CHAPTER

5 Criteria of Malignancy and Diagnostic Pitfalls

The criteria of malignancy are structural alterations of cancer cells seen at the microscopic level compared to normal cells, as well as biochemical and molecular alterations induced by accumulation of cooperative genetic alterations that affect key molecular pathways in cancer cells and modify microenvironment interactions. The correlation of these molecular aspects with morphological changes is essential for better understanding of carcinogenesis, and progression of cancer.

Cancer is essentially diagnosed based on morphological features identified by histopathology and/or cytopathology. Molecular markers in cancer pathology are useful tools to improve early detection and diagnosis, and assess prognosis and response to therapy. Flow cytometry is one of the tools used to highlight acquired capabilities of cancer, including unrestricted growth, extended cell survival, and genetic instability. The application of flow cytometry and other molecular tests in cancer pathology requires sensible selection and careful morphological correlation.

The criteria defining tumors as acquired from the pathologic diagnosis form the basis of management of cancer patients. The sources of diagnostic pitfalls are mostly avoidable. Knowledge of such pitfalls leads to reliable implementation of pathologic findings in cancer management.

MORPHOLOGIC CRITERIA OF MALIGNANCY

Histopathology still remains the most accurate method for cancer diagnosis. The recognition of malignancy is based on both direct and indirect histologic criteria. Direct criteria are conclusive of malignancy, whereas, indirect criteria are only suggestive of malignancy. Since some features of malignancy are simulated by a variety of hyperplastic processes, the diagnosis of malignancy must be based on more than one direct criteria. Recent advances in histochemistry, immunopathology and DNA analysis have added new biologic parameters to the standard morphologic study of tumors.

Direct Criteria

Cellular Changes

1. Anaplasia. The term literally means reversion of form, but in practice it refers to the nuclear morphologic features of malignancy. It includes enlargement of the nucleus (karyomegaly) due to increase in DNA (polyploidy), increase in nucleoproteins and nuclear edema. There is increase in the nucleocytoplasmic ratio (N/C ratio) partly due to enlargement of the nucleus and partly due to reduction in cytoplasmic volume. The normal N/C ratio of 1/4 may be increased to almost 1/1. The nuclei are hyper-chromatic or darkly stained by basophilic stains (Hematoxylin) due to increase in the amount of heterochromatin and DNA.

The nuclei appear pleomorphic or variable in size and shape with irregular clumping and distribution of chromatin which appears coarsely granular. Parachromatin clearing may be marked resulting in abnormal vesicular nuclei. The nucleoli may increase in size and number. Macronucleoli more than 5 microns in diameter are strongly consistent with malignancy and is due to blockage of transport of nucleolar products to the cytoplasm. Multiple nucleoli are attributed to polyploidy. Multinucleated giant cells may occur as a result of nuclear division without cytoplasmic division. Differentiation between malignant and benign multinucleation is important. In benign multinucleation, the nuclei are uniform and more numerous.

2. Increased mitosis. The increase of mitotic figures in a tumor is partly due to an increase in growth fraction and partly due to prolongation of metaphase. The mitotic activity is assessed by one of two methods; namely: a) The mitotic index, expressed as number of mitosis per 1000 cells or b) Mitotic count in 10 microscopic high power fields (HPF). The number of mitotic figures in a tumor usually correlates with biologic behavior. Thus the mitotic index is about 1/1000 in benign tumors, but may increase to 20/1000 in malignant tumors. The mitotic count per 10 high power fields is usually below 1/10 HPF in all benign tumors, except cellular leiomyoma in which a count up to 4/10 HPF may be observed. In

malignant tumors, they are classified into those with low mitotic activity (2-5 mitoses/10 HPF) and those with high mitotic activity (6 and more per 10 HPF). In some very active tumors such as high grade non-Hodgkin's lymphoma, the mitotic count may be over 50/10 HPF. The mitotic count is an important criterion for the diagnosis of smooth muscle tumors. Thus, in benign leiomyoma it is 1-4/10 HPF and in case of leiomyosarcoma it is 10 and more/10 HPF. Myomatous tumors with intermediate counts (5-9/10 HPF) are considered to be of borderline malignancy.

Proliferation rate may also be quantitated by Ki-67 labeling of malignant cells. Ki-67 is a nuclear protein which can label cells in all active phases of the cell cycle using immunohistochemistry. It yields higher percentages of positively labeled proliferating cells when compared with proliferating cell nuclear antigen (PCNA) or mitotic indices. The proliferative rate is assessed by counting multiple regions with highest immunolabeling density for the proliferation marker Ki-67 and reporting the mean of the percentage. It can be more accurate than mitotic count in small biopsies.

In benign tumors the Ki-67 indices range from 0-3% while malignant tumors most frequently show an index above 10%. In high grade lymphomas like Burkitt lymphoma, almost 100% of the nuclei are stained which may serve as a diagnostic feature. The Ki-67 proliferation rate is particularly helpful to separate well-differentiated (low or intermediate grade) tumors from poorly differentiated (high grade) neuroendocrine carcinomas, which usually have dramatically different Ki-67 labeling rates. There are no definitive cut-points, and it is recommended to specify the actual proliferative rate in the pathology report, in addition to designating a tumor grade.

Proliferation markers have also been used by classification systems for prognosis in other tumors as GIST. The added prognostic information provided by Ki-67 immunostaining is now well established with regard to astrocytic tumors. The latest WHO classification of central nervous system tumors uses Ki-67 indices to differentiate between low grade (index 0-5%) and high grade gliomas (index above 10%).

Women with hormone receptor(s) positive breast carcinoma and "high" Ki-67 (i.e. >15%) should receive chemotherapy in addition to hormone therapy, those with "low" Ki-67 (<15%)) should receive hormone therapy alone. Moreover, Ki-67 may have a prognostic value specially when it attains very high level.

3. Abnormal mitosis. This refers to mitotic figure with abnormal distribution of chromosomes or to mitosis with more than two spindles. The result of abnormal mitosis is either aneuploid cells or multinucleated giant cells. Aneuploid cells contain abnormal number of chromosomes either below normal (hypoploidy) or above normal (hyperploidy and polyploidy). In hyperploidy there is an increase of one or more chromosomes above the normal number, whereas in polyploidy there is three or multiple of the normal haploid number of 23 chromosomes. Tumor cells with increased number of chromosomes may remain viable and capable of division, but, multinucleated giant cells are usually sterile end cells which will ultimately die. Hypoploid abnormal mitosis is usually a lethal change due to possible deficiency of some vital genes in daughter cells.

Abnormal mitosis may result through one of the following mechanisms (Fig 5-1): (a) Asymmetrical separation of chromosomes in anaphase due to chromosomal sticking resulting in a hypoploid and hyperploid cells (Fig 5-1A). (b) Chromosomal lagging in anaphase resulting in polar chromosomes (= micronuclei) in daughter cells and hypoploidy (Fig 5-1B and C). (c) Chromosomal bridge occurs when two chromosomes break and heal by translocation forming a dicentric chromosome. It subsequently forms a bridge in anaphase with the ends of the double chromosome migrating to opposite poles of the cell (Fig 5-1D). This kind of abnormal mitosis is commonly seen after irradiation of tumors and is not compatible with cell viability, (d) Endomitosis or non-disjunction in which duplication of chromosomes occurs in metaphase without accompanying spindle formation or cytoplasmic division resulting in a polyploid nucleus (Fig 5-1E). (e) Multipolar mitosis is characterized by the formation of more than two spindles in metaphase (usually tripolar or quadripolar), and results in polyploidy (Fig 5-1F). (f) "Hollow metaphase" or "colchicine effect" characterized by widely scattered chromosomes in metaphase (Fig 5-1G). g) Failure of Cytokinesis; nuclear division without cytoplasmic division results in multinucleated giant cells (Fig 5-1H).

Population Changes

1. Hypercellularity. Malignant tumors are characterized by increase of cell population as compared to normal. This results in a crowded cellular

pattern, deformation of cell shape from mutual pressure resulting in cellular moulding and anisocytosis.

2. Disorganized pattern. The regular arrangement of normal cells (polarity) is lost with the development of cancer. Malignant cells are irregularly piled up in a disorganized pattern.

3. Invasion. Infiltration of the stroma is an important direct criterion of malignancy. Two qualitatively different patterns of invasion are recognized, namely: (a) cohesive sheet or cylinder invasion, versus (b) single cell invasion. The invasive property of malignant tumors may also be manifested as lymphatic permeation, angioinvasion, perineural space invasion and capsular inva sion.

Indirect Criteria

1. Necrosis

Malignant tumors are usually degenerated and necrotic. Massive or focal coagulation necrosis is liable to occur due to vascular occlusion or as a result of the tumor overgrowing its limited blood supply. The pattern of tumor necrosis is related to the pattern of its blood supply (Fig 5-2). Thus, some tumors are organized as cords having a central blood vessel (Fig 5-2A) as in the case of Ewing sarcoma and papillary carcinomas. The tumor cells around the vessel appear viable for a distance of 150 micrometers, beyond which necrosis of tumor cells is observed. This pattern of necrosis correlates well with the diffusion path length of tissue oxygen from blood supply. Other tumors, such as comedocarcinoma of breast, exhibit a different tumor cord-vascular pattern, with the blood vessels arranged around the periphery of tumor nodule (Fig 5-2B). As the tumor nodule grows beyond 300 micrometers, central necrosis develops.

2. Structural Dedifferentiation

This denotes the reversion of the cell to its immature embryonic form with loss of cytoplasmic structures. The cytoplasm becomes scanty with ill-defined cell membrane and loss of surface organelles such as cilia and intercellular junctions (desmosomes). On the other hand, dedifferentiation refers to the appearance of abnormal ciliation in ovarian serous adenocarcinoma, abnormal keratinization in squamous cell carcinoma and enormous mucus production with signet ring cell formation in adenocarcinoma. Dedifferentiation usually correlates with biological malignancy. However, there are exceptions to this rule. For example, melanotic malignant melanoma and mucus-secreting adenocarcinomas are functionally rather differentiated, but behave as aggressive tumors. On the other hand, basal cell carcinoma of the skin is an undifferentiated tumor but is biologically a favorable malignant tumor.

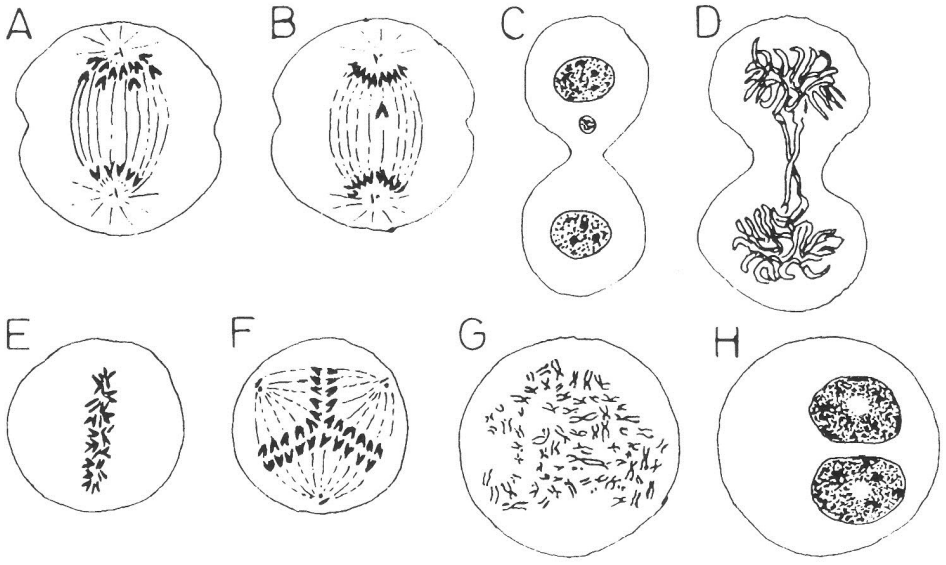

Fig 5-1 *The cytomorphology of abnormal mitosis in malignant tumors (A) Asymmetrical separation of chromosomes (B) Chromosomal lagging (C) Micronucleus (D) Double chromosomal bridge (E) Endomitosis or non-disjunction (F) Tripolar mitosis (G) Colchicine-like effect (H) Multinucleated giant cells*

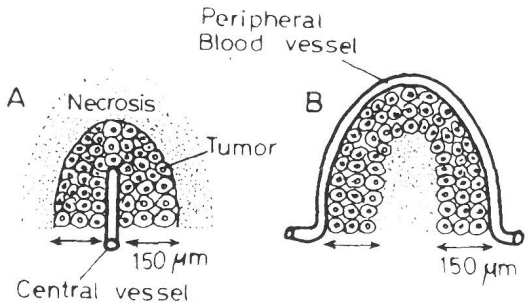

Fig 5-2 *Patterns of necrosis in malignant tumors (A) peripheral necrosis with central blood supply and (B) central necrosis with peripheral blood supply.*

CYTOPATHOLOGY

Cytopathology is the method applied for diagnosis of malignancy in cells exfoliated from the epithelial surfaces of the human body or are removed from tissues by various methods including; washing (bladder), aspiration (bile ducts and pancreas), brushing (bronchial), scraping (cervicovaginal smear), or fine-needle aspiration cytology (FNAC). Among the criteria suggestive of malignancy in cytology material are the increase in cellularity, poor adhesiveness between cells, variable size and shape of the cells, abnormalities in nuclear size and shape with increased N/C ratio, nuclear hyperchromasia, coarse granular chromatin, and prominent nucleoli.

FNAC is widely practiced as a painless, rapid, and cost-effective method of diagnosis. Although FNAC can be applied to almost any organ or body site, a definitive specific diagnosis is not always possible by cytology which however can provide the category of the disease and a differential diagnosis in most of the cases.

The category of atypical or borderline nuclear change (BNC) assigned to a group of lesions in cytology is similar to the category of atypical squamous or glandular cells of undetermined significance (ASCUS/AGUS) in the Bethesda system of cervical cytopathology. Under diagnosis or false negatives may be encountered in low grade malignancies where differences from benign lesions are subtle or due to the hypocellular nature of the smear. Over diagnosis, on the other hand, or false positives may be caused by reactive or therapy-induced changes in cytological features, metaplasia, or benign tumors with remarkable nuclear atypia.

The Bethesda reporting terminology for cervical smears is divided into 5 categories: benign cellular changes, atypical squamous or glandular cells of undetermined significance, low grade intraepithelial lesion, high grade intraepithelial lesion, and cancer. A reporting terminology was proposed by Worsham for aspirates from other body sites, divided into 6 categories:

Category 1:	Acellular specimens
Category 2:	Stromal elements only
Category 3:	Hypocellular aspirate with only rare epithelial cells
Category 4:	Adequate cellularity, may or may not have a specific diagnosis
Category 5:	Atypical/suspicious aspirates
Category 6:	Aspirates diagnostic of malignancy

The limitations of FNAC include the absence of a tissue pattern in smears, the lack of experience in some less common conditions, and the relatively high level of expertise required in the interpretation and application of ancillary studies on cytology material. The benign, atypical, or malignant nature of certain lesions, for example, follicular lesions in thyroid and papillary lesions in breast cannot be reliably diagnosed from cytology. Aspirates of low cellularity should not be reported as frank malignant.

An accurate diagnosis can be established by FNAC through adequate representative sampling, optimum processing, and correlation of interpretation with clinical and radiological studies. A second smear or even a tissue biopsy is recommended in cases with borderline nuclear changes to avoid diagnostic pitfalls leading to a missed diagnosis of cancer or overtreatment of benign condition.

FLOW CYTOMETRY

Flow cytometry allows rapid quantitative analysis of various constituents of individual cells with a high degree of accuracy. By application of specific fluorescent dyes, it is possible to evaluate the following tumor parameters: (a) DNA ploidy level of cell population after staining with propidium iodide (PI), (b) The proportion of cells in each phase of the cell cycle, particularly the S-phase fraction, (c) The proportion of necrotic cells in the tumor after staining with ethidium bromide which

can only enter degenerated cells, (d) RNA analysis of tumor cells after specific staining with acridine orange stain, and (e) Determination of S-phase time and potential doubling time, by injection of iodode-oxyuridine, which is incorporated only into cells in S-phase, then serial tumor tissue samples are obtained for DNA analysis.

Flow cytometry has become essential for the diagnosis of hematopoietic and lymphoid disorders using antibody panels to identify the cell type (hematopoietic, lymphoid, or non-hematopoietic), cell lineage (B- and T cells, natural killer cells, myeloid/monocytic cells, neuro/neuroendocrine cells, and epithelial cells), cell maturation stage (precursors vs. matured cells), and B-cell clonality (immunoglobulin light chain restriction).

Technique

Flow cytometry requires the preparation of a single-cell suspension. The cells are stained with one or several fluorescent labels. The cells are then passed, one-by-one, through one or more laser beams to excite the cells and the fluorescent labels. The resulting fluorescence is collected by the corresponding detectors, separated, measured, and the resulting digitized impulses are transmitted for computer analysis (Fig 5-3). The data are mostly displayed in the computer as single-parameter histograms or two parameter plots . A total of 10,000 cells are usually analyzed within few seconds. Specimens suitable for flow cytometry include peripheral blood, bone marrow, body fluids, cerebrospinal fluid, urine, fine-needle aspirates, and any fresh tissue after mechanical mincing.

DNA Analysis

DNA analysis of tumors shows either a diploid or aneuploid pattern (Fig 5-4). For calibration and use as normal standard, normal human lymphocytes (diploid cells), are processed in a similar manner in order to work the 2C position of DNA value corresponding to normal diploid cells (G1+G0 phases). The diploid pattern [Fig 5.4A] is similar to that of normal cells with a large peak to the left representing resting cells (G1+G0 phases) corresponding to the normal 2C DNA content. A small peak to the right is also seen at 4C, corresponding to cycling cells which have doubled their DNA content (G2+M phases). The area under the curve between the two peaks represents cells in S-phase.

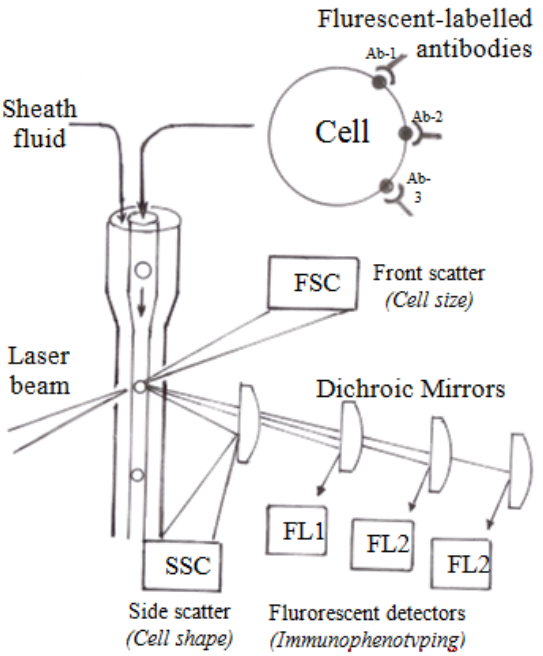

Fig 5-3 *The basic components of a multi-parameter flow cytometer. Forward scatter (FSC) determines cell size, side scatter (SSC) determines cell shape, and different fluorescent signals determine phenotyping (Rosai, 2012).*

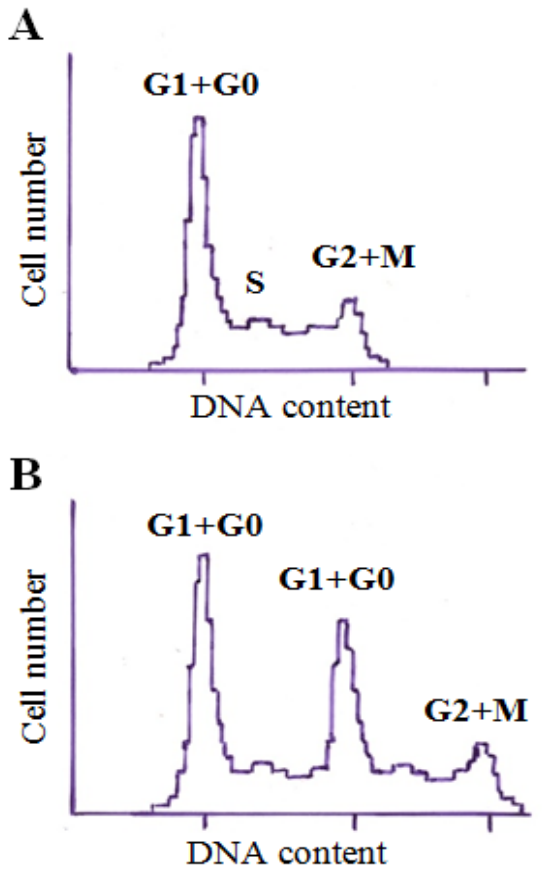

Fig 5-4 *DNA quantitative histograms measured by flow cytometry (A) Normal pattern (B) aneuploidy pattern (Silverberg, 2006).*

Aneuploidy

Aneuploidy implies abnormal (usually increased) quantities of DNA content. The (G1+G0) peak is shifted to the right of 2C DNA value, and similarly the (G2+M) peak is also shifted to the right of 4C DNA value. The term DNA index (DI) is used to express tumor ploidy and is obtained by dividing the DNA value of (G1+G0) peak for the tumor by the DNA value for (G1+G0) peak for normal standard cells. Tumors are not classified as aneuploid unless the DI exceeds 1.15 (i.e. more than 15% increase than normal diploid value).

Aneuploid tumors show DNA peaks away from the normal modes (Fig 5-4B) and fall into three main patterns: (a) Tetraploid pattern with (G1+G0) peak at 4C DNA value and DNA index of about 2, (b) Non-tetraploid pattern with (G1+G0) peak in an intermediate position between 2C and 4C (about 3C DNA content), and (c) Multiploid pattern showing multiple peaks of (G1+G0) indicating the presence of more than one tumor cell line (subclonal), each of which fulfilling the criteria of aneuploidy (DI more than 1.15).

A study of ploidy of bladder carcinomas by DNA flow cytometry (Tribukait, 1984) revealed that 32% of the tumors were diploid, 11% tetraploid, 35% nontetraploid and 22% multiploid. Generally, patients with diploid tumors have a more favorable prognosis than those with aneuploid tumors. Tumors can be arranged in the following order with increasing degree of malignancy: diploid tumors, tetraploid, nontetraploid and multiploid tumors.

From the diagnostic point of view, aneuploidy is considered an atypical phenomenon, highly suggestive but not conclusive of malignancy, because it may be observed in dysplastic lesions and is even present in some normal cells, such as the megakaryocytes and syncytiotrophoblast. However, once the diagnosis of malignancy is established, the presence of aneuploidy has important prognostic value.

Immunophenotyping

Immunophenotyping analysis by flow cytometry is done through gating cell populations. To accurately target and gate the cell population(s) of interest: As the first step, it is most important to determine whether the cells of interest are hematopoietic/lymphoid. Generally speaking, all hematopoietic/lymphoid cells express CD45 antigens (CD45+). Thus, a histogram of CD45 vs. side light scatter (SSC) is indispensable as a starting point of flow cytometry analysis (Fig 5-5). When distinct cell populations cannot be established due to limited and or mixed cells, histograms can gate cells based on cell size using lymphocytes (small) and monocytes (intermediate) as an internal size control. Once the cells of interest are gated, further analysis of cell lineage can be performed. Identification of cell lineage by flurochrome-conjugated antibodies is also needed when particular CD45+ cell windows cannot be specified in the CD45 vs. SSC histogram.

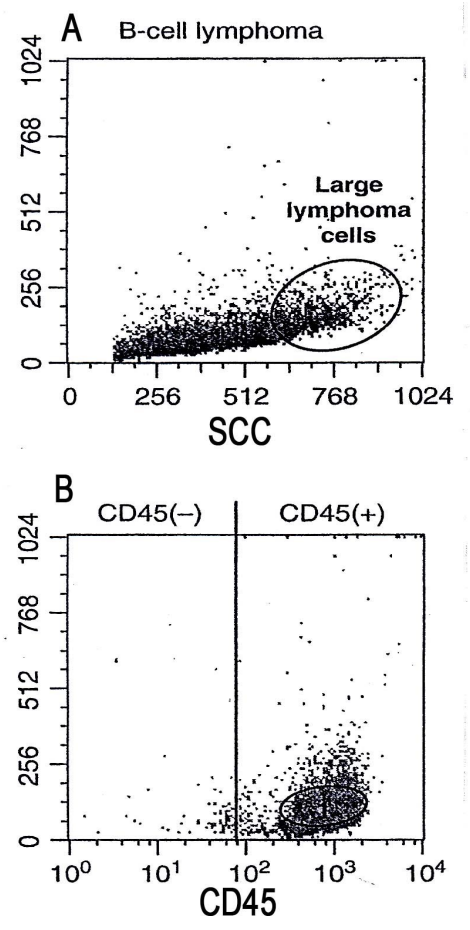

Fig 5-5 *Scatter diagram of large cell NHL in bone marrow as measured by flow cytometry. (A) Identification of cell population through cell size by FSC vs. SSC. (B) Immunophenotyping of tumor cells by CD45.*

In order to make an accurate interpretation of flow cytometry data, one must correlate the patient's history, clinical presentation, and laboratory tests, and the clinician's concerns and possible pathology findings.

IATROGENIC CHANGES IN MALIGNANT TUMORS

No or Minimal Effect

The appearance of the treated cancer in the surgical specimen may vary between completely viable tumor tissue (P 5-1 to 5-4) versus a spectrum of changes in neoplastic tissue in response to preoperative chemotherapy or radiotherapy, which can present a diagnostic difficulty to the pathologist, especially in the absence of relevant data regarding previous treatment.

Differentiation

Examples include *germ cell tumors* and ovarian epithelial tumors. Residual rhabdomyosarcoma of childhood sometimes shows increased proportion of rhabdomyoblasts and strap cells originally present as minor elements in the tumor before therapy. Other types of soft tissue sarcoma show a lower histologic grade after chemotherapy because of the selective destruction of high grade population by this kind of therapy. These changes underline the importance of applying the tumor grade to the pretreatment biopsy for accurate prognostic evaluation.

Differentiation of *neuroblastoma* to ganglioneuroblastoma with predominance of differentiating or ganglion cells over immature neuroblasts is also found after chemotherapy (P 5-5). Mature heterotopic elements may be present in residual tumors after preoperative chemotherapy. Such elements include cartilage, fat, and skeletal muscle in Wilms tumor, and osteoid in hepatoblastoma.

The induction of granulocytic differentiation in one of *AML* subtypes by differentiation-inducing agent, all-trans retinoic acid (ATRA) formed the basis of subsequent trials to develop new and better differentiation-based combined therapies. Such therapies can be targeted against specific abnormalities underlying the pathogenesis of a given AML subtype, or possibly take advantage of characteristics shared by different AML.

The effect of radiation therapy on tumors is rather similar to chemotherapy. Differentiation induced in tumors after radiation therapy is seen in *squamous carcinoma* showing more keratin in the post-irradiated tumor with or without intact tumor cells (P 5-6). More frequent alterations after radiation therapy include cytomegaly with abnormal shapes and hyperchromatic nuclei (P 5-7 and P 5-8).

Degeneration

Chemotherapy-induced changes in malignant tumors do not necessarily show degenerative necrosis. Morphologic changes include vacuolization of neoplastic cells simulating foamy histiocytes with or without associated inflammatory cell reaction and/or areas of fibrohyaline or fibrous tissue. One or more of these features can be induced by chemotherapy or radiotherapy in breast carcinoma (P 5-9), non-Hodgkin lymphoma (P 5-10), osteosarcoma (P 5-11 and P 5-12), Wilms tumor (P 5-13), PNET (P 5-14), GIST (P 5-15), and others.

Eradication of Tumor

Complete histologic response to lethal chemotherapy or radiotherapy may have major implications for additional therapy, as evident in certain instances of breast carcinoma (P 5-16) and osteosarcoma (P 5-17) showing absence of intact or viable tumor cells. Detailed scoring of the extent of histologic response to therapy in breast carcinoma and osteosarcoma is discussed in chapters 18 and 20. Complete eradication of tumor cells is also reported in several tumor types such as lymphoma (P 5-18), GIST (P 5-19), metastatic carcinoma of breast origin (P 5-20), thymoma (P 5-21), and germ cell tumors (P 5-22).

The predictive value of histologic response to pre-operative therapy in surgical specimens varies among tumors and is reported to be specific to certain treatment protocols. Altering the duration and the dose of chemotherapy may affect the sensitivity and the specificity of the response rates as a measure of progression-free survival.

SURGICAL MARGINS

The evaluation of the surgical margin presents a challenge to the pathologist to ensure complete tumor removal which generally dictates the need for further local therapy, such as additional surgery, radiation therapy, or chemotherapy. The surgical goal is to remove a margin of uninvolved

tissue, but the amount of resected uninvolved tissue that is considered adequate has not been clearly defined for all forms of cancer.

When evaluating a margin microscopically, a positive margin is identified by the presence of invasive carcinoma at the surgical margin. However, in certain organ systems, this may not be adequate. For example, in head and neck oral carcinoma, primary skin melanoma, or breast carcinoma, a margin is considered inadequate (close or positive) if tumor is within a certain distance from the margin (Table 5-1). The relevance of severe dysplasia or carcinoma in situ may be organ specific but should always be reported.

Small samples from the resection bed which are closest to the resection margin can be submitted by the surgeon to the pathologist for evaluation. If given an en bloc resection, the pathologist evaluates the margin status by either a perpendicular or en face (tangential) section (Fig 5-6).

Most pathologists are familiar with perpendicular margin as the exact distance of the tumor from the margin can be determined on microscopic examination. Perpendicular margins are preferred when narrow margins (less than 0.2 mm) are considered as negative. The only disadvantage with perpendicular margin is that little tissue is sampled in large resections.

Table 5-1 Recommended Clearance of Excised Tumors Based Upon Tumor Site, Type, and Size

Tumor site	Tumor type	Clearance	Frozen section
Skin	Melanoma		
	Melanoma in situ	5 mm	
	0-2 mm	1 cm	Not indicated
	2-4 mm	1-2 cm	
	>4 mm	2-3 cm	
Oral/Oropharynx*	NOS	>5 mm	Indicated
Breast (applies to invasive and intraductal carcinoma)	NOS	>1 cm	Indicated

**High grade dysplasia and carcinoma in situ should also be reported in margins*

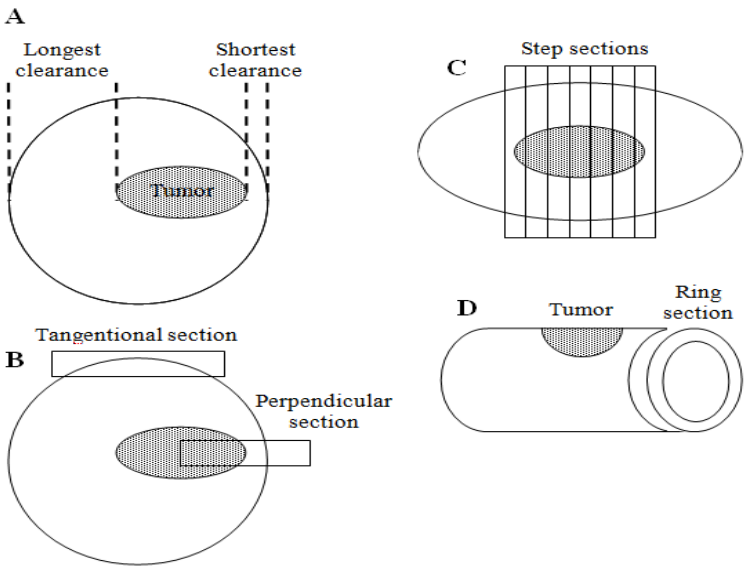

Fig 5-6 *Evaluation of adequacy of surgical excision (A) Definition of terms (B) Perpendicular sections are preferable than tangential sections (C) Step sections in malignant melanoma (D) Ring section for surgical margin in tubular structures*

BIOMARKERS OF CANCER

In spite of intensive research efforts, there is so far no single biochemical change that could distinguish malignant from normal cells. However, some malignant tumors are associated with increased production of biochemical products (tumor markers or biomarkers) which have been most useful in cancer detection or diagnosis. Tumor biomarkers are classified into 2 classes, namely; tissue biomarkers and serum biomarkers while others may be detected in urine or other body fluids of patients with cancer. Tissue markers are more accurate and are studied in tumor tissue (Chapter 4) by either immunohistochemical or molecular genetic methods.

Certain markers are used for screening purpose, e.g. alpha-fetoprotein (AFP) in chronic hepatitis B and C and cirrhosis patients, in conjunction with ultrasonography, for detection of hepatocellular carcinoma. Human chorionic gonadotropin (beta HCG) is an excellent example of a screening test in gestational trophoblastic tumors developing after molar pregnancy. Others are only used in the presence of a mass lesion, e.g. CA-125 in suspected ovarian cancer among the differential diagnosis of a pelvic mass.

False negative results provide limitations to the use of some markers, e.g. up to 20% false negative results for CA 19-9 in pancreatic carcinoma. Most markers are not specific for individual tumors, besides, they are elevated in some nonmalignant conditions such as: pregnancy, autoimmune disease, inflammatory conditions and liver disease, giving false positive results.

In patients with established diagnosis of cancer, levels of serum markers may serve as prognostic indicators because the serum concentration of tumor markers increases with tumor progression and metastasis. Highly sensitive serum biomarkers are most useful in monitoring the response to therapy and to detect recurrence as early as possible. The following is a summary of the main clinically useful serum tumor markers.

1. Oncofetal antigens: These are normally present in fetal and embryonic tissue and their production is repressed after birth, to be present in only minute quantities in the circulation of adults. Oncofetal protein production is increased again in the serum with the onset of malignancy, being expressed by tumor cells. Examples are AFP in hepatocellular carcinoma and gonadal tumors, carcinoembryonic antigen (CEA) in colon, lung, pancreas and breast cancers.

2. Enzymes: Examples are prostatic acid phosphatase in prostatic cancer, alkaline phosphatase in metastases and osteosarcoma, neuron-specific enolase (NSE) in neuroendocrine tumors especially small cell lung cancer and neuroblastoma, and lactate dehydrogenase (LDH) in lymphoid malignancies and other cancer types. Tumor angiogenesis is an inefficient system to provide adequate blood supply, hence it results in anoxic areas in the tumor. This is reflected in a shift of glucose metabolism from aerobic into anaerobic (glycollysis) with production of lactic acid.

3. Proteins: Examples are CA-125 in ovarian cancer, CA-19-9 in colon and pancreatic tumors, CA 15-3 and CA 549 in breast cancer, and prostate specific antigen PSA in prostatic cancer. Others include monoclonal immunoglobulins in multiple myeloma and lymphomas with gammopathies.

4. Eutopic hormones (normally secreted by the tissue): Examples are beta HCG in trophoblastic tumors, catecholamines in pheochromocytoma and neuroblastoma, serotonin in carcinoid tumors, gastrin in gastrinoma, thyroglobulin in thyroid carcinoma of follicular origin, and calcitonin in medullary thyroid carcinoma.

5. Ectopic hormones (not normally secreted by the tissue, a paraneoplastic syndrome): Examples are ACTH by small cell lung cancer and erythropoietin in renal cell carcinoma.

Table 5-2 displays malignancies frequently associated with the commonly used serum markers. Awareness of the limitations of the use of such markers is essential to avoid inappropriate consumption of economic resources and spare the patients unnecessary anxiety or non-indicated invasive diagnostic tests.

CANCER CELLS IN VITRO

Malignant cells exhibit many characteristic features in vitro distinctive from normal cells. These are observed during malignant transformation of normal cells in tissue culture under the effect of various carcinogenic agents, or by growing in vitro of cell lines from various malignant tumors. Cancer cells demonstrate the following differences from normal cells:

1. Immortality: Malignant cells can grow indefinitely in culture, mainly due to elevated levels of telomerase that maintains telomere length. Normal cells will die after about 60 doublings due to continual shortening of chromoso-

Table 5-2 Some Useful Serum Biomarkers for the Diagnosis of Malignancies

Class	Malignancy frequently associated	Other malignancies or non-malignant conditions
Enzymes		
Lactic dehydrogenase (LDH)	Non-Hodgkin lymphoma/Leukemia	Hepatic disease, infarcts, injuries
Neuron-specific enolase (NSE)	Neuroendocrine tumors	Benign liver disease
Alkaline phosphatase (ALP)	Bone secondaries, osteosarcoma	Paget's disease, normal pregnancy
Hormones		
Human chorionic gonadotropin (HCG)	Gestational trophoblastic tumors	Abortion, other cancers
Adrenocorticotropic hormone (ACTH)	Adrenal cortical carcinoma and paraneoplastic syndromes in other carcinomas	
Serotonin	Carcinoid tumors	Certain Foods (meat and fruits)
Oncofetal antigens		
Alpha-fetoprotein (AFP)	Hepatocellular carcinoma	Viral hepatitis, pregnancy
Carcinoembryonic antigen (CEA)	Metastatic colon cancer	Pregnancy, inflammatory bowel disease
Proteins		
CA 15-3 antigen	Metastatic breast cancer	Benign breast disease
Prostate-specific antigen (PSA)	Prostate cancer	Prostatitis, hyperplasia
CA 19-9	Pancreatic carcinoma	Cholecystitis, cirrhosis
CA 125	Ovarian carcinoma	Pregnancy
Immunoglobulins (M protein)	Multiple myeloma	Cirrhosis, Gaucher disease

Fischbach and Dunning, Manual of laboratory and diagnostic tests, 2009

mal telomeres with each cell division.

2. Reduced requirement of growth factors: Malignant cells generally grow well in media containing less serum than that needed by normal cells. Malignant cells, in contrast to normal cells, produce their own growth factors which stimulate their own growth by autocrine mechanism.

3. Anchorage - independence: Malignant cells can grow in fluid medium or soft agar. However, normal cells will grow only when they are attached to a solid surface.

4. Round cell morphology is a feature of malignant cells whereas normal cells have a flattened cell morphology.

5. Chromosomal abnormalities and DNA aneuploidy are features of malignant cells maintained in culture.

6. Loss of contact inhibition is a feature of malignant cells in vitro. This results in formation of multilayered collection of cells (foci). Conversely, normal cells continue to divide until they contact each other, when further cell division ceases with the formation of confluent monolayer.

7. Reduced cohesiveness and invasion: Cancer cells are less cohesive due to loss of intercellular adhesive molecules. Malignant cells also show invasion of gel-foam matrix in organ culture.

8.. Transplantability: In contrast to normal cells, malignant cells grown in vitro can form tumors when injected into syngeneic host animals or nude mice (immune deficient).

PITFALLS IN CANCER DIAGNOSIS

The diagnostic features of malignancy assist in correct diagnosis of cancer in most cases during routine daily practice. However, some features overlap with benign, atypical, or even inflammatory lesions. In addition, information pertaining to tumor grade, depth of invasion, adequacy of resection margins, and lymphatic invasion are subject to disagreements in reporting. Only disagreements that need to be followed by corrected reports are considered as clinically significant. Missing a diagnostic finding (false negative error) is more common than unjustified diagnosis of malignancy (false positive error).

Table 5-3 Errors Related to Clinical and Technical Causes (preanalytical)

1. Lack of clinical information
2. Lack of radiological picture (especially in bone, brain, chest, or abdominal masses)
3. Lack of information from previous pathologic studies
4. Scanty, superficial, or non-representative samples
5. Poor fixation
6. Mislabeled samples
7. Inadequate processing
8. Superficial or thick tissue sections
9. Under or over staining by Hematoxylin and Eosin
10. Outdated reagents or poor quality control in immunohistochemistry

Sources of Error

They are classically classified into 3 main groups:

1. *Pre-analytical*: deal mainly with adequacy of specimens, adequacy of demographical and clinical data, technical elements of sample identification, specimen handling including proper orientation or choice of sections in grossing stations, processing of samples, or defective application of ancillary diagnostic studies (Table 5-3).

2. *Analytical*: knowledge-based errors in the interpretation leading to false diagnosis of cancer (false positive), missed diagnosis of cancer (false negative) or misclassification (Table 5-4).

3. *Post-analytical*: including errors in transcription of reports, delayed report delivery, or missing/incorrect non-diagnostic information. Identifying the source of error in pathology diagnosis helps understand the root cause and accordingly apply measures to improve quality and reduce errors.

Types of Errors

There are three types of diagnostic pathologic errors of cancer, namely: false negative error, false positive error and mistyping error. Obviously, a false positive diagnosis of cancer is most serious since it results in unnecessary intensive therapy or mutilating surgery.

Frequency of Errors

Intraoperative frozen section diagnosis has a false negative error of 2% and false positive errors of 0.2% (Rosai and Ackerman, 2011). In case of diagnostic cytology, false negative error is about 15% in cervical screening and up to 70% in low-grade transitional carcinoma of the urinary bladder (Anthony, 1998). The false negative results of fine needle aspiration cytology of breast cancer is about 5% and 2% in tissue core biopsy. Discrepancy of diagnosis (mistyping) upon review is about 3% in cytology and up to 5% in histopathology (Anthony, 1998)

Methods to Detect Error

The objective of quality assurance in pathology is to ensure the quality of the submitted pathology report based on the interpretation of optimal technical preparation. Among the methods adopted to detect errors in pathology are:

1. Double reader or peer review: resulted in a mean discrepancy rate of 6.7% of which only 5.4% had moderate to marked effects on patient care. Review can be done for all cases known to present diagnostic challenge or all cases newly diagnosed as positive or negative for malignancy. Preliminary review can be done by pathology residents or fellows. Corrected reports based on re-review should document both the change in diagnostic findings and the reference to what was changed and why it was changed.

2. Subspecialities: a pathologist in a group taking interest in one or two subspecialties will better cope with all but a small number of challenging cases.

Table 5-4 The Ten Principal Causes of Analytical Diagnostic Errors

1. Mistaking normal structures for pathologic processes
2. Mistaking non-neoplastic diseases for tumor
3. Mistaking one tumor type for another
4. Unfamiliarity with rare tumor types
5. Failure to recognize common tumors in uncommon sites
6. Unfamiliarity with recently described disease entities
7. Misleading artifacts
8. Failure to recognize an inadequate or non-representative biopsy
9. Failure to assess the need for further consultation
10. Failure to formulate an appropriate differential diagnosis

3. Cytology-histology and frozen-final correlation: clinicians should be notified of any discrepancies after correlation immediately, and with thorough documentation in the surgical pathology report.

4. Pathology group meeting: with presentation of all cases with issues such as corrected diagnosis or major discrepancies between final diagnosis and cytology or frozen section.

5. Institutional re-review: secondary review of outside material is necessary for any patient being seen for treatment purposes.

6. Clinicopathologic conference reviews: can be done for selected cases after sign-out.

Audits

Regular reviews of a set of cases based on well-defined criteria (consecutive cases, malignant cases, breast cases, etc.) are done in a retrospective or prospective manner. Audits should be performed on cases signed out no more than 48 hours before to reduce the liability of errors identified. A review of 500 cases is usually enough to identify clinically significant error rates between 0.5% and 2%, which the range of errors between excellent and average laboratories.

Quality control is achieved through documentation "If it isn't written, it didn't happen" of procedures taking place from the time the specimen is received until a report is signed out (procedure manuals), written recording of consultations and communications, and careful review of reports in general and diagnoses in particular.

Pathologists must make a balance between submitting a meaningful and timely diagnosis relevant to patient care and making an over-confident diagnosis in spite of inadequate or unsatisfactory diagnostic material, missing clinical/radiologic information, or the need for further consultation. Finally, most pathology diagnoses represent one arm, though essential, in the decision-making process. Adequate and clear communication between the clinician and the pathologist is mandatory to provide the best possible care for the patient.

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