Challenges in Immune Profiling of Cancer Immunotherapy

Abstract
The pathologist’s decision drives effective cancer treatment decisions for patients. This is possible only through the availability of high quality tissue samples and appropriate screening methodologies. Further, with the explosion of technology development in genomics and proteomics and interest in immunological research and immunotherapy, the importance of appropriately collected tissue specimens has heightened. Some of the crucial factors that need to be considered in immune analysis of cancer immunotherapy are discussed.

Keywords: Genomics; Proteomics; cancer immunotherapy; Tumor microenvironment; Enzyme linked Immunospot; IncRNA; Long non-coding; Optimal cutting media; Ribonucleic acid

Abbreviations: PBMC: Peripheral Blood Mononuclear Cells; TME: Tumor Microenvironment; FFPE: Formalin-Fixed Paraffin-Embedded; OCT: Optimal Cutting Media; RNA: Ribonucleic Acid; DNA: Deoxy Ribonucleic Acid; TCR: T Cell Receptor; TIL: Tumor Infiltrating Lymphocytes; IHC: Immunohistochemistry; Cytof: For Cytometry by Time of Flight; Elispot: Enzyme Linked Immunospot, Mirna: Non-Coding Small RNA; IncRNA: Long Non-Coding RNA

Introduction
A number of scientific advances in diagnosis and treatment were made through the study of blood, bone marrow, tumor, or other tissue samples from cancer patients [1-3]. The goal of immunotherapy is to leverage the body’s own immune system to help fight cancer. Therefore, understanding the status and interaction between cancer tissue and the immune system in tumor is important for developing effective cancer immunotherapy strategies. Tumors are comprised of heterogeneous cell populations, including transformed cells and untransformed cells (such as stromal, endothelial and immune cells), which have essential functions in their microenvironment. The complexity and heterogeneity of the interaction between the immune system and tumor cells, particularly in the tumor microenvironment (TME), underlies the immune status of each individual tumor. Recent advances in collection and pathologic examination of specimens and technologies have provided tools that will facilitate an in depth understanding of this interaction and will help guide the development of future personalized cancer immunotherapies.

Specimen Type
Collection and preservation of high-quality specimens from patients is critical as these are used for pathological diagnosis as well as to determine their molecular signature for therapy. Numerous studies made recommendations for specimen collection and handling [4-5]. Consistent and valid expression data can only be accomplished when

- a. The type and timing of sample collection
- b. Method of sample processing and storage
- c. Optimal laboratory procedures and
- d. Platforms selection is carefully planned.

Peripheral Blood
Peripheral blood is routinely used for immune analysis because of its accessibility. Several diagnostic elements were determined in peripheral blood using appropriate assays. The major advantage of using peripheral blood is that pharmacodynamics changes can be measured at various time points before and after therapy. However, these changes do not always reflect solid tissues as selective T cells are enriched in tissues. In addition to blood cells, serum samples are used for multiple functional parameters including cytokines, chemokines and antibody titers (Table 1).

The concept of the liquid biopsy and their clinical relevance, such as early detection, prognosis, monitoring and outcome of cancer patients, have been well recognized [6]. The tumor (circulating tumor cells, CTC) and non-tumor cell free nucleic acids (tumor DNA, mRNA, miRNA) can be obtained from blood of cancer patients. This strategy is less invasive and allows for repeated samplings and real-time monitoring of tumor dynamics in individual patients. The CTCs are captured using antibodies specific to tumor-specific antigens [7] or molecularly detected by RT-PCR. While circulating cell-free DNA and RNA in plasma/serum had long been known [8,9], cell-free miRNA (non-coding small RNAs) or even IncRNA (long non-coding RNA) provide potential as a non-invasive biomarker in clinical application [10-11].

Tissues
Fresh tumor tissues are more valuable than frozen tissues. However, surgical access to fresh tissue, especially prior to treatment, is challenging. The type of fresh tissues collected
from patients at bedside range from fine needle aspirates, punch biopsies and image guided biopsies (X-ray, computerized tomography, magnetic resolution imaging or ultrasound) to laser capture microdissected samples. Advances in sample collection tools made collection of a variety of specific fresh tissue samples from surgical biopsies and cancer resection specimens possible. Tumor and tissue banking provide biological and clinical information necessary to develop various diagnostic and therapeutic strategies [12].

**Table 1: Summary of sample types and screening methods.**

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Assays</th>
<th>References</th>
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<tbody>
<tr>
<td>Peripheral blood PBMC</td>
<td>Multiparameter flow cytometry</td>
<td>[13,16]</td>
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<tr>
<td>Blood serum/plasma/liquid biopsy</td>
<td>Protein microarray, Cytokine and chemokine analysis</td>
<td>[19-20]</td>
</tr>
<tr>
<td>Blood clots/Frozen blood</td>
<td>Leukocyte ratio assessment, Immune cell lineage specific epigenetic modification</td>
<td>[14]</td>
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<tr>
<td>Ascites/pleural effusion</td>
<td>Multiparameter flow cytometry Cytokine analysis</td>
<td>[13,16]</td>
</tr>
<tr>
<td>Tissues Fresh</td>
<td>Multiparameter flow cytometry of TILs</td>
<td>[13,15-16]</td>
</tr>
<tr>
<td>Tissues Frozen (FFPE)</td>
<td>Multicolor IHC staining Multiplexed tissue imaging</td>
<td>[10]</td>
</tr>
<tr>
<td>DNA/RNA/miRNA</td>
<td>PCR, qPCR, Next generation sequencing, Genome wide microarray, RNA sequencing and Transcriptome analysis, T cell and B cell receptor sequencing</td>
<td>[6-11] [21-23] [12] [19] [25]</td>
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Frozen tissues are collected in the form of formalin-fixed paraffin-embedded (FFPE) or preserved in optimal cutting media (OCT) media or snap frozen. Protocols for the collection and cryopreservation of tumor tissues are necessary to avoid introducing potential variation during sample collection and storage.

In addition to blood cells and tissues, RNA and DNA samples isolated from them are banked for testing genetic, transcriptomic and proteomic analyses not only for diagnosis but also to address identification of patients suitable for different treatment approaches.

**High throughput Technologies**

These are valuable tools for testing i) gene signature and epigenetic modification of tumor and immune cells, ii) the function, diversity and iii) clonality of T lymphocytes the breadth of antibody responses. Flow cytometry has been one of the most powerful techniques because of its ability to analyze unique cells of interest from the heterogeneous populations of cells using multiple parallel probes [13-15]. Flow cytometry is useful to find correlates of clinical response in patients by its ability to i) identify tumor antigen-specific T cells with the help of peptide-MHC multimers; ii) Evaluate phenotypic and functional parameters of rare subpopulations of cells; iii) Measure intracellular antigens such as cytokine, chemokine and degranulation in response to antigen specific stimulation; iv) Sasses cell viability, necrosis, apoptosis, cell cycle analysis, etc. Mass cytometry (or CyTOF, for Cytometry by Time of Flight) has also been used for high-dimensional single cell analysis [16].

**Immunohistochemistry (IHC)** assays detect antigens or proteins in tissues and cells. Multicolor IHC staining provides spatial localization and distribution of phenotypic and functional biomarkers within the TME [17]. These *in situ* multiplexed methods add greater depth to our understanding of tumor pathogenesis and immunity related analytes in the TME that could be used to optimize the efficacy of immunotherapy protocols.

**Classic immune monitoring assays**, such as ELISpot, tetramer and intracellular cytokine staining, are very valuable to assess as
well as correlate T cell response to therapy [18]. They generally offer high sensitivity and specificity, and they make use of patient specimens that are readily available. However, the results of these immunoassay monitoring assays can be more reliable if they are targeted to tumor-specific antigens.

Gene expression technologies are currently being used to precisely identify the immune status of tumors from tumor specimens that were properly collected and processed [19-20].

DNA microarray analysis has been successfully used to identify the pathways that interplay many immunosuppressive and immunostimulatory components. Protein microarray is used to track the interactions and activities of immune related proteins and peptides, and to determine their function, and determining function on a large scale.

Whole exome and RNA sequencing examines all protein coding regions of the genome and enables to find the genes most likely to affect phenotype in each individual tumor [21-22]. From these nucleotide variants or neoaigens, prediction algorithms and the tandem minigene library enable the identification of high pMHC affinity mutant class-I and-II neoeptopes, respectively.

T and B cell receptor deep sequencing provides the full spectrum of T and B cell repertoire and can be used to potentially identify immunosequencing biomarkers in peripheral blood and tumor tissue for clinical diagnostics and therapeutic development to improve patient care [23].

Immune Markers Analysis

While immunotherapies hold promise, however, they also usher in new complexities. Individualized immunotherapies are based on evaluation of patients’ immune status: function, phenotype and signature/pattern. The correlations between genetics, tumor phenotype, and patient response to chemotherapy and immune therapy are important. In particular, there is a need for better, faster ways to assess patient immune profiles in order to guide the application of immunotherapies.

Tissue specimens, novel technologies and high dimensional data analysis platforms are the three pivotal components for immune marker analysis. Firstly, pathologists look to tissue analysis for information about where the cancer originated, how it is evolving, and how certain therapies are affecting the disease and its progression. As availability of patient’s tissue samples is limited, more tissue-based diagnostics are necessary that make use of available patient specimens. Further, tissue gathering and handling protocols need to be standardized across laboratories and institutions across globe.

Secondly, new high-dimensional analysis tools for both fresh and preserved tissues are now available to assess the phenotypic and functional tumor or immune microenvironment with unprecedented depth and complexity. However, the clinical significance of most genomic alterations detected by transcriptome analysis such as whole exome sequencing is unknown because non-tumor specific, germline variants associated with disease will also be detected.

Thirdly, immune marker analyses require testing in large clinical studies and a broader database. Data mining and crosslab data-sharing, such as through the NIH repositories, will be important for the success of immune marker analysis in immunotherapy. Such activities will enable to make decisions based on larger datasets, rather than coming to conclusions based on a narrow view of clinical response data from individual labs.

Finally, in order to get a complete picture of the immune status of the TME, it may be best to integrate data from multiple assays by correlating the results from different technologies, such as complementing gene expression analysis with flow cytometry staining and T and B cell receptor deep sequencing with multiplex IHC. This integrated approach would yield a powerful method to accurately evaluate the immune profile of human tumors.

References

8. Ashworth T (1869) A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Aus Med J 14: 146-149.


