DNA Ploidy and Cell Cycle Analysis in Cancer Diagnosis and Prognosis

This review focuses on the clinical utility and potential value of cell cycle analysis and DNA ploidy interpretation in the diagnosis of human tumors, the application of these techniques to cytologic diagnosis, and their capability for predicting disease outcome in human neoplasia.

Introduction

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 techniques of flow cytometry and image analysis for the determination of DNA total content in human tumor cells have been at the forefront of these new developments. This review will focus on the clinical utility and potential value of cell cycle analysis and DNA ploidy interpretation in the diagnosis of human tumors, their application to cytologic diagnosis, and their capability for predicting disease outcome in human neoplasia.

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. Cells in the resting diploid state (G0) phase contain 7.14 picograms of DNA and enter the cell cycle as gap 1 (G1) cells. During the synthesis phase (S phase), cells increase their DNA content continuously from 7.14 to 14.28 pg/cell until they reach the tetraploid state with twice the diploid DNA content. The second gap (G2 phase) refers to the tetraploid, premitotic fraction of cells that undergo mitosis in the M phase to generate two diploid G0 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 G0 and G1 cells. The G2 and M cells feature a 2.0 DNA index that corresponds to a 4N chromosome count of 92.

The distribution of a population of cells within the cell cycle generates a pattern known as a histogram and represents DNA ploidy. A DNA histogram is defined as diploid when the predominant or G0/G1 peak is equal to the G0/G1 peak of a known diploid reference cell population and the S and G2M phase contents are relatively low (Figure 1). In normal tissues and most low-grade or slowly proliferating neoplasms, approximately 85% of the cell population forms the G0/G1 peak and 15% of the cells are in the S phase and G2 and M phases.

DNA aneuploidy is defined as a DNA content of the G0/G1 peak of a cell population that varies significantly from the mean peak of the known diploid reference cell population. The DNA index of an aneuploid cell population may rarely be < 1.0 (hypodiploid) or > 1.0 (hyperdiploid). Aneuploid cell populations with a DNA index near 2.0 must be differentiated from diploid cell populations with significant G2M phases. Table 1 summarizes the terminology used for DNA ploidy definitions.

Techniques for Measuring DNA Content

Flow cytometry is a technique that features simultaneous measurement of multiple characteristics of single cells stained with excitable dyes moving in a fluid stream exposed to laser beam light. Computerized analysis of light scattering and cell fluorescence produces data analyzed by the on-board computer, resulting in the production of a histogram. In addition to the well-described immunophenotyping functions, when cells are stained with the dye propidium iodide, fluorescence is proportional to the nuclear DNA content. Requiring a cell suspension of individual cells, when solid human neoplasms are analyzed by flow cytometry, the tissue must be disaggregated by mechanical or enzymatic techniques.

Computer-based image analysis applies digital technology to quantitative measurements performed on static cytopathologic and histopathologic specimens. In contrast to flow cytometry, image
analysis features simultaneous morphologic assessment of cells measured for DNA content by video imaging of nuclear optical density after Feulgen staining. A comparison of the histogram generated from the computer reconstruction of the digitized images of a population of cells measured by image analysis with that determined from a flow of similar cells through a flow cytometer is shown in Figure 1.

Technical Issues in DNA Ploidy Measurements
Various technical issues impact on the measurement of total DNA content in human neoplasms. Specimen volume is important; image analysis determination is available for as few as 100 cells, whereas flow cytometry requires a minimum of 5,000 to 10,000 cells. Specimens must be stored in a standard fashion and fixed in 10% neutral buffered formalin, the optimal fixative for the DNA ploidy study.

As mentioned above, flow cytometry requires tissue disaggregation, which is best performed by direct needle aspiration or mechanical techniques on fresh tissue. Retrospective flow cytometric studies utilizing enzymatic disaggregation of tissue can produce significant errors in DNA ploidy measurement.

The tissue section image analysis method has become a preferred technique for small needle biopsies of such organs as the prostate and breast. It should be emphasized that heterogeneity of DNA content is common in many types of human neoplasia, and sampling issues can be significant when searching for aneuploid populations in a large neoplasm. The proper use of diploid controls and standards, the expertise of the instrument operator, and the experience of the histogram interpreter are all critical issues in creating high standards of performance for DNA content analysis.

Flow Cytometry vs Image Analysis
Major reviews of DNA content analysis have highlighted the relative advantages and disadvantages of both technologies [1-5]. These comparative studies have highlighted an excellent overall performance of both techniques, with approximately 95% of samples showing similar diploid or aneuploid histograms when measured by either image analysis or flow cytometry (Figure 2) [6]. The selective nature of the image analysis technique has led workers to conclude that it is slightly more sensitive than flow cytometry [7]. A comparison of the two methods, including their relative advantages and disadvantages for the determination of DNA content in human neoplasms, is included in Table 2.

Determination of Cell Proliferation Rate
The earliest methods of estimating cell proliferation in human neoplasia were based on mitosis counting. During the past 10 years, various newer methods have been applied to determining the cell proliferation rate, or percentage of cells actively synthesizing DNA (Table 3). In addition to determining DNA ploidy, flow cytometry and image analysis also provide estimates of the percentage of cells in the S phase by histogram evaluation and mathematic modeling. Although flow cytometry is more accurate than image analysis for this purpose, both techniques have serious drawbacks with regard to the accuracy and reproducibility of S-phase determinations. Mitotic figure counting is the easiest method to perform. However, lack of reproducibility and standardization are important problems with this method. Human tumors that currently feature mitosis counting in the standard pathology report include breast cancer, smooth muscle sarcoma, and malignant melanoma.

Tritiated Thymidine Labeling--This method directly measures the S phase of proliferating cells but requires viable fresh tissue incubated with radioactive thymidine and interpreted after autoradiography. It also suffers from interobserver variation and is generally cumbersome and rarely used clinically.

Bromodeoxyuridine assay uses a monoclonal antibody that measures cells in S phase that incorporate the bromodeoxyuridine thymidine analog. Although considered an accurate proliferation marker when measured by routine immunohistochemistry or flow cytometric technique, this nonradioactive method is not generally utilized in most laboratories.

Ki-67 Immunostaining--The antibody Ki-67 was raised against a Hodgkin's disease cell line and detects an antigen in the nucleus associated with cell proliferation [8]. Ki-67 immunostaining has been applied to a wide variety of human neoplasms and has been judged to be superior to bromodeoxyuridine assays and tritiated thymidine uptake in the assessment of cell proliferation in human neoplasms (Figure 3) [9]. The original Ki-67 antibody could be utilized only in fresh and frozen tissues. The newer MIB-1 monoclonal antibody developed through recombinant techniques is reactive to a selective part of the
Ki-67 antigen and can be utilized in archival formalin-fixed paraffin-embedded specimens. Comparative studies indicate that the MIB-1 marker accurately reflects an estimate of the S-phase fraction [10]. Currently, the MIB-1 antibody is considered the most easy-to-read and widely applicable cell cycle marker available.

**Proliferating cell nuclear antigen (PCNA)**, also known as cyclin, is a nonhistone nuclear protein cofactor for DNA polymerase-delta. Although this marker was originally believed to be an ideal cell proliferation label that could be applied to archival specimens, more recent studies suggest that it is less sensitive than Ki-67 [11] and subject to significantly variable results when specimens are exposed to microwave antigen retrieval procedures [12]. In addition to this marker, immunoreagents for the detection of cyclin A and cyclin D have recently become available. These may prove to be of substantial interest as potential indicators of aggressive neoplasms.

**DNA Polymerase-Alpha** is a cell cycle-related enzyme detected by monoclonal antibodies that requires fresh-frozen tissue sections and may be relatively insensitive.

**p105** detects a nuclear antigenic epitope involved with RNA synthesis. In early studies, p105 has shown promise as a potential marker of aggressive malignant lymphomas and solid tumors.

**Nucleolar organizer regions (NORS)** are loops of DNA that encode ribosomal RNA production [13,14]. Nucleolar organizer region staining is accomplished by a silver impregnation technique that can be performed on paraffin sections; this technique has correlated with cell proliferation in a wide variety of human neoplasms.

### Cancer Diagnosis and Diagnostic Cytology

In general, the use of DNA ploidy status as a stand-alone diagnostic signal of malignancy has been unsuccessful. However, in a variety of body sites and human tissue samples, DNA content and cell cycle analysis have achieved value in augmenting the standard diagnostic process. The introduction of flow cytometric determination of DNA content to bladder washing and urine cytology represented the first major application of this technique in daily diagnostic pathology. Originally conceived as an adjunct for the diagnosis of low-grade transitional-cell carcinoma in cytology specimens, flow cytometry ultimately proved to be no more sensitive or specific for this diagnosis than conventional cytologic methods [15]. DNA ploidy has shown promise in differentiating reactive atypia associated with intravesical chemotherapy from recurrent transitional-cell carcinoma ([Figure 4]) [16]. The ability of aneuploid DNA content to predict adverse outcome in bladder cancer is discussed below.

DNA ploidy analysis has not proven clinically useful in the interpretation of Pap smears, in that it cannot differentiate among the different grades of cervical intraepithelial neoplasia; cannot predict regression, persistence, or regression of these lesions; and cannot specifically identify invasive carcinoma [17].

DNA analysis can complement conventional cytology in the diagnosis of lung cancer, with aneuploidy or high S-phase fractions serving as an indicator of malignancy. However, there has been little clinical use of the technique for lung cancer screening [18].

DNA content analysis has achieved variable success in augmenting routine cytologic diagnosis of malignancy in serous effusion specimens. Some studies have indicated high false-negative and false-positive rates [19], whereas others have found DNA ploidy determination to be a useful adjunctive technique [20]. It must be emphasized that certain diploid primary tumors, particularly tumors of the breast, colon, and prostate, may feature diploid metastases to body cavities; this provides an explanation for so-called false-negative DNA ploidy results ([Figure 5]).

Ploidy determination is a sensitive measurement of malignancy in spinal fluid; aneuploid populations are highly specific for metastatic lesions [21]. Small specimen sample size generally limits this application, however.

Application of DNA ploidy to the diagnosis of malignancy in fine-needle aspiration biopsy cytology specimens has been limited by the frequent finding of aneuploidy in nonmalignant and even nonneoplastic conditions ([Table 4]) [22-24]. DNA ploidy and cell cycle analysis has, however, been useful in predicting outcome of malignant tumors diagnosed by conventional methods and assessed for DNA content on fine-needle aspiration specimens.

DNA ploidy analysis has proved valuable in the confirmation of severe dysplasia and risk of invasive malignancy in borderline biopsies of Barrett's esophagus and gastric ulcers ([Figure 6]) [25-27]. Cell cycle analysis using the MIB-1 antibody has also been useful in differentiating dysplasia grades in Barrett's esophagus [28]. DNA analysis has been less successful in predicting the presence of
dysplasia in chronic ulcerative colitis [29].

**Prediction of Tumor Aggressiveness and Clinical Outcome**

The rest of this review will consider cell proliferation and S-phase measurements as prognostic factors in human neoplasia, according to tumor type.

**Breast Cancer**—A substantial number of studies designed to test whether DNA aneuploid content predicts disease outcome in breast cancer have been performed during the past 8 years (Table 5). The results of these studies have been mixed: Some investigators have found aneuploidy in breast cancer to be an independent predictor of disease relapse and short survival on multivariate analysis, whereas others have found either no independent correlation of ploidy status with disease outcome or, in rare instances, a complete lack of correlation [30-45].

With regard to cell proliferation and S-phase measurements in breast cancer, a larger number of studies have correlated disease outcome with high proliferative compartments on univariate analysis. However, there has been a similar lack of consensus as to the independent status of this predictor on multivariate analysis [38,46-50] (Figure 7).

**Gynecologic Cancers**—DNA aneuploidy has been uniformly associated with high grade and stage in endometrial cancer. Patients with diploid tumors have a better prognosis than those with aneuploid lesions [51-52]. For uterine sarcomas, DNA ploidy patterns have correlated with tumor grade and size, mitotic index, and general overall survival [53].

Similar to endometrial cancer, DNA ploidy patterns have been predictive of disease outcome in ovarian cancer and have achieved independent status on multivariate analysis as a predictor of prognosis [54]. Large tumors may show heterogeneous ploidy patterns, which may be indicative of disease aggressiveness in itself [55]. DNA content analysis has not been successful in predicting malignancy in ovarian, sex cord, and stromal tumors [56].

**Genitourinary Tract Cancer**—Results of studies assessing DNA aneuploidy in genitourinary tract cancers have varied. In general, ploidy patterns have shown a high predictive value for disease outcome in cervical cancer [57] and less predictive value in vulvar, vaginal, and penile squamous cell carcinoma [58,59].

**Germ-Cell and Trophoblastic Tumors**—DNA ploidy analysis has not proved valuable in the prediction of disease outcome in germ-cell tumors. Although adult benign teratomas are generally diploid, virtually all aggressive germ-cell tumors, including germinomas, embryonal carcinomas, and endodermal sinus tumors, are uniformly aneuploid [60]. For gestational trophoblastic neoplasia, a major clinical use of DNA analysis involves confirmation of the presence of partial hydatidiform mole by the determination of a triploid DNA content histogram indicating a DNA index of 1.5 and the fertilization of one ovum by two spermatozoa (Figure 8) [61].

**Prostate Cancer**—Virtually all of the major retrospective studies utilizing flow cytometry and image analysis have confirmed the significant predictive value of DNA aneuploidy for outcome in prostatic adenocarcinoma (Table 6) [62-79]. Recent studies have focused on applications of DNA ploidy to the original prostate cancer needle biopsy; results of these studies have shown an excellent correlation with ploidy patterns on radical prostatectomy specimens and independent predictive significance for extraprostatic disease, distant metastasis, and disease recurrence [77]. Needle-biopsy ploidy status (Figure 9) ultimately may be combined with tumor grade and other prognostic markers to stratify patients into treatment protocols.

**Bladder and Kidney Cancers**—Although unsuccessful for the identification of low-grade transitional-cell carcinoma on urine cytology due to its uniform diploid nature, the presence of aneuploidy in urine cytology or bladder biopsy specimens indicates high risk for muscle-invasive disease and adverse patient outcome [80-82]. Aggressive bladder cancers, in addition to featuring high nuclear grade and aneuploid DNA content, have also been recently linked to mutation of p53, as well as to various other changes in growth factors and invasion and metastasis markers [83] (Table 7). In a recent study, Ki-67 and PCNA immunostaining did not independently predict disease aggressiveness [84].

Early retrospective studies of flow cytometry and more recent prospective studies have shown DNA ploidy pattern to have independent significance for outcome in renal-cell carcinoma [85]. Ploidy findings appear significant for clear-cell renal carcinoma, and also tend to separate granular-cell renal carcinomas, which may be aneuploid, from benign oncocytomas of the kidney, which are uniformly diploid [86].

**Upper Aerodigestive Tract Tumors**—Early studies of DNA content by flow cytometry in squamous
cell carcinomas of the head and neck showed variable results, with some studies indicating a favorable and others an unfavorable implication of aneuploidy [87,88]. More recently, aneuploidy has been associated with disease progression in these tumors [89,90]. DNA analysis of salivary gland tumors has received limited attention. Noteworthy is the finding that, although aneuploidy may be seen in benign pleomorphic adenomas, its presence indicates a higher rate of recurrence after surgery and subsequent malignant progression [91].

GI Cancers--Although earlier studies failed to demonstrate a significant correlation between ploidy patterns and outcome in esophageal cancer, most of the more recent studies have shown both univariate and multivariate statistical significance for the presence of aneuploidy and disease outcome [92-94]. For gastric adenocarcinoma, the majority of studies have correlated ploidy patterns with disease outcome on univariate analysis but have been less successful in demonstrating independent status on multivariate analysis [95-97]. Ploidy patterns have predicted prognosis in gastric cancer and have correlated with proliferation rates and growth factor expression [98]. For gastrointestinal (GI) stromal tumors, DNA ploidy has been found to predict tumor grade and was an independent indicator of poor prognosis [99-101].

DNA content analysis in colorectal adenocarcinoma has generally correlated with disease outcome, but the results of numerous earlier studies have not been unanimous. Similar to esophageal and gastric cancer, ploidy patterns in colorectal carcinoma have nearly uniformly shown significance in the prediction of disease outcome on univariate analysis but have not always achieved independent status on multivariate analysis [102-105]. DNA aneuploidy has recently been described in normal colonic mucosa in patients with colorectal cancer, suggesting that abnormal DNA content may be an indicator of early transformation in the large intestine [106]. In diploid colorectal cancer cases, elevation of the S-phase percentage has been associated with higher stage disease and decreased patient survival (Figure 10) [102]. Although DNA aneuploidy is associated with significantly shortened patient survival in pancreatic carcinoma, the clinical utility of ploidy patterns is limited due to the disease's virtually universal poor outcome. In one study, patients with diploid pancreatic carcinoma had a mean survival of 17 months, as compared with just 8 months for patients with aneuploid tumors [107]. Although inconsistent results have been published, most studies indicate that aneuploidy in primary hepatocellular carcinoma is predictive of adverse outcome on univariate and multivariate analysis [108-110]. Although the premalignant potential of liver cell dysplasia has been under investigation, one recent study indicated a significant frequency of aneuploidy in dysplastic liver cells, which may further support the role of dysplasia in the evolution of hepatocellular carcinoma [111]. However, nondiploid patterns may be seen in chronic hepatitis and cirrhosis, possibly as the result of tissue regeneration. Therefore, aneuploidy cannot be used to specifically identify malignant hepatocytes on liver biopsies and aspiration cytologies [112,113].

Lung Tumors--For non-small-cell lung cancer, the association of DNA ploidy pattern with disease outcome has been somewhat variable, although most studies indicate adverse outcome for aneuploidy on univariate analysis [114-116]. For undifferentiated small-cell carcinoma, DNA analysis has been of limited use given the nearly uniform finding of aneuploidy in these tumors (Figure 11) [117]. For malignant mesothelioma, DNA analysis has similarly been of limited value in diagnosis and the prediction of prognosis [118].

Bone and Soft-Tissue Tumors--DNA ploidy determination has generally correlated with survival rates in bone and soft-tissue sarcomas [119]. In osteosarcoma, for example, near-diploid histograms are associated with favorable prognosis and response to therapy (Figure 12) [120]. In general, ploidy analysis has correlated with tumor grade for sarcomas, with diploid lesions denoting low histologic grade and localized disease, and aneuploid tumors indicating high-grade disease and increased risk of recurrence and distant metastasis. DNA ploidy and cell cycle analysis cannot, however, differentiate between malignant and benign soft-tissue tumors, in that benign proliferations and nonneoplastic disorders, such as fibromatosis and wound healing, can feature aneuploid histograms.

CNS Tumors--For gliomas, the results of DNA content analysis as a prognostic indicator are similar to the results for pancreatic cancer; namely, aneuploid tumors have an extremely short survival of < 1 year and diploid tumors have a survival ranging from 2 to 3 years in some cases [121]. In meningiomas, DNA ploidy status does not appear to correlate with recurrence rate and risk of metastasis, although proliferation index may be helpful for determining aggressiveness and, possibly, for planning adjuvant therapy [122,123].

Endocrine Tumors--DNA content analysis has not proved successful in predicting outcome for papillary and follicular thyroid carcinomas. Aneuploidy may be seen in encapsulated thyroid
adenomas, and both papillary and follicular neoplasms with metastatic foci may be diploid [124,125]. On occasion, high DNA index has been associated with progressive disease and death in differentiated thyroid cancer [126]. Nonetheless, DNA content analysis has been ineffective in the diagnosis of differentiated thyroid cancer on fine-needle aspiration specimens (Figure 13). For adrenocortical tumors, aneu-ploidy may be identified in encapsulated benign lesions, and thus, cannot be utilized to diagnose malignancy [127]. Similarly, for parathyroid and pancreatic islet-cell tumors, aneuploidy may be identified in encapsulated benign lesions. Thus, ploidy status cannot be utilized to predict outcome in patients with these tumors [128,129]. Likewise, DNA aneuploidy is common in carcinoid tumors and is not useful in predicting metastasis or survival time [130]. Interestingly, for pheochromocytomas and paragangliomas, DNA ploidy pattern may be an important independent prognostic variable [131]. For oncocytic neoplasms of the salivary gland, kidney, and thyroid, diploid DNA content has generally been associated with a benign clinical course [132]. On occasion, aneuploidy in these tumors has been associated with the potential for invasion and metastasis.

**Malignant Melanoma**—Although surgical pathology microstaging methods have generally been reliable predictors of outcome in malignant melanoma, a significant percentage of cases may fall into intermediate-thickness level III lesions, which are of uncertain clinical potential. DNA ploidy assessment has proved valuable in differentiating among this latter group, in that diploid intermediate-thickness lesions tend to behave like thin lesions and aneuploid lesions act similar to more clinically aggressive thick tumors [133]. Aneuploidy cannot, however, be utilized as a diagnostic tool to differentiate atypical pigmented lesions from melanoma because benign melanocytic nevi occasionally exhibit aneuploid DNA patterns.

**Hematopoietic Malignancies**—Table 8 shows DNA ploidy and cell cycle analysis distribution in non-Hodgkin's lymphoma according to the Working Formulation. In general, the prognostic significance of DNA ploidy patterns in this disease has been variable, and S-phase calculations or direct cell cycle proliferation measurements have proved more valuable clinically [134,135]. In leukemia, limited data are available concerning ploidy status and disease outcome. Similarly, there is little information to suggest that ploidy status predicts disease outcome in Hodgkin's disease.

**Pediatric Neoplasms**—In pediatric round cell neoplasms, DNA aneuploidy has generally been associated with an improvement rather than a diminution in survival. Similarly, for neuroblastoma, abnormal DNA content has been associated with improved response to therapy and significantly increased survival (Figure 14) [136,137]. Studies of medulloblastoma also have shown an association of aneuploidy with favorable clinical outcome [138], although recent studies have failed to confirm the improved survival associated with abnormal DNA content [139].

**Summary**

Table 9 summarizes the diagnostic and prognostic value of DNA ploidy and cell cycle analysis in a variety of human neoplasms. This summary highlights the substantial clinical value of DNA ploidy and cell cycle analysis in the diagnosis of cancer and, more importantly, in the prediction of outcome.

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