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Pathological and Biological Relevance of Cytophotometric DNA Content to Breast Carcinoma Genetic Progression

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Abstract Correlating cytophotometrically detectable genetic alterations to events of known biological and pathological significance in breast carcinoma has been challenging, in large part owing to the difficulty in isolating and analyzing premalignant (*i.e.*, hyperplastic) or preinvasive (*i.e.*, *in situ* carcinoma) lesions. This problem may be addressed by using histologically directed evaluation of intact, paraffin-embedded tissue sections. Using image cytophotometry in preserved sections, we have identified clonal DNA content abnormalities (*i.e.*, aneuploidy) in up to three-fourths of preinvasive breast carcinomas. Moreover, comparison of ploidy determinations between residual *in situ* and corresponding invading neoplastic populations suggests that host invasion is accompanied by measurable DNA content shifts in many cases. Image cytophotometric DNA content abnormalities are also detectable in florid/atypical proliferative lesions, albeit less frequently (~25% of cases) and to a lesser extent (*i.e.*, near-diploid) than *in situ* carcinomas. Taken together, these findings imply an association between clonal DNA content aberrations and histologic disease progression. Although the sensitivity of cytophotometric ploidy assessments in tissue sections is limited by nuclear sectioning artifact and overlap, the presence of genomic instability in precursor lesions is supported by evidence of individual chromosome aneuploidy, which can be demonstrated in tissue sections by interphase cytogenetics with fluorescent, centromere-specific probes. Further, presence of intra-tumoral clonal DNA content heterogeneity is confirmed by cytogenetic studies showing co-existing near-diploid chromosome number modes in many tumors with hyperdiploid stemlines. Karyotypic stemline analyses imply *polyploidization* events are an important mechanism of clonal evolution leading to genetic heterogeneity. Recent studies also demonstrate predictable relationships between cytophotometric and karyotypic alterations, as well as between cytophotometric ploidy and molecular level events. Therefore, we conclude that cytophotometrically detectable DNA content anomalies may precede unequivocal morphologic transformation in breast neoplasia. Moreover, clonal DNA content evolution via endoreduplication may not only accompany biologically critical steps in histologic progression of breast tumors, but may also be reflected in DNA histogram patterns.

Key words: Breast carcinoma, DNA analysis, neoplastic progression

Progression of neoplastic disease is caused by an accumulation of structural genetic aberrations which, in aggregate, result in a malignant and eventually metastatic phenotype through abnormal gene expression [1]. There are several important corollaries of this theory. First, neoplasms will be genotypically heterogeneous, since step-

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wise accumulation of abnormalities results in admixture of subclones. Second, pathologically or clinically recognizable steps of neoplastic progression (such as host invasion or drug resistance) are associated with causal genetic anomalies. Finally, the biological extent of disease progression, and thereby prognosis, theoretically reflects the sum total of causal genetic changes.

Genetic pathology in neoplasia may be studied at the molecular, chromosomal (karyotypic), and cellular (DNA content) levels, each of which is associated with its own rapidly developing technologies. For the most part, however, these technologies have been employed individually and separately to evaluate neoplastic diseases. Despite the logical inference that genetic aberrations at the molecular level will eventually be reflected at the chromosomal or cellular levels, the nature of such relationships is incompletely defined. Furthermore, most evaluations of genetic pathology, particularly in neoplasia of the breast, have not been performed in the context of histopathologic disease progression. Thus, the biological relevance and inter-relationships between genetic lesions observed at various technical levels of observation remain largely unresolved in breast neoplasia.

Cytophotometric DNA content (*i.e.*, ploidy) determinations, although widely evaluated in clinical and research settings, typify these limitations. Two-thirds to three-fourths of invasive breast carcinomas are characterized by cytophotometrically detectable clonal DNA content abnormalities, or so-called "DNA aneuploid" populations [2]. Innumerable publications have established relationships between DNA aneuploidy and disease outcome, although simultaneous associations with poor differentiation and/or high proliferative fraction raise doubts concerning the actual prognostic significance of ploidy [2]. It is noteworthy that a variety of abnormal DNA content patterns are cytophotometrically defined, including hypodiploid, hyperdiploid, tetraploid, and hypertetraploid histogram types—implying either divergent mechanisms or sequential evolution of aneuploid clones during neoplastic progression. Few studies, however, evaluate the clinical significance of various aneuploid subsets. Moreover, the need to perform DNA quantitation on disaggregated tissue samples has obscured relationships between clonal DNA content and pathologic features of disease progression. This is because suspensions or smears of dissociated neoplasms mix benign, host-derived cells with neoplastic populations from various components of the tumor, including *in situ* and invading neoplastic components.

We will review the accumulating literature which addresses relationships between cytophotometric DNA aneuploidy and histologic disease progression, as well as karyotypic genetic changes in breast neoplasia. Our objective is to define the sequence, biological relevance, and pathological correlates of clonal DNA content aberrations. Abnormal DNA content may thereby be understood more completely in the context of an endpoint in translational studies of breast tumor development and behavior.

It must be noted before starting that genetic analyses of breast neoplasia at any level are complicated by the well-documented clinical, pathological, and epidemiological heterogeneity of this disease. Various disease subsets are not necessarily analogous with respect to genetic pathology or even mechanisms of genetic progression. This problem is underscored by the apparently indirect relationship between proliferative breast disease (PBD) and malignant neoplasia [3]. Lengthy natural history of breast tumors combined with obvious problems in serial sampling of breast tissue add to the difficulty of establishing and studying precursor lesions. Finally, the microanatomical architecture of the breast duct lobule complicates microdissection approaches to selective analyses of both precursor and "evolved" neoplastic populations.

KARYOTYPE VERSUS DNA CONTENT IN BREAST NEOPLASIA

Chromosomal anomalies in neoplastic cells may be numerical or structural. The former result from mitotic malsegregation, resulting in individual aneusomies, or from endoreduplication (polyploidization), resulting in gross DNA content aneuploidy. The causes and mechanism(s) of structural chromosome rearrangements in solid tumors are poorly understood. They are generally unbalanced, resulting in net change (commonly loss) of genetic material. This is documented by the large number of allelic losses demonstrated in adult solid tumors of various types, including breast carcinoma [4].

Until recently, karyotypic analyses of human breast tumors have been largely unrewarding. Metaphase spreads have a low rate of success in solid tumors and are limited by obvious sampling artifacts. Moreover, in breast carcinoma they are characterized by extreme complexity involving most chromosomes, and by considerable inter-tumoral variability. To a great extent, this reflects the genetic heterogeneity of this tumor system, as previously noted. It also implies a significant frequency of non-causal, or so-called "random," genetic aberrations. Combined, these factors have frustrated attempts to establish common denominators of breast tumor development at the level of individual gene loci. Establishing a sequence of clonal genetic progression is additionally complicated by the striking intratumoral heterogeneity repeatedly documented in solid tumor karyotypic analyses [5]. Such observations, it should be noted, stand in disturbing contrast to cytophotometric analysis, which are typically characterized by one or two dominant "clonal" populations.

The landmark studies of Dutrillaux *et al.* [6] have, in large part, reconciled the cytophotometric and karyotypic pathology of human breast neoplasia. These authors compared the flow cytometric DNA index of numerous breast tumors to modal chromosome number and total number of rearrangements. They observed three distinct karyotypic subsets: tumors with near-diploid (<50) modal chromosome number (n = 32), tumors with "hyperdiploid" (>50) modal chromosome number (n = 48), and tumors with both near-diploid and hyperdiploid modes (n = 33). With respect to frequency of hyperdiploid stemlines, data correlate well with flow cytometric studies of breast carcinoma, which report abnormal DNA content in approximately two-thirds to three-fourths of cases. Interestingly, the mean chromosome number (71) of hyperdiploid cases reported by these authors corresponds to a flow cytometric DNA index of approximately 1.75, similar to the mean DNA index of DNA aneuploid breast tumors frequently documented in the cytometry literature.

However, Dutrillaux *et al.* [6] reported the presence of a near-diploid mode in approximately 60% of tumors, and the cytometry literature reports show a 20–40% incidence of diploid-range histograms. This discrepancy is explained by the inability of conventional flow cytometric analyses to resolve diploid-range stemlines from benign, host-derived cells that contaminate suspensions of dissociated neoplasms. Thus, all flow cytometric DNA histograms contain a diploid-range population. In conventional histogram interpretation algorithms, however, this peak is generally assumed to be derived exclusively from host-derived nuclei. Extensive multi-site sampling protocols, however, can identify neoplastic foci with unimodal diploid-range DNA histograms in most breast tumors [7]. In addition, our laboratory has demonstrated the presence of diploid-range neoplastic populations in approximately 40% of DNA aneuploid breast carcinomas using multiparametric analysis of intact cell suspensions (see Table I) [8]. This may be accomplished by computer "gating" of populations labeled for cytokeratin, a cytoplasmic marker of epithelial differentiation. Both karyotypic and cytophotometric analyses thus demonstrate that near-diploid clones are present in many, if not most, breast carcinomas with highly aneuploid clones.

Dutrillaux and colleagues [6] made two other observations worthy of emphasis. First, so-called "near-diploid" cells are often profoundly abnor-

TABLE I. Cytometric Versus Karyotypic Stemline Analysis

	Near Diploid Only	Near Diploid + Hyperploid	Hyperdiploid Only
Karyotype* (n = 113)	32 (28%)	33 (29%)	48 (42%)
Flow Cytometry (n = 168)	60 (36%)	38⁺ (23%)	70 (41%)

*[6] Dutrillaux *et al.*

⁺≥20% of all cytokeratin + events in diploid range in G_0/G_1 population

mal from a karyotypic standpoint. Modal chromosome numbers less than 32 are described by these and other authors [9]. Cytophotometric DNA indicies of corresponding clones, however, rarely if ever fall below 0.85, since hypodiploid karyotypes are primarily the result of rearrangements with limited net DNA loss (*i.e.*, as opposed to true chromosomal monosomy). Due to technical limitations of conventional cytophotometry, neoplastic populations with a DNA index of 0.8–0.95 are difficult to resolve from diploid host cells, accounting for the relative lack of hypodiploid cases compared to cytogenetic analyses [10]. Thus, karyotypic heterogeneity of cytophotometrically diploid-range cases may account for the variable clinical outcome of breast tumors with "normal" DNA content.

The other important observation made by Dutrillaux *et al.* [6] is that the appearance of hyperdiploid clones correlated with decreased chromosome number, and thus an increasing number of rearrangements within the near-diploid stem line. It has been proposed by these and other investigators [9] that hyperdiploid clones are initiated by endoreduplication of near-diploid precursors. Endoreduplication is believed to be driven by failure to undergo mitosis after DNA synthesis (or so-called "tetraploidization"). This observation suggests that DNA aneuploid populations observed cytophotometrically are more genetically "evolved" on average than diploid-range stemlines, possibly accounting for the often-reported adverse prognostic significance of DNA aneuploidy in breast carcinoma. Empirical observations in flow cytometric DNA histograms lend support to the notion of ancestral relationships between concurrent stemlines detected cytophotometrically. In our laboratory, DNA hypodiploid breast carcinomas are often accompanied by a DNA hyperdiploid population. In all such cases [8] the DNA index of the hyperdiploid stemline is a nearly exact multiple of the hypodiploid population, suggesting the hyperdiploid clone arose by clonal expansion after a hypodiploid progenitor doubled its genome. Analogously, DNA tetraploid (DNA index = 2.0) breast carcinomas are accompanied in many cases by a diploid-range stemline (DNA index = 1.0).

Following endoreduplication, genetic progression may proceed with more chromosomal rearrangements, accounting for stemlines having DNA indices between 1.2 and 1.6, followed by additional endoreduplication events (accounting for hypertetraploid DNA stemlines). Clinicopathologic associations documented in the cytometry literature also provide evidence in favor of this genetic progression scenario. First, neoplasms with hypertetraploid DNA content have been reported to be more clinically aggressive than hyperdiploid cases, a finding compatible with a greater degree of genetic evolution [11]. In addition, DNA tetraploid breast carcinomas are less aggressive than hyperdiploid cases [2]. A plausible explanation for this is that endoreduplication in tetraploid neoplasms occurrs early in the course of genetic evolution, before extensive genomic rearrangement.

In summary, accumulated cytogenetic and cytophotometric data are largely reconcilable and seem to suggest breast tumors evolve *genetically* through chromosomal rearrangement with net genomic loss, leading to one or more endoreduplication/polyploidization events (Fig. 1). This does not account for genetic events at the molecular level which accompany this process, nor does it address the factors which lead to or favor clonal expansion and eventual clonal dominance. Finally, we have yet to address this hypothesis in the context of morphologically recognizable steps in tumor progression.

MORPHOLOGICAL VERSUS GENETIC PROGRESSION IN BREAST NEOPLASIA

The requirement for tissue dissociation has largely obscured the morphological correlates of genetic events in breast tumor progression, accounting for the relative dearth of literature addressing cellular (or molecular) level genetic alterations in preneoplastic or preinvasive breast lesions. Anecdotal reports in the literature suggest genetic instability preceeds unequivocal malignant transformation. Our group and others [12–15] have identified cytophotometric DNA aneuploidy (using Feulgen-stained smears or sections) in at least some examples of atypical hyperplasia (AH). These findings imply that risk for subsequent invasive carcinoma associated with some forms of PBD reflect a partially transformed state, or true dysplasia. This interpretation is in accordance with the lengthy natural history of breast neoplasia. It does seem to contradict other epidemiologic features of PBD,

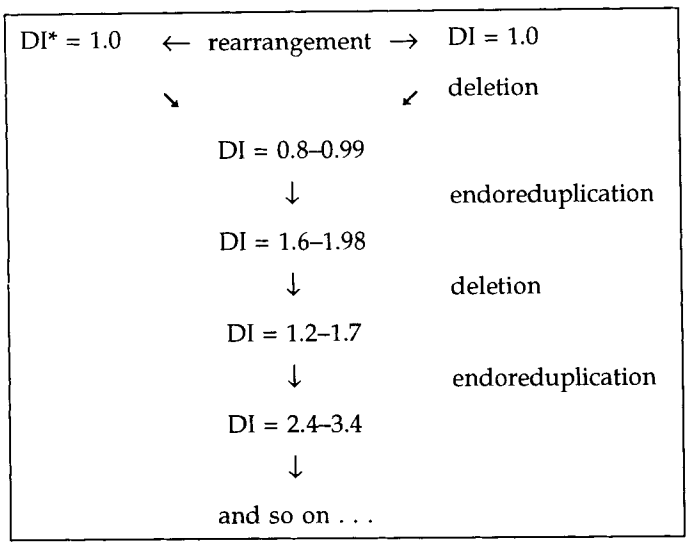

Fig. 1. Proposed sequence of clonal ploidy evolution as a function of genetic progression.

*DI: DNA Index

namely the lack of anatomic association between foci of PBD and subsequent malignant neoplasms [3].

Although image cytophotometric technology, particularly in tissue sections, is subject to criticism on the basis of selection bias or nuclear slicing artifact, the notion of genetic instability in "premalignant" breast lesions is buttressed by occasional reports of abnormal karyotypic analyses putatively obtained from "benign" epithelial proliferations [16]. From a histomorphologic standpoint, the presence of genetic pathology in at least some examples of PBD would be in keeping with the well-documented, continuous histologic spectrum which characterizes intraepithelial proliferations of the breast. Certainly additional objective data concerning genetic features of PBD would be of considerable value with, of course, the caveat that some breast carcinoma subsets appear to evolve without pathologic evidence of widespread or "atypical" intraductal proliferation.

In contrast to PBD, there is uniform agreement that intraductal carcinoma [ductal carcinoma *in situ* (DCIS)] is characterized by unequivocal genetic pathology, including clonal abnormalities of DNA content. This may be readily inferred from the cytologically bizarre appearance of many DCIS lesions. Indeed, our image cytophotometric analyses of DCIS have revealed cases with apparently hypertetraploid populations [15]. In contrast, abnormal DNA content in atypical proliferative lesions was limited to near-diploid or near-tetraploid DNA content. The presence of hypertetraploidy in some DCIS is a noteworthy finding in view of the genetic progression hypothesis we outlined; it suggests that some breast lesions may be characterized by an "advanced" state of genetic progression before host invasion, much less metastasis.

We do not mean to imply that *in situ* and invasive breast lesions are karyotypically and

Fig. 2. Resolution of diploid-range neoplastic stemlines using multiparametric DNA analyses. **Top:** Ungated DNA histograms. Both neoplasms display a near tetraploid population and a near-diploid population. **Middle:** Dot plots (FL-2 = Propidium iodide, FL-1 = FITC – cytokeratin). Cytokeratin positive cells are numerous in the diploid-range population of the case on the left (arrow). The case on the right, in contrast, contains few cytokeratin-positive diploid-range events. Vertical line designates a gate established with use of non-immune isotype control. **Bottom:** Cytokeratin-gated DNA histograms. Using the gates established above, the cytokeratin-positive DNA histograms display presence of a diploid-range stemline on the case on the left, but not in the case on the right.

FIGURE 2

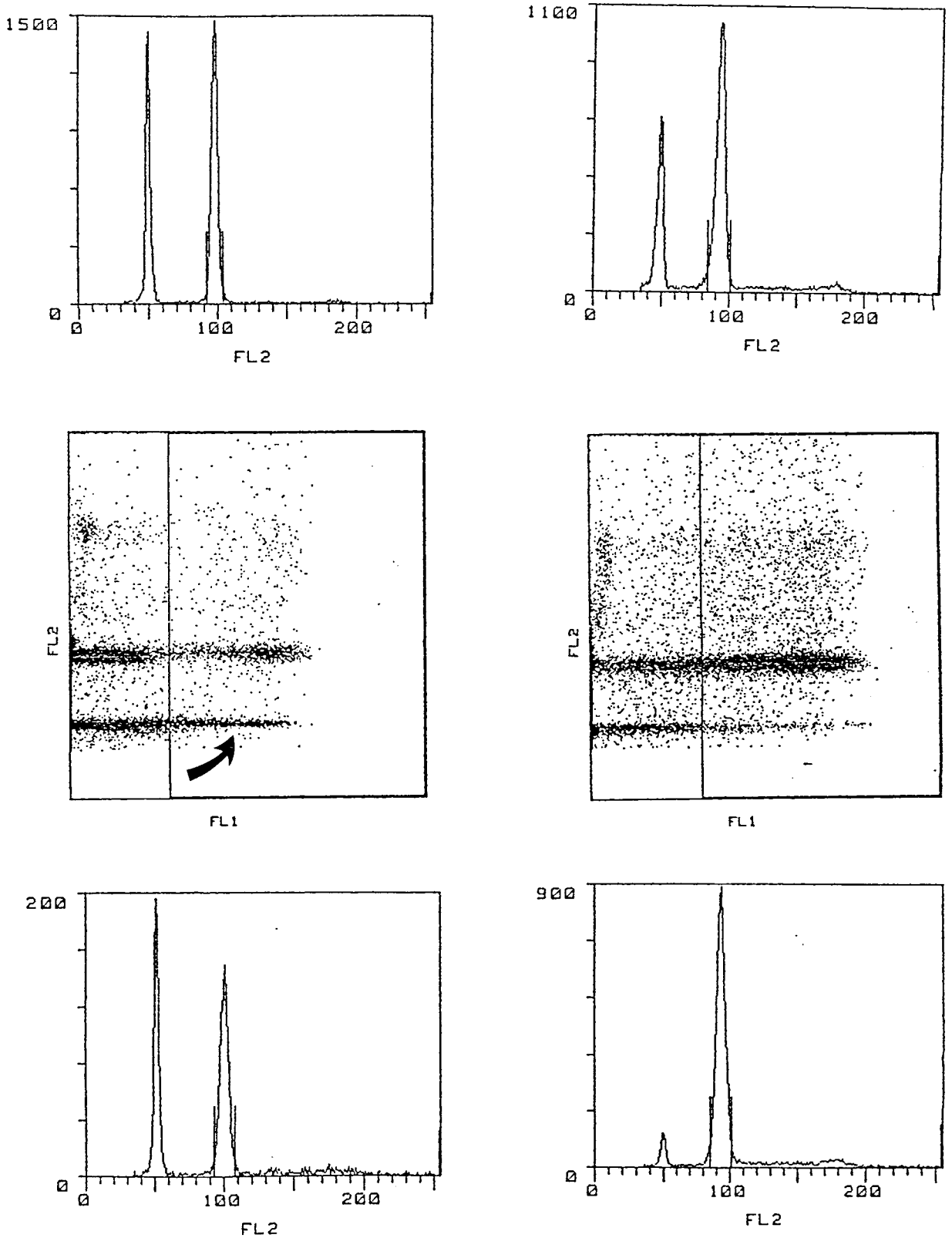
1500
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TABLE II. Pattern of Individual Chromosome Aneusomy as a Function of Disease Progression in One Breast Carcinoma [24]

	CHROMOSOME				
Histology	1	16	17	18	X
Atypical Hyperplasia	0	GAIN	?loss	?loss	0
Intraductal Carcinoma	GAIN	GAIN	GAIN	GAIN	GAIN
Invasive Carcinoma	GAIN	GAIN	LOSS/GAIN	LOSS/?gain	LOSS

cytophotometrically identical. Both types of analyses have, in fact, revealed substantial differences between pre-invasive and invasive carcinomas. First, Nielsen *et al.* [17] reported that the modal chromosome number of invasive lesions is lower than *in situ* lesions, compatible with the working hypothesis of genetic progression outlined earlier. Flow cytometric studies, moreover, report DNA aneuploid populations in 38–46% of DCIS, [18–20] significantly less than the 60–75% incidence of DNA aneuploidy repeatedly demonstrated in invasive lesions. It is not entirely clear, though, whether this difference reflects less frequent endoreduplication in DCIS or merely a lack of clonal expansion and/or dominance following this event. The latter phenomenon is more difficult to demonstrate flow cytometrically in DCIS lesions when compared to more cellular invasive tumors.

Due to obvious changes required in cell phenotype, the initiation of host invasion would almost certainly be accompanied by, or follow, significant molecular level genetic alterations. Whether such alterations are reflected at the cellular level is, however, largely unexplored. Using image cytophotometry of Feulgen-stained intact tissue sections, our laboratory observed frequent DNA content shifts between the intraductal and invasive components of breast tumors [21]. These data are supported by comparing flow cytometric DNA breast tumor histogram patterns with histopathology. We have observed that neoplasms with heterogeneous DNA content (*i.e.*, multiple stemlines) are significantly more likely to harbor a prominent intraductal component or mixtures of histologic patterns [22]. Such data do not necessarily suggest that either host invasion or metastasis is predictably associated with cytophotometrically detectable evidence of clonal evolution [23]. Instead, it seems that neoplasms which lack a phenotypically dominant population are characterized by clonal DNA content heterogeneity. This interpretation is also supported by a correlation between DNA content heterogeneity and histopathologic growth pattern (*i.e.*, grade) variability [22].

Using interphase cytogenetic analysis with centromeric fluorescent-labelled probes, our group has collected preliminary data which corroborate the mechanisms of genetic evolution implied by cytophotometric ploidy determinations [24]. Table II shows selective interphase genetic analysis of a breast tumor characterized by focal atypical hyperplasia and DCIS near the edge of the invasive component. The area of AH displayed trisomy for chromosome 16, but counts for chromosomes 1, 17, 18, and X were near normal. In contrast, areas of DCIS showed gains in signal counts of all probes tested, suggestive of an endoreduplication event. Finally, cells in the invasive region not only retained signal gains for probes to chromosomes 1, 16, and 17, but also displayed populations that had signal loss compatible with monosomy for probes 18 and X. Data such as these are anecdotal and preliminary, but appear to provide additional support to cytometric and karyotypic data which distinguish intraepithelial from invasive neoplastic populations in the human breast.

Finally, it is by no means clear that hyperdiploid clones will inevitably become the dominant population in every breast carcinoma. Recall that

Dutrillaux *et al.* [6] noted near-diploid stemlines in approximately one-half of hyperploid neoplasms. Near-diploid clones may conceivably continue to "evolve" after giving rise to hyperploid stemlines via endoreduplication, thereby becoming the most aggressive (and numerically dominant) clone. This scenario may explain the presence of diploid-range stemlines in metastatic lesions or the clinical recurrences of DNA aneuploid primary breast carcinomas noted by various authors. It further emphasizes that, on occasion, diploid-range stemlines numerically dominate accompanying aneuploid clones in cytokeratin-gated DNA histograms.

EPILOGUE

It is fashionable in some quarters to discount the biological relevance of cellular level genetic pathology and focus exclusively on molecular level aberrations. We can hardly dispute that neoplasia is, in essence, a disease of abnormal gene expression. However, the evidence that cellular level genetic events such as endoreduplication are relevant to solid tumor progression seems indisputable. We would argue that clinicopathologic correlates of these events largely remain to be defined, and that refinement of cytophotometric technology (particularly improved analysis of near-diploid populations) will further our understanding of genetic progression in breast neoplasia. A more detailed approach to histologic microdissection will be required to gain significant insight into the relationship between chromosomal or clonal DNA content anomalies and pathological development of breast neoplasia. It goes without saying that similar considerations apply equally to molecular level analyses.

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