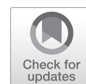

2 Advances in Diagnostic Procedures and Their Applications in the Era of Cancer Immunotherapy

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Abstract

Diagnostic procedures play critical roles in cancer immunotherapy. In this chapter, we briefly discuss three major diagnostic procedures widely used in immunotherapy: immunohistochemistry, next-generation sequencing, and flow cytometry. We also describe the uses of other diagnostic procedures and preclinical animal models in cancer immunotherapy translational research.

Keywords

Immunotherapy · Diagnostic procedure · Biomarker · Immunohistochemistry · Next-generation sequencing · Genetic diagnostics · T-cell receptor sequencing · Flow cytometry · Polymerase chain reaction · Microarray · Southern blot · Western blot · Proteomic profiling · Preclinical animal models

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Introduction

Cancer immunotherapy (also called immuno-oncology) is a cancer treatment designed to stimulate and utilize the body's own immune system, or to block immune escape or immune inhibitory pathways, to fight cancer. In the past few decades, with the advancement of understanding of immunity in cancer biology and the tumor immune microenvironment, immunotherapy has demonstrated tremendous clinical progress in various cancer types [1, 2]. However, only a subset of patients have responded to and benefited from immunotherapy. Moreover, immunotherapeutic drugs have been associated with immune-related adverse events, some of them severe and even life threatening. For the diagnosis and identification of patients whose disease is likely to respond to immunotherapy without severe toxicity, diagnostic procedures must be accurate, sensitive, robust, and versatile as well as applicable in various tumor types to guide the selection of the most suitable treatment regimens. In this chapter, we briefly discuss several diagnostic techniques, preclinical animal models, and their relevance in immunotherapy.

Immunohistochemistry

Immunohistochemistry (IHC) is a simple diagnostic procedure that is well established and widely used to detect and visualize antigen (that

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is, protein) expression levels and cellular or subcellular patterns using highly specific antigen–antibody reactions in tissue sections [3]. The term "immunohistochemistry" comprises three parts, "immune", "histo", and "chemistry": (1) "immune" indicates antibody–antigen recognition; (2) "histo" indicates tissue morphology preservation; and (3) "chemistry" indicates the antibody–antigen reaction, resulting in staining. IHC assays can be performed on formalin-fixed and paraffin-embedded (FFPE) or fresh frozen (FF) tissue sections. IHC has been widely used not only for diagnostic pathology classification, but also anti-tumor drug development in cancer immunotherapy [4]. IHC provides cancer diagnostic, prognostic, and predictive guidance for immunotherapy [5–9].

IHC Applications

Cancer Pathology Diagnostics

Both adjuvant to and independent of conventional hematoxylin and eosin staining, IHC staining of cells and tissue provides comprehensive histologic and morphologic information using highly specific antibody markers. In cancer pathology diagnostics, these markers include tumor cell proliferating antigens, growth factors, tumor-specific signaling pathway factors, and tumor-infiltrating lymphocytes. IHC assays have contributed to the pathologic classification and diagnosis of a variety of cancer types, including breast, lung, and prostate cancers [4, 5, 10–13], using well-established, specific tumor markers [14].

Predictive Biomarker Tests

In addition to cancer diagnostics, IHC assays have been increasingly used for predictive biomarker tests for targeted immunotherapy, especially immune checkpoint inhibitors [15]. For instance, PD-L1 assessed by IHC has served as a predictive biomarker for identifying patients more likely to benefit from anti–PD-1/PD-L1 immunotherapy. The PD-L1 IHC 28-8 and PD-L1 IHC 22C3 pharmDx assays (Agilent) have been approved by the U.S. Food and Drug Administration as diagnostic or companion tests for anti–PD-1 therapies [16–19]. However, patients with PD-L1− status can still benefit from anti–PD-1 or anti–PD-L1 treatment.

In addition, IHC-assessed tumor-infiltrating lymphocytes (TILs) play an important role in predicting immunotherapy outcomes. Several clinical studies have shown that increased TIL density is associated with improved survival rate in patients receiving immunotherapy for melanoma, colorectal cancer, lung cancer, breast cancer, and other tumor types [15, 20–24]. TIL density can be estimated using routine hematoxylin and eosin staining without distinguishing lymphocyte types and populations [25]. With IHC, immune-related markers can be used to assess specific immune cell types and subsets of TILs, including CD3+, CD4+, CD8+, CD20+, CD45RO+, and FOXP3+ lymphocytes, as well as ratios between these subsets etc., thereby providing more comprehensive information about the tumor microenvironment. For instance, higher CD8+ TIL T-cell density and CD8+/FOXP3+ ratio were associated with clinical outcomes [26].

Another application of IHC assays in immunotherapy diagnostics involves microsatellites, which are special repeat sequences in the DNA. When DNA repair genes are not functional, microsatellite sequences can acquire or lose nucleotides, which is known as microsatellite instability (MSI). High neoantigen load or tumor mutational burden caused by deficiency in the DNA mismatch repair protein function, also interpreted as MSI-High (MSI-H), is a well-known indicator of genomic instability. Patients with high MSI or DNA mismatch repair protein deficiency (dMMR) usually carry a high number of genetic mutations in tumors. MSI-H/ or dMMR has been associated with increased T-cell activation and immune cell infiltration and therefore better response, especially in patients with colorectal tumors or metastatic colorectal cancer treated with immunotherapy [27–30].

MSI test can be assessed by detecting microsatellite DNA loci in the genome using polymerase chain reaction (PCR) or by examining loss of expression of mismatch repair proteins MLH1, MSH2, MSH6, and PMS2 using

IHC. The PCR test provides direct evidence for MSI status, whereas IHC assesses the expression levels of mismatch repair proteins, an indirect indicator of MSI. Nonetheless, comparison studies suggested high concordance between IHC testing and DNA-based MSI PCR testing (>90% coincidence rate) [28, 31–34]. Moreover, IHC assays are more feasible and economical for the clinical setting. IHC analysis requires only 4 × 3 μm formalin-fixed, paraffin-embedded sections, whereas PCR requires more biopsy material for DNA extraction. Therefore, IHC has been used as a primary detection method: if no deficiency of any repair protein is detected, indicating microsatellite stability, then no further PCR is required. On the other hand, if any repair protein is found to be deficient on IHC, PCR can be used as a complementary, secondary detection assay to further determine MSI status. Although a few studies have shown discordance between IHC and PCR tests for MSI in ovarian cancer [35], most studies have suggested that IHC is a reliable and economical method for MSI diagnostics.

Single-Marker Versus Multiplexed IHC

While conventional chromogenic IHC can detect only one targeted antigen per experimental run using chromogens such as 3,3′-diaminobenzidine tetrahydrochloride, multiplexed IHC enables the detection of multiple targeted antigens simultaneously on a single tissue section or assembled tissue microarray section. Current multiplexed IHC platforms use either fluorescence-labeled antigens (up to eight) detected by fluorescence microscopy [9] or, in "next-generation" IHC, metal-conjugated antigens (up to 60) detected by mass spectrometry [36] to maximize antigen detection capacity and the quality and resolution of image acquisition.

Compared with traditional, single-marker IHC, multiplexed IHC offers several advantages. First, by labeling multiple antibodies on a single section and obtaining maximal data sets from one sample, multiplexed IHC saves precious samples, including clinical samples with limited availability. Second, traditional IHC stains use one antibody per section, so spatial and co-localization data from multiple-antibody staining are obtained by staining serially cut sections individually and aligning the serial images. The more sections stained, the less accurate the spatial information. However, for multiplexed IHC, multiple-antibody staining is performed simultaneously or sequentially on one section, providing accurate spatial information that is easily assessable. Finally, multiplexed IHC enables the introduction of housekeeping protein markers as references for normalization, eliminating potential errors between batches and producing more accurate IHC data. Nonetheless, despite its comprehensive insights into tissue context and microenvironment, multiplexed IHC has some technical limitations and complications. Highly specific, high-quality IHC antibodies are required for a multiplexed IHC platform to produce accurate results; antibody cross-reactivity leads to unreproducible, unreliable results. Additionally, interpreting multiplexed IHC data can be challenging and more time consuming.

IHC has been an essential diagnostic procedure in cancer diagnosis and therapy for many years. With technical advancements, automation, and standardization of IHC techniques, as well as the application of multiplexed IHC platforms, IHC will play an expanding role in cancer diagnostics, predictive biomarker testing in the era of immunotherapy.

Next-Generation Sequencing

Next-generation sequencing (NGS) techniques have enabled fast, affordable analysis of the genome and transcriptome in immunotherapy. There are three major sequencing platforms: 454 sequencing, SOLiD, and Solexa. 454 sequencing, among the first NGS platforms, has the longest read length and is very fast but is expensive and has a high error rate. The SOLiD platform is the most accurate but has shorter reads. Solexa has the lowest price and highest throughput and has a low error rate but uses short reads [37–39]. Recently, third-generation sequencing has

emerged, which can generate very long reads (1–100 kb). Two such techniques are PacBio and Oxford Nanopore. Long reads at this scale are essential when a reference genome is lacking or for identification of a novel gene or isoform. However, these platforms also have high error rates [40].

Most genetic diseases are not caused by a single mutation in a single region. Instead, complex diseases are the result of variations in many different genomic regions. For years, researchers tried to connect genomic variations to complex diseases using genome-wide association studies [41]. Because individual genetic variations with a large effect size are very rare and hard to detect in these studies, studying gene interactions with this method is challenging, and epigenetic causes are often overlooked [42]. Identifying these rare variants became more achievable with NGS technologies. This approach can powerfully contribute to personalized medicine, as each cancer patient has a distinct mutational signature, and tailoring treatment to each patient can improve clinical outcomes.

High-throughput sequencing has enabled fast, unbiased genetic comparisons of patients and healthy controls. However, although NGS technologies have substantially decreased the costs of sequencing, these technologies did not render large-scale sequencing of the entire genome affordable. Thus, targeted enrichment techniques were developed to limit sequencing to areas of interest, reducing cost and time spent. Targeted enrichment techniques can be PCR- or hybridization-based. If the genomic region of interest is known, researchers can use flanking PCR primers to amplify specific regions before library preparation for sequencing. As longer PCR products have more errors, PCR enrichment requires many parallel reactions with shorter products, which increases cost. Despite its limitations, PCR enrichment can be very useful in the clinic, as sequencing of enriched regions leads to higher accuracy, which is essential in the clinical setting. In hybridization-based methods, the target regions are captured after the sequencing library is prepared, using complementary oligonucleotides. These oligonucleotides can be attached to an array or can be in solution. Generating microarrays can be costly and requires large amounts of input DNA, while hybridization to labeled oligonucleotides in solution is more affordable and can be performed using a small amount of input DNA [42].

A widely used method of targeted enrichment is whole-exome sequencing (WES). The exome comprises the protein-coding regions of the genome, and sequencing only the exome can still give essential insights about genetic diseases, many of which are caused by mutations in these regions, while sequencing only approximately 2% of the sequences required for whole-genome sequencing (WGS) [43]. So far, many studies have identified mutations that cause genetic diseases and cancers using whole-exome sequencing [42, 44].

The causes of many diseases lie not only at the DNA level but also at the RNA level. For this reason, RNA sequencing (RNA-Seq), which uses NGS technologies to analyze RNA transcripts (the transcriptome), is essential for understanding the changes in tissues and cells under different conditions. RNA sequencing quantifies the abundance levels of both mRNAs and non-coding RNAs. RNA seq is very advantageous while studying complex diseases, as it can efficiently detect gene fusions, allele-specific expressions, and non-coding RNAs, which can have regulatory functions [42, 45]. Recently, platforms such as 10x Genomics started to sequence RNAs from single cells instead of bulk samples. Single-cell RNA sequencing has the potential to identify and analyze rare cell populations that might be missed in pooled analysis [46, 47].

Many diseases involve epigenetic abnormalities [48]. Epigenetic means utilization of the genomic information to establish specific gene expression patterns. The cellular states in development and disease rely on a particular gene expression program that is facilitated by transcribing the genetic code on the DNA and making RNA [49]. This particular RNA program can then be translated to a particular protein program, which executes cellular functions and phenotypic features. It is important to understand how epigenetic mechanisms control diverse cel-

lular fates through establishing unique gene expression programs [50]. Chromatin in eukaryotic cells consists of DNA that is wrapped around highly conserved histone proteins [51]. The amino-termini tails (N-terminal tails) of histones undergo posttranslational modifications that alter the nucleosome structure [52]. Proteins that recognize these modifications or the changes in nucleosome structure play an important role in regulating gene expression [53, 54] and are frequently mutated in cancer [49].

A common technique for studying the epigenome is chromatin immunoprecipitation followed by sequencing (ChIP-seq) [55–57]. In this technique, samples are fixed to maintain DNA–protein interactions; DNA that is not bound to any protein is digested and removed; and protein–DNA complexes are precipitated using an antibody specific to the protein of interest. Then, the cross-links are reversed, and DNA sequences that bind to the protein are sequenced using NGS technologies. ChIP-seq reveals histone modifications and binding patterns of DNA-binding proteins such as transcription factors [58]. Thus, ChIP-seq can be used to determine the molecular causes of complex diseases [59].

Another technique for studying chromatin state is called Assay for Transposase-Accessible Chromatin followed by sequencing (ATAC-seq) [60]. In this method, open chromatin is mapped using a special type of transposase that can insert certain sequences into chromatin regions that are open or accessible. The inserted sequences are then used for PCR amplification followed by NGS. As DNA accessibility affects gene expression, ATAC-seq from clinical samples can identify many clinically relevant epigenetic changes [61].

NanoString gene expression panels (also known as nCounter panels) offer a distinct way of analyzing gene expression. This method does not involve any enzyme: no reverse transcription or amplification. Instead, individual mRNAs are labeled with DNA barcodes. Each barcode has a sequence of six fluorescent spots that can be one of four colors, as well as complementary sequences for the gene of interest. mRNA transcripts are hybridized to these barcodes and then imaged on a slide. Instead of quantifying the overall fluorescence intensity, the assay counts individual barcodes, which is equivalent to counting individual mRNA transcripts. This direct, single-molecule counting method is precise and reproducible and works well with formalin-fixed, paraffin-embedded tissue samples. NanoString offers many different gene panels targeted for oncology, immunology, and neuroscience. Each panel contains up to 800 targets and can be customized to an extent [62].

Most human T cells have T-cell receptors (TCRs) that comprise alpha and beta chains. TCR chains are highly diverse as a result of recombination and can detect millions of antigens. Complementarity-determining region 3 (CDR3) is a site of antigen contact in the variable region of TCRs and thus is often studied to determine T-cell repertoire diversity [63]. Most commonly used for characterizing the T-cell repertoire are NGS-based assays that involve sequencing the CDR3 region of the TCR beta chain. These assays can be used to analyze T-cell clones in formalin-fixed, paraffin-embedded tumor samples. More recently, advances in single-cell genomics have enabled single-cell TCR sequencing to identify the T-cell repertoire in patients [64]. There is growing interest in identifying T-cell clones in cancer that can be expanded to respond to specific tumor antigens after immunotherapy. Importantly, when combined with tumor cell sequencing and T-cell phenotyping, TCR sequencing can provide comprehensive information for monitoring and predicting immunotherapy response [65].

Response to immunotherapy varies widely between individuals, so there is a dire need to identify predictive biomarkers for immunotherapy response. A potential predictor of this response is a high tumor mutational burden, or the proportion of nonsynonymous mutations in the tumor genome [66]. Tumor mutational burden can be determined by whole-exome sequencing or gene-targeted sequencing. Initially, whole-exome sequencing, by comparing tumor data with matched non-tumor tissue, was preferred; however, gene-targeted sequencing can be more advantageous owing to lower costs and higher

sensitivity. Tumor mutational burden has the potential to play a key role in the immuno-oncology field [67].

NGS can also be used to predict immunotherapy response via microsatellites. MSI is observed in many cancer types and is a potential predictive marker for immunotherapy response. While MSI testing often uses PCR amplification of known repeat regions, MSI status can also be determined using NGS methods such as targeted sequencing using gene panels [68].

Tumors that lack functional DNA repair genes acquire many mutations and are thus more immunogenic and sensitive to immunotherapy. However, not all tumors that lack DNA repair genes respond to immunotherapy. It was recently shown that this variable immunotherapy response can be explained by the extent of MSI. This study suggests the potential of analyzing MSI intensity using NGS techniques as a means to predicting immunotherapy response [69].

Flow Cytometry

Clinicians have recently started to use flow cytometry techniques as flow cytometers have become smaller and more affordable, allowing the rapid analysis of many characteristics of a wide variety of samples, including blood and bone marrow [70]. Flow cytometers take cells in suspension, focus the cells into a stream using a fluidics system, and create liquid droplets that each contain a single cell. Thus, each cell can be analyzed individually. To analyze the cells, flow cytometers use lasers to record single cells' optical and fluorescence properties. While light scattering patterns can indicate the size and internal complexity of cells, fluorescence can be used to analyze many different properties that the researchers are interested in through the use of fluorescence-labeled antibodies, which can stain cell-surface proteins and internal proteins. Samples can be stained with several antibodies at once, so many different properties can be obtained simultaneously. After optical and fluorescent signals are detected, amplification and conversion steps enable data analysis on computers. These data are often visualized using two-dimensional dot plots and histograms.

Flow cytometry can assess the DNA content of cells using dyes that stain DNA. The signal from these DNA-intercalating dyes is directly proportional to the amount of DNA, allowing ploidy and cell cycle kinetics of tumor cells to be determined. DNA analysis can also have prognostic value in several types of cancer [71]. A frequently used application of flow cytometry in the clinic is immunophenotyping, which characterizes cell populations according to the antigens they express either on their surface or intracellularly. Immunophenotyping is used to diagnose and classify lymphoma and leukemia, diagnose immune deficiency disorders, quantify stem cells in the blood, monitor HIV+ patients, and so forth [71].

Immune cells have many different subtypes that express various cell-surface markers. Recent advances allow analysis of many different antigens simultaneously using antibodies that are tagged with different fluorescent colors. This multicolor analysis allows precise gating of cell populations. For instance, one can quantify the proportions of B cells and different subtypes of T cells using sequential gating based on the markers these cells are known to express. In the clinic, CD4+ T cells can be quantified by flow cytometry to monitor the infection stage of HIV+ individuals [71]. However, not all antigens are on the cell surface. Other recent advances in flow cytometry allow staining of intracellular antigens as well, by permeabilization of the samples before staining. This intracellular staining of lymphoid and myeloid differentiation markers can be very useful in leukemia diagnosis [72].

Flow cytometry can also be used to assess the functionality of immune cells. Cell proliferation can be measured using fluorescently labeled antibodies that recognize the thymidine analog 5-bromo-2′-deoxyuridine (BrdU), as proliferating cells incorporate BrdU into their DNA. The cytotoxicity of natural killer cells can be measured using fluorescently labeled target cells; as the target cells are killed by natural killer cells, the amount of fluorescence decreases. Moreover, neutrophil function can be measured by analyzing phagocytosis, which is done by incubating

neutrophils with fluorescently labeled bacteria and then quantifying the neutrophils' fluorescence levels.

Tumor-specific T-cell responses are often studied in immunotherapy patients, as antigen-reactive T cells are crucial for a successful antitumor response. Antigen-specific T cells can be detected either directly through their TCRs or by functional assays measuring cytokine secretion, proliferation, cytotoxicity, and so forth. Flow cytometry can be used for direct detection of antigen-specific T cells using fluorescently labeled major histocompatibility complex–peptide complexes, although this direct detection does not give information about cell function. Flow cytometry can also analyze various functionality parameters using in vitro stimulation of cells with peptides or protein lysates. One way to assess T-cell activation is to quantify cytokine secretion. By inhibition of cytokine secretion using chemicals such as brefeldin A, intracellular cytokines can be quantified by flow cytometry as discussed above. Another method of measuring T-cell activation is to quantify cell-surface molecules that are known to be upregulated upon T-cell activation, such as CD69 and CD25. T-cell function can also be studied by measuring their proliferation and cytotoxicity with flow cytometry [73].

Fluorescence-activated cell sorting separates cells according to their characteristics. The analysis of particles by this method is the same as that used for flow cytometry with some additional steps. After the properties of each droplet are determined by the computer, each droplet is charged and deflected in a specific direction based on its properties. For instance, cells that express green fluorescence can be directed into one tube, and cells that express red fluorescence can be directed into another tube. Using multiple colors allows more precise separation of cells based on the markers they express. Although sorting is not yet a common clinical procedure, it has significant clinical potential, as it can allow high-purity isolation of very specific cell types, which can then be cultured and expanded in vitro and reinfused into patients in cell-based therapies. For instance, while chemotherapy can be highly toxic to the hematopoietic compartment, autologous transplant of hematopoietic stem cells sorted by fluorescence-activated cell sorting can increase the survival of cancer patients [74].

Mass cytometry, also known as cytometry by time-of-flight (or CyTOF), is a fusion of flow cytometry and mass spectrometry that allows the simultaneous characterization of over 40 properties of single cells. In mass cytometry experiments, cells are labeled with antibodies of interest. Unlike in flow cytometry, these antibodies are not labeled with fluorescence but with heavy metals. Samples that are labeled with antibodies are charged and deflected in a magnetic field. Their time of flight in the magnetic field is then recorded. Lighter ions deflect more than heavier ions, and the specific heavy-metal probes can be identified using their mass-to-charge ratio. These signals are recorded for each cell, and the quantity of probes in each cell corresponds to the expression levels of the antigen that was labeled with the specific antibody–heavy metal complex. As the signal overlap with different heavy metals is minimal, many parameters can be quantified simultaneously with mass cytometry. In contrast, emission spectra of fluorophores can overlap easily, limiting the number of antigens that can be characterized in a flow cytometry experiment [75].

Furthermore, mass cytometry can identify molecular changes that cause diseases and thus has potential in the clinic for observing disease progression and predicting therapy response [76]. For instance, Yao et al. analyzed inflammatory cells in the airway from patients with cystic fibrosis and asthma patients using mass cytometry and found differences in the frequencies and functions of different immune cell subtypes [77]. In another study, Corneau et al. investigated CD4+ T cells from healthy and HIV+ individuals for activation, differentiation, exhaustion, and cell cycle markers. The researchers concluded that many "resting" cells express cell cycle markers or co-inhibitory receptors, which challenge the current definition of resting T cells in the HIV context [78]. Mass cytometry is often used to characterize immune cells but can also be applied to other cell types from any tissue [79].

Another modified version of flow cytometry is imaging flow cytometry, which captures fluorescence, bright-field, and dark-field images of each cell as it flows through the cytometer. Imaging flow cytometry includes many magnifying objectives, two cameras, and up to 10 fluorescence channels, allowing the measurement of thousands of parameters of a single cell. This method can be used to diagnose leukemia from even unstained blood samples, which would not only make sample preparation in the clinic easier but also allow analysis of samples that are close to their native state. Moreover, imaging flow cytometry can be used to study rare cell types in liquid biopsy and can efficiently identify circulating tumor cells [80].

Other Preclinical and Clinical Diagnostics Techniques in Immunotherapy Research

Besides the major diagnostic procedures we discussed above, here, we briefly present additional preclinical methods and clinical diagnostic techniques and concepts used in immunotherapy research.

PCR is a frequently used, fundamental molecular biological technique that amplifies a DNA region of interest. While NGS uses massive, simultaneous deep sequencing to generate comprehensive genomic information with low cost and fast turnaround time in many clinical genetic diagnostic applications, routine PCR (including reverse transcriptase PCR (RT-PCR)) is still a very sensitive molecular genetic test for cancer diagnosis and has a wide application in cancer clinics. On the other hand, before the explosion of NGS techniques, hybridization-based gene expression microarray (also known as chip assay) technologies, including RNA and DNA microarrays, have been extensively used in cancer diagnosis to evaluate alterations in the expression of large numbers of cancer-related pathway gene sets, in many types of cancer [81–84].

Another well-established molecular technique to examine gene expression patterns is Southern blot (also called Southern blot hybridization), named after Edwin Southern, who developed this technique in the mid-1970 s [85]. In brief, Southern blot detects and locates specific gene sequences using designated labeled DNA probes that hybridize with denatured DNA fragments that have been pre-transferred and immobilized on a supporting membrane from an electrophoresis separation gel.

Like other blotting techniques, Western immunoblotting emerged from the Southern blot and is a semi-quantitative biological technique for detecting protein–protein interaction via a highly specific antibody–antigen binding blot. Western blot has been widely used in biology research since its development in the late 1970s [86, 87]. In addition, Western blot has been used for clinical diagnosis, including the detection of infectious diseases such as HIV, bovine spongiform encephalopathy, feline immunodeficiency, hepatitis B, and hepatitis C as well as autoimmune diseases such as paraneoplastic disease and myositis conditions. Western blot has also been used to identify malignant lymphoma and stomach cancer antigens [88]. Nonetheless, to date, Western blot has had limited clinical diagnostic use in cancer immunotherapy. The labeled probes, detection targets, and applications of five blotting techniques using similar principles are shown in Table 2.1.

Like Western blotting, *enzyme-linked immunosorbent assay* (ELISA) is an antibody-based bioassay with extensive uses, from basic research to clinical diagnostics. However, unlike other antibody-based assays, ELISA is a plate-based, cell-based quantitative bioassay that detects not only proteins but also other protein-binding ligands, including hormones, drugs, small-molecule compounds, and cytokines. As a fast, sensitive quantitative immunoassay, ELISA has been widely used in preclinical and clinical cancer immunotherapy research [89–91].

mRNA-based arrays and sequencing assays are usually carried out to monitor mRNA or gene expression profiles at the transcriptional level and infer protein expression levels. However, in some circumstances, RNA levels are not consistent with protein levels. Thus, direct detection of protein level and activity is desirable. Nowadays,

Table 2.1 Summary of five blotting techniques

Blotting	Labeled probe	Detection targets	Applications
Southern	DNA oligonucleotides complementary to target DNA sequence	DNA	Detection or identification of DNA or gene of interest
Northern	DNA or RNA oligonucleotides complementary to target RNA sequence	RNA	Detection of gene expression pattern or profile
Western	Protein, antibody, or peptide	Protein	Detection of protein expression level and pattern
Eastern	Protein, antibody, or peptide	Protein post-translational modifications	Detection of post-translational modifications such as phosphorylation and glycosylation
Southwestern	DNA oligonucleotides	DNA-binding protein	Detection of DNA–protein interactions

with the advancement of quantitative mass spectrometry techniques, proteomic arrays (also called proteomic profiling) provide more direct protein measurement for discovery of tumor-specific and tumor-associated antigens as predictive diagnostics biomarkers [92–94]. Proteomic arrays also have specific antibody–antigen recognition-based clinical diagnostic applications. As with other array assays, the complex data sets from proteomic arrays are usually recorded and visualized as comprehensive heat maps [95].

Western blotting, IHC, flow cytometry, ELISA, and proteomic arrays are all based on antibody–antigen interaction. In IHC, formalin fixation preserves tissue section morphology and architecture, but antigen retrieval is required to break the cross-link introduced by fixation and unmask antigen sites and therefore may limit antibody usage. IHC assays are multiplexible, but standardizing IHC assays is a challenge. On the other hand, Western blot detects target proteins from cells or tissue extraction, so cell morphology and tissue architecture information are lost, and Western blot is not multiplexible, although target protein data can be semi-quantified or quantified. With recent advances in mass spectrometry, proteomic arrays offer more an effective, global, and direct way to measure, monitor, and identify immune-related proteins in the tumor microenvironment. Proteomic arrays thus play an increasingly important role in the discovery of tumor-specific and tumor-associated antigens and potential drug targets in immunotherapy [96–99].

Preclinical Tumor Models in Immunotherapy Research

In the research and development of new immunotherapeutic drugs, in vivo preclinical data from animal tumor models are critical for evaluation of drug activity, understanding drug action mechanisms, and optimizing drug administration plans before drugs enter clinical trials. Because only a subset of patients respond to immunotherapy, it is critical to develop and establish animal models with functional immune systems and tumors that resemble human cancer as closely as possible for the testing of novel immunotherapeutic treatments. Common techniques for generating animal models used in cancer immunotherapy research and cancer biology include spontaneous tumors, genetic engineering, graft transplantation, and carcinogenesis induced chemically, physically, virally, or by radiation [100–103]. Below, we describe preclinical animal model types and related concepts in immunotherapy.

Immunodeficient and Immunocompetent Mouse Models, Nude Mouse

In general, preclinical animal models can be divided into two categories: immunodeficient and immunocompetent. Immunodeficient models include nude mice, which have a T-cell production deficiency, and severe combined immunodeficiency (SCID) mice, which have defects in both T-cell and B-cell function, but normal natural

killer cell and macrophage function [104, 105]. An even more severely immunodeficient mouse strain, developed by the Jackson Laboratory, is nonobese diabetic/SCID mice. In addition to T-cell and B-cell deficiency, nonobese diabetic/SCID mice also have reduced natural killer cells and reduced mature macrophage populations [106, 107].

A nude mouse, or athymic nude mouse, is a laboratory mouse bearing a spontaneous deletion in the *FOXN1* gene. Phenotypically, nude mice lack body hair (hence their name) and have no functional thymus gland, leading to a defective immune system for production of mature T cells [108–110]. In cancer immunotherapy research, since nude mice are immunodeficient and incapable of rejecting tumor cells or transplants from humans or other species, these mice are often used to grow grafted tissue to test novel therapies.

In immunocompetent models, however, the immune system is preserved or reconstituted. With the success of cancer immunotherapy agents, the development of immunocompetent models is urgently needed to test novel immunotherapeutic agents. There are three major immunocompetent mouse model types, as follows.

Syngeneic tumor models are generated by inoculating allografts (also called homografts) of mouse cancer cell lines into host mice from the same inbred strain to induce and establish a tumor-bearing system. Through the use of syngeneic allografts, immune rejection of transplants can be avoided. Syngeneic tumor models are fully immunocompetent.

In genetically engineered mouse models, the tumor-bearing system is introduced by genetic manipulation techniques, such as transgenic methods, knock-in, or knock-out to develop endogenously arising tumors, genetically mimicking human disease that is caused by gene mutation, deletion, insertion, or other alteration. For instance, the introduction of double deletion of the *Trp53* and *Pten* genes in mice leads to invasive bladder cancer [111].

Carcinogen-induced tumor models develop tumors after carcinogenic induction by chemicals, virus, radiation, physical stress, etc. For instance, Fantini et al. developed a muscle-invasive bladder cancer mouse model induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine, bearing histologic resemblance to human tumor as well as a competent immune system [112].

Xenograft Tumor Models

Xenograft tumor models are generated by inoculating xenograft tumors from a different species into a host animal to establish a tumor-bearing system, including patient-derived xenografts and cell line–derived xenografts.

A translational cancer patient-derived xenograft model is a humanized tumor model, in which human tumor grafts or primary human cancer cells are transplanted to a host animal. Xenograft mouse models of human cancer can be generated heterotopically (usually subcutaneously) or orthotopically; however, orthotopic tumor models, in which the specific tissue site of the tumor remains the same, are preferred.

Immunocompetent humanized xenograft models are of particular value for immuno-oncology research, allowing human tumors to be assessed in a functional immune system. Since graft transplantation requires an immunodeficient recipient as host, the immunocompetency of humanized xenograft models can be achieved by reconstitution of the host immune system via co-engraftment. The transplant types used to generate these preclinical animal models are summarized in Table 2.2.

Common Translational Research Techniques and their Biospecimen Requirements

Biospecimen types used in various translational tests are summarized in Table 2.3 [113].

Table 2.2 Transplant types for generating preclinical animal models

Transplant type	Description
Autograft	The transplantation of cells, tissues, or organs from one part of the body to another in the same individual
Allograft/Homograft	The transplantation of cells, tissues, or organs, to a recipient from a genetically non-identical donor of the same species
Xenograft	The transplantation of cells, tissues, or organs, to a recipient from a different species

Table 2.3 Summary of biospecimens used in common translational research techniques

Common translational tests		Biospecimen
Nucleotide-based assays	DNA-sequencing, RNA sequencing	DNA/RNA extracted from any type of tissue, liquid biopsy
	Cell-free DNA sequencing	Total cell-free DNA isolated from plasma, cell-free samples
	TCR sequencing	DNA/RNA extracted from any type of tissue, liquid biopsy
	Southern blot	DNA extracted from any type of tissue, liquid biopsy
	Northern blot	RNA extracted from any type of tissue, liquid biopsy
	Microarray	DNA/RNA extracted from any type of tissue, liquid biopsy
Antibody-based assays	Western blot	Homogenized tissue, liquid biopsy
	Proteomic array	Homogenized tissue, liquid biopsy
	IHC	Tissue specimen (FFPE, FF, FNA, etc. section)
	Flow cytometry	Any tissue specimen, liquid biopsy

NGS next-generation sequencing, *TCR* T-cell receptor, *IHC* immunohistochemistry, *FFPE* formalin-fixed paraffin-embedded, *FF* fresh-frozen; fine-needle aspirated

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