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ORIGINAL ARTICLE

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Improvement of breast cancer prognostication using cell kinetic-based silver-stainable nucleolar organizer region quantification of the MIB-1 positive tumor cell compartment

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Abstract Recently, it was stated that the proliferative activity (P) of a cell population could be indirectly calculated by multiplying the MIB-1 immunopositivity and silver-stainable nucleolar organizer region (AgNOR) features extracted exclusively in MIB-1 positive (pos.) nuclei: P=MIB-1×$AgNOR_{MIB\text{-}1pos.}$. To study the prognostic significance of this hypothesis, MIB-1 immunohistochemistry and AgNOR staining were applied on a series of 89 cases of breast cancer with an 8-year follow-up period. The mean MIB-1 immunopositivity ($MIB\text{-}1_{mean}$) was evaluated immunohistometrically on paraffin sections using a TV image analysis system CM-2 (Hund, Wetzlar, Germany). Later, a combined MIB-1/AgNOR staining was applied and evaluated using a TV image analysis system AMBA (IBSB, Berlin, Germany). The AgNOR features of 150 randomly chosen tumor nuclei were investigated, irrespective of their MIB-1 status (AgNOR count, AgNOR area). Later, a second measurement was performed on 100 MIB-1 positive tumor nuclei exclusively ($AgNOR\ count_{MIB\text{-}1pos.}$, $AgNOR\ area_{MIB\text{-}1pos.}$). AgNOR count and $AgNOR\ count_{MIB\text{-}1pos.}$ showed a different data distribution [2.7±0.7 (mean±SD) vs 3.9±1.1; r=0.315, P=0.014]. Similar results were obtained for AgNOR area and $AgNOR\ area_{MIB\text{-}1pos.}$ (5.1±2.1 μm² vs 7.5±2.4 μm²; r=0.501, P<0.001). Kaplan–Meier survival curves revealed significant differences for $MIB\text{-}1_{mean}$ (P=0.0018) and $AgNOR\ area_{MIB\text{-}1pos.}$ (P=0.0340). In Cox models, both parameters provided independent prognostic information. Using their combination, the P, three groups of patients with statistically different survival could be separated (P=0.0014). Thus, the combination of MIB-1-immunopositivity and AgNOR measurements in MIB-1 positive nuclei appears to be more useful in breast cancer prognosis than the exclusive application of one of the two methods. By this combined application, probably effects of tumor biology are represented more precisely.

Keywords AgNOR · MIB-1 · Image cytometry · Breast cancer · Survival analysis

Introduction

The analysis of cell cycle components has become a well-established method in clinical pathology, mostly focusing on its prognostic value in invasive carcinoma. The immunohistochemical analysis of the MIB-1 or proliferating cell nuclear antigen (PCNA) expression revealed prognostic validity in different tumor entities [10, 11, 12, 20, 24, 26, 29, 41, 42], including carcinoma of the breast [1, 4, 38]. As recently reviewed, also the analysis of the silver-stainable nucleolar organizer regions (AgNORs) has been frequently found to be prognostically useful [35]. The standardization of the conventional staining procedure [37] by the introduction of wet autoclaving in 1994 [32, 31] has especially improved the meaning of AgNOR analysis, and different studies on the prognostic significance of this new staining protocol have been reported since then on carcinoma of the breast [34], oral cavity [36], lung [48], stomach [17, 39], prostate [9], and colorectum [16, 33]. However, the analysis of either the MIB-1 immunoreactivity or the amounts of AgNOR describes a static situation only. While the number of cells in G_1, S or G_2/M phase of the cell cycle may be estimated, the duration of the cell cycle phases and the generation time of the cell population are not accessible under in vitro conditions. Thus, also, the proliferative activity cannot be calculated sufficiently.

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A recent recommendation to overcome this problem emphasized that a combined analysis of MIB-1 expression and AgNOR staining could provide more realistic kinetic information about tumor proliferation [6, 7]. This hypothesis, formulated by Brugal, was based on the idea that MIB-1, an indicator of the growth fraction (G), almost exclusively labels cycling cells and, that the amount of AgNORs as a result of amplification and activation of ribosomal genes, is inversely proportional to the cell cycle duration, thus representing an indicator of the generation time (T). The faster the cycle, the higher the amount of AgNORs [13, 14, 19, 30, 40, 44, 49]. Thus, the proliferative activity (P) of a cell population might be estimated more precisely if AgNOR analysis was restricted on MIB-1 positive cells ("$AgNOR_{MIB\text{-}1\text{-}pos.}$"). This means to interpret the traditional equation for the proliferative activity P=G/T as P=MIB-1/(1/$AgNOR_{MIB\text{-}1pos.}$) or as P=MIB-1×$AgNOR_{MIB\text{-}1pos.}$. This proposal was accepted at the recent meeting of the European Committee on AgNOR Quantitation within the European Society of Pathology in Innsbruck, Austria (1–3 October 1999). However, clinical investigations are still mostly lacking. In this study, we present our experience from a series of 89 clinically well-defined breast cancer cases with an 8-year follow-up period. The AgNOR quantification of the MIB-1 positive tumor cell compartment revealed a higher prognostic significance than MIB-1 or single AgNOR parameters.

Materials and methods

Clinical data

Formalin-fixed and paraffin-embedded tumor material from 89 female breast cancer patients aged 59.8±14.1(SD) years was investigated. Nineteen patients with stage-I tumors received a breast-conserving surgical therapy with later local irradiation (52 gy). The remaining 70 were treated by simple mastectomy. In all cases, axillary lymphadenectomy was performed. Postmenopausal patients with positive estrogen receptor (ER) status received tamoxifen (30 mg daily) for at least 2 years. Node-positive premenopausal patients and postmenopausal ER-negative patients received six cycles of chemotherapy [cyclophosphamide, methotrexate, 5-fluorouracil (CMF-scheme)]. At the end of the follow-up period, 49 patients were alive (mean observation period 6.3±0.7 years), and 40 had died after 3.1±2.0 years. For all of the patients, the TNM staging, the histomorphological grading, and the biochemical estrogen and progesterone receptor status (ER, PR) were available.

MIB-1 analysis

Immunohistochemical staining was performed on 2-µm paraffin sections from the longest tumor diameter, using a standard protocol with a 1:10 diluted primary MIB-1 antibody (Dianova 505) and a 1:100 diluted peroxidase-labeled goat-anti-mouse secondary antibody. Diaminobenzidine (DAB) was used as a chromogen. For immunohistometry, a TV image analysis system, CM-2 (Hund, Wetzlar, Germany), which has been explained in detail previously [4], was used. Measurements were performed using a 20× objective magnification (numerical aperture 0.40), investigating the tumor representatively in a meandering way and sampling both peripheral and central tumor areas. A total area of 1.94 mm^2 from 40 viewing fields was analyzed per slide. On average, 6338 tumor nuclei were investigated per case. The MIB-1 immunoreactivity was expressed as the mean immunopositivity of all 40 viewing fields ($MIB\text{-}1_{mean}$).

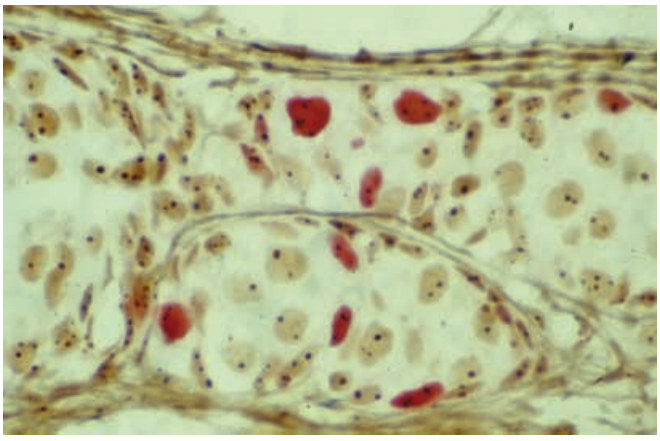


Fig. 1 Combined MIB-1/AgNOR (silver-stainable nucleolar organizer region) staining of a case of breast carcinoma. MIB-1 positivity is indicated by a *red* nuclear signal. MIB-1 negative nuclei are slightly *brown*. AgNORs are characterized by *small black dots* within the nucleus. 25× objective magnification

Combined MIB-1/AgNOR staining and AgNOR analysis

The combined MIB-1/AgNOR staining was performed on 2-µm paraffin sections from the same block that had been taken for MIB-1 immunohistometry. The slides were incubated with a 1:50 diluted primary MIB-1 antibody (Dianova 505; working dilution 1:50), followed by a 1:500 diluted biotin-labeled goat-anti-mouse secondary antibody. Alkaline phosphatase-linked streptavidin was used as a chromogen. After counterstaining with an aqueous fast-red solution, AgNOR silver staining was performed using a standard protocol [23]. For AgNOR analysis, a modular TV image analysis system AMBA (IBSB, Berlin) was available. This system has previously been described in detail [5]. Each slide was analyzed twice using a 40× objective magnification (numerical aperture 0.75). First, the AgNOR parameters of 150 randomly chosen tumor nuclei were investigated irrespective of their MIB-1 status. In a second measurement, the AgNORs of 100 MIB-1 positive tumor nuclei (Fig. 1) were analyzed exclusively. The second series of AgNOR measurements included 67 cases only, because 21 slides revealed such a low MIB-1 immunoreactivity that fewer than 100 MIB-1 positive nuclei were available. Per slide, the mean number of AgNORs and the mean sum area of the AgNORs were calculated (AgNOR count, AgNOR area, $AgNOR\ count_{MIB\text{-}1pos.}$, and $AgNOR\ area_{MIB\text{-}1pos.}$). The $MIB\text{-}1_{mean}$ values from MIB-1 immunohistometry were multiplied with the values of the second series of AgNOR measurements, resulting in the parameters P_{count} ($MIB\text{-}1_{mean}$×$AgNOR\ count_{MIB\text{-}1pos.}$) and P_{area} ($MIB\text{-}1_{mean}$×$AgNOR\ area_{MIB\text{-}1pos.}$).

Statistics

For statistical purposes, the BMDP package (Statistical Software Inc., Los Angeles, Calif.) was used. To analyze interrelationships between different parameters, coefficients of correlation were calculated. Multi-field tables were analyzed using the Pearson χ^2 test. Univariate survival analysis was performed according to Kaplan and Meier and evaluated using the Wilcoxon–Breslow test. For multivariate survival analysis, Cox models were calculated. Statistical significance was accepted for $P<0.05$ (Cox models $P<0.10$).

Table 1 Mean values, ranges, and median values for MIB-1 immunohistometry and silver-stainable nucleolar organizer region (AgNOR) analysis; *n*=67. *pos.* positive

	Mean values (±SD)	Range	Median value	95% Confidence limits
$MIB\text{-}1_{mean}$ (%)	10.8±9.7	0.4–40.8	8.1	8.5–13.2
AgNOR count (*n*)	2.7±0.7	1.4–4.9	2.6	2.5–2.9
AgNOR area (μm²)	5.1±2.1	2.8–10.8	4.3	4.6–5.6
$AgNOR\ count_{MIB\text{-}1pos.}$ (*n*)	3.9±1.1	1.7–6.6	3.6	3.6–4.1
$AgNOR\ area_{MIB\text{-}1pos.}$ (μm²)	7.5±2.4	3.1–14.1	7.1	7.0–8.1
P_{count}	45.4±45.6	0.8–227.4	31.4	33.2–54.6
P_{area}	89.9±93.6	1.6–422.6	67.8	65.1–109.2

Table 2 Statistical analysis of $MIB\text{-}1_{mean}$ and silver-stainable nucleolar organizer region (AgNOR) parameters. Right/top: coefficients of correlation, left/bottom: *P* values; *n*=67. *pos.* positive

	MIB-1 (mean)	AgNOR (counts)	AgNOR (area)	AgNOR (counts) MIB-1pos.	AgNOR (area) MIB-1pos.	P_{count}	P_{area}
$MIB\text{-}1_{mean}$	–	0.530	0.535	0.192	0.243	0.938	0.924
AgNOR count	<0.001	–	0.625	0.315	0.234	0.600	0.515
AgNOR area	<0.001	<0.001	–	0.072	0.501	0.527	0.665
$AgNOR\ count_{MIB\text{-}1pos.}$	>0.05	0.014	>0.05	–	0.428	0.446	0.254
$AgNOR\ area_{MIB\text{-}1pos.}$	0.047	>0.05	<0.001	<0.001	–	0.280	0.502
P_{count}	<0.001	<0.001	<0.001	<0.001	0.029	–	0.885
P_{area}	<0.001	<0.001	<0.001	0.048	<0.001	<0.001	–

Results

MIB-1 and AgNOR data distribution

The data distribution and the correlation matrix of the variables are presented in Table 1 and Table 2. AgNOR count and $AgNOR\ count_{MIB\text{-}1pos.}$ revealed different mean values and standard deviations but still significantly correlated (r=0.315, P=0.014). AgNOR area and $AgNOR\ area_{MIB\text{-}1pos.}$ were related more closely (r=0.501, P<0.001), although the mean values and standard deviations were different. The upper limits of the 95% confidence intervals of the AgNOR count and the AgNOR area were significantly lower than the lower limits of the 95% confidence intervals of $AgNOR\ count_{MIB1\text{-}pos.}$ and $AgNOR\ area_{MIB\text{-}1pos.}$, respectively.

The AgNOR variables revealed higher values if analyzed in MIB-1 positive nuclei exclusively (P<0.01, Fig. 2). The $MIB\text{-}1_{mean}$ values highly and significantly correlated with the AgNOR count (r=0.530, P<0.001) and AgNOR area (r=0.535, P<0.001) but only weakly with $AgNOR\ count_{MIB\text{-}1pos.}$ (r=0.192, P>0.05) and $AgNOR\ area_{MIB\text{-}1pos.}$ (r=0.243, P=0.047).

Univariate survival analysis

Significant differences between two groups of patients (1:1 distribution; threshold: median value) were found for $MIB\text{-}1_{mean}$ (P=0.0018, Fig. 3) and $AgNOR\ area_{MIB\text{-}1pos.}$ (P=0.0340, Fig. 4). No significant differences in survival were observed for AgNOR count, AgNOR area (Fig. 5), and $AgNOR\ count_{MIB\text{-}1pos.}$ (P>0.05). The lymph node status (P=0.0267) and the biochemical ER status (P=0.0098) revealed statistical significance, while the tumor size, the histomorphological grading and the PR-status revealed only a remarkable, but non-significant trend of a better survival probability for lower sized, higher differentiated, or PR-positive patients (P>0.05).

Multivariate survival analysis

$MIB\text{-}1_{mean}$ (P=0.0025) and $AgNOR\ area_{MIB\text{-}1pos.}$ (P=0.0775) provided statistically independent prognostic

Fig. 2 Significantly different distribution (P<0.001) of the single values of silver-stainable nucleolar organizer region (AgNOR) counts (*n*) and AgNOR area (μm²) in all tumor cells (first measurement) or in MIB-1 positive tumor cells only (second measurement) ▶

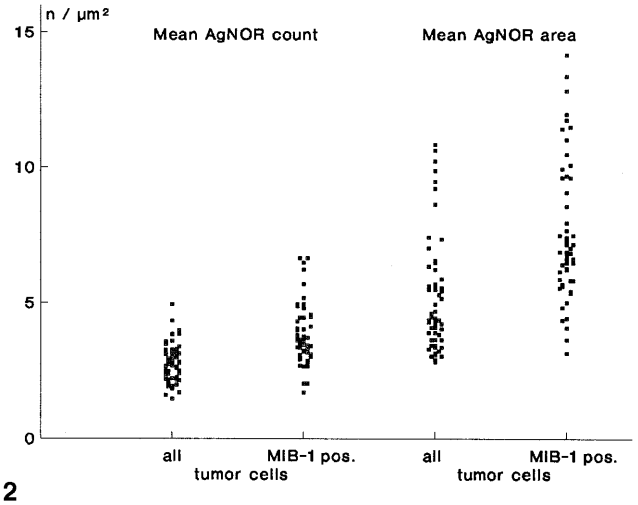
Fig. 3 Kaplan–Meier survival curves for 67 breast cancer patients, stratified in two groups according to the median value of $MIB\text{-}1_{mean}$ (P=0.0018)

Fig. 4 Kaplan–Meier survival curves for 67 breast cancer patients, stratified in two groups according to the median value of silver-stainable nucleolar organizer region (AgNOR) area (P>0.05)

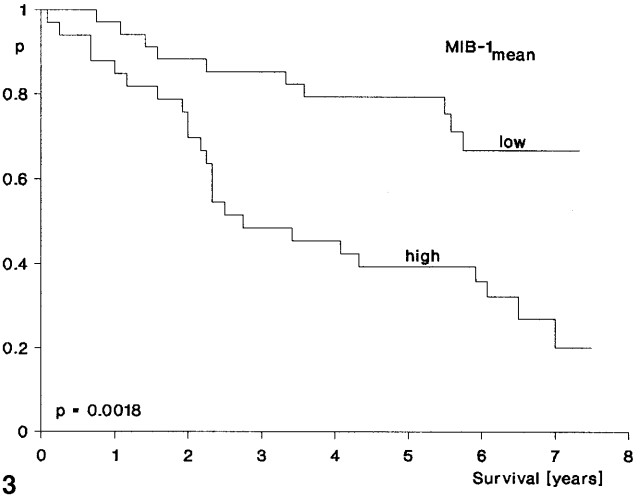
Fig. 5 Kaplan–Meier survival curves for 67 breast cancer patients, stratified in two groups according to the median value of silver-stainable nucleolar organizer region (AgNOR) $area_{MIB\text{-}1positive}$ (P=0.0340)

Fig. 6 Kaplan–Meier survival curves for 67 breast cancer patients, stratified in three groups according to P_{area} after 1:2:1 quantilization (P=0.0014)

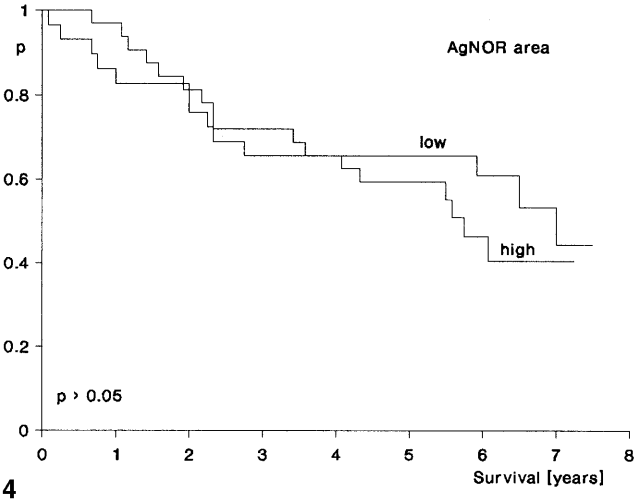
Fig. 7 Kaplan–Meier survival curves for 67 breast cancer patients, stratified in four groups according to P_{area} after 1:1:1:1 quantilization (P=0.0034)


n / µm²
Mean AgNOR count
Mean AgNOR area
15
10
5
0
all
MIB-1 pos.
tumor cells
all
MIB-1 pos.
tumor cells

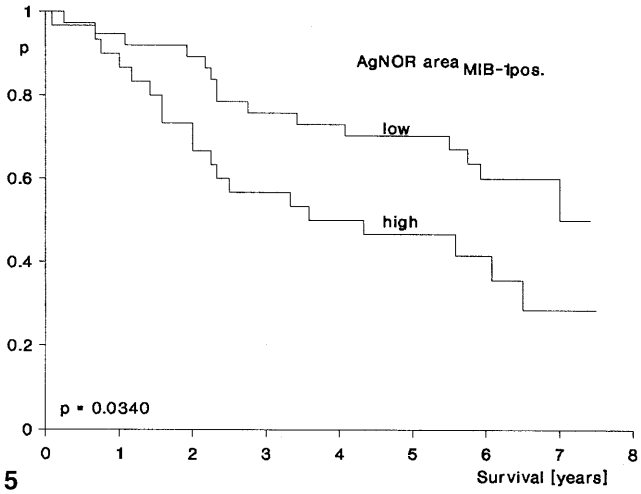
2


p
MIB-1mean
low
high
p = 0.0018
Survival [years]

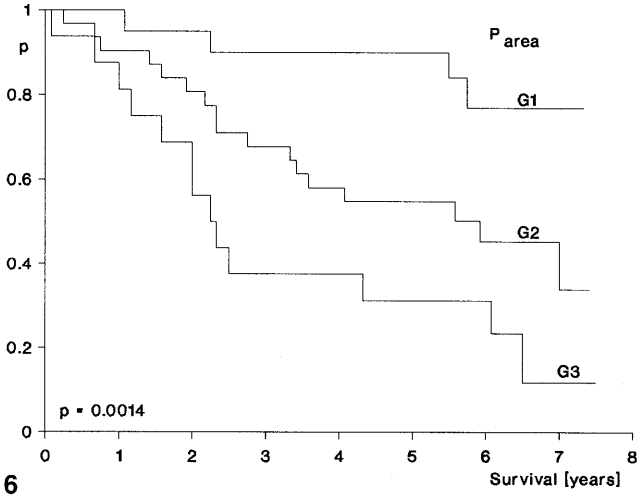
3


p
AgNOR area
low
high
p > 0.05
Survival [years]

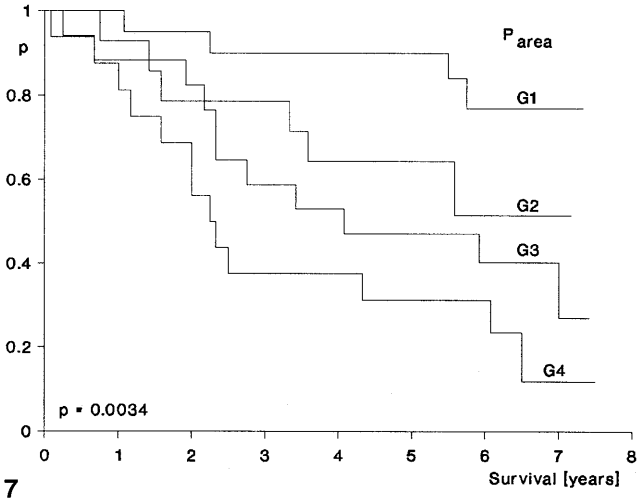
4


p
AgNOR area MIB-1pos.
low
high
p = 0.0340
Survival [years]

5


p
P area
G1
G2
G3
p = 0.0014
Survival [years]

6


p
P area
G1
G2
G3
G4
p = 0.0034
Survival [years]

7

information. AgNOR $count_{MIB\text{-}1pos.}$, AgNOR count, and AgNOR area revealed no multivariate significance. The low concordance of MIB-1_{mean} and AgNOR $area_{MIB\text{-}1pos.}$ (36/67=53.7%; $P>0.05$) underlined the independence of the two variables. P_{area} enabled the separation of three or four groups of patients with statistically different survival probabilities ($P=0.0014$ and $P=0.0034$; Fig. 6 and Fig. 7).

In a Cox model, P_{area} was entered exclusively ($P=0.0001$), while MIB-1 or AgNOR features, staging parameters, grading, or the ER or PR status were not considered ($P>0.10$). As the number of cases is restricted, the results of the multivariate survival analysis have to be interpreted carefully, especially concerning the clinical variables.

Discussion

Since 1994, when Öfner proposed to use wet autoclaving of the specimens [32, 31], AgNOR staining has been looked upon as a standardized method within the spectrum of quantitative morphology. However, conventional AgNOR analysis, investigating a tumor cell population as a whole, means a phenomenologic description only. To establish AgNOR analysis based on tumor biology, three important findings were pioneering.

First, in the early 1990s, the strongly inverse correlation between the amount of AgNORs and the cell cycle duration in cell culture experiments could be well documented by various groups [13, 14, 19, 30, 40, 44, 49]. Later, in concordance with our recent results, clinical studies on the correlation between AgNOR features and the immunoreactivity of tumor tissue for Ki-67, MIB-1, or PCNA showed a not strongly linear but still remarkably correlated distribution of the single values and statistically significant coefficients of correlation for acute leukemia [43], non-Hodgkin's lymphoma [18], oral squamous cell carcinoma [36, 50], renal cell carcinoma [46], or breast carcinoma [2, 3, 15, 22, 27]. Furthermore, after multilabeling techniques for the demonstration of AgNORs and immunohistochemical proliferation markers had been established, the amounts of AgNORs were compared between the immunopositive and the immunonegative compartment of the cell population. For Ki-67 [21], MIB-1 [2, 8, 28, 47], and PCNA [8, 45], the AgNOR amounts were markedly higher in the immunopositive cells.

These landmarks were the basis for Brugal's hypothesis in 1994 [7]. Unfortunately, however, only a theory was derived but no convincing clinical data presented. Since then, to the best of our knowledge, there have been only two studies that evaluated its clinical value. Investigating 50 cases of pTa- or pT1-staged urothelial carcinoma of the bladder with a recurrence rate of 30% (15/50), the AgNOR pattern of the MIB-1 positive tumor cell compartment was the best multivariate predictor for recurrence, compared with grading, MIB-1 immunoreactivity, and the analysis of AgNOR features in the whole tumor cell population [47]. In a small breast cancer study ($n=39$) with a 5-year follow-up period, higher metastasis-free or recurrence-free survival rates were obtained for lower P_{area} values [25]. However, the results of this study are difficult to interpret. Comparing the mean values of patients with a bad prognosis (patients with visceral metastasis or dead from disease) and a good prognosis (all other patients), the mean AgNOR area in all tumor cells or in MIB-1 negative tumor cells surprisingly differed more clearly than the mean AgNOR area of MIB-1 positive tumor cells. Furthermore, the MIB-1 values revealed no univariate prognostic significance, and survival differences between the mean AgNOR area in all tumor cells and the mean AgNOR area of MIB-1 positive tumor cells could not be documented for a low or a high MIB-1 expression.

In our study, for the first time, the amount of AgNORs was compared between the whole tumor cell population and its MIB-1 positive compartment. This comparison appeared to be more appropriate for clinical purposes than to compare the MIB-1 positive and the MIB-1 negative compartment as it had been proposed previously. It could be demonstrated that AgNOR analysis should be restricted to MIB-1 positive tumor nuclei due to its higher prognostic significance (Fig. 4 and Fig. 5). Additionally, the prognostic significance of the combined MIB-1-/AgNOR parameter, P_{area}, was higher than its single components, MIB-1_{mean} and AgNOR $area_{MIB\text{-}1pos.}$ (Fig. 3, Fig. 4, Fig. 6, and Fig. 7). Therefore, in the future, combined MIB-1/AgNOR evaluation should be applied on well-documented clinical studies to support our findings. Some further, yet unpublished oral presentations at the Innsbruck workshop gave encouraging data that should motivate clinical pathologists to combine AgNOR analysis and MIB-1 immunohistochemistry more frequently.

Additionally, in order to achieve an even better quality of image analysis, a new development of MIB-1/AgNOR double staining procedures should be further evaluated. Taking into account segmentation drawbacks that sometimes could arise if both components of the double staining are evaluated within the spectrum of visible light, an approach was made to combine AgNOR staining with a fluorescein isothiocyanate (FITC)-labeled MIB-1 staining. The authors reported about a stable staining product over years and presented convincing microphotographs [23]. However, this method requires more expensive equipment, including a fluorescence microscope.

Finally, a methodological restriction concerning studies with a combined MIB-1-/AgNOR analysis should be mentioned in order to give a correct view to the reader. This application cannot be performed on every case within a clinical series, which means a risk of selection. Preferably, cases with low MIB-1 values, indicating a good clinical course normally [4], will have to be excluded. If, for example, the MIB-1 immunoreactivity is 0.5% (or 1%) only, the identification of at least 100 MIB-1 positive nuclei for the AgNOR analysis means the need to screen

20,000 (or 10,000) tumor nuclei at all. Thus, from our series, only 67 cases (75%) could be analyzed; the remaining ones revealed too small a number of MIB-1 positive nuclei only, due to a low immunoreactivity or to a restricted number of tumor nuclei on the section, for example, in cases with a scirrhous-like or lobular growth pattern. Due to the restricted number of cases, the results of our study, especially those of multivariate survival analysis, have to be interpreted carefully. The survival curve of the 22 patients whose tumors could not be analyzed was almost identical to that of the patients with low MIB-1 expression documented in Fig. 3.

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