Quantity of AgNOR in gastric endocrine carcinoid tumours as a potential prognostic tool

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In order to assess if the quantity of silver-stained nucleolar organizer region (AgNOR) proteins represents a prognostic tool in gastric carcinoids, a standardised AgNOR analysis was performed on 24 samples collected from the pathology archives of the Universities of Messina and Parma; the samples were taken at surgery from 11 males and 13 females (mean age 55 yrs, age range 28-77 yrs); 13 cases were defined as Type I, 1 case as Type II and 10 cases as Type III; 16 cases showed a diameter <1 cm, 8 >1 cm. Only 6 tumours were deeply invasive, breaking through the muscularis propria or the subserosa. The proliferative status of carcinoids performed by Ki67 protein antibodies was available in 20/24 cases. The quantification of AgNORs was performed according to the guidelines of the Committee on AgNOR Quantification and the mean area (μm^2) of AgNORs per nucleus (NORA) was determined by means of image analyser and specific software programs. The relationship between NORA values and Ki67 data was investigated by Spearman correlation test. The mean NORA value of all 24 gastric carcinoids was 1.279 μm^2 (SD 0.404); values ranged from 0.734 to 2.142 μm^2. A significantly higher ($p<0.001$) mean NORA value (1.736 μm^2; SD 0.283) was found in tumours larger than 1 cm, in comparison to the smaller neoplasms (1.051 μm^2; SD 0.214); moreover, cases showing deep wall invasion exhibited a mean NORA value of 1.765 μm^2 (SD 0.276), significantly higher ($p<0.001$) than those with superficial growth (1.118 μm^2; SD 0.296). Finally, a similar highly significant difference was seen between type III carcinoids (1.615 μm^2; SD 0.375) and type I-II (1.040 μm^2; SD 0.208). A linear relationship between Ki67 and corresponding NORA values was obtained by the Spearman correlation test ($p=0.001$). No other significant correlations were found between mean NORA values and other clinico-pathological parameters. The AgNOR method seems to be an additional tool potentially able to predict the prognosis of this kind of endocrine tumour, facilitating the identification of fast-growing tumours and being able to directly correlate with the size, deep invasion of gastric wall and tumour type, generally considered as the best prognostic indicators.

Key words: AgNORs, standardised AgNOR analysis, gastric carcinoid, Proliferation, prognosis

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The AgNOR technique allows the visualisation at the light microscopic level of a set of argyrophilic non-histone proteins localised in the nucleolar organizer regions (NORs) (Ploton *et al.,* 1986; Derenzini and Ploton, 1991). 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 (Derenzini *et al.,* 1989; Trerè *et al.,* 1989; Delahunt *et al.,* 1991; Öfner *et al.,* 1992)

In histopathology, the analysis of the interphase AgNOR proteins has been extensively used to differentiate preneoplastic from neoplastic lesions (Crocker and Skilbeck, 1987; Derenzini and Trerè, 1991; Muscarà *et al.,* 1991; Tuccari *et al.,* 1993; Muscarà *et al.,* 1997), although the diagnostic usefulness of the AgNOR technique has been limited by the overlap of AgNOR values between benign and malignant neoplastic cases (Derenzini and Trerè, 1991; Öfner and Schmid, 1995). Moreover, other studies concerning this histochemical method have shown the prognostic value of the AgNOR amount as being an independent variable able to predict the recurrence and/or the overall survival in various kinds of malignancies (Rüschoff *et al.,* 1990; Delahunt *et al.,* 1991; Eusebi *et al.,* 1991; Pich *et al.,* 1992; Aubele *et al.,* 1994; Pich *et al.,* 1994; Öfner *et al.,* 1995; Antonangelo *et al.,* 1997; Giuffrè *et al.,* 1997; Giuffrè *et al.,* 1998; Tuccari *et al.,* 2000). However, the AgNOR quantity has been found to also be increased in the active phase of non-neoplastic diseases (Tuccari *et al.,* 1993; Muscarà *et al.,* 1997), and in regenerating cells (Tuccari *et al.,* 1999). Moreover, the introduction of international guidelines for AgNOR analysis have permitted the investigation of the proliferation rate on routinely processed archival material in a reproducible manner (Öfner *et al.,* 1995).

Carcinoid tumors originate from the neuroendocrine cells throughout the body and are capable

of producing various peptides. Their clinical course is often indolent, but they can be aggressive and resistant to therapy (Oberg, 2003; Schnirer *et al.,* 2003). As regards neuroendocrine tumours, only few studies based on a non-standardised AgNOR procedure have been performed, and only exclusively in bronchial, pulmonary and pancreatic neoplasms (Benbow and Cromie, 1989; Soomro *et al.,* 1991; Bohm *et al.,* 1993; Rüschoff *et al.,* 1993). The aim of the present study was to perform a standardised AgNOR analysis in gastric carcinoids in order to assess its potential prognostic role, which heretofore had not been determined.

Materials and Methods

Twenty-four gastric endocrine neoplastic samples were collected from the pathology archives of the Universities of Messina and Parma; they had been removed at surgery from 11 males and 13 females (mean age 55 years, age range 28-77 yrs). On the basis of clinico-pathological guidelines (Rindi *et al.,* 1993), 13 cases (54.1%) were defined as Type I (associated with chronic atrophic gastritis, with or without pernicious anemia), 1 (4.2%) as Type II (associated with MEN1 and/or Zollinger-Ellison syndrome) and 10 (41.7%) as Type III (sporadic tumours, not related to hypergastrinemia, mainly localized in the antrum or corpus).

According to WHO classification of gastropancreatic endocrine tumours (Solcia *et al.,* 2000), tumour size, histological features and clinical data were taken into account to classify these cases. The tumour size was <1 cm in 16 cases, and >1 cm in 8 cases. The level of gastric wall invasion was also noted and is reported in Table 1; in particular, 6 tumours were deeply invasive, breaking through the muscularis propria or the subserosa. Microscopic features were characterized by a solid, trabecular or microglandular pattern composed of small regular cells with finely granular cytoplasm and round or oval nuclei. Moreover, immunohistochemical reactivity for chromogranin A and synaptophysin was encountered in all cases. The proliferative status of carcinoids, performed by Ki67 protein antibodies (Cattoretti *et al.,* 1993), was available in 20/24 cases and is reported in Table 1 together with other main clinico-pathological data.

All surgical samples had been fixed in 10% neutral formalin for 12-24 hrs at room temperature and then embedded in paraffin at 56°C. From each

Table 1. Clinico-pathological data and corresponding NORA values in gastric carcinoids.

Case	*Sex*	*Age*	*Carcinoid Type*	*Size (cm)*	*Gastric wall invasion*	*Ki67(%)*	NORA (μm^2)	NORA (mean±SD)
1	F	43	1	0.3	Submucosa	NA	1.505	
2	M	72	1	0.3	Submucosa	0.4	0.784	
3	M	77	1	0.4	Mucosa	0.4	0.734	
4	F	63	1	0.4	Submucosa	0.6	1.04	
5	F	72	1	0.5	Submucosa	15	0.947	
6	F	71	1	0.5	Submucosa	NA	0.845	
7	M	59	1	0.5	Mucosa	0.7	0.941	
8	F	39	1	0.5	Submucosa	0.3	1.023	
9	F	55	1	0.5	Mucosa	0.7	0.914	
10	F	58	1	0.5	Submucosa	1	1.1	
11	M	49	1	0.8	Mucosa	0.9	1.118	
12	F	53	1	0.9	Mucosa	0.1	1.141	
13	F	63	1	1	Submucosa	1	1.135	
14	M	30	2	2	Mucosa	0.9	1.33	
								1.040 (±0.208)
15	F	28	3	0.4	Submucosa	NA	0.973	
16	M	43	3	0.5	Mucosa	NA	1.126	
17	F	47	3	0.6	Muscularis propria	2	1.49	
18	M	47	3	1.1	Muscularis propria	15	1.845	
19	F	33	3	1.5	Muscularis propria	14	1.956	
20	M	64	3	1.6	Muscularis propria	1	1.735	
21	M	66	3	2.1	Subserosa	30	1.421	
22	M	58	3	2.2	Submucosa	3	1.542	
23	M	71	3	2.5	Muscularis propria	28	2.142	
24	F	57	3	2.5	Mucosa	2	1.92	
								1.615 (±0.375)

NA: Not available; SD: Standard deviation.

tissue block, two consecutive 4 μm-thick sections were cut and mounted on silane-coated glass slides, then dewaxed in xylene, rehydrated in graded ethanols and submitted to haematoxylin and eosin (H&E) stain and to the AgNOR technique according to guidelines of the Committee on AgNOR Quantification (Öfner *et al.,* 1995), respectively. In detail, sections were immersed in sodium citrate buffer (pH 6.0) and incubated in 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. Subsequently, the slides were immersed in a freshly prepared silver-staining solution containing one part (v:v) 2% gelatin in 1% formic acid and two parts 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 ascending ethanols, clarified in xylene and mounted with a synthetic medium (Permount).

The quantification of AgNORs was performed by an image analysis system consisting of an optical Leitz microscope fitted with a single chip colour CCD video camera (Ikegami ICD-840PDC, Ikegami Tsushinki Co. Ltd., Tokyo, Japan) having a resolution of 460 x 420 (horizontal x vertical) TV lines, a colour monitor and an image processing unit installed in a 486/33MHz processor-based personal computer. For each slide examined, microscopic fields representative of the lesion were assessed, excluding areas in which regressive changes, frank necrosis or technical artefacts were present, as compared with the corresponding H&E stained section. The mean area (μm^2) of AgNORs per cell (NORA) was evaluated on neoplastic cells at one focal plane with a x40 objective lens in at least 100 nuclei per specimen (mean 140); specific softwares, IM 5200 (Microscience Inc.) and AgNOR (Immagini e Computer, Rho-Milan, Italy), were utilised to determine mean NORA values *per* cell and *per* case, respectively.

After testing the normal distribution of NORA values in all the samples by the Kolmogorov-Smirnov test, a statistical descriptive analysis was performed (mean and standard deviation) and differences among categories were assessed by analysis of variance and t-test. Moreover, the relationship between NORA values and Ki67 data was investigated by the Spearman correlation test.

Results

All silver-stained specimens of tumour tissues showed an adequate staining intensity, homogeneously distributed throughout the section. In the nuclei, and also within the nucleoli, of neoplastic cells, the AgNORs were clearly distinguishable as black dots (Figure 1a); an increase of extranucleolar silver dots scattered throughout the nucleus was found in some specimens (Figure 1b), where the AgNORs were often clustered in irregularly shaped structures. Mature lymphocytes, whenever present, exhibited a single round-shaped, centrally localised AgNOR.

The gastric carcinoids showed a mean NORA value of 1.279 μm2 (SD 0.404), with the quantity of NORA ranging from 0.734 to 2.142 μm^2. When the cases were stratified on the basis of tumour size (>1 cm or <1 cm), the level of gastric wall invasion (mucosa or submucosa *versus* muscularis propria or subserosa) and the type of carcinoids (I-II *versus* III), different NORA values were obtained. In fact, a significantly higher ($p<0.001$) mean NORA value (1.736 μm^2; SD 0.283) was found in tumours larger than 1 cm, in comparison to the smaller ones (1.051 μm^2; SD 0.214); moreover, cases showing a deep wall invasion exhibited a mean NORA value (1.765 μm^2; SD 0.276) significantly greater ($p<0.001$) than those with superficial growth (1.118 μm^2; SD 0.296). Finally, a similar highly significant difference was seen between type III carcinoids (1.615 μm^2; SD 0.375) and type I-II (1.040 μm^2; SD 0.208). A linear relationship between available Ki67 data and corresponding NORA values was obtained with a Spearman correlation coefficient of 0.6752 ($p=0.001$).

No additional significant correlations were found among mean NORA values and other clinico-pathological parameters .

Discussion

In neuroendocrine tumours of the gastrointestinal tract, the differential diagnosis between benign and malignant tumours, as well as the prediction of their biological behaviour, are still open questions. In particular, clinico-pathological criteria of malignancy in well-differentiated neoplasms are partially organ-specific (Solcia *et al.,* 2000); moreover, the recently released WHO classification further subdivides non overtly malignant tumours into two subgroups,

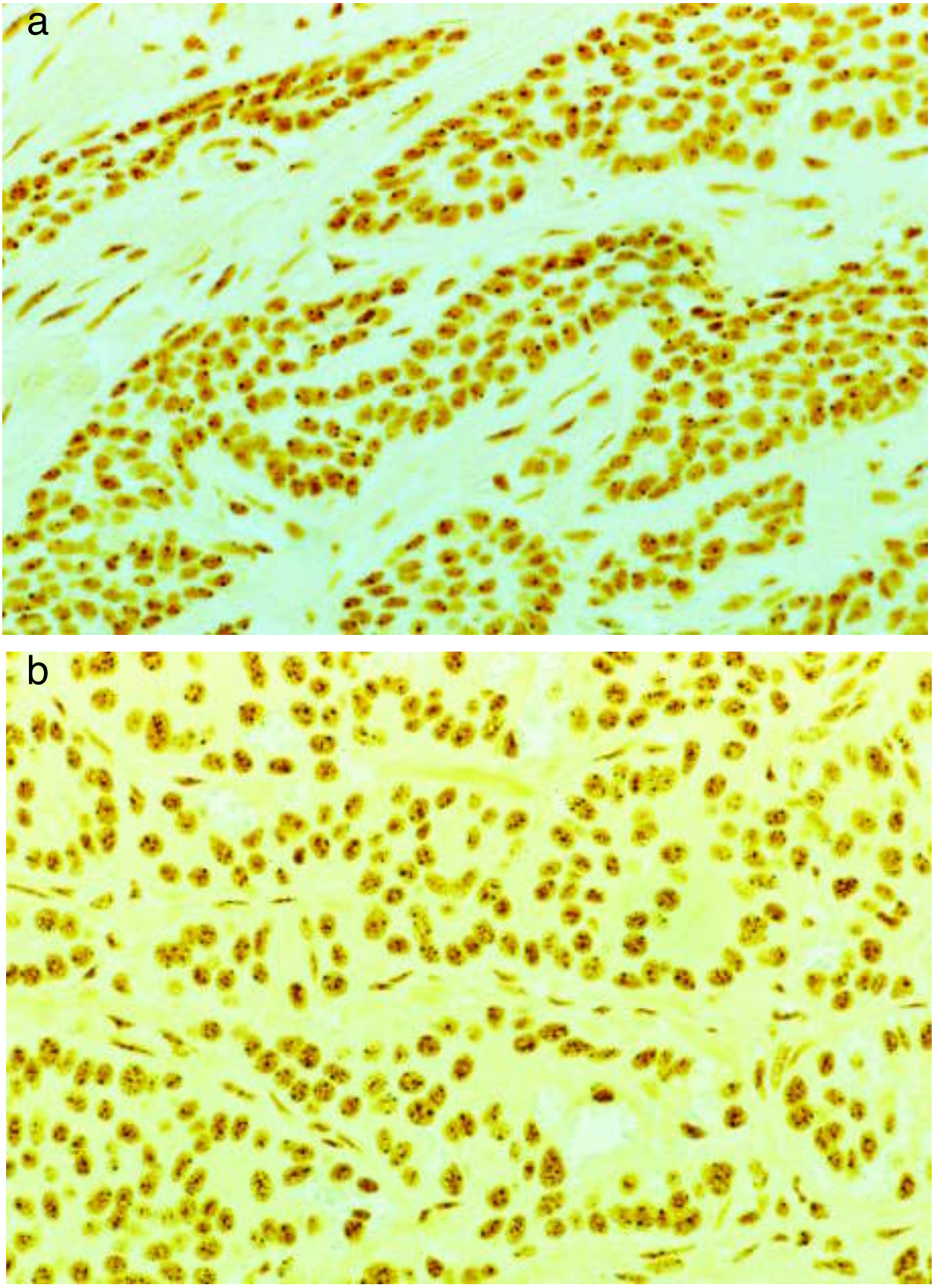

Figure 1. AgNOR method. Few single black dots are seen in the nucleus of neoplastic cells arranged in cords (a, x200); AgNORs are sometimes clustered in irregularly shaped collections inside nuclei of neoplastic cells (b, x200).

those with benign behaviour and those with uncertain, possibly malignant behaviour. The criteria for the identification of this latter subgroup in the stomach are based on tumour size, occurrence of angioinvasion, gastric wall invasion and growth fraction immunohistochemically expressed by Ki67 (Rindi *et al.,* 1999). Although a high Ki67 index has been considered able to correlate with morphological predictive parameters in gastric endocrine tumours, the routine use of this prognostic indicator in carcinoids is affected by several limitations concerning the standardization of the methodology used for the determination of the Ki67 index, the use and the role of antigen unmasking procedures and the definition of cut-off levels for differential recognition of tumours with benign or unfavourable outcome (Bordi *et al.,* 2002).

By the AgNOR method, only few studies have been carried out in order to discriminate between benign and malignant endocrine tumours, mainly in parathyroids (Boquist, 1990; Kanematsu *et al.,* 1997; Tuccari *et al.,* 2000), although a variable degree of overlap has been found; moreover, the inability of AgNOR counts to differentiate between bronchial carcinoid tumours and small cell carcinoma of the bronchus has been reported (Benbow and Cromie, 1989). The present study is the first in which the standardised AgNOR analysis has been performed in gastric carcinoids according to the guidelines of the Committee on AgNOR Quantification (Öfner *et al.,* 1995); in particular, by wet-autoclave pre-treatment, we have obtained a constantly high staining quality of single-interphase AgNORs, irrespective of the duration of formalin fixation and archival storage, similarly to that reported elsewhere (Öfner and Schmid, 1996). The AgNOR histochemical reaction is mainly due to the affinity for silver of the major nucleolar proteins involved in the control of rRNA synthesis and processing, such as nucleolin (C23 protein), nucleophosmin (B23 protein) and RNA polymerase I (Derenzini, 2000). In particular, nucleolin and RNA polymerase I are present in nucleolar regions associated with fibrillar centers and adjacent regions, thus corresponding to NORs; nucleophosmin is present mostly in nucleolar regions with pre-ribosomes (dense granular components) in the rest of the nucleolar body. Although some findings suggest that parameters of nuclear architecture functionally interface with components of transcriptional control (Stein *et al.,* 2004), all of these silver-stained proteins reflect the quantity of AgNORs and the ribosome biogenesis rate, as demonstrated in studies with neuroblastoma and breast cancer cell lines, in which the quantity of interphasic silver-stained NOR proteins is strictly related to the doubling time of tumour cells (Trerè *et al.,* 1989; Öfner *et al.,* 1992); thus, the greater the quantity of NORA, the faster the cell proliferation. Moreover, in order to attempt a further step in the standardisation of AgNOR analysis, we have performed the quantification of AgNORs area by an image analyser system, which is more objective and free of observer bias than counting silver precipitates by eye, similarly to that reported elsewhere (Rüschoff *et al.,* 1990; Öfner *et al.,* 1995; Öfner and Schmid, 1996).

In this series of cases, a highly significant difference in the AgNOR quantity was found in gastric carcinoids larger than 1 cm in comparison to the smaller ones, thus suggesting a progressive increase in the proliferation rate in those tumours, which, in the literature have been considered as possibly being malignant. Moreover, a significantly higher mean NORA value was noticed in cases with deep gastric wall invasion, which has been considered a predictor parameter of malignant outcome in gastric carcinoids (Rindi *et al.,* 1999). In addition, a highly significant difference in the AgNOR quantity was encountered when type III carcinoids were compared with those of type I-II, in agreement with the observations that type III carcinoids are *more active* lesions, with an aggressive course in terms of clinical setting (Rindi *et al.,* 1999). Finally, to obtain a much better insight into the proliferation kinetics of carcinoids, we have analysed NORA values in comparison to Ki67 data, which brought out a positive linear relationship between the respective dynamic and static parameter; therefore, the increased rate of cell proliferation, as well as the percentage of proliferating cells, explain the tumour mass growth rate, thus determining a worse prognosis.

On the basis of the present results, the AgNOR method seems to be an additional potential tool for making a prognosis in gastric carcinoids tumours; it facilitates the identification of fast-growing neoplasms and correlates with already recognized prognostic indicators such as the size, the level of gastric wall invasion, the type of these tumours and their Ki67 immunoexpression. Thus, the AgNOR analysis is recommended and should be applied to routine work due to its high significance, low cost, adequate standardization, simplicity and rapidity.

In particular, this approach may be useful preoperatively when only endoscopic biopsies are available, without any appropriate information on the tumour staging (size, lymph node involvement, metastasis).

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