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REVIEWS

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CHARACTERIZATION OF BREAST CANCER DNA CONTENT PROFILES AS A PROGNOSTIC TOOL

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Worldwide, breast cancer in women remains to be the most common malignancy that in a considerable proportion shows the resistance to genotoxic treatments and poor outcome. Chromosomal instability manifested as aneuploidy represents an integral characteristics of the malignant genotype not only because of the selection of mutated aneuploid sub-clones that stipulate the tumor progression, but also because of the reversible endopolyploidy of tumor cells that serves for the endless maintenance of therapy-resistant tumor stem cells. Therefore, cytometric determination of DNA content in tissue samples for detecting malignancy, monitoring responses to therapy, and prognosing disease outcome needs to be revived. Both flow and image cytometry are most frequently used for generation of DNA content profiles (histograms), interpretation of which, however, may have some caveats. This review presents the major characterization criteria and analysis tools for breast cancer DNA histograms.

***Key Words*: breast cancer, aneuploidy, DNA content analysis, DNA histogram, flow cytometry, image cytometry.**

INTRODUCTION

Breast cancer is among the most common malignancies in women worldwide, and its manifestation considerably varies based on cell proliferation state, karyotype and nuclear DNA content testifying aneuploidy. There is also the well-known cancer hallmark such as genome instability resulting in aneuploidy-linked elevated mutagenicity [1, 2]. The latest research conducted on cell cultures and patient tumors as well showed that stemness of tumor cells is tightly associated with polyploidization, the process that results in the numerical gain of the whole set of chromosomes [3–5]. This property stipulates the resistance to anti-cancer treatments and poor outcome [6]. It is supposed that the new treatment strategies aimed to eradicate the highly evolvable polyploid cells would help to maintain tumors in a drug sensitive state [7]. In light of this newly emerged aspect in tumor cell biology, cytometric determination of DNA content in tissue samples "gets a second breath", thus becoming a very important and demanding method not only for detecting malignancies, but also for assessing responses to therapy and disease outcome. Both flow and image cytometry are most frequently used for generation of DNA content profiles (histograms). Their interpretation, however, may have some caveats, and therefore needs to be univocal and accurate. The aim of this review is to present the major characterization criteria and analysis tools for breast cancer DNA histograms that together can be used for gaining prognostic and treatment response information.

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Abbreviations used: 7-AAD – 7-aminoactinomycin D; BRCA1 and 2 – breast cancer associated, types 1 and 2; CCD – charge-coupled device; CIN – chromosomal instability; DAPI – 4′,6-diamidino-2-phenylindole; DI – DNA index; HER2 – human epidermal growth factor receptor, type 2; IF – integrated fluorescence; IOD – integrated optical density; MAD2 – mitotic arrest deficient, type 2; NANOG – homeobox transcription factor; OCT4 – octamer-binding transcription factor 4; PCNA – proliferating cell nuclear antigen; SOX2 – SRY (sex determining region Y)-box 2; SPF – S-phase fraction; TOP2A – DNA topoisomerase, type 2-alpha.

ANEUPLOIDY AND BREAST CANCER

Chromosomal instability (CIN) is the main reason of aneuploidy in cancer cells [8, 9]. Aneuploidy implies a condition in which the chromosome number is not exact multiple of the haploid karyotype. As a result of improper segregation of chromosomes during division of mother cell, two daughter cells become aneuploid. For example, one daughter cell has chromosomal gain (2n+*x*), whereas another daughter cell has chromosomal loss (2n–*x*). However, in some other cases chromosomal gains and losses may not necessarily be concordant. The fact that cells in the majority of aneuploid tumors have 40–60 chromosomes (while diploid cells have 46 chromosomes) indicates that the accumulation of chromosome imbalances generated by the sequential loss and gain of single chromosomes through CIN may be the most common pathway to aneuploidy [9]. Missegregation of chromosomes can be due to defects in the kinetochore-microtubule attachments and dynamics, centrosome number, spindle-assembly checkpoint, and chromosome cohesion [2]. The disruption of multiple genes and pathways is believed to play in the aforementioned defects [10].

Aneuploidy that was first proposed as a cause of cancer by D. Hansemann and Th. Boveri at the turn of 20th century was based on observations of multipolar cell divisions and bipolar asymmetric segregation of chromosomes in large polyploid cells [11, 12]. With the beginning of the molecular biology era the most attention was switched to elementary genetic and molecular changes in cancer, while during the last two decades aneuploidy was again recognized as a main driver of cancer progression, and from some views,

aneuploidy together with CIN is considered as an origin of cancer [13–16].

Both structural and numerical chromosomal abnormalities, that can currently be detected by conventional karyotyping and other more advanced cytogenetic techniques such as fluorescence *in situ* hybridization, spectral karyotyping and array-based comparative genomic hybridization, are characteristic of cancer genomes. Contrary to structural chromosomal abnormalities, the role of numeric chromosomal abnormalities (termed also as "whole-chromosome aneuploidy" or simply "aneuploidy") in tumor development is much less well-understood largely because of difficulties to identify tumorigenesis related genes on aneuploid chromosomes [2, 17]. Although at least at low frequency aneuploidy is likely to promote tumorigenesis, it is currently observed in most tumors including breast cancer (≈ 90% of solid tumors and 85% of hematopoietic malignancies) [16]. Understanding the role of aneuploidy specifically in these tumors is of great importance to uncover pathogenesis of disease and develop new strategies for treatment [2, 18, 19] and prognostication [20].

In breast cancer, the spindle-assembly checkpoint appears to be affected causing CIN [9]. Expression of MAD2 gene is essential to control this checkpoint [21]. Mutations in BRCA1 and BRCA2 genes can also contribute to CIN. While the former gene is required for the proper spindle checkpoint function [22], the latter gene is required for proper cytokinesis [23]. Of 200 breast cancer cases, there are ≈ 15% of tumors having cells with 46 chromosomes (these cells are not free of translocations, inversions, deletions and/or additions), 70% of tumors having cells with > 46 but ⩽ 68 chromosomes, and ≈ 15% of tumors having cells with ⩾ 69 chromosomes [16]. Among those chromosomes that can be affected [24–27], chromosome 17 is affected most frequently (both numerical and structural abnormalities in this chromosome are common in breast cancer [27]). The majority of breast tumors (54%) have whole-chromosome 17 aneuploidy, of which 14% are monosomic and 86% are polysomic [28]. Numerical aberrations of chromosome 17 (either gains or losses) are linked to breast cancer initiation and progression, and possibly to treatment response [27]. Notably, this chromosome contains such genes as HER2, BRCA1, P53, and TOP2A, whose alterations are of importance in breast cancer pathogenesis [27].

Polyploidization that occurs due to unscheduled whole-genome duplications has been proposed to constitute an important step in the development of cancer aneuploidy since it holds the probability to amortize consequences of chromosome damage or even loss [29, 30]. Transient and reversible polyploidy works as a pro-survival mechanism after genotoxic treatment by activating pluripotency and self-renewal cassette (OCT4/SOX2/NANOG) characteristic for most aggressive tumors [6] and giving rise to resistant survivals after de-polyploidization [3,4].

In breast cancer cell lines as well as in breast cancer primary specimens, ionizing radiation can induce dose-dependent polyploidization together with manifestation of breast cancer stem cell phenotype in polyploid cells [5]. Self-renewal activation in polyploid cells displays the property to overcome therapy-induced cell senescence [31]. Moreover, the development of rare polyploid cells in normal senescent fibroblasts correlates with self-renewal signaling, the fact that suggests that these polyploid cells can be potential cancer candidates [32]. Because of the definite role of polyploid cells in therapy resistance and tumor repopulation after therapy, they are currently considered as critical drug targets for tackling cancers [7]. Interestingly, tetraploidy is more frequent in BRCA2-mutated than in sporadic breast carcinomas, the fact that prompts to propose that BRCA2 mutations can facilitate polyploidization through cytokinesis failure as well as formation of chromosome bridges [33].

BASIC DEFINITIONS IN DNA CYTOMETRY

DNA histogram is the distribution of the frequency of integrated optical density (IOD) or integrated fluorescence (IF) values obtained by cytometric measurements of cells stoichiometrically stained for DNA. In DNA histogram ***diploidy*** is shown by the position of the modal value of the peak corresponding to G_0-/G_1-phase cells having diploid chromosomal set 2n (this position is usually expressed as 2c). In case if the modal value of the peak differs from that of normal diploid cells (< 2c or > 2c, excluding 4c), one could conclude that sample contains cells with ***aneuploidy***. The term "aneuploidy" also implies a biological phenomenon (concisely considered in the previous section). Appearance of additional peak(s) (4c, 8c, 16c, etc.) corresponding to cells with geometrically doubled set of chromosomes is characteristic to ***polyploidy***. In case if DNA distribution in the examined sample cannot be differentiated from that of normal (resting, proliferating, or polyploidizing) cell population, there could be ***euploidy***. Diploid (2c) and tetraploid (4c) tumors are often considered as euploid. ***DNA stemline*** is the G_0-/G_1-phase cell fraction of proliferating cell population with a unique chromosomal outfit. In DNA histogram stemline shows a distinct peak (*X*c) with a second doubling one (2*X*c) [20].

TYPING OF DNA HISTOGRAMS FOR BREAST CANCER PROGNOSIS (Auer's classification)

Based on the analysis of Feulgen method-stained tumor biopsy material, Auer *et al.* [34] first proposed classification of breast cancer DNA histograms. These histograms are empirically divided into 4 groups. The ***type I histogram*** is characterized by a single distinct modal DNA value in the diploid (or near-diploid) region (2c) of normal cells with only a minor fraction of cells exhibiting higher DNA values (> 2c). The ***type II histogram*** has either a distinct peak in tetraploid (or near-tetraploid) region (4c) of normal cells or a couple of well-defined peaks in 2c and

4c regions, although the presence of the minor peak corresponding to octoploid cells (8c) is also possible (< 5%). There are no at all or there are few cells that have DNA amounts corresponding to the DNA synthesis phase of normal cells (< 5%). Although ***type III histogram*** like type II histogram often shows two peaks in 2c and 4c regions, it is similar to that of proliferating normal cell population with DNA values scattered between the normal 2c and 4c region. There is a sizable number (> 5%) of cells with DNA amounts similar to those of normal S-phase cells. Later, it has been recognized that this type of histogram is most difficult to delineate [35, 36]. The ***type IV histogram*** is characterized by the large fraction of aneuploid tumor cells with highly increased and scattered DNA values significantly exceeding the normal 4c region. There is a distinct correlation between the type of DNA distribution pattern of breast cancer and the survival time of the individual. Histograms of types III and IV are indicative of worst prognosis. Auer *et al.* [37] also demonstrated that metastases and primary tumors shared the same DNA histogram pattern. Thus, while tumors exhibiting DNA values within the limits of normal tissue correlate with favorable prognosis, tumors with increased and scattered DNA values are indicative of poor prognosis.

ADDITIONAL PARAMETERS FOR SUCCESSFUL EVALUATION OF BREAST CANCER DNA HISTOGRAMS

Opfermann *et al.* [36] noted that Auer's classification is limited in that "it did not take into account general important biologic parameters which could alter the biologic behavior of cells" (assuming the necessity to estimate individual cells based on the phase of mitotic cycle, and cell population growth fraction). In this regard, the authors proposed to introduce two additional parameters such as ploidy balance and proliferation index that can be derived by distributing breast cancer biopsy cells in 10 different ploidy intervals ranging from 2c to > 8c. This way of histograms classification is free of uncertainties related to the statements such as "near-diploid" or "near-tetraploid" used in the definition of Auer histogram patterns. The ***ploidy balance*** is simply calculated by subtraction of the percentage of aneuploid cells (2.5c, 3c, 3.5c, 5c, 6c, 7c, and > 8c) from the percentage of euploid cells (2c, 4c, and 8c). Accordingly, the difference can fluctuate from +100% (all cells are euploid) to −100% (all cells are aneuploid). ***Proliferation index*** is the percentage of cells between major and related peaks (regardless of the ploidy level of the major peak). Although these parameters demonstrate that aneuploidy and cell proliferation does correlate with tumor aggressiveness as derived from the "tumor aggressiveness triangle", at least 15% of the patients failed to be correctly classified in terms of long *vs.* short survival time [36]. Therefore, additional parameters should be taken into consideration.

There is more precise approach based on calculation of ***DNA index*** (DI) which is defined as the ratio of the modal DNA value of the tumor cells to the modal DNA value of the internal control cells (2c). This parameter that was first introduced by Barlogie *et al.* [38, 39] is being actively used since the early 80th of the last century when the flow cytometry era started to bloom. Fernö *et al.* [40] proposed to categorize the ploidy of breast cancer cell populations based on DI distribution as follows: hypodiploid (DI < 0.95), diploid (DI = 0.95–1.04), near-hyperdiploid (DI = 1.05–1.14), hyperdiploid (DI = 1.15–1.91), tetraploid (DI = 1.92–2.04), hypertetraploid (DI ⩾ 2.05), and multiploid (in case if DNA histogram has ⩾ 2 peaks corresponding to aneuploid/polyploid cell population). This type of DI categorization compared with those proposed by other authors also includes the class of near-hyperdiploid tumors whose cells can yet be distinguished from diploid (2c) cells.

Determination of DI is often supplemented with detection of cell proliferation level by measurements of ***S-phase fraction*** (SPF), which is expressed as a percentage of tumor cells in DNA synthesis phase of the cell-cycle. This parameter correlates with proliferative activity of tumor cells. At present, quantification of SPF is usually performed by means of special mathematical models in computer programs for cell-cycle analysis. Notably, the earliest method that was actively used for SPF quantification is a planimetric method of Baisch *et al*. [41] assuming that the S-phase compartment constitutes a rectangle distribution between the modal values of G_0/G_1 and G_2 peaks. Flow cytometrically measured SPF correlates with the labeling index measured by autoradiography of tumor biopsies pulse-labeled with tritiated thymidine [42, 43].

SPF parameter appears to be prognostically very informative for some human cancers, including breast cancer [44, 45]. Starting from the middle of 80th of the last century, SPF became an object of extensive exploration for delineating of its prognostic value in breast cancer. In one of the earliest works on this issue, Hedley *et al*. [46] demonstrated that disease-free survival with SPF ⩽ 10% was significantly longer than that with SPF > 10%, and the latter SPF value strongly correlated with high tumor grade and abnormal DI but weakly correlated with nodal, hormone (estrogen) receptor, and menopausal status. The fact that SPF values are significantly higher in aneuploid tumors than in diploid tumors is confirmed in other reports [47–51]. According to Clark *et al*. [48], SPF is highly predictive of disease-free survival in patients with diploid tumors but does not provide additional prognostic information for aneuploid cases. Sigurdsson *et al.* [49, 50] proposed to gain prognostic information by dividing the SPF into 3 prognostic categories: low (< 7.0%), intermediate (7.0–11.9%) and high (⩾ 12%). These categories allow grouping the patients according to their level of risk. The risk of death or recurrence for diploid and non-diploid cases is up to 50% higher for the high S-phase category than for the intermediate category, and approximately 50% higher for the intermediate category than for the low category [49]. In this

situation, ploidy does not provide additional prognostic information with reference to any of S-phase categories. In spite of different techniques (e.g., whether the tissue is fresh, frozen, or paraffin-embedded, etc.) and cut-points (e.g., whether the SPF is di- or trichotomized, etc.), correlations between SPF and other prognostic markers are relatively consistent across studies: higher SPF is generally associated with worse tumor grade, negative receptor status, larger tumors, and positive axillary nodes [52]. Moreover, higher SPF is generally associated with worse disease-free and overall survival in both univariate and multivariate analyses [52].

Since polyploidy can correlate with aggressiveness of cancer, the detection and analysis of polyploid cells in DNA histograms is of importance. Diploid (including near-hyperdiploid) and hyperdiploid tumors are most common comprising about 45 and 36% of all breast cancer ploidy types, respectively [40]. Favorable prognosis is more characteristic for diploid and near-hyperdiploid cases (low-risk group) [40, 53, 54], though Fernö *et al.* [40] claim that prognosis for near-hyperdiploid tumors that comprise only about 4.0% of all cases is even somewhat better than for diploid tumors. As for hypodiploid, hypertetraploid, and multiploid cases, all of them fall into the category of worse prognosis (high-risk group). Interestingly, in this group hypodiploid cases can be most aggressive [40, 55]. Hyperdiploid cases are probably to fall between low- and high-risk groups [40, 54–57]. However, there is some controversy regarding prognosis of tetraploid cases (≈ 5.0% of all cases). Some authors reported better prognosis for tetraploid cases [53, 56–58] than other authors did [40, 54]. Notably, Stål *et al*. [56] reported even a slightly longer survival in tetraploid cases than in diploid cases. Ewers *et al*. [59] found that the recurrence rate in early-stage disease was twice as low among patients with euploid (diploid and tetraploid) tumors as among patients with aneuploid tumors.

Although some investigators did report the lack of prognostic significance of DNA ploidy and/or SPF in breast cancer, those reports, however, are not numerous [52, 60]. In fact, the assignment of DI and SPF can be subjected to a number of various technical pitfalls that may take place starting with specimen preparation/processing and ending with instrument alignment and cell-cycle analysis [52, 61]. Also, tumors may not be usually consistent in their ploidy (there is variation in DNA content between different areas of the tumor) [62]. In this case, examination of tissue samples from several different areas of the tumor may be needed.

FLOW CYTOMETRY *VERSUS* IMAGE CYTOMETRY

Numerical and structural chromosomal abnormalities together can lead to DNA aneuploidy which is quantified cytometrically in a cell population. There is a variety of DNA staining methods using light-absorbing or fluorescent stains that stoichiometrically bind to DNA, so DNA content can be measured based on IOD or IF values. As for staining of DNA with light-absorbing dyes, the Feulgen reaction by using of Schiff reagent is still most popular, although this staining technique was developed almost a century ago [63]. As for fluorescence staining of DNA, there is a variety of nucleic acid fluorescent dyes, and some of them (e.g., Hoechst dyes, DAPI, and 7-AAD) selectively or preferably bind to DNA [64]. Both flow and image cytometry are frequently used for generation of DNA histograms of DNA-stained cell samples, although none of these techniques is free of disadvantages (Table).

Table. Major differences between flow and image cytometry that can affect the quality of assessment of DNA content histograms obtained from cancer samples

Flow cytometry	Image cytometry*
1. Few thousand cells or cell nuclei can be analyzed for a short time (≈ 1 min)	At least few dozen cell nuclei can be analyzed for a short time (≈ 1 min)
2. While performing analysis, morphological assessment of cells is impossible	While performing analysis, morphological assessment of cells is possible
3. Quantification of S-phase cells is relatively precise	If DNA histogram peaks are too broad, additional staining of S-phase cells may be needed
4. More sensitive in detection of near-diploid aneuploidy	Less sensitive in detection of near-diploid aneuploidy unless a larger cohort of nuclei is analyzed
5. Analysis requires cell suspension	Samples can be prepared from cell suspension, tissue imprints or histological sections

Note: *image cytometry implies software-based analysis of IOD from digitalized images obtained by microscope equipped with CCD camera.

Nevertheless, flow cytometry is superior in terms of speedy acquiring a large cohort of cells or cell nuclei in sufficient numbers for precise statistical certainty. Because of this advantage, DNA histogram peaks look sharper (since coefficients of variation of the peaks become lower), and thus S-phase cells can be quantified with a special DNA content/cell-cycle analysis software. Several models have been proposed to estimate S-phase in overlapping populations that may take place in some tumors [43, 65]. Taking into account the aforementioned advantages, flow cytometry is generally well-described and widely used technique [66]. Contrary to flow cytometry, a few hundred cells are usually analyzed by image cytometry resulting in broader DNA histogram peaks (coefficients of variation of the peaks are relatively high). In this regard, the assessment of S-phase seems somewhat problematic, although there could be a good option for solving this situation by specific staining of those cells that proliferate (e.g., using antibodies to proliferation-associated antigens such as Ki-67 and PCNA [67, 68]). While performing analysis, flow cytometry compared with image cytometry, however, is incapable of discriminating non-malignant cells of other types (lymphocytes, granulocytes, macrophages, and some stromal cells such as fibroblasts) unless they are specifically labeled. Using microscope, an experienced operator can easily discriminate

these cells mainly based on size and shape of their nuclei and some nuclear texture features. Thereby, image cytometry-based generation of DNA histograms is less likely to be interfered by "contamination" with unwanted cells. As for detection of aneuploid cell populations, a combination of both techniques is superior to either of them alone [69]. However, none of these techniques can necessarily detect gains or losses of single intact chromosomes or deletions in individual chromosomes especially if a tissue sample contains a very small fraction of aneuploid cells. In general, there is a good correlation in DNA content analysis data between flow and image cytometry [69], although a thorough understanding and appreciation of the methodological problems associated with both techniques may be needed [70].

In 2001 there was the last consensus report on diagnostic DNA image cytometry proposed by the group of prominent European analytical cytologists [20]. With regard to identification of neoplasia and grading of tumor malignancy, this report contains several main clinically-oriented recommendations such as short description of DNA histogram (histogram type), interpretation of DNA histogram concerning the occurrence of DNA aneuploidy and/or the histogram type, prognostic interpretation of DNA histogram, and summarized morphologic/cytometric diagnosis. In that time when this report was written the usual precision of DNA image cytometric measurements should at least allow DNA stemlines to be identified as abnormal (or aneuploid), if they deviate more than 10% from the diploid (2c) or tetraploid (4c) region, i.e., if they are outside 2c ± 0.2c or 4c ± 0.4c.

CONCLUDING REMARKS

Like two decades ago, DNA content profile remains to be a subject of thorough examination to gain the information on prognosis of disease and response to treatment. For this purpose, combination of various characterization criteria and analysis tools for DNA histograms may be needed. Determination of consensus values in interlaboratory comparisons is required.

Finally, it is worth to note the another principally different analytical approach of analysis of DNA-stained cancer specimens that currently shows promising data from diagnostic and prognostic points of view. This is nuclear texture measured by digital image analysis that allows detailing the phenotype by objective classifying of visual and sub-visual changes in nuclear chromatin [71]. Qualitative and quantitative changes in chromatin structure are of importance for understanding the neoplastic process as well as for identifying structural changes that may indicate functional alterations [72]. Nuclear textural features have been proven to be successful in clinics for prognosis in several cancers including breast cancer [73]. However, the nuclear texture analysis should be performed on undistorted nuclei. For this purpose, monolayers of nuclei carefully isolated from the sections of formalin-fixed and paraffin-embedded tissues are usually used [74].

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