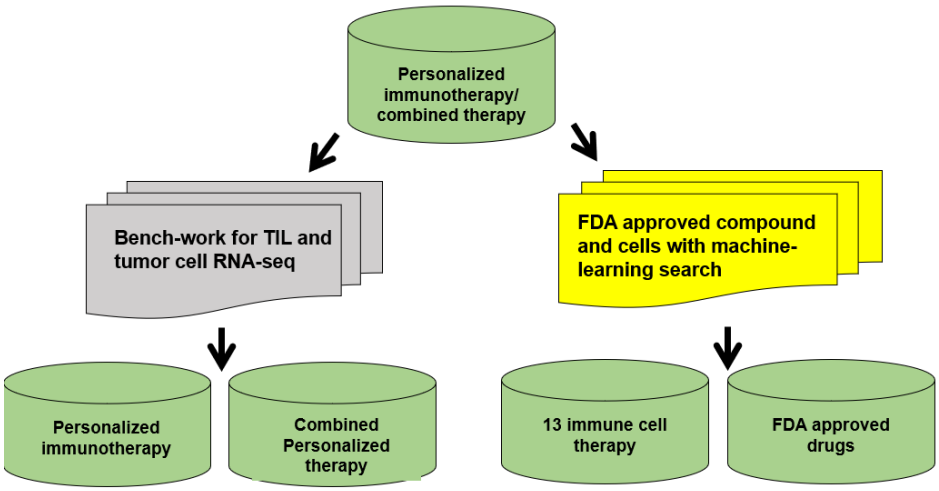

Fig-4 New strategies and design for personalized immunotherapy

Additionally, combination therapy also can improve efficacy and decrease adverse responses. In order to study many factors determining the individualized immune response with their treatments, a machine-learning model is guided to study the evaluating system[71]. Machine-learning models can use single-cell genomic data from both TIL and tumor cells to discover targeting compounds and optimal cells. Machine learning is the study of computer algorithms automatically defining "known data" to "unknown data." Currently, machine-learning analysis can study two databases, including an immune-cell therapy database coming from FDA with different clinical trials such as genes/biomarkers related to TAA or TSA (neoantigens from autogenous tumor cells) for CAR-T and TCR T-cell and the second database built for machine-learning analysis to support immune checkpoint targeting: TME related gene expression including ADO and IDO pathway; TIME-related gene expression; HDACI and NDMT inhibitor-related gene expression, and gene expression related to chemotherapy approved by FDA as well. Finally, we combine both data with a

machine-learning model to perform personalized immunotherapy for each patient so that good immunotherapy consists of the characteristics of high efficacy, few side effects, and low cost for each tumor patient.

Current Protocols for Personalized Immunotherapy

As noted above, there are at least three main reasons to cause the differing efficacy of TILs in the treatment of tumor diseases. Now, more and more procedures of T-lymphocyte treatment will be discovered to treat solid tumors. Moreover, after the development of genomics to clinical immunotherapy, more and more targeted drugs with their mechanisms have been discovered for the treatment of tumor diseases. Now is a good time to develop personalized immune therapies for tumor disease. This report includes 1) Single-cell RNA-seq and 2) artificial intelligence analysis to address the clinical selection for patients with solid tumor diseases as Fig-5.

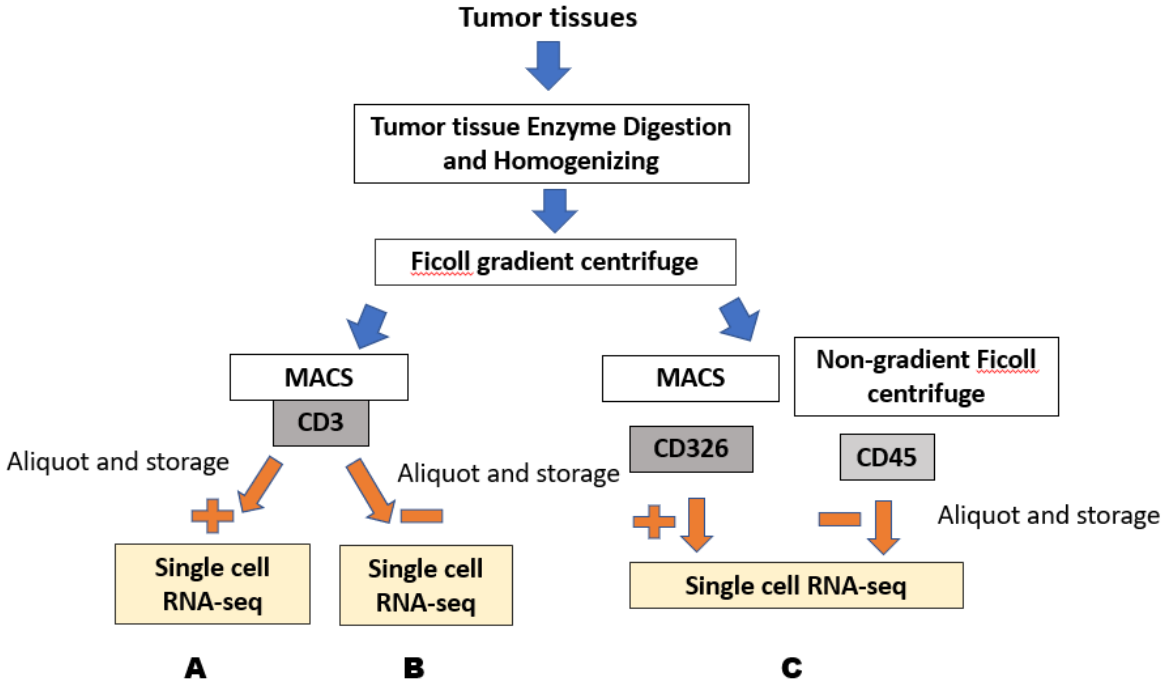

Fig-5. Protocol for personalized immunotherapy. A) and B) Harvesting TIL from solid tumor tissues to run RNA-seq to discover quiescent genes, and C) Harvesting tumor-cell from solid tumor tissues to run RNA-seq to discover neo-antigen or other related genes.

1. SINGLE-CELL RNA-SEQ

Isolation of immune-cell and autologous tumor cells: TIL and primary tumor cells were isolated from solid tumors as previous report [72-73]. Before isolation, voiding normal tissue and necrotic areas and then tumor tissues were washed in phosphate-buffered saline, cut into small pieces, and digested with 0.25mg/ml of collagenase IV at 4°C overnight [74]. In detail, TIL and tumor cells were centrifuged in Ficoll-Hypaque solution at 500g for 30 min. The buffy-coat with PBMN was to use magnetic anti-$CD3^+$ microbeads (MACS technology, Miltenyi Biotech, Foster City, CA, USA) while the depleted $CD3^+$ parts can be further used for $CD3^-$ immune cells such as $CD19^+$ cells, $CD16^+/CD56^+$ cell, and $CD11b^+$ cell. The primary tumor cells below the buffy coat containing primary tumor cells were purified on a two-step MACS isolation method, depleting $CD45^+$ cells and positively selecting $CD326^+$cell binding to achieve primary tumor cells. If tumor cells with unknown tumor biomarkers, we can use two steps, or a non-gradual centrifuge combined with depleting $CD45^+$ cells.

RNA sequencing from immune-cell and tumor cells: cDNA library from $CD3^+$ cells (or $CD8^+$ cells) and $CD3^-$ cells, and cDNA library from primary tumor cells was generated by using a protocol as previously reported[72,73, 75-77]. Briefly, after the DNA was fragmented with the downstream end-repair process and a single 'A' base adding, the fragment was ligated to adapters, purified by 2% agarose gel, and then enriched by PCR to create the final sequencing library. Finally, RNA single-end sequencing was performed using Solexa/Illumina Genome Analyzer II using the standard protocol. The sequencing library was loaded on a single lane of an Illumina flow cell. The image was performed using CASAVA 1.6

module to transfer BCL format into FASTQ format. Sequenced reads were generated by base calling using the Illumina standard pipeline. Alignment of sequenced reads was performed using a Galaxy platform with the references sequenced reads from the hg19 were aligned to human transcript reference sequences for the expression analysis at gene/transcript levels by Tophat and differential analysis by Cufflinks and Cuffdiff in Galaxy platform. Evaluation of gene expression among genes of RNA-seq was used by RPKM (reads per kilobase of transcript per million mapped reads) in this study. To discover SNP from tumor cells, RNA-seq was performed by SAM tool for BWA alignment, and GATK tool for discover variances to mine coding SNP related to neoantigens from tumor cell RNA-seq.

2. MACHINE-LEARNING ANALYSIS

Database establishment for immune-cell therapy: Two databases were built for machine-learning analysis: (A) currently different immune therapy coming from FDA with different clinical trials and (B) genes/biomarkers related TAA or TSA including neoantigens from autogenous tumor cells for CAR-T and TCR T-cell. Database establishment also includes molecular targeting therapy with five additional databases: (A) immune checkpoint targeting: (B) TME-related gene expression including ADO, IDO pathway; (C) TIME-related gene expression; (D) HDACI and NDMT inhibitor-related gene expression and (E) gene expression related to chemotherapy approved by FDA.

Results for Personalized Immunotherapy

1. ISOLATION OF IMMUNE CELLS AND PRIMARY TUMOR WITH SINGLE-CELL RNA-SEQ

An isolation performance with immune-cells results is demonstrated in our previously mentioned reports[64,70, 78-80] as Fig-5. After isolating $CD3^+$ cell and $CD3^-$ cell from TIL with pairing immune-cell, library preparation, and RNA-sequencing, three sets of quality control (QC) of sequencing libraries and techniques were performed, including 2100 bioanalyzer with 200–450 bp molecular weight for mRNA QC (if the specimens need small RNA, QC can harvest both >200 bp and <200 bp range), sequence reads 30 million for RNA-seq QC regarding the High-Seq 2500, and analysis QC for each base pair score >30 generated by base calling using the Illumina standard pipeline data with triple gene expression from immune-cells. After RNA-seq data came out, based on a list of higher expression genes of quiescent genes and lower expression CTL genes either from TIL $CD3^+$ cell or from TIL $CD3^-$ cell, we further used quantitative rtPCR to define quiescent genes from isolated cells with $CD8^+$cell from $CD3^+$ cell or $CD16^+/56^+$ cell, $CD19^+$ cell, or $CD11^+$ cell from TIL $CD3^-$ cell so that the results can guide distinct ACT for each patient as Table-1. RNA-seq from tumor cells can discover some tumor antigens on the surface of tumor cells, including CT antigen, DA, and OFA in TAAs, viral antigen, or neoantigens, as shown in Table-2. Some chemotaxis gene changes for moving, migrating, and infiltrating will be studied for personalized immune therapy.

Table-1. Biomarker and quiescent gene expression from RNA-seq related to prediction for immune-cell therapy

Source-1 (TIL)	Marker (low)	Higher Expression of Quiescent genes and other genes	Prediction of immune-cell therapy
$CD3^+$ cell	CD3/CD4/CD8	Tob, KLF2, TGF-beta et al	TIL
	CD3/CD4/CD8	IL10/TGF-beta et al	Functional TIL
	CD3/CD4/CD8	Neoantigen	TCR T-cell
$CD3^-$ cell	CD19/CD20	Tob, KLF2, TGF-beta et al	TIL-B cell
	CD11b/CD68	IL4/IL13	Macrophage (TAM1)
	CD16/CD56	Tob, KLF2, TGF-beta et al	NK
	CD16/CD56/CD3	Tob, KLF2, TGF-beta et al	NKT
	CD11b	IL4/IL13	Leukocyte Infusion Therapy

Table-2. Individual phenotype expression and genotype characteristics from shared neoantigen

Gene	AA change	Pancreas	Colorectal	ESCC	Liver	Lung adeno	Lung squ	Ovarian	Stomach	Cervical
TP53	R175H	Yes	Yes	Yes	-	Yes	-	Yes	Yes	-
	R173H	Yes	Yes		-	Yes	Yes	Yes	Yes	-
	R273C	Yes	Yes	Yes	-	Yes	Yes	Yes	Yes	-
	R248W	Yes	Yes	Yes	Yes		Yes	Yes	Yes	
	R2480	Yes	Yes	Yes	Yes	-	Yes	Yes	Yes	
	R282W	Yes	Yes			Yes	Yes	Yes	Yes	
	Y220C	Yes		Yes	Yes	Yes	Yes	Yes	Yes	
	V157F	Yes		Yes	Yes	Yes	Yes	Yes		
	G2455	Yes	Yes		-	-	Yes	Yes	Yes	-
	Y163C	Yes		Yes		-	Yes	Yes	-	-
	R2495	Yes	-	-	Yes	-	Yes	-	Yes	-
KRAS	012D	Yes	Yes		Yes	Yes			Yes	Yes
	G12V	Yes	Yes	-	-	Yes	-	Yes	Yes	Yes
	G12C	Yes	Yes		Yes	Yes		-	Yes	Yes
	G12R	Yes	Yes	-	-	-	-	-	-	-
	G130	Yes	Yes						Yes	Yes
	Q61H	Yes					Yes		Yes	
	012A	Yes	Yes			Yes				
	012S		Yes		-	Yes			Yes	
PIK3CA	E542K	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes	Yes
	E545K	Yes	Yes	Yes	Yes	Yes	Yes	Ovarian	Yes	Yes
	H1047R	Yes	Yes	Yes	Yes	Yes	Yes	Ovarian	Yes	Yes
CTNNB1	S45P	-	-	-	Yes	-	-	-	-	-
	T41A				Yes	Yes				
EGFR	L858R	-	-	-	-	Yes	-	-	-	-
	T790M			-		Yes	-	-	-	-
BRAF	V600E	-	Yes	-		Yes	-	-	-	-
GNAS	R201C	Yes			Yes				Yes	
	R201H	Yes	Yes			Yes			Yes	Yes

B. MACHINE-LEARNING ANALYSIS

The genes were input into *the Cytoscape platform* for discovering the network profiles of quiescent $CD8^+$ cells (or quiescent $CD16^+/56^+$ cell or quiescent $CD11b^+$ based on RNA-seq with rtPCR support), including their seed-proteins and their neighbors (which are derived) for a T-cell or B-cell or macrophage network[64,70]. All networks are displayed in a configuration including Betweenness Centrality (BC), Connectivity Degree (CD), and Clustering coefficient (CC). The quantitative network indicates a higher degree of BC, lower CD, and lower CC, as genes with higher inducing to the immune cells for cell therapy and lower toxicity to induce the immune cells. If we want to find a combination with therapeutic targeting, including drug targeting and small molecule targeting, as shown in Fig-6, the machine-learning analysis is involved in the best optimal combination. If we want to combine CAR-T and targeted therapeutic drugs, we also use the machine-learning model to discover CAR-T therapy

combined with chemotherapy or targeted therapy, for example, as in Fig-6A. Network and layout from patient genomic biomarkers with red color indicate inducing network; Fig-6B. IL12 with IL2 can induce γ-IFN higher expression in the patient; Fig-6C. After personalized immune therapy, the primary hepatocellular cancer (51 years old, female) with a size of about 8x9 cm mass before treatment; Fig-6D. Tumor mass decreased to 6x7 cm with liquefaction after five months of personalized immunotherapy[64, 81-82].

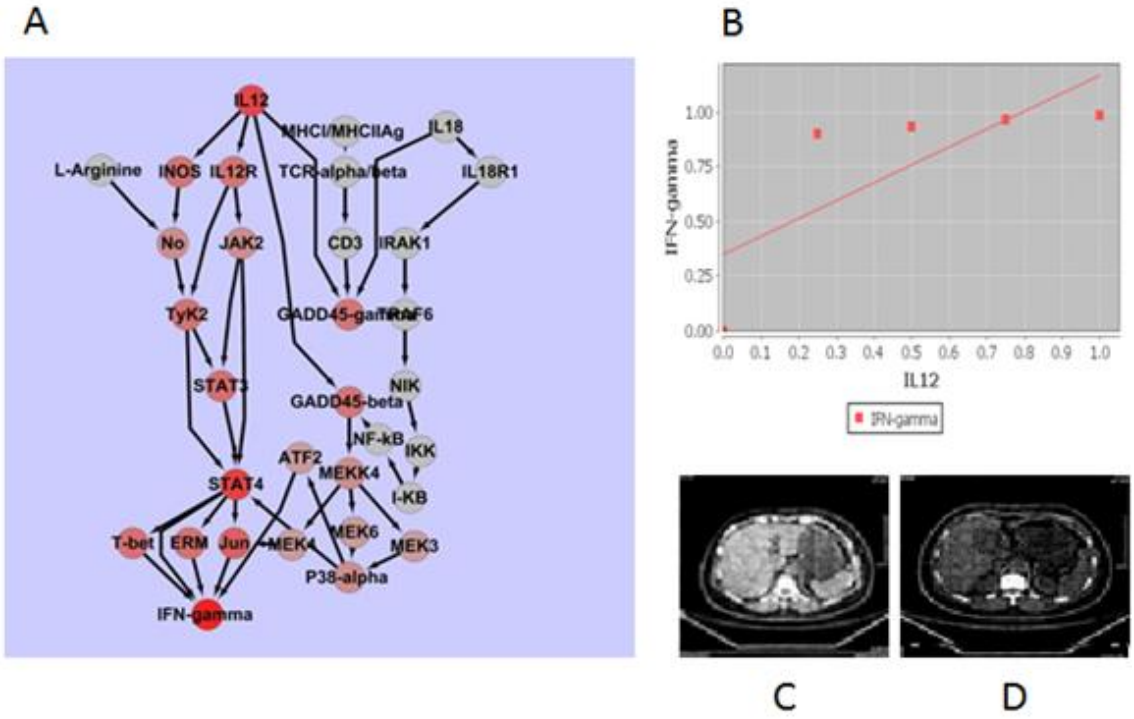

Fig-6. Machine-learning for personalized immunotherapy. A). Network and layout from patient genomic biomarkers with red color indicates inducing network; B). IL12 with IL2 can induce γ-IFN higher expression in the patient; C). A after personalized immune therapy, the primary hepatocellular cancer (51 years old, female) with size about 8x9 cm mass before treatment; D). Tumor mass decreased to 6x7 cm with liquefaction after 5 months of personalized immunotherapy.

Discussion and Conclusion

In this manual, after we set up single-cell genomic techniques and a machine-learning model, we can discover an optimal therapy for each patient. The treatment model combined at least three sources: (I) immune cell therapy such as $CD8^{+}$T-cells or $CD16/56^{+}$ NK cell or CD11b (macrophage or neutrophil) including current all clinical trials for immune cell therapy, (II) targeting immunotherapy with all clinical trials, (III) other all different treatment such as HDACI or DNMA inhibitors and FDA approved drugs so that we can use the machine-learning analysis to resolve three challenge issues from immunotherapy: safe treatment voiding cytokine releasing syndrome (CRS); specific targeting treatment free off-targeting tumor cell; satisfying payment avoiding confounded to clinical patients for higher payment. This study will improve clinical immunotherapy for each patient, or the increase of efficacy, the decrease of adverse effects and the decrease of the cost in clinical applications.

Acknowledgments

Study TIL and primary tumor cells for more than 30 years so that the works were funded by different funds such as the National Science Foundation of China (No. 39370706), USA IGR-91-022-9 for single-cell genomics from TIL; tumor cell genomics analysis (PO-1 75606-3 and RO1 CA60085) to co-work with Dr. Preisler. I have studied and set up TIL and primary tumor cells culture from solid tumors for clinical application for more than 30 years. All acknowledgments are for my colleagues. The mention of trade names or commercial products in this article is solely to provide specific information and does not imply recommendation.

Competing interest statements

The authors declare no financial interests.

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