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Prognostic significance of standardized AgNOR analysis in early and advanced gastric carcinomas

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Abstract To assess the prognostic significance of silver-stained nucleolar organizer region (AgNOR) proteins, a standardized AgNOR analysis was performed on 78 patients affected by early (EGC, *n*=24) or advanced (AGC, *n*=54) gastric carcinomas. The histopathological diagnosis, grading and staging were done according to WHO and UICC recommendations; the mean follow-up time was 56.9 months. Visualization and quantification of AgNORs were made in formalin-fixed, paraffin-embedded sections as specified in the guidelines of the Committee on AgNOR Quantification (1995). Statistical analysis was performed on the mean AgNOR area values (NORA). Highly significant differences ($P<0.001$) were found in NORA values between EGC and AGC, between low- and high-grade gastric carcinomas and between patients dead from gastric cancer and living patients. In addition, significant *P* values were found on comparison of NORA values relating to pT status, pN status and stage. Comparison of Kaplan-Meier survival curves revealed that patients affected by gastric carcinomas with higher NORA values (>5.213 μm^2) had a worse prognosis. Finally, using Cox multiple regression analysis, the AgNOR quantity emerged as a useful independent prognostic variable to predict the final outcome of patients affected by EGC or AGC.

Key words AgNORs · Standardized AgNOR analysis · Gastric carcinoma · Proliferation · Prognosis

Introduction

The AgNOR technique is a simple and inexpensive silver-staining procedure that allows visualization of a set of argyrophilic nonhistone proteins localized in the nucleolar organizer region [6, 34]. These silver-stained nucleolar organizer region (AgNOR) proteins are associated with ribosomal genes, and their quantity has been demonstrated to be strictly related to the rate of cell proliferation [8, 9, 27].

In histopathology, analysis of the interphase AgNOR proteins has been used to differentiate preneoplastic and neoplastic lesions [4, 7, 22, 24, 43], but the diagnostic utility of the AgNOR technique has been limited by the overlap of AgNOR values in benign and malignant neoplasms [7, 25]. In recent years, studies concerning the AgNOR applications in neoplastic pathology have stressed the prognostic value of this histochemical method [6, 13, 23, 25, 37].The AgNOR content has been proven to be an independent predictive variable in various malignancies such as thymoma, renal cell carcinoma, breast carcinoma, pharyngeal carcinoma, squamous cell carcinoma of the lung and colorectal carcinoma [1, 2, 5, 10, 30, 32, 33, 37]. Moreover, recently proposed standardized AgNOR staining protocol and quantification methods offer the possibility of investigating this prognostic variable in a reproducible manner on routinely processed archival material [26, 28, 29, 38]. Successful AgNOR analysis has been performed in colorectal carcinoma, breast cancer and lung carcinoma [30, 31, 42].

We tested human early and advanced gastric carcinomas both to evaluate their cell cycle speed and to verify the prognostic value of AgNOR proteins.

Materials and methods

Surgical samples from 78 patients (44 male, 34 female; mean age 61.6 years, ranging from 26 to 84 years) resected for gastric carcinoma were included in this study: in 24 early gastric carcinoma (EGC) was diagnosed and in 54, advanced gastric carcinoma (AGC).

The carcinomas were classified and staged according to WHO criteria [44] and the UICC recommendations [14], respectively; the main clinico-pathological data are summarized in Table 1. Preoperative radiation and/or chemotherapy were not performed in any of these cases. Data concerning follow-up and cause of death were obtained from city registry offices; 2 patients who died dur-

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Table 1 Clinico-pathological data and corresponding mean NORA values (μm²) in 78 gastric carcinomas cases (*NS* not significant)

Parameter	No.	NORA	SD	*P* value
Sex				
Male	44	5.126	±1.411	NS
Female	34	5.325	±1.816	
Tumour location				
Antrum	53	5.248	±1.519	NS
Antrum, body	9	5.813	±1.549	
Body	13	4.557	±1.823	
Cardia	3	5.626	±1.836	
Type of tumour				
Early gastric carcinoma	24	4.166	±1.099	<0.001
Advanced gastric carcinoma	54	5.678	±1.563	
Histological type				
Tubular adenocarcinoma	40	4.908	±1.415	NS
Papillary adenocarcinoma	7	5.690	±1.391	
Mucinous adenocarcinoma	9	5.064	±1.583	
Signet-ring cell carcinoma	22	5.675	±1.892	
Histological grading				
Low	47	4.688	±1.293	<0.001
High	31	6.009	±1.689	
TNM class				
pT1	24	4.166	±1.099	<0.05
pT2	21	5.060	±1.463	(pT1, pT3, pT4 *vs* pT2)
pT3	27	5.947	±1.401	<0.001
pT4	6	6.632	±2.010	(pT1 *vs* pT3, pT4)
pN0	30	4.172	±1.043	<0.001
pN1	24	5.237	±1.630	
pN2	24	6.490	±1.158	
Stage				
Stage Ia	17	4.236	±1.028	<0.001
Stage Ib	17	4.032	±1.204	(Ia, Ib *vs* II, IIIa, IIIb, IV)
Stage II	12	5.561	±1.365	
Stage IIIa	10	5.929	±1.512	
Stage IIIb	12	5.941	±1.290	
Stage IV	10	6.874	±1.301	
Clinical course				
Alive	32	3.796	±0.886	<0.001
Death from gastric cancer	44	6.210	±1.137	

ing surgery were excluded from the survival study. At the time of the analysis, 44 (57.9%) patients (5 EGC and 39 AGC) had died of disease, and 32 (42.1%) (19 EGC and 13 AGC) were censored. The mean follow-up time of all patients was 56.9 months (ranging from 3 to 197), while for censored patients it was of 101.5 months (ranging from 36 to 197); in detail, for this latter group, the mean follow-up time was 105.3 months (ranging from 36 to 197) for EGC and 95.9 months (ranging from 48 to 144) for AGC.

All surgical samples were fixed in 10% neutral formalin for 12–24 h at room temperature and then embedded in paraffin at 56°C. From each tissue block, two consecutive 3-μm-thick sections were cut and mounted on silane-coated glasses, then dewaxed in xylene and rehydrated in graded ethanols; one was then subjected to haematoxylin-eosin (H&E) staining and one to the AgNOR technique according to the guidelines of the Committee on AgNOR Quantification [27]. In particular, sections were immersed in sodium citrate buffer (pH 6) and incubated in a wet autoclave at 120°C (1.1–1.2 bar, at sea level) for 20 min and then allowed to cool down to 37°C; the slides were then immersed in a freshly prepared silver-staining solution containing one part by volume of 2% gelatine in 1% formic acid and two parts of 25% aqueous silver nitrate solution at 37°C in a thermostatically controlled environment for 11 min. The reaction was then stopped by washing the slides with double-distilled deionized water to remove unwanted silver precipitates. Finally, all sections were dehydrated in increasing concentrations of ethanols, clarified in xylene and mounted with a synthetic medium (Permount).

Quantification of AgNORs was performed by an image analysis system (Immagini e Computer, Rho-Milan, Italy) consisting of an optical Leitz microscope fitted with a single chip colour CCD video camera (Ikegami ICD-840PDC, Ikegami Tsushinki, Tokyo, Japan) with a resolution of 460×420 (horizontal×vertical) TV lines, a colour monitor and an image processing unit installed in a 486/33 MHz processor-based personal computer. For each slide examined, microscopic fields representative of the lesions were assessed excluding, on the basis of the corresponding H&E-stained section, any areas in which regressive changes, frank necrosis or technical artefacts were present. The peripheral infiltrating portion of any neoplasias was preferred as an area of study. The mean area (μm²) of AgNORs per cell (NORA) was evaluated in one focal plane with a ×40 objective lens in at least 100 (mean 121) nuclei per specimen; specific software, namely IM 5200 (Microscience) and AgNOR (Immagini e Computer, Rho-Milan, Italy), were used to determine mean NORA values per cell and per case, respectively.

After the normal distribution of NORA values in all patients had been tested by the Kolmogorov-Smirnov test, a cut-off point was determined utilizing the mean NORA value. A statistical descriptive analysis was performed for each clinico-pathological variable; differences among categories were assessed by analysis of variance and the Newman-Keuls' test, while correlations between continuous parameters were investigated by Spearman's rank test. Survival analysis was performed by the Kaplan-Meier method, and for comparison of the survival curves the Mantel-Cox

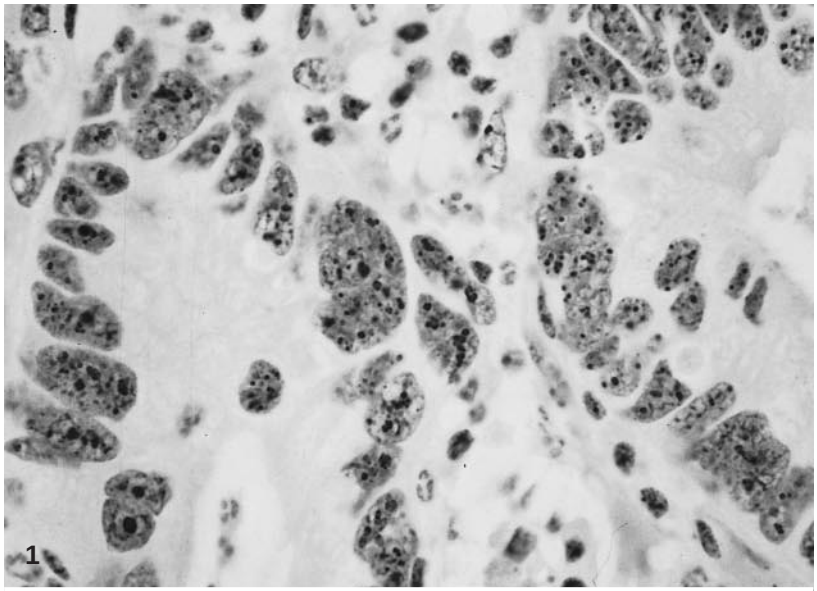

Fig. 1 Tubular adenocarcinoma – numerous black dots scattered throughout the nucleus in the neoplastic elements. Ag-NOR technique, ×157

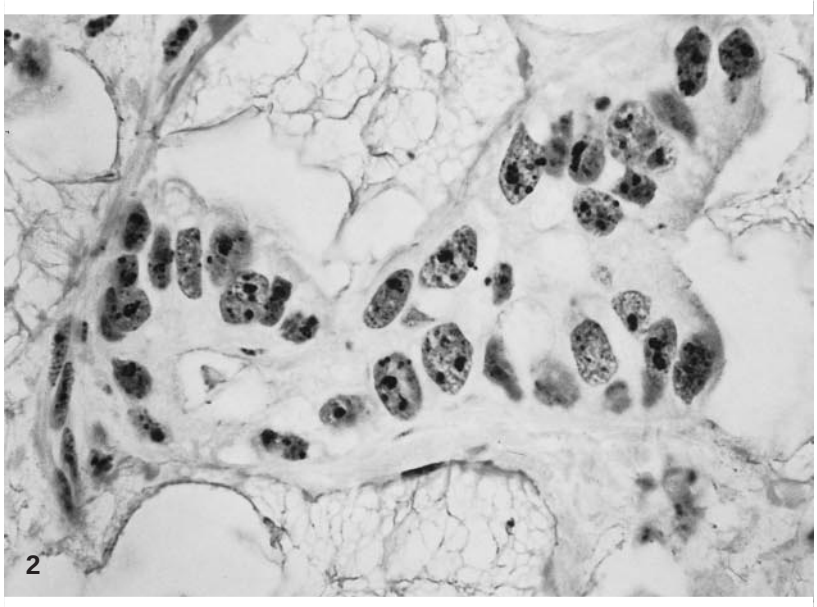

Fig. 2 Mucinous adenocarcinoma – coarse argyrophilic precipitates in nuclei present in a neoplastic islet. AgNOR technique, ×157

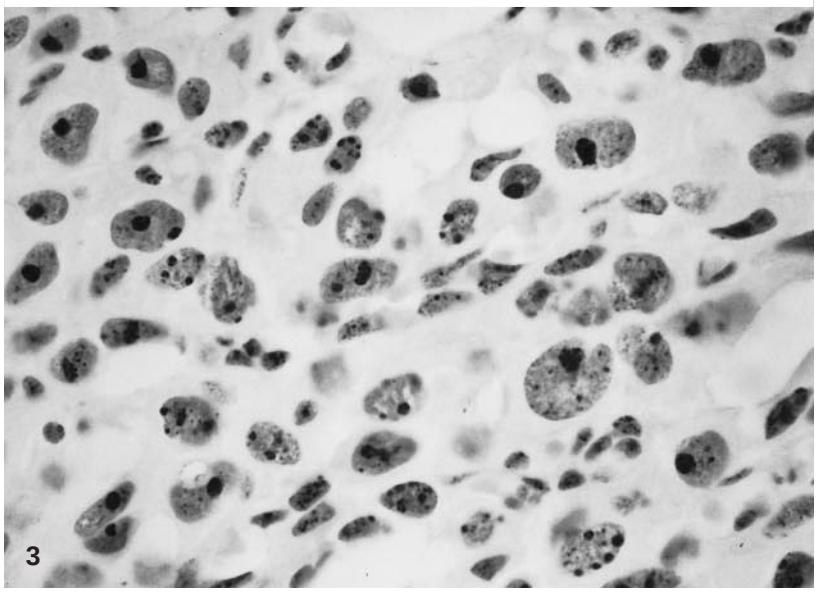

Fig. 3 Signet-ring cell carcinoma – AgNORs clustered in irregularly shaped collections. AgNOR technique, ×157

Table 2 Prognostic parameters examined in gastric carcinoma cases: a univariate analysis of cancer-specific mortality by Mantel-Cox log-rank test (*NS* not significant, *df* degrees of freedom)

Parameter	χ^2	*df*	*P* value
All patients (*n*=76)			
Sex	1.58	1	NS
Type of tumour	19.32	1	<0.0001
Histological type	1.50	3	NS
Histological grading	9.11	1	<0.0025
pT status	43.36	3	<0.0001
pN status	54.36	2	<0.0001
Stage	64.66	5	<0.0001
NORA	61.32	1	<0.0001
EGC patients (*n*=24)			
Sex	0.18	1	NS
Histological type	6.97	3	NS
Histological grading	3.65	1	NS
pN status	0.33	1	NS
Stage	0.33	1	NS
NORA	25.06	1	<0.0001
AGC patients (*n*=52)			
Sex	0.70	1	NS
Histological type	3.35	3	NS
Histological grading	0.86	1	NS
pT status	15.57	2	<0.0004
pN status	28.03	2	<0.0001
Stage	30.68	4	<0.0001
NORA	24.94	1	<0.0001

log-rank test was used. Finally, a multivariate analysis (Cox regression model) was utilized to determine the independent effect of each variable on survival. A *P*-value less than 0.05 was considered statistically significant.

Results

All silver-stained specimens of gastric carcinoma showed an adequate staining intensity homogeneously present throughout the whole section; in particular, the AgNORs were clearly distinguishable as black dots, even within nucleoli (Figs. 1–3). In carcinomatous cells, the AgNORs were often clustered in irregularly shaped collections, with an increase of extranucleolar silver dots scattered throughout the nucleus (Figs. 1–3). In lymphoid aggregates, which were encountered in some samples, lymphocytes exhibited a single, round, centrally localized AgNOR.

NORA values related to clinico-pathological data are given in Table 1. A significant *P*-value was achieved in the correlation of NORA with type of tumour, histological grading, TNM class, tumour stage and clinical course, while no relationships were found with sex, tumour location and histological type (Table 1); moreover, the AgNOR quantity in gastric carcinomas was unrelated to the patient's age.

In gastric carcinoma specimens, NORA values showed a normal distribution (two-tailed *P*=0.543) with a mean of 5.213±1.592 μm^2. In particular, 4/24 EGC and 36/52 AGC cases had NORA values greater than the cut-off point. Data obtained by univariate analysis of prognostic parameters examined in all 76 patients and in selected EGC and AGC cases are reported in Table 2. Among the prognostic variables examined, type of tumour, histological grading, pT and pN status, stage and NORA showed significant *P*-values when all patients were considered; the survival curves of all patients with low and high NORA values are illustrated in Fig. 4. Moreover, in stratified analysis, the significant relationship between NORA parameter and prognostic outcome was also maintained when the type of tumour (EGC or AGC) was used to define the strata (Table 3). Three multivariate survival analyses were performed by the Cox regression model. With the first model, including all 76 patients, NORA and stage of carcinoma were identified as independent prognostic variables; in the second model including only EGC cases, NORA was the only exclusively selected variable, while in the third model, including only AGC cases, NORA and stage were again revealed as independent variables (Table 3).

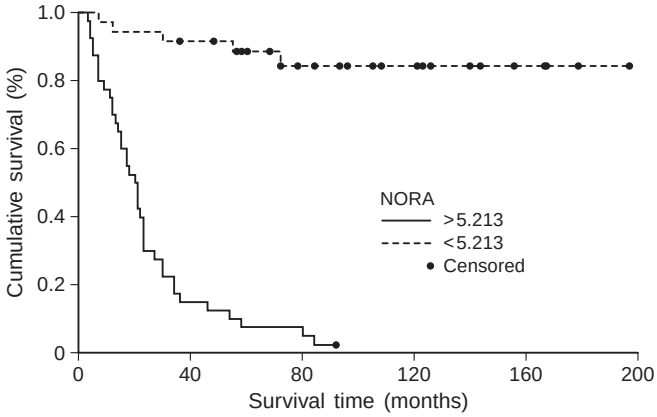

Fig. 4 Kaplan-Meier survival curves of all patients with low and high NORA values

Table 3 Multivariate survival analysis by Cox regression model in gastric carcinoma cases (β regression coefficient, *SE* standard error, *Exp(β)* ratio of risk)

Variable	β	SE	Exp(β)	*P* value
All patients (n=76)				
NORA	2.348	0.534	10.465	<0.0001
Stage	0.462	0.125	1.588	<0.0002
EGC patients (n=24)				
NORA	3.584	1.129	36.047	<0.0015
AGC patients (n=52)				
NORA	1.762	0.583	5.826	<0.0025
Stage	0.513	0.153	1.671	<0.0008

Discussion

In gastric pathology, routine use of the AgNOR technique for diagnosis has been considered questioned because of the overlap of AgNOR number in hyperplastic, dysplastic and neoplastic lesions [3, 11, 36, 40, 41].

Even if a significantly higher AgNOR count is found in dysplastic mucosa than in nondysplastic mucosa [3] or in hyperplastic mucosa [11] the AgNOR area can only be considered as one discriminant variable between low- and high-grade gastric dysplasias [21]. No significant correlations have been found between the percentages of MIB-1 and of PCNA dysplastic elements and the corresponding AgNOR area and number [21]. However, the AgNOR count has been shown to be a useful predictor of nodal metastasis in gastric carcinomas regardless of tumour size, depth of invasion and histological type [17, 18]; in addition, the prognostic value of the AgNOR count has been verified in endoscopically biopsied specimens at the margin of invasive gastric carcinomas [19] and in patients with AGC with serosal invasion [15].

In order to achieve good and reproducible results, especially when the AgNOR analysis is utilized in the prediction of tumour aggressiveness, a standardized staining protocol and method of evaluation must be applied, as reported elsewhere [29]. Wet-autoclave preteatment has brought about a notable improvement in staining quality of single-interphase AgNORs, regardless of tissue origin and of the duration of formalin fixation and archival storage [26]. To ensure better comparisons between different laboratories, we have performed AgNOR staining at 37°C in a thermostatically controlled environment, and quantification by an image analyser system is a further contribution to standardization, being more objective and free of observer bias than counting AgNORs by eye [27], which is clearly only best and should be restricted to adequately stained slides with cells exhibiting distinct AgNORs as definite substructures of nucleoli.

We have documented here significantly higher mean NORA values in advanced gastric carcinoma than in the early type, in high-grade carcinomas than in low-grade ones, and in patients dead of gastric cancer than in living patients with an uneventful clinical course. In addition, significant *P*-values have been found on comparison of NORA values relating to pT and pN status and to tumour stage. In our opinion, the differences in NORA values may be due to a slower cell cycle time in EGC than AGC; it is well known that a considerable number of EGC have a relatively slow rate of growth [16, 45]. However, it has been noted that in the natural history of gastric carcinoma the duration of early phase is extremely variable: some cancers begin to invade very soon, while others take more than 10 years to enter the advanced phase [12, 35]. Carcinomas of the latter type are more likely to be detected at the stage of EGC, while cases with a rapid growth have already become AGC before they are diagnosed [16, 35]. Nevertheless, studies performed by cell kinetic methods have not found any differences in the growth fraction between EGC and AGC [12, 20, 39]. A greater number of AgNORs has been found in AGC than EGC; this difference was not statistically significant [41], but perhaps this was due to the small number of EGC cases examined.

Univariate analysis of various potential prognostic factors in our cases of gastric carcinoma showed that NORA, the type of tumour, the histological grading, pT and pN status and the stage were significant prognostic factors. Moreover, when the cases were stratified according to early or advanced type of tumour, the prognostic significance of the NORA parameter was maintained. Finally, multivariate analysis by the Cox regression model clearly documented that NORA was an independent variable in EGC or AGC, suggesting that the AgNOR method may be included in the list of prognostic factors with a high rank order of influence on final outcome.

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