DNA Histogram Analysis for Node-Negative Breast Cancer

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The combination of DNA ploidy and S-phase is one of the strongest general prognostic indicators for node-negative breast cancer (1). The costs associated with this prognostic power are relatively minimal; laboratory total processing time is usually less than 10 min and reagents are relatively inexpensive. The test can be standardized world-wide as long as strict guidelines are followed, and the test's result can be presented in a way that is easily understood by oncologists. DNA ploidy and S-phase prognostic strength can be further augmented by other well-known prognostic indicators, such as menopausal status and primary tumor size.

Figures 1 and 2 provide evidence for the above statements (1). Figure 1A shows highly significant relapse-free survival (RFS) stratifications for over 935 node-negative patients in the Baylor study. The stratifications are based on a prognostic model composed of an optimal combination of adjusted DNA ploidy and S-phase. When the same prognostic model and stratification boundaries are applied to other independent studies (see Fig. 1B and C), similar highly-significant patient stratifications are observed. Figure 2 demonstrates how the prognostic model can be further augmented by primary size and menopausal status.

These patient stratifications are potentially important for managing this prevalent and deadly disease. In 1999, the incidence of breast cancer in the United States was over 170,000; greater than leukemia, lymphoma, and AIDS combined. In 2003, it is predicted to be over 200,000. Most of these cases are discovered before the tumor has infiltrated into the patient's nodes (node-negative); therefore, this diagnostic test can potentially impact the strategy for optimal treatment for this large patient group.

The DNA histogram test can be divided into four processing steps: sample preparation, DNA histogram acquisition, cell-cycle analysis, and final processing. In order for cytometrists to perform this test to its full capability, the following guidelines for each of these steps are recommended.

SAMPLE PREPARATION

Published staining methods that produce DNA histograms with DNA diploid %CVs averaging 3% to 4% or less and relatively low debris and aggregates are required (2). Samples should be either fresh or frozen (as those were that are presented in Fig. 1). Histograms from paraffin-embedded material have characteristically high CVs and debris, which mitigates their potential usefulness; however, there are some emerging technologies that show great promise in producing high-quality DNA histograms from paraffin-embedded material (3). An internal standard such as trout RBCs is highly recommended, since it provides an internal DNA reference, making it possible to distinguish DNA hypo- and hyper-diploids (2). The proportion of the standard in the histogram can also serve as an additional quality control for various types of pipetting errors (4).

DNA HISTOGRAM ACQUISITION

If possible, set the cytometer's trigger and discriminator to be the DNA content signal (e.g., integrated red fluorescence). The discriminator or threshold should be as low as possible for adequate debris modeling but high enough to avoid diluting the listmode file with low fluorescent particles (e.g., channel 10 for 1,024-channel scale). The sample flow rate should be slow enough to optimize CV. The internal standard should be placed in the channel that results in the DNA diploid peak being centered at channel 50 (256-channel scale) or 200 (1,024-channel scale). If the cytometer has a time parameter, display the DNA content parameter–versus–time histogram during acquisition (5). If there is a partial obstruction during the run, this plot will clearly show a peak shift and the area of instability can be eliminated by gating if the sample can not be reacquired. Do not use light scatter gating, because the distribution is generally too heterogeneous for gating to be useful. Do not use pulse-processing gating, since aggregates are normally not distinguishable from nonaggregates in these samples, and arbitrarily setting a diagonal gate creates an unwanted source of variability in S-phase estimation. Also, many times tumor nuclei are not spherical and can be selectively eliminated by pulse processing gates. Acquire at least 15,000–20,000 events.

CELL-CYCLE ANALYSIS

All cell-cycle analyses should be done with 256-channel DNA histograms. Best results are achieved when the average number of events per channel between the lowest G1 and highest G2 is >100 (6). The software used to
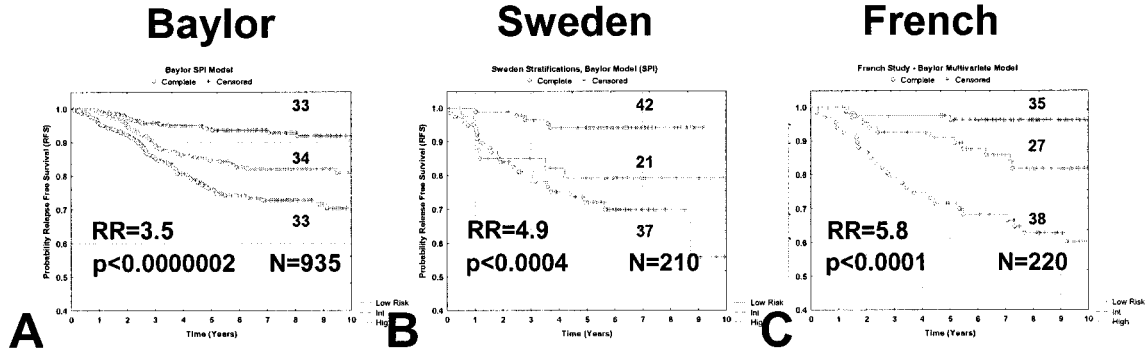
FIG. 1. DNA ploidy and S-phase prognostic model. Relapse-free survival (RFS) stratifications for 935 node-negative breast cancer patients in the Baylor study (A). The same prognostic model and stratifications were applied to the Sweden and French studies (B and C).

analyze the histogram needs to accurately model both aggregates and debris, since they form continuous distributions that overlap with S-phase. Visually inspect the model fit to evaluate these two important requirements. Make sure the analysis software can fit complex DNA histograms (such as: Multi-cycle, Phoenix Flow Systems; or ModFitLT, Verity Software House, Topsham, ME), since DNA aneuploidy and multiploidy are quite prevalent in solid tumors such as breast cancer.

If at all possible, use automatic analysis to initially model the data. Automatic analysis is efficient and the results are reproducible. A set of rules and guidelines are available to determine whether the correct model was chosen for the given DNA ploidy pattern (7). Do not change range positions unless absolutely necessary, since any interaction with the modeling process will add some level of variability. Model only clearly identifiable peaks that have a CV near that of the DNA diploid G0G1 peak; sharp spikes in the data are generally artifacts. Shoulders are common in solid tumor DNA histograms and can simply reflect slight differences in cell population stainability. Do not try to model shoulders, because the results will be highly variable. Do not worry about being too aggressive in modeling clearly identifiable peaks, since the final processing step will appropriately convert these DNA ploidy patterns into low- and high-risk categories.

FINAL PROCESSING

Much of the variability that has been associated with this test has been due to investigators making decisions based on "feel" and intuition rather than hard statistical evidence. There are seven DNA ploidy adjustments (8) necessary to obtain highly significant risk groups. These adjustments were found by a specially-designed statistical process involving the clinical end-point and distant metastatic relapse, and are *not* intuitively obvious, which explains why DNA ploidy has commonly been found a weak or insignificant prognostic indicator.

There are two necessary S-phase adjustments to eliminate a spurious correlation with DNA ploidy (8). The first is to scale the DNA diploid S-phase to be comparable to the DNA aneuploid S-phase. The second is to compensate for the aneuploid fraction effect when lower aneuploid fractions favor high S-phase estimates.

Once DNA ploidy and S-phase have been properly adjusted, they can be included in a prognostic model that was originally derived from the Baylor study population (see Fig. 1A). The output of the model is the relative risk index (RRI), which classifies a given patient into a specific risk group. An RRI of 1 represents a patient that is close to the average RFS in the Baylor study. An RRI of 2 is a patient that has twice the risk of relapse as the average,
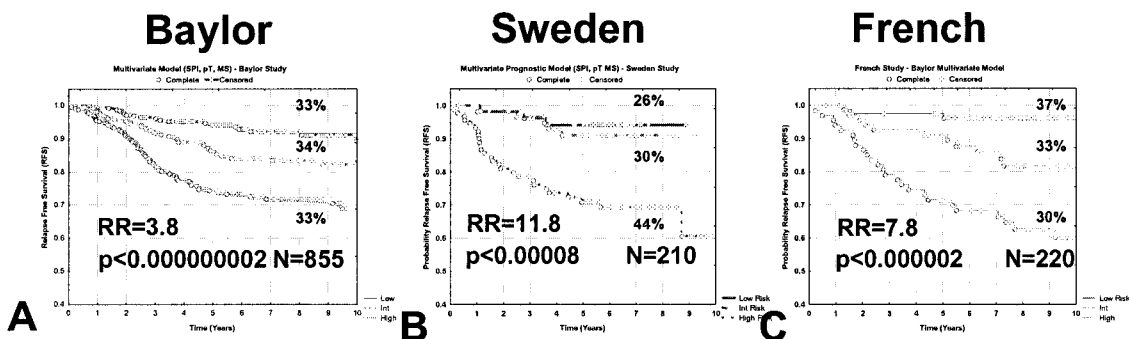

FIG. 2. DNA ploidy and S-phase, primary size, and menopausal status prognostic model. Relapse-free survival (RFS) stratifications for 855 node-negative breast cancer patients in the Baylor study (A). The same prognostic model and stratifications were applied to the Sweden and French studies (B and C).

and a patient with an RRI of 0.5 has one-half the risk. RRI provides the oncologist with clearly-defined information about the patient's risk of relapse.

The first key for reacceptance of this test as a viable modality for patient prognosis is for other investigators to reproduce the findings summarized in the final processing section. A hypothesis or method is only valid if it is reproducible by the scientific community. If these adjustments do in fact eliminate the variability that this test has endured for the last 20 years, the sooner it is validated by the cytometry community, the better.

If and when the findings are validated, an editorial/paper from this society should be presented and/or published and addressed to oncologists, explaining why the test did not work well in the past and why and how it works now. If this is not done, then any papers written or presented concerning DNA histogram analysis will forever be diluted in a sea of other papers demonstrating variable results.

All the guidelines and processing discussed above should also be extensible to other solid tumors such as prostate and colon. The potential patient benefit of this test is enormous.

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