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Ag-NOR protein distribution correlates with patient survival in stage I endometrial adenocarcinoma

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Summary. The relationship between argyrophil nucleolar organizer region (Ag-NOR) protein quantity and prognosis was studied in 33 cases of stage I endometrial adenocarcinoma. Ag-NOR protein quantity was measured by image analysis in silver-stained sections from paraffin-embedded samples of curettings. Patients had a minimum 10-year follow-up. Only 2 out of 25 patients exhibiting a mean Ag-NOR protein area of less than 3 μm^2 died of cancer, whereas 5 of the 8 patients with a mean Ag-NOR protein area of more than 3 μm^2 died of the disease. The present results demonstrate that the Ag-NOR protein value is closely related to patient survival in stage I endometrial carcinoma and that it is a reliable prognostic indicator in this type of carcinoma.

Key words: Interphase argyrophil nucleolar organizer regions – Proliferation – Prognosis – Endometrial adenocarcinoma

Introduction

Endometrial carcinoma is one of the most frequent cancers in women and prognosis depends critically on the stage at presentation. Stage I disease accounts for 80% of cancers (Malkasian et al. 1977, 1980) and the 5-year survival rate of 90% is found in cases with clinical stage I (DiSaia and Creasman 1986). Prognostic indicators in patients with stage I disease are the histological grade, depth of myometrial invasion and DNA ploidy pattern (Van der Putten et al. 1989). There is increasing evidence that the cell proliferation rate is directly related to the degree of malignancy (Quinn and Wright 1990). Recently the quantity of silver-stained proteins of interphase nucleolar organizer regions (Ag-NOR proteins) has been found to be a new variable for measuring cell kinetics (Derenzini and Ploton 1991; Derenzini and Trerè 1991). The Ag-NOR proteins are a set of acidic proteins, located in the nucleolus during interphase, where they are associated with ribosomal genes. These proteins are selectively stained by silver methods and easily visualized in routine paraffin-embedded sections (Derenzini et al. 1990b; Derenzini and Ploton 1991). The quantity of Ag-NOR proteins is related to the proliferative activity of cancer cells: the greater the quantity of Ag-NOR proteins, the more rapid is cell proliferation (Trerè et al. 1989; Derenzini et al. 1990a). These data are also in agreement with previous findings demonstrating a higher quantity of Ag-NOR proteins in cancer cells than in the corresponding hyperplastic or normal elements (Crocker 1990). In the present study we have measured, using an automated image analyser, the amount of Ag-NOR proteins in 33 cases of stage I endometrial adenocarcinoma of patients with a follow-up of at least 10 years. The aim of this study was to ascertain whether Ag-NOR protein quantity might represent a prognostic factor in stage I endometrial adenocarcinoma.

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Materials and methods

Thirty-three cases of stage I endometrial adenocarcinoma diagnosed at the Clinic of Obstetrics and Gynaecology of the University of Bologna between 1975 and 1977 were studied. All were treated with total abdominal hysterectomy and bilateral salpingo-oophorectomy and adjuvant radiation therapy. Patients who had died of other diseases (9 cases), and patients who were lost to follow-up (15 cases) were not included in this study.

The evaluation of interphase Ag-NOR protein content was performed on curettings obtained before therapy. All tissues had been fixed in alcohol for 24 h and paraffin-embedded. From each block two consecutive sections (3 μm^2) were cut and stained with haematoxylin and eosin (H & E) and silver for Ag-NOR proteins according to the method of Ploton et al. (1986). With this method Ag-NOR proteins appear as black stained dots.

Sections were dewaxed in xylene and ethanol, post-fixed for 30 min in 3:1 (vol/vol) absolute ethanol: acetic acid solution and then rehydrated. Ag-NOR staining was carried out using a solution of one volume 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate. Silver staining was performed for 10 min at 32° C.

The area occupied by the Ag-NOR proteins within nucleoli of 200 cells per patient was measured using a specific program of a computer-assisted image analysis system (IBAS Kontron 2000). The system consists of a Zeiss microscope, a Sony camera, a digitizer tablet used by the operator to define the nuclear area and the grey level, a host computer based on a 280 processor with operative system CP/M. The principal stages of image processing were as follows. The neoplastic zone to be studied was carefully selected in the H & E section and then retraced in the silver-stained section. A field was selected by the operator at the microscope using a ×40 oil-immersion objective lens. The selected image was then captured into digital memory and visualized on the monitor of the image analyser. By moving the mouse on the digitizer tablet, the operator interactively defined the grey threshold which permitted the automatic quantification of only the black dots corresponding to the interphase Ag-NORs. By using a zoom function the operator had the possibility of looking at two images at the same time: the first one showed the original field, the second one only the structures selectively defined by the different grey thresholds. The measurement was performed only when the structures defined by the chosen grey threshold were superimposable on the silver-stained proteins in the original field.

Statistical analysis of data was performed using a specific computer programme. Survival curves were calculated according to the method of Kaplan and Meier (1958), and differences between curves were analysed by the log-rank test. Multivariate analysis of survival was estimated according to the Cox proportional hazards regression model (Cox 1972).

All cases examined were endometrioid adenocarcinomas; they were graded according to Poulsen et al. (1975) on the curettings.

Myometrial invasion was evaluated on the surgical specimen. On the basis of the maximum depth of infiltration in the myometrium by the tumour, cases were classified as follows: M0, no myometrial invasion; M1, tumour involving up to one-third of myometrium; M2, tumour involving up to two-thirds of myometrium; M3, tumour involving the whole thickness of the myometrium, reaching the tunica serosa and extending to the cervix. No cases were present with extension of the disease beyond the uterus. In the 3 cases in which no myometrial invasion was present, the neoplastic glands merged together and were easily distinguished from those of atypical endometrial hyperplasia.

Table 1. Ag-NOR values of the endometrial carcinomas compared with follow-up time, histological grade and myometrial invasion

Case	Ag-NOR area (μm^2)	Follow-up (years)		Histological grade	Myometrial invasion
1.	1.61	a&W	14	G1	M1
2.	1.73	a&W	14	G2	M1
3.	1.60	a&W	14	G1	M1
4.	2.10	a&W	14	G1	M1
5.	2.26	a&W	14	G1	M1
6.	2.41	a&W	14	G1	M1
7.	2.67	a&W	14	G2	M2
8.	2.29	a&W	14	G1	M1
9.	2.71	a&W	14	G1	M3
10.	1.85	a&W	14	G1	M0
11.	2.64	a&W	13	G1	M1
12.	2.81	a&W	13	G2	M1
13.	2.15	a&W	13	G3	M1
14.	1.94	a&W	13	G1	M1
15.	2.33	a&W	13	G1	M1
16.	2.24	a&W	13	G1	M3
17.	1.58	a&W	13	G2	M1
18.	1.82	a&W	13	G1	M1
19.	1.58	a&W	13	G1	M0
20.	3.58	a&W	12	G2	M1
21.	1.40	a&W	12	G1	M0
22.	4.11	a&W	12	G2	M1
23.	2.02	a&W	12	G1	M1
24.	2.23	a&W	10	G1	M1
25.	4.49	a&W	10	G2	M1
26.	1.67	a&W	10	G1	M1
27.	5.58	dod	6	G2	M2
28.	2.38	dod	4	G2	M1
29.	2.46	dod	4	G1	M1
30.	3.60	dod	3	G2	M1
31.	4.35	dod	2	G2	M2
32.	5.85	dod	1	G2	M2
33.	4.77	dod	1	G3	M1

a&W, Alive and well; dod, died of disease

Results

The patients' ages ranged from 35 to 77 years (mean ± SD: 58 ± 7 years). In Table 1, patients are distributed according to length of follow up and not by date of entry into the study. In 3 patients the carcinoma was confined to the mucosa, while in most of the cases (25 cases) the tumour involved up to the internal third of the myometrium. In 4 cases the carcinoma penetrated most of the myometrial wall, while in 2 cases extension was seen up to the tunica serosa or the cervix.

Nineteen cases displayed histological grade I, 12 grade II and the remaining 2 cases showed histological grade III.

Twenty-six of the 33 patients were alive and well at least 10 years after presentation, while 7 had died as a consequence of the tumour from 1 to 6 years after curetting.

In sections selectively stained for the Ag-NOR proteins the nucleolar structures were the only cell components which were darkly stained. In Figs. 1 and 2 2 cases of endometrial carcinoma, characterized by different quantitative distributions of Ag-NOR proteins (2.24 μm^2 and 5.85 μm^2, respectively), are shown. The silver-stained structures appear as black dots in the nucleolar area. The Ag-NOR proteins are infact selectively located in the fibrillar centres of the nucleolus, which are roundish structures.

In Table 1 the mean Ag-NOR area for each case is shown, together with the follow-up time, histological grade and depth of myometrial invasion. The Ag-NOR protein areas ranged from 1.40 to 4.49 μm^2 (mean ± SD: 2.30 ± 0.77 μm^2) in surviving patients, and from 2.38 to 5.85 μm^2 (mean ± SD: 4.14 ± 1.39 μm^2) in non-surviving patients. In order to define the relationship between interphase Ag-NOR protein quantity and survival, the 33 patients were divided into two groups according to their Ag-NOR protein values. Group A ($n=25$) included cases with mean Ag-NOR protein area lower than 3 μm^2. Group B ($n=8$) included cases with mean Ag-NOR protein areas greater than 3 μm^2. The Kaplan and Meier survival curves for patients of the two groups are reported in Fig. 3. Of patients from group A 92% survived at least 10 years in contrast to only 37% of group B patients. The differences between the survival

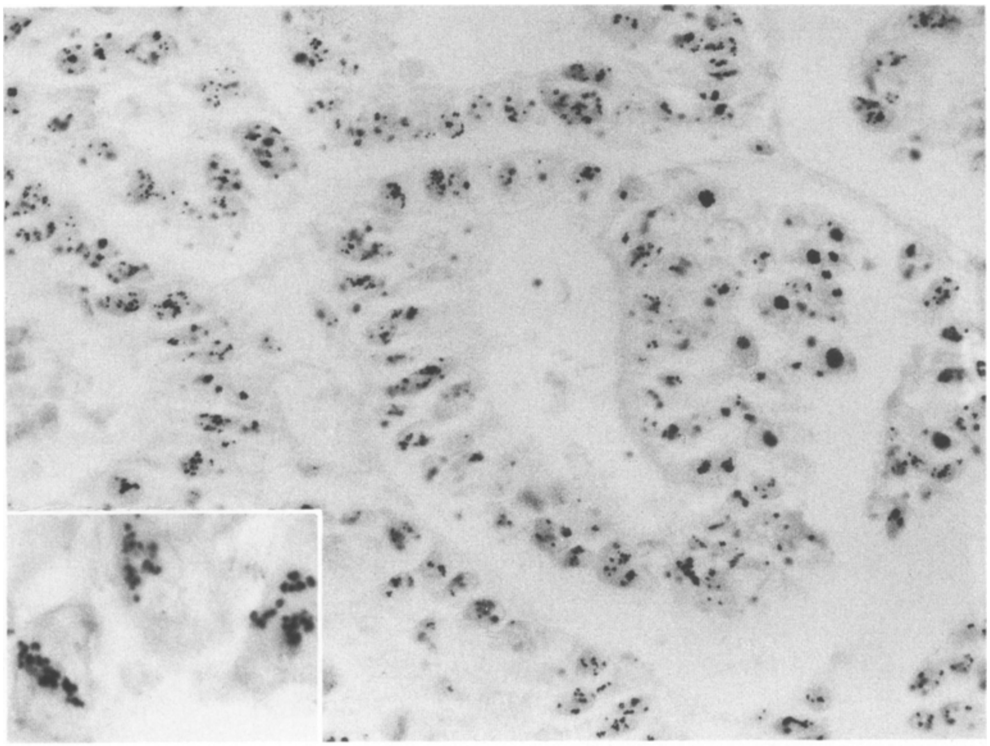

Fig. 1. Endometrial grade 1 carcinoma (case 16) with a mean Ag-NOR protein value of 2.24 μm². The patient is still alive and well 13 years after hysterectomy. Selective staining for the Ag-NOR proteins. No counterstaining, ×400. *Insert:* detail at higher magnification; ×800

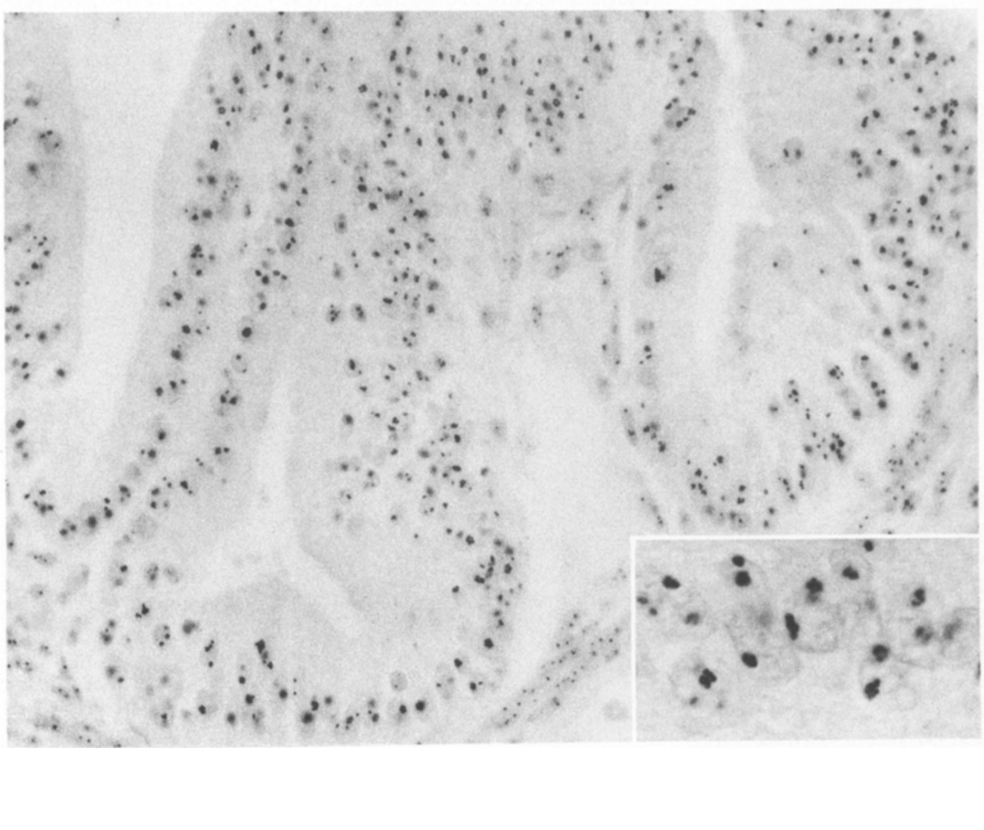

Fig. 2. Endometrial grade 2 carcinoma (case 27) with a mean Ag-NOR protein value of 5.85 μm². The patient died of the disease 1 year after the hysterectomy. Selective staining for the Ag-NOR proteins. No counterstaining, ×400. *Insert:* detail at higher magnification; ×800

curves, calculated by the log-rank test, was highly significant (chi-squared = 16.79; $P < 0.001$).

When histological grading and depth of myometrial invasion were correlated with patient survival, the 10-year survival rate was 95% in patients with G1 cancer (19 cases) versus 57% in patients with G2 and G3 cancer (14 cases) (chi-squared = 8.05, $P < 0.005$), and 96% in patients with M0 and M1 cancer (27 cases) versus 50% in patients with M2 and M3 cancer (6 cases) (chi-squared = 4.73, $P < 0.05$). Univariate analysis of survival showed therefore that Ag-NOR area had the highest prognostic significance.

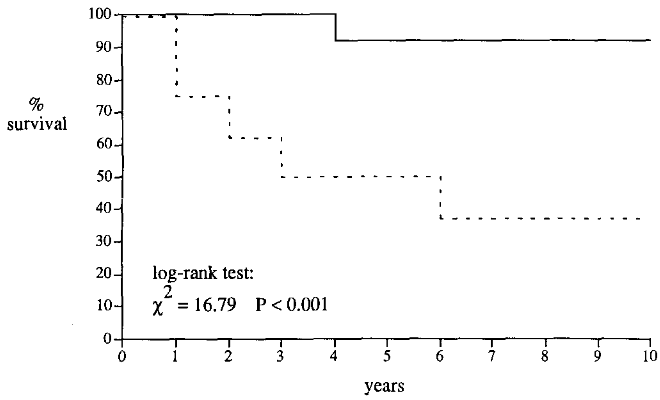

Fig. 3. Ag-NOR protein area and survival in 33 patients with stage I endometrial adenocarcinoma. (Ag-NOR area $<3\ \mu m^2$ = ——; Ag-NOR area $>3\ \mu m^2$ = ····)

In the multivariate analysis of survival, the joint effect of all the three variables considered (Ag-NOR area, histological grading and depth of myometrial invasion) was highly significant (chi-squared = 11.42, $P<0.01$). The results also indicated that the three variables were probably linked, and that only the Ag-NOR area showed a separated effect greater than its standard deviation.

Discussion

Identification of cases of stage I endometrial adenocarcinomas that may be fatal is of vital clinical importance. In these tumours, apart from cell kinetic evaluation, flow cytometric measurements have been shown to represent an important prognostic indicator (Britton et al. 1989; Rosemberg et al. 1989; Van der Putten et al. 1989). Diploid cancers account for only about 10% of cancer death whereas aneuploid tumours are responsible for about 40% of the 5-year cancer mortality (Britton et al. 1989). DNA content, in combination with the evaluation of the shortest axis of cancer cell nucleus and the degree of myometrial invasion, is also of prognostic value (Van der Putten et al. 1989).

High values for the cell proliferation rate are related to the progression of the disease as demonstrated by Rosemberg et al. (1989). They measured the percentage of S-phase cells in paraffin-embedded early-stage endometrial cancers by DNA flow cytometry and found that patients with an S-phase fraction below 5% had a 5-year cancer mortality of 7%, whereas 49% of those with an S-phase fraction above 10% died of the disease.

Our results have shown that the mean areas of Ag-NOR protein in stage I endometrial adenocarcinomas range from 1.40 μm^2 to 5.85 μm^2. In addition, cases with low Ag-NOR protein values appear to have a better prognostic outcome than patients with high values of Ag-NOR proteins.

The evidence indicates that a relationship exists between Ag-NOR protein quantity and cell proliferation rate. The quantity of the Ag-NOR proteins increases when the cell enters the mitotic cycle, reaching its greatest value during the S-phase (Pession et al. 1991). Ag-NOR protein values are directly related to the percentage of S-phase cells, determined by either DNA flow cytometry or bromodeoxyuridine (BrdU) incorporation, in non-Hodgkin's lymphomas (Crocker et al. 1988), breast tumours (Giri et al. 1989) and meningiomas (Orita et al. 1990). A linear relationship between Ag-NOR protein value and rapidity of cell proliferation has also been demonstrated in tumour cell lines (Trerè et al. 1989; Derenzini et al. 1990a). A highly significant correlation between Ag-NOR protein quantity and cell proliferative indices evaluated by BrdU labelling and Ki-67 immunostaining was also shown in cancer tissues of different origins (Trerè et al. 1991). In the latter study it was observed that those tumours exhibiting a mean Ag-NOR protein value greater than 3.0 μm^2 were constantly characterized by very high BrdU and Ki-67 labelling indices. This is the reason why in the present paper the value of 3.0 μm^2 was chosen as the cut-off line for distinguishing rapidly proliferating from moderately or slowly proliferating endometrial adenocarcinomas.

In our study 92% of patients with a mean Ag-NOR area lower than 3.0 μm^2 were still alive and well after at least 10 years from the initial diagnosis. In contrast 63% of patients with a mean Ag-NOR area greater than 3.0 μm^2 died of the disease. These data indicate, like other studies, that the clinical outcome of stage I endometrial adenocarcinoma is closely related to the proliferative activity of the cancer cells.

The prognostic utility of Ag-NOR distribution has been shown in neuroblastomas (Egan et al. 1988), meningiomas (Orita et al. 1990), colorectal cancers (Moran et al. 1990; Öfner et al. 1990; Rüschoff et al. 1990), renal carcinomas (Delahunt et al. 1991), oral squamous cell adenocarcinomas (Sano et al. 1991), gastric cancers (Kakeji et al. 1991), soft-tissue sarcomas (Kuratsu et al. 1991), pharyngeal carcinomas (Pich et al. 1991) and sarcomatoid carcinomas of the breast (Eusebi et al. 1991). Among the methods used for cell kinetic evaluation, Ag-NOR protein quantification is by far the simplest, most repetitive and least expensive (Derenzini and Trerè 1991). Quantification of Ag-NORs might therefore be very helpful as a prognostic indicator of clinical outcome in stage I endometrial adenocarcinoma.

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References

Britton LC, Wilson TO, Gaffey TA, Lieber MM, Wieand HS, Podratz KC (1989) Flow cytometric DNA analysis of stage I endometrial carcinoma. Gynecol Oncol 34:317–322

Cox DR (1972) Regression models and life tables. J R Stat Soc Br 34:187–220

Crocker J (1990) Nucleolar organizer regions. Curr Top Pathol 82:91–149

Crocker J, Macartney JC, Smith PJ (1988) Correlation between DNA flow cytometric and nucleolar organizer regions in non-Hodgkin's lymphoma. J Pathol 154:151–156

Delahunt B, Ribas JL, Nacey JN, Betwaite PB (1991) Nucleolar organizer regions and prognosis in renal cell carcinoma. J Pathol 163:31–37

Derenzini M, Ploton D (1991) Interphase nucleolar organizer regions in cancer cells. Int Rev Exp Pathol 32:150–192

Derenzini M, Trerè D (1991) Importance of interphase nucleolar organizer regions in tumour pathology. Virchows Arch [B] 61:1–8

Derenzini M, Pession A, Trerè D (1990a) The quantity of nucleolar silver-stained proteins is related to proliferating activity in cancer cells. Lab Invest 63:137–140

Derenzini M, Thiry M, Goessens G (1990b) Ultrastructural cytochemistry of the mammalian cell nucleolus. J Histochem Cytochem 38:1237–1256

DiSaia AR, Creasman WT (1986) Management of endometrial adenocarcinoma stage I with surgical staging followed by tailored adjuvant radiation therapy. Clin Obstet Gynecol 13:751–765

Egan MJ, Raafat F, Crocker J, Williams D (1988) Comparative study of the degree of differentiation of neuroblastoma and mean numbers of nucleolar organizer regions. J Clin Pathol 41:527–531

Eusebi V, Cattani MG, Lamovec J, Trerè D, Ceccarelli C, Veronesi C, Clemente C, Derenzini M (1991) Prognostic relevance of silver stained nucleolar proteins in sarcomatoid carcinomas of the breast. Ultrastruct Pathol 15:203–214

Giri DD, Nottingham JF, Lawry J, Dundas SAC, Underwood JC (1989) Silver binding nucleolar organizer regions (AgNORs) in benign and malignant breast lesions: correlation with ploidy and growth phase by DNA flow cytometry. J Pathol 157:307–313

Kakeji Y, Korenaga D, Tsujitani S, Haraguchi M, Maehara Y, Sugimachi K (1991) Predictive value of Ki-67 and argyrophilic nucleolar organizer region staining for lymph node metastasis in gastric cancer. Cancer Res 51:3503–3506

Kaplan EC, Meier P (1958) Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457–481

Kuratsu S, Aozasa K, Myoui A, Tsujimoto M, Ueda T, Uchida A, Hamada H, Ono K, Matsumoto K (1991) Prognostic significance of argyrophilic nucleolar organizer staining in soft-tissue sarcomas. Int J Cancer 48:211–214

Malkasian GD Jr, McDonald TW, Pratt JH (1977) Carcinoma of the endometrium: Mayo Clinic experience. Mayo Clin Proc 52:175–180

Malkasian GD Jr, Annegers JF, Fountain KS (1980) Carcinoma of the endometrium: stage I. Am J Obstet Gynecol 136:872–888

Moran K, Cooke T, Forster G, Gillen P, Sheehan S, Dervan P, Fitzpatrick JM (1990) Prognostic value of nucleolar organizer regions and ploidy values in advanced colorectal cancer. Br J Surg 76:1152–1155

Öfner D, Tötsch M, Sandbichler P, Hallbrucker C, Margreiter R, Mikuz G, Schmid KW (1990) Silver-stained nucleolar organizer regions (Ag-NORs) as a predictor of prognosis in colonic cancer. J Pathol 162:42–49

Orita T, Kajiwara K, Nishizaki T, Ikeda N, Kamiryo T, Aoki H (1990) Nucleolar organizer regions in meningioma. Neurosurgery 26:43–46

Pession A, Farabegoli F, Trerè D, Novello F, Montanaro L, Sperti S, Rambelli F, Derenzini M (1991) The Ag-NOR proteins and transcription and duplication of ribosomal genes in mammalian cell nucleoli. Chromosoma 100:242–250

Pich A, Pisani P, Kzengli M, Cappello N, Navone R (1991) Argyrophilic nucleolar organizer region counts and prognosis in pharyngeal carcinoma. Br J Cancer 64:327–332

Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ (1986) Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem J 18:5–14

Poulsen HE, Taylor CW, Sobin LH (1975) Histological typing of female genital tract tumours. International histological classification of tumours, vol 13. World Health Organization, Geneva, pp 64–65

Quinn CM, Wright NA (1990) The clinical assessment of proliferation and growth in human tumours: evaluation of methods and applications as prognostic variables. J Pathol 160:92–102

Rosemberg P, Wingren S, Simonsen E, Stal O, Risberg B, Nordenskjol B (1989) Flow cytometric measurement of DNA index and S-phase on paraffin-embedded early stage endometrial cancer: an important prognostic indicator. Gynecol Oncol 35:50–54

Rüschoff J, Bittinger A, Neuman K, Schmitz Moormann P (1990) Prognostic significance of nucleolar organizer regions (NORs) in carcinomas of the sigmoid colon and rectum. Pathol Res Pract 186:85–91

Sano K, Takahashi H, Fujita S, Inokuchi T, Pe MB, Okabe H, Tsuda N (1991) Prognostic implication of silver-binding nucleolar organizer regions (Ag-NORs) in oral squamous cell carcinoma. J Oral Pathol Med 20:53–56

Trerè D, Pession A, Derenzini M (1989) The silver-stained proteins of interphasic nucleolar organizer regions as a parameter of cell duplication rate. Exp Cell Res 184:131–137

Trerè D, Farabegoli F, Cancellieri A, Ceccarelli C, Eusebi V, Derenzini M (1991) Ag-NOR protein quantity in human tumours correlates with the proliferative activity evaluated by bromodeoxyuridine labeling and Ki 67 immunostaining. J Pathol 165:53–60

Van der Putten HW, Baak JP, Koenders TJ, Kurven PH, Stolk HG, Stolte LA (1989) Prognostic value of quantitative pathologic features and DNA content in individual patients with stage I endometrial adenocarcinoma. Cancer 63:1378–1387