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Expression of interphasic nucleolar organizer regions in normal, dysplastic and neoplastic colorectal mucosa

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Summary. Silver-binding nucleolar organizer region (Ag-NOR) expression in interphasic nuclei was studied in normal, dysplastic and neoplastic colorectal mucosa at the light microscope level and by means of an image analyser (IBAS II). Both methods showed a progressive increase in the mean number of Ag-NOR sites per nucleus from mild dysplasia to invasive carcinoma. Ag-NOR counts differed significantly in the various classes of lesions ($P<0.001$), except between moderate and severe dysplasia ($P>0.05$). Severe dysplasia showed a mean number of NORs lower than that for invasive carcinoma though an overlap in the respective frequency distributions was observed. The mean of the variances of the mean dot areas per cell nucleus (pooled variance) also showed a step-wise increase from normal to neoplastic lesions, indicating a greater variability in NOR size as a characteristic of malignant cells. A similar increase was observed in the percentage of nuclear area occupied by Ag-NORs. The mean area per silver-stained dot was also measured in the different classes of lesions by IBAS II. Data obtained showed no significant differences among the values. In conclusion, the wide overlap between the frequency distributions does not allow consideration of the Ag-NOR count alone to be a reliable marker of malignant transformation in a single cell. It appears that the study of Ag-NOR number needs to be evaluated together with dot anisometry in order to be a useful criterion in distinguishing the biological behaviour of neoplastic lesions in colorectal mucosa.

Key words: Nucleolar organizer regions – Quantification – Large intestine – Dysplasia – Invasive carcinoma

Introduction

Nucleolar organizer regions (NORs) contain ribosomal DNA loops that are mainly localized in the nucleolar fibrillar centers of interphase nuclei (Morton et al. 1983). By virtue of associated argyrophilic proteins, NORs can be easily identified as black dots (Ag-NORs) using a silver-staining technique performed on paraffin sections (Ploton et al. 1986). Recent data suggest that the mean number of interphase NORs may reflect cell kinetics (Di Stefano et al. 1991). It has been shown that neoplastic cells are characterized by a larger number of smaller sized and irregularly distributed Ag-NORs when compared with normal or hyperplastic cells (Crocker and Nar 1987; Crocker and Skilbeck 1987; Egan and Crocker 1988; Smith and Crocker 1988). Accordingly, the Ag-NOR technique has been suggested for possible use in diagnostic histopathology.

In a previous study, we demonstrated striking differences in the number and distribution of Ag-NORs among hyperplastic polyps, adenomatous polyps and invasive carcinomas of colorectal mucosa (Derenzini et al. 1988). In order to investigate whether variations in Ag-NOR expression can be correlated with different degrees of colorectal dysplasia (mild, moderate and severe) and if this technique can be helpful in differentiating severe dysplasia from overt invasive carcinoma, we evaluated in each class of lesions (a) the mean number of Ag-NORs per nucleus; (b) the percentage of the mean nuclear area occupied by Ag-NORs; (c) the mean individual dot area and (d) the mean area of Ag-NORs per single cell nucleus, together with the relative variance. This enabled us to obtain the overall mean weighted variance (pooled variance) which represents the measure of variability of Ag-NOR size within a single cell.

Materials and methods

Slides of normal, dysplastic and carcinomatous large bowel specimens recovered from the files of the Histopathological Laboratory of the First Surgical Clinic of the University of Rome were re-examined independently by three pathologists (P.L.M., F.M.A., V.M.) and the grade of dysplasia was assessed according to the criteria suggested by Morson and Dawson (1990). Only cases where

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the three observers agreed were selected as clearly representative of their respective pathological type.

The 30 selected cases included 18 adenomatous polyps with mild (6), moderate (6) and severe dysplasia (6), invasive carcinomas (6) and normal colonic mucosa (6) as control.

For the Ag-NOR technique, tissue was routinely fixed in 10% formalin, dehydrated and embedded in paraffin. Sections 4 μm thick were dewaxed, treated with 3:1 ethanol/acetic acid solution for 30 min and rehydrated. The silver solution was obtained by adding 1 volume of 2% gelatin in 1% aqueous formic acid to 2 volumes of 50% silver nitrate. Silver staining was performed at room temperature for 14 min in the dark. NORs were visualized as distinct black intranuclear dots.

In each case, 100 randomly selected epithelial cell nuclei were examined on slides by light microscopy (LM) using a 100× oil-immersion lens and the mean number of Ag-NORs per nucleus was determined. A morphometric analysis was carried out in order to evaluate the areas of the argyrophilic dots and the nuclear areas. The counting procedure was performed in 50 nuclei for each case on micrographs of the same fields observed by LM by means of an IBAS II Image Analyser at a final magnification of ×1250. On micrographs nuclei containing dots greater than 1 μm^2 and clumps where the dots could not be easily resolved were not considered given that the areas of the Ag-NORs range from 0.2 to 0.4 μm^2 as determined by transmission electron microscopic studies (Derenzini et al. 1986).

For each class of lesions the following variables were evaluated: the mean number of Ag-NORs per nucleus; the percentage of nuclear area occupied by Ag-NOR proteins; the mean individual dot area; the mean Ag-NOR area per nucleus with the corresponding variance. Finally, the mean of the variances (pooled variance) was calculated. With the exception of the pooled variance, statistical analysis was performed applying the Kolmogorov-Smirnov non-parametric test (two-tailed). Tests based on the confidence intervals of the pooled variances were employed to investigate the presence of significant differences between pairs of variances.

Results

For each class of lesion, the mean number of dots counted on micrographs was smaller when compared with the values obtained by LM. On microscopy a finer resolution is allowed by continuous focusing and dots lying in different planes can be resolved (Fig. 1).

Both counting procedures showed a progressive increase in the mean number of argyrophilic dots from normal mucosa to dysplasia to invasive carcinoma (Fig. 2). The differences in NOR numbers counted by IBAS were statistically significant ($P<0.001$), except when moderate dysplasia was compared with severe dysplasia ($P>0.05$). Similar values of significance were obtained in the analysis of LM results, yet a lesser degree was observed between moderate dysplasia and invasive carcinoma ($P<0.01$) (Table 1).

The comparison of the respective frequency distributions revealed a wide overlap of the ranges in the number

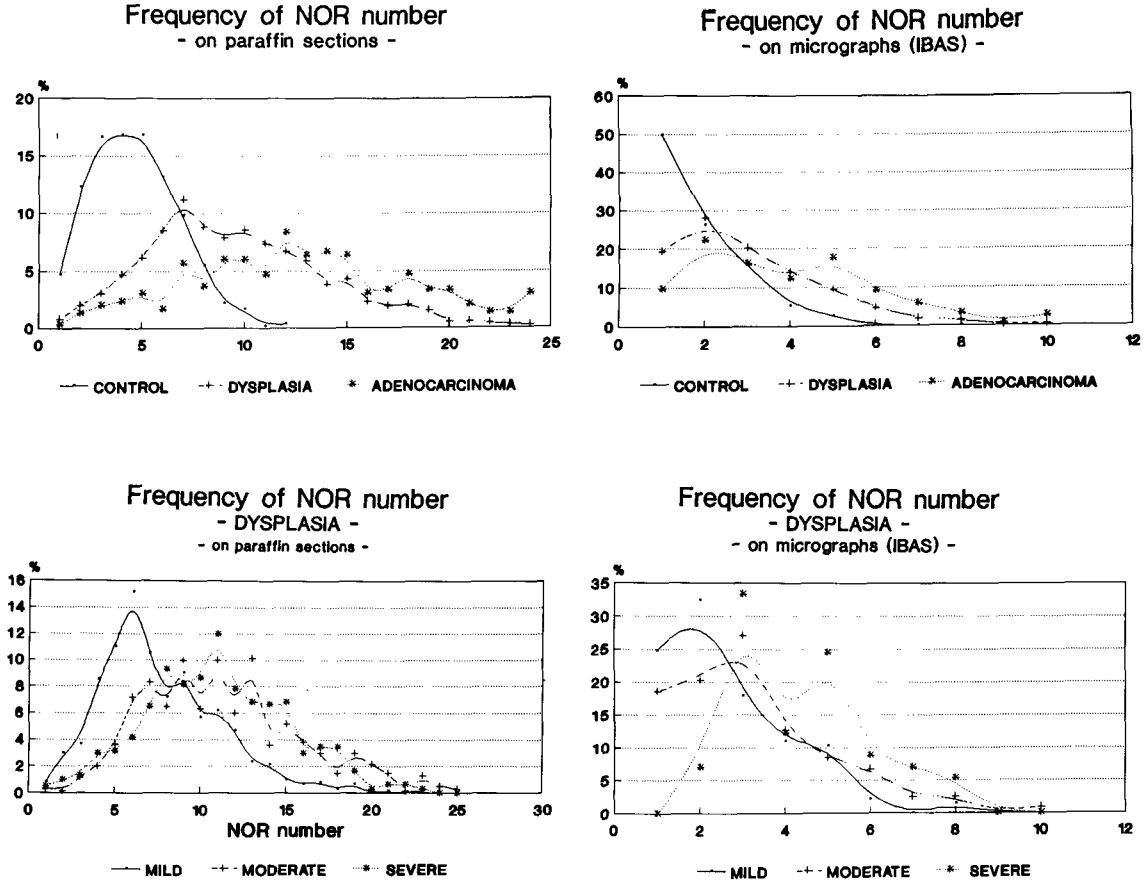

Fig. 1A–D. Frequency distributions of silver-binding nucleolar organizer regions (Ag-NORs) counted from paraffin sections and from micrographs. Frequency of NORs on paraffin sections (**A, C**) and on micrographs (**B, D**)

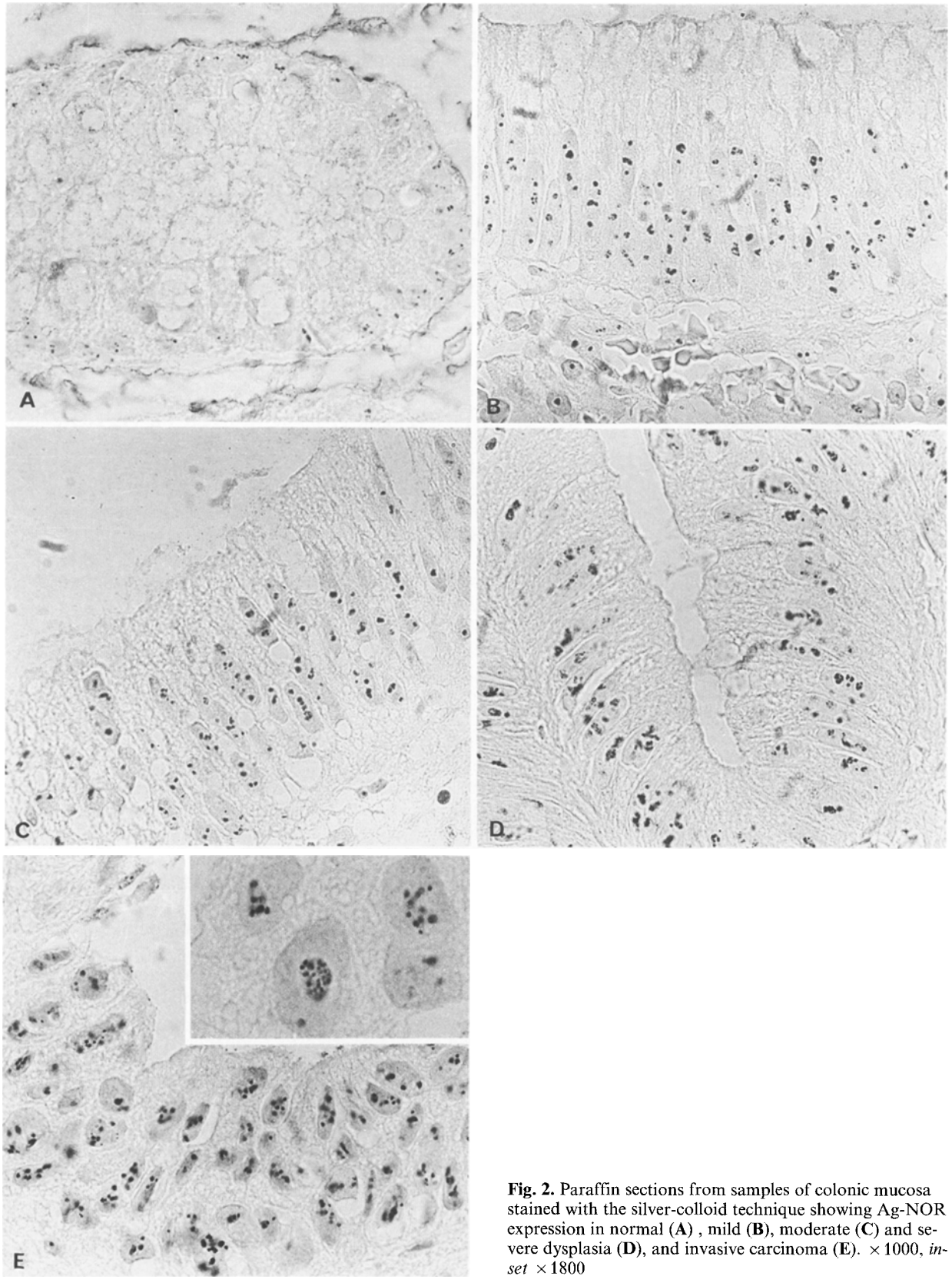

Fig. 2. Paraffin sections from samples of colonic mucosa stained with the silver-colloid technique showing Ag-NOR expression in normal (**A**) , mild (**B**), moderate (**C**) and severe dysplasia (**D**), and invasive carcinoma (**E**). ×1000, *inset* ×1800

Table 1. Ag – NOR numbers in colorectal dysplastic and neoplastic lesions

	Light microscopy	Measured nuclei	Mean	SD	Range
	Control	600	3.64	2.13	1 – 11
	Mild dysplasia	600	6.98	3.51	1 – 21
*	Moderate dysplasia	600	11.01	5.6	1 – 31
+	Severe dysplasia	600	10.01	4.25	1 – 27
	Carcinoma	600	13.56	7.72	1 – 49

	IBAS	Measured nuclei	Mean	SD	Range
	Control	300	1.84	1.05	1 – 5
	Mild dysplasia	300	2.62	1.49	1 – 8
*	Moderate dysplasia	300	3.21	1.86	1 – 10
	Severe dysplasia	300	3.52	1.97	1 – 10
	Carcinoma	300	3.95	2.15	1 – 10

The comparisons between pairs of samples are significant ($P<0.001$) ($+=P<0.01$) with the exception marked by * ($P>0.05$)

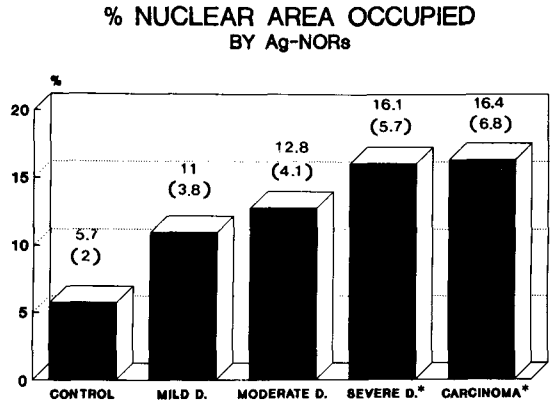

Fig. 3. Modifications of the percentage of mean nuclear area occupied by Ag-NORs in normal colonic mucosa, dysplastic and neoplastic lesions. Standard deviation values are given in parentheses. The comparisons between pairs of samples are significant ($P<0.05$) with the exception marked by * ($P>0.05$)

Table 2. Analysis of the mean area per silver-stained dot

	Mean area of Ag – NORs (μm^2) ± SD	Pooled variance of Ag – NOR areas	
Control	0.256 ± 0.208	0.007	
Mild dysplasia	0.302 ± 0.154	0.014	
Moderate dysplasia	0.228 ± 0.105	0.011	*
Severe dysplasia	0.382 ± 0.336	0.012	
Invasive Carcinoma	0.329 ± 0.203	0.025	

The comparisons of the mean area values between pairs of samples are not significant ($P>0.05$). The differences between pooled variances are significant ($P<0.005$) with the exceptions marked by * ($P>0.05$)

of silver-stained black dots in the various classes of lesions.

The percentage of the mean nuclear area occupied by Ag-NORs increased from normal epithelium to dysplastic lesions ($P<0.05$) whereas no differences were observed between severe dysplasia and invasive carcinoma (Fig. 3).

The mean of the variances of single cell mean dot area (pooled variance) showed a progressive increase from normal to dysplastic to carcinomatous lesions, indicating a greater variability in NOR size in malignant cells ($P<0.005$) (Table 2). The mean area of single interphasic NORs tended to overlap in the different classes of lesions (Table 2), without significant differences.

Discussion

In 1986, Ploton et al. showed that the Ag-NOR technique can be applied to routinely processed, paraffin-embedded tissues. Since then, the value of Ag-NOR enumeration in differentiating benign from malignant neoplastic lesion has been emphasized (Crocker and Nar 1987; Crocker and Skilbeck 1987; Derenzini et al. 1988) but its limited diagnostic value has been reported in some tumours (Derenzini et al. 1990; Nairn et al. 1988; Suarez et al. 1989).

In this study, we compared the expression of Ag-NORs in different grades of colonic epithelial dysplasia and in invasive carcinoma. We used a short incubation time in the silver solution (14 min) in order to avoid overstaining, which may cause coalescence of dots and/or non-specific reactivity of the whole nucleolus, as recently pointed out by Rüschoff et al. (1990). Silver dots were counted both directly in paraffin sections at the optical level and on micrographs of the corresponding fields with an IBAS II image analyser system. The mean number of dots counted using the image analyser was smaller than that obtained by LM, where focusing allows the identification of dots lying in different planes. Both counting methods showed a significant increase in the mean number of argyrophilic dots from normal mucosa to dysplasia to invasive carcinoma ($P<0.001$), the only exception having been observed between moderate and severe dysplasia ($P>0.05$). Nonetheless we found a wide overlap in frequency distributions of the respective number of dots. Such an overlap made it impossible to identify a single cell as malignant except for those cells showing a marked increase in the number of dots.

A progressive increase in the percentage of the mean nuclear area occupied by silver-stained dots was observed in the sequence normal to dysplastic lesions to invasive carcinomas.

Further data (mean weighted variance, single dot mean area) have been provided by measurements of Ag-NOR areas within a single cell. The analysis of the overall weighted variance of the Ag-NOR areas in different colonic lesions suggests that a greater variability in the dimension of dots characterizes a neoplastic cell from a non-neoplastic one. This is in agreement with the re-

sults of Love and Soriano (1971), who, after studying the nucleolini (as the fibrillar centers were initially called), first suggested "isonucleolinosis" and "anisonucleolinosis" as criteria apt to distinguish benign from malignant cells (Love et al. 1973).

In contrast, single dot mean area did not give significantly different results in the various lesions. This finding differs from those of Rüschoff et al. (1990) who found significant differences in the size of Ag-NORs between normal and neoplastic epithelium. The procedure of segmentation of the clumps which they adopted to resolve single dots appears to be subjective and was found to be difficult to reproduce reliably.

Our data suggest that the Ag-NOR count is useful in identifying different grades of epithelial dysplasia and infiltrating colonic adenocarcinoma, although its predictive value is reliable only when a suitable number of cells is evaluated. In addition to the modifications in number, another useful criterion is furnished by the variability in dot size in any one cell, easily recognized on LM without further morphometric studies.

The Ag-NOR technique does not represent a method to achieve an automatic evaluation of malignancy, but provides, in our opinion, a valid addition to the criteria already available to the histopathologist.

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