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Original Research Paper

MIB-1, AgNOR AND DNA DISTRIBUTION PARAMETERS AND THEIR PROGNOSTIC VALUE IN NEUROENDOCRINE TUMOURS OF THE LUNG

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ABSTRACT

One of the most important questions in clinical routine is to find out patients with good or worse prognosis to apply an optimal therapy scheme for each patient. In this study 58 patients with different neuroendocrine tumours of the lung were investigated. Histological sections were prepared with different stainings (MIB-1, AgNOR, Feulgen). By means of high resolution image cytometry stereological parameters were derived which are indicators for proliferation, ploidy and kinetics of the tumours. Cox regression analysis was calculated to test the significance of the parameters with regard to prognosis. The best parameter was MIB-1 which can easily be applied as a clinical standard staining and measurement.

Keywords: AgNOR, carcinoid, DNA distribution, image analysis, MIB-1, neuroendocrine lung tumours, prognosis, small cell carcinoma.

INTRODUCTION

Lung cancer is in industrial countries the most frequent cause of death for men and women. The overall 5-year survival rate is only about 15%. One part of lung tumours are neuroendocrine tumours divided in subtypes with different malignant potential (benign or low-grade malignant tumours, called typical carcinoids (TC) and on the other side the high-grade malignant tumours, poorly differentiated of small (SCLC) or large cell type (LCLC). Between these tumour types, the well-differentiated carcinoma with a lower grade of malignancy (WDNEC) take place (WHO, 1998). In clinical routine it is important to distinguish patients with better and worse prognosis. The aim of this study was to test the markers MIB-1, AgNOR and DNA distribution parameters, which are applied as different biological indicators of proliferation, ploidy and kinetics, with regard to the survival of patients and to the improvement of their therapy.

MATERIAL AND METHODS

In this study 32 cases of SCLC, 13 of WDNEC and 14 of TC with a follow-up time up to 7 years were collected. In Table 1 the complete clinical data set is shown. The tumour block was sliced into 4 μm thick sections from paraffin embedded tissue from routine and were stained afterwards according to MIB-1 (Fig. 1) (Böhm *et al.,* 1996), AgNOR (Fig. 2) (Aubele *et al.,* 1994a; Böhm *et al.,* 1993) and Feulgen (Fig. 3) (Aubele *et al.,* 1994b; Jütting *et al.,* 1999).

DATA ACQUISITION

MIB-1. CAS 200 image analysis system, (63x objective, n.a. 0.8, pixel size of 0.32x0.25 μm^2), 2 to 7 fields randomly selected within the tumour area. Two different filters (500 nm and 620 nm) were used for thresholding the nuclei area and the MIB-1 reactivity area, respectively.

AgNOR. SAMBA 2005 image analysis system, (Zeiss microscope, 40x objective, n.a. 0.65, optovar 1.6x, CCD-camera (Hamamatsu)). 4-7 fields randomly selected within the tumour area (100-150 cells/slide, digitized image 512x512 pixels, resulting pixel distance of 0.165 μm).

DNA. Zeiss Axiomat-microscope, (Bosch TV-camera (128x128 pixel), 100x objective (oil immersion, n.a. 1.3, 548 nm filter, pixel distance 0.25 μm)). 100 single tumour cell nuclei and 20 leukocytes (diploid peak) per slide, scanned in transmission with shading correction, recalculated to extinction (Haroske *et al.,* 1994a, b). Thickness of the sections was measured by a laser scanning microscope at 5 locations. The mean thickness was 6.3 μm (2.5 - 11 μm range).

Table 1. *Clinical data of 58 tumour patients of the lung separately for each tumour type.*

32 SCLC:	
sex	4 female, 28 male
age	MV = 59.7 years, range (38 to 77 years)
cell type	16 OAT, 16 ITM
tumour size	5 T1, 19 T2, 7 T3, 1 Tx
lymph node involvement	8 N0, 8 N1, 11 N2, 2 N3, 3 Nx
metastasis	21 M0, 10 M+, 1 Mx
stage	10 EXT, 22 LIM
therapy	OP and/or R and/or CH
survival time	MV = 25.3 months, range (2 to 130 months) 29 patients deceased
13 WDNEC:	
sex	7 female, 6 male
age	MV = 51.5 years, range (19 to 71 years)
tumour size [cm]	MV = 3.2 cm, range (0.9 to 9 cm)
lymph node involvement	7 N0, 6 N+
therapy	OP and/or R and/or CH
survival time	MV = 51.7 months, range (4 to 90 months) 5 patients deceased
13 TC:	
sex	5 female, 8 male
age	MV = 56 years, range (22 to 69 years)
tumour size [cm]	MV = 2.7 cm, range (1.0 to 6 cm)
lymph node involvement	10 N0, 3 N+
therapy	OP
survival time	MV = 60.2 months, range (12 to 101 months) 2 patient deceased

LIM = limited disease, EXT = extensive disease, OP = operation, CH = chemotherapy, R = radiotherapy, OAT = oat cell type, ITM = intermediate cell type

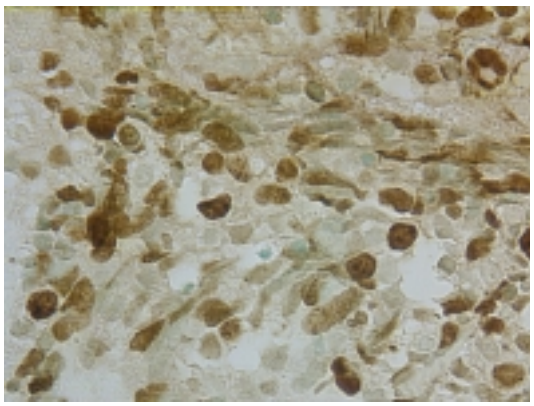


Fig. 1. *MIB-1 stained nuclei.*

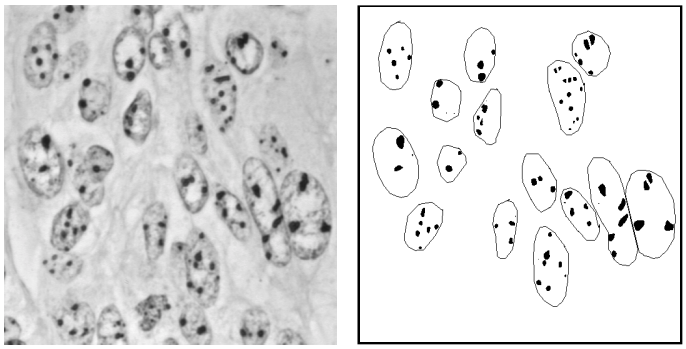


Fig. 2. *AgNOR stained nuclei and segmentation mask.*

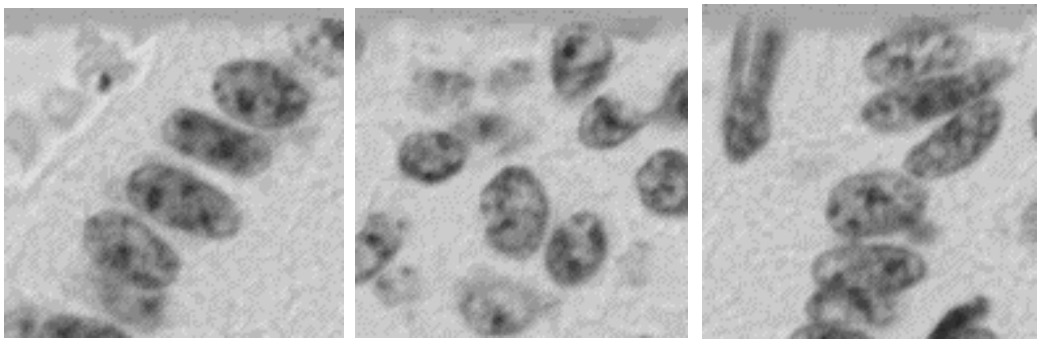
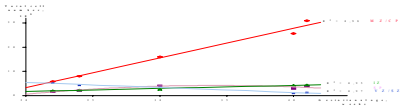


Fig. 3. *Feulgen stained nuclei.*

About 100 nuclei per case and staining were randomly gathered to extract stereological features as volume density, numerical volume density and different DNA parameters (Schenck *et al.*, 1997).

Stereological parameters

$$V_V(\mathrm{MIB}-1) = \frac{\sum \mathrm{Area}_{\mathrm{marked}}}{\sum \mathrm{Area}_{\mathrm{total}}} \qquad (1)$$



(2)

$$N_V(\mathrm{AgNOR}) = \sqrt{\frac{\left(\sum \mathrm{Number}_{\mathrm{AgNORs}}\right)^3}{\left(\sum \mathrm{Area}_{\mathrm{nucleus}}\right)^2 \sum \mathrm{Area}_{\mathrm{AgNORs}}}} \qquad (3)$$

For evaluating the 'real' DNA distribution from sections with different thickness following formulae have to be taken into account with the assumption that the nuclei are nearly round objects:

$$\mathrm{fac} = \begin{cases} \dfrac{1}{1-\dfrac{3r}{8t}} & ;\ \text{if } r < t \\ \dfrac{1}{\dfrac{3t}{4r} - \dfrac{1}{8}\left(\dfrac{t}{r}\right)^3} & ;\ \text{if } r > t \end{cases} \qquad (4)$$

r = radius of nucleus
t = thickness of section

IOD = integrated optical density (in extinction), normalization factor of lymphocytes = 1.6 due to the underestimation of IOD of the dark and condensed lymphocytes, then

$$\mathrm{DNA}_{\mathrm{nucleus}} = \frac{\mathrm{IOD}_{\mathrm{nucleus}}\ \mathrm{fac}}{\mathrm{Mean}\left(\mathrm{IOD}_{\mathrm{lymphocytes}}\right)}\ 1.6 \qquad (5)$$

From the DNA distribution the mean value, 5c-exceeding rate, entropy, 2c-deviation index, value of stemline peak, proliferation status and the euploid/ aneuploid status (Ploidy) were calculated (Schenck *et al.*, 1997). In Fig. 4 some typical DNA distributions for the different histological tumour types are shown.

Entropy: Entropy describes the information content and disorder of a histogram. Its lowest value is reached if all DNA-values are found in the same channel of the histogram. Its maximum will be reached, if all measured cells are equally distributed over all channels.

$$\mathrm{Entropy} = -\sum_{i=1}^{N} p(i)\ \mathrm{ld}(p(i)) \qquad (6)$$

2c deviation index: In this histogram feature the cells are weighed with the square of their distance to the normal DNA content of 2c.

$$2c\ \mathrm{DI} = \frac{1}{N}\sum_{i=1}^{N}(c_i - 2c)^2 \qquad (7)$$

c_i = c-value of nuclei$_i$

STATISTICS

Stepwise Cox regression analysis was applied to search for features correlated with survival time (Lee, 1980). To demonstrate the results Kaplan-Meier curves for different strata are plotted. SAS and BMDP statistical packages were used. All statistical evaluations were done at 95% level.

RESULTS

All calculated features were univariately tested by Cox regression analysis. Following features are significant:

V_V(MIB-1)	$p < 0.0001$
V_V(AgNOR)	$p = 0.0123$
DNA-Proliferation status	$p = 0.0090$
Entropy	$p = 0.0003$
5c-Exceeding Rate	$p = 0.0046$
DNA-Mean value	$p = 0.0073$
Ploidy (eu/aneuploid)	$p = 0.0265$

To demonstrate the results Kaplan-Meier survival curves for V_V(MIB-1), V_V(AgNOR) and proliferation status for different strata are plotted.

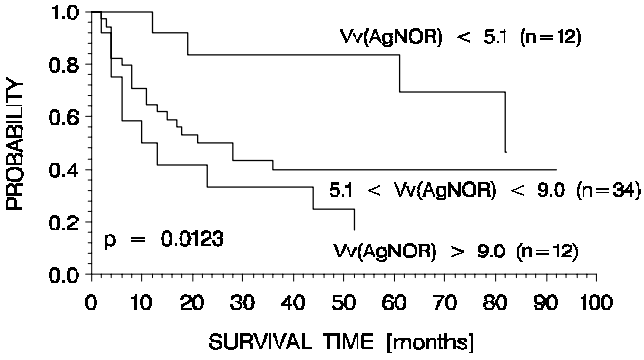


Fig. 6. *Kaplan-Meier survival curves for V_V(AgNOR) for 3 different strata.*

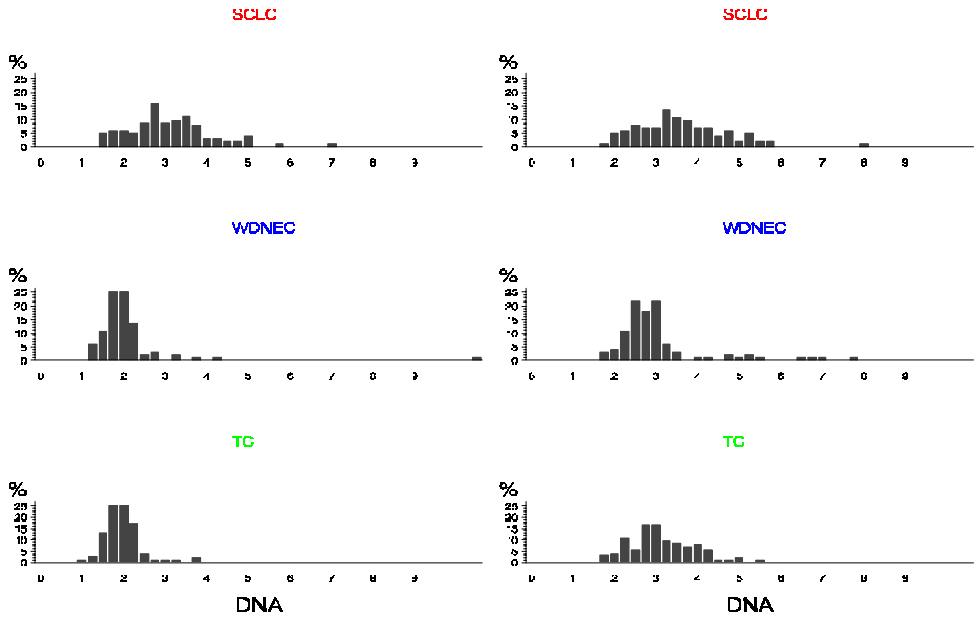


Fig. 4. *Typical DNA distributions.*

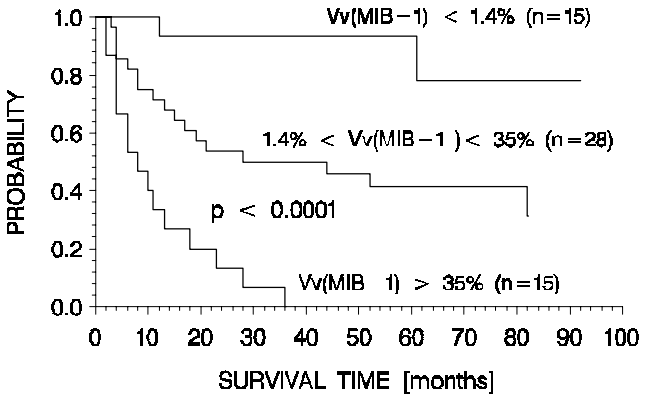


Fig. 5. *Kaplan-Meier survival curves for V_V(MIB-1) for 3 different strata.*

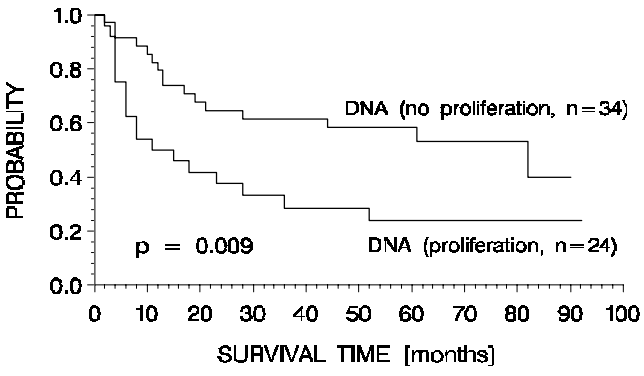


Fig. 7. *Kaplan-Meier survival curves for proliferating and non proliferating DNA distributions.*

SUMMARY

DNA distribution parameters, V_V(AgNOR) and V_V(MIB-1) are significant to predict the survival of patients suffering of neuroendocrine tumours of the lung. To demonstrate the results Kaplan-Meier survival curves for three parameters are plotted for different strata. In clinical routine the application of MIB-1 staining is recommended due to its highest significance and quickest realisation. This investigation can be done before surgery even if only a biopsy exists and the histological diagnosis and the tumour staging (tumour size, lymph node involvement, metastasis) are not clear. According to the measured MIB-1 value an individual treatment scheme for each patient can be applied.

A preliminary report of some of the data (Jütting *et al.,* 1999) was presented at the X^{th} International Congress for Stereology, Melbourne, Australia, 1-4 November 1999.

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