DNA Ploidy and Cell Cycle Analysis in Breast Cancer

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Abstract

During the past 10 years there has been considerable interest in the application of new technologies to identify human malignancy and predict disease outcome. Markers of cell proliferation and the technologies of flow cytometry and image analysis for the determination of DNA total content in human tumor cells have been studied in breast cancer for 20 years. In this review, the uses and limitations of these technologies for the determination of ploidy status are discussed. This review also considers the prognostic significance and potential clinical utility of ploidy measurements, S phase calculation, and individual cell cycle regulatory biomarker expression levels.

The use of markers of cell proliferation and the technologies of flow, laser scanning, and image cytometric analysis for the determination of DNA total content in human tumor cells have been among the most frequently used ancillary methods for the detection of neoplasms and the prediction of cancer outcome. This review focuses on the clinical usefulness of cell cycle analysis and DNA ploidy measurements in the diagnosis of human tumors, their applications to cytologic diagnosis, and their potential for predicting disease outcome.

The Normal Cell Cycle and DNA Ploidy

Human neoplasms actively synthesizing DNA replicate through a process similar to that of normal cells known as the cell cycle[1,2] ▮**Figure 1**▮. Cells in the resting diploid state (G_0 phase) contain 7.14 pg of DNA and enter the cell cycle as the gap 1 (G_1) cells. During the synthesis phase (S phase), cells increase their DNA content continuously from 7.14 to 14.28 pg per cell until they reach the tetraploid state with twice the diploid DNA content. The second gap (G_2 phase) refers to this tetraploid, or premitotic, fraction of cells that undergo mitosis in the M phase to generate 2 diploid G_0 cells, which may reenter the cell cycle or persist in the resting state. A DNA index of 1.0 corresponds to a 2N or 46 chromosome number characteristic of G_0 and G_1 cells. The G_2 and M cells feature a 2.0 DNA index that corresponds to a 4N chromosome number of 92.

The distribution of a population of cells within the cell cycle generates a pattern known as a histogram and represents DNA ploidy.[2] A *DNA histogram* is defined as DNA diploid when the distribution of total DNA content in the predominant or G_0/G_1 peak of the tumor cell population is

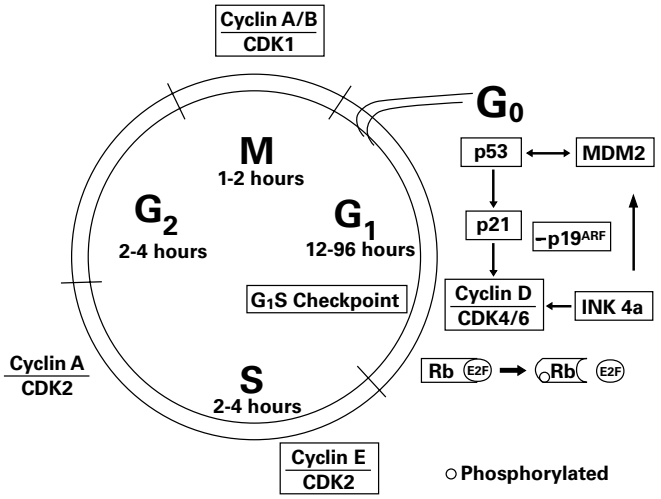

Figure 1 The normal cell cycle. CDK, cyclin-dependent kinase; Rb, retinoblastoma.

equal to that of the total DNA content of the G_0/G_1 peak of a known-to-be-diploid reference cell population and the S and G_2M phases of the tumor cell population are relatively low. In normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population forms the G_0/G_1 peak and 15% of the cells are in the S and G_2M phases.

DNA aneuploidy, also known as nondiploidy, is defined as a DNA content of the G_0/G_1 peak of a cell population that varies substantially from the G_0/G_1 peak of the known diploid reference cell population **Table 1**. The DNA index of an aneuploid cell population rarely might be less than 1.0 (hypodiploid) and far more commonly is greater than 1.0 (hyperdiploid). Nondiploid cell populations featuring a DNA index of the G_0/G_1 main peak at or near 2.0 must be differentiated from diploid tumors with significantly increased G_2M phases. Some investigators refer to these nondiploid tumors with DNA indices near 2.0 as tetraploid tumors. **Image 1** illustrates the 4 most frequent types of DNA histograms.

Table 1
DNA Ploidy Definitions

G_0/G_1 Peak DNA Index	DNA Ploidy Definition
= 1.00 (1.00 ± 0.15)	Diploid
<0.85 or >1.15 (excluding tetraploid)	Aneuploid
<0.85	Hypodiploid
1.15-1.9	Hyperdiploid, hypotetraploid aneuploid
1.9-2.1	G_2M population, tetraploid; G_0/G_1 population, tetraploid aneuploid
>2.1	Hypertetraploid aneuploid
Multiple peaks at 1.0, 2.0, 3.0, 4.0, etc	Multiploid or polyploid

Aneusomy and Aneuploidy

Aneusomy is defined as a single cell with an abnormal number of chromosomes. Loss of a single chromosome is termed *monosomy*; gain of a single chromosome is termed *trisomy.* In contrast, *aneuploidy* is defined as a condition of a population of cells when the average DNA content per cell in the cells comprising the G_0/G_1 phase is substantially different from the normal diploid content (7.14 pg). Aneusomy can be detected by classic cytogenetics or, more recently, in interphase nuclei of smears and tissue sections by fluorescence in situ hybridization.[3] Although aneusomy and aneuploidy often coexist, it has been reported that fluorescence in situ hybridization is more sensitive than flow cytometric– or image cytometric–derived DNA ploidy measurements for the detection of abnormal DNA content in human tumors.[4,5]

Techniques for Measuring DNA Content

Techniques used to demonstrate DNA content include flow cytometry, image analysis, and laser scanning cytometry.

Flow Cytometry

The flow cytometer uses an optical-to-electronic coupling system and records how a cell interacts with a focused laser beam in terms of scattering of incident light and the cell's ability to emit fluorescence. The photons of light scattered and emitted by a cell following its encounter with the laser beam are separated into various wavelengths by a series of filters and mirrors. Detectors then generate electrical impulses that are converted into digital signals that then are accumulated in a frequency distribution, or histogram. DNA content is studied most commonly by staining cells with propidium iodide, a DNA-binding dye that can be excited with a standard argon laser. Combined DNA and RNA measurements can be made with the metachromatic fluorochrome acridine orange.[6] A technique also has been described to simultaneously study DNA and RNA by measuring DNA staining with Hoechst 33342 and RNA staining by pyronin Y.[7]

DNA analysis by flow cytometry provides fast results, permits multiparameter analysis correlating DNA content with antigen expression, and provides the sensitivity for detecting near-diploid aneuploid peaks. However, because flow cytometry requires disaggregation of the tissue sample, there is no simultaneous morphologic comparison.[8] The presence of aneuploid populations might be masked by the inclusion of numerous benign, nontumor cells. Flow cytometry also has the advantage of permitting retrospective studies of paraffin-embedded tissue samples. Although the best histograms are obtained from fresh or frozen tissue samples,

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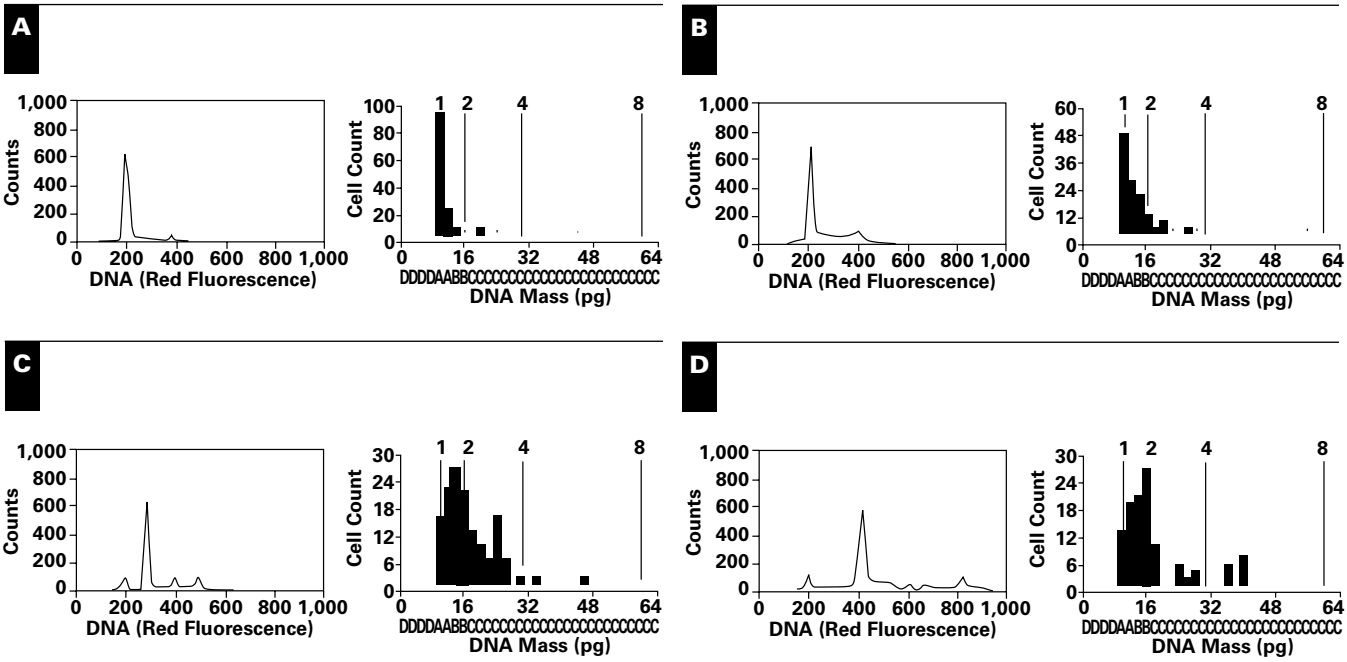

Image 1 Types of DNA ploidy histogram. These paired flow cytometric (left) and image analysis (right) histograms show examples of diploid pattern with low S phase (**A**), diploid pattern with high S phase (**B**), hyperdiploid hypotetraploid aneuploid (**C**), and tetraploid aneuploid (**D**).

Hedley and coworkers[9] developed a technique that permitted flow cytometry to be performed on formalin-fixed, paraffin-embedded tissue sections. Fixed sections are cut from tissue blocks, dewaxed in xylene, and rehydrated through alcohol solutions. Single-cell or nuclear suspensions are obtained after incubation with a 0.5% pepsin-saline solution. Cells are counted, washed, and stained with 6-diamidino-2-phenylindole.[10] Modifications of the Hedley technique have permitted good correlations between paraffin-embedded specimens and histograms from fresh specimens,[11] although histograms obtained from paraffin-embedded tissues have wider coefficients of variation and are, therefore, less precise.[12] Techniques also have been described for performing chromosome analysis by flow cytometry.[13]

Measuring the S Phase by Flow Cytometry

S-phase measurement usually is obtained through a computer program that estimates the proliferation fraction by using a variety of models to fit the raw data. The models used to analyze asynchronous populations of specimens often model the G_0/G_1 and G_2/M phases by gaussian distributions, while the S-phase region is modeled by a polynomial equation, several small gaussian distributions, a trapezoid, or a broadened rectangle.[14-17] A rectangular model is used most often for calculating asynchronous populations with a relatively low S phase.[18] The mathematical models for measuring the S phase might be problematic. The intercalating dyes provide static measurements of the percentages of cells having an S-phase amount of DNA. This does not indicate which cells are cycling or which cells are noncycling. Problems exist in estimating S-phase fractions in tumors with overlapping populations.[14] Examples of S phase calculation problems are cases in which a diploid G_2/M overlaps with an aneuploid S phase, an S phase overlaps from a near-diploid population, or multiple aneuploid populations have close DNA content.

The most common problem occurs when a diploid G_2/M population overlaps with an aneuploid S phase, as often is seen in breast cancer and in other solid tumors with a DNA index in the range of 1.3 to 1.8.[14] Several modeling systems, such as those described by Dressler and coworkers[19] and McDivitt and colleagues,[20] attempt to estimate S phase in overlapping populations. There also can be difficulty interpreting the G_2/M area of a diploid histogram caused by 2 simultaneous cells, termed *doublets,* being interpreted as 1 cell with tetraploid DNA. Software has become available to permit discrimination of doublets from true G_2/M cells.

Image Analysis

Computer-based image analysis applies digital technology to quantitative measurements in cytopathologic and histopathologic examination. The most widespread uses of this technology are in the quantitation of DNA content and in the quantitation of immunofluorescence and immunohistochemical staining. The availability of image-processing instruments gives the pathologist direct visualization of the

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cell population being studied for DNA measurement, providing simultaneous correlation between DNA studies and morphologic features.

The image analysis system consists of a modified optical microscope, a video camera with digitizer, and a high-speed computer. The image is scanned at intervals known as *pixels*. At each pixel, a photometric device measures the amount of light absorbed or transmitted. These measurements are recorded in an electronic or mechanical device. A photoelectric cell is used to transform the optical values of light absorbed or transmitted into electronic signals of various amplitude or intensity. The computer assigns a number between 1 and 255 to each pixel in the image, depending on its optical density. Once the image is digitized, a computer reconstruction of the image can be made. From this digital image, morphometric and optical density measurements are performed.

DNA content is studied easily by the image analysis system using Feulgen-stained preparations. DNA can be measured in cytologic preparations or in tissue sections.[21] The dye binds stoichiometrically to the double-stranded DNA molecule. The computer-based image analysis system uses the summation of the optical density of each Feulgen-stained nucleus to calculate the amount of DNA present based on the Beer-Lambert law. The operator reviews the morphologic characteristics of a given cell or nucleus on the monitor and can reject nontumor cells or artifactually distorted cells. As the DNA is measured, a histogram is plotted on a second monitor. The instrument calculates the DNA index from peaks on the histogram selected by the operator and also calculates the coefficient of variation. This method requires strict control of the staining procedure and rigorous preparation of normal control standards.[22]

Because image-based DNA ploidy measurements permit the visual recognition of abnormal cells, this technique provides increased sensitivity when low numbers of tumor cells are present admixed with large numbers of normal cells. Image analysis, therefore, may be more sensitive for detecting aneuploid tumor populations than flow cytometry when small numbers of tumor cells are present. In addition, image analysis can permit DNA studies on small specimens, such as biopsy and aspirate specimens, that might not contain enough cells for flow cytometric analysis. However, the method is slow and requires a skilled morphologist. In addition, this method may be insensitive for an aneuploid peak that is near diploid. The majority of comparative studies have shown excellent correlation between analysis of DNA content by flow cytometry and the computer-based image method.[23] Flow cytometric studies may fail to detect an aneuploid population in 10% of cases in which such a population can be detected by the image analysis method.[24] However, 3% of aneuploid cases detected by flow cytometry, particularly the near-diploid aneuploid peaks, will not be detected by image analysis. In general, image analysis is the more sensitive method; however, the combined use of image and flow cytometry is the most sensitive.

Quantitative immunohistochemical analysis, initially designed for the analysis of tumor estrogen and progesterone receptor content, is performed on the computer-based image analysis system permitting an automated assessment of the degree of reactivity of an immunologic marker based on the summation of optical density measurements with a particular chromogen.[25,26] A threshold level for the instrument is set to eliminate nonspecific background staining, and, thus, true immunoreactivity can be analyzed. The image analysis method can be used to quantitate immunohistochemical measurements of cell cycle regulatory proteins (see "Cell Cycle Regulators").

Laser Scanning Cytometer

The laser scanning cytometer was developed recently to take advantage of the sensitivity of fluorescence-based assays and the specificity of on-slide measurements. The system uses fluorochromes such as propidium iodide applied to cell smears and tissue sections that are analyzed by a high-speed computer. The operator selects the cells for study in a manner similar to that for an image analysis system, or the entire cell population on the slide can be measured automatically. Given its versatility and the ability to study small samples, the laser scanning cytometer shows considerable potential to become a major instrument for the future cytometry laboratory.[23,27,28]

Major reviews of DNA content analysis highlighted the relative advantages and disadvantages of all of these technologies.[8,29-31] These comparative studies have highlighted excellent overall performance of all techniques, with approximately 95% of samples showing similar diploid or aneuploid histograms. A comparison of flow cytometry and image analysis for the determination of DNA ploidy in human neoplasms and the relative advantages and disadvantages of each method[24,25,32] is provided in **▮Table 2▮**. A variety of technical issues can affect DNA ploidy measurements **▮Table 3▮**. In particular, when solid tumors are disaggregated into single-cell suspensions for flow or image cytometry,[8,29-31] both false-negative and false-positive (aneuploidy) results may be obtained **▮Table 4▮**. Comparisons of flow and image cytometry in the determination of DNA ploidy status in breast cancer also are shown in **▮Image 2▮**, **▮Image 3▮**, and **▮Image 4▮**.

DNA Ploidy in Breast Cancer

It is estimated that, based on current incidence rates, an American woman has a 1 in 9 chance of developing breast

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Table 2
Comparison of Flow Cytometry and Image Analysis for the Determination of DNA Ploidy

	Advantages	Disadvantages
Flow cytometry	High-speed, fast results	No simultaneous morphologic comparison of cells during DNA ploidy analysis
	High number of cells counted	Low numbers of aneuploid peaks of tumor cells possibly masked by large numbers of benign cells, stromal cells, and inflammatory cells
	Can perform multiparameter analysis to subdivide cells for DNA analysis into epithelial and nonepithelial, lymphoid, etc	Requires disaggregation
	High cell count enhances sensitivity for near-diploid aneuploid peaks	
Image analysis	Simultaneous morphologic evaluation of cells during DNA ploidy determination	Relatively slow, tedious work and requires skilled morphologist
	Small samples (eg, biopsy specimens, small aspirates, curetting specimens, and needle core specimens) may be used	May be insensitive for near-diploid aneuploid cell populations
	Does not require disaggregation	Coefficient of variation percentages of G_0/G_1 peaks may be high for small samples
	Pathologist-directed histogram increases sensitivity	

Table 3
Technical Issues That Affect DNA Ploidy Analysis

Specimen volume
Specimen storage
Fixatives
Disaggregation
Stains
Tissue section techniques
Diploid controls and standards
Operator expertise
Quality assurance programs
Tumor heterogeneity

Table 4
Benign Neoplasms and Nonneoplastic Conditions Associated With Aneuploid DNA Content

Leiomyoma
Colonic adenoma
Melanocytic nevi
Thyroid adenoma
Pituitary adenoma
Parathyroid adenoma
Adrenal cortical adenoma
Schwannoma
Fibromatosis
Foreign body granuloma
Normal seminal vesicle
Benign tumors with high lymphocyte content

cancer at some time during her life.[1] As would be expected for such a major disease, there is great interest in the discovery, development, and clinical testing of biomarkers and prognostic factors that can be used to guide the selection of therapy for the disease.[33-38]

A large number of prognosis-related studies using flow cytometry and static cell image analysis have been performed on breast cancer specimens to measure total DNA content and determine the S-phase fraction.[39-73] The first major retrospective flow cytometric study of DNA content as a predictor of prognosis in breast cancer by Auer et al[73] divided histograms into 4 groups ranging from pure diploid to pure aneuploid, the former with an excellent prognosis and the latter with a high relapse and mortality rate. The results of a variety of studies on the prognostic significance of ploidy and S-phase status have been notably variable, with some investigators finding significant prediction of disease-free and overall survival on both univariate and multivariate analyses and others finding no effect on disease outcome **Table 5**.[43-72] Of the 34 selected studies highlighted in Table 5, 15 (44%) found that aneuploid DNA content in primary breast cancer was a significant adverse prognostic factor on multivariate analysis, 6 studies (18%) found ploidy status predictive on univariate analysis only, and 11 studies (32%) found no prognostic significance.

Despite numerous large studies indicating DNA ploidy status as an independent and powerful prognostic indicator in breast cancer,[39,40,42,44,47,50,55,60,61,63,65-68,72] many investigators have reported that the association of both ploidy and S-phase fraction status with prognosis was variable and not conclusive.[41,48,51-54,57,59,62,64,69] Thus, the clinical use of DNA content measurement in breast cancer patient management has been controversial. Other reviews have questioned the independent prognostic value of DNA ploidy in breast carcinoma.[59,74,75] In large retrospective studies using disaggregation of paraffin blocks and flow cytometry, aneuploidy was identified in approximately 50% of breast adenocarcinomas.[45,48]

Separate determination of DNA analysis for prognosis prediction for node-negative and node-positive status in patients with breast cancer has been performed.[41,49,63] In 1 report of patients with node-negative status, multivariate analysis showed that neither DNA index nor elevated S-phase percentage were of predictive significance alone, but they achieved independent status to predict survival when

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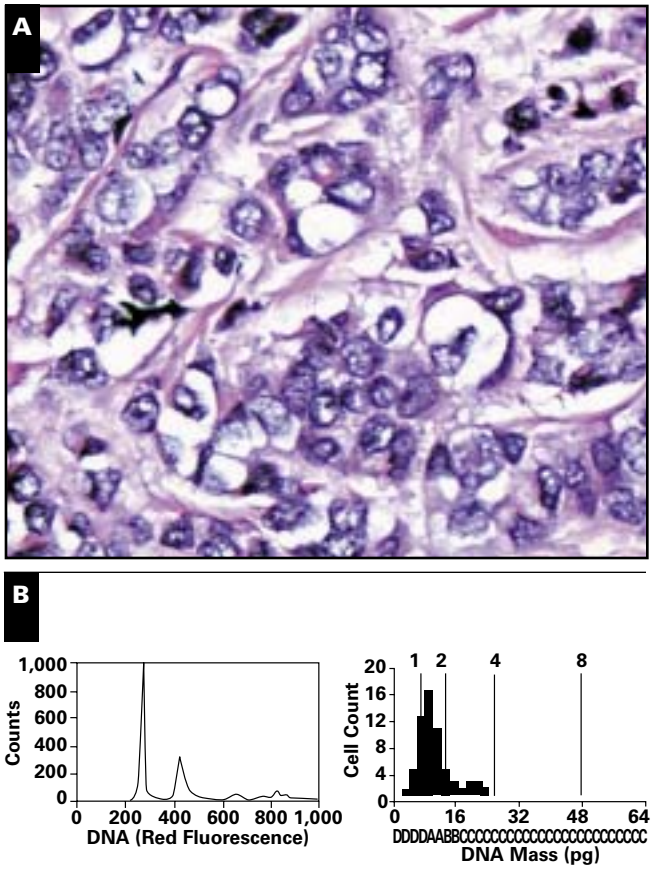

Image 2 This specimen features a poorly differentiated infiltrating ductal breast adenocarcinoma (**A**, H&E, ×100) in which the image analysis histogram (**B**, right) and flow cytometric histogram (**B**, left) are essentially identical. Both instruments indicate a hyperdiploid, hypotetraploid aneuploid tumor. In addition, the flow cytometric histogram shows diploid stromal and inflammatory cells with separate G_0/G_1 and G_2M peaks at fluorescence channels 200 and 400, respectively. The aneuploid tumor cell G_0/G_1 population peak is at the 300 fluorescence channel (DNA index, 1.44), and its corresponding G_2M is at 600. The image analysis histogram shows only the tumor cell population, with DNA index of the aneuploid peak similarly at 1.41.

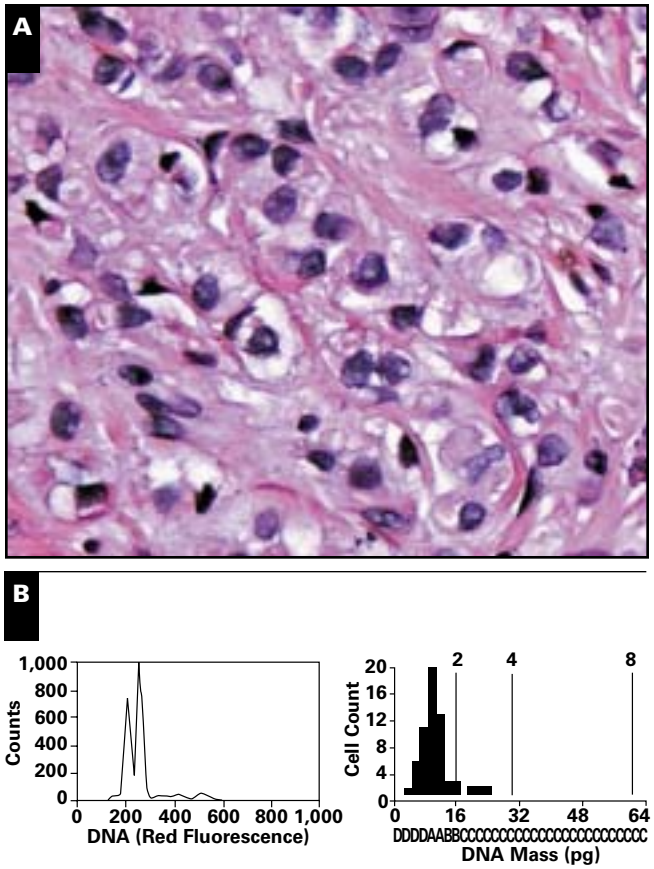

Image 3 In this infiltrating ductal breast adenocarcinoma (**A**, H&E, ×100), the image analysis histogram (**B**, right) is diploid with a DNA index of 1.08, whereas the flow cytometric histogram (**B**, left) features a split G_0/G_1 peak; a near-diploid aneuploid population is identified with a DNA index of 1.21. This tumor illustrates the increased sensitivity of flow cytometry for near-diploid aneuploidy, as the instrument can separate the diploid stromal, benign epithelial, and inflammatory cell populations from the near-diploid aneuploid tumor cells.

used as a combination variable.[41] In another study of node-positive breast cancer patients, DNA ploidy was a significant univariate factor but failed to achieve prognostic significance in a multivariate analysis.[49]

A potential clinical role for DNA ploidy analysis in breast cancer has been to aid in the selection of patients with lymph node–negative status for adjuvant chemotherapy. In a large study including 1,665 patients from the National Surgical Adjuvant Breast and Bowel Project, statistical analysis did not confirm the ability of the DNA index and S-phase fraction percentage to independently predict overall survival and discouraged the use of DNA content measurement for the selection of patient-specific therapies.[48] Although the study was promising, the National Surgical Adjuvant Breast and Bowel Project concluded that DNA analysis could not detect patients with prognosis good enough to preclude their receiving adjuvant chemotherapy. A study from India, however, concluded that ploidy status was an independent prognostic factor predicting recurrence and could be used to identify a subset of node-negative breast cancer patients who could be selected to receive or not receive adjuvant treatment.[50] Interestingly, in patients with recurrent disease studied by flow cytometry who had received hormonal or cytotoxic therapy or both, DNA analysis was unsuccessful for predicting the future clinical course, which suggested that treatment could conceivably

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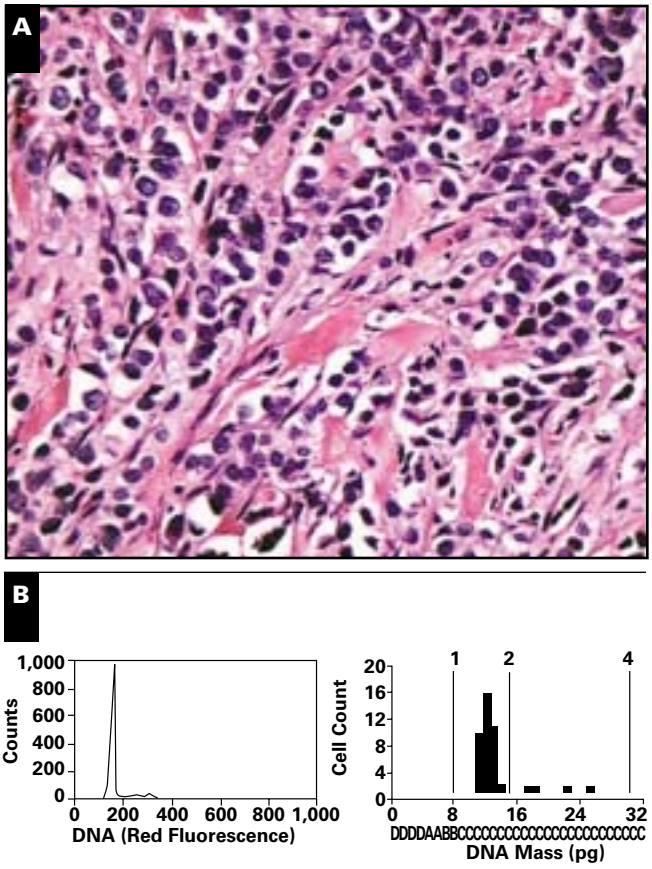

Image 4 This breast cancer (**A**, H&E, ×100) reveals an aneuploid histogram when evaluated by image analysis (**B**, right) of tumor cells only with a DNA index of 1.62. The cell suspension obtained by fine-needle aspiration of the resected tumor was not examined by routine microscopy before analysis. On flow cytometric analysis (**B**, left), a diploid pattern was obtained with DNA index of 1.03 representing the predominant benign epithelial cell, inflammatory cell, and stromal cell components. A second aspirate from this specimen was examined microscopically and contained predominantly stromal and inflammatory cells with apparently insufficient numbers of tumor cells for resolution of an aneuploid peak. The relative insensitivity of flow cytometry for identification of aneuploid cell populations when the tumor cell concentration in the suspension is low can be improved by microscopic study of the suspension before evaluation and by 2-color labeling using an epithelial marker antibody to selectively gate for epithelial cells and correspondingly enhance the sensitivity for aneuploidy.

cause divergent results and diminish the ability of DNA ploidy status to predict subsequent outcome in treated patients.[50]

Although image analysis and flow cytometry have been used to determine DNA content in breast carcinoma, some investigators believe that image analysis might have advantages, including lower cost, the ability to analyze small specimens, and the capability for detecting rare aneuploid cell populations and characterize them according to a specific morphologic appearance without the potential impact of enzymatic or mechanical tissue digestion.[76,77] In lobular breast carcinoma, ploidy and cell cycle analysis have not successfully predicted disease outcome.[78]

In summary, DNA ploidy and S-phase fraction status have been correlated generally with high tumor grade, negative hormone receptor status, and amplification of certain molecular markers indicating unfavorable prognosis, such as the Her-2/*neu* and c-*myc* genes.[79] Although it currently is of uncertain use as a prognosis marker, at many hospitals and cancer centers, ploidy status often is included as a component of a panel of prognostic markers for patients with breast cancer. In general the S-phase calculation by flow cytometry generally has outperformed the DNA ploidy status as a prognostic factor in breast cancer. However, neither the American Society of Clinical Oncology[80] nor the College of American Pathologists[81] includes ploidy and S-phase measurements in the lists of recommended prognostic factors approved for breast cancer patient management.

Cell Proliferation Markers in Breast Cancer

A wide variety of procedures and biomarker measurements have been used to measure cell proliferation in breast cancer **Table 6**. Most studies in breast cancer have used immunohistochemical techniques with antibodies to proliferating cell nuclear antigen and the MIB-1 clone of the Ki-67 cell proliferation antigen.

Ki-67 Immunostaining

The antibody Ki-67 was raised against a Hodgkin disease cell line and detects an antigen in the nucleus associated with cell proliferation.[82] The original antigen epitope cannot be detected in formalin-fixed, paraffin-embedded tissue samples and, therefore, immunostaining requires the use of fresh tissue samples with cryostat sections or touch imprints.[83,84] The antigen detected by Ki-67 begins to be expressed in the mid-G_1 phase and is present through the M phase. Noncycling cells and early G_1 cells do not demonstrate immunostaining. Cell proliferation labeling measured by Ki-67 immunostaining correlates with the S-phase levels calculated by flow cytometry but generally is higher, reflecting the fact that the Ki-67 antigen also is expressed in late G_1 as well as in early G_2/M phases of the cell cycle.[85] A number of studies testing Ki-67 labeling have shown mixed results, with some investigators finding independent prediction of prognosis and others finding no significant correlation.[86-91] Several studies have shown good correlation

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Table 5
DNA Ploidy and S-Phase Measurements in Breast Cancer

Year/Reference	Specimen Type	Study Type/ Analysis Method	Disease Outcome Statistical Correlation		Comment
			Univariate Significance	Multivariate Independent Significance	
1988					
Kallioniemi et al[39]	Paraffin	R/FC*	Yes	Yes	Ploidy combined with S phase
Fallenius et al[40]	FNA	R/FC	Yes	Yes	Auer histogram classification; no correlation with node status
1989					
Muss et al[41]	Frozen	R/FC*	No	No	DNA index not associated with time to progression or survival
Clark et al[42]	Frozen	R/FC*	Yes	Yes	S phase did not correlate
Christov et al[43]	Fresh	R/FC*	No	No	Correlation with grade
1990					
Lewis[44]	Paraffin	R/FC*	Yes	Yes	Only ploidy predicts survival
Toikkanen et al[45]	Paraffin	R/FC*	Yes	No	55% aneuploid rate
Kute et al[46]	Paraffin	R/FC*	Yes	No	Ploidy discriminates patients with estrogen receptor–negative cancer
Joensuu et al[47]	Paraffin	R/FC*	Yes	Yes	Independent when combined with S phase
1991					
Fisher et al[48]	Paraffin	R/FC*	No	No	1,665 cases; S phase correlated
Witzig et al[49]	Paraffin	R/FC*	Yes	No	Separate analysis of node-negative and node-positive patients
Sharma et al[50]	Paraffin	R/FC*	Yes	Yes	To identify patients for adjuvant treatment
1992					
Arnerlov et al[51]	Paraffin	R/FC*	No	No	Ploidy not a significant predictor
Bosari et al[52]	Paraffin	R/FC*	No	No	All multiploid and hypertetraploid patients had recurrence
1994					
Leivonen et al[53]	Paraffin	R/FC*	No	No	Study of patients with metastatic or recurrent diseases
Witzig et al[54]	Paraffin	R/FC*	No	No	S phase remained significant
1995					
Camplejohn et al[55]	Paraffin	R/FC*	Yes	Yes	Ploidy and S phase status independent predictors of outcome
Dieterich et al[56]	Fresh	P/IA*	Yes	No	DNA ploidy status had prognostic significance but was not independent of tumor grade
Pfisterer et al[57]	Fresh	R/FC*	No	No	Neither ploidy nor S-phase fraction a significant prognostic factor
1996					
Romero et al[58]	Paraffin	R/FC*	Yes	Yes	S-phase fraction an independent predictor in DNA diploid tumors
1997					
Bergers et al[59]	Fresh	R/FC*	No	No	1,301 patients; no prognostic significance of ploidy
Shiao et al[60]	Paraffin	R/FC*	Yes	Yes	Ploidy independent predictor of survival in white but not black women
1999					
Midulla et al[61]	Paraffin	R/FC*	Yes	Yes	Ploidy an independent predictor of survival
Wong et al[62]	Paraffin	R/FC*	No	No	Ploidy and S phase did not predict survival
Pinto et al[63]	Fresh	R/FC*	Yes	Yes	Ploidy independently predicted disease-free survival; S phase predicted overall survival
2000					
Mandard et al[64]	Paraffin	R/FC* and IA*	No	No	Mitotic index outperformed S phase calculation; ploidy not significant
2001					
Bracko et al[65]	Fresh	R/FC*	Yes	Yes	Ploidy independently predicted disease-free and cancer-specific survival
Pinto et al[66]	Fresh	P/FC*	Yes	Yes	S phase outperformed Ki-67 labeling index
Bagwell et al[67]	Paraffin	R/FC*	Yes	Yes	After 10 adjustments to reclassify histograms, ploidy and S phase were independent predictors in node-negative cancer patients
Tsutsui et al[68]	Fresh	FC	Yes	Yes	Ploidy an independent predictor in node-negative cancer patients
Chassevent et al[69]	Paraffin	R/FC*	No	No	Ploidy not significant; S phase an independent predictor in node-negative cancer patients
2002					
Chavez-Uribe et al[70]	Paraffin	FC	Yes	No	Ploidy status a significant predictor
2003					
Chang et al[71]	Paraffin	FC	Yes	No	In 346 patients, ploidy status significant on univariate analysis, and S-phase fraction significant on both univariate and multivariate analyses
Tsutsui et al[72]	Paraffin	FC	Yes	Yes	DNA ploidy independent predictor in 998 patients

FC, flow cytometry; FNA, fine-needle aspirate; IA, image analysis; P, prospective; R, retrospective.
* Disaggregated specimen.

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Table 6
Markers of the Cell Cycle

Cell Cycle Marker	Cell Cycle Phase(s) Detected
Mitosis counting	M
Tritiated thymidine uptake	S
Bromodeoxyuridine labeling	S
Flow cytometry	S (calculated)
Image analysis	S (estimated)
Ki-67 (fresh, frozen tissue)	G_1, S, G_2/M (part)
Ki-67 MIB-1 (paraffin)	G_1, S, G_2, M (part)
PCNA (paraffin)	G_1, S, G_2
p105 (paraffin)	G_1, S. G_2/M
AgNOR (paraffin)	S, G_2/M (part)
Cyclins	
A	S
B	S (part), G_2, M (part)
D	G_1 (part)
E	G_1 (part), S (part)

AgNOR, silver-staining nucleolar organizing region; PCNA, proliferating cell nuclear antigen.

between immunohistochemical staining for Ki-67 and thymidine and bromodeoxyuridine labeling indices,[92-95] as well as with flow cytometric determinations of the S-phase fraction.[96-99] Ki-67 labeling has been shown to correlate with tumor grade in cancers of the breast.[86,100-102]

Ki-67 MIB-1 Immunostaining

Problems with reproducibility in the staining of formalin-fixed, paraffin-embedded tissue samples with the original Ki-67 antibody led to the discovery of the MIB-1 clone capable of measuring cell proliferation in processed tissue samples.[103,104] The MIB-1 clone has proved to be a reliable marker of proliferating cells in a large variety of human neoplasms, including cancers of the lung, breast, gastrointestinal tract, kidney, and prostate and soft tissue sarcomas.[85] In breast cancer, MIB-1 immunostaining **Image 5** has become the most frequently used technique to measure cell proliferation. However, the results of studies to correlate MIB-1 expression with disease outcome have varied in the literature.[90,91,105] Some studies have failed to link MIB-1 expression rates with prognosis,[90] while others have found substantial correlation.[91,105]

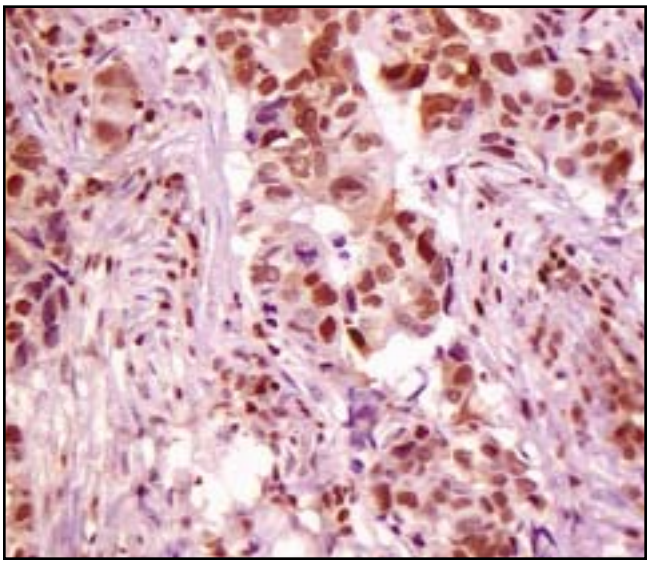

Image 5 Cell proliferation measured by Ki-67 (MIB-1) immunostaining. Note the high (>50%) nuclear staining of this rapidly proliferating infiltrating ductal adenocarcinoma (peroxidase/antiperoxidase, ×100).

In summary, cell proliferation measurements seem to feature a greater incidence of statistically significant correlation with breast cancer outcome on both univariate and multivariate analyses than DNA ploidy alone. Although neither the American Society of Clinical Oncology[80] nor the College of American Pathologists[81] guidelines include Ki-67 labeling, many laboratories provide this testing at the request of oncologists for use in planning the treatment of breast cancer.

Cell Cycle Regulators

A variety of cell cycle regulators active at the G_1/S and G_2/M phases have been implicated as prognostic factors in breast cancer.

Cyclin D

Cyclin D1 (*PRAD*1; *bcl*-1) is a gene localized to chromosome 11q13. In addition to studies revealing amplification of cyclin D1 in experimental breast cancer cell lines, amplification also has been identified in 20% of clinical breast cancers.[106-109] Cyclin D1 amplification is associated with enhanced cell proliferation via complex formation of the cyclin protein subunits with the cyclin-dependent kinase 4 that stimulates cell cycle progression during the G_1 phase.[107] Cyclin D expression has been linked to the expression of the estrogen receptor[79] and the transition from in situ to invasive ductal breast cancer.[109] Large-scale studies validating the prognostic significance of cyclin D overexpression in breast cancer have not been published to date.

Cyclin E

In a recent study, high levels of the low-molecular-weight isoforms of cyclin E, measured by Western blot analysis, correlated strongly with decreased disease-specific survival[110]; moreover, levels of total cyclin E also were highly correlative with poor outcome, consistent with the findings of previous studies performed by immunohistochemical analysis.[111] Confirmatory data for cyclin E as a prognostic marker in primary breast cancer is necessary before formal recommendations can be made.

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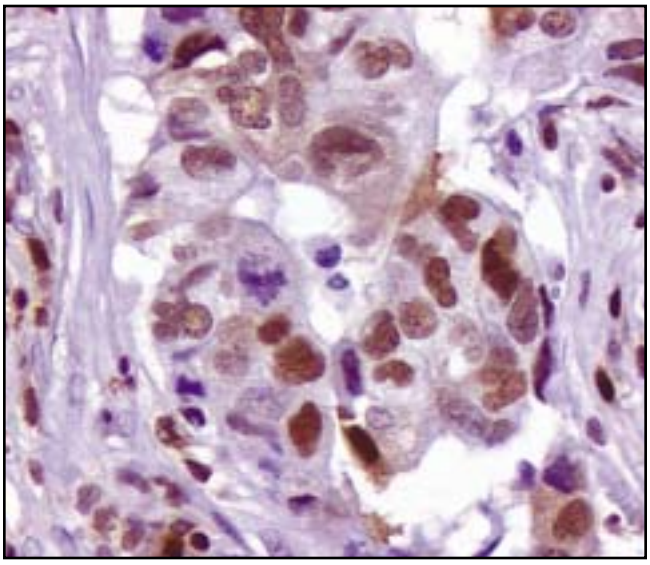

Image 6 Expression of p27 in breast cancer. In this high-grade infiltrating ductal adenocarcinoma, most of the nuclei show abundant p27 immunostaining, a nevertheless favorable prognostic finding (peroxidase-antiperoxidase, ×200).

p21

The p21 protein (p21/WAF1/Cip1) is an inhibitor of cyclin-dependent kinases and serves as a critical downstream effector in the p53-specific pathway of cell growth control.[112] Some studies have linked altered expression of p21 with adverse outcome in breast cancer,[113,114] whereas others have not.[115]

p27

p27 (kip1) is a cell cycle regulator that acts by binding and inactivating cyclin-dependent kinases.[115] Low p27 expression **Image 6** has been correlated with poor prognosis in many (but not all) studies of patients, especially patients with small primary tumors.[116-119] However, most experts agree that additional studies are warranted to assess the prognostic significance of p27 loss.

skp2

The S-phase kinase-associated protein skp2 is required for the ubiquitin-mediated degradation of various proteins, including the cyclin-dependent kinase inhibitor p27.[120] Expression of skp2 is inversely proportional to the expression of p27. A recent report suggested an important role for skp2 overexpression in the pathogenesis of estrogen receptor–negative, Her-2–negative breast carcinoma,[120] consistent with the proposal that skp2 can serve as a proto-oncogene. Limited data are available concerning skp2 expression as a prognostic marker in breast cancer.

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