ACTA STEREOL 1985; 4/2: 237—242
PROC ESS IV-1 GÖTEBORG 1985
ORIGINAL SCIENTIFIC PAPER

PARTICLE SIZE ANALYSIS OF NUCLEI (NUCLEAR UNFOLDING) IN TUMOUR PATHOLOGY

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ABSTRACT

The widely accepted use of basic statistics of karyometrical parameters to describe nuclear polymorphism may encounter serious objections and more informative and sensitive methods are desirable.

In this study we have investigated the applicability of size analysis of cell nuclei (nuclear unfolding) for the quantitative description of nuclear polymorphism. The method is able to give a good description of polymorphism due to polyploidization in hepatocytes; it can detect small differences between nuclear size distributions of normal salivary gland cells and epithelial cells in benign salivary gland tumours. It is also shown that the method is suitable to detect nuclear changes due to aneuploidy in malignant tumours and to establish the proportional contribution of different cell populations to the tumours.

Keywords: Aneuploidy, nuclei, polymorphism, subpopulations, tumour pathology, unfolding technique.

INTRODUCTION

Nuclear polymorphism plays an important role in the diagnosis of tumours, particularly in the discrimination between benign and malignant and in the determination of the grade of malignancy.

In quantitative pathology, several subjective planimetrical parameters of the cell nucleus (karyometrical parameters) and related derivatives have been introduced to quantify size and shape of cell nuclei. Some of these parameters are: area, perimeter, short and long axis respectively shape factor, nuclear contour index, roundness factor and the ratio of long and short axis of nuclei (Baak and Oort, 1983).

Quantitative histopathological studies of several types of tumours have shown that the mean values and standard deviations of these parameters are of diagnostic importance and may provide the pathologist with more reliable and reproducible prognostic information than currently used subjective grading systems (Baak and Oort, 1983).
Notwithstanding these advantages, the quantitative description of nuclear polymorphism with basic statistics of the karyometrical parameters, such as mean value and standard deviation, suffers from the severe drawback that the size of cell nuclei is considered to be normally distributed. When a karyometrical parameter is not normally distributed, the use of the above mentioned basic statistics may give rise to results devoid of any significance, especially in cases where multimodality occurs.

Quantitative DNA studies of malignant tumours have revealed that aneuploidy is a well recognized feature of several tumour types (Learum and Farsund, 1981; Friedlander et al, 1984). In normal tissue cells of several organs (e.g. liver), polyploidy is also a well-known phenomenon (Brodsky and

Uryvaeva, 1978). Therefore, it is not correct to treat karyometrical parameters as normally distributed quantities.

Consequently, in tumour pathology, more sensitive and informative methods to quantify nuclear polymorphism are needed. A sensitive nuclear polymorphism describing method must be able to detect the presence of subpopulations constituting the ruling population of cell nuclei. It must also be able to register cell kinetic features which give rise to alterations in the nuclear volume.

More information regarding nuclear polymorphism may be derived from the analysis of nuclear size distributions using statistical non-parametric density estimates. Several stereological methods, well-known as unfolding techniques, enable the estimation of the size distribution of spherical nuclei in a tissue from the circular profile size distribution of sectioned nuclei in histological sections (Cruz-Orive, 1983; Knol et al, 1985).

MATERIAL AND METHODS

The investigation comprises studies on the nuclei of serous and striated duct cells of three normal human salivary glands, the hepatocytes of the liver of two rats, three adenolymphomas and three oxyphilic adenomas of the salivary glands as benign tumours as well as three adenocarcinomas of the salivary glands and two seminomas as malignant tumours.

All tissues in this preliminary study were embedded in paraffin after fixation in a 10% formal-saline solution. A haematoxylin-eosin staining was performed on routinely obtained sections of approximately 7 µm thickness.

A systematic sampling technique was used in the acquisition of the karyometrical data from the histological sections, with a data-acquisition rate of 200 nuclear contours per hour. Nuclei with damaged or ill-defined boundaries were excluded.

The equipment used in this study consists of a microscope furnished with a drawing tube and a graphic tablet connected to a commercially available semi-automated analyzing system (MOP-Videoplan, Kontron, Munich, FRG). The measurements are performed at high magnifications, realized with a *100 oil immersion objective (Planapo, NA = 1.3).

The values of the short and long axis, derived from the tracings of 300 nuclear profiles, were used to calculate the equivalent profile radius. The latter was used to determine the radius and volume distributions of the cell nuclei by means of the unfolding technique. Details on the mathematical and physical aspects of the unfolding method used are given by Knol et al (1985).

To obtain a set of numerical data characterizing the unfolded size distributions, a χ^2-minimization procedure was used to optimize the agreement between these distributions and a combination of up to five lognormally distributed functions (Bevington, 1969). The positively skewed lognormal distribution has been proposed and fitted for a wide variety of empirical distributions. It can be seen as a convenient model to describe positively skewed data; these are data that show a tendency to have a pronounced tail to the right. In this way, up to five lognormally distributed subpopulations were obtained. The subpopulation with the highest contribution to the overall nuclear size distribution will further be denoted as the principle subpopulation. Its proportional contribution is defined as the ratio of the area under the curve of the lognormal distribution function of the principle subpopulation and the area under the curve of the unfolded nuclear size distribution. The mode of the volume of the principle subpopulation will further be denoted by V_0; the 'mode' is defined as the value of the most frequent occurrence. The radius, volume and proportional contribution of each subpopulation is used in the quantitative description of nuclear polymorphism.

RESULTS

Qualitative description of the nuclear features

The nuclei of serous and striated duct cells of the submandibular gland can be qualitatively described as uniform in size, polymorphism is absent. Small numbers of striated duct cells with small hyperchromatic nuclei have been observed in two of the investigated salivary glands.

The nuclei of the hepatocytes are obvious variable in size. The dispersion of large and small nuclei is not homogeneous throughout the liver.

The majority of cells in the oxyphilic adenomas have vesicular nuclei and are called 'light cells' in salivary gland pathology. The nuclei of these cells are uniform in size. Occasionally, there are cells with a small hyperchromatic nucleus and more deeply eosinophilic cytoplasm. These cells are called 'dark cells' (Lucas, 1976). In this study, only two tumours contain 'dark cells'.

The nuclei of the epithelial cells in the adenolymphomas show minimal variations in size.

The three malignant tumours of the salivary glands and the seminomas exhibit considerable variations in nuclear size.

Quantitative description of nuclear features

Nuclear unfolding of the salivary gland cells results in slightly positively skewed unimodal distributions of the nuclear radii.

The radius distributions of the striated duct cells, with some hyperchromatic nuclei, exhibit a minute disturbance in the left-hand tail (figure 1A). There is a very good agreement between the nuclear size distributions of the normal salivary, serous- and striated duct cells, notwithstanding a hardly visible disturbance in the left part of the distribution of the striated duct cells.

The nuclei of the hepatocytes are distributed according to a bimodal function, which indicates the existence of clearly separated subpopulations (figure 1B).

Nuclear unfolding of the adenolymphomas reveals unimodal distributions that are more positively skewed. The two oxyphilic adenomas with 'dark cells' exhibit a bimodal distribution with a low peak on the left and a high maximum on the right. The bimodality of the curves suggests the existence of two subpopulations (figure 1C). The principle subpopulation shows a more positively skewed distribution when compared with the nuclei of normal salivary gland cells.

The malignant tumours have more complex and variable nuclear size distributions with an extended width as most notable characteristic (figure 1D).

Approximation of the unfolded distribution by a composition of lognormally distributed populations results, in general, in very good fits. Discrepancies between the approximation and the original size distribution are very small as can be seen in figures 1A-1D, which contain both curves.

In the bimodal distributions of the hepatocytes and oxyphilic adenomas, the decomposition of the nuclear size distributions into lognormal populations can be used to estimate the proportional contributions of these subpopulations. Applying this approximation method on the bimodal distributions of the hepatocytes of the two investigated livers reveals that the first (left hand) populations have proportional contributions of 29% respectively 40%, whereas the second (right hand) populations have a proportional contributions of 71% respectively 60%.

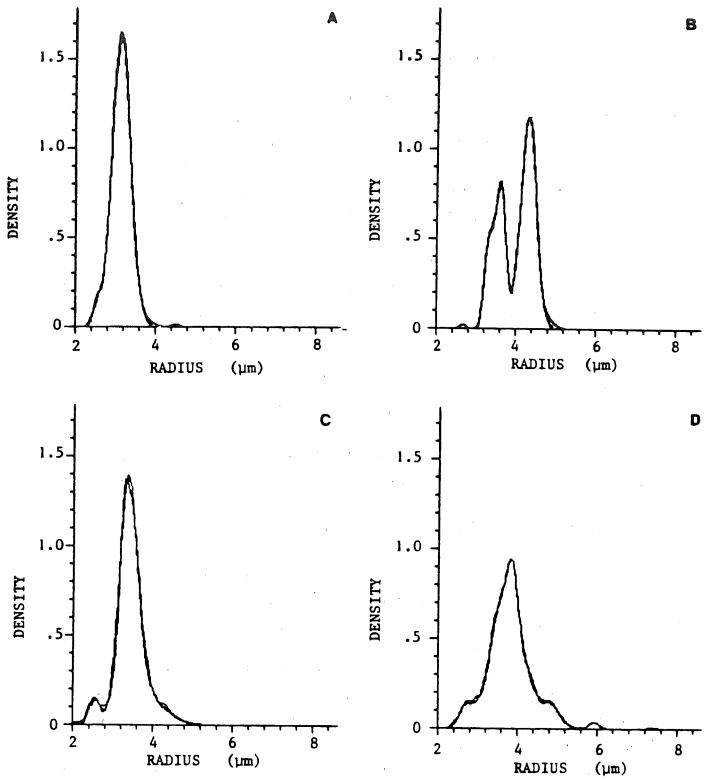

Figure 1. Radius density distribution of the cell nuclei of:
A: striated ducts B: hepatocytes
C: oxyphilic adenoma D: adenocarcinoma

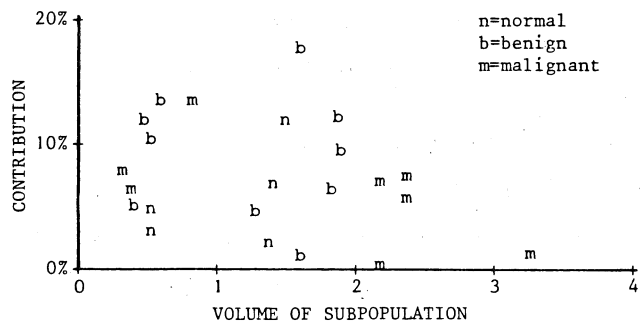

Figure 2. Nuclear volume of the secondary subpopulations in terms of V_0 vs. their contribution.

The volumes of the latter populations expressed in terms of the volume V of the first are given by 1.9V respectively 1.8V.
A decomposition of the nuclear size distribution of the oxyphilic adenoma in figure 1C, discloses that 14% of the whole population of epithelial cells consists of 'dark cells'.

Table 1 summarizes the mean values and standard deviations of the nuclear volumes of the principle subpopulations of the normal salivary gland cells, the benign and malignant tumours. This table also contains the extreme values, given in parentheses.
The Student-t test reveals that the difference between the nuclear volumes of the principle subpopulation of normal cells and benign tumour cells is statistically significant (two tailed P = 0.005). The difference between the nuclear volumes of the principle subpopulation of benign and malignant tumour cells also is statistically significant (P = 0.02).

Table 1. The nuclear volume of the principle subpopulations.

Normal cells	Benign tumours	Malignant tumours
$(V_0 \pm SD)\ \mu m^3$	$(V_0 \pm SD)\ \mu m^3$	$(V_0 \pm SD)\ \mu m^3$
124±14 (101-138)	156±17 (127-171)	568±242 (229-884)

To assure transparency of the acquired data, a compilation of the most informative descriptive measures is given in figure 2. It contains a graph of the volume of the secondary subpopulations in terms of the volume V_0 of the principle subpopulation versus their proportional contribution. From figure 2 it is seen that subpopulations with a nuclear volume exceeding $2V_0$ exclusively stem from the malignant tumours. In the benign tumours, 5 out of 6 have a secondary subpopulation with a volume between $1.5V_0$ and $2V_0$. In the normal salivary gland cells, only three cases reveal a secondary subpopulation with a value greater than V_0 with a upper limit of $1.5V_0$.

DISCUSSION

Unfolding of the karyometrical data acquired from routinely obtained paraffin embedded and haematoxylin-eosin stained histological sections results in appropriate, continuous and smooth size-distributions of cell nuclei. The nuclear unfolding method has disclosed that the size of cell nuclei in normal tissues and tumours is not normally distributed and that in the same cell type more than one population of nuclei may occur.

These findings underscore our fundamental criticism on the widely accepted use of basic statistics of karyometrical parameters to quantify nuclear polymorphism.

Approximation of the nuclear size distribution with lognormal distributions enables the estimation of the volume and proportional contributions of the individual subpopulations in multimodal nuclear size distributions and can be used in the quantitative description of nuclear polymorphism.

The bimodal nuclear size distributions of the hepatocytes reveal subpopulations of nuclei with volume V respectively nearly 2V. This is in accordance with the results of DNA measurements which have substantiated diploid and tetraploid nuclei in rat's hepatocytes (Brodsky and Uryvaeva, 1978). Thus, nuclear polymorphism, originating from polyploidization, can

excellently be described with the unfolding method, in contrast to the use of basic statistics of karyometrical quantities, which then leads to senseless results.

From this preliminary study it appears that the nuclear unfolding technique can register small differences in the appearance of nuclear size distributions of normal salivary gland cells and cells of benign salivary gland tumours. The constituting subpopulations, obtained from an approximation with lognormal curves, can be used to quantify these differences. Although the biological significance of these subpopulations in skewed unimodal nuclear size distributions remains somewhat obscure, they can be used to discriminate between the nuclei of normal cells and tumour cells.

Nuclear unfolding of the malignant tumours has disclosed that the size distributions of malignant tumour cells are much more complex, possibly due to aneuploidy which has been described in literature (Learum and Farsund, 1981; Eneroth and Zetterberg, 1974).
The approximation with subpopulations has revealed significant differences in the nuclear volumes of benign and malignant tumours.

It seems likely that the unfolding technique of the nuclear size distribution, followed by a decomposition into subpopulations, is suitable to detect and describe small differences and disturbances, which are possibly a consequence of cell kinetic phenomena. Further clinico-pathological studies are necessary to substantiate whether our method scores success in diagnostic and prognostic possibilities where other methods have failed.

ACKNOWLEDGEMENT

The authors wish to thank the 'Utrechtse Stichting tot Bevordering van de Mondheelkunde' for the financial support that effectuated this research project.

REFERENCES

Baak JPA, Oort J. A manual of morphometry in diagnostic pathology. Berlin: Springer-Verlag, 1983.

Bevington Ph R. Data reduction and error analysis for the physical sciences. New York: McGraw-Hill, 1969.

Brodsky WY, Uryvaeva IV. Cell polyploidy: Its relation to tissue growth and function. Int Rev Cytol 1978; 50: 275-332.

Cruz-Orive LM. Distribution-free estimation of sphere size distributions from slabs showing overprojection and truncation, with a review of previous methods. J Microsc 1983; 131: 265-90.

Eneroth CM, Zetterberg A. Microspectrophotometric DNA analysis of malignant salivary gland tumours. Acta Laryng 1974; 77: 289-94.

Friedlander ML, Hedley DW, Taylor IW. Clinical and biological significance of aneuploidy in human tumours. J Clin Pathol 1984; 37: 961-74.

Knol RGF, de Wilde PCM, Slootweg PJ. A physically more realistic method for unfolding sphere size distributions of nuclei in eukaryotic cells. Book of extended abstracts of IV European Symposium for Stereology. Goteborg: Chalmers University of Technology, 1985.

Laerum OD, Farsund T. Clinical application of flowcytometry: a review. Cytometry 1981; 2: 1-13.

Lucas RB. Pathology of tumours of oral tissues. London: Churchil Livingstone, 1976.