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DNA Flow Cytometry Measurements as Surrogate Endpoints in Chemoprevention Trials: Clinical, Biological, and Quality Control Considerations

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Abstract DNA flow cytometric evaluation of S-phase fraction (SPF) is a strong and consistent predictor of relapse-free survival in the node-negative breast cancer patient. As such, it can be implicated as a marker of tumor aggressiveness and has been shown to be an independent predictor of outcome in a multivariate setting. Measurement of ploidy status is less well-defined as a marker of prognosis, but may be an important marker of response to therapy. Estimation of DNA ploidy and proliferative capacity by flow cytometry can be obtained from virtually any type of specimen, including fine needle aspirates, fresh or frozen material, as well as formalin-fixed, paraffin-embedded material, as long as there is a sufficient number of tumor nuclei for assay. Therefore, the assay has clinical relevance in predicting relapse, as well as providing flexibility for sample preparation.

In addition, flow cytometric measurements are biologically relevant markers. In general, DNA index is a good estimate of total chromosome number. SPF, using sophisticated modeling algorithms, shows good correlation with thymidine labeling index and/or bromodeoxyuridine incorporation, two standard assays used to measure DNA synthesis in fresh tissue. Recently, preliminary data in locally advanced breast cancer have indicated that ploidy and/or S-phase may also be useful in predicting cellular response to chemotherapy.

Although there is good justification for measuring these parameters, appropriate quality control and quality assurance measures must be incorporated into all aspects of the assay—from sample handling and preparation to interpretation of cell cycle and histogram data. Using commercially available software programs and recommended guidelines for standardization and interpretation, data obtained from this assay can be quality controlled and reproducible from lab to lab. In many laboratories, however, quality control issues are not always addressed. A sufficient framework exists to employ these guidelines to achieve better standardization provided they are monitored and regulated. The most difficult aspect of the assay, especially in a chemoprevention setting, is to ensure that sufficient representative nuclei are obtained for evaluation.

CONCLUSION: DNA flow cytometry measurements show clinical and biological relevance in early stage breast cancer, and can be quality controlled to provide reliable data.

Key words: Breast cancer, chemoprevention, flow cytometry, kinetics, ploidy, quality control, S-phase

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DNA flow cytometry (FCM) offers at least two measures of tumor aggressiveness and potential disease progression. One is an estimate of a cell's proliferative activity measured as the percentage of cells with a DNA content consistent with those in S-phase, or DNA synthetic phase, of the cell cycle. The S-phase fraction (SPF) is usually described as high, intermediate, or low. The other measure is an estimate of tumor ploidy, measured as the total nuclear DNA content. FCM does not allow us to identify specific genetic abnormalities afforded by cytogenetic analysis; therefore, the term "DNA" precedes the ploidy description when obtained by FCM [1,2]. Using FCM, a tumor with a "normal" amount of DNA is "DNA diploid," and a tumor with an abnormal or non-diploid amount of DNA is "DNA aneuploid." Using a DNA diploid control, one can estimate the relative amount of DNA in the non-diploid population and refer to this value as the DNA index (DI). By definition, DNA diploid tumors have a DI = 1.00 and DNA aneuploid tumors have a DI greater than or less than 1.00 (*e.g.*, DNA hyperdiploid, DI > 1.00; DNA hypodiploid, DI < 1.00) [2].

Measurements of DNA ploidy, and especially proliferative activity, have had an impact in the clinical setting due to their ability to predict risk of tumor recurrence and early death in early stage breast cancer patients [3,4]. Investigators now evaluate the importance of these markers in predicting response to treatment [3,5–7]. In addition to their clinical relevance, both ploidy status and SPF are biologically relevant markers that help to characterize the heterogeneity of a tumor population. In general, DNA index is a good estimate of total chromosome number [8–10]. SPF, using sophisticated modeling algorithms, shows good correlation with thymidine labeling index (TLI) [11] and, in preliminary studies, with bromodeoxyuridine (BrdU) incorporation [12], two standard assays to measure DNA synthesis in fresh tissue.

Although good justification exists for measuring these parameters, there is a critical need to incorporate appropriate quality control and quality assurance practices into all aspects of the assay. A sufficient framework exists to employ recommended guidelines for standardization and interpretation of cell cycle and histogram data [13–16].

The following discussion details aspects of clinical and biological relevance of DNA FCM measurements and discusses issues critical to quality control and quality assurance of this assay.

CLINICAL RELEVANCE

FCM measurements have been extensively described as prognostic factors in breast cancer [2,3]. Because prognostic factors can best benefit the node-negative patient, this discussion will be limited to studies focusing on this early stage breast cancer population.

Similar to reports on later stage disease, data conflict regarding the clinical significance of ploidy status in predicting either disease-free survival or overall survival in early stage breast cancer patients [3]. Results from the ancillary study (INT0076) of the Intergroup clinical trial (INT0011) showed that DNA ploidy status did not discriminate for time-to-recurrence in those node-negative breast cancer patients who did not receive adjuvant therapy [4]. SPF, however, was a significant predictor of time-to-recurrence in this study, and was closely correlated with tumor size. Using a modified software program, S-phase measurement was also able to predict recurrence within a low-risk subpopulation of this study, comprised of patients with estrogen receptor-positive (ER+) tumors <3 cm in diameter [17].

At least eight other studies in the literature evaluate DNA FCM measurements in node-negative patients [3]. These studies range in patient number from 149 to nearly 350, with 4–10 years of median follow-up time. Three studies report that patients with diploid tumors have a significantly longer relapse-free survival compared to patients with aneuploid tumors [18–20], while five other studies failed to observe that ploidy status significantly predicted recurrence [4,21–24]. It is difficult to compare data in some of these studies due to variability in definition and interpretation of DNA ploidy status.

In contrast, proliferative capacity consistently shows a significant association with risk of recurrence and/or early death, even though this measurement is technically more difficult to estimate and reproduce [3]. On average, estimates of five-year disease-free survival show a 22% difference in patients with low versus high S-phase tumors [3].

Measurement of proliferative capacity by FCM has been an independent predictor of time-to-recurrence in four of the five studies reporting multivariate analysis [18,19,21,22]. Although each study looked at somewhat different variables, tumor size and ER status were common to all. In one of the studies [18], a high SPF was an independent predictor of recurrence in DNA diploid tumors only. O'Reilly *et al.* [21] and Clark *et al.* [18] reported that S-phase predicted recurrence of both DNA diploid and aneuploid tumors. In the study which did not find that S-phase predicted recurrence, it was observed to predict overall survival [23]. However, since follow-up was relatively short (4.25 years), full interpretation awaits further follow-up.

In the United States, several clinical trials have or are using DNA FCM measurements as part of a clinical or ancillary laboratory protocol [3]. Objectives of these studies include evaluating DNA ploidy and S-phase as markers of prognosis and predictors of response to therapy. In the current node-negative intergroup trial (INT0102), SPF helped determine eligibility in an otherwise good prognosis group. Patients with ER+, progesterone receptor-positive (PR+), <2 cm tumors that had a high S-phase were eligible for randomization to one of two treatment arms. Patients whose tumors had a low SPF (ER+, PR+, <2 cm size) were assigned to observation only. Different cutpoints were used to define high and low SPF for DNA diploid and DNA aneuploid tumors. This trial has recently reached its accrual goal, but analysis has not yet been performed.

The NSABP-B-04 natural history study [24] with 10 years of follow-up included clinically node-positive and -negative breast cancer patients who did not receive any adjuvant therapy. DNA ploidy status did not predict either disease-free survival or overall survival. Measurement of SPF, however, showed a significantly longer time-to-recurrence in patients with low SPF tumors compared to high SPF tumors [24]. This relationship was strongest in the node-negative patients. A multivariate analysis including both clinically node-negative and -positive patients showed only SPF and tumor size to be independent predictors of disease-free survival [24].

RESPONSE TO THERAPY

It is too early to generalize about using these measurements to predict response to therapy. In locally advanced disease, several investigators have reported that DNA aneuploidy and/or a high SPF was associated with objective regression to combination chemotherapy [3,5–7]. In these studies, patients had undergone pre-surgical chemotherapy; tissue was available for both pre- and post-chemotherapy comparison. Preliminary data from Dressler and colleagues [25] showed that post-treatment, the DNA aneuploid, high SPF population was no longer detectable in approximately one-third of the patients studied. However, a DNA diploid, low SPF, malignant population remained. Histologic evaluation confirmed the presence of malignant cells throughout the post-chemotherapy specimen. Although this was a small study, the data suggest that the inherent DNA diploid population in some aneuploid tumors may include malignant, DNA diploid, low SPF cells unresponsive to the therapy given [25].

Results from randomized trials and other ongoing studies are required to better evaluate the role DNA ploidy and S-phase may play in tumor biology and in predicting response to therapy. Still unanswered is whether or not these markers of prognosis will translate into markers of tumor progression. TLI studies have not found an association between a high labeling index and prediction of metastasis [26]. Conceptually, the presence of high SPF by FCM may be interpreted as a high-risk marker for existing micrometastases. Thus, a high SPF predicts early recurrence.

BIOLOGICAL RELEVANCE

The technique of single parameter DNA FCM (measuring only DNA fluorescence) allows flexibility in sample preparation; virtually any sample can be assayed for ploidy if a sufficient number of tumor nuclei are present. Therefore, we can use fresh, frozen, or formalin-fixed, paraffin-embedded material, as well as needle aspirates or core biopsies. FCM requires that a single cell or nuclear suspension be prepared prior to staining with the DNA fluorochrome. This can be achieved by enzymatic digestion, mechanical dissociation, or a combination of both [2]. The stained cells/nuclei are then run on a flow cytometer to estimate ploidy status relative to a known DNA diploid control sample, and sample events are collected either as a histogram or a list

mode file. Cell cycle analysis is performed using sophisticated software programs based on modeling algorithms that attempt to "fit" to the sample raw data and deconvolute overlapping populations [2,27].

The most commonly used DNA fluorochrome in single color assays is propidium iodide, a fluorochrome which intercalates between double-stranded nucleic acids. Therefore, in order to measure DNA incorporation of dye, the cells are treated with RNase to remove contaminating RNA [2]. Incorporation of the dye is stoichiometric and depends on the conformation, *i.e.*, unwinding, of the DNA [2,15]. In general, the more dye incorporated, the more DNA is in the cell. Because FCM gives an estimate of total DNA in a cell as compared to specific chromosomal abnormalities detected by cytogenetic analysis, it was of interest to evaluate the biologic accuracy of the FCM-derived DNA index [8]. Also interesting was to perform this comparison using formalin-fixed, paraffin-embedded specimens, the most common tissue specimen obtainable in hospitals. In a study of 56 pediatric solid tumors, Dressler and colleagues [8] recently reported a good correlation between DNA index obtained by FCM on archival tissue and total chromosome number obtained by cytogenetic analysis on corresponding fresh specimens. Similar correlations have been observed by other investigators using fresh or frozen tissue from colon cancer [9] and non small-cell lung cancer cell lines [10]; however, weaker associations have also been reported [28]. Overall, the DNA index obtained by DNA FCM appears to be a biologically relevant estimate of total chromosome number.

Estimation of SPF is a technically more complex measurement compared to DNA index, and yet as stated earlier, SPF shows more consistent results in predicting prognosis [3]. SPF values are obtained from a variety of software programs which take various approaches to estimating the number of cells with S-phase DNA, *i.e.*, cells in the DNA synthetic phase of the cell cycle. Due to overlapping populations of DNA diploid S- and G_2M-phase cells with DNA aneuploid G_0/G_1-, S-, and occasionally G_2M-phase cells, early reports of S-phase were restricted to DNA diploid tumors, *i.e.*, those tumors with no overlapping abnormal populations [2,27]. Indeed, some programs currently in use still cannot accurately estimate cell cycle in DNA aneuploid populations, and will hopefully soon be replaced with more sophisticated programs which not only mathematically model overlapping populations, but also compensate for debris and aggregates. Because of the complexity of the programs and the fact that we are obtaining a static measure of proliferative capacity, it is important to evaluate the accuracy of the SPF obtained by FCM by comparing it with other techniques, such as TLI and BrdU incorporation.

In an elegant study performed by Meyer *et al.* [11], parallel samples obtained from breast cancer patients were assayed by TLI and DNA FCM and evaluated for their ability to predict survival. Labeling index values and SPFs were divided into three groups to characterize proliferative activity: high versus intermediate versus low. Kaplan-Meir survival curves obtained from each set of data showed the same pattern of behavior: patients with low labeling indices or low SPF had the longest survival; patients with high labeling indices or high SPF had the shortest survival, and patients whose tumors showed intermediate values had intermediate survival times. Although relatively few deaths occurred in this study, SPF appeared to be a stronger predictor of early death compared to TLI ($p = .009$ versus $p = .046$). It was interesting to note that DNA aneuploid tumors showed higher proliferation estimates by both TLI and FCM than DNA diploid tumors. Since TLI morphologically identifies and counts only malignant cells, this study supported the finding that the low SPF observed in DNA diploid tumors by FCM was accurate and not a result of dilution effects caused by contaminating non-malignant cells in the sample.

An additional study by Seamer and colleagues [12] compared SPF values obtained by three software programs used in FCM to SPF values obtained using BrdU-anti-BrdU uptake in a mixed population of DNA aneuploid breast cancer cell lines and DNA diploid human peripheral blood lymphocytes. In this study, a good correlation was observed between BrdU incorporation and software-generated SPF. The study also showed that the software programs tested were accurate provided the DNA aneuploid population comprised at least 10% of the sample population. Considerable variability in SPF estimation was observed when the aneuploid population comprised only 5% of the sample. Although this study was performed with cell lines,

it suggested that the SPF values obtained by software programs may be a biologically relevant reflection of cells in DNA synthesis. Obviously, this type of experiment needs to be repeated with solid tumor specimens.

QUALITY CONTROL/QUALITY ASSURANCE CONSIDERATIONS

The routine use of FCM techniques to evaluate DNA content and cell cycle parameters requires quality control and standardization of the entire assay, from sample preparation to instrumentation, acquisition, analysis, and interpretation of DNA histogram data. Recently published guidelines and recommendations of the DNA cytometry consensus conference addressed broad issues in utility and standardization of the technique [13]. General recommendations were made for sampling, sample processing, instrument performance, and data and histogram analysis [13,14]. Specific guidelines and criteria for evaluability and quality assurance have also been reported for DNA histogram interpretation, optimized for breast cancer but applicable to a wide variety of solid tumors [15,16]. These reports present a framework for standardization and reproducibility of this technology.

Three essential factors must be considered for appropriate quality assurance and control of DNA histogram interpretation. These include histopathologic/cytologic review of sample material; data-generated guidelines for evaluability and interpretation of DNA ploidy and cell cycle parameters; and utilization of appropriate software programs for cell cycle analysis [see 2,15,16 for a more detailed description].

The first area of quality assurance requires histologic or cytologic confirmation by a pathologist that the sample material used for the assays reflects representative tumor tissue. This is easily accomplished when using paraffin block material by evaluating the 4 μm sections immediately preceding and following the thick sections cut for FCM [2]. When fresh or frozen tissue is used, a cytospin preparation of dissociated cells before fluorochrome staining offers a representative sample [2]. Without this first critical step of quality assurance, there is neither validity nor acceptable quality of the assay results.

The second area, data-generated guidelines to evaluate and interpret DNA ploidy and cell cycle parameters, constitutes an essential component in providing reproducible and accurate data from laboratory to laboratory. This involves quality control checks in data acquisition, including flow rate, number of events collected, concentration of the sample, and thresholds set to maximize collection of nuclear/cellular events while still allowing for evaluation of debris contamination [16]. It also involves the use of data-generated guidelines for evaluability and definition of DNA diploidy versus DNA aneuploidy. These guidelines include: (1) acceptable ranges for G_0/G_1 coefficient of variation (CV) for both DNA diploid and DNA aneuploid populations; (2) confirmation of the DNA diploid peak position; (3) definition of the minimum number of events that constitute a real peak versus an artifact; and (4) minimizing doublet or aggregate formation, especially in the interpretation of tetraploid tumors [2,15,16]. These parameters are also critical to the evaluability and reproducibility of cell cycle analysis. For example, a wide CV will increase the variability of the SPF estimation [16, 27], and an inability to confirm the diploid position can cause a histogram to be analyzed as a DNA hyperdiploid population by one user and DNA hypodiploid by another user, resulting in different SPF estimates. In addition, Seamer and colleagues [12] reported that SPF estimates on very small DNA aneuploid populations were not accurate or reproducible.

When trying to standardize cell cycle analysis for reproducibility of SPF between laboratories, it is essential that each laboratory first set its own cutoff values to define high versus low or high versus low versus intermediate SPF values; cutpoints cannot be extrapolated from the literature [2]. Median values reported in the literature show considerable variability, and can range from 5.0% to 14.0% [2]. In addition, there are several factors that can affect SPF estimation, including methodology, instrumentation, histogram interpretation, and choice of modeling algorithm. Because of these variables, it may be more realistic and clinically relevant if we consider SPF measurements in the context of the risk groups that they define (high, intermediate, or low risk) versus the absolute value of the measurement. This approach may help to attain standardization more efficiently, as well as be more informative.

The third area involves the use of appropriate software programs that have been validated and provide reliable, accurate estimates of cell cycle parameters [12,27]. Although numerous software programs are available and have been used to analyze DNA histograms, a select few are sophisticated enough to handle complex histograms occurring in most non-diploid tumors. Software programs must allow for accurate estimation of SPF when overlapping populations exist, which is the case for virtually every DNA aneuploid histogram. Although imperfect, there are at least two commercially available software programs that use sophisticated mathematical algorithms to model not only overlapping populations, but the contribution by debris and aggregate formation as well. These software programs also offer the user a menu for "automated" analysis in which the software will first interpret the histogram and then select the most appropriate model to fit the raw data. By definition, the variability of the values obtained in the "automated" mode is 0%. The programs also allow the user to select and/or build a model, or modify certain parameters of the existing model. Even with this increased flexibility, and using an older version of one of the software programs, Dressler and colleagues [27] reported only a 3% variability which did not vary with the magnitude of the SPF value. Recently, reproducibility studies were applied to archival material using an updated version of this software program and showed, on average, less than a 1.5% variability when user-selected and user-generated models were employed (Dressler and Peña, unpublished results).

In summary, DNA FCM measurement of SPF and DNA ploidy status provide clinically useful information that is biologically relevant and can be quality controlled. Measurement of S-phase is an independent marker of recurrence in early stage breast cancer patients, and measurement of both parameters may be useful to predict response to treatment and more clearly define tumor heterogeneity. Quality control and standardization of cell cycle measurements can be achieved by incorporating guidelines and criteria for evaluability that have been previously validated. Cell cycle programs with sufficient accuracy and reproducibility of SPF may prove more informative and reliable if considered in the context of the defined risk groups versus absolute value.

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