Review Article

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Quantitative Pathology: Historical Background, Clinical Research and Application of Nuclear Morphometry and DNA Image Cytometry

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Quantitative analysis of histo- and cytochemical components such as DNA, RNA or chromatin pattern on one hand (cytometry) and the quantitative analysis of geometric non-chemical cell and tissue components (morphometry and sterology) on the other, have developed somewhat independently. Today, many different techniques, such as morphometry, sterology, and static image and flow cytometry are well established and routinely used in diagnostic quantitative pathology. The potential significance of these techniques in the individualization of care in cancer patients include the objective distinction between benign, borderline and malignant lesions, objective grading of invasive tumours, prediction of prognosis, and therapy response.

The first description of cell nucleus was given by Brown in 1833 and the first microscopic description of human malignant tumours by Müller in 1838 (1). Among the first to apply the microscope to the study of human cells was the French microscopist Donné, whose work culminated in an atlas published in 1845 (2). In 1870

in Basel, Miescher isolated nucleic acids from Salmon sperm. As early as 1890 (3), David von Hansemann postulated all cancers are characterised by asymmetrical cell division that ultimately leads to cancer. Sterobe in 1892 (4) found asymmetrical mitoses in regenerative tissues and non-malignant tumours. In 1904, Kohler constructed a monochromatic microscope and described the absorption of ultraviolet light (UV) by the nuclei. In the same year, Dhere in Paris demonstrated nucleic acids ability to absorb ultraviolet light. In contrast to Hansemann, Boveri's (1914) (5) hypothesis on cancer relied on qualititative changes in chromosomes of cancer cells. In 1933 (6), Haumeder proved that cancer cells have nuclei larger than normal, which suggested a higher DNA nuclear content. In 1924 (7), Feulgen produced the chromogenic stoichiometric reaction for DNA, which allowed the measurement of nuclear DNA content in cells on microscopical slides. The work of Caspersson and his colleagues in Stockholm between 1932 and 1939 marked the beginning of modern quantitative cytometry. They combined the observations of Kohler and Dhere with microscopic measurements and determined the amount of DNA in the nuclei. Due to the advent of improved electronic equipment and digital computers in the 1950s and 1960s, rapid DNA cell analysis, cell sorting, and quantitative chromatin pattern analysis could be applied at a much larger scale than before the Second World War.

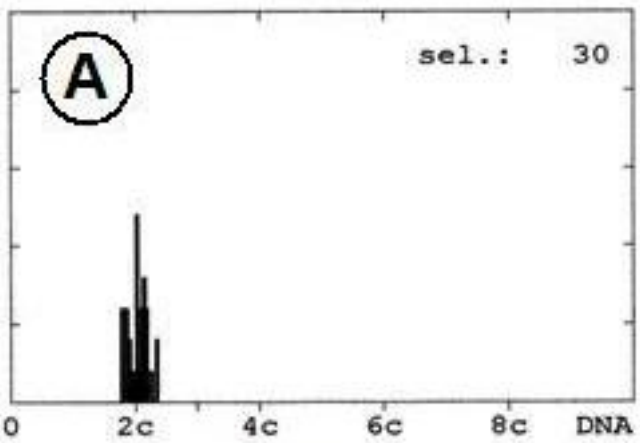
As early as 1925, morphometric analysis started. Jacobi, in 1925 (8), found that the volume of a normal cell doubles before cell division. Heiberg and Kemp, in 1929 (9), were probably the first to substantiate the subjective impression that cancer nuclei are larger than those of normal cells. In the 1950s and 1960s, an increased interest amongst anatomists and biologists gave a strong impetus to morphological and stereological analysis in biomedicine. In the late 1970s and early 1980s, the application of morphometric analysis to pathologically changed tissues became increasingly popular and widely applied, particularly in cancer. Morphometric techniques are fairly simple and inexpensive, but sometimes time-consuming. On the other hand, DNA cytometry is more expensive, but highly reproducible.

The sharp increase in interest in the application of quantitative pa-

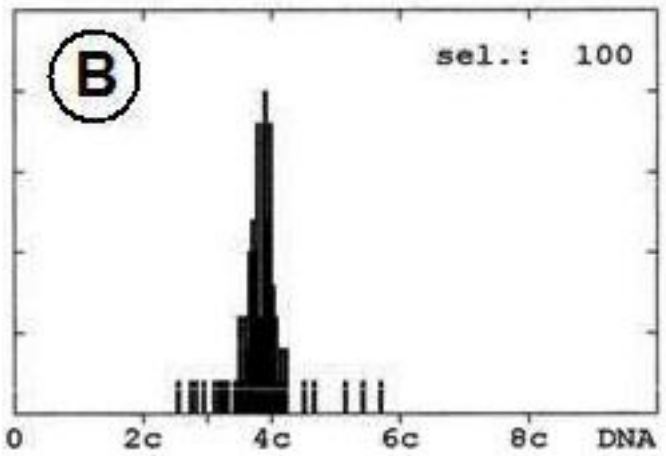
thology to cancer diagnosis and prognosis is mainly due to the following reasons: (a) the increased social demands of quantitation and objectivity; (b) the improvement in, and widespread availability of, adequate technology; (c) the awareness that changes can be detected with quantitative analysis which would otherwise escape observation; (d) the improvement of therapeutic possibilities for cancer patients. Finally, the opinions of pathologists have not always proved consistent or reproducible while quantitative pathological analyses are more reproducible and capable of preventing under- and over treatment. For a detailed historical account the reader is referred to (Koss 1982 (10), 1987 (11), Caspersson 1987 (12), Baak 1991 (13), Mariuzzi and Collan 1995 (14).

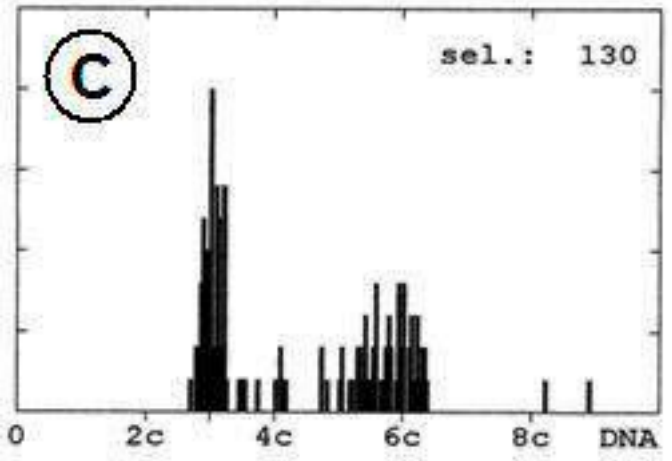
DNA cytometry: Cancer develops through a sequence of cellular events reflected by various degrees of atypia (Brawer 1992 (15)), and numerous reports have indicated that such events are associated with alterations in nuclear DNA contents and cellular morphometric size and shape features (Malinin et al. 1988 (16), Merkel and McGuire 1990 (17), William and Daly 1990 (18).

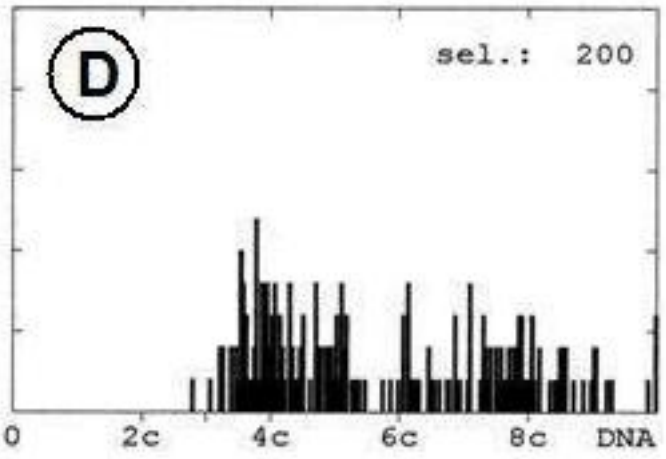
Both flow cytometry and static image cytometry analysis have been used to determine DNA ploidy of cancer. But both of these techniques have some limitations. Flow cytometry cannot be performed successfully when only a small amount of tumor cells are present in the needle biopsy. This is because plain flow cytometry has practically no ability to distinguish tumor from non-tumor cells. Therefore, a small number of non-diploid (aneuploidy) cells may be diluted to insignificance by larger numbers of benign diploid cells (19). In contrast, static image analysis allows determination of ploidy in both cytological smears and tissue sections with relatively small amounts of tumor. Unfortunately, the interpretation of results is still hampered by the lack of standard methodologies (20, 21). Simple (22-25) and com-



(A) Diploid Histogram







(D) Histogram of "New York Skyline". Multiple broad aneuploid peaks of numerous DNA values

plex algorithms (26, 27), and/or classification strategies (28-30) which could make the interpretation of histograms more objective for diagnosis, prognostication, and therapy planning of the neoplasm were created.

Figure 1: (A) There are no values outside the diploid range. Even though the number of cells studied is low, this type of histogram without any evidence of non-diploidy can be considered diploid. (B) Dominant tetraploid peak, only a few nuclei outside the peritetraploid region (3.4c-4.4c). There are no diploid nuclei. (C) Prominent peak at 3c region with a broad peak at 6c that may reflect the proliferative cells of dominant population. (D) Multiple broad aneuploid peaks of numerous DNA values are seen over the whole range of the histogram. (c=haploid DNA content).

On the basis of flow cytometry, Tribukait prepared a theory on the progression of prostate cancer (31, 32). The model is a three-compartment model of ploidy progression describing how a diploid tumor progresses to tetraploid tumours and subsequently becomes aneuploid.

This theory was furnished by repetitive flow cytometric study of FNAB specimens (33). This evidence is much in line with that of Auer in breast cancer

(34) and also supported by the evidence of static image cytometry by Buhmeida and Collan (35), but the early phases may include near diploid cases more often than flow cytometry detects them.

DNA studies have shown that patients with diploid cancers (Figure 1. A) have longer disease-free intervals and survival times than those with non-diploid tumors (Figure 1. B, C, and D) (36). However, they may not be so helpful in predicting stage for an individual patient. The first report on the relationship of DNA ploidy of prostate carcinoma with prognosis appeared in 1966 (37).

It has been suggested that cytological smear preparations are more suitable than tissue sections for determination of DNA content and morphometric parameters such as nuclear shape, size, and texture due to less overlap between cells and between cell nuclei (38). In a multivariate analysis, Forsslund et al (39) showed that DNA ploidy was a better predictor of survival than histological grade and tumour stage. Frankfurt and his colleagues (40) examined 45 patients with prostate cancer and noted that all 11 patients with organ confined cancer had diploid tumors. None of the aneuploid tumors were organ confined.

The most convincing evidence of the prognostic role of DNA content comes from a study by Forsslund and Zetterberg (41) where DNA was measured in a series of patients with a long-term follow-up. Patients who died within 3 years of diagnosis consistently had DNA stemlines at 3c and 6c, whereas long-term survivors (>15 years) had stemlines at 2c and 4c. In the Mayo Clinic prostatectomy series, ploidy was one of the significant predictive factors found in multivariate analysis of tumour characteristics (42).

Several clinical and pathological variables are useful in assessing the prognosis of cancer patients. Therefore, an active search is ongoing for powerful new prognostic and predictive tools capable of identifying high-risk patients who would benefit from individually tailored treatment options (43). As a part of this ongoing search, focus has been recently made on DNA quantification, which might provide useful prognostic information (44). Indeed, abnormalities in DNA ploidy are seen in many human tumors, and determination of ploidy and proliferative activity has been shown to provide prog-

nostic information in several solid tumors (45, 46). While several studies have suggested that DNA ploidy is an independent prognostic factor (47-49) others have reported that DNA content is not associated with clinical outcome (50,51). Part of these discrepant observations might be explained by the inconsistencies and true differences in the technical aspects of recording the DNA contents. Also, it is well known that some cancer tumours consist of many different subpopulations of tumor cells with different DNA content (52,53).

To overcome this problem, the introduction of some other quantitative tools, such as immunohistochemical staining, RT PCR, and DNA microarray etc., might help find biological markers that combined together to form biological models that could help in knowing more about the biology and behavior of cancer.

Aneuploidy is one of the features of cancer cells that distinguish them from normal cells (54). Because aneuploidy has been recognized as a cardinal feature of many cancers, it plays an important part in tumourigenesis and is considered as a potential therapeutic target once the causes are revealed by further investigations.

Genomic instability is observed in the majority of human tumors. Dysregulation of the mitotic spindle checkpoint is thought to be one of the mechanisms facilitating aneuploidy in tumor cells (55). However, the mechanisms behind genetic instability and aneuploidy still remain unexplored (56).

Nuclear Morphometry: During the past several years, it has been well established that several clinical and histopathological variables are helpful in predicting the clinical outcome of cancer patients. Such prognostic predictors include tumour stage (57,58), histological type, tumour differentiation, ploidy, proliferative activity, p53 expression, apoptosis, and vascular and lymphatic invasion. Among the most powerful prognostic determinants in colorectal cancer, for example, is the histological tumour stage, including the depth of local invasion into the bowel wall and the infiltration in the regional lymph nodes (59-61). Despite this fact, the clinical staging of colorectal cancer is currently based on information not obtainable by histological examination of the primary tumour, particularly when done only in bi-

opsies, where the exact depth of tumour infiltration into the bowel wall, LNN involvement, and the data on distant metastases cannot be obtained. There is increasing recent evidence, however, that light microscopic examination of the primary tumour by quantitative measurements could provide useful prognostic information (62).

Currently, computer-assisted image analysis (nuclear morphometry) provides a new powerful tool for high-precision measurement of several variables characterising the size and shape of cancer cell nuclei in conventional tissue sections (63, 64). Several of these nuclear profiles seem to be useful prognostic predictors in various human malignancies (65, 66). Until now, however, few studies have used morphometric measurements to determine the nuclear size and shape profiles in normal and neoplastic colorectal tissues (67). Not unexpectedly, the nuclear size is usually larger and its shape is more often irregular in cancer cells (68, 69).

In 1982, Diamond and associates introduced nuclear morphometry to aid in prediction of prognosis among patients with prostate cancer (70, 71). He and his colleagues observed that nuclear roundness was very useful in separating long survivors among stage B patients from those who develop metastasis. They observed no overlap in nuclear roundness between the two groups. Since then, many histological studies (72-76) have used nuclear morphometry to predict prognosis in patients with prostate cancer. Eichenberger and associates (73) calculated 12 shape descriptors including nuclear roundness, ellipticity factors, and concavity factors. They used discriminate analysis to select the major morphometric parameters which best distinguished patients with good or poor prognosis. Elliptical shape measurement was found to be the best in this respect.

To critically evaluate the usefulness of nuclear morphometry for prediction of prognosis, Partin et al (75) developed a morphometric evaluation system called Hopkin's Morphometry System, and produced and compared 15 different shape descriptors in stage A2 prostate cancer. These were analyzed by 17 different statistical tests. The best separation was provided by the lower quartile analysis of the ellipticity shape descriptor ($p<0.01$). These studies revealed that the elliptical

shape of the nuclei is very important as a prognostic factor.

The results of the study by Martinez-Jabaloyas et al (77) revealed that mean nuclear area and other factors proved to have a prognostic value in the univariate analysis and concluded (78) that nuclear morphometry in the primitive tumor provides independent prognostic information in survival analysis for patients with metastatic prostate cancer. The combined evaluation of high nuclear morphology, ploidy, and cell survival parameters such as Bcl-2 expression might better identify patients with poor prognosis among early stage prostate carcinomas diagnosed by FNA biopsies (79).

Besides the prognostic and predictive power of morphometry, Buhmeida et al (80) revealed that the nuclear size features are useful in distinguishing between different atypia groups of the prostate gland in fine needle aspiration biopsies, particularly if the sample-associated means of the size features (area, diameter, perimeter, short and long axes) are used for the interpretation of data. The study suggested if the upper range limit of sample-associated mean areas of nuclei is below 27μm2, it is most probable that we are dealing with benign cells. If the upper range limit is above 39μm2, it is possible that there are malignant cells in the sample. However, values above 52μm2 represent malignant samples with certainty. Further studies will be necessary for associating nuclear size features with Gleason grades.

IN SUMMARY

Cytometric analysis of cellular DNA content can be performed rapidly and with relative ease and there is accumulating evidence that it provides an objective assessment of the inherent malignant potential in a number of human cancers. It seems likely that determination of tumors' ploidy will add significantly to the clinical and pathological assessment. Unfortunately DNA ploidy measurements from biopsies are rare in clinical practice, in spite of the extensive literature that supports their use (81). This, in fact, needs to be emphasized in more educational courses to those who are dealing with cancer. Unfortunately, there is a gap between the scientific researchers and clinicians who are treating cancer patients. Our target is to reduce this gap and enhance people to

learn more about the importance of implementing such tools in routine clinical practice to help them in taking the right treatment decisions.

Compound prognostic factors based on the gene expression profiles (tested by DNA arrays) are promising and will accelerate the discovery of new predictive and prognostic molecules, but clinically relevant data up to this moment are still lacking (82). Multivariate analyses of prognostic factors are enough, and multivariate models for prediction of compound prognosticators or predictors have not been well tested in clinical practice.

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