The primary audience for this work is the practicing pathologist and those studying to be pathologists, and it has 4 objectives. The first objective is to function as a convenient reference for problematic situations that are commonly encountered in the daily practice of surgical pathology. I believe there is a need for an accessible single volume that quickly addresses 95% of the difficult situations a pathologist is likely to encounter. The second objective is to present recent developments in surgical pathology, especially those that relate to molecular techniques, and to provide a thorough integration of morphologic and molecular diagnosis. The third objective is to render a reasonably comprehensive treatment of molecular pathology and the new tools that are revolutionizing our profession, in a stepwise manner, beginning with basic molecular biology. The fourth objective is to provide a clear and succinct general reference that can be read cover-to-cover, in the very limited time available to practicing pathologists, for general education.

The intent is to blend a survey of the literature with my own experience to offer a useful guide to daily practice in a rapidly evolving discipline. My hope is that by omitting the common and simple entities that are very unlikely to be challenging, and the very rare entities that are unlikely to be encountered, a more practical, readable, and accessible work has resulted. This view of pathology is intended to allow busy readers to feel anchored and secure in the rapidly shifting ground beneath their feet.

In keeping with the desire for a shorter and more accessible work that focuses on recent developments, references have been generally restricted to review articles, foundational papers, and recent publications that propose and support fundamental changes in practice. I have generally omitted citations for assertions that most would consider uniformly accepted, and which are likely to be found in other books. I apologize to those whose work could not be cited because of the spatial limitations.

The integration of traditional morphologic pathology with newer molecular concepts has been especially challenging. I have chosen to discuss specific findings in molecular pathology for each organ system with the corresponding discussion of morphologic pathology, so as to provide a complete and integrated discussion of all aspects of pathology for each organ system in one location, while placing the detailed treatment of cell and molecular biology, and its applications, at the end of the work. No approach or organization is perfect, and I have no doubt that others might have done it differently, but my hope is that this approach permits the most complete discussion of individual entities, while the final chapters provide a standalone guide to molecular pathology.

No book is ever perfectly accurate or complete, and readers are invited to share any thoughts regarding the accuracy, comprehensiveness, or “user-friendliness” of the discussion. This work was born of the personal experience of its author, and can be augmented and improved by the personal experience of its readers.

Kirk Heriot

Special User Notes

Geneticists express gene names as italicized capital letters, while proteins are expressed in nonitalicized capital letters. This work uses capitalized italics for gene names, and nonitalicized small letters for protein names with a few exceptions due to convention (e.g., MLH1, MSH2, ATP7B, etc).

We have implemented in this text a new reference citation system that takes advantage of the accessibility of universal online identification numbers for publications. PubMed identification numbers (PMID) are used for journal articles, and international standard book numbers (ISBN) are used for books throughout the body of this text. We also make available a complete list of traditional reference citations. Download either HeriotSurgicalPathologyRef.xlsx or HeriotSurgicalPathologyRef.pdf at www.ascp.org/PressUpdates
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Practical Surgical Pathology: Integrating Molecular Pathology into Your Morphologic Practice
Benign Processes That Mimic Invasive Carcinoma

The most common benign simulator of breast cancer is adenosis. Adenosis exists in both a localized and generalized or florid form. The localized form has a cellular proliferation of acini, often accompanied by stromal cells, and frequently has a low-power “whorled” appearance i4.1. The florid form has multiple such nodules, often with coalescence, that may result in a clinically distinct mass i4.2. Because the proliferation and crowding of lobules in florid adenosis distorts the normal lobular architecture, it may resemble infiltrating carcinoma. The diagnosis of carcinoma—even a minute focus of carcinoma—should be made with caution when it is within or adjacent to an area of adenosis. A helpful feature is that a focus may appear to be infiltrative at high power, but at lower power, it seems more circumscribed. If a putatively invasive area is encircled in the imagination and has a fundamentally smooth contour, a diagnosis of florid adenosis may be more appropriate. Very close examination of the nuclei in this area will disclose that they are smaller than in most cases of carcinoma, even lobular carcinoma.

The distinction of florid adenosis from carcinoma is particularly challenging on a thin needle biopsy, which unlike a mammotome, affords little opportunity to appreciate the retained lobular architecture of adenosis. Indeed, on a needle biopsy, florid adenosis may so strongly resemble carcinoma that immunohistochemical stains are not even be helpful in the distinction of florid adenosis from carcinoma and laminin—components of basement membranes—may also be helpful in the distinction of florid adenosis from carcinoma [PMID3817761], though this approach is not used as often as the identification of myoepithelial cells.

For myoepithelial cells in the breast because some breast cancers (called basal-type) are well known to express these 2 keratins. Whatever stains are used, in evaluating these stains, it should be remembered that the normal myoepithelial layer is discontinuous. A well formed myoepithelial layer is a strong point for a benign process, but the inability to demonstrate a continuous myoepithelial layer in a small problematic focus does not prove invasion.

The greatest challenge in biopsies of benign, cellular breast lesions is to appreciate the need to obtain immunohistochemical stains. The clues that an apparent carcinoma may contain myoepithelial cells and requires immunohistochemical stains to exclude a benign lesion include:

1. The apparent infiltration of clusters of cells with any indication of a dimorphic population, such as cells on the periphery that have clear cytoplasm or smaller uniform nuclei
2. The apparent infiltration of glands whose lining is multilayered
3. Infiltration largely as rounded clusters, as neoplastic glands tend to be flattened or misshapen, while those of adenosis are usually more rounded
4. Infiltration by cells with clear cytoplasm or small, spindled nuclei
5. The absence of an in situ component

The demonstration of an intact, well developed basement membrane by immunohistochemical stains for type 4 collagen and laminin—components of basement membranes—may also be helpful in the distinction of florid adenosis from carcinoma [PMID3817761], though this approach is not used as often as the identification of myoepithelial cells.

The term “apocrine adenosis” has been used in different ways [PMID19147303] and is somewhat confusing, as apocrine metaplasia and adenosis often occur together as part of the spectrum of fibrocystic changes. In apocrine adenosis, there is often myoepithelial hyperplasia in addition to adenosis and apocrine metaplasia, accounting for the alternative name of adenomyoepithelial adenosis. Apocrine adenosis is a combination of adenosis, apocrine metaplasia, and myoepithelial hyperplasia. The difficulty in distinguishing this process from carcinoma may be augmented by the prominent nucleoli of apocrine cells i4.6, but the clue to a
benign diagnosis, or the need for immunohistochemical stains, is circumscription 4.7.

The tubular adenoma is rarely specifically diagnosed, but refers to a benign, circumscribed proliferation of innocuous glands whose increased cellularity may simulate an invasive carcinoma 4.8. Its circumscription and innocuous-appearing nuclei usually make immunohistochemical stains unnecessary, but if needed, the identification of myoepithelial cells as described above, is recommended.

Microglandular adenosis (MGA) is an unusual entity that is felt by some to be related to adenosis of the usual type, but by others to be a distinct process that may be an early form of tubular carcinoma because of its apparently infiltrative pattern 4.9. MGA usually (but not always) has only 1 layer of epithelial cells, without a myoepithelial layer. There are eosinophilic secretions in the lumens, and the cytoplasm is often clear 4.10.

In microglandular adenosis, the glands have an infiltrative pattern, but the nuclei appear benign. If the differential diagnosis is between microglandular adenosis and carcinoma, myoepithelial cells can be sought by immunohistochemistry, as MGA occasionally has a few such cells and their presence is a strong indicator of a benign process, though a negative stain has no meaning. A search for a confluent basement membrane may be informative, as this is retained in microglandular adenosis but is generally lost in invasive carcinoma. Occasionally a periodic acid-Schiff (PAS) stain may sufficiently highlight the basement membrane [PMID19824897], but more often immunohistochemical staining for either collagen type 4 or laminin will be needed [PMID1944007] (immunohistochemical stains are more sensitive to tiny papillae with very subtle fibrovascular cores; those arising in the breast parenchyma. The most common diagnostic dilemma in this region is the so-called nipple adenoma, which was elucidated by my teacher, David Jones, in 1955 [PMID143517] as a florid, cellular, and complex papillomatosis of the nipple ducts, often associated with hyperplasia of the adjacent ducts and increased stromal cellularity 4.12. Although initially worrisome, the appearance is that of an increased number of ducts, each of which has hyperplasia and papillomas, rather than true infiltration, and nuclei are unremarkable.

**Nonpapillary Intraductal Proliferations**

In assessing intraductal proliferations, it is helpful to first look for nuclear atypia. If this is pronounced, the diagnosis of high-grade ductal carcinoma in situ (DCIS, also called intraductal carcinoma) can be made without consideration of architectural features 4.13. Necrosis is often taken as an additional diagnostic criterion of high-grade DCIS, but it is not a criterion and its significance is simply that it should heighten the search for significant nuclear atypia.

If there is no high-grade nuclear atypia, then both a characteristic nuclear morphology and an architectural pattern are needed for the diagnosis of low-grade DCIS. Low-grade intraductal carcinomas of all types are composed of a monomorphic population of cells with relatively uniform, evenly spaced, round nuclei, in one of several recognized growth patterns. In contrast, a benign intraductal proliferation (hyperplasia) is likely to have oval nuclei separated by irregular distances 4.14. If there are secondary lumens, they are irregularly shaped. Hyperplasias are characterized by a process colloquially called “streaming,” in which the intraluminal epithelial cells to “run together” in a direction that is more parallel to the wall of the duct than the normal perpendicular arrangement 4.15.

There are several patterns to low-grade DCIS, all of which have cells with rounded, hyperchromatic nuclei, which are paradoxically more uniform than in intraductal hyperplasias (possibly reflecting clonal origin). In the cribriform type of low-grade DCIS, the nuclei are more evenly spaced and cytoplasmic boundaries are sharper than in hyperplasias. There is no streaming. It is often said that the spaces are rounder and more sharply defined in DCIS, as if they had been “punched out” by a rounded cookie cutter. This is true, but the real difference between the spaces of hyperplasia and those of cribriforming low-grade DCIS concern the cells around the lumen. In cribriforming low-grade DCIS, the cells are palisaded around the lumen. They have a basal nucleus and apical cytoplasm, resulting in a radial orientation around the lumen 4.16. The result is that in low-grade DCIS, there is a more orderly radial arrangement around the lumen than in hyperplasia.

In micropapillary low-grade DCIS (“micropapillary” refers to tiny papillae with very subtle fibrovascular cores), there is nuclear uniformity and hyperchromasia, as is the case for other forms of low-grade DCIS. There is often a club-like appearance, with the apical portion of the papillae being rounder than the portion near the duct wall, in contrast to the tapering appearance of most benign microcancers 4.17. Whereas benign micropapillary proliferations rarely distend the duct or acinus, micropapillary carcinoma often does. The milieu is often helpful. In micropapillary carcinoma, there is often an associated proliferation, close to the problematic micropapillary focus, that is compatible with nonpapillary DCIS, whereas benign micropapillations are often associated with benign hyperplasias in the adjacent tissue.

Additional features, usually less helpful, are that benign micropapillae are relatively uniform in size and shape, whereas their malignant counterparts show more variability. Benign micropapillae are cohesive, whereas their malignant counterparts are dyscohesive, with exfoliation of cells into the lumen. In benign micropapillae, there is maturation, with the cells at the tip being smaller than those at the base, while there is uniformity throughout the micropapillae in micropapillary DCIS. The “Roman bridge” form of low-grade intraductal
carcinoma is a variant of the micropapillary type in which there is connection between the micropapillae at their tips \(i4.18\).

The least common type of low-grade DCIS is the solid type, which must be distinguished from high-grade DCIS. In the solid type of low-grade DCIS, there is nuclear monotony, as is the case for all forms of low-grade DCIS of nuclei, rather than the large and variable nuclei of high-grade DCIS.

Special mention should be made of the entity called “clinging carcinoma,” first described by Azzopardi [PMID20306829], and now called “flat epithelial atypia” or “flat intraductal neoplasia.” Flat epithelial atypia is characterized by a single layer of cells with high-grade nuclei lining the duct \(i4.19\). These cells fulfill the criteria for high-grade DCIS and are considered as such by some observers, but because there is only a single layer of such cells and the architectural features of all other forms of DCIS are lacking, some prefer to regard this as a form of atypical hyperplasia. It now appears that this finding does indicate an increased incidence of invasive carcinoma, vindicating Azzopardi’s thinking [PMID20306828, PMID20306829].

Considerable variation in the diagnosis of problematic intraductal lesions has been shown among so-called “expert” breast pathologists [PMID1847606]. To my mind, this study not only indicates the extremely difficult nature of difficult intraductal proliferations, but also exposes the fallacy that the so-called “expert” supersubspecialist pathologist is necessarily superior to the general pathologist. Clearly, the case for the esteemed academic specialist is heavily diluted when these individuals cannot agree among themselves. Neither the clinician nor the general pathologist should search for a “magical guru” who can provide “the” answer in diagnostically difficult situations, but should rather seek a thorough understanding of current knowledge, with a willingness to indicate and accept the uncertainty that is sometimes an inherent and inescapable part of medicine. Since this study, standardization in the interpretation of difficult lesions has improved with better immunohistochemical stains, but these stains strengthen the case for the general pathologist, as they are readily available to all pathologists and provide a far more standardized algorithm than was heretofore possible.

There is a consensus that a diagnosis of low-grade DCIS requires some degree of geographical extent. While high-grade DCIS can be diagnosed in a single duct of any size, the diagnosis of low-grade DCIS generally requires that \(>1\) minute focus be present. Some suggest that the total extent of the process be \(0.2\) cm (2 mm) or more, while others suggest that 3 or more ducts be involved.

There is considerable confusion over the term “atypical hyperplasia.” It is not clear if this is a true, reproducible stage in the progression of carcinoma, or if it simply represents a variety of patterns that cannot be unanimously accepted as benign or malignant. To diagnose atypical hyperplasia (ADH), some require the full diagnostic criteria of DCIS, but insufficient extent (less than \(2\) mm or \(<3\) ducts \(i4.20\), while others do not require that any part of the questionable area resemble fully developed DCIS and will diagnose ADH for a proliferation that has some characteristics of DCIS, but no part of which clearly fulfills the criteria for DCIS—for example, intraductal proliferations that lack both the nuclear atypia of a high-grade DCIS and the architectural characteristics of a low-grade DCIS \(i4.21a, i4.21b\).

Once DCIS and atypical hyperplasia have been excluded, the process is diagnosed as an intraductal hyperplasia of the usual type. Previously, hyperplasias were graded as mild, moderate, or severe, depending on the degree of epithelial proliferation and the extent to which the duct was filled. For example, \(>4\) layers of epithelial cells was considered to be moderate hyperplasia. Newer thinking pays less attention to the degree of proliferation and simply seeks to differentiate the process from atypical hyperplasia. If there is no atypia and no architectural features of any form of DCIS, such that the diagnosis of DCIS is not tenable even for a part of the intraductal process, the diagnosis should be hyperplasia of the usual type. Because the extent of the proliferation is not critical, no comment need be made as to whether the process is mild, moderate, or severe. These terms may be used if the ducts are nearly completely filled but the criteria for atypical ductal hyperplasia and intraductal carcinoma are not fulfilled. However, the process can simply be designated as hyperplasia of the usual type without comment on the cellularity because if there is no atypia, the malignant potential is minimal for any cellularity (1.5 times normal) [PMID17058097].

While all carcinomas develop along a continuum that defy distinct “breakpoints,” the risk for invasive cancer is clearly greater for ADH than for usual hyperplasia, suggesting that the clinically important breakpoint is between any hyperplasia of the usual type—of any cellularity—and atypical hyperplasia, not between atypical hyperplasia and intraductal carcinoma. There is little need to differentiate between ADH and low-grade DCIS because such a distinction is arbitrary, subject to substantial interobserver variation, and the biologic potential of the 2 processes is similar. Indeed, there is a proposal to conflate atypical ductal hyperplasia and low-grade intraductal carcinoma under the designation of intraductal neoplasia grade 1 (ie, to essentially consider ADH as a low-grade DCIS) [PMID17058097].

In situations that are equivocal between ADH/DCIS and usual intraductal hyperplasia, obvious apocrine metaplasia is often taken as a reassuring indicator, but this is not a primary criteria. In equivocal situations, a focus of streaming favors usual hyperplasia. Processes that run along the ductal system and undermine or replace the normal epithelium of ducts or lobules suggest ADH/DCIS.

The presence or absence of a myoepithelial layer, while of immense value in identifying subtle manifestations of invasive carcinoma, may not be definitive in the assessment of intraductal lesions because many foci of intraductal carcinoma retain some degree of myoepithelial cells around the periphery. However, myoepithelial cells are often attenuated or partially lost in a population of neoplastic cells, and the clear presence of a well-defined myoepithelial layer is a reassuring finding in situations where other criteria have not yielded a definitive diagnosis, especially if they can be found accompanying a problematic epithelial proliferation into the lumen and not just on the periphery. The presence of a myoepithelial layer in excluding ADH/DCIS is most helpful in macrocircular intraductal processes, where a benign process can be assumed if myoepithelial cells are found throughout the proliferation.

High molecular weight keratin (CK903; also called 34\(\beta\)E12) is generally present in hyperplasia of the usual type and is absent in 90% of cases of ADH/low-grade DCIS. Likewise,
cytokeratins 5 and 6 are found in nearly all cases of usual ductal hyperplasia but are rarely seen in ADH/DCIS [PMID1922316], but as we will see in our discussion of molecular phenotyping of breast cancers in Chapter 47, those invasive carcinomas that do express cytokeratins 5 and 6 (ie, have a basal phenotype) tend to be negative for estrogen and progesterone receptors and negative for HER2 overexpression, and may be more aggressive [PMID1746782].

To my mind, the diagnosis of either ADH or DCIS on a biopsy should generally prompt a complete excision. The sole exception to this appears to be a situation where there is a biopsy of microcalcifications (not a mass) and both of the following 2 conditions are met: there are only 1 or 2 foci in the biopsy that most observers would classify as atypical ductal hyperplasia and not DCIS and postbiopsy mammogram reveals no residual calcifications at all [PMID1909574].

The pathologist often encounters a situation in which extensive intraductal carcinoma, with nearly confluent clusters of intraductal tumor, must be differentiated from early invasion. Because foci of intraductal carcinoma generally retain some myoepithelial cells (though this layer may be attenuated), the immunohistochemical demonstration of a well defined myoepithelial layer suggests absence of invasion. The same panel of antibodies employed in distinguishing florid adenosis from invasion is recommended in this situation. Likewise, basement membrane material is generally intact in intraductal carcinoma and attenuated, but not necessarily lost altogether, in invasive foci, and in my experience, a well defined and essentially continuous basement membrane layer may also suggest the absence of invasion.

While immunohistochemistry can be of great help in addressing this question, the pathologist must again remember the twin caveats: The myoepithelial layer is discontinuous and myoepithelial cells may not be readily demonstrable in noninvasive cases, and invasive cases may still have some amount of basement membrane. The experienced observer weaves together the H&E appearance, the general presence or absence of a myoepithelial layer, and perhaps the degree of basement membrane formation into a determination of the degree of invasion, if any. In cases that remain problematic after the application of immunohistochemical stains, a useful rule on H&E examination is to lean away from invasion if the putative invasive focus is very close to a focus of DCIS because of the possibility that the focus of putative invasion is actually a tangential section through DCIS simulating invasion. If invasion can be confidently established, but extends <1 mm into the stroma, the term “microinvasion” can be used.

A number of detailed classifications of DCIS have been proposed, based primarily on nuclear grade and the presence or absence of necrosis. Of these, the Van Nuys system [PMID722355] and the Nottingham system [PMID80275] are the most commonly used. The Van Nuys system was expanded into a prognostic index [PMID63704] that factors in size and margin status, and was subsequently updated to include patient age [PMID4353464]. However, at the end of the day, the exclusion of small foci of invasion and margin status seem to be the most important considerations because the incidence of lymph node metastases from high-grade DCIS is only about 1% [PMID2020234] and that of low-grade DCIS is negligible. Recently, higher numbers of lymph node metastases in high-grade DCIS have been suggested, ranging from about 4% if the DCIS is negative for p16, cyclooxygenase-2, and Ki-67 to about 20% if the DCIS is positive for all 3 markers [PMID2422045], but there is always the difficulty of an unappreciated small focus of invasion in these studies, and even if the validity of these markers and these higher numbers are verified, the principal difficulty with DCIS with negative margins is the risk of local recurrence, not metastases.

If a focus of DCIS is estrogen receptor positive, attenuation of estrogen effect, either by receptor blockade or by inhibition of the aromatase enzyme involved in estrogen production, seems to reduce local recurrence, but with this exception, no additional studies beyond H&E seem to have routine relevance in DCIS at this writing. Even allowing for discontinuous foci, a margin of 1 cm (10 mm) appears adequate for DCIS and if achieved, likely obviates the need for radiation therapy in most cases [PMID1020203].

Macropapillary Intraductal Proliferations

Benign papillomas usually have uniform nuclei, less cellularity, prominent fibrovascular cores with only a few fronds [4.22], less nuclear crowding, and a simpler architecture than intraductal papillary carcinoma, but none of these criteria are absolute. Benign papillomas maintain a 2-cell population, epithelial and myoepithelial [4.23].

Papillary intraductal carcinomas tend to have delicate fibrovascular cores with numerous, thin, or variable papillations that are much longer than they are wide [4.24]. Nuclear crowding, atypia, enlargement, or hyperchromatism are suggestive of malignancy, as are numerous mitoses [4.25]. Intraductal papillary carcinomas tend to have a monomorphic population of cells without myoepithelial cells [4.26].

An important clue to the possibility of an intraductal papillary carcinoma is nuclear crowding. Such crowding may impart either stratification or columnarity to the cells [4.26], and for this reason, columnar cells that are oriented perpendicularly to the core, with or without stratification, while not diagnostic of papillary DCIS, should elicit a search for other features. In problematic cases, the appearance of epithelium that is in proximity to the papillary process can be helpful. If the epithelium in the portion of the duct wall not involved by the papillary proliferation is clearly innocuous, one can lean toward a benign diagnosis for the whole duct, whereas an intraductal papillary carcinoma might be favored if the remaining epithelium resembles nonpapillary DCIS. Likewise, if the adjacent ducts are benign, the problematic duct is probably best regarded as benign, whereas it is likely malignant if surrounded by malignant intraductal proliferations of any sort.

The term “intracystic papillary DCIS” is somewhat unclear, but usually refers to papillary DCIS in a dilated duct [PMID2020027]. The only significance of this is that it is more likely to present as a mass. The term “solid papillary carcinoma” refers to a large, cellular intraductal papillary carcinoma whose intraductal location and papillary nature may not be immediately obvious, causing concern for invasive carcinoma [4.27a]. On close examination, subtle fibrovascular cores can be seen [4.27b], but the primary clue to this process is that low-power examination shows a well circumscribed process, suggesting a dilated duct [4.27c].
In equivocal cases, the best test currently available is the presence or absence of myoepithelial cells, as the demonstration of these cells in reasonable numbers essentially precludes a malignant diagnosis. A number of markers have been proposed to highlight myoepithelial cells in papillary lesions, but as discussed earlier in benign mimics of invasive carcinoma, a combination of nuclear staining for p63 and cytoplasmic staining for smooth muscle actin, calponin, and myosin heavy chain offer the greatest combination of sensitivity and specificity [PMID1725623].

As is always the case, immunohistochemical stains must be interpreted in conjunction with the H&E appearance and the presence of myoepithelial cells in breast lesions does not guarantee benignity since the myoepithelial cells at any location within a duct or lobule may persist as carcinoma spreads to that location from another focus in the duct. Myoepithelial cells will also be found in the rare cases of myoepithelial carcinoma. Likewise, their absence does not ensure malignancy if the H&E appearance does not suggest carcinoma.

An intraductal papillary proliferation may be clearly benign in some areas but suspicious or clearly malignant in others i4.28. Immunohistochemical staining for myoepithelial cells may parallel this appearance, being demonstrable in the benign-appearing areas and absent in the areas of concern. After the presence of a focus of DCIS is established within a papilloma, there are differences in terminology, but good agreement on basic concepts. Tavassoli uses the term “atypical papilloma” if the criteria for DCIS are satisfied in <1/3 of the lesion, and “carcinoma within a papilloma” when the involved area is between 33% and 90% of the papillary mass [PMID13680220], but has more recently avoided the term “carcinoma” in such situations in favor of “ductal intraepithelial neoplasia” for foci occurring in a papilloma [PMID19492881]. Page feels that any area that could be regarded as DCIS elsewhere and is larger than 3 mm should be called “DCIS within a papilloma,” while such areas that are smaller than 3 mm should be called “ADH within a papilloma” [PMID8674001]. Either of these nomenclatures is perfectly acceptable, and there are advantages to each, but to my mind, there are 2 central points. First, the same cytologic, architectural, and size criteria should apply in a papillary lesion as elsewhere, and if the criteria for low-grade DCIS are met, I favor a diagnosis of low-grade DCIS within a papilloma because the use of different nomenclatures for foci within and not within papillomas is likely to confuse the clinician. Second, to my mind, a patient should not be labeled with a “cancer” diagnosis—even in situ—if the size criteria for DCIS are not met.

Papillary intraductal carcinomas may invade beyond the duct in which they arose, resulting in stromal invasion and a truly invasive carcinoma. As is the case for any form of DCIS, it can be difficult to determine if there is focal invasion immediately adjacent to the duct. The clues to focal stromal invasion in papillary intraductal carcinoma are those employed in other situations—irregularly shaped or angulated clusters of atypical cells and a desmoplastic stromal reaction, though the latter finding may be difficult to appreciate in the breast, where the stroma is often fibrotic. In questionable situations, immunohistochemical proof of loss of myoepithelial cells in a small cluster of cells suggests stromal invasion.

The Breast Biopsy: Potentials and Limitations

The breast biopsy begins with a suspicious finding on a mammogram or an MRI. Mammographically suspicious findings are reported using the BI-RADS (Breast Imaging-Reporting and Data System) developed by the American College of Radiology [PMID7458080]. This system is as follows:

0: Incomplete or compromised study.
1: Negative.
2: A findings that is comfortably felt to be benign; normal surveillance indicated.
3: A finding that is probably benign, but a followup within 4-6 months is suggested.
4: A suspicious finding; biopsy should be considered.
5: A highly suspicious finding; biopsy indicated.
6: A finding that has been shown to be malignant by biopsy.

Carcinoma of the breast is rising in incidence and will now affect 1 in 9 women at some point in their lives. Mammography (and increasingly MRI) in conjunction with biopsy remains our best defense against this alarming trend. The breast biopsy has performed admirably in the early diagnosis and treatment of breast cancer, but there are several caveats in forming conclusions about a breast mass or areas of radiographic abnormality from a biopsy.

A useful way to minimize both overdiagnoses and underdiagnoses is to remember that the breast biopsy—like any biopsy—simply serves to stratify patients into 3 categories—clearly positive, clearly negative, and a third group for which a definitive diagnosis cannot be rendered at the time.

The third group of breast biopsies—those that are indeterminate—include 2 broad situations. The first group of indeterminate biopsies is those cases in which there is atypical breast tissue, such that it can safely be assumed that the area of interest has been sampled, but the interpretation of the biopsy is problematic, even after following the algorithms and special stains discussed above. While there are a variety of situations in which interpretation of a breast biopsy can be inconclusive, 2 of the most frequent areas of problematic interpretation involve papillary lesions and foci of atypical ductal hyperplasia.

Papillary lesions that are identified on aspiration or core biopsy material are challenging, and there is not yet a clear consensus on this matter. Some believe that any papillary process identified in such a sample, even if it appears clearly benign, should prompt an excision [PMID1949281, PMID1356929]. On the other hand, because stereotactic (mammotome) biopsies remove much more tissue, an argument can be made that histologically benign-appearing papillary lesions biopsied by this means might be followed without excision if the postprocedure mammogram suggests no residual lesion. If the patient and her physician cannot reach a clear decision on the next step, immunohistochemical stains that suggest benignity may permit a “wait and see” approach, provided that the lesion does not enlarge or become radiographically more worrisome, but because papillary lesions are often heterogeneous, and there may be unsampled areas of atypia or carcinoma adjacent to benign areas that were sampled,
this approach is potentially problematic. In any case, in a core biopsy or fine needle aspiration (FNA) with any papillary process, it is generally wise to comment that a focal papillary carcinoma cannot be excluded with complete certainty in that material, even if myoepithelial cells are found, and to recommend either an excision or close surveillance.

Also in this first group of indeterminate biopsies, in which the target has been sampled but interpretation is problematic, are those that have atypical ductal hyperplasia. As discussed above, excision is prudent unless the biopsy was done for microcalcifications, there are only 1 or 2 foci of ADH, and there are no residual calcifications. Even in this situation, some are more comfortable with an excision. As mentioned, florid hyperplasia of the usual type is no longer considered a major risk factor, but the difficulty with this diagnosis on a biopsy is the possibility that there may be a more significant process that was not sampled, especially if the biopsy was of a distinct mass. These situations must be individualized, but clearly, there must either be an excision or surveillance.

The second group of potentially indeterminate breast biopsies consists of those situations in which the biopsy is clearly benign, but there is the possibility that the target may not have been sampled. There are a variety of situations in which the pathologist can suspect that the target area may not have been sampled, and should therefore take care to avoid imparting a false sense of security, but 3 situations are commonly encountered and deserve special mention. The first is a biopsy for a mass in which there is normal breast tissue that would not seem to form a clinical mass, in which case it is prudent to comment that the tissue is benign, but verification that the target was sampled is needed.

The second concerns biopsies that are benign, but do not have breast parenchyma (eg, contain only fat). In these cases, it is problematic as to whether the clinical mass is mesenchymal or the biopsy captured only surrounding fibroadipose tissue (eg, does the patient have a lipoma, or did the biopsy sample only adipose tissue adjacent to a neoplasm?). In these cases, a comment should be made that the mass may be a mesenchymal lesion, such as a lipoma, but that clinical correlation is needed to ensure that the target area was sampled.

The third situation in which the biopsy is benign but there is a hazard of a false negative are those biopsies done for microcalcifications and no calcifications are seen in the biopsy. In these cases, I recommend a comment on the presence or absence of calcifications in the biopsy so that the physician who performed the biopsy can ensure that the target area was sampled. However, the primary defense against a false negative from failure to sample the target area lies with the physician performing the biopsy, not with the pathologist. The physician performing a biopsy for calcifications should radiograph both the tissue removed before it is sent to pathology to ensure that it contains the target calcifications, and also the patient after the procedure to ensure that calcifications are no longer present or are reduced in number. This postprocedure double-radiographic examination is superior to radiographing the paraffin blocks because there may be tissue in the patient, adjacent to that which was removed, that may contain calcifications that are of greater concern, in which case the pathologist’s affirmation of calcifications in the paraffin blocks may lead to a false sense of security regarding specimen adequacy. Only the radiologist can confirm that the most significant calcifications were sampled.

About 10% of microcalcifications are of the calcium oxalate type, and cannot be detected on H&E examination. These are birefringent, requiring examination with polarized light to be detected, and are usually associated with benign processes [PMID2589847]. While the pathologist should comment on the presence or absence of calcifications, he or she should not be expected to determine, based on either paraffin block radiography or microscopic examination, if the target was sufficiently sampled.

Assessment of Invasive Ductal Adenocarcinomas

It is generally recommended to remove each inked margin in a lumpectomy en bloc, transect it at right angles, then place each fragment in the cassette on its side. If the tissue contains tumor, it can then be determined how close it is to the margin, whereas simply laying the tissue face down in a cassette prevents the examiner from determining the distance of the tumor from the margin. Some advocate fixing lumpectomy specimens overnight prior to taking margins. While this delays the case for a day, some feel this provides better morphology. The caveat here, however, is that if this procedure is employed, it might be prudent to ink and section or incise the tumor to allow fixative to access to the tumor to permit prompt fixation.

Any breast specimen that has been radiographed prior to being sent to the laboratory is likely to have distorted dimensions, especially in the dimension that was compressed. This compression may compromise the assessment of margins and a comment regarding this distortion and its possible effect on the assessment of margins might be made in these situations. Evaluation of margins in lumpectomy specimens may also be complicated by the fact that the ink applied to the specimen by the pathologist often runs into crevices that are caused by the trauma of excision, thus coating surfaces that are not true margins. A third area of concern in margin assessment is that cautery artifact (linear streaking of cells and chromatin) clearly indicates a surgeon’s margin, but does not necessarily indicate a final margin, as surgeons often “back out” and excise around an area that was previously penetrated. Thus, a surface may have both cautery artifact and India ink and still not be a final margin. A fourth difficulty is that the final assessment of margins is done in a 2-dimensional microscopic field, which does not perfectly reflect the 3-dimensional ramifications of the ductal system. No absolute numbers have emerged for minimum requirements for a margin to be considered negative, but in general, it should be negative by at least 2 mm [PMID22075613] and preferably by 5 mm [PMID17439024]. Even if these margins are achieved, postoperative radiation therapy is generally given in lumpectomy cases. It is generally prudent to report the margin status of the invasive and in situ components separately.

The submission of separate margins by the surgeon from the cavity after the lumpectomy has been advocated as a method of assessing margins that is superior to attempting to obtain margins from the lumpectomy itself [PMID6322345]. These separate margins from the cavity should overlap as much as possible, inked, cut at right angles, and submitted for
4: The Breast

i4.1 A nodule of adenosis. Note the sharp circumscription.

i4.2 Multiple coalescing nodules of adenosis, forming a clinically worrisome mass. Note the circumscription of the individual masses.

i4.3 A needle biopsy of a distinct breast mass with worrisome cellularity and what appears to be an infiltrative pattern.

i4.4 The same biopsy as in i4.3 stained for p63, identifying the nuclei of a myoepithelial layer, suggesting a diagnosis of adenosis rather than invasive carcinoma.

i4.5 The same biopsy as in i4.3 stained for calponin, identifying myoepithelial cytoplasm. The identification of a myoepithelial layer by both p63 and calponin secures a diagnosis of adenosis rather than invasive carcinoma.

i4.6 Sclerosing adenosis with apocrine metaplasia. The nuclear enlargement, and in particular, the prominent nucleoli, can easily cause a misdiagnosis of carcinoma.

i4.7 The same specimen as in i4.6, but shown at lower power to illustrate the circumscription of the process.

i4.8 A so-called tubular adenoma, another process of extensive cellularity that can simulate a carcinoma unless attention is given to its circumscribed overall architecture.

i4.9 Microglandular adenosis often have clear cytoplasm and eosinophilic secretions in the lumens.

i4.10 Microglandular adenosis simulating a tubular carcinoma because of the infiltrative pattern.
A problematic breast proliferation that received a consensus diagnosis of atypical hyperplasia because this was the only duct involved, despite the fact that the criteria for low-grade intraductal carcinoma of the cribriform type are essentially fulfilled.
4: The Breast

**i4.21 a** A cellular, complex intraductal epithelial proliferation, which at low power lacks the nuclear uniformity, even spacing, and well-defined round secondary lumens of low-grade cribriform intraductal carcinoma

**i4.22** Benign intraductal papilloma with well-developed fibrovascular cores and low cellularity.

**i4.23** A high-power view of the process in **i4.22**, illustrating the retention of the spindle-shaped myoepithelial cells.

**i4.24** Papillary intraductal carcinoma with areas of hypercellularity, with numerous epithelial cells that are not directly connected to a papilla. Note the mitotic figure in the right center.

**i4.25** Papillary intraductal carcinoma with areas of great cellularity, with numerous epithelial cells that are not directly connected to a papilla. Note the mitotic figure in the right center.

**i4.26** Papillary intraductal carcinoma demonstrating the lack of myoepithelial cells compared to the benign papilloma in **i4.23**, which retains myoepithelial cells.

**i4.27 a** Papillary intraductal carcinoma of great cellularity with small, subtle fibrovascular cores, which out of context can simulate invasive carcinoma.

**b** The same process as in **i4.27 a**, but subtle fibrovascular cores, suggesting a papillary process, can be seen.

**c** The same process as in **i4.27 a**, but this low-power view demonstrates circumscription and shows that the process is occurring in a dilated duct.
4.30 Small clusters of tumor cells, within the main tumor mass, that simulate lymphatic invasion because of retraction artifact.

4.31 A micrometastasis of breast carcinoma to an axillary lymph node.

4.32 Mucinous (colloid) carcinoma of the breast.

4.33 Medullary carcinoma of the breast with pleomorphic nuclei, minimal gland formation, and lymphocytes. Note the absence of an intraductal component.

4.34 Medullary carcinoma of the breast with sharp circumscription.

4.35 Tubular carcinoma of the breast with small, angulated glands and minimal nuclear pleomorphism. Although the individual glands appear innocuous, note the infiltrative pattern.

4.36 A high-power view of tubular carcinoma of the breast, demonstrating the angulated outline with occasional protrusions.

4.37 a A metaplastic carcinoma of the breast with a benign-appearing stromal component. 

b Same case as in 4.37a with positive immunohistochemical staining for cytokeratin 7, identifying the stromal component as having epithelial characteristics.

4.38 The same case as in 4.37 with p63 staining of the nuclei, additional evidence of a metaplastic carcinoma.
There are 3 common nonneoplastic conditions of the proximal small bowel that can endoscopically simulate neoplasia: Brunner gland hyperplasia, heterotopic gastric mucosa, and heterotopic pancreas. Heterotopic gastric mucosa occurs in the proximal small bowel. Heterotopic pancreas may occur in the stomach or proximal small bowel. The distribution of acinar and islet elements is irregular in heterotopic pancreas, and normal pancreatic architecture may not be apparent.

In the small bowel, gastrointestinal stromal tumors (GISTs), lymphomas, neuroendocrine tumors, serosal mesotheliomas, and metastatic tumors are all more common than primary adenocarcinomas, and all of these should be excluded before a diagnosis of primary small bowel adenocarcinoma is made.

**Intestinal Stromal Neoplasms**

Because the H&E morphology, the immunohistochemical characteristics, and the molecular biology of intestinal GISTs are the same as those in the stomach, they are diagnosed in the same way and with the same differential diagnosis as their gastric counterparts. However, the criteria for malignancy are different, and GISTs in the intestinal tract are more likely to be malignant than those in the stomach. Duodenal GISTs, for example, are usually malignant if they are very cellular, have 2 or more mitoses per 50 high-power fields (HPF), or are >4.5 cm in size, whereas these findings would be of far less concern in the stomach. In the jejunum, ileum, and colon, definitive criteria for malignancy have been elusive. A benign course can rarely be guaranteed if a GIST in the small bowel is >5 cm or has ≥1 mitoses per 10 HPF, it is likely malignant

As is the case for gastric GISTs, the differential diagnosis of intestinal GISTs includes muscle and nerve sheath neoplasms, spindle cell carcinoma, inflammatory myofibroblastic tumor, mesothelioma, melanoma, and angiosarcoma. In addition, 2 other entities are encountered in the mesentery and enter the differential diagnosis for intestinal GISTs. These are mesenteric fibromatosis and sclerosing mesenteritis.

Although mesenteric fibromatosis and sclerosing mesenteritis occur in the mesentery rather than the wall of the gastrointestinal tract, they may clinically, grossly, and microscopically simulate an intestinal GIST. As is the case with fibromatoses in general, mesenteric fibromatosis consists of uniform spindled cells in a stroma that may be focally myxoid or collagenous, but is not inflamed. Mesenteric fibromatosis is unencapsulated and may sometimes infiltrate into the wall of the bowel, closely simulating a GIST.

Sclerosing mesenteritis is an inflammatory condition of unknown etiology, but possibly associated with abdominal trauma or surgery, that eventuates in fibrosis through the normal reparative process. It has several appearances depending on when it is examined. Early lesions have a myxoid stroma with fat necrosis. Neutrophils are rare, but scattered eosinophils may be seen in some cases. Chronic inflammation, perhaps with germinal centers and lipid-laden macrophages, follows. The necrotic fat may be separated into lobules by septae. Sclerosis, perhaps with calcification, is seen in old lesions. Fat necrosis and inflammation, consequences of the healing process, separate sclerosing mesenteritis from mesenteric fibromatosis. While sclerosing mesenteritis may initially simulate a GIST, it is not infiltrative and will not involve the bowel wall.

The distinction of mesenteric fibromatosis from sclerosing mesenteritis can be important, but it is much more important to separate these entities from a GIST. GISTs are usually positive for CD117 and DOG1. Also, GISTs rarely express β-catenin. Sclerosing mesenteritis may express this protein, but not in the nucleus. Nuclear staining for β-catenin strongly suggests mesenteric fibromatosis over either GIST or sclerosing mesenteritis.

**Intestinal Lymphoid Proliferations**

Proliferations of mucosa-associated lymphoid tissue may be reactive or neoplastic and can be immunohistochemically characterized, if necessary, in the same way as lymphoid proliferations elsewhere. Reactive lymphoid hyperplasia is particularly common in the terminal ileum. Low-grade lymphoid neoplasms of the intestines are often called MALITomas, but this term is not helpful clinically, and a specific immunophenotype is needed.

Immunoproliferative small-intestinal disease (also called Mediterranean lymphoma or α chain disease) is a lymphoma characterized by diarrhea, malabsorption, weight loss, and...
abdominal pain, and is usually found in the second or third decade. On biopsy, there is a diffuse plasma cell infiltrate mixed with small lymphocytes that immunohistochemically stain as marginal zone cells. Both the plasma cells and the small lymphocytes express monoclonal α chains without a light chain. Antibiotics may cause regression, and for this reason, this diagnosis should not be made without an antibiotic trial, as is the case for apparent extranodal marginal zone lymphomas of the stomach. Those cases that are truly neoplastic generally stain as marginal zone lymphomas [Nakamura ISBN 978-9283224327, PMID30603173].

Enteropathy-associated T-cell lymphoma (EATCL) is a T-cell lymphoma, often of the small intestine, that is associated with ulceration and malabsorption (23.4a, 23.4b). A disproportionate number of patients with EATCL have a history of celiac disease [Müller-Hermelink ISBN 978-9283224327]. EATCL should be excluded in situations where there is malabsorption or ulceration in the presence of a lymphoid infiltrate or large numbers of apparent macrophages, as the neoplastic lymphocytes in EATCL may resemble macrophages. Some cases of EATCL have gains of 9q [PMID12414511].

Mantle cell lymphomas are also common in the gastrointestinal tract, where they are often polypoid. Most small lymphoid polyps are benign (23.5), but mantle cell lymphomas are also common in the gastrointestinal tract and are often polypoid. Many cases of polyposis of the bowel secondary to lymphoma are mantle cell lymphomas (23.6a, 23.6b).

### Colonic Polyps and Adenocarcinoma

Little will be said about the interpretation and reporting of common colonic carcinoma, as this topic is well covered in other works.

Juvenile polyps are hamartomas of the lamina propria and have cystically dilated glands separated by expanded lamina propria, often with appreciable acute inflammation (23.7). They are most commonly seen in children but are sometimes seen in adults. Because of the concomitant acute inflammation, juvenile polyps were formerly called inflammatory polyps, but the latter term is no longer used because it is ambiguous and may connote a poly of a different type, especially an inflammatory pseudopolyp secondary to inflammatory bowel disease, and might mislead the clinician into believing there is another pathologic process. A juvenile poly may be a sporadic finding, but juvenile polyposis is an autosomal dominant syndrome.

Peutz-Jeghers polyps are hamartomas of the small intestine. While the epithelial component may predominate, the core of smooth muscle results in a characteristic low-power arborizing appearance that is more orderly than a juvenile poly (23.8). Sporadic Peutz-Jeghers polyps occur, but these are less common than sporadic juvenile polyps, and most patients with a Peutz-Jeghers polyp will have Peutz-Jeghers syndrome, which is autosomal dominant and occasionally associated with malignancies, especially of the breast and gastrointestinal tract [PMID20561235, PMID20659341].

The hyperplastic poly is the simplest of all colonic polyps and it characterized by epithelial hyperplasia that has a "sawtooth" appearance on the surface (23.9a). In the interior, the hyperplasia results in glands with infoldings (23.9b). Nuclei remain small and basal (23.9c). There is no increase in malignant potential in the hyperplastic poly.

The serrated adenoma has a lower power appearance similar to that of a hyperplastic poly, with "sawtooth" epithelial hyperplasia and retention of ample cytoplasm (23.10a). But whereas the nuclei of a hyperplastic poly are small and basal, those of the serrated adenoma are enlarged, round to ovoid, with a somewhat vesicular (peripheralized) chromatin pattern and occasional nucleoli (23.10b). The serrated adenoma may also have focal nuclear stratification (23.10c) and mitoses, some of which may be close to the luminal surface. Some have defined a serrated adenoma architecturally, on the basis of the location of the serrations relative to the surface or lower layers of the epithelium. However, to my mind, it is a postulate in pathology that malignant potential is derived more from the appearance of the nucleus than from cellularity or architecture—a lesson first taught by Dr Papanicolaou—and I recommend that the diagnosis of serrated adenoma be based on nuclear morphology.

The biologic potential of the serrated adenoma appears to be that of an adenomatous polypl, and it may be helpful to state in parentheses that the serrated adenoma is essentially a type of adenomatous polypl [PMID6635156, PMID12355124].

In general, the nuclei of adenomatous polyps are enlarged and elongated, with coarse chromatin and small nucleoli (23.11a, 23.11b). They are larger than the nuclei of hyperplastic polyps, though a hyperplastic poly may artifactorially appear to be adenomatous if the section is thick (23.12a, 23.12b). Typical adenomatous polyps have only mild stratification of nuclei, largely limited to the lower half of the epithelial thickness (23.11b).

The terms "adenoma" and "adenomatous polyp" are interchangeable. Some adenomatous polyps have a villous architecture (23.13), and these may be called villous adenomas if entirely composed of a villous pattern or tubulovillous adenomas if partially of such a pattern. In this nomenclature, the term "tubular adenoma" refers to an adenomatous polypl without a villous component. However, because all of these types of adenomatous polyps have the same malignant potential and the only architectural features that are important to note are glandular crowding, irregular shapes, and nuclear stratification (as discussed in the following section on dysplasia in adenomatous polyplps), these subclassifications of adenomatous polyplps serve little point and potentially confuse the clinician, who simply needs to know the degree of dysplasia, and if possible, the completeness of removal.

(23.13a-c) compare the nuclei of hyperplastic polyps, serrated adenomas, and adenomatous polyps. While there are exceptions among individual cells in any given polypl, and sweeping statements are to be avoided, in general, the nuclei of hyperplastic polyps are smaller than the other types (23.14a). The nuclei of serrated adenomas and adenomatous polyps are of roughly equal size, but in most cells, the nuclei of the former type are rounder and have a more open chromatin pattern (23.14b), while those of an adenomatous polypl are more spindled, with coarser chromatin and less prominent nucleoli (23.14c).

There are situations in which it is difficult to definitively classify a polypl. Indeed, some polyplps are a mixture of 2 or even all 3 types (23.14d). In situations where it is difficult to distinguish between types of colonic polyplps, I recommend leaning toward the "higher" diagnosis (eg, serrated adenoma or adenomatous polypl instead of hyperplastic polypl or adenomatous polyp).
polyp instead of mixed hyperplasticadenomatous polyp) so as to ensure adequate surveillance.

Mucosal prolapse may act alone and cause a polyp that endoscopically simulates one of the more conventional polyps i23.15a, or it may be superimposed on an existing polyp of a routine type i23.15b. Less common colonic polyps include those caused by leiomyomas of the muscularis mucosa i23.16, lymphoid follicles i23.5, and ganglioneuromas i23.17a-c.

Dysplasia in Adenomatous Polyps

Few areas are more controversial than the matter of dysplasia in colonic adenomatous polyps. The first concept to realize is that by definition, all adenomatous polyps have low-grade adenocarcinoma-directed dysplasia. For this reason, specific mention of low-grade dysplasia in a typical adenomatous polyp is unnecessary and is likely to confuse the clinician into believing that the polyp has a greater biologic potential than it actually has. The lowest degree of dysplasia that should be specifically mentioned is intermediate-grade dysplasia, but the WHO classification recognizes only the categories of low-grade dysplasia (ie, no “added” dysplasia) and high-grade dysplasia [Hamilton ISBN 978-9283224327]. Any mention of dysplasia should include a comment that ordinary adenomatous polyps have low-grade dysplasia, so the clinician can see how many “steps” above an ordinary adenomatous polyp this particular polyp is.

Assessment of dysplasia in polyps must work hand-in-glove with increased colon cancer screening. If the pathologist can identify those polyps that have a higher degree of dysplasia, the complete removal of those polyps and more frequent surveillance of those patients augment the effectiveness of colon cancer screening. The 3 criteria that are most consistently useful in identifying adenomatous polyps that have increased dysplasia are irregularly shaped or crowded glands, nuclear stratification, and severe nuclear atypia [Hamilton ISBN 978-9283224327, PMID11354160, PMID795660]. “Irregularly shaped or crowded glands” refers to glands that have irregular contours or a cribriform appearance, or abut each other without intervening stroma i23.18a, i23.18b. Nuclear stratification refers to nuclei that are found in the upper half of the epithelium i23.18c. Severe nuclear atypia refers to a nuclear appearance that is indistinguishable from that of an invasive carcinoma. Specifically, the nucleus of an adenomatous polyp with high-grade dysplasia is large and round, rather than the elongated nucleus of the usual adenomatous polyp, and often has vesicular chromatin and a prominent nucleolus i23.18d. Large numbers of mitoses, especially in the upper portion of the epithelium, may also be considered, though this tends to reflect the degree of stratification. Villous architecture is no longer generally regarded as independently significant.

Although these 3 broad criteria are generally agreed upon, there is wide interobserver variation in how they are applied. Some will diagnose high-grade dysplasia if only 1 of the above findings is present, and others will diagnose intermediate-grade dysplasia if 1 or 2 criteria are present. To my mind, it is prudent to accept 1 of the above findings as still within the spectrum of a routine adenomatous polyp (ie, low-grade dysplasia), while 2 or more suggest high-grade dysplasia, which is essentially synonymous with adenocarcinoma in situ (AIS). In borderline situations, it might be reasonable to make a “lower” diagnosis if there is a well defined negative stalk or base, and a “higher” diagnosis if the lesion is incompletely removed.

There is no consensus on the term “intramucosal carcinoma.” Some do not use the term since the distinction of AIS from intramucosal invasion is unlikely to be clinically significant because the mucosa lacks lymphatic vessels except for rare lymphatic channels in the muscularis mucosa [PMID296428], and therefore, there is no chance of metastases in intramucosal carcinoma. Others believe that intramucosal invasion is a recognizable step between AIS and submucosal invasion. Among the latter group, some believe that the diagnosis of invasion into the mucosa requires the same criteria as are used to identify invasion anywhere, including a desmoplastic stroma i23.19a. Others believe intramucosal invasion can be inferred from extensive, confluent areas of AIS in the mucosa, even without a stromal reaction i23.19b, i23.19c. The important point is to verify that there has not been invasion into the submucosa, because invasion into the submucosa introduces the possibility for metastases. The muscularis mucosa can be difficult to identify in polyps, making it potentially problematic to determine if an adenocarcinoma is invasive only into the mucosa or also into the submucosa. In questionable cases, a trichrome stain or an immunohistochemical stain for actin may highlight the muscularis mucosa.

The management of situations in which carcinoma in a polyp invades into the submucosa is not completely settled. Many feel it is permissible to manage these cases by polypectomy alone if the carcinoma is not poorly differentiated, there is no vascular invasion, and there is a stalk or base that can be shown to be negative [PMID35987]. Not everyone is comfortable with this approach, however, and there is a risk of nodal metastases in these situations, ranging from 2-25% in differing studies [PMID735483]. The precise risk of management by polypectomy alone is difficult to assess, probably because of disagreement regarding whether or not the stalk is invaded, and if so, whether or not its base is truly negative, and how thoroughly lymph node dissection is performed after resection.

Although there are a number of syndromes that involve colonic neoplasms, there are 2 major colon carcinoma syndromes, one with polyposis (familial adenomatous polyposis) and one without polyposis (hereditary nonpolyposis colon cancer syndrome; also called Lynch syndrome). Gardner syndrome is familial adenomatous polyposis with extraintestinal manifestations. Both of these syndromes are inherited in an autosomal dominant manner.

Colorectal Cancer Screening

At this writing, traditional colonoscopy is far and away the best method of early detection. The colonoscopic detection of flat premalignant lesions is challenging, but newer endoscopic methods using dyes (so-called chromoendoscopy) or magnification are promising. Colonography (the “virtual” colonoscopy by CT scan) requires the same preparation as the traditional procedure—universally acknowledged as the most trying part of the test—and is therefore only slightly less inconvenient to the patient. Colonography also introduces the dilemma of what to do if a polyp is found. Must it be removed? The literature is settling on the matter at this writing, but there is support for a protocol of recommending colonoscopy and removal of polyps.
Stool testing for occult blood is a very poor surrogate for colonoscopy and simply serves to identify a group of patients who need further testing. When testing for occult blood is indicated, a fecal immunochemical test is recommended over the traditional hemoccult cards because it uses a monoclonal antibody that is specific for human hemoglobin and therefore avoids interference from dietary hemoglobin [PMID1699808].

Testing of plasma for methylated DNA sequences that are associated with malignancies is another promising avenue of screening. Since methylation of a promoter site diminishes the transcription of a gene (this is a form of epigenetic regulation of the genome, discussed in Chapter 47), hypermethylation of promoters of tumor suppressor genes can silence them and thereby lead to neoplasia. The detection of methylated DNA for the gene septin 9 (SEPT9) in plasma may be helpful in screening for colon cancer [PMID19406918]. For reasons that are not yet clear, hypermethylation of genes that do not serve a known tumor suppressor function may also be associated with neoplasia. The vimentin gene, which codes for an intermediate filament structural protein and is not a known tumor suppressor, is hypermethylated in many colon carcinomas, and the identification of this in stool specimens is a promising means of early colon cancer detection [PMID1875824].

The Colectomy Specimen

In addition to an assessment of tumor differentiation in general, I recommend that the pathologist comment on the degree of differentiation of the tumor at the advancing front in colon carcinomas, as this may be less differentiated than the bulk of the tumor i23.20a, i23.20b. Reduced differentiation or buds of tumor cells branching from a larger cluster at the advancing front correlate with increased lymph node metastases in T1 (submucosally invasive) colon carcinomas [PMID2047227].

As is the case for so many other tumors, attempts to identify important prognostic variables other than tumor grade and stage have met with mixed results at best. Supposedly independent prognostic variables in colon carcinoma that may portend an aggressive course include microacinar architecture, neuroendocrine differentiation (if confirmed by immunohistochemical stains), tufting of tumor cells, and CD10 expression. A Crohn-like lymphoid or granulomatous reaction has been suggested as a favorable finding. As is the case for other sites, firmly established independent prognostic variables are elusive and even when successfully identified, add only indirectly to patient care.

An area of controversy is the number of lymph nodes that the pathologist should recover in the gross examination of a colon cancer resection. Some pathologists recommend that no less than 12 lymph nodes be recovered, while others argue that this number is arbitrary and does not increase the detection rate of positive lymph nodes. In some cases, the pathologist may be called upon to assess the effect of the neoadjuvant therapy after resection. A 5-tiered system has been proposed [PMID21771024], but in most cases, an evaluation of viable tumor cells and fibrosis suffices i23.21a-c.

Intestinal Carcinomas with Neuroendocrine Differentiation

Most intestinal neoplasms with neuroendocrine differentiation are pure, low-grade neuroendocrine carcinomas (carcinoid tumors) in the appendix i23.22 or terminal ileum i23.23. The rectum is also a relatively common site. Low-grade neuroendocrine carcinomas are usually indolent, especially in the appendix, though some in the terminal ileum may be aggressive i23.24a, i23.24b.

The World Health Organization currently lists 3 categories of tumors with features of both a neuroendocrine tumor and an adenocarcinoma [Klimstra ISBN 978-9283224327]. The first of these is a carcinoid with a tubular pattern, in which a lumen is enclosed by a single layer of cells with the same uniform appearance as those forming low-grade neuroendocrine carcinomas in a trabecular pattern i23.25. This neoplasm is probably a pure carcinoid (ie, low-grade neuroendocrine carcinoma) that can resemble an adenocarcinoma because the neuroendocrine rosettes resemble glands. It is relatively indolent, especially if it occurs in the appendix.

The second neoplasm with morphologic features of both a neuroendocrine carcinoma and an adenocarcinoma is one in which individual cells have features of both a neuroendocrine tumor and an adenocarcinoma. This is often manifested as the so-called goblet cell carcinoid, because
individual cells may have both neuroendocrine features and a distinct cytoplasmic vacuole \textsuperscript{23.26a, 23.26b}. Although not as aggressive as a conventional adenocarcinoma, goblet cell carcinoids have metastatic potential and warrant a hemicolectomy \textsuperscript{[PMID12534685]}. The third tumor in this category is the mixed neuroendocrine carcinoma-adenocarcinoma, which consists of separate areas of each tumor type \textsuperscript{23.27a-c}. Because of its pure adenocarcinoma component, it is more aggressive than a goblet cell carcinoid and will behave as a conventional adenocarcinoma. This category includes neoplasms that initially appear to be goblet cell carcinoids, but that are actually a mixture of a conventional carcinoid and a mucinous signet ring adenocarcinoma \textsuperscript{23.28a-c}.

Neoplasms with features of both a neuroendocrine tumor and an adenocarcinoma may overlap, both morphologically and immunohistochemically. The unqualified term “adenocarcinoid” is confusing, and is used by some as synonymous with goblet cell carcinoid and by others as a mixed neuroendocrine carcinoma-adenocarcinoma. I recommend avoiding this term, or at least descriptively qualifying it if used.

Very rarely, there will be a high-grade neuroendocrine carcinoma (small cell carcinoma) that is primary in the gastrointestinal tract \textsuperscript{23.29a-d}.

### Unusual Intestinal Neoplasms

The medullary carcinoma is a form of undifferentiated colonic carcinoma. It resembles a breast medullary carcinoma, with highly pleomorphic nuclei and occasionally prominent nucleoli. There is very little gland formation, if any \textsuperscript{23.30}. Cytoplasmic mucin is inconspicuous and usually cannot be demonstrated. Lymphocytes may be adjacent to or within the tumor. The medullary carcinoma should be excluded in any colorectal carcinoma that has minimal gland formation or a neuroendocrine appearance but is negative for neuroendocrine markers. Medullary colonic carcinomas tend to arise from defective DNA repair and have both microsatellite instability and a deficiency of the mismatch repair protein MLH1. In addition, they differ from conventional colon carcinomas in that they are usually positive for calretinin, a marker that is used to stain mesothelial cells and is negative for CDX2 \textsuperscript{[PMID8992917]}. Despite the alarming nuclear appearance and the minimal gland formation, the colonic medullary carcinoma has a better prognosis than conventional colon carcinomas.

The appendix may be the site of an ordinary colonic adenocarcinoma, of the full range of neuroendocrine tumors described above, or of mucinous tumors that are difficult to evaluate. Although small mucocoele do exist, most mucinous distention of the appendix is caused by a neoplasm, and certainly, any such distention or any pools of mucin found in an appendix should prompt the submission of the entire specimen. Appendiceal mucinous neoplasms may be adenomas, mucinous tumors of uncertain malignant potential (UMP), or fully malignant mucinous adenocarcinomas \textsuperscript{23.31a, 23.31b}.

Appendiceal mucinous neoplasms are the most common cause of pseudomyxoma peritonei, even in the presence of a mucinous ovarian neoplasm \textsuperscript{[PMID1751804]}. Any peritoneal mucinous deposit should be closely examined for epithelial cells, using immunohistochemical stains if necessary, but even if none are found, it is wise to examine the appendix.

### Molecular Pathology in Colorectal Cancer

Many colon carcinomas have some chromosomal abnormality. Among the numerous chromosomal abnormalities that have been reported are losses of 8p or 18q \textsuperscript{[PMID15756695]}. However, most colon cancers arise through 1 of 2 mechanisms related to individual genes, not to chromosomal abnormalities. 80% of colon cancers and the vast majority of sporadic cases are thought to arise from accumulated abnormalities of tumor suppressor genes, such as \textit{p53}, \textit{APC}, \textit{K-RAS}, and \textit{SMAD4}. This pathway to neoplasia has been well described by Vogelstein (eg, \textsuperscript{[PMID15090690]}). A minority of colon cancers arise as part of a syndrome, the 2 most common of which are familial adenomatous polyposis (FAP; also called adenomatous polyposis coli [APC]) and hereditary nonpolyposis colon cancer syndrome, both of which are discussed in Chapter 46.

While there has long been anecdotal feeling that rectal adenocarcinomas may have a different behavior from those arising elsewhere in the colon, recent work in the molecular biology of adenocarcinomas from the rectum and colon has shown that this is not the case, and that rectal adenocarcinomas are not different from general colonic adenocarcinomas \textsuperscript{[PMID22880696]}. Increased understanding of the molecular biology of colorectal carcinoma is beginning to find clinical application. The \textit{ERBB} family of genes encodes transmembrane proteins that contain an intracellular tyrosine kinase that is important in initiating the intracellular response to extracellular growth factors. The epidermal growth factor receptor gene (\textit{EGFR}) and \textit{HER2} are members of this family. Just as some cases of breast cancer overexpress \textit{HER2} and are candidates for treatment with trastuzumab (Herceptin), some cases of colon cancer overexpress the \textit{EGFR} protein. If overexpression is found (immunohistochemistry is usually used to identify these cases), cetuximab (Erbitux) or panitumumab (Vectibix), monoclonal antibodies that bind to the extracellular domain of the \textit{EGFR} protein and diminish its signaling capacity, may be beneficial \textsuperscript{[PMID17948503]}. These drugs have a roughly comparable effect on the \textit{EGFR} protein to that of Herceptin on the \textit{Her2} protein.

As is the case for lung cancer, the Ras protein family, of which K-ras is the most important, receives input from the \textit{EGFR} protein. The normal, wild-type form of the \textit{K-RAS} gene is found in 50-70% of colorectal cancer patients. In these patients, the signaling pathway distal to \textit{EGFR} is normal, and blockade of the receptor may be helpful. However, 30-50% of colorectal carcinoma patients have a gain of function mutation in \textit{K-RAS}, usually from a mutation in codons 12 or 13 \textsuperscript{[PMID19644023]}. These patients do not benefit from \textit{EGFR} blockade, presumably because the mutant K-ras protein is autonomous and does not require activation by the \textit{EGFR} pathway. For this reason, before therapy with cetuximab or panitumumab is instituted to blockade \textit{EGFR}, colon cancers are often tested for a mutation in \textit{K-RAS} \textsuperscript{[PMID9917867]}. In addition to \textit{K-RAS}, mutations in the genes \textit{BRCA} or \textit{PIK3CA} may result in proteins that function independently of the \textit{EGFR} protein and therefore negate the benefit of \textit{EGFR} blockade \textsuperscript{[PMID19765664]}. It is worthwhile to retest a colorectal carcinoma for these mutations if a metastasis occurs because the genotype of a metastasis may differ from the primary \textsuperscript{[PMID23740278]}.

Gefitinib (Iressa) and erlotinib (Tarceva) are inhibitors of the intracellular tyrosine kinase moiety of \textit{EGFR}. At this writing, they are used in some cases of advanced non-small cell
carcinoma of the lung and are in phase II trials for colorectal carcinomas [PMID20524863]. They, or similar drugs, may find their way into use in some cases of colon cancer, but since they still target part of the EGFR molecule, there presumably will still be the problem of mutations conferring autonomy to downstream genes.

The V600E mutation in the BRAF gene found in some cases of thyroid cancer, is also found in some cases of sporadic colorectal carcinomas that have microsatellite instability (discussed in Chapter 46) [PMID248914] f23.1. Attention is being focused on this subgroup because there is an inhibitor of the Braf enzyme (vemurafenib).

The Anal Canal

“The Anal Canal intraepithelial neoplasia” (ACIN or AIN) is accepted terminology for squamous dysplasias in this location, reflecting the movement toward the terminology “intraepithelial neoplasia” instead of “dysplasia” in all sites. AIN is graded on a scale of 1-3, using the same criteria as are used in the cervix i23.32a-c. Anal canal squamous carcinomas are strongly associated with human papillomavirus, especially types 16 and 18 [PMID12231953, PMID12231960]. Interestingly, as is the case for high-grade cervical intraepithelial neoplasia, 90% of cases of AIN 2 or 3 are positive for the protein P16INK4a (usually just called p16, discussed in Chapter 46). Many cases of AIN also demonstrate staining for the proliferation marker Ki-67 in cells above the basal layer [PMID3639520].

The anal canal is one of the sites in which the verrucous carcinoma may be found i23.33. The diagnosis of verrucous carcinoma is made here as in other locations, but as in other sites, the precise terminology is less important than identifying invasion and assessing resection margins.

The terms “transitional” and “cloacogenic” are not used now, and these carcinomas are best thought of as squamous carcinomas of the basaloid type, as are encountered in other sites i23.34. Invasive carcinomas of the anal canal should simply be called squamous carcinomas, with a comment on whether it is of the common or basaloid type.

Core Points for Intestinal Neoplasms

- Intestinal GISTs: morphologic, immunohistochemical, and molecular characteristics parallel those in the stomach.
- Intestinal lymphomas: evaluated as in other sites.
- Mantle cell lymphomas of the gastrointestinal tract often present as polyps, but most lymphoid polyps are benign.
- Immunoproliferative small-intestinal disease (Mediante lymphoma): a low-grade lymphoma, with plasma cells
  - Often accompanied by diarrhea and malabsorption
  - Predominantly affecting patients in the second or third decade
- Enteropathy-associated T-cell lymphoma (EATCL): a high-grade lymphoma associated with ulceration and malabsorption
- Juvenile polyps: hamartomas of the lamina propria
  - Characterized by dilated glands and acute inflammation
- Peutz-Jeghers polyps: hamartomas of smooth muscle
- Hyperplastic polyp: “sawtooth” epithelial hyperplasia, with nuclei that are small and basal
- Serrated adenoma: serrations of a hyperplastic polyp, but nuclei are enlarged and round, with vesicular chromatin and occasional nucleoli
  - Nuclear stratification and mitoses are common. The individual nuclei of a serrated adenoma are larger than those of a hyperplastic polyp, and rounder, with more vesicular chromatin, than those of an adenomatous polyp.
- Adenomatous polyps: nuclei are elongated, with coarse chromatin and small nucleoli, and differ from those of either the hyperplastic polyp or the serrated adenoma
  - Low-grade dysplasia by definition.
- Adenomatous polyps with high-grade dysplasia: irregularly shaped or crowded glands, nuclear stratification, and severe nuclear atypia.
- Most cases in which carcinoma in a polyp invades into the submucosa can be managed by polypectomy if:
  - The carcinoma is not poorly differentiated
  - There is no vascular invasion
  - There is a negative stalk or base
- Most colon cancers are sporadic and arise from abnormalities of tumor suppressor genes, such as K-RAS and P53.
- Some colon cancers overexpress epidermal growth factor receptor (EGFR)
  - Blockade of its extracellular portion or inhibition of its intracellular tyrosine kinase portion may be beneficial.
- But 30-50% of colorectal carcinoma patients have a gain of function mutation in the K-RAS gene
  - The K-ras protein is decoupled from EGFR and the tumor is refractory to either extracellular blockade of EGFR or inhibition of its intracellular tyrosine kinase.
23.1 Heterotopic gastric mucosa in the duodenum

23.2 Heterotopic pancreatic tissue in the duodenum

23.3 a A gastrointestinal stromal tumor of the colon
b A higher-power view of the same case as in 23.3a. This neoplasm was positive for CD117 and was of uncertain malignant potential.
c Mesenteric fibromatosis. As is the case for fibromatosis in other sites, there is a uniform proliferation of fibroblasts in a stroma that varies from myxoid to collagenous.
d A high-power view of the same case as in 23.3c, illustrating the nuclear uniformity.
e Mesenteric fibromatosis infiltrating lobules of fat in the mesentery. This process may also infiltrate the bowel wall, simulating a GIST.

23.4 a Enteropathy-associated T-cell lymphoma of the small intestine
b Enteropathy-associated T-cell lymphoma. A higher-power view of the same case as in 23.4a.

c A gastrointestinal stromal tumor of the duodenum

23.5 Lymphoid hyperplasia of the colon, resulting in an endoscopically evident polyp

23.6 a Mantle zone lymphoma of the colon, resulting in an endoscopically evident polyp
b A higher-power view of the same case as in 23.6a, illustrating the uniform population of small lymphocytes that is characteristic of mantle cell lymphoma. These cells were positive for bcl-1.
23: Intestinal Neoplasms

23.7 A juvenile polyp of the colon, with sharply expanded lamina propria and acute inflammation

23.8 A Peutz-Jeghers polyp. Although the bulk of the polyp is composed of epithelium, the fundamental process is hyperplasia of the smooth muscle from the muscularis mucosa, resulting in an arborizing appearance.

23.9 A hyperplastic colonic polyp with “saw tooth” hyperplasia of the surface epithelium. The interior of a colonic hyperplastic polyp, where the epithelial hyperplasia has resulted in infolding.

23.10 A serrated adenoma. The low-power appearance is virtually identical to that of a hyperplastic polyp. The same case as in 23.10a, illustrating the nuclei of a serrated adenoma, which are significantly larger than those of a hyperplastic polyp. Note the generally open chromatin pattern, with small nucleoli.

23.11 An adenomatous polyp. A high-power view of the nuclei of an adenomatous polyp. The nuclei are significantly larger than those in a hyperplastic polyp, but they are more spindled, with coarser chromatin and less distinct nucleoli than the nuclei of a serrated adenoma.

23.12 A colonic polyp that appears adenomatous in a thick section but hyperplastic in the thinner section.

23.13 An adenomatous polyp with a villous architecture (villous adenoma).

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**23.14** Side-by-side comparisons of the nuclei in **a** a hyperplastic polyp, **b** a serrated adenoma, and **c** an adenomatous polyp.

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**23.15** Mucosal prolapse that resulted in an endoscopically evident polyp. Mucosal prolapse superimposed on a hyperplastic polyp in the sigmoid. Note the stromal fibrosis in the left-center.

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**23.16** A leiomyoma of the muscularis mucosa

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**23.17** A ganglioneuroma of the colon, presenting as a polyp. **a** is a low-power view illustrating the schwannoma-like stroma that pushes aside the glands. **b** illustrates the stroma and the ganglioneuroma cells.

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**23.18** The 4 images of **23.18** illustrate the features of an adenomatous polyp with high-grade dysplasia. **a** Glandular crowding in an adenomatous polyp.

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**23.19** Intramucosal adenocarcinoma. A small amount of muscularis mucosa can be seen in the upper right. The remainder of the photograph is mucosa but the normal lamina propria has been displaced with a fibrotic stroma, an indication of invasion.

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**23.20** **d** shows a polyp in which a gland has an abrupt transition between an area of adenomatous change and an area of serration and nuclear stratification. In the lower right corner of the photograph, still in the polyp, there is a gland with the small nuclei characteristic of a hyperplastic polyp.

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**23.21** **c** illustrates the characteristic prominent nuclei and abundant amphophilic cytoplasm of ganglioneuroma cells.

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**23.22** **c** Nuclear stratification in an adenomatous polyp

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**23.23** **d** An adenomatous polyp with rounded nuclei, open chromatin, and nucleoli, nuclear features that are not usually seen in an adenomatous polyp.

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**23.24** **b, c** Other examples of intramucosal adenocarcinoma inferred by the glandular crowding rather than by stromal desmoplasia. The muscularis mucosa is to the upper left in **b** and to the lower right in **c**.

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A neuroendocrine carcinoma with cytoplasmic vacuolization (so called goblet cell carcinoid).

A low-grade neuroendocrine carcinoma which was treated with neoadjuvant chemotherapy and radiation therapy prior to resection. There is appreciable necrosis and elimination of the surface component, but residual viable tumor remains in the stroma.

A low-grade neuroendocrine carcinoma (carcinoid tumor) in the appendix.

A low-grade neuroendocrine carcinoma of the terminal ileum metastatic to a lymph node. A higher-power view of the same case as in i23.24a. Note the extensive lymphatic invasion adjacent to the lymph node and also note that despite the aggressive behavior the neoplasm continues to have low-grade nuclei.

A neuroendocrine carcinoma with cyttoplasmic vacuolization (so called goblet cell carcinoid).
23.17 A mixed neuroendocrine carcinomadenocarcinoma. 
\( \text{a} \) illustrates a high-grade adenocarcinoma with subtle gland formation in the upper right.
\( \text{b} \) illustrates a routine low-grade neuroendocrine carcinoma, with a trabecular growth pattern, immediately adjacent to the focus in 23.27a.

23.18 Another example of a mixed neuroendocrine carcinomadenocarcinoma in which the adenocarcinoma component (seen in \( \text{a} \) and \( \text{d} \)) initially appears to be a goblet cell carcinoid. However, in addition to the distinctly mucinous nature of the cytoplasm, unusual in a lower-grade goblet cell carcinoid,

23.29 a-c A primary high-grade neuroendocrine carcinoma (small cell carcinoma) of the colon. In a note the paucity of cytoplasm and the hyperchromasia of the nuclei.
\( \text{b} \) is a positive synaptophysin immunohistochemical stain, confirming the neuroendocrine nature of the process.
\( \text{c} \) is Ki-67 immunohistochemical stain to assess proliferation. Almost all of the neoplastic cells are positive, with a nuclear staining pattern. The rounded cluster that is negative in the lower right corner is a lymphoid nodule. This very high proliferation rate identifies the carcinoma as being high grade, and is not seen in a grade 1 or grade 2 neuroendocrine carcinoma.

23.30 A colonic medullary carcinoma with high-grade nuclei, prominent nucleoli, and no gland formation.

23.31 A mucinous adenocarcinoma of the appendix. \( \text{a} \) illustrates an in situ component while \( \text{b} \) illustrates a poorly differentiated, signet ring invasive component.
i23.32 Anal intraepithelial neoplasia. 

a illustrates AIN1. Note the resemblance to CIN1 of the cervix, with diminished maturation and mild nuclear enlargement. The prominent perinuclear halos reflect the association of AIN with human papillomavirus, as is the case for CIN. 

b illustrates AIN2. Note the sharp reduction in maturation, the mitotic figure in the center, well above the basal layer, and the dyskeratotic cells near the surface. 

c illustrates AIN3/ squamous carcinoma in situ. Note the almost complete absence of maturation and the mitotic figures.

i23.33 A verrucous carcinoma of the anal canal. As is typical of this entity there is a large bulbous protrusion into the stroma, but the interface of the epithelium and the stroma remains smooth and there is no stromal reaction.

i23.34 A squamous cell carcinoma, primarily of the basaloid type, of the anal canal (formerly called transitional cell or cloacogenic carcinoma of the anus)
FISH Preparation

FISH probes must be labeled, and both the probe and the target must be prepared for hybridization. Most clinical laboratories purchase their FISH probes, but for the daring that wish to make them, the steps in the process involve producing the DNA that is to be used as the probe (using either cloning technologies or PCR), labeling it with a fluorochrome, and subjecting the probe to preannealing steps so that it will be able to anneal to the target sequence.

Labeling the probe consists of ensuring that some of its nucleotides will be labeled with a reporter molecule. There are 2 ways to include a reporter molecule. The first is an indirect method, in which reporter compounds that are not fluorescent, but which can subsequently be attached to fluororescent labels, are joined to the nucleotides of the probe. The 2 most commonly used nonfluorescent reporter compounds are biotin, a naturally occurring vitamin in the B-complex family, and digoxigenin, a plant compound that like digoxin, is obtained from *Digitalis*. Biotin has a natural affinity for avidin, and in a biotin-based system, avidin is attached to fluorochromes. In a biotin system, after probe-target hybridization, avidin-fluorochrome is added. If the probe has bound, the avidin-fluorochrome will bind to the biotin on the probe. In the other common indirect labeling method, digoxigenin is attached to the probe and after hybridization to the target, antibodies to digoxigenin, attached to fluorochromes, are added. If the probe has bound, the digoxigenin will bind the antibody-fluorochrome.

If the target sequence is short, or if interphase cells are used, it may be necessary to amplify the signal. If the original reporter molecule was biotin, additional layers of biotin that will react with the reporter must be introduced into the digoxigenin. Such procedures of colloquially referred to as “stacking.”

The second method of labeling is direct labeling, in which a fluorochrome is directly attached to the nucleotides that will be incorporated into the DNA probe. This leads to immediate fluorescence as soon as the probe binds the target. These probes are more expensive and typically generate a slightly weaker signal, but they are quicker and easier to use. “Stacking” is rarely used in direct labeling.

Whether indirect or direct labeling is employed, the nucleotides carrying the reporter molecules or the compounds that will react with the reporter must be introduced into the double-stranded cloned DNA that is to become the probe. One could simply use an enzyme to add a labeled nucleotide to one end of the probe, but this results in a probe that carries only 1 label and will therefore generate a weak signal. It is better to use a process that continually incorporates label during synthesis, so that the probe will carry many labeled nucleotides. This is often done by PCR, in which case, if the primers are correctly designed, labeled nucleotides will continuously be incorporated into the growing chain.

Another way to incorporate label throughout the probe is a process called nick translation. Nick translation uses 2 enzymes. The first is a eukaryotic DNase that nicks 1 of the 2 strands of DNA. The second enzyme is the prokaryotic enzyme DNA polymerase I, which in prokaryotes functions in DNA replication both to excise the RNA primer and to replace those bases with DNA. In nick translation, DNA polymerase I attaches to the site that has been nicked by the eukaryotic DNase, removes about 10 base pairs, then fills in the gap using the labeled nucleotides.

Less commonly, probe labeling occurs by random primer extension, in which the DNA to be labeled is denatured and labeled nucleotides are added in the presence of random primers. If this method is used, it is necessary to use a DNA polymerase that does not have exonuclease activity in the 5' to 3' direction, as this is the direction of synthesis and any such exonuclease activity will negate incorporation. Prokaryotic DNA polymerase I has exonuclease activity in both directions, but if it is treated with a protease, it is split into 2 fragments, the larger of which retains polymerase activity and an exonuclease activity in the 3' to 5' direction, but not in the 5' to 3' direction. This is called a Klenow fragment and will add labeled nucleotides to DNA in the presence of oligonucleotide primers (a Klenow fragment can also be produced from a gene that has been truncated).

Before the probe is ready for use, it must undergo a number of preparatory steps. Because repetitive sequences are found throughout the genome, both probe and target DNA may contain repetitive sequences. If both probe and target do contain repetitive sequences, when they are denatured and exposed to each other, hybridization of counterpart sequences in the repetitive regions will cause nonspecific hybridization that interferes with the specific hybridization signal that is sought. Signals from repetitive sequences can be substantially diminished by adding a mixture of commonly found repetitive DNA sequences to the probe prior to exposure to the target. Such DNA is called blocking DNA. By binding to repetitive DNA sequences in the probe, blocking DNA makes them unavailable for binding to the target. Cot-1 is a fragment of human DNA that is composed primarily of repeating sequences and is often used as blocking DNA. Most commercially available probes have already been “blocked.”

The target must also be prepared. There are 2 requirements for the target. It must be fixed, so that its morphology is preserved and can be visualized, and its cell membranes must be rendered permeable, so that the probe can enter the cell and find its way to the chromosomes. Targets such as blood and bone marrow smears can generally be prepared with simple, time-honored techniques that are very similar to those used for the old G-banding procedures. Archival materials, such as formalin-fixed, paraffin-embedded tissue blocks require special techniques to remove the paraffin and permit access of the probe to the target. Considerable experience has been gained in this area and established, reasonably effective procedures are now well in hand for many commonly used probes.

The second phase of target preparation is a pretreatment that will facilitate hybridization with the probe. There are a number of protocols for specific situations. For example, in some situations, positive charges on the target must be neutralized to permit access of the probe. In other situations
FISH Is Limited to Characterized Abnormalities

A major application of FISH is in cytogenetics, but it can only detect previously characterized abnormalities. In all of the many applications of FISH, because the probe must be specifically designed, it can only be used to detect the presence or absence of previously identified chromosomal aberrations. It cannot be used to identify new abnormalities.

Many of the uses of FISH pertain to cytogenetics, and FISH is in essence the next generation of cytogenetic techniques. In addition to the opportunity to correlate with morphology, FISH is also more sensitive than conventional metaphase cytogenetics and permits the detection of abnormalities that are beyond the sensitivity of the older technique. FISH can be done on paraffin section, on smears, or on disaggregated nuclei from a paraffin block, and may be used to assess DNA or mRNA. The major types of FISH probes in cytogenetics are locus-specific (looking for a particular abnormality, often translocations), centromeric (used for chromosome identification), subtelomeric, telomeric, and whole chromosome paints.

When FISH is used to detect monosomy or trisomy, metaphase spreads are used and a fluorescent probe that is complementary to the centromere of the chromosome to be tested for is applied. It is not simply a matter of counting signals in the test cells; normal controls are run concurrently and the cells of interest are compared to the control group. The mean number of signals for the control group should certainly be close to 2 but will barely be exactly 2. For the control group, the mean and standard deviation is obtained. If a cell being tested has a number of signals that is above or below 2 standard deviations from the control group, it is considered to have an abnormality in the number of that chromosome. On the other hand, if the tested cell has a signal number that is within 2 standard deviations of the control mean, it is considered to have a normal number of that chromosome. Care must be taken to count only in areas in which the cells are not overlapping and in which the nuclei are intact and the chromosomes are well seen. 2 signals that are very close together or are separated by only a thin fluorescent strand are counted as a single signal.

In microdeletion analysis, metaphases are generally studied and a fluorescent DNA probe that is complementary to the DNA that may be deleted is used. After hybridization, a significant loss of fluorescent signal implies a microdeletion. Again, one cannot simply examine the test cells in isolation. A fluorescent DNA probe for part of the chromosome, typically the centromere, is labeled with a fluorochrome of a different color to distinguish it from the test DNA probe and used as a control to ensure that an apparent deletion is not caused by a diminished number of chromosomes.

Whereas metaphase chromosomes are used in determinations of chromosome number and in microdeletion studies, translocations are usually detected with interphase chromosome painting. Using FISH for translocations is more problematic from an interpretational viewpoint because it is not just a matter of presence or absence of signal. A common procedure is to employ DNA probes that are specific for the portions of DNA that are on either side of the possible translocation, not for the abnormal DNA itself. In one form of testing, a different color conjugate is used for each side. In normal situations, where there is no translocation, the 2 colors are so close together that they merge into a single, intermediate color. In
situations where the translocation has occurred, the 2 colors are separated and will appear as 2 distinct colors (this is called break apart FISH) f45.11a-b, f45.12.

The process can also be reversed, so that in the normal situation, the probes are hybridized to areas that should be far apart, with 2 distinct colors. If there has been a translocation, it will bring the 2 colors together and merge them into a new color (this is called double fusion strategy) f45.13a-b, f45.14. A common example of this is that a red probe is used for the normal ABL gene on chromosome 9 and a green probe for the normal BCR gene on chromosome 22. If there has been a BCR/ABL translocation, the 2 probes are inappropriately fused, resulting in a yellow color.

One can also employ a probe that is specific for the post-translocation DNA. This is a single-color test, in which the probe, labeled with a color that is different from the background color, simply binds if the translocation is present and fails to bind if it is not (this is called single fusion strategy).

FISH can also be used to detect inversions. The detection of these abnormalities is based on the same principles as in the detection of translocations. In one approach, portions of DNA that should be adjacent are labeled with different color probes. As long as the relevant portions remain adjacent, these colors will merge into a single, intermediate color. An inversion causes the component colors to separate, resulting in 2 separate signals of different colors f45.15. Another approach to

![Image](image1.png)

![Image](image2.png)

![Image](image3.png)

![Image](image4.png)

![Image](image5.png)
detecting inversions with FISH is to use a single color probe that spans the breakpoint for the inversion. If the inversion is present, the presence of the probe indicates that there has been an inversion.

As is always the case in FISH studies, overlapping nuclei should be avoided, though software is available to sort out signals in such situations. Interpretation is especially difficult when there has been previous treatment (for example, looking for \textit{BCR/ABL} in a previously diagnosed CML who has been treated with imatinib). As is the case for immunohistochemistry, the interpretation of FISH is primarily manual, but automated methods, hopefully reducing interobserver variability in interpretation, are emerging [PMID 19430291].

There are some terms that may be used only in cytogenetic studies of malignancies. “Hypodiploid” is usually used to indicate that there are 43 or fewer chromosomes, and “hyperdiploid” refers to 47 or greater, though some will say there must be 48 chromosomes to be truly hyperdiploid. Some cancers, such as some cases of childhood ALL may have >50 chromosomes per cell. These are called high hyperdiploid. Pseudodiploid refers to the normal number of 46 chromosomes, but with aberrations.

Rarely, the probe and target in FISH will be reversed and a probe will be prepared from a patient sample and applied to a commercial or constructed target, such as a metaphase spread. This is called reverse FISH. The principal example of reverse FISH is comparative genomic hybridization (CGH), discussed momentarily.

FISH results are usually reported in the International System for Human Cytogenetic Nomenclature (ISCN) nomenclature that is used in G-banding in clinical genetics. For FISH results performed on immobilized metaphase chromosomes, the traditional G-banding result, if available, is given first, followed by a period, followed by the letters “ish,” followed by a more detailed description of the abnormality as elucidated by FISH. For normal findings, the FISH results that follow the G-banding results are reported as the number of the chromosome followed by the band in which the tested locus resides, followed in parentheses by the name of the locus, with a multiplication sign and a number indicating the number of signals. For example, 46,XX.\textit{ish} Xcen (DXZ1x2) is a normal female G-band karyotype (46,XX), followed by FISH results that indicate that the centromeric region of the X chromosome was specifically studied by FISH. This study revealed 2 copies of the locus DXZ1, which is found at the centromere. In normal findings, the chromosome is not in parentheses.

For abnormal findings, the G-banding pattern is again given first and followed by a period, after which the letters “ish,” specific type of abnormality is given (eg, del for deletion, inv for inversion, t for translocation, dup for duplication, etc), and the affected chromosome follows, but is in parentheses. The breakpoint or breakpoints follows the affected chromosome, again in parentheses. The specific locus is then given, followed immediately by a “–” sign if it is absent and a “+” sign if it is present, also in parentheses. For example, 46,XX.\textit{ish} del(22)(q11.2q11.2) (D22S75-) refers to a male that was normal with G-banding, but upon FISH studies, was shown to have a deletion in the D22S75 locus in the 11.2 region of the long arm of chromosome 22 (this is the DiGeorge syndrome critical region).

Unless the nomenclature specifically indicates otherwise, it is conventional to assume that the other chromosome is normal. In the above example, the deletion of the D22S75 locus occurs on only 1 chromosome, while 46,XY.\textit{ish} del(22)(q11.2q11.2)(D22S75–x2) indicates such a deletion on both chromosomes. Likewise, 46,XY.\textit{ish} dup(17)(p11.2p11.2) (RAI1++) indicates a duplication of the RAI1 locus on only 1 copy in the 11.2 region of the short arm of chromosome 17 (a single plus sign indicates a single copy of the locus on that chromosome, which is normal). The other chromosome is not mentioned and is therefore presumed to be normal. On the other hand, 46,XY.\textit{ish} dup(17)(p11.2p11.2)(RAI1++x2) indicates that the locus is duplicated on both chromosomes, for a total of 4 copies.

Unlike conventional G-banding, which requires metaphase spreads, FISH may also be done on intact nuclei, where the chromosomes will be in interphase. This is called nuclear ISH (nuc ish). Because this involves interphase chromosomes rather than metaphases, nuclear ISH is also called interphase ISH. FISH is the leading manner in which interphase cytogenetics is performed. There are 3 advantages to interphase cytogenetics.

- The chromatin in interphase chromosomes is more open and accessible to probes than those in metaphase. Investigations for translocations, for example, are more easily done in the relative openness of an interphase chromosome.
- There are many situations in which metaphase spreads cannot practically be obtained. For example, some tissues, such as muscle and nerve, have a very low mitotic rate and metaphases will be very rare at best. Even in some tissues that do undergo mitoses with reasonable frequency, metaphases may be difficult to obtain.
- Interphase cytogenetics can be done more quickly because one does not have to wait for a significant number of mitoses to develop or use a drug like colchicine to attempt to increase the number of metaphases. Rapid turnaround time is particularly important in many obstetrical settings, where decisions must be made quickly.

ISCN nomenclature also applies in nuc ish, but because G-banding is impossible in interphase chromosomes, G-banding nomenclature does not precede the nuc ish nomenclature unless it has been performed separately on metaphase spreads. Without separate G-banding, the nomenclature begins with nuc ish, after which the locus is designated, followed by a multiplication sign, after which the number of signals seen is given. For example, nuc ish(DXZ1x2) indicates that there were 2 fluorescent signals for this locus on interphase FISH testing. If the chromosome band at which the locus resides is known, it is included after the chromosome number and before the locus. For example, nuc ish 8cen(DBZ2x2) indicates that there were 2 signals from locus DBZ2 on the centromeric region of chromosome 8.

In the testing of neoplastic cells, the number of nuclei examined is indicated in brackets at the end. For example, nuc ish(NMYCx4)[50] means that 50 nuclei from a tumor were examined, and there was an average of 4 signals of the NMYC oncogene per nucleus. In reporting the nuc ish findings for
translocation studies, the precise nomenclature depends slightly on the methodology and the number of probes used (ie, it will differ slightly if single fusion, double fusion, or break apart probes are used). But in a typical single fusion study for possible CML, nuc ish(ABL1,BCR)x2[200] means that 200 nuclei were examined, with the finding that they each had 2 ABL1 loci and 2 BCR loci (normal finding, no translocation). But nuc ish(ABL1x2),(BCRx2),(ABL1 con BCRx1)[200] means that while the expected number of signals from ABL1 and BCR were found, the examination revealed a juxtaposition of the ABL1 and BCR signals (indicative of a translocation) on 1 chromosome in each of the 200 nuclei that were studied. In this case, the first 2 sets of parentheses indicate the total number of signals for ABL1 and BCR (the normal 2 signals for both) and the third set of parentheses, (ABL1 con BCRx1), indicates the number of signals that are juxtaposed per nucleus (1). nuc ish(ABL1x2),(BCRx2),(ABL1 con BCRx2)[200] indicates that the cells have the expected number of ABL1 and BCR signals (2), but that a translocation has occurred on both chromosomes in all 200 nuclei that were examined. nuc ish(ABL1x2),(BCRx2),(ABL1 con BCRx1)[50/200] describes a translocation in only 50 of the 200 nuclei that were examined.

An example of nuc ish is testing for HER2, which resides on 17q21. Because this procedure is often done on formalin-fixed, paraffin-embedded tissue that cannot be treated to increase the number of metaphases, nuc ish is necessary. In this procedure, a fluorescent probe specific for the HER2 locus is used and the number of signals per neoplastic nucleus is tabulated. Because amplification of chromosome 17 is relatively frequent in breast cancer cells (reports range from about 10% to 50%), it is necessary to control for abnormal copy number of this chromosome. As subsequently discussed, α DNA is a form of repetitive found primarily at centromeres. Most chromosomes have a characteristic number of repeats of the basic unit in their α DNA and can be identified by fluorescent probes that hybridize to a known number of repeating units. These probes are called chromosome enumerator probes (CEP). By using a CEP for chromosome 17, which is stained with a fluorochrome of a different color from that used to detect HER2, one can determine if an increased HER2 signal results from amplification on 1 or both chromosomes, or from an extra chromosome. A typical dual-probe testing procedure uses direct labeling of both the HER2 locus and the chromosome 17 CEP, the former with rhodamine and the latter with FITC. The final results are then expressed as the ratio of the number of signals for both HER2 per neoplastic nucleus divided by the number of signals for CEP17. Ratios >1.8 are considered negative (ie, no amplification of HER2 per chromosome). Ratios between 1.8 and 2.2 are considered equivocal, while ratios >2.2 indicate HER2 amplification per chromosome [PMID17159189]. In addition to the use of a ratio of 2.2 as an indication of amplification, it is also important to determine the number of cells expressing this ratio, as breast cancers are often heterogeneous. A ratio of HER2/CEP 17 that equals or exceeds 2.2 in 30% of tumor cells appears to be a prognostic breakpoint [PMID21759603].

Difficulties with any FISH study performed on formalin fixed, paraffin-embedded tissue are nuclear truncation artifact, where copy number is underestimated because a particular section is thinner than an intact nucleus and may
not contain the necessary DNA for the probe to bind to [PMID20657674]. This is a rapid, reproducible, and quantitative technique that in conjunction with the judicious choice of fluorescent probes, can provide excellent information, but it requires metaphase spreads, it is expensive, inversions and translocations will not be detected because they do not change the DNA content, and the instrument cannot determine where on the spreads the probe has bound.

Laser capture microdissection (also called laser-assisted microdissection) is used in FISH studies to separate cells of interest from surrounding cells in paraffin blocks (such as tumor cells from stromal cells). In this technique, an ethylene vinyl acetate film is placed over the area of interest in a paraffin block. A laser then melts the film, after which the molecules of the film diffuse into the block and surround the area of interest, permitting it to retain its integrity after removal from the block. This permits tiny areas of a biopsy or particular tissue types to be isolated and studied [PMID18989416]. For example, one could theoretically separate the epidermis from the dermis in a paraffin block of a skin biopsy and study it by FISH without interference from other tissues.

Another developing area is the use of FISH on extended chromatin fibers (fiber-FISH). DNA is packed in an extraordinarily complex manner, with a basic unit, called a nucleosome, consisting of a 147-base-pair unit of DNA wound around a collection of 8 histone proteins. A spacer sequence of DNA connects each nucleosome, resulting in a "string of beads" that can be seen in the electron microscope. Multiple levels of complex coiling of this string produce the normal interphase chromosome. In extended chromatin fibers, the chromosomes are physically stretched so that they are no longer in the coiled configuration that is characteristic of interphase chromosome. FISH can be used with multiple probes of different colors that collectively "paint" an entire chromosome (this is called whole chromosome painting). Many such libraries have been constructed by needle-tip dissection and removal of specific bands from a chromosome (called chromosome microdissection), followed by the construction of a FISH probe that specifically hybridizes to that band, whether or not it contains any known abnormalities. Because traditional FISH is useful only for known abnormalities, whole chromosome painting may occasionally disclose abnormalities that traditional banding or FISH techniques may not reveal.

In reverse chromosome painting, abnormal or marker DNA (a marker chromosome or fragment is one that is of unknown origin) is amplified and labeled, after which it is hybridized to normal metaphase spreads [PMID1984099]. The location of hybridization reveals the origin of the unknown sequence. In a related technique, if the sequence of a gene is known, but not its location, a FISH probe can be designed that is complementary to the gene and hybridized to metaphase spreads. More advanced techniques using hybridization of the probe to interphase nuclei might provide a more precise location of the gene.

FISH can be combined with immunohistochemistry, allowing a particular population of cells in a section to be studied. For example, if one wants to study T lymphocytes in a mixed population of cells, one can ignore all FISH signals except those emanating from cells that have previously immunohistochemically stained for CD3 [PMID2066547].

FISH can also be combined with flow cytometry. Metaphase chromosomes can be stained with a fluorescent probe that binds a known sequence of DNA and subjected to flow cytometry, which can measure the fluorescence intensity relative to normal metaphases and determine the amount of that particular DNA sequence in the sample relative to normal controls [PMID20657674].

Comparative Genomic Hybridization
CGH is an extension of FISH that has applications in both tumor pathology and cytogenetics. CGH provides an assessment of DNA gains or losses in populations of cells without cell cultures. In a typical situation, tumor (or other potentially abnormal) DNA and control DNA are extracted and labeled with fluorochromes. Often, the normal DNA is labeled with a red fluorochrome and the test DNA with a green fluorochrome. The normal and abnormal DNA populations are then mixed 1:1 in a buffered solution and applied to a substrate that contains normal metaphase spreads. These immobilized normal metaphase spreads are called the target. The 2 DNA populations will then compete for hybridization to the normal metaphase spreads. The ratio of green to red at a given
and for each interval, a mean and a standard deviation for spread. Multiple metaphase spreads are measured in this manner tally convert the counterstain fluorescence from the metaphase control and test hybridizations can be properly assessed band by band. This ensures that some way, usually by digital imaging.

DNA. Because of signal fading, results must be preserved in data of color that occur as the 2 fluorochromes blend in myriad to some extent at virtually all locations along the metaphase from the 2 fluorochromes as both normal and test DNA hybridize is that in CGH, it is not simply a matter of the presence or absence of a signal. Rather, results are in the form of a blending of color from the 2 fluorochromes as both normal and test DNA hybridize to some extent at virtually all locations along the metaphase spreads. The eye is not sensitive enough to detect the many gradations of color that occur as the 2 fluorochromes blend in myriad possible combinations. Therefore, digital imaging is needed to differentiate and quantitate the 2 colors of the control and test DNA. Because of signal fading, results must be preserved in some way, usually by digital imaging.

The first step in analyzing data from CGH studies is to digitally convert the counterstain fluorescence from the metaphase target spreads into a pseudo-Giemsa banding. This ensures that each chromosome in the target is properly labeled so that the control and test hybridizations can be properly assessed band by band.

Imaging software then measures the fluorescent signals from the test and control probes at intervals along a target metaphase spread. Multiple metaphase spreads are measured in this manner and for each interval, a mean and a standard deviation for fluorescence intensity are calculated for the normal sample. The software is generally set to flag any interval for which the fluorescence of the test DNA is more or <2 standard deviations from the mean fluorescence of the normal sample. That interval is then considered to have abnormally high or low expression of DNA.

CGH permits the study of a pathogenic organism's genome relative to the genome of a related, nonpathogenic organism. It also permits the investigation of changes in its gene expression during pathogenesis or as a response to antibiotics. However, there are limitations of CGH.

- It is difficult to obtain good data from intervals near a centromere, from regions of heterochromatin (condensed chromatin that is not often transcribed) or from the p arm of an acrocentric chromosome (numbers 13, 14, 15, 21, and 22) because the chromatin is condensed in all of these areas. Likewise, signals from telomeres are fainter from other regions, making CGH less reliable in these areas. Suspected telomeric abnormalities should be confirmed by a FISH study specifically targeted at those areas.

- CGH cannot detect inversions or balanced translocations since, despite their potential clinical significance, they do not create an actual gain or loss of DNA and will hybridize to the same degree as normal DNA.

- Statistically significant differences in signal ratios generally require a gain or loss of 10 million base pairs or more of DNA. Smaller changes frequently escape detection, and SNPs will surely be missed.

- While CGH will certainly detect monosomies or trisomies (changes in the number of 1 chromosome in an otherwise normal number of chromosomes, such as 47,XXX), it will not detect generalized ploidy changes such as triploidy or tetraploidy, where all of the chromosomes are present in an abnormal number because the ratios of the 2 signals does not change (the term “-somy” refers to 1 chromosome, while the term “-ploidy” refers to the entire set).

- Even with the use of blocking DNA, it is difficult to use CGH to assess copy number in any area where repetitive DNA is common.

- CGH may indicate an abnormality without being able to provide specific information on the nature of the precise structural anomaly.

- Because the DNA from many cells within the 2 groups being tested is pooled (for example, one might pool the DNA from 1,000 normal cells to create the control group and from 1,000 tumor cells to create the test group), low-level mosaicism may be missed. For example, if 10% of the tumor cells have an abnormality at a particular location and the other 90% do not, the overall fluorescence emission from the tumor population may appear within 2 standard deviations of the normal DNA, and the minority population may be missed.

Most applications of CGH have been in solid tumors, though execution of an acrocentric chromosome (numbers 13, 14, 15, 21, and 22) because the chromatin is condensed in all of these areas. Likewise, signals from telomeres are fainter from other regions, making CGH less reliable in these areas. Suspected telomeric abnormalities should be confirmed by a FISH study specifically targeted at those areas.

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Most applications of CGH have been in solid tumors, though it is also used to supplement conventional cytogenetics if insufficient metaphases are recovered or if there is maternal DNA or bacterial contamination. Many applications of CGH are now carried out on microarrays (aCGH) because of its much higher resolution (see discussion of microarrays).

Nomenclature in CGH is also governed by the ISCN. In conventional FISH, a commercially available probe is used to hybridize to the patient's DNA. In contrast, in CGH, patient DNA is used, along with control DNA, to hybridize to reference
metaphase spreads. For this reason, CGH is considered a type of reverse FISH. If a conventional karyotype has been performed, it is given first, followed by a period, followed by the phrase “rev ish” to indicate that the remainder of the nomenclature is from CGH. The designations “enh” and “dim” indicate that CGH has identified an enhanced or a diminished (respectively) number of copies of a chromosome or a part of a chromosome from the test DNA. For example, 47,XX,+mar is a conventional G-banding designation for a female with an extra chromosome and further indicates that the nature of that extra chromosome is unknown (marker chromosome). 47,XX,+mar,rev ish enh (1)q indicates that the extra chromosome is derived from the long arm of chromosome 11. In 46,XX,add(7)(q25).rev ish del(7)(q25)q25), enh(21)(q22), the notation in front of the period indicates that there is a female karyotype, but that there is additional material on chromosome 7, which has an expanded region 2, band 5, on the long arm. After the period, we see that there is a derivative chromosome, based on chromosome 7, that is derived from a translocation between chromosomes 7 and 21, causing extra material from region 2, band 2, of the long arm of chromosome 21 to be translocated to, and thus to enhance region 2, band 5, of the long arm of chromosome 7. The additional material on chromosome 7 that was identified on G-banding is shown by CGH to be derived from the long arm of chromosome 21. 46,XY,rev ish dim(18)(q23) indicates that a male whose conventional karyogram is normal, but who has reduced DNA in region 2, band 3 of the long arm of chromosome 18.

**Multicolor FISH and Spectral Karyotyping**

FISH probes of different colors can collectively “paint” an entire chromosome in a technique called whole chromosome painting. In 1996, 2 groups described slightly different methods of simultaneously painting all 24 different human chromosomes (the 22 autosomes and the 2 sex chromosomes). One technique is called multicolor (or multiplex) fluorescence ISH (M-FISH) [PMID8630489]. The second technique is called spectral karyotyping (SKY) [PMID8662537].

M-FISH and SKY are related techniques in which the target is the patient’s metaphase chromosomes, denatured and immobilized on a substrate. Sets of probes that are specific for each chromosome are prepared by PCR and fluorescently labeled. All of the probes in each set have a unique color. For example, all the probes for chromosome 1 might be yellow, all those for chromosome 2 might be green, all those for chromosome 3 might be purple, etc. Because there are not enough separate fluorochromes to generate 24 unique hues, combinations of 5 fluorochromes are used. For a number of fluorochromes given by N, used in every possible combination, \(2^N - 1\) different hues are possible. With 5 unique fluorochromes mixed in every possible combination, we can have 31 separate hues with combinatorial labeling because \(2^5 - 1 = 31\). This is called combinatorial labeling. For example, all of the probes to chromosome one might be labeled with fluorescent dye number 1, which is yellow, and all of those that hybridize to chromosome 2 may have a combination of dyes 1 (yellow) and 2 (blue), making green. All of those specific to chromosome 3 may be tagged with dyes 2 (blue) and 3 (red), making purple, etc. The result is a set of probes for each chromosome, which collectively hybridize to most areas of the chromosome and give it a unique color 4f5.19.

M-FISH uses sets of excitation filters and corresponding dichroic mirrors (that reflect light of 1 color and transmit light of other colors). One set of filters and mirrors is used for each of the 6 wavelengths of the individual fluorochromes (the 5

![Multicolor fluorescence in situ hybridization (M-FISH) in a child with ALL. Using combinatorial labeling, each chromosome has a unique color.](image1)

![Spectral karyotyping (SKY) is similar to M-FISH in that it also uses combinatorial labeling, but the splitting of light beams and mathematical analysis are used to allow a single imaging to suffice. Although mathematically more complicated than M-FISH, the end result is the similar, and SKY offers the convenience of a single exposure.](image2)
M-FISH allows the operator to favor a particular fluorochrome counterstained with DAPI. As is the case for CGH, the target must either be confidently classified by hand or discarded. Any target spreads that the software cannot confidently classify must either be confidently classified by hand or discarded.

The individual chromosomes in the target metaphases are then scanned to detect the amount and color of each fluorochrome for each interval of each chromosome. This is called a fluorescence intensity profile. For each chromosome, the fluorescence intensity profile consists of a separate graph for each fluorochrome for each homologue. For example, if chromosome 1 is labeled only with 1 fluorochrome, then its fluorescence intensity profile will consist of only 2 graphs, 1 for each homologue of the pair, since the other 4 fluorochromes will be absent. On the other hand, some chromosomes are labeled with 4 fluorochromes, and their fluorescence intensity profiles consist of 8 graphs, 1 of each of the 4 colors for each of the 2 homologues. The summation of the fluorescence intensity of each fluorochrome at each interval along the fluorescence intensity profile results in a composite image for each chromosome. This composite image is then used to generate an artificial pseudocolor for that chromosome, making it possible to distinguish the different chromosomes.

As is always the case, most errors of interpretation in M-FISH and SKY stem from suboptimal material or preparation, such as poor fixation of the metaphase spreads, poor denaturation so that the probe and target cannot anneal properly, insufficient or excessive washing after hybridization, etc. One of the potential problems that may occur in even well-prepared material is called flaring (or sandwich effect). In flaring, a translocation between 2 chromosomes may result in a mixing of fluorochromes at the site of translocation that initially suggests the presence of DNA from a third chromosome. For example, if chromosome 1 is labeled with fluorochrome 1, and chromosome 3 is tagged with fluorochromes 3, 4, and 5, and there is a translocation between chromosomes 1 and 3, at the site of translocation, we may see fluorochromes 1, 3, 4, and 5. If this is the combination used to highlight chromosome 7, then we may believe that a portion of chromosome 7 has translocated to chromosome 1 or chromosome 3. While small translocations and insertions may unavoidable simulate the presence of a third chromosome, many cases of flaring can be resolved by realizing that if the colors are from a third chromosome, they will be intimately mixed, but if the mixture of colors results from flaring (ie, from a translocation), close study often reveals that they are juxtaposed but do not intermingle.

Because M-FISH and SKY can determine multiple parameters at one time, applications include analysis of multiple abnormalities, assistance in understanding complex karyotypes, identification of abnormalities for which there is no specific probe, and identifying marker chromosomes in cancers. M-FISH and SKY have been used effectively in elucidating chromosomal aberrations in many tumors.

A significant limitation of M-FISH and SKY is that they must be performed on patient metaphase chromosomes and are not yet suitable for interphase applications. The necessity for metaphase spreads from the patient requires that the patient's cells be cultured and that a sufficient number of metaphases be recovered.

A second limitation of M-FISH and SKY is that at this writing, they have relatively low resolution. Inversions, translocations, and duplications are likely to escape detection unless they are larger than about 2 million base pairs. While these techniques provide a “bird's eye” view of the genome and are excellent for situations where there are multiple abnormalities, they are not techniques of choice in looking for a specific, previously characterized abnormality.

M-FISH and SKY are also more effective in detecting abnormalities among different chromosomes than within a single chromosome, which makes them particularly useful for assessing tumors in which there are chromosomal aneuploidies or translocations between different chromosomes. However, the low efficiency in identification of intrachromosomal abnormalities has led to the development of higher-resolution techniques.

One approach to increasing the sensitivity of detecting abnormalities within individual chromosomes is multicolor banding (M-banding). M-banding is similar to M-FISH except that it uses a larger number of fluorochromes and shorter probes for each chromosome. These probes hybridize to smaller, sequences of DNA, the end result being the painting of shorter segments of chromosomes. For example, in M-FISH, a maximum of 4 fluorochromes are used for any one chromosome, but in M-banding, 8 or more fluorochromes may be combined to paint a single chromosome. The short labeled probes used in M-banding are called region-specific partial chromosome paints (RPCP). Each RPCP overlaps with the adjacent RPCP, so that the entire chromosome can be painted.

In M-banding, after hybridization with immobilized patient or test cell metaphases, the fluorescent signals of each fluorochrome are obtained, and they are summed as is the case for M-FISH. Again, artificial pseudocolors are assigned to the fluorescent pattern.Unlike M-FISH, in M-banding, individual chromosomes are not all painted in a single color. 20-30 different bands are obtained for each chromosome, and about 500 distinct bands can be obtained for the haploid genome in M-banding [PMID:93418]. The number of different colors that can be obtained from n different fluorochromes is 2^n - 1, so achieving 500 different colors is not as daunting as might be thought. 9 fluorochromes will do the job (2^9 = 512).

M-banding is an ideal follow-up step once M-FISH has implicated a particular chromosome, since it permits the study of that chromosome in much greater detail. Processes such as deletions and inversions that involve a single band can often be identified on M-banding, whereas these are likely to be missed with M-FISH or SKY. Also, because M-banding focuses on an individual chromosome, it can identify specific breakpoints much
more effectively than M-FISH. Many M-FISH studies suggest abnormalities that are either confirmed by ordinary FISH—if the suggested abnormality in known and a probe for it is either available or can be made—or are studied in greater detail by M-banding.

Another higher-resolution technique is ArmFISH, a 42-color application of multicolor FISH that has specific colors for the p arm and the q arm of each chromosome (excepting the y chromosome and the p arms of the acrocentric chromosomes) [PMID1107174].

DNA Sequencing

The most common mechanism for DNA sequencing has been the chain termination method, typically using dideoxy (dd) nucleotides, pioneered by Frederick Sanger [PMID271968]. In this procedure, the DNA to be sequenced is divided into 4 samples. Each of these samples is denatured and incubated in a solution of DNA polymerase, primers, and the 4 bases in nucleoside triphosphate (NTP) form, so that a complementary strand can be generated. But in each of the 4 reactions, a small amount of a modified NTP, a dideoxy NTP, is added. A dideoxy NTP contains the sugar dideoxyribose, which lacks hydroxyl groups at both the number 2 and the number 3 carbon atoms. Ribose, used in RNA, has hydroxyl groups at both of these positions, and deoxyribose, used in DNA, has an hydroxyl group only in the number 3 position. Extension of a nucleic acid requires the bonding from the 5' carbon of one sugar to the 3' carbon of the adjacent sugar via the binding of a phosphate group attached to the 5' carbon to an hydroxyl group attached to the 3' carbon. Hence, either ribose or deoxyribose can sustain elongation of the nucleic acid, but dideoxyribose cannot because it lacks an hydroxyl group at the 3' position of the sugar.

Each of the 4 reactions uses copious amounts of all 4 normal bases and a small amount of 1 base in dideoxy form. Each of these dideoxy NTPs (ddNTPs)—ddATP, ddGTP, ddCTP, or ddTTP—is labeled. The original label was radioactive, but now the label is usually a fluorescent tag. Synthesis of the complementary strand terminates when a dd base is encountered because the dideoxy structure prevents any further addition. The double-stranded DNA so created is then denatured. The complementary strands in each of the 4 reactions will be a series of truncated fragments, each of which was terminated when a ddNTP of the one type that is present in that reaction mixture successfully competed with its normal analogue. For example, in the reaction containing ddATP, the shortest fragment is a complementary strand that was terminated quickly when a ddATP replaced a normal ATP very soon after replication began. A slightly longer fragment results from a complementary strand that was terminated a little later by the incorporation of another ddATP. For each of the 4 reactions, the longest fragments are those in which the dd analogue did not compete successfully with its normal counterpart—and thus terminate synthesis—until the complementary strand was almost completed.

The products of all 4 reactions are then separately subjected to electrophoresis. In a gel system, for each of the 4 reactions, the incorporation of another ddATP. For each of the 4 reactions, the longest fragments are those in which the dd analogue did not compete successfully with its normal counterpart—and thus terminate synthesis—until the complementary strand was almost completed.

The products of all 4 reactions are then separately subjected to electrophoresis. In a gel system, for each of the 4 reactions, the smallest of these fragments, at the bottom of the gel, represents a complementary sequence that was terminated soon after replication began, close to the point of origin of the replication. The largest fragment, at the top of the gel, represents a complementary sequence in which the dideoxy nucleotide was not incorporated until near the end of the sequence. By comparing all 4 wells, one can read the sequence by walking up the ladder from the bottom of the gel. In fact, in the reaction that

![Image](469.508_ch45.indd 497)