Digital Image Analysis of Mean AgNOR Area Objectively Discriminates between Reactive Plasmacytosis and Multiple Myeloma in Cytologic Smears from Bone Marrow Aspirates

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Key Words

AgNOR area · Bone marrow aspirates · Digital image analysis · Multiple myeloma · Reactive plasmacytosis

Abstract

Objective: To differentiate between reactive plasmacytosis (RP) and multiple myeloma (MM) in bone marrow aspirates by assessment of silver-stainable nucleolar organizer regions (AgNORs). **Methods:** Archival cytologic smears ($n = 69$) from bone marrow aspirates were retrospectively investigated. Twenty cases had RP and 49 cases MM (24 low-grade, 22 intermediate-grade and 3 high-grade MM). Diagnoses were confirmed by evaluation of the corresponding bone marrow trephine biopsies. Cytologic smears were subjected to AgNOR staining using a standardized silver-staining protocol and assessed by two independent clinical histopathologists using digital image analysis. AgNOR count and area were analyzed in 100 plasma cell nuclei per sample. **Results:** The mean AgNOR area was found to be significantly ($p < 0.001$) higher in low-grade MM (mean: $23.4 \mu\text{m}^2$; range: 19.2 – $26.7 \mu\text{m}^2$) than in RP cases (mean: $6.2 \mu\text{m}^2$; range: 3.8 – $8.4 \mu\text{m}^2$). In contrast, mean AgNOR count failed to disclose significant differences between low-grade MM (mean: 5.36; range: 1–7) and RP (mean: 3.68; range: 1–6). **Conclusion:** Dig-

ital image analysis of the mean AgNOR area might be used as a diagnostically useful adjunct to aid objectively in the challenging differentiation between RP and MM in bone marrow aspirates.

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Introduction

Approximately 15% of all hematological malignancies affect patients with a median age of 68 years in males and 70 years in females in the United States [1]. Amongst these, multiple myeloma (MM) is a bone marrow-based disease resulting from the proliferation of a clone of neoplastic cells which are closely related to plasma cells (PC) occurring in the middle-aged and elderly population.

For bone marrow trephine biopsies, conventional major and minor criteria required for MM diagnosis have been well established [2]. Immunohistochemistry and in situ hybridization [3, 4] can be applied to equivocal cases. However, using cytologic smears from bone marrow aspirates, the differential diagnosis between low-grade MM and reactive plasmacytosis (RP) might be challenging [5], underlining the need for objective morphological criteria facilitating the conventional discrimination.

Nucleolar organizer regions (NORs) are nucleolar components containing loops of DNA actively transcribing into ribosomal RNA (rRNA) genes. These are selectively stained by silver methods [6], which stain NORs as black dots of different size, shape and number. In the past, AgNORs have attracted considerable attention in tumor biology because of their relation to cell proliferation [7].

In MM, mean AgNOR counts have been found to be reliable predictors of survival [8] and suggested to reflect the myeloma cell mass and the kinetics of myeloma cells [9], but a thorough comparative analysis of the mean AgNOR count and area has not yet been performed with respect to the challenging differential diagnosis of PC proliferations encountered in bone marrow aspirates. This so far neglected evaluation was defined to be the goal of the present study.

Materials and Methods

Specimen Selection

Retrospective computerized database analysis was performed in order to identify all patients with newly diagnosed MM and RP between 1995 and 1998, from whom bone marrow trephine biopsies and aspirates were available at the Institute of Pathology, HELIOS Clinics Bad Saarow (the former HUMAINE Clinics), Germany. A total of 69 archival cytologic smears previously stained with the Papanicolaou technique and their corresponding 69 trephine biopsies were retrieved from the files of the Institute of Pathology. The reference collective comprised 20 cases of RP (mean age: 54.2 years; range: 31–82 years), and 49 cases had MM (mean age: 59.3 years; range: 28–86 years). The latter group included 24 low-grade (G1), 22 intermediate-grade (G2) and 3 high-grade (G3) MM based on the grading criteria devised by Bartl et al. [10]. Patients assigned to the reference collective ($n = 20$) suffered from hemopoietic malignant diseases other than MM and were found to have accompanying reactive increase in PC. These patients were consistently kept under regular clinical follow-up at the Department of Hematology and Oncology, HELIOS Clinics Bad Saarow, in order to exclude any progress to PC dyscrasia (mean follow-up 4.2 years; range: 3–7 years).

MM were preliminarily categorized in terms of patterns of medullary involvement (interstitial, nodular and/or diffuse) and percentage of PC in aspirates and bone marrow biopsies. The 'nodular pattern' of infiltration was defined as a random infiltrate with a well-defined round border between hematopoietic and fat cells.

Immunohistochemistry and in situ Hybridization

Bone marrow trephine biopsy specimens were wax embedded and cut into 4- μ m-thick serial sections and prepared for this study following decalcification according to the approach described by Schaefer [11]. For immunohistochemistry, the sections were deparaffinized with graded series of alcohol and

xylene. Thermal antigen demasking (100°C) was performed in 0.01 M citrate buffer for 30 min, automatically performed by the Bond™ system (Vision BioSystems, Mount Waverley, Vic., Australia). Monoclonal anti-CD138 (clone: 5F7, 1:80 dilution; Novocastra Laboratories, Newcastle-upon-Tyne, UK) was applied as the primary antibody for decoration of PC, and was incubated at room temperature for 1 h. Subsequently, sections were washed with PBS and incubated with rabbit anti-mouse IgG (1:50) followed by mouse peroxidase-antiperoxidase conjugate (1:200). The enzymatic reaction was developed in a freshly prepared solution of diaminobenzidine (0.5 mg/ml; Sigma, Deisenhofen, Germany) and 0.01% hydrogen peroxide in water. Negative controls were prepared by omitting the first-layer antibody. Trephine biopsies were subsequently subjected to the detection of immunoglobulin light-chain mRNA by means of Bond in situ hybridization employing lambda (catalog No. PB0669) and kappa (catalog No. PB0645) probes according to the manufacturer's instructions.

AgNOR Staining

Smears were simultaneously processed under the same conditions according to the standardized approach described by Fiorella et al. [12]. Briefly, following removal of coverslips from the glass slides, destaining was performed with 5% aqueous trichloroacetic acid for 5 min. After rinsing with distilled water and drying, the slides were stained with two drops of a working solution consisting of two parts 50% aqueous silver nitrate and one part 2% gelatin in 1% formic acid. They were then again covered with a coverslip and kept in a dark room for 20 min. Thereafter, coverslips were again removed and the slides were washed in distilled water, dehydrated and mounted after counterstaining with Giemsa (fig. 1).

AgNOR Count and Morphometry

Morphometric analysis was performed on silver-stained cytologic smears of bone marrow aspirates using a semiautomatic computer-assisted digital interactive image analyzer (IBAS-2000; Kontron, Oberkochen, Germany) which consisted of a magnetic tablet connected with a microcomputer and light-emitting diode. Smears were viewed by means of a microscope equipped with a camera lucida, using an oil immersion objective with a final magnification of $\times 1,800$ in order to make AgNOR separation straightforward. Following accurate outlining of AgNOR boundaries using the light-emitting diode projection onto the microscopic field of study, AgNOR areas were calculated automatically. Additionally, the total number of AgNORs per PC nucleus were counted considering a total of 100 different PC nuclei in each specimen. All smears were independently assessed by two clinical histopathologists (P.A.M. and S.K.) and reassessed later in order to analyze intra- and interobserver variability.

Statistical Analysis

The nonparametric Mann-Whitney U test was used to statistically compare AgNOR count and area among the different groups (RP, low-grade and intermediate-grade MM) except for high-grade MM due to the limited number of cases available ($n = 3$). Receiver-operating characteristic curve (ROC) analysis was performed in order to determine the optimal cutoff point in terms of differentiation between RP and MM. Intra- and interob-

Fig. 1. Silver-impregnated Giemsa-stained cytologic smears from bone marrow aspirates. Note the steady increase in AgNOR areas when comparing RP (a), low-grade (b), intermediate-grade (c) and high-grade (d) MM. $\times 100$ objective.

server reliability was established by means of Cohen weighted κ statistics. $p < 0.05$ was considered significant. All calculations were performed using the statistical software package SPSS 13.0.

Results

Histopathology of Medullary Involvement of MM and RP

All MM cases showed approximately 25–30% of neoplastic PC in bone marrow trephine biopsies and aspirates, consistently displaying an interstitial pattern of medullary involvement. A nodular and/or diffuse pattern of medullary infiltration was not seen in any of the

cases investigated. The same holds true concerning medullary fibrosis and osteolytic changes. Based on in situ hybridization, all MM cases showed monoclonal light-chain restriction, which was vice versa excluded in all RP cases.

AgNOR Area and Count

The results of AgNOR area and count are summarized in tables 1 (AgNOR area) and 2 (AgNOR count). Due to the limited number of high-grade MM available in this study ($n = 3$), no attempt was made to statistically compare this group with the other groups. Therefore, in tables 1 and 2 no p values are provided concerning high-grade MM.

Table 1. AgNOR area in RP and MM in cytologic smears

Specimen	AgNOR area, μm^2					p value
	mean	range	95% CI	SE	SD	
RP (n = 20)	6.2	3.8–8.4	4.7–7.3	1.3	5.8	<0.001
Low-grade MM (n = 23)	23.4	19.2–26.7	20.1–25.6	2.1	10.1	
RP (n = 20)	6.2	3.8–8.4	4.7–7.3	1.3	5.8	<0.001
Intermediate-grade MM (n = 22)	36.2	28.5–42.3	29.3–38.1	2.6	12.2	
Low-grade MM (n = 23)	23.4	19.2–26.7	20.1–25.6	2.1	10.1	0.02
Intermediate-grade MM (n = 22)	36.2	28.5–42.3	29.3–38.1	2.6	12.2	
High-grade MM (n = 3)	90.3	66.2–101.5	76.1–98.4	3.8	6.6	

CI = Confidence interval for mean; SE = standard error; SD = standard deviation.

Table 2. AgNOR count in RP and MM in cytologic smears

Specimen	AgNOR count					p value
	mean	range	95% CI	SE	SD	
RP (n = 20)	3.68	1–6	1.8–5.2	0.2	0.9	0.7
Low-grade MM (n = 23)	5.36	1–7	3.2–5.9	0.4	1.9	
RP (n = 20)	3.68	1–6	1.8–5.2	0.2	0.9	0.02
Intermediate-grade MM (n = 22)	6.52	2–8	5.4–7.0	0.3	1.4	
Low-grade MM (n = 23)	5.36	1–7	3.2–5.9	0.4	1.9	0.03
Intermediate-grade MM (n = 22)	6.52	2–8	5.4–7.0	0.3	1.4	
High-grade MM (n = 3)	7.21	3–9	7.1–7.8	0.7	1.2	

CI = Confidence interval for mean; SE = standard error; SD = standard deviation.

The mean AgNOR area was found to be significantly higher in low- and intermediate-grade MM compared to RP ($p < 0.001$). There was also a significant ($p = 0.02$) difference in terms of the mean AgNOR area between low- and intermediate-grade MM, accompanied by a trend toward a further increase in the mean AgNOR area in high-grade MM (fig. 1, table 1).

Using receiver-operating characteristic curve (ROC) analysis, the optimal cutoff point was found to be 13.8 (95% confidence interval: 11.2–15.6) for the differentiation between RP and MM.

The mean AgNOR count failed to differ significantly between RP and low-grade MM, although there were significant differences between RP and intermediate-grade MM ($p = 0.02$), and between low- and intermediate-grade

MM ($p = 0.03$), accompanied by a trend toward a further increase in the mean AgNOR count in high-grade MM (table 2).

Reproducibility of the Data

Reproducibility analysis revealed substantial intra- and interobserver concordance concerning AgNOR area and count (table 3).

Discussion

Evaluation of interphase AgNORs represents an independent prognostic parameter for various types of human malignancies, e.g. pharyngeal carcinoma,

Table 3. Intra- and interobserver variability in terms of AgNOR area and count determination

	Agreement	κ value
Intraobserver variability		
Count	84%	0.76
Area	85%	0.77
Interobserver variability		
Count	82%	0.75
Area	86%	0.78

prostate cancer, renal cell carcinoma and MM [13]. Concerning the latter, AgNOR counts have been reported to correlate with histologic grading [14] and to be reflective of myeloma cell mass and myeloma cell kinetics [9].

We present the first study focusing on the biological principle of comparing AgNOR area and count in bone marrow aspirates in the setting of reactive and neoplastic PC proliferations to elucidate so far neglected conventional morphologic parameters, aiming to expand the multiparametric approach established in this scenario. Since the lack of a standardized silver-staining protocol has led to misinterpretation [6], a standardized silver-staining procedure [12] was employed in this study.

We found a significantly increased mean AgNOR area in MM compared with RP. Among the MM cases investigated, conventional dedifferentiation seemed to

follow a trend towards higher mean AgNOR areas, confirming the role that has been attributed to the mean AgNOR area in various other malignancies [15–19]. Our results are also in line with studies on the association between mean AgNOR count and histologic grading in MM [9, 14].

The mean AgNOR count has been suggested to be an index for M protein synthesis rather than an independent prognostic factor in MM [20]. Therefore, from a morphological point of view, AgNOR area and count appear to morphologically reflect different aspects behind the neoplastic progression of MM. This might explain the conflicting reports on the questionable prognostic significance of the mean AgNOR count in MM [8, 20].

Routinely, sufficient data are commonly obtained by flow cytometry of bone marrow aspirates. However, since the latter represents an ‘amorphological’ approach, clinical histopathologists might feel more comfortable when evaluating conventional morphological parameters such as AgNORs.

A possible limitation of our study is the limited number of cases evaluated which might explain the lack of the nodular and/or diffuse pattern of marrow infiltration in any of the trephine biopsies investigated. Therefore, although our data strongly hint at a significant discriminating ability of the mean AgNOR area with respect to differentiation between RP and MM in bone marrow aspirates, these novel findings clearly need to be confirmed by larger prospective clinical trials.

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