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Phenotypic and Genotypic Diagnosis of Malignancies

An Immunohistochemical and Molecular Approach

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Dedicated to the light of my life
My father and mother
Sami and Haya
For their love and support

Contents

Preface

Recent years have seen an explosive increase in new methods in diagnostic tumor histopathology, especially in the immunohistochemical, genetic and molecular profiling of tumors. The idea for this book began with the first edition in 2002, when I published my personal notes and experience in immunohistochemistry and molecular pathology, which I frequently use in my daily work as a histopathologist. The continuous development of new antibodies and molecular diagnostic methods make it necessary to constantly update the information in this book; and the second updated edition was published in 2005.

In this edition the majority of the chapters have been reviewed, the immunohistochemical profile of the different tumor groups is updated and the most recent WHO classification of tumors is considered. Also, new chapters in immunohistochemistry, molecular protocols and molecular diagnosis have been added.

The book is designed to be a practical, easy to use bench reference in immunohistochemistry and molecular pathology, useful for the diagnosis and differential diagnosis of tumors. This book should be of value to histopathologists, oncologists, hematologists and laboratory technicians.

Amman, December 2007 *Muin S.A. Tuffaha*

1
Introduction to Immunohistochemical and Molecular Methods in Tumor Diagnosis, and the Detection of Micrometastases and Circulating Tumor Cells

Solid and hematological tumors make a large heterogeneous group with different histogenesis and pathogenesis, different biological and clinical behaviors and consequently different responses to various therapeutic methods, which make accurate diagnosis and precise tumor classification essential for tumor management.

During recent years, classic surgical pathology has rapidly developed and now, beside conventional microscopy and electron microscopy, there are a number of additional highly sensitive diagnostic tools, including immunohistochemistry, cytogenetics and molecular pathology. These methods provide further objectives and reproducible criteria for diagnosis, classification and follow-up of tumors.

Since the late 1980s, immunohistochemistry has presented a sensitive and uncomplicated method to screen the immunophenotypic profailes of different tumors to determine their histogenesis by immunological detection of a specific cellular antigens on tissue sections prepared from frozen tissue or formalin-fixed paraffin-embedded tissue blocks. Nowadays we have several hundreds of monoclonal and polyclonal antibodies specific of cellular and extracellular structures. The use of these antibodies gives us the possibility to classify many undifferentiated tumors including solid tumors and hematological neoplasia, in addition to the possibility to determine the sensitivity of some tumors to specific therapeutic agents.

In the past decade, the rapid development of molecular biology provided other sensitive and powerful molecular tools for the recognition and classification of tumors. Examples are tumors derived from tissue types characterized by unique tissue-specific antigens. These antigens or tissue-specific markers are encoded by the cellular genome and translated into complementary mRNA. The presence of these tissue-specific mRNAs can be detected by RT-PCR-based methods, representative examples are tumors derived from thyroid, prostate and neural tissue. Other tumors are associated with recurrent specific chromosomal and genetic aberrations or rearrangements, which can be used as specific genotypic markers. The detection of tissue-specific transcripts or tumor-specific genetic aberrations by molecular methods offers the basis for precise tumor diagnosis. This method is now used for the diagnosis of tumors exhibiting similar morphology and non-specific immunohistochemical phenotype such as synovial sarcoma, PNET and melanoma.

Table 1.1 Stages of metastatic process.

Tumor development and evasion of immune response
Separation of the tumor cells from the original tumor
Erosion of basement membrane overcoming of the physiological barriers
Invasion of blood and lymphatic vessels
Adhesion and invasion at different distant sites
Organization of metastatic tissue with the induction of tumor stroma

It is also used for the diagnosis of many hematological malignancies such as chronic myeloid leukemia, follicular lymphoma and myelodysplastic syndrome.

Another major problem facing the modern oncology beside the diagnosis and classification of tumors is the metastatic spread of malignant tumors and tumor relapse after therapy. Metastases can be the first manifestation of some tumors and represent an important factor determining patient's final outcome as they cause about 90% of deaths from solid tumors.

Additionally, the presence of micrometastases or individual tumor cells in surgical margins is the major cause of tumor relapse after surgical excision of tumors. The detection of such submicroscopic micrometastases or disseminated tumor cells in surgical margins, lymph nodes, bone marrow or peripheral blood is difficult, impractical or impossible by conventional microscopic examination and needs more sensitive and specific methods like immunohistochemistry and molecular methods. Furthermore, a large number of patients with distant metastases are clinically asymptomatic, which means that more sensitive methods are needed to monitor tumor progression.

Metastatic spread is an important aspect in tumor biology and usually determines the clinical behavior. The metastatic process is a long complicated multistep process which begins with tumor development and evasion of immune response and ends with the organization of metastatic tissue and the induction of tumor stroma and neovascularization (Table 1.1).

An additional cause of metastatic spread is assumed to be the dissemination of tumor cells due to tumor manipulation during surgical intervention or diagnostic assays. Many reports suggest that the manipulation of tumors could provoke shedding of tumor cells into blood or lymphatic vessels causing metastatic spread. To prevent this risk factor the "no-touch isolation technique" with primary lymphovascular ligation was suggested many years ago.

One of the first steps to go behind the metastatic cascade is the examination of peripheral blood to detect potential circulating malignant cells. This method is useful to follow-up malignancies spreading via the blood stream such as sarcomas, melanoma, neuroblastoma, prostatic, thyroid and hepatocellular carcinomas. PCR- and RT-PCR-based molecular techniques are found to be the most efficient assay to detect circulating tumor cells in peripheral blood, and allow the detection of one tumor cell in up to10^5 background nucleated blood cells. However, it is important to consider that the presence of tumor cells in peripheral blood is only one step in a multistep process and not all tumor cells circulating in peripheral blood are able to

attach and proliferate to develop metastases. Many studies show that the majority of circulating tumor cells are killed by different mechanisms including immunological and mechanical ways. Experimental studies demonstrate that approximately 0.01% of circulating tumor cells arising from solid tumors are able to establish metastatic colonies after extravasation. Animal experiments show that only about 0.01% of cancer cells injected into the circulation are able to form metastatic colonies with tumor stroma. In contrast, the presence of tumor cells in peripheral blood is considered to be a negative prognostic factor correlating with tumor progression and an essential step to the development of organ metastases. Many clinical observations suggest that tumor cells detected by sensitive methods during remission may reflect a latent tumor stadium under the control of the immune system and tumor cells may be reactivated after suppression of the immune system.

The next station in the metastatic spread after invasion of the lymphatic vessels is the locoregional lymph nodes, which act as clearing sites for foreign antigens and tumor cells, mainly for tumors originated from epithelial tissue. Among many prognostic factors, the status of the locoregional lymph nodes is one of the most important and useful indicators for most types of carcinomas, such as breast, lung, esophageal and gastrointestinal carcinomas, and generally the presence of lymph node metastases is a significant predictor of poor prognosis in all cancers. Many studies showed that 30–60% of lung, breast, and gastrointestinal cancers already have lymph node or distant metastases at the time of diagnosis. Recent studies showed that breast cancer with minimal involvement of a single axillary node, even in form of disseminated tumor cells, notably correlates with worse prognosis compared with cases with no axillary node involvement. Regional lymph nodes are subjected to routine study by conventional microscopy by examination of H&E stained sections from each removed lymph node, but many studies show that about 20% of the cases of gastrointestinal carcinoma with histologically negative lymph nodes (pN0) suffer tumor relapse in less than five years. Immunohistochemical staining of lymph nodes performed on frozen or formalin-fixed paraffin-embedded tissue are found to be more sensitive than the conventional microscopy alone in detection of lymph node metastases. Recently, molecular methods based on PCR and RT-PCR proved to be very sensitive tools for the detection of submicroscopic metastases and disseminated tumor cells with a sensitivity ratio up to one target cell in 10^6 background cells. Because molecular methods are costly and time-consuming to be done on all removed lymph nodes, the alternative examination of the sentinel lymph node has been suggested. The sentinel lymph node is defined as the first locoregional lymph node receiving lymph from the tumor mass and can be detected by dye or radioactive scanning. This method is effective in the management of malignant melanoma and breast carcinoma.

Another important station for tumor cells is bone marrow, which also acts as a clearing and filtration site for antigens and malignant cells. Many studies found a marked correlation between the presence of micrometastases or disseminated tumor cells in bone marrow and the progress of malignant tumors. Several studies showed that even the presence of a small number of tumor cells in bone marrow is associated with poor prognosis in several tumor types such as carcinomas of GIT, breast,

prostate and neuroblastoma and considered as an independent indicator of tumor relapse.

The detection of malignant cells in different body fluids is also important for tumor diagnosis and tumor staging. Examination of liquor can be useful in the therapy of various malignancies such as lymphoma/leukemia, melanoma, breast carcinoma or primary brain tumors. Examination of pleural effusion and sputum is also essential in cases of lung and breast carcinoma or primary mesothelioma. The study of ascitic fluid also gives valuable information in pancreatic and hepatocellular carcinomas.

In conclusion, the modern approach in the diagnosis of tumors is a multistep process, which includes conventional histology, immunohistochemistry, cytogenetics and molecular methods. The early detection of micrometastases and circulating tumor cells before metastatic disease becomes clinically evident gives a better chance for the eradication of residual tumor cells to prevent tumor relapse in addition to its prognostic importance.

2
Diagnostic Immunohistochemistry

2.1
Introduction

Immunohistochemistry is a sensitive method to detect tissue- and tumor-specific antigens using highly specific monoclonal or polyclonal antibodies against different cellular and extracellular antigens. The antigen–antibody complex is detected by various sensitive, direct and indirect detection systems such as the peroxidase-anti-peroxidase (PAP) system, the alkaline phosphatase–anti-alkaline phosphatase (APAAP) system and the avidin (streptavidin)–biotin horseradish peroxidase (ABC) system. The immunohistochemical reaction is performed on tissue sections prepared from formalin-fixed paraffin-embedded tissue blocks or frozen tissue, allowing the study of tumor morphology combined with the immunohistochemical phenotype and the pattern of antigen expression. At the present time a large number of monoclonal and polyclonal antibodies are used in immunohistochemistry, covering all types of histogenetic differentiation essential for the diagnosis of tumors and also helpful for determining the strategies of tumor therapy.

An additional important benefit from immunohistochemical methods is the detection of minimal residual tumor cells, since the immunohistochemical stain of tumor cells is more sensitive than conventional histopathology, allowing the possibility to detect small groups of tumor cells or even a single tumor cell in tissue sections or smears. For example, cytokeratin-positive tumor cells can be visualized as isolated tumor cells or micrometastases in lymph nodes or as disseminated tumor cells in bone marrow in up to 50% of patients with stage I and stage II breast cancer. In addition to the diagnostic value and the prognostic information obtained by immunophenotyping of tumors, immunohistochemical methods can give valuable information to evaluate the sensitivity of different tumors to various therapeutic agents used for tumor therapy (so-called pharmacopathology), discussed in the following section.

2.2 Pharmacopathology

Modern immunohistochemical methods play an important role in the therapy management of many types of malignancies. Using highly specific antibodies it is possible to detect the expression of specific epitopes which are specific targets for immunotherapy, such as HER-2, EGRF-1 and CD20. Specific antibodies are also used to detect the expression of hormone receptors and enzymes which are targets for receptor antagonists and enzyme inhibitors. Nowadays the semi-quantitative estimation of steroid receptors, HER-2, EGFR-1, CD20 and CD117 are widely used for cancer therapy.

2.2.1 Evaluation of Steroid Hormone Receptor Status by Semi-Quantitative Estimation of Receptor Expression (Estrogen, Progesterone and Androgen Receptors)

The aim of this assay is to evaluate the expression of steroid receptors (receptor status) in hormone-dependent tumors, since these receptors (estrogen, progesterone and androgen receptors) are essential for the proliferation and differentiation of tumors derived from hormone-dependent tissues such as breast, ovarian and uterine tissue.

2.2.1.1 Estrogen Receptor

Estrogen receptor (ER) is a member of the steroid family of ligand-dependent transcription factors. There are two isoforms of the estrogen receptor (ER), the alpha form (ER α) and beta form (ER β). ER α and ER β isoforms have a ~95% homology in the DNA-binding domain but only ~60% homology in the ligand-binding domain. These isoforms show different expression in different tissue types: the ER α isoform is mainly expressed in both epithelial and stromal cells of the breast, uterus, placenta, liver, CNS, and bone, whereas the ER β isoform is mainly expressed in the prostate, testes, ovary, spleen, thymus, skin and endocrine glands, including the thyroid and parathyroid glands, adrenal glands and pancreas. Both isoforms are expressed on the nuclear membrane of normal and tumor cells and can be detected by immunohistochemical staining using specific monoclonal antibodies (such as the clones 1D5, 6F11) on tissue sections prepared from frozen or paraffin-embedded tumor tissue. Most antibodies used in immunohistochemistry are directed to the alpha isoform of the estrogen receptor. Antibodies directed to the beta form of the receptor molecules are rarely used but overexpression of ER β is noted during the progression of breast tumors and is thought to be a sign of tamoxifen resistance. This assay is essential for the therapy management of breast and endometrial cancers to determine the response of these tumors to the anti-estrogen therapy (i.e. tamoxifen) or oophorectomy. Statistical observations reveal that about 70% of the patients with estrogen receptor-positive tumors showed a significant clinical response to anti-estrogen therapy, whereas about 85% of the patients with estrogen receptor-negative tumors showed no or little response to this therapy.

Table 2.1 Scoring system according to Remmele.

	Percentage of positive cells		Intensity of stain
0	No positive cells	0	No detectable stain
1	<10%	1	Weak nuclear stain
2	10–50%	2	Moderate nuclear stain
3	51–80%	3	Strong nuclear stain
4	>80%		

Since the estrogen receptor is an unstable molecule with a short half-life, biopsies must be handled to preserve the receptor structures. Rapid fixation of tumor tissue after surgical excision using buffered neutral formalin for 16–24 h is essential for optimal staining.

Few semi-quantitative scoring systems were suggested, whereas the modified scoring system suggested in 1987 by Remmele, the modified scoring system suggested in 1985 by McCarty and the Allred scoring system proved to be the most practical and simple systems. The three systems depend on the evaluation of the stain intensity and the percentage of positive cells.

Remmele Scoring System This simple scoring system has a 12-point scale (0–12). To calculate the score one of the numbers 0, 1, 2 or 3 is given according to the intensity of the nuclear stain and one of the numbers 0, 1, 2, 3 or 4 is given according to the percentage of positive tumor cells (Table 2.1). The score is calculated by multiplying the number reflecting the dominant stain intensity by the number reflecting the percentage of these positive tumor cells with a maximum score of 12 (3×4). Tumors with a score of less than 3 show usually a poor response to the anti-estrogen therapy corresponding to 10–20 fmol/mg of cytosol tumor protein using the biochemical assay. Clinical observations demonstrate a good correlation between the results of this scoring system and the tumor behavior, especially the response to anti-estrogen therapy.

McCarty Scoring System This scoring system has a 300-point scale (0–300), calculated by multiplying the percentage of positive cells (0–100) by the number reflecting the intensity of the immunohistochemical stain (0: no detectable staining; 1: weak nuclear staining; 2: moderate nuclear staining; 3: strong nuclear staining). The total score is explained as follows:

- Negative (−): 50 or less
- Weakly positive (+): 51–100
- Moderately positive (++): 101–200
- Strongly positive (+++): 201–300

Allred Scoring System The Allred scoring system has an eight-point scale (0–8). This scoring system is calculated by adding the number representing the proportion of positive cells 0, 1, 2, 3, 4 or 5 to number reflecting the intensity of the nuclear stain

Table 2.2 Allred scoring system.

	Percentage of positive cells		Intensity of stain
0	No positive cells	0	No detectable stain
1	<1%	1	Weak nuclear stain
2	1–10%	2	Moderate nuclear stain
3	10–33%	3	Strong nuclear stain
4	33–66%		
5	>66%		

0, 1, 2 or 3 (Table 2.2). Tumors with a score of less than 3 show usually a poor response to the anti-estrogen therapy.

Different breast cancer types exhibit different expression levels of steroid receptors depending on the histological subtype of the tumor, differentiation and proliferation grade in addition to other cytogenetic features. Papillary, tubular, invasive lobular and mucinous breast carcinomas are usually associated with a high expression level of estrogen receptors, whereas medullary, apocrine and metaplastic carcinomas lack estrogen receptors or show very low expression levels. Note that breast cancers associated with BRCA-1 and BRCA-2 mutations usually lack the expression of estrogen receptors. Breast cancer in men is more frequently positive for estrogen and progesterone receptors and is supposed to be more sensitive to anti-hormonal therapy. Furthermore the expression level of bcl-2 oncoprotein is markedly associated with the expression level of steroid receptors, whereas there is an inverse relationship between the expression of HER-2, EGFR, mammaglobin and Ki-67 (MIB-1) proliferation index and the expression of steroid receptors.

2.2.1.2 **Progesterone Receptors**

Progesterone is a steroid hormone involved in the differentiation of breast parenchyma and endometrium in addition to milk protein synthesis. Progesterone receptor (PR) is another member of the steroid family expressed on the nuclear membrane. The progesterone receptor molecule is a 946-amino-acid protein, which has two homologous isoforms PR-A and PR-B. Both isoforms are expressed in breast parenchyma, but PR-A is the dominant isoform in endometrial stroma and PR-B is the dominant isoform in endometrial glands. Progesterone receptors can be detected by immunohistochemistry using specific polyclonal and monoclonal antibodies (such as the 1A6 clone). Progesterone receptor status is one of the important prognostic factors in breast, endometrial and ovarian cancers. A high expression level of both estrogen and progesterone hormone receptors is a positive prognostic factor for breast and endometrial cancers and predicts a good response to anti-estrogen therapy. Several clinical observations show that estrogen and progesterone receptor-positive breast cancers show a good response to tamoxifen therapy in about 77% of cases, whereas estrogen-positive but progesterone-negative breast cancers are more likely to be poorly differentiated, with more aggressive behavior. The estrogen-positive, progesterone-negative breast cancers show a better response to aromatase

inhibitors but are more resistant to tamoxifen therapy, with an optimal response to the tamoxifen therapy in only 27% of cases; and the response to this therapy is usually shorter than estrogen-positive, progesterone-positive tumors. It is interesting that estrogen-negative but progesterone-positive breast cancers show a good response to tamoxifen therapy in about 45% of cases, while breast cancers negative for both estrogen and progesterone receptors show an optimal response to the tamoxifen therapy only in about 10% of cases.

2.2.1.3 Androgen Receptors

Androgen receptor (AR) is also a member of the steroid family of ligand-dependent transcription factors, which can be detected by immunohistochemistry using specific antibodies. Androgen receptor is expressed in the majority of estrogen-positive and in about 50% of estrogen-negative breast carcinomas in addition to DCIS, furthermore the majority of metastatic breast tumors are immunoreactive for androgen receptor. Clinical studies show also that ER-negative/AR-positive tumors have a better prognosis and longer disease-free survival than ER- and AR-negative tumors, which suggests a possible role of the androgen receptors in the breast cancer growth and differentiation. Androgen receptor is also highly expressed in the prostatic tissue and in the majority of prostatic tumors. The expression of the androgen receptor is an important prognostic factor for the cancer of the prostate and correlates with the grade of prostatic tumors and can give additional information useful for the treatment of prostatic carcinoma, namely the response to the anti-androgens or LHRH-analogs.

2.2.2 Estimation of Human Epidermal Growth Factor Receptor-2 Expression

*H*uman *e*pidermal growth factor *r*eceptor-*2* (HER-2 neu) is a member of the type 1 receptor tyrosine kinase family with domains on the cell surface, functioning as growth factor receptors. The type 1 receptor tyrosine kinase family includes the receptors HER-1 (EGFR or c-erbB1), HER-2 (c-erbB-2), HER-3 (c-erbB-3) and HER-4 (c-erbB-4).

The HER-2 neu oncoprotein, also known as p185 or c-erbB-2 (*c*hicken *e*rthro*b*lastic virus) is a 185-kDa transmembrane glycoprotein encoded by the HER-2 proto-oncogene located on chromosome 17 q12–21 consists of an extracellular domain (P105), a single transmembrane segment, and a cytoplasmic tyrosine kinase domain. In mammals, the HER-2 receptor plays an important role in the development of neural tissue and cardiac muscle. HER-2 is also normally expressed on the membrane of normal epithelial cells and 20×10^3 to 50×10^3 receptors are generally found on the surface of normal breast epithelial cells, nevertheless some types of epithelial tumors are associated with HER-2 overexpression and 2×10^5 to 3×10^6 receptors may be expressed on the cell membrane of these tumor cells. HER-2 overexpression is found in various human carcinomas, mainly breast carcinoma in addition to some other cancer types such as non-small cell carcinoma of the lung, salivary gland tumors and gastrointestinal tumors. Remarkable is the HER-2 overexpression in the

epithelioid components of synovial sarcoma but this phenomenon is not associated with gene amplification.

About 30% of unselected breast carcinomas are associated with HER-2 overexpression; most of them are invasive ductal carcinoma, inflammatory breast cancer, Paget's disease as well as breast cancer arising in pregnant women, whereas invasive lobular carcinoma, mucinous carcinoma and medullary carcinoma of the breast are rarely accompanied by HER-2 overexpression. HER-2 overexpression is also found in a significant percentage of male breast carcinomas (about 30%).

Another considerable observation is the relationship between the expression of HER-2 oncoprotein and the expression of steroid hormone receptors. Tumors with HER-2 overexpression show usually a low expression level of estrogen and progesterone receptors, while tumors with a high expression level of estrogen and progesterone receptors often lack overexpression of the HER-2 oncoprotein. Moreover, there is an inverse relationship between the expression of the bcl-2 oncoprotein and the expression of both HER-2 and EGFR. It is also interesting to mention that BRCA-1- and BRCA-2-related breast carcinomas are rarely associated with HER-2 overexpression.

The overexpression of the HER-2 oncoprotein is an important prognostic factor for breast cancer and the following aspects must be considered:

- Breast cancers with HER-2 overexpression have a poor prognosis with a median survival rate of 3 years compared with tumors lacking the HER-2 overexpression having a median survival rate of 6–7 years.
- Tumors with HER-2 overexpression are frequently poorly differentiated and a high percentage of the tumor cells is in the S phase.
- A high percentage of tumors with HER-2 overexpression is associated with P53 mutations and the tumor cells are usually bcl-2-negative.
- Tumors with HER-2 overexpression are usually resistant to anti-estrogen therapy and CMF (cyclophosphamide/methotrexate/5-fluorouracil) chemotherapy.
- Tumors with HER-2 overexpression are usually highly sensitive to the anthracycline chemotherapy (Doxorubicin, Epirubicin) and have an enhanced response to the taxane chemotherapy (paclitaxel).
- Tumors with HER-2 overexpression are sensitive to the specific immunotherapy and specific tyrosine kinase inhibitors.

The extracellular domain of the HER-2 neu molecule is the therapeutic target for the specific IgG1κ humanized chimeric monoclonal antibody Trastuzumab (Herceptin®), approved 1998 and found to be effective in malignancies with HER-2 overexpression. An important aspect of this specific immunotherapy includes the efficiency against non-proliferating disseminated tumor cells (dormant cells), which are usually in the G0 phase of the cell cycle, where conventional chemotherapy is less effective. The HER-2 neu molecule is also the target for specific tyrosine kinase inhibitors such as Tykerb (lapatinib ditosylate), which block the kinase-substrate interaction and the extracellular tyrosine kinase receptors on tumor cells.

For optimal therapy management and to verify the sensitivity of tumors to the specific antibody a three-point semi-quantitative scoring system to determine the HER-2 overexpression is used. The scoring is estimated after immunohistochemical staining of tumor sections. This immunohistochemical reaction is applicable on routinely processed sections from formalin-fixed paraffin-embedded tissue using specific polyclonal (HercepTest™, Dako) or monoclonal (such as the clones PN2A, CB11, SP3, TAB 250 and B10) antibodies. The antibody included in the HercepTest™ and other clones such as SP3 and CB11 are directed to the extracellular domain of the HER-2 molecule, while some other clones are directed to the intracellular domain. Because of the quick damage of the HER-2 molecules due to oxidation or other factors (slide aging), fresh prepared tissue slides must be used. Tissue after frozen section is not suitable for this reaction. Critical for accurate evaluation is the standardization of the immunohistochemical reaction, which includes standard tissue pretreatment, adequate antigen unmasking (40 min in a water bath at 95–100 °C) and the use of certified antibodies. The hallmark of the three-point scoring system is the interpretation of the intensity and continuity of the membrane staining and the percentage of positive cells (Table 2.3).

For precise scoring the following factors must be considered:

- The interpretation of the immunohistochemical stain must begin with the evaluation of standardized control slides with the scores 0, 1+ and 3+.
- Only membrane staining should be evaluated. Cytoplasmic or nuclear stain must be neglected. Staining caused by edge artifacts must also be ignored.
- Only invasive tumor components must be considered.
- Primary tumors with heterogeneous cell populations exhibiting different expression intensity of the HER-2 oncoprotein and consequently different scores, must be particularly documented. In such cases, the assessment of metastatic tissue to determine the metastasizing cell population is recommended. This phenomenon is described in about 5% of breast tumors.

Tumors with scores of 0 or 1+ have no gene amplification and no HER-2 overexpression and consequently show a poor sensitivity to the specific immunotherapy. Tumors with a score of 3+ are associated with HER-2 overexpression and show a good

Table 2.3 The three-point scoring system.

Score	HER-2 overexpression	Staining result
0	Negative No gene amplification	No detectable staining or membrane staining in less than 10% of tumor cells
1+	Negative No gene amplification	A faint partial membrane staining in more than 10% of tumor cells
2+	Positive	A weak to moderate staining of the entire membrane in more than 10% of tumor cells
3+	Positive High gene amplification	A strong staining of the entire membrane in more than 10% of tumor cells

response to the specific antibody therapy, whereas tumors with a score of 2+ needs a further confirmation to estimate the number of gene copies in the tumor cells to justify this type of therapy because of possible side-effects and high cost. The confirmation of the HER-2 overexpression in tumors with a score of 2+ can be achieved by genetic or chromosomal studies such as fluorescent *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH) and real-time PCR assays. The presence of <2 gene copies in the examined cells indicates no gene amplification, whereas 2–6 copies signifies low-level amplification and >6 gene copies signifies strong gene amplification. Many studies show that the concordance between immunohistochemical score 0, 1+ and 3+ and the FISH method is more than 96%, whereas only 10–33% of tumors with a score of 2+ show HER-2 gene amplification in the FISH assay, which emphasize the necessity of further confirmation of HER-2 overexpression in tumors with a score of 2+.

2.2.3
Estimation of Epidermal Growth Factor Receptor Expression

*E*pidermal *g*rowth *f*actor *r*eceptor-1 (EGFR, Erb1) is another member of type 1 receptor tyrosine kinase family mentioned in the previous section functioning as growth factor receptors. EGFR is a transmembrane 170-kDa glycoprotein encoded by the c-erbB1 proto-oncogene and composed of the three following major domains:

- An extracellular ligand-binding domain, which binds to different growth factors (see below);
- A transmembrane lipophilic region;
- An intracellular domain characterized by tyrosine kinase activity initiated in response to ligand binding.

Epidermal growth factor receptor-1 (EGFR) binds to epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin-binding epidermal growth factor (HB-EGF), amphiregulin, betacellulin and epiregulin. It plays an essential role in the development of many normal tissue types and is normally expressed on the membrane of normal epithelial cells and up to 25×10^4 receptors can be found on the surface of non-neoplastic epithelial cells such as endometrial cells. The activation of EGFR pathways is important for cell cycle progression and cell proliferation in addition to cell differentiation, but the expression or overexpression of EGFR in tumors is usually a sign of aggressive behavior and poor prognosis. The deregulation of cell cycle coupled with the overexpression of EGFR has been observed though the growth and the progression of different tumors of different histogenesis, mostly epithelial malignancies including carcinoma of the breast, non-small cell carcinoma of the lung, squamous cell carcinoma of head and neck, renal cell carcinoma, colon and pancreatic adenocarcinoma, ovarian and bladder carcinoma, however some other non-epithelial tumors such as glioblastoma multiforme are also associated with EGFR overexpression. Note that the EGFR overexpression in glioblastoma multiforme is a favorable prognostic factor.

The inhibition of EGFR activity causes the disruption of EGFR pathways and the blockage of the cell cycle progression leading to cell apoptosis, inhibition of tumor angiogenesis and suppression of the metastatic activity. EGFR is now the therapeutic target of different EGFR tyrosine kinase inhibitors such as lapatinib ditosylate and specific monoclonal antibodies such as the chimeric IgG1κ antibody Cetuximab (Erbitux®), the human IgG4κ antibody Panitumumab (Victibix®) and the humanized IgG1 antibody Nimotuzumab (TheraCIM®). The specific antibodies are now approved and used for the therapy of EGFR positive tumors such as colorectal and head and neck carcinomas.

The overexpression of EGFR can be estimated by the immunohistochemical staining using different specific polyclonal or monoclonal primary antibodies such as the clones E30, EGFR.25 and 31G7. For optimal immunohistochemical staining, unmasking of EGFR antigen by enzymatic digestion is needed (optimally 5 min digestion with 0.1% proteinase K). For the immunohistochemical staining and the evaluation of EGFR overexpression, we have to take the same cautions listed in the previous HER-2 section.

Two scoring systems were proposed for the semi-quantitative evaluation of EGFR overexpression in order to estimate the response of related tumors to the specific immunotherapy:

- The first system is identical to the three-point scoring system used for the estimation of HER-2 overexpression.
- The second system is a modification of the scoring system suggested by Remmele used for the evaluation of the estrogen receptor status. This scoring system has a nine-point scale (0–9) and calculated by multiplying the number reflecting the dominant intensity of the membrane stain 0, 1, 2 and 3 by the number reflecting the percentage of these positive tumor cells 0, 1, 2 and 3, as in Table 2.4.

According to this scoring system tumors having a score of less than 4 considered to have low EGFR expression and show a poor response for the specific therapy. The presence of cells showing cytoplasmic stain must also be mentioned.

Other similar pharmacopathologic diagnostic systems are now developed for other growth factor receptors, which can be the target for immunotherapy agents or specific

Table 2.4 The nine-point scale (0–9) is calculated by multiplying the number reflecting the dominant intensity of the membrane stain (0, 1, 2, 3) by the number reflecting the percentage of these positive tumor cells (0, 1, 2, 3).

	Percentage of positive cells		Intensity of stain
0	No positive cells	0	No detectable stain
1	<10%	1	Weak nuclear stain
2	<50%	2	Moderate nuclear stain
3	>50%	3	Strong nuclear stain

inhibitors such as the vascular endothelial growth factor receptor (VEGFR) and the platelet-derived growth factor receptor (PDGFR).

The vascular endothelial growth factor receptor binds to the vascular endothelial growth factor stimulating the proliferation of the vascular endothelium and increasing the vascular permeability, which may cause tumor progression. VEGFR is the therapeutic target for the humanized IgG1-specific antibody Bevacizumab (Avastin®) found to be effective in metastatic colorectal carcinomas.

The platelet-derived growth factor receptor is a further growth factor receptor, which binds to the platelet-derived growth factor causing the proliferation of mesenchymal cells. PDGFR is also the target of specific therapeutic antibodies.

2.2.4 Detection of CD 20

CD20 is a non-glycosylated 33-kDa phosphoprotein encoded by a gene located on chromosome 11q12–13, next to the site of the t(11; 14)(q13; q32) translocation associated with mantle cell lymphoma. This transmembrane antigen is expressed in the late pre-B cell stage after CD19/CD10 expression but before CD21/CD22 and surface immunoglobulin expression. CD20 remains as a constant antigen throughout the course of B-lymphocyte maturation but is absent in plasma cells.

CD20 (also known as L26) acts as a receptor playing role in the differentiation, activation and proliferation of B-lymphocytes. CD20 is also expressed on neoplastic B-lymphocytes and found almost in all lymphomas/leukemias of B-lymphocyte derivation. The extracellular domain of this antigen can be a suitable target for specific antibodies for the treatment of malignancies carrying this antigen. Rituximab (Rituxan®) a chimeric monoclonal IgG1κ antibody, Y-90 radiolabeled Ibritumomab (Zevalin®) murine monoclonal IgG1κ antibody and the I-131 radiolabeled Tositumomab (Bexxar®) murine monoclonal IgG2aλ antibody are examples for CD20-specific antibodies. These specific antibodies are used for the treatment of various types of CD20-positive B-cell lymphoma especially low-grade lymphomas mostly follicular lymphoma. This antigen can be detected on tissue sections using specific monoclonal antibodies (such as clone L26) to determine the sensitivity of these malignancies to the specific immunotherapy.

Many other lymphocyte-, leukocyte- and plasma cell-specific antigens are also now the target for specific therapeutic antibodies and can be detected by immunohistochemistry or flow cytometry such as CD22, CD 33, CD38 and CD52.

CD22 CD22 (also called as B-lymphocyte cell adhesion molecule or BL-CAM) is another B-cell-associated antigen, a type 1 integral membrane glycoprotein expressed in early B-lymphocytes differentiation, almost in the same stage as CD19 expressed. CD22 is also highly expressed in many types of B-cell lymphomas such as hairy cell lymphoma and expressed in about 90% of high-grade B-cell lymphoma. CD22 can be detected by immunohistochemistry using specific antibodies. CD22 is the therapeutic target of specific antibodies such as the humanized IgG1 antibody HLL2