
Recent Trends in
Biotechnology
MEDDOCS
International

Bioinformatics Tools and Validation Methods for Immune Infiltrates in Tumor Microenvironments and Immunogenomics

Yuchen Liu; Li Wang; Hoi-Lam Ngan; Vivian Wai Yan Lui*

School of Biomedical Sciences, Faulty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR.

Corresponding Author: Vivian Wai Yan Lui

School of Biomedical Sciences, Faulty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong SAR.

Email: vlui002@cuhk.edu.hk

Published Online: Sep 07, 2020

eBook: Recent Trends in Biotechnology

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Abstract

Recent advances in bioinformatics and experimental sciences have expanded researchers' capability to study the interactions of genomic events in cancer with tumor microenvironments. These include a wide range of bioinformatics analyses or estimation of tumor immune cell infiltrates, immune activation or immune suppression activities. These bioinformatics tools, together with validations in the right *in vivo* models, could provide valid and important knowledge to advance the understanding of immunogenomics, which may benefit future design of immunotherapies for cancers. Here, we summarize recent advancements in bioinformatics tools for immune infiltrate estimation in human tumors, and emphasize on how they can be applied for the understanding of tumor genetic changes within the tumor immune microenvironment. Lastly, we will discuss several experimental methods to validate these immuno-genomic bioinformatics findings *in vivo*.

Keywords: Bioinformatics; Immunogenomics

Introduction

There is a tremendous need in the research field to understand how the genetic events in human tumors affect the tumor immune microenvironment. Tumor immune microenvironment is defined as the immune cell components, as well as immune signaling events or interactions found in a tumor. Generally speaking, a tumor can be potentially classified as "immune-hot" or "immune-cold" based on the levels of immune cells or their activation statuses inside the tumor. Besides the fact that various immune cell types can be "attracted" to the tumor side, these immune cells in the tumor can be "active", "inactive" or even "suppressed". Their activities are usually regulated , in a very dynamic manner, by various cytokines secreted by the tumor cells, other tumor-associated cells, as well as other immune cells present in the tumor microenvironment. Such a diverse array of immunological activities and the presence or levels of various immune cell infiltrates can only be analyzed with large-omics studies, such as transcriptomics data, which theoretically contain gene expression profiles of all immune cells and cells within the tumor microenvironment. Though recent advance in flow cytometry can allow purification and partial quantitation of the levels of many immune cells in a tumor, these immune cells, upon isolation, are nevertheless "removed" from the direct tumor microenvironment and may have change in their functional profiles compared to real *in situ* environments. There are methods under development to simultaneously examine many immune cell types and their activities on a tumor slide, yet, the number of cell types and activation status assessable are potentially limited currently. These new methods under development may help further validation of immunogenomics interactions in various cancers, yet, as of today, transcriptome (RNA-seq)-based computational methods remain the main ways of immune infiltrate quantification for immunogenomics analyses. Per large scale genomic efforts of the Cancer Genome Atlas (TCGA Pan-Cancer Atlas), transcriptomic data are already available for 33 pan-cancers [1]. Therefore, a timely review of the methods or tools for transcriptome-based tumor

immune microenvironment for human cancers will be very useful for beginners of the cancer immuno-genomic field. At the same time, growing knowledge on how tumor genetic events can shape the tumor immune microenvironment will help the design of new immunotherapies and likely adjuvants to enhance the antitumor efficacies of nowadays immunotherapies. Thus, we will also introduce some research methods recently employed by researchers to validate the bioinformatics results related to tumor genetic events regulating tumor immune microenvironment.

Bioinformatics tools and methods in the quantification and estimation of immune infiltrates in human tumors

In human, there are 6 major immune cell types, namely B lymphocytes, T lymphocytes, Natural Killer (NK) cells, macrophages/monocytes, Dendritic Cells (DC) and neutrophils. In terms of activity to fight against the foreign bodies or tumor cells, B lymphocytes, T lymphocytes, NK cells, macrophages, and neutrophils are the key players. Dendritic cells are the ones responsible for antigen presentation. While B and T lymphocytes are capable of developing memories over time to target the same "enemy" that they were previously exposed to. While NK cells, macrophages and neutrophils can engulf foreign bodies and eliminate them from our body. Many of these immune cells can release cytokines to communicate with each other and elicit other regulatory functions among them. There are various subtypes for these immune cells being characterized and each of them are known to elicit different specific functions in the immune system. In fact, as of today, there are still immune cell types being identified and the list will surely keep increasing in the near future.

In cancer, there is an increasing need to understand the tumor immune landscape to facilitate the development of new and effective immunotherapy. Over the past decade, prompted by the availability of "information rich" transcriptomic data (RNA-seq) for over 30 cancer types by TCGA, there have been increasing efforts to use computational methods to dissect the immune tumor microenvironment in various cancers, as well as in individual patient tumors using these data. Several major computational methods have been specifically developed to estimate or calculate the tumor-infiltrating levels of multiple immune cell types, ranging from anywhere around 6 immune cell types (TIMER) to as many as 64 immune and non-immune cell types (xCell) [2-5].

Currently, there are mainly two classes of immune cell estimation methods: marker gene-based methods and deconvolution-based methods (**Table 1**). Maker genes methods calculate immune cell infiltration scores with the expression values of marker gene lists that characterize respective immune cell types. The marker gene lists are extracted from multiple immune cell RNA-Seq datasets, extensive literature reviews and most likely are experimentally validated. The single sample Gene Set Enrichment Analysis (ssGSEA) developed by Hanzelmann *et al.* in 2013, [6] and its extension, xCell [3] are the most widely-known marker gene-based methods. Both methods calculate the enrichment scores for marker gene pre-defined cell types. ssGSEA is a simple technique that can calculate the enrichment score of any genesets, including immune genesets, in a simple sample according to the ranking of their expression levels. The xCell method defines gene signatures (marker genes) for as many as 64 cell types (of which 62 are immune cells or immune cell progenitors, 2 are fibroblast and endothelial cells), calculates and adjusts the ssGSEA scores of all these cell types. The ssGSEA score adjustment will transform the scores to linear scales and avoid the mixture of closely related cell types. Earlier tumor immune infiltrate estimation has employed ssGSEA and characterized the immune landscape in Head and Neck Squamous Cell Carcinoma (HNSCC) [7], clear cell renal cell carcinoma [8] and breast cancer [9]. In 2016, Becht *et al.* developed the Microenvironment Cell Populations-counter (MCP-counter) method [10], which is another marker gene-based method for immune cell quantification of 8 immune cell types, cytotoxic score as well as fibroblasts and endothelial cells within the tumor microenvironment. This method generates an MCP-counter score by calculating the log2 geometric mean of marker genes for each immune cell types. In principle, the three major marker gene methods can quantify each immune cell type individually, and prevent possible signal spill over by modifying the marker gene list. However, there exists major caveat that all the marker-gene based methods could only generate semi-quantitative scores instead of exact immune cell fractions within a tumor, thus the scores could only be compared between samples semi-quantitatively. These methods cannot be used to compare the quantitative levels of different immune cell types within one sample.

Table 1: Summary of Immune Cell Estimation Methods.

Method	Approach	Algorithm	Estimated Cell types	Year	Advantage	Limitati-on	Website/source ode	Citations by June-2020
CIBEROS-RT	Deconvolution with signature matrix	*v*-support vector regression	22 immune cell types: B cell naïve, B cell memory, B cell plasma, T cell CD8+, T cell CD4+ naïve, T cell CD4+ memory resting, T cell CD4+ memory activated, T cell follicular helper, T cell regulatory (Tregs), T cell gamma delta, NK cell resting, NK cell activated, Monocyte, Macrophage M0, Macrophage M1, Macrophage M2, Myeloid dendritic cell resting, Myeloid dendritic cell activated, Mast cell activated, Mast cell resting, Eosinophil, Neutrophil	2015	1) The most widely used deconvolution-based immune cell estimation method. 2) Web interface, user friendly.	Result can only be compared between cell types within sample, but not between samples.	https://cibersort.stanford.edu/	1546
CIBEROS-RT abs. mode	Deconvolution with signature matrix	*v*-support vector regression	22 immune cell types: B cell naïve, B cell memory, B cell plasma, T cell CD8+, T cell CD4+ naïve, T cell CD4+ memory resting, T cell CD4+ memory activated, T cell follicular helper, T cell regulatory (Tregs), T cell gamma delta, NK cell resting, NK cell activated, Monocyte, Macrophage M0, Macrophage M1, Macrophage M2, Myeloid dendritic cell resting, Myeloid dendritic cell activated, Mast cell activated, Mast cell resting, Eosinophil, Neutrophil	2018	1) Results can be compared both between cell types within sample and across samples. 2) Web interface, user friendly.	Results reflect the absolute proportion of each cell type, but are not immune cell fraction.	https://cibersort.stanford.edu/	116
TIMER	Deconvolution with reference sample expression dataset	linear least square regression	6 immune cell types: B cell, T cell CD4+, T cell CD8+, Neutrophil, Macrophage and Myeloid dendritic cell	2016	1) The only method takes tumor purity into immune cell estimation by now. 2) Web interface, user friendly.	1) Result can only be compared between samples within cancer type, but not be compared between cell types or cross cancer types. 2) Result of one sample depends on all samples submitted together in a single run.	http://timer.cistrome.org/	387
EPIC	Deconvolution with signature matrix	constrained least square regression	5 immune cell types, fibroblasts, endothelial cells and uncharacterized cells. Immune cells are: B cell, T cell CD4+, T cell CD8+, Macrophage, NK cell	2017	1) Results are absolute immune cell fraction relative to total amount of cells, which can be compared both between cell types within sample and across samples. 2) Web interface, user friendly	Limited immune cell types.	http://epic.gfellerlab.org	84

quan-TIseq	Deconvolution with signature matrix	constrained least square regression	10 immune cell types and uncharacterized cells. Immune cell types are: B cell, Macrophage M1, Macrophage M2, Monocyte, Neutrophil, NK cell, T cell CD4+ (non-regulatory), T cell CD8+, T cell regulatory (Tregs), Myeloid dendritic cell	2019	1) Results are absolute immune cell fraction relative to total amount of cells, which can be compared both between cell types within sample and across samples. 2) Provide entire pipeline that can analyze raw RNA-Seq data.	Most recent deconvolution tool, which is not widely used and broadly validated.	http://icbi.at/quantiseq	31
MCP-counter	Marker Gene	mean of marker gene expression	8 immune cell types, cytotoxicity score, fibroblasts, endothelial cells. Immune cell types are: T cell, T cell CD8+, NK cell, B cell, Monocyte, Macrophage/Monocyte, Myeloid dendritic cell, Neutrophil	2016	Highly specific signature with low background prediction.	Result can only be compared between samples, but not be compared between cell types	http://github.com/ebecht	284
xCell	Marker Gene	expression ssGSEA	64 immune and non-immune cell types, 3 scores. Immune cell types are: B cell, B cell memory, B cell naive, B cell plasma, T cell CD4+ memory, T cell CD4+ naïve, T cell CD4+ (non-regulatory), T cell CD4+ central memory, T cell Cd4+ effector memory, T cell CD8+ naïve, T cell CD8+, T cell CD8+ central memory, T cell CD8+ effector memory, Class-switched memory B cell, Common lymphoid progenitor, Common myeloid progenitor, Myeloid dendritic cell, Myeloid dendritic cell activated, Eosinophil, Granulocyte-monocyte progenitor, Hematopoietic stem cell, Macrophage, Macrophage M1, Macrophage M2, Mast cell, Monocyte, Neutrophil, NK cell, T cell NK, Plasmacytoid dendritic cell, T cell gamma delta, T cell CD4+ Th1, T cell CD4+ Th2, T cell regulaory (Tregs). Non-immune cell types: Endothelial cell, fibroblast. 3 scores: immune score, stroma score, microenvironment score	2017	1) Low background prediction. 2) Provide as much as 64 immune and non-immune cell types with 3 scores. 3) Web interface, user friendly	1) Result can only be compared between samples, but not be compared between cell types. 2) Result of one sample depends on all samples submitted together in a single run	https://xcell.ucsf.edu	307

The second class of computational methods are the deconvolution methods. For deconvolution methods, the gene expression profile of a single sample is considered as the convolution of the gene expression of different cell types, and the deconvolution methods estimate the fraction of each immune (and non-immune) cell types in a sample through regression with cell type-specific signature matrix or expression profile. Cell-type Identification by Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) [11], CIBERSORT Absolute mode (CIBERSORT-ABS) [12], Tumor Immune Estimation Resource (TIMER) [2,4,5], Estimating the Proportion of Immune and Cancer cells (EPIC) [13] and quantification of the Tumor Immune contexture from human RNA-seq data (quanTIseq) [14] are the recently developed methods with different deconvolution algorithms (detailed in **Table 1**).

CIBERSORT is the most widely used deconvolution method with ν-support vector regression algorithm. It can estimate as many as 22 immune cell types and calculate the relative immune cell fraction within a patient tumor. However, the results of CIBERSORT can only be used for comparison of levels of various immune cell types within a single sample, but not meant for comparisons across different samples [11]. The results are expressed as fractions relative to total immune-cell content within a single sample. An extension of CIBERSORT, known as CIBERSORT-ABS (absolute mode of CIBERSORT) was developed in 2018 by Chen et al. [12] to allow comparisons of levels of 22 immune cell types both within one sample, as well as between samples. The results of CIBERSORT-ABS are scores that reflect the absolute proportion of immune cell types.

TIMER, developed by Li et al., was the first, and still the only method by now that takes tumor purity into account when estimating the abundancy levels of immune cell infiltrations. In this method, the immune marker genes are selected dynamically according to the tumor purity of the input sample, that the immune genes whose expression levels are negatively correlated with tumor purity are considered as immune markers. It uses the linear least regression method for deconvolution with reference sample (immune cell line) expression datasets for 6 immune cell types (B cell, $CD4^+$T cell, $CD8^+$T cell, neutrophil, macrophage and myeloid dendritic cell). The results of TIMER are in arbitrary unit and only comparable between samples of the same cancer type [2,4,5].

Two other deconvolution methods, EPIC and quanTIseq, both perform constrained least square regression to calculate immune cell fractions with immune cell signature gene expression matrix (signature matrix). The main difference between EPIC and quanTIseq is that EPIC can calculate the abundances of 5 immune cell types, fibroblasts and endothelial cells, while quanTIseq calculates the levels of 10 immune cell types. Since both methods estimate the absolute immune cell fraction in a sample, they can be utilized for comparisons of various immune cell types within one sample, as well as for samples within the same cancer types or even across cancer types [13,14].

A comprehensive analysis using single cell RNA-seq datasets to simulate bulk RNA-seq data has been performed to assess and compare the accuracy of different immune cell estimation methods [15]. Moreover, the experimental gold standard (fluorescence-activated cell sorting and immunohistochemistry) to evaluate immune cell infiltrations has also been included in this study. In general, the deconvolution methods are more accurate for well-defined immune cell types, while the marker gene-based methods show lower background prediction (background prediction means a false positive prediction when an immune cell type is actually absent from a tumor but the computational method turns out to predict its presence) and more suitable for absence/presence prediction. Therefore, for a simple present or absent calculation of various immune cell types, one can simply employ MCP-counter and xCell. For a more reliable quantitative comparison within the same cancer type, CIBERSORT, CIBERSORT-ABS, TIMER, EPIC and quanTIseq could be used. While for cross cancer type comparisons, CIBERSORT-ABS, EPIC, quanTIseq, xCell and MCP-counter are more suitable. Many of these methods are supported by user-friendly online tools and they can be accessible for online analysis in very simple ways: The respective websites are listed here: CIBERSORT/CIBERSORT-ABS (https://cibersort.stanford.edu/), TIMER (http://timer.cistrome.org/), EPIC (http://epic.gfellerlab.org) and xCell (https://xcell.ucsf.edu/).

As mentioned above, currently, we can use multiple computational methods (or even open tools) available for the estimation of immune cell infiltration abundance for different scenarios or comparisons. However, these methods are still not perfect. The marker gene-based methods (xCell and MCP-counter) only generate semi-quantitative scores that represent the enrichment of cell types, but not the real immune cell fractions within a sample, and the deconvolution methods (CIBERSORT, CIBERSORT-ABS, TIMER, EPIC and quanTIseq) have the background prediction problem especially for the low-abundance cell types. So in the future, it would be ideal to develop the new marker gene based-algorithm that could estimate immune cell fractions directly. Vice versa, to overcome the low abundance immune cell estimation problem in deconvolution method, it would be important to refine reliable signature matrix to avoid possible false-positive background calculation. Furthermore, with the emergence of even more new immune cell subtypes or subtypes with various activation statuses, new methods for the estimation of even a wider variety of immune cell types with various activation statuses should be developed.

Immune-related scores in the estimation of immune-hotness of a tumor

Tumors with higher immune cell infiltration levels are known as the “immune hot” tumors. Emerging clinical findings in the area of immunotherapy showed that patients whose tumors are heavily infiltrated with immune cells, in particular, $CD8^+$T cells, appeared to have higher response rates to Immune Checkpoint Inhibitor (ICI) treatment (**Table 2**) [16]. The reason is the presence of high levels of immune cell army, especially those that can efficiently eradicate tumor cells, can elicit effective antitumor responses at the tumor site once the anti-immune suppressive immune checkpoint signals are removed by ICI [17]. Thus, there exists an important and practical need to help define an “immune hot” tumor in clinical setting. As for cancer biology, unlocking the molecular or pathologic reasons that drive the shaping of the immune-hotness of a tumor can promote our understanding of human tumorigenesis and cancer progression.

Table 2: Summary of immune checkpoint molecules.

Classification	Immune checkpoints	Binding parters	Reference
Inhibitory checkpoint molecules	PD-1	PD-L1/PD-L2	[18]
	CLTA-4	B7-1/B7-2	[19]
	?	B7-H3	[20]
	?	B7-H4	[21]
	VISTA	?	[22]
	TIM-1	?	[23]
	TIM-3	CEACAM1 /Galectin-9	[24]
	LAG-3	MHC	[25]
	BTLA	HVEM	[26]
	CD160	HVEM	[27]
	LIGHT	HVEM	[28]
	CD47	SIRPα	[29]
	2B4 (CD244)	CD48	[30]
	KIR	MHC Class I	[31]
	IDO	?	[32]
Stimulatory checkpoint molecules	TCR	MHC	[33]
	CD40L (TNFSF4)	CD40	[34]
	OX40	OX40L	[35]
	ICOS	ICOSL	[36]
	GITR	GITRL	[37]
	CD28	B7-1/B7-2	[38]
	CD27	CD70	[39]
	4-1BB	4-1BBL (TNFSF9)	[40]
	?	B7-H3	[41]

Currently, there are several immune-related scores being utilized for the estimation of immune-hotness of a tumor (**Table 3**). Some of which are based on the knowledge that key anti-tumor cytokines, such as IFN-γ, are crucial for the activation of anti-tumor immune cells, including NK cell, Cytotoxic T cell and DC [42]. Whilst others examined immune-related genes to look at the levels of genes essential for eliciting anti-tumor cytolytic activity by T cells.

Table 3: Summary of immune-related scores.

Immune-related score	Gene used in calculation (If applicable)	Algorithm	Source
IFNG-score			
6-gene score	IDO1, CXCL10, CXCL9, HLA-DRA, STAT1, IFNG	weighted arithmetic mean of gene expression (TPM or quantile normalized, log10 transformed raw counts)	[43]
18-gene score	CD3D, IDO1, CIITA, CD3E, CCL5, GZMK, CD2, HLA-DRA, CXCL13, IL2RG, NKG7, HLA-E, CXCR6, LAG3, TAGAP, CXCL10, STAT1, GZMB		
Cytolytic score (CYT)	GZMA, PRF1	geometric mean of gene expression (offset 0.01)	[44]
Cytotoxic score	CD8A, EOMES, FGFBP2, GNLY, KLRC3, KLRC4, KLRD1	mean of marker gene expression	MCP-Counter [10]
T-effector (T-eff) score	GZMA, GZMB, PRF1, IFN-γ, EOMES, and CD8A	geometric mean of gene expression (offset 0.01)	[45]

Immune score	NA	sum of all immune cell types calculated in xCell	xCell [3]
microenvironment score	NA	sum of all immune and stromal cell types calculated in xCell	xCell [3]

IFN-γ is secreted by activated T cell, NK cell and Natural Killer T (NKT) cells. It plays a critical role in stimulating $CD4^+$ T helper 1 cells, $CD8^+$ cytotoxic T cell, NK cells, DCs and macrophages and inhibition of regulatory T cells (Treg), $CD4^+$T helper 2 cells and Th17 cells in the antitumor processes [42]. To define immune-related gene expression signature associated with response to pembrolizumab (PD1 inhibitor), IFN-γ signaling was found. Thus, IFN- γ score was developed [43]. The 6 gene and extended 18 gene IFN- γ scores have been validatd in HNSCC [46] and gastric cancer [43] that tumors displaying high IFN-γ scores are associated with statistically significant improved patient prognosis, respectively [43,46], and the IFN- γ scores could predict better anti-PD-1 therapy response [43].

Activated $CD8^+$ T and NK cells will elicit tumor cell lysis, thus achieving antitumor acitivity in a patient. The cytolytic (CYT) score which indicates cytolytic activity of infiltrated immune cells has been develped by Rooney et al. [44]. This score was first utliized to identify genetic abberations (amplification and recurrent mutations) that positlvely associated with cytolytic activity. CYT scores are strongly associated with cytolytic lymphocytes markers as expected. Moreover, high CYT-score alone could predict better patient outcome in pan-cancer, as well as in HNSCC [44,46].

The effector T-cell signature (T-effector score), which was first developed by Bolen et al.. It can reflect functional immune response elicited by $CD8^+$T cells [45]. This T-effector score is computed based on the expression levels of *GZMA, GZMB, PRF1, IFNG, EOMES* and *CD8A* genes in a tumor. Among which, *GZMA, GZMB, PRF1* are genes that are upregulated for anti-tumor responses elicited by $CD8^+$T cells. Several studies has employed this T-effector score. In melanoma, the T-effector score was found to be highly correlated with Tumor-Induced Plasmablast-like-enriched B cell population (TIPB) signature [47]. In pan-cancers, this score was found to be closely associated with a tumor-infiltrating B cell marker, *GPR18* expression, which indicated a functional crosstalk between *GRP18* and effector T cells in as many as 28 cancer types, including HNSCC, lung, liver, kidney and breast cancers [48].

In addition to the above three immune activity scores, several other immune cell infiltration estimation methods, such as MCP-counter and xCell can also calculate cytotoxic score and immune/microenvironment scores. The cytotoxic score calculation in MCP-counter is based on the geometirc mean of *CD8A, EOMES, FGFBP2, GNLY, KLRC3, KLRC4* and *KLRD1* expression levels, which represents the cytotoxic lymphocyte levels in a patient tumor [10]. In xCell, the immune and microenviroment scores are the sum of all immune cell types and all immune and stromal cell types, respectively [3]. These immune-related scores may help identifying “immune hot” tumors vs. “immune cold” tumors for clinical and preclinical settings.

The IFN-γ, CYT, T-effector scores, as well as the cytotoxic scores are simple mathematical or geometric means of short gene lists. They are easily to be used for multiple purposes, such as for the prediction of tumor hotness and ICI treatment response with limited gene expression data (instead of whole set of RNA-Seq data). However, these immune scores also have their limitations, e.g. unclear cutoffs for a clear definition of immune “hot” or “cold” tumors in different cancer types. Thus, further systematic analysis may be required to clarify the classification or definition of immune-hotness with these scores in pan-cancers.

Somatic mutations shaping tumor immune microenvironment

The current ICI has shown good clinical efficacy in a wide range of cancer types, demonstrating success of cancer immunotherapy. Though the response rate is currently around 20% in most cancer, it nevertheless, represents a new direction for anticancer therapy, especially for patients who exhausted treatment options. In general, Programmed death-ligand 1 (PD-L1) expression in tumor cells and Tumor Mutation Burden (TMB) are considered as clinical biomarkers for the use of ICIs. However, these biomarkers are far from predictive of ICI clinical outcomes in patients as reported by many. This is likely due to multifactorial involvement at the tumor immune microenvironment which enables ICIs to elicit good antitumor responses in patients [49]. For instance, the immunogenicity of the tumors, which affects the tumor immune microenvironment, can potentially influence ICI treatment outcomes. Recently, emerging efforts in the field of immunogenomics are trying to identify and discover genomic determinants that can shape a patient’s tumor immune microenvironment. These studies could help identify clinically effective peptides or neo-antigens which can shape or prepare the tumor immune microenvironment of a patient to potentially increase the antitumor efficacy of ICIs in cancer patients, By integrating the tumor immune infiltration levels, or the immune-related scores with a single gene mutation or multiple mutations of question, one can determine the association of genetic events with the TIL levels and immune-related scores introduced above. With further validations in animals, one can then confirm the immune activating or tumor immune microenvironment-shaping ability of these peptides or neo-antigens. Here, we summarize some recently identified genetic mutations that have been found to be correlated with tumor immune microenvironment activities.

Mitogen Activated Protein Kinase (MAPK) pathway mutations have recently been reported to cause marked tumoral infiltration of several anti-tumor lymphocytes in HNSCC. These include: $CD8^+$ T cells, dendritic cells and neutrophils [46]. Moreover, significantly enhanced T cell-related cytolytic immune response gene signatures were observed in MAPK pathway mutated vs wild type HNSCC tumors, suggesting active T cell recruitment and activity in MAPK pathway-mutated HNSCC tumors. The study further validated in immunocompetent HNSCC mouse models that the mouse equivalences of human *HRAS* p.G12V mutation and *MAPK1* p.E322 and p.D321 mutations could directly recruit $CD8^+$ T cell infiltration into the HNSCC tumors, and cause increased apoptosis of tumor cells. Of note, two independent pan-cancer ICI cohorts showed that MAPK pathway mutated patients appeared to have better clinical outcomes upon ICI treatments. This study suggested that MAPK pathway mutations could shape a T cell-inflamed immune microenvironment in HNSCC, which may potentially benefit T-cell-based immunotherapies. These findings warrant future clinical investigations in related immunotherapy settings.

The immunogenicity of MAPK pathway mutations in HNSCC is further supported by a study by Lyu et al. The author reported that *HRAS* mutated HNSCC tumors were associated with higher expression of *HLA*-genes, $CD8^+$ T cell marker, and cytolytic/pro-inflammatory markers including granzyme B and perforin 1 when compared with wild type [50]. The study further identified that *TP53*–mutated HNSCC tumors were associated with reduced expression of Human Leukocyte genes (*HLA*) genes (including *HLA-DOA, HLA-DOB, HLA-DRA, HLA-DMA, HLA-DMB, HLA-DRB1, HLA-DRB5, HLA-DRB6, HLA-DQB1, HLA-DQB2, HLA-DQA1, HLA-DQA2, HLA-DPA1, HLA-DPB1, HLA-DPB2*), tumor-infiltrating markers (including B cell, $CD8^+$ T cell and NK cell markers), as well as the cytolytic markers indicative of an "immune-cool" microenvironment. Whilst *HRAS-mutated* HNSCC appeared to have increased HLA expressions (including *HLA-F, HLA-C, HLA-J, HLA-DQB1, HLA-DRB5, HLA-DPA1, HLA-DRB1, HLA-DOB, HLA-DMA, HLA-DRQ, HLA-DRBB, HLA-DQA1, HLA-DPB1).*

In colorectal cancer, Tran *et al.* showed that mutation of one of the MAPK pathway genes, *KRAS* p.G12D mutation, represented a neo-epitope well-recognized by HLA-C*08:02 allele, thus recognized by HLA-C*08:02-restricted tumor-infiltrating lymphocytes with demonstrated specificity against such a mutation [51]. Interestingly, subsequent *in vivo* experiment showed that tumors that progressed with infusion of these neo-antigen specific lymphocytes had further acquired the loss of chromosome 6 event that corresponded to HLA-C*08:02 (Class I MHC molecule), thus resulting in immune evasion. In gastrointestinal cancer, Li *et al.* found that in stomach cancer and colon cancer, *ARID1A* mutations were associated with significant increases in tumoral infiltrations of $CD8^+$T cells, NK cell, activated CD^+4 cells, and activated dendritic cells, as well as increased in cytolytic activity as revealed by ssGSEA. These *ARID1A*-mutated tumors also displayed elevated PD-L1 expression, and appeared to demonstrated favorable responses with immune-checkpoint inhibitors [52].

The impact of mutational landscape on tumor immune microenvironment in lung cancer is well-studied. *KRAS* mutations represent common driver mutations in Non-small Cell Lung Cancer (NSCLC) and indicates poor prognosis. Recent clinical trials identified that this subgroup of *KRAS* mutated tumors were actually more responsive to ICIs then conventional chemotherapies [53]. Many studies demonstrated that these *KRAS* mutated tumors are associated with increased PD-L1 expression, $CD8^+$ T cell/$CD66b^+$ cell infiltration and immune signature expression [54,55]. However, robust efficacy of ICIs on patients with specific *KRAS* mutations are still to be fully elucidated in clinical settings (KEYNOTE-042) [55]. Further genomic characterization identified that when a loss-of-function mutation of *STK11/LKB1* gene was found to be co-mutated with the *KRAS* gene, PD-L1 downregulation was observed, causing resistance to ICI in *KRAS* mutated lung cancer [56,57]. The clinical relevance of these findings is still being examined carefully at the moment. While *KRAS* and *TP53* co-mutations were associated with higher PD-L1 expression, PD-1 expression, CTLA-4 expression, higher infiltration of $CD3^+$/$CD8^+$/$CD45RO^+$ lymphocytes and high TMB predicting better ICI outcomes [56,58]. *TP53* mutation alone in lung cancer can also drive upregulation of T-effector and interferon-γ gene signatures reflecting the immune-reactive nature of the *TP53* mutant tumors [58]. These studies demonstrated the complex interplay of co-mutated genomic events that heavily modulate the tumor immune microenvironment, can influence patient response to ICIs. Besides, *NOTCH1/2/3* mutations are associated with anti-tumoral M1 macrophage infiltration, $CD4^+$ T cell/$CD8^+$ T cell/NK cell activation as well as enhanced antigen processing/antigen presentation signatures and can help predict ICI response [59]. Of note, the immuno-active phenotype is not observed in *NOTCH4* mutated NSCLC tumors.

In melanoma, loss-of-function mutations of *ATR* gene were found to trigger infiltration of pro-tumorigenic M2 macrophage, reduction of $CD4^+$ T cells and upregulation of PD-L1 expression [60]. *BRAF* p.V600E/K mutations are common driver mutations in melanoma. Using *in vivo* models, Chen *et al.* demonstrated that upon combination therapy of BRAF inhibitor and MEK inhibitor, immune competent mice with *BRAF* p.V600E mutant tumors survived longer than those in immune compromised NOD/SCID gamma (NSG) mice, and such a good response to the combination therapy appeared to be associated with immune-stimulatory cell death of BRAF p.V600E mutated melanoma (i.e. immunogenic pyroptotic cell death) [61]. This was one of the first reports demonstrating drug-induced cell death of tumors in active shaping of the tumor immune microenvironment.

To conclude, the somatic mutations accumulated during tumor evolution can heavily modulate the tumor immune microenvironment by regulating the expression of PD-L1, immuno-active gene signatures as well as TILs infiltration. The immune-modulating effects of different somatic mutations may outweigh each other when co-occurred within the same tumor. Owing to the heterogeneity nature of a tumor, a single genomic mutation, in some cancers or some individuals, may not be a strong enough immunostimulant or neo-antigen. Therefore, more efforts are required to identify the immuno-modulating effects of somatic mutations of human tumors. Nevertheless, the currently identified immuno-modulating somatic mutations do serve as essential clues to study genomic influences on immunotherapy responses in patients. In the future, comprehensive genomic characterization of a patient tumor may facilitate actual precision use of immunotherapies for patients.

Methods of validation for immuno-genomic interactions *in vivo*

Over the years, various methods have been developed to determine the immunogenicity of a specific protein sequence, or a specific gene. The protein sequence or gene of interest can be derived from hypothesis-driven approaches by investigators, or by *in silico* (computational) prediction of antigenic peptides. The peptide or DNA vector expressing the gene of interest will be then delivered into an immunocompetent mouse model, such as that of C57BL/6, or into autologous antigen-presenting cells co-cultured with patient-derived T cells, to see if such peptides or genes would mount an antigen-specific immune response (**Figure 1**). Successful mounting of an anti-neoantigenic response will result in the production of cytokines (e.g. IFN-γ, TNF-α) and an antigen-specific $CD8^+$T cell response upon neoantigen re-exposures. This can be quantitatively measured by Enzyme-Linked Immunospot assay (ELISpot) [62] or by intracellular cytokine staining followed by measurements with MHC multimer assays (an assay which helps identifying antigen-specific T cells by quantifying the antigenic peptide-MHC tetramer/pentamer-TCR complex formation) [63]. In addition to these standard methods, sequencing and analysis of the cognate T-Cell Receptors (TCR) can also be applied for the identification of clonal T cell responses to neo-antigens [64]. Using these methods, studies have validated a variety of neo-antigens, such as *MTFR2* p.D326Y, *CHTF18* p.L769V and *MYADM* p.R30W for non-small cell lung cancer [65], *B-RAF* p.V599E mutation for melanoma [66], *TP53* p.Y220C and *TP53* p.G245S for ovarian cancer

[67], *KRAS* p.G12D and *ERBB2IP* p.E504G for metastatic colon cancer and cholangiocarcinoma [51]. More importantly, to further confirm the anti-tumor immunogenicity of a neoantigen of interest, mice could be prophylactic vaccinated with the neoantigenic peptide prior to tumor implantation to determine whether the neoantigen-specific immune response could protect the animals from tumor establishment. Furthermore, co-cultures of neoantigen-activated T cells with neoantigen-expressing cancer cells can also be used to determine cytolytic activity of T cell towards the cancer cells expressing the neoantigen in question [68]. Another approach is Adoptive Cell Transfer (ACT) therapy, which utilizes purified neoantigen-reactive $CD4^+$T cells and $CD8^+$ cytotoxic T cells which have been genetically engineered to express tumor-reactive TCRs or Chimeric Antigen Receptors (CAR) to help these antitumor immune cells reaching the tumor cells more effectively, thus eradicating the tumor [69]. Recently, Trans *et al*. have shown that $CD8^+$ T cells specific to *KRAS* p.G12D mutation could result in a nearly complete tumor regression in metastatic colon cancer [51].

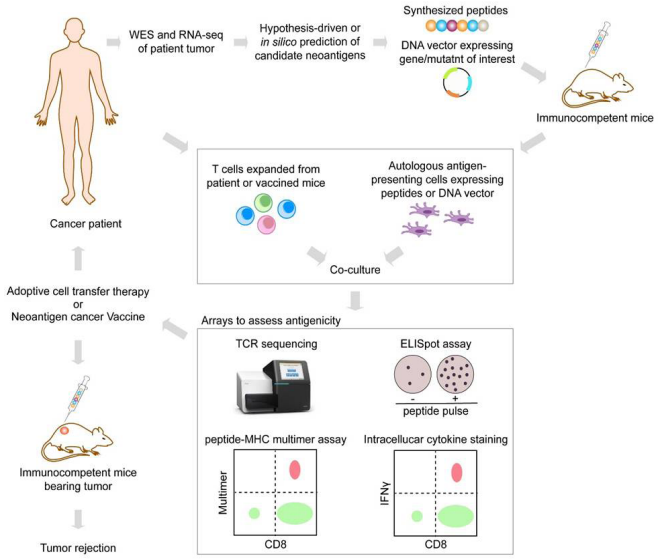

Figure 1: Overview of identifying and evaluating the immunogenicity of candidate neoantigens.

Whole-exome sequencing data and RNA-sequencing data from patient tumor and blood were obtained to identify and predict the candidate neoantigen of interest. Tandem minigenes inserted in DNA vector or synthesized peptides for candidate neoantigen could be expressed in Antigen-Presenting Cells (APCs) or immunocompetent mice. T cells from patients' tumor or blood, or from the splenocytes of vaccinated mice can be co-cultured with autologous APCs pulsed with neoantigen peptides to identify antigen-specific T cells using enzyme-linked immunospot (ELISpot) assay, intracellular cytokine staining, or peptide-MHC multimer assay. The antigen-reactive T cells could then be sequenced to identify the antigen-specific T-Cell Receptor (TCR). Candidates are then selected to generate neoantigen-based cancer vaccine or develop TCR-engineered adoptive T-cell transfer therapy, which could be applied in immune competent mice or patients to assess the T cell response. Successful immunogenic candidate-based therapy can, theoretically, achieve tumor rejection.

It is of increasing realization that the antigenic nature of a gene or a mutant gene does not only lie in the protein sequence itself. In fact, the biological activity of a wild type or mutant gene or protein could also contribute to its ability to shape the tumor immune microenvironment. This is exemplified by recent discovery of an array of immune checkpoint molecules, such as PD-L1, PD-L2, Galectin-9, VISTA, B7-H3, B7-H4, B7-1, B7-2 and MHC on tumor cells or antigen-presenting cells in the tumor could shape and set the tone of immunoactive or immunosuppressive state in a tumor [18-22,24,25,33-38,40,41,70]. A list of those immune checkpoint molecules and their interacting partners are shown in **Table 2**. These molecules, upon interaction with their respective binding partners, will elicit immune suppressive action in the tumor. It was hypothesized that gene amplification or overexpression of PD-L1, for example, could cause immunosuppression *in situ*. In fact, an experimental validation has been performed to demonstrate that when PD-L1 overexpressed tumor cells were introduced into mice, it will cause immunosuppression by inducing T-cell apoptosis [71]. Conversely, overexpression of immune activating genes, mostly antitumor cytokines, such as *IL-2* and *IL-12* could result in increased immunogenicity against cancer, validated *in vivo* [72-74].

Most recent findings also highlighted an increasing number of unconventional signaling molecules regulating the tumor immune microenvironment. For example, in HNSCC, an ErbB3 (HER-3) analog peptide with a 73% amino acid homology to ErbB3 amino acid 872-866 (peptide sequence: KTPIKWMALESIHFG) has been demonstrated to induce $CD4^+$T-cell response which directly recognize and kill HNSCC cells [75]. In breast cancers that express HER2, they have been found to be associated with tumoral infiltration by $FoxP3^+$ regulatory T cells, suggesting the presence of an immunosuppressive environment in these tumors [76]. *N-MYC* amplification has also been demonstrated to be associated with reduced T-cell infiltration and suppressed interferon pathway activity in metastatic neuroblastoma [77]. Similarly, in pancreatic ductal adenocarcinoma, amplification of *FGFR1, NOTCH2 and MYC* have all been shown to be associated with suppressed cytotoxic T cell function [78]. Furthermore, NF1 deficiency has also been reported to be linked with increased levels of macrophages in glioblastoma [79]. All these recent findings reveal the need for better modeling of the entire gene/protein, entire mutant gene/protein, perhaps not just a peptide stretch. Therefore, to validate such an immunoregulatory activities of a gene/protein-of-interest, one would be required to express the entire protein in a cellular context relevant to the tumor type under study.

Due to the increasing knowledge on how a specific gene or a specific mutation could cause changes in a tumor microenvironment, there is a demanding need to engage immunocompetent tumor models as they largely mimic immune response patterns in humans (**Figure 2**). Although the tumors carried by these models are not directly derived from human, these models do carry syngeneic tumors of the same tumor type under investigations. For instance, C3H/HeJ syngeneic immunocompetent mouse model has recently been utilized to validate the immunoreactive effect and $CD8^+$T cell-attracting activities of MAPK pathway mutations when transplanted with an engineered mouse HNSCC cell line SCCVII [46]. The immunogenicity and specificity of multiple genetic events (e.g. *Tubb3* p.G402A, *Kif18b* p.K739N, *Mthfd11* p.F294V, *Gnas* p.S112G and *Tm9sf3* p.Y382H) from B16F10 mouse melanoma cells have been experimentally validated in C57BL/6 mice [80]. Using syngeneic FVB/n mice, Noblitt *et al.* showed that MT1A2 cells infected with EpherinA1 failed to form tumors in those mice, validating a role of EpherinA1-EphA2 complex in inhibiting the tumorigenic potential of breast cancer [81]. The advantage of using immunocompetent syngeneic mouse models easy experimentation with reproducible results. However, the fast kinetics of tumor growth of many of these syngeneic mouse tumor models often

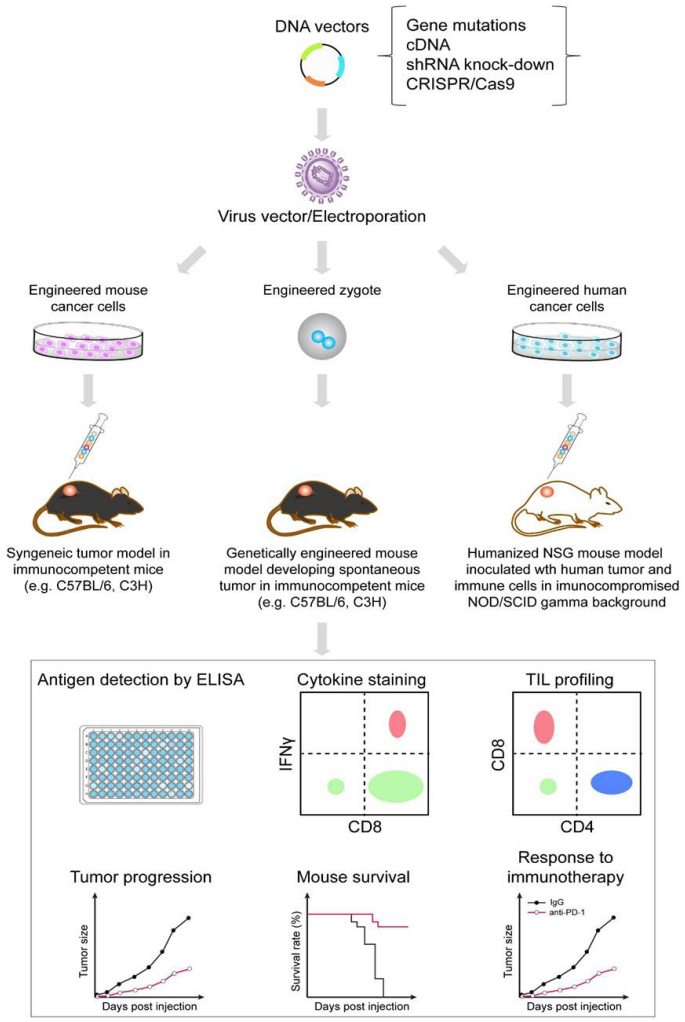

Figure 2: The process for validating the immunoregulatory role of candidate genetic events with different mouse tumor models.

First, specific DNA vectors for the generation of the candidate neo-antigenic genetic events (e.g. overexpression, mutation, suppression or deletion of candidate gene) are constructed and transferred into cells through virus infection or electroporation. The engineered cells will then be selected and injected into three major different mouse models. Syngeneic tumor models are generated by transplanting the engineered mouse cancer cell into immune competent mice (e.g. C57BL/6, C3H). Genetically engineered tumor models are generated by specific modification of the genome of the immunocompetent mice usually at the zygote stage, which will be implanted into the uterus of a pregnant mouse. Spontaneous tumors can be observed in various stage of development of the newborn mice. Potentially, tissue specific gene expressions or inducible gene expressions can be achieved using tissue-specific promoters or inducible promoter (e.g. tetracycline-inducible promoter) driving gene expression and tumor formation. For the humanized tumor model, the human immune system could be established in the mice by injecting purified human peripheral blood mononuclear cells or hematopoietic stem cells into immune compromised NOD/SCID/IL2Rγ (NSG) mice. After that, the human cancer cells or human patient-derived tumor could be transplanted into the NSG mice. To investigate the immune response induced by the candidates, the mouse blood or spleen could be collected for ELISA assay for various cytokine measurements, tumor collected for cytokine staining and Tumor Infiltrating Lymphocyte (TIL) profiling with specific antibodies. In addition, the in vivo tumor growth, mouse survival rate as well as their response to drug treatment can be monitored.

prohibit the observation or evaluation of the full effectiveness of immunotherapy at earlier stages of tumor development. Moreover, the syngeneic mouse models, largely being engineered models, often lack the true genomic heterogeneity of the human tumors and the related microenvironment, making them not-yet-ideal in predicting immunotherapy responses.

Genetically Engineered Mouse Models (GEMMs) that can intrinsically mimic the process of tumorigenesis or tumor progression upon manipulation of certain cancer-related gene(s) are also important tools for the evaluation of how tumor genetics may affect the tumor immune landscape [82]. For example, *Kras p.G12D; Trp53 p.R172H* mutant mouse is found to develop pancreatic tumors through *JAK2-STAT3*-dependent immune evasion [83], indicating that mutant p53 could shape the tumor microenvironment. A breast cancer GEMM with Mouse Mammary Tumor Virus (MMTV) promoter -driving expression of *HRAS* and *MYC* genes, mimicking co-amplification event, was found to result in accelerated formation of mammary adenocarcinomas compared to un-engineered control, and single gene amplified *KRAS* or *MYC* models [84]. Recently, CRISPR/Cas9-mediated genome editing has been employed for the generation of GEMMs for various cancers such as invasive lobular breast carcinoma [85] and lung cancer [86], suggestive of a powerful platform for rapid *in vivo* validation of loss of candidate tumor suppressors and multigenic cancer genomic events. As GEMMS develop tumors in a natural immune-proficient microenvironment, GEMMs allow the study of many immune-related processes *in vivo*, including immune tolerance, immune-editing and/or immunosuppression [82]. The ability to study the involvement of immunity in multi-stage tumor development is an obvious advantage of GEMMs compared to syngeneic tumor models. Importantly, since in GEMMs, the tumors are often developed spontaneously over time, the tumors developed appear to represent heterogeneous tumors as in human patient tumors, which will allow the investigations of the immunogenomics interactions between the tumor genetics and the immune system *in vivo*.

Lastly, another widely used model for immunology and therapeutic studies are the humanized mouse models. The humanized NOD SCID gamma (NSG) mouse models allow both the implantation of human tumors as well as immune cells from human. These NSG mice themselves are deprived of T cell, B cell with extremely low NK cells due to the loss-of-function mutation of *Prkdc* and null mutation of *IL2Rγ* in NOD/ShiLtJ background [87]. Yet, human immune cells such as human T cells and $CD34^+$ Hematopoietic Stem Cells (HSC) can be purified from patients directly, gene-modified and being re-introduced into the mice. The humanized mouse models can be used for the assessment of therapeutics using patient's tumor and immune cells (of the same patient or from other human donors), creating a more humanized tumor immune microenvironment relevant to the treatment under investigation. For instance, the NSG mice bearing human solid tumors have been used to evaluate the therapeutic effect of anti-CD47 antibody in bladder cancer, breast cancer and liver cancer [88]. The engraftment of *MYC* p.T58A and *BCL2* overexpressing human HSCs into NSG mice led to rapid development of B-cell lymphomas, validating a combined role of *MYC and BCL2* in producing B-cell malignancy [89]. Infection of HSC-engrafted NSG HLA-A2- transgenic mice with Epstein-Barr Virus (EBV) has led to HLA-A2-restricted $CD8^+$ T cell-mediated immune protection against EBV, suggestive of a model mimicking the pathological effects of EBV infection in B

cell lymphoma in human [90]. The main advantage of humanized NSG models is that they can be used as a preclinical whole animal testing for immunotherapy efficacy studies such as gene therapy and for assessing new vector technologies *in vivo* since they provide a "humanized" immune response with human immune cells. However, a big challenge of using this model to establish a full human chimera is that the human granulocytes, platelets and red blood cells are not able to circulate appropriately in the murine host and their life-spans are limited in the grafted host (NSG mice). Other practical disadvantages include the relatively high cost of labor and time for the generation of these models. There are also reported problems of spontaneous murine tumor formation not of human tumor origin, such as the noted formation of murine lymphoma of a B-cell subtype positive for EBV and mammary tumors, in NSG mice when they were transplanted with patient-derived tumors [91]. Therefore, great care needs to be taken to ensure human origin of the tumor model when NSG mice are used.

Though each of these mouse models appears to have their respective advantages and disadvantages, nevertheless, they are useful *in vivo* models which can help addressing various immunogenomics questions in cancer, such as validating the immunogenicity of candidate neoantigen, investigating the immunomodulating effect and deciphering the underlying mechanism of candidate genetic events, as well as evaluating the effectiveness of immunotherapy.

Conclusions

There are arrays of bioinformatics tools, and biological methods to examine and determine how the tumor immune microenvironments are for each cancer type, or even each tumor. How these immune activities within each tumor are being shaped, especially by aberrant gene expression of mutations in each tumor will inform us the mechanism of tumor immune shaping, as well as immunotherapy design for individual patient tumor. With our expanding knowledge on tumor immunity and immunotherapy responses in patients, it is envisioned that more advanced tools, likely based on improvements of the current algorithms, can help address more diverse questions in the immunogenomics field. The anticipated identifications of immune cells at single cell RNA-seq level would definitely impact our understanding of the complexity of multi-immune cell involvement in the tumor immune microenvironment.

Acknowledgements

V.W.Y.L. receives fundings from Lee Hysan Foundation Research Grant and Endowment Fund Research Grant Schemes 2018-2019 (CA11281) and 2019-2020 (CA11286), United College, the Chinese University of Hong Kong, General Research Fund, Research Grant Council, Hong Kong government, Hong Kong SAR (#17121616, #1416857), Research Impact Fund (#R4017-18), the Health and Medical Research Fund (HMRF#15160691, the Health and Medical Research Fund, the Food and Health Bureau, The Government of the Hong Kong Special Administrative Region), University-Industry Collaboration Program (UIM/329; Innovation and Technology Fund, Hong Kong government, Hong Kong SAR), and the Hong Kong Cancer Fund, Hong Kong SAR. Y.L. receives funding supports (Postdoctoral Hub PH-ITF Ref.: PiH/052/18 of UIM/329), from the Innovation and Technology Fund, Hong Kong government, Hong Kong SAR. WL receives funding supports (Faculty Postdoctoral Fellows Ref: FPFS/18-19/44) from Chinese University of Hong Kong, Hong Kong SAR.

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