32 Immunohistochemistry and Biomarkers for Targeted Tumor Therapy

Contents

A large number of tumor-associated antigens are now the target for specific antitumor agents, including specific antibodies and specific inhibitors, including selective kinase inhibitors. As a morphology-based method that highlights the targets with its cellular localization, immunohistochemistry is a very useful tool that can detect many of these targets on sections from formalin-fixed paraffin-embedded tumor tissues. For many tumors with different histogenesis, several molecular targets are now established. The following list includes the most common targets that can be detected by immunohistochemistry.

- Lymphoproliferative neoplasia: CD19, CD20, CD22, CD30, CD33, ALK.
- Pulmonary non-small cell carcinoma: PD-L1, ALK, c-MET, ROS-1, NTRK, HER2.
- Breast carcinoma: ER, PR, AR, HER2, PD-L1, TROP-2, NTRK.
- Thyroid carcinoma: BRAF-$_{V600E}$, NRAS-$_{Q61R}$, TROP-2.
- Gastrointestinal carcinoma: microsatellite instability (MSI/MMRD), PD-L1, HER2, BRAF-$_{V600E}$, NRAS-$_{Q61R}$, NTRK.
- Pancreatobiliary adenocarcinoma: microsatellite instability (MSI/MMRD), PD-L1, HER-2, IDH1.

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- Carcinoma of the female genital system: ER, PR, HER-2, folate receptor alpha (FRα), microsatellite instability.
- Transitional cell carcinoma: PD-L1.
- Brain tumors: IDH1.
- Melanoma: BRAF-$_{V600E}$, NRAS-$_{Q61R}$, NTRK.
- Renal cell carcinoma (ALK rearrangement RCC): ALK.

The majority of the abovementioned antigens were listed in previous related chapters. In this chapter, PD-L1, BRAF, RAS, NTRK, and the mismatch repair proteins are listed as additional biomarkers for the assessment of personalized tumor therapy.

32.1 Mismatch Repair Proteins and Assessment of Microsatellite Instability (MSI)

DNA mismatch repair is a highly conserved biological pathway that plays a key role in maintaining genomic stability and preventing mutations from becoming permanent in dividing cells [1]. Microsatellites are short and tandemly repeated simple DNA sequences composed of 1–8 nucleotide bases scattered throughout the coding and noncoding human genome that can be up to 100 times repeated and consequently are liable for errors during the DNA replication due to endogenous or exogenous toxic agents. Two types of DNA mismatches are described within the microsatellites and include base/base mismatches and replication errors with deletion or insertion (indel). The mismatched DNA sequences can be identified and corrected by the mismatch repair protein orchestra, in mammalians including the MSH2, MSH3, MSH6, MLH1, and PMS2 proteins, as well as other proteins, including DNA polymerases and DNA ligase, exonuclease 1 (EXO1), proliferation cell nuclear antigen (PCNA), replication factor C (RFC), and regulation of replication protein A (PPA), which are encoded by different genes on different chromosomes. The MSH2, MSH3, and MSH6 proteins recognize and bind to the mismatched DNA sequence and form a heterodimeric complex, whereas the MLH1 and PMS2 proteins excise the mismatched nucleotides. The loss of one or more of the mismatch repair proteins (MMR proteins) usually occurs due to hypermethylation of the MLH1 promoter with epigenetic silencing or due to mutations within the genes encoding these proteins. The loss of these proteins leads to the accumulation of DNA replication errors in the areas of short repetitive DNA sequences, known as microsatellite instability. The microsatellite instability plays a causal role in HNPCC/Lynch syndrome and other related syndromes such as Muir-Torre syndrome, Turcot syndrome, and constitutional mismatch repair deficiency syndrome with colorectal and endometrial carcinomas and in many other sporadic malignant tumors, including skin and brain tumors. MMR protein deficiency and microsatellite instability (MSI) are detected in ~15% of all colorectal adenocarcinomas and ~40% of endometrial and ovarian endometrioid carcinoma. Colorectal adenocarcinomas with mismatch repair deficiency show distinct morphology with increased intratumoral-activated T lymphocytes due to the accumulation of mutated peptides. These carcinomas are commonly localized in the right hemicolon and usually show a good response to immune checkpoint inhibitors. Mucinous and medullary adenocarcinomas are frequently associated with microsatellite instability.

Two methods are now available for the detection of MMR protein deficiency/microsatellite instability in tumor tissue. The DNA-based molecular methods include PCR or NGS and immunohistochemistry. The molecular methods detect the changes in the DNA sequences, including insertion and deletion errors—compared with the DNA from normal tissue—caused by the loss of function of the MMR proteins. Immunohistochemistry is a good and low-cost alternative for molecular testing with high concordance based on detecting the MMR proteins in the tumor cells. The immunohistochemical reaction must be performed on well-fixed tissue, preferably preoperative biopsies. The MMR proteins show in stained sections a nuclear expression pattern which must be compared with the

expression in normal mucosal cells and stromal inflammatory cells, mainly lymphocytes, as a constant mandatory positive internal control for the precise interpretation (Fig. 32.1). A cytoplasmic or membranous staining pattern should be considered as an artifact. In routine immunohistochemistry, the four MLH1, MSH2, MSH6, and PMS2 mismatch repair proteins are the most informative targets and commonly used for the evaluation of mismatch repair deficiency in different tumor types:

32.1.1 Human Mut L Homolog 1 (MLH1)

Is a mismatch repair protein encoded by the MLH1 tumor suppressor gene located on chromosome 3. MLH1 heterodimerizes with PMS2, PMS1, or MLH3 to form MutLα, MutLβ, or MutLγ, respectively.

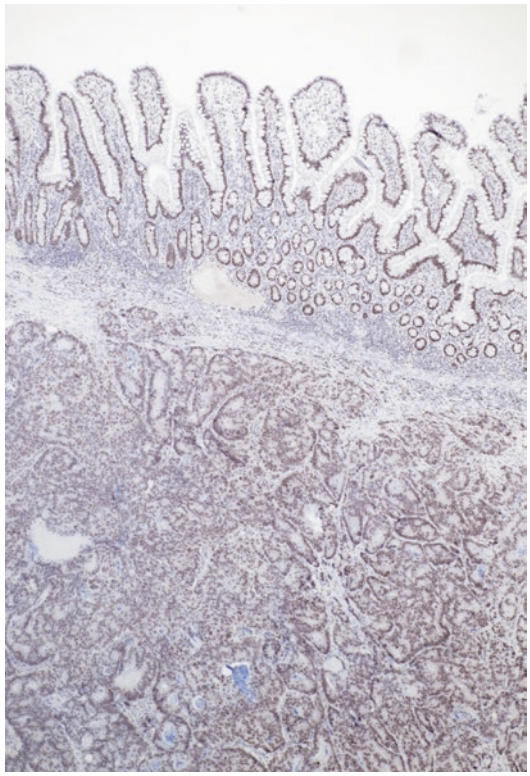

Fig. 32.1 MLH1 expression in primary adenocarcinoma of the small intestine. Nuclear MLH1 expression in normal mucosa and neoplastic cells

In examined tumor sections stained by immunohistochemistry, the loss of MLH1 is usually associated with the loss of PMS2, mainly due to MLH1 gene inactivation by hypermethylated gene promoter or mutation. The loss of MLH1 and PMS2 with the absence of the MLH1 promoter hypermethylation can be sporadic, as well as the manifestation of Lynch or related syndromes. The loss of both MMR proteins associated with hypermethylation of the MLH1 gene promoter is considered sporadic. The loss of MLH1 alone is also possible but rare and must be confirmed by more sensitive molecular methods.

In colorectal carcinomas, the combination of the loss of MLH-1 and BRAF mutation is suggestive of the sporadic nature of the neoplasia and most likely to be developed through the serrated pathway. The association between MLH1 deficiency and BRAF mutations is not a feature in gynecological carcinomas (Fig. 32.2).

32.1.2 PMS1 Homolog 2 (PMS2)

Is a mismatch repair endonuclease encoded on chromosome 7. The endonuclease activity of PMS2 causes single-strand breaks near the mismatch bases, presenting entry points for the exonuclease EXO1 to degrade the mismatched DNA sequence.

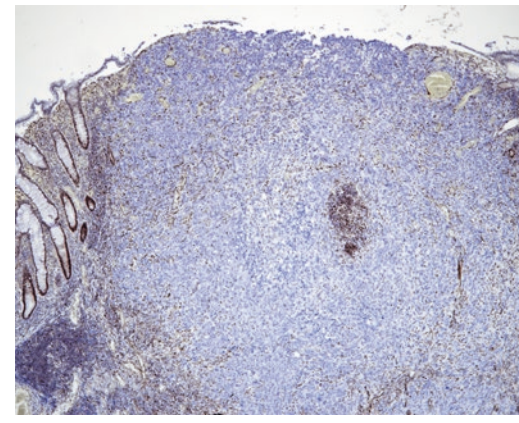

Fig. 32.2 Poorly differentiated colorectal adenocarcinoma. The tumor cells lack the expression of MLH1. Strong MLH1 expression is seen in normal mucosal and stromal cells

The loss of PMS2 is usually associated with the loss of MLH1. The isolated loss of PMS2 expression due to mutations within the PMS2 gene is also possible but less common and found in ~4% of tumors with MMR protein deficiency and can be associated with Lynch or related syndromes.

32.1.3 Human Mut S Homolog 2 (MSH2)

Is a DNA mismatch repair protein encoded on chromosome 2 and heterodimerizes with MSH6 or MSH3 to form the MutSα or MutSβ complex, respectively. These complexes recognize and bind to the mismatched dsDNA to initiate the repair of mismatched DNA.

In most cases, the loss of MSH2 occurs due to mutations within the MSH2 gene or promoter hypermethylation. Rarely, in up to 3% of the cases, the loss of MSH2 appears as a result of a germline deletion of the 3′ end of the EPCAM gene, located upstream of the MSH2 gene leading to gene silencing.

The loss of MSH2 is generally associated with the loss of MSH6 and usually appears as the manifestation of Lynch or related syndromes. The loss of MSH2 alone is rare and must be confirmed by more accurate molecular methods (Fig. 32.3).

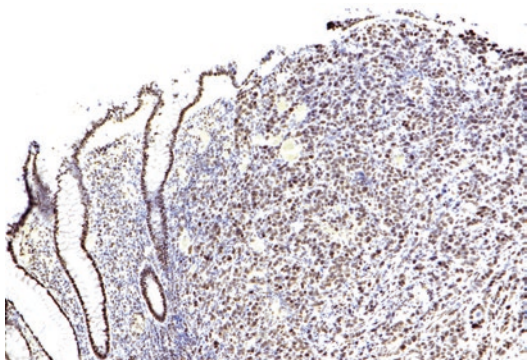

Fig. 32.3 Poorly differentiated colorectal adenocarcinoma with strong MSH2 expression in mucosal-stromal and tumor cells

32.1.4 Human Mut S Homolog 6 (MSH6)

Is a DNA mismatch repair protein encoded on chromosome 2 that heterodimerizes with MSH2 to form the MutSα complex.

In examined tumor sections, the loss of MSH2 is usually associated with the loss of MSH6; however, the loss of MSH6 alone due to mutations within the encoding gene is also common and can be associated with Lynch or other related syndromes [2].

The subclonal loss of the MMR proteins in the tumor sections can rarely be noticed in some tumor types, usually as a result of MLH1 promoter hypermethylation due to tumor progression, and must be mentioned in the final report [3]. The loss of MSH6 expression can be noted in tumors after neoadjuvant therapy causing false results.

The nuclear expression of the four proteins (MLH1, PMS2, MSH2, and MSH6) is the normal pattern indicating no evidence of mismatch repair deficiency. The loss of all MMR proteins must be considered an artifact, and the reaction must be repeated or retested by other molecular methods.

32.2 Programmed Death-Ligand 1 (PD-L1)

PD-L1 (clustered as CD274) is a member of the B7 family of cell surface ligands, a type I transmembrane protein composed of extracellular domains, transmembrane domain, and intracellular domains and expressed on activated immune cells and different tumor cells. PD-L1 is an immune checkpoint protein that plays an important role in the modulation of the immune reaction by binding to its receptor-programmed cell death protein 1 (PD-1) (see Chap. 16), expressed on activated CD4+ and CD8+ T lymphocytes, B lymphocytes, and myeloid cells. As a major immune checkpoint protein, PD-L1 mediates the antitumor immune response and eliminates the effects of the cytotoxic T lymphocytes that cause

the activation of the host immunity against tumor cells. In routine immunohistochemistry, the detection of PD-L1 in tumor tissue is widely used as an important biomarker to predict the clinical response to PD-1 and PD-L1 selective checkpoint inhibitors. The immunohistochemical detection of PD-L1 status (TPS, IC, CPS) is now required for the therapy of many malignant tumors, including squamous cell carcinoma of the head and neck, non-small cell lung carcinomas, mesothelioma, triple-negative breast carcinoma, gastrointestinal adenocarcinoma, hepatocellular carcinoma, renal cell carcinoma, and transitional cell carcinoma of the kidney and urinary bladder in addition to carcinomas of the uterine cervix [4, 5].

Different antibody clones with different specificity are now available for the immunohistochemical stain of the PD-L1 molecule. The choice of the antibody depends on the target tissue and the staining method [6]. The immunohistochemical reaction can be optimized and standardized using control tissue such as tonsillar or placental tissue and reference tumor slides. For adequate evaluation of the PD-L1 score by immunohistochemistry, a minimum amount of 100 well-preserved viable tumor cells must be present in the examined section. The heterogeneity of PD-L1 expression in different tumor parts must be considered when interpreting stained sections. To predict the response to the anti-PD-L1/PD1 checkpoint inhibitor therapy, different PD-L1 scores are required for different tumors and include the following scores:

- *Tumor proportion score (TPS)* is the percentage of viable tumor cells with partial or complete membranous PD-L1 staining of any intensity.
- *Immune cell score (IC)* is the proportion of tumor area occupied by all PD-L1-positive tumor-infiltrating immune cells (lymphocytes, macrophages, granulocytes, dendritic cells) of any staining pattern and any intensity.
- *Combined positive score (CPS)* is the amount of PD-L1-positive cells (invasive tumor cells with membranous staining in addition to lymphocytes, macrophages with any staining pattern) divided by the total number of viable tumor cells multiplied by 100.

$$\mathrm{CPS} = \frac{\text{Total amount of PD-L1 positive cells (tumor cells, lymphocytes, macrophages)}}{\text{Total amount of viable tumor cells}} \times 100$$

32.3 RAS

The Ras proteins are a group of closely related proteins that belong to the family of small G proteins with high sequence homology and overlapping functions with GTPase activity. These proteins are expressed in all mammalian cells and encoded by different genes located on different chromosomes [7, 8]. Mutations within the RAS genes cause the deregulation of the RAS-MAPK signaling pathway and uncontrolled kinase activity affecting cell proliferation and differentiation. As the RAS mutations are the most common mutations associated with human neoplasia, mutations within the encoded genes are used as therapy-related biomarkers, whereas KRAS, NRAS, and HRAS are the most common targeted biomarkers in this group.

- The KRAS gene (*K*irsten *ra*t *s*arcoma) located on chromosome 12p12.1 shows the most frequent mutation rate and driver mutations in this gene are commonly found in colorectal and pancreatic adenocarcinomas. Mutations within this gene are usually detected by molecular sequencing or NGS.
- The HRAS gene (*H*arvey *ra*t *s*arcoma) is located on chromosome 11p15.5. Mutations within this gene can be found in the urinary bladder, salivary gland, and thyroid carcino-

mas in addition to melanocytic tumors. Mutations can be detected by molecular sequencing or NGS.

– The NRAS gene (*N*euroblastoma Ras) is located on chromosome 1p13.2. Mutations within this gene are frequently found in melanomas, follicular thyroid tumors, and adrenocortical tumors. The NRAS 182 A > G mutation is one of the most common mutations found in the NRAS gene and encodes an anomalous amino acid sequence where glutamine is substituted by arginine at position 61 (NRAS-Q61R). NARAS mutations are found in 15–25% of melanomas, whereas the NRAS-Q61R mutation is found in ~35% of all NRAS-mutated radiation-induced cutaneous melanomas. RAS mutations are also described in up to 50% of thyroid tumors with follicular morphology, including follicular carcinoma and follicular variant of papillary thyroid carcinoma, in addition to ~20% of adrenocortical tumors, a subset of acute myeloid leukemia and multiple myeloma [9]. The NRAS-Q61K mutation is a further common mutation variant associated with the tumors mentioned above. Mutations within the RAS genes can be detected by molecular sequencing. In routine histopathology, the NRAS-Q61R protein can also be detected by specific antibodies with high sensitivity and specificity as a surrogate marker for this mutation.

32.4 BRAF

BRAF is a member of the RAF kinase family and a cytoplasmic serine-threonine kinase that plays an important role in the RAS-RAF-MAPK kinase signaling pathway. Different mutations within the BRAF gene are considered diagnostic, prognostic, and therapeutic biomarkers for various tumors, including melanoma and thyroid and colorectal carcinomas. BRAF is listed in detail in the chapters on thyroid and melanocytic tumors (see Chaps. 14 and 21). Only a few mutation variants of the BRAF gene, mainly $BRAF_{V600E}$, can be detected by routine immunohistochemistry using specific antibodies.

32.5 Neurotrophic Tropomyosin Receptor Kinase (NTRK)

*N*eurotrophic *t*ropomyosin *r*eceptor *k*inase (NTRK) A, B, and C are highly homologous proteins composed of extracellular, transmembrane, and intracellular domains encoded by three different genes, NTRK1, NTRK2, NTRK3, located on the chromosomes 1q, 9q, and 15q, respectively. Each TRK receptor binds to a specific member of the neurotrophin family of ligands that takes part in developing the central and peripheral nervous systems. The expression of all three TRK proteins may be activated by different genetic anomalies caused by the fusion of one of the NTRK genes to a second gene with a potent promotor, causing abnormal activation of the intracellular tyrosine-kinase domain of the TRK receptor. Nowadays, more than 80 NRTK fusion partners are described. Diagnostically important is the t(12;15)(p13;q25) translocation generating the ETV6-NTRK3 fusion transcript associated with congenital fibrosarcoma and cellular mesoblastic nephroma and secretory carcinoma of the breast and salivary glands, in addition to a subset of acute lymphoid and myeloid leukemia, mainly pediatric papillary thyroid carcinoma, gliomas, and inflammatory myofibroblastic tumor. TRK overexpression is also found in a small percentage of other different tumors, such as NSCLC, gastrointestinal and colorectal adenocarcinomas, cholangiocarcinoma, and melanoma, due to sporadic mutations in the promotor region, which can be used as targeted tumor therapy using one of the available selective TRK inhibitors [9].

The immunohistochemical detection of the TRK proteins in tumor cells is a surrogate for an NTRK gene fusion, which should be later con-

firmed by one of the molecular biology methods. In routine immunohistochemistry, a Pan-TRK antibody is used to stain the TRK molecules, which binds to all three TRK A, B, and C molecules. Tumors associated with NTRK1 or NTRK2 gene fusions usually have a cytoplasmic expression pattern, but rare perinuclear and nuclear membrane staining has been reported. Tumors harboring NTRK3 fusions show both cytoplasmic and nuclear expression pattern [10].

32.6 Anaplastic Lymphoma Kinase (ALK)

Anaplastic lymphoma kinase (ALK) is a membrane-associated receptor tyrosine kinase encoded on chromosome 2p23 and clustered as CD246 listed in previous chapters (see Chaps. 3 and 16). The ALK molecule is composed of extracellular, transmembrane, and intracellular domains playing an important role in the regulation of the cell cycle by activation of different cellular signaling pathways, including the *m*itogen-*a*ctivated *p*rotein *k*inases (MAPK; Ras and RAF), PI3K/AKT/mTOR, JAK, and STAT, which are responsible for the regulation of cell proliferation, transformation, and apoptosis. Multiple genetic mechanisms are discovered causing the abnormal activation of the ALK molecule, including translocations (anaplastic large cell lymphoma), inversions (non-small cell carcinoma), gene amplifications, and point mutations (neuroblastoma). Tumors harboring activating ALK genetic anomalies are sensitive to specific ALK tyrosine-kinase inhibitors. The expression of ALK in tumor tissue can be detected by immunohistochemistry using different specific antibodies.

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