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AgNORs AND PCNA IMMUNOREACTIVITY IN EARLY AND ADVANCED GASTRIC CARCINOMA

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ABSTRACT

In order to point out differences in the biological behaviour between early and advanced gastric carcinoma, their proliferative activity is evaluated on paraffin sections by silver staining of NOR particles and by immunohistochemical detection of PCNA.
AgNOR particles were measured by a Leitz-Texture Analyzing System. The following variables were considered: nuclear area; area, number and percentage area of AgNOR; mean area of each AgNOR particle. The percentage of PCNA-positive nuclei was calculated. The results failed to show any significant difference of EGC compared to AGC. The possibility that differences in biological behaviour, other than the proliferative activity, could exist between EGC and AGC, is discussed.

Key words: AgNOR, early gastric cancer, PCNA, proliferative activity.

INTRODUCTION

The natural history of early gastric cancer (EGC) is not yet well understood: in particular, it is not known how long is needed for EGC to evolve into advanced gastric cancer (AGC) and whether such evolution is inevitable. Cases of EGC with slow evolution have remained unaltered for up to 7 years (Eckardt et al., 1984). In other cases the EGC seems to evolve to an advanced stage in a short time (Inokuchi, 1984).
If the biological behaviour of EGC is heterogeneous such heterogeneity could be correlated with the proliferation rate of the neoplastic cells. In this study EGC cases which may be differentiated on the basis of their clinico-morphological properties are considered. They are compared with AGC cases, by means of silver-stained nucleolar organizing regions (AgNORs) measurement and anti-proliferating cell nuclear antigen (PCNA) antibody positivity.

MATERIAL AND METHODS

Fifty-seven well-differentiated, intestinal EGCs were selected from a total of 340 cases seen from 1974 to the present date at the University of Ancona, Institute of Morbid Anatomy. The study also included 14 well-differentiated intestinal AGC cases and 5 cases of normal gastric mucosa.
The 57 EGC cases were subdivided according to the following variables:
1. Depth of invasion: 36 EGCs were intramucosal and 21 submucosal. In 16 cases of submucosal EGC it was possible to measure the 2 components (intra- and submucosal) of the neoplasia separately. Therefore, the total number of observations was 73.

2. Size: 29 EGCs were large (greater than 2 cm.), 17 small (1-2 cm.) and 11 minute (less than 1 cm.).
3. Presence of neoplastic invasion of the submucosal lymphatic vessels (5 cases).
4. Presence of lymphnode metastasis (9 cases).

In all cases the stomach, opened along the greater curvature, was fixed in 10% buffered formalin and the samples were embedded in paraffin. Histological sections, representative of the lesion, 3-4 thick, were stained with Haematoxylin-Eosin, for AgNORs as described by Smith and Crocker (1988) and for PCNA using PC10 monoclonal antibody (DAKO, Glostrup, Denmark) according to Sternberger (1979).

The AgNOR-stained sections were examined with a Leitz-TAS image analyzing system, measuring the following parameters for each nucleus for a total of 100 nuclei per case: nuclear area, area and number of the AgNOR particles, percentage area of AgNOR particles compared to the nucleus, mean area of each AgNOR particle per nucleus. A 63x objective was used. The number of pixels/ on the Leitz-TAS monitor was 2.56.

The sections stained for PCNA were observed with a Leitz-Orthoplan microscope. Since in our experience the staining intensity depends on the fixation procedure, the germinative centre of lymphatic follicles was taken as a positive internal control. Thirty-six EGC and 8 AGC cases were selected, for each of which 1000 nuclei were examined, calculating the percentage of positive nuclei.

The numerical data were processed with the BMDP statistical package running on an IBM 4381 computer, using the programs BMDP2D (detailed data description), BMDP7D (variance analysis), BMDP3D (comparison of two groups with t-test) and BMDP7M (stepwise discriminant analysis).

Table 1 : Results of the descriptive statistical analysis (SD = standard deviation; SE = standard error)

		Nuclear Area	AgNORs Area	N° AgNORs	% Area AgNORs	Area per AgNORs	% PCNA
Mean value	EGC	59.87	6.41	2.805	11.64	2.68	66.94
	AGC	72.18	7.14	2.69	11.07	2.89	61.25
	Normal Mucosa	34.06	2.39	1.16	12.15	2.16	--
SD	EGC	13.52	1.69	0.62	2.11	0.71	20.13
	AGC	14.34	1.903	0.401	2.507	0.59	21.99
	Normal Mucosa	3.84	0.38	0.09	1.93	0.34	--
SE	EGC	1.58	0.19	0.073	0.24	0.08	3.35
	AGC	3.83	0.508	0.107	0.67	0.15	7.77
	Normal Mucosa	1.72	0.17	0.04	0.86	0.15	--
Max	EGC	94.53	11.78	4.54	17.21	4.71	95.0
	AGC	93.26	10.68	3.41	18.34	4.28	95.0
	Normal Mucosa	36.99	2.73	1.29	13.95	2.63	--
Min	EGC	30.76	3.08	1.75	7.21	1.5	7.0
	AGC	48.85	4.47	2.07	8.98	1.7	30.0
	Normal Mucosa	28.03	1.77	1.07	9.03	1.68	--
N° observ.	EGC	73	73	73	73	73	36
	AGC	14	14	14	14	14	8
	Normal Mucosa	5	5	5	5	5	0

Table 2: Results of the variance analysis indicating significance of the differences between the 3 classes (EGC, AGC and normal mucosa)

	SUM OF SQUARES		SQUARE OF MEAN		F	p
	between groups	within groups	between groups	within groups		
NUCLEAR AREA	5431.72	15899.06	2715.86	178.64	15.20	< 0.00001
OVERALL AgNOR AREA	86.97	254.93	43.48	2.86	15.18	< 0.00001
No OF AgNOR PART.	12.65	29.84	6.32	0.33	18.88	< 0.00001
%AGNOR AREA	5.43	419.16	2.71	4.709	0.58	0.56 n.s.
AREA / AgNOR PART.	1.95	42.31	0.97	0.47	2.06	0.13 n.s.
PCNA	212.24	17579.29	212.24	418.55	0.51	0.48 n.s.

RESULTS

The results of the descriptive statistical analysis are reported in Table 1.The parameters were normally distributed. The variance analysis has shown a significant difference between the groups (EGC, AGC and normal mucosa) for the following parameters: nuclear area, AgNORs area and AgNORs number (Table 2).

Table 3: Results of comparison between means of the 3 classes (Student's t-test)

		Nuclear Area	AgNORs Area	N° AgNORs	% Area AgNORs	Area per AgNORs	% PCNA
EGC vs normal mucosa	t student	11.04	15.33	19.82	- 0.56	2.94	/
	P	< 0.00001	< 0.00001	< 0.00001	0.59 n.s.	0.02	/
AGC vs normal mucosa	t student	9.07	8.84	13.45	- 0.98	3.28	/
	P	< 0.00001	< 0.00001	< 0.00001	0.35 n.s.	0.006	/
EGC vs AGC	t student	- 2.97	-1.34	0.83	0.79	- 1.16	0.67
	P	0.008	0.19 n.s.	0.41 n.s.	0.44 n.s.	0.25 n.s.	0.51 n.s.

However, the variance analysis found no significant differences between the various EGC subgroups: intramucosal vs submucosal, large vs small and minute, lymphnode metastasis vs no metastasis, lymphatic invasion vs absence of invasion.
A comparison between the means, performed with Student's t-test, revealed no significant differences between EGC and AGC. However, the normal mucosa proved to be significantly different from both EGC and AGC for the following parameters: nuclear area, AgNORs area and AgNORs number (see Table 3).
The discriminant analysis performed on all cases confirmed the results of the comparison between means: while it was possible to correctly classify all cases of normal mucosa, there is a notable overlap of values between EGC and AGC, with low percentages of correct classification.

DISCUSSION

The latest literature reports (Kodama, 1983; Inokuchi, 1983) seem to support the hypothesis that EGC comprises a heterogeneous group of lesions: some cases would appear to be carcinomas diagnosed by chance in an early phase but with the same aggressive biological behaviour as AGC; other EGCs would seem to have biological characteristics of their own, corresponding to slow growth and localized malignancy with low tendency to metastatize.
This hypothesis could be supported by studies on the biological characteristics of neoplastic cells. The proliferative activity of a tumor is one of the parameters which determines its growth rate and influences its aggressiveness.
PCNA positivity correlates well with the proliferative activity of the tissue and also AgNORs seem to be associated with proliferation, although not in specific manner (Leek et al., 1991; Treré et al., 1991).
To our knowledge, no PCNA studies on EGC cases have so far been published. Jain et al. (1981) studied AGC and found a correlation between PCNA and tumor-related death rate. They also observed considerable variability in the staining intensity within the tumor itself. In our experience, the staining intensity varies particularly from case to case and seems to be linked to the different ways of fixing the tissue. Anyway, when evaluating PCNA positivity, we recommend the use of the cells of the germinal centre of the lymphatic follicles present in the same section as reference cells and sample 1000 nuclei from different areas of the tumor.
In order to quantify the AgNORs, most authors have used the number of positive particles per nucleus (Derenzini et al., 1990; Egan et al., 1988). In fact, this number increases moving from cells at rest to proliferating cells, whether normal or neoplastic. In the cells at rest the sites of the NORs are probably grouped together, whereas in the proliferating cells they are scattered and can thus be more easily identified individually (Rosa et al., 1990). The increase in AgNORs would thus only be apparent. A true increase may occur in cases of aneuploidy or polyploidy. Since the possibility of identifying the individual NOR sites also depends on the techniques used in the staining process, the counting differences reported by various authors may indeed be due to technical problems. The number of AgNORs we observed in EGC, AGC and normal mucosa (2.8, 2.6 and 1.16, respectively) agree with the data of Suarez et al. (1989) (2.75 for the tumor cells and 1.3 for normal epithelium) while Rosa et al. (1990) found higher values (3.66 and 2.1).
The area occupied by the AgNOR particles is a variable which, although unquestionably related to the number of NORs, is independent of the capacity to separate the individual sites visually. The results obtained show higher values in the neoplastic lesions than in the normal mucosa, especially for the overall area, but also for the mean area of each AgNOR particle. We may

therefore conclude that a real increase in the NORs occurs in gastric carcinoma (both EGC and AGC).

The results of the statistical analysis showing no differences in proliferative activity (measured by both AgNOR and PCNA) between EGC and AGC are in agreement with the results of Suarez et al. (1989) and may be interpreted in various ways:

a) this study may not be representative. Only well-differentiated cases of EGC of the intestinal type have been chosen because in diffuse-type adenocarcinomas the nucleus often becomes compact and the AgNORs become difficult to measure. Nevertheless, Kodama et al. (1983) have shown that "Pen A" - type EGC, whose biological behaviour is more aggressive, is often histologically of the intestinal type. It thus remains to be seen whether differences in proliferative activity exist between AGCs and EGCs of the diffuse type.

b) EGC and AGC may be undistinguishable biologically. EGC may simply be the initial phase of AGC.

c) EGC and AGC may be biologically different while still having the same proliferative activity. Fujita (1978) showed that EGC and AGC have the same labelling index whereas the doubling time of the neoplastic mass is clearly different: 28 days for AGC, 2-3 years on average for EGC. This phenomenon is explained by the elevated cell loss occurring through desquamation or ulceration which is prominent for neoplasms lying at the mucosal surface. However, when the tumor infiltrates the deeper layers of the gastric wall, the cell loss becomes minimal and the doubling time decreases abruptly.

The crucial difference between EGC and AGC may be the speed of moving from a superficial growth phase to in-depth invasion. Further variables must be sought which can detect differences in the infiltrative capacity and thus in the aggressiveness of the tumor.

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