

Argyrophilic nucleolar organizer regions and bromodeoxyuridine and ³[H]-thymidine labelling indices in colorectal cancer

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Abstract. The count of argyrophilic nucleolar organizer regions (AgNORs) has been proposed as a useful method for evaluating cell replication in human tumours. The current study was undertaken to compare AgNOR values in colorectal cancers with two better established methods for investigating cell proliferation such as bromodeoxyuridine (BrdUrd) and ³[H]-thymidine (³[H]dT) labelling indices (LIs).

Because some concern still exists regarding accuracy and reproducibility of AgNOR quantifying methods, we carried out a control study by independently repeating the same measurements (number, area and area per silver-stained NOR particle) in two centres with different operators and computer-assisted image analysers on 40 colorectal carcinomas. AgNOR values recorded in the two centres were strictly correlated ($r = 0.75$; $P < 0.001$ for number; $r = 0.62$, $P < 0.01$ for area; $r = 0.63$, $P < 0.001$ for area per silver-stained NOR particle) and the range of values were almost identical. Then, AgNOR values were compared with BrdUrd and ³[H]dT LIs, respectively obtained by *in vivo* incorporation and *in vitro* incubation in the same series of colorectal carcinomas. No correlation was found between AgNOR values and BrdUrd or ³[H]dT LIs. BrdUrd and ³[H]dT LIs were instead reciprocally significantly correlated. No evident correlation was seen between LIs or AgNOR values and clinico-pathological parameters of the tumour. In conclusion, in colorectal neoplasms, AgNOR values did not appear to relate with more direct parameters of cell proliferation. It follows that AgNOR reliability as a biomarker of cell proliferation remains questionable.

Nucleolar organizer regions (NORs) are loops of nucleolar DNA containing genes coding for ribosomal RNA. During interphase the NORs are located in the fibrillar component of the nucleolus (interphase NORs). They are associated with specific proteins (AgNOR proteins) which are selectively stained by silver methods (Ploton *et al.* 1986, Derenzini *et al.* 1990b).

It has been shown that the number of AgNORs within the nuclei of benign lesions is significantly different from that of malignant lesions (Crocker & Skilbeck 1987, Derenzini *et al.* 1988, Di Gregorio *et al.* 1991). A general relationship between the interphase quantity of

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AgNOR proteins and the proliferative activity of cells has been suggested (Trere' *et al.* 1989, Vagner-Capodano *et al.* 1987), particularly in myogenic stromal tumours of the stomach and in cervical intraepithelial neoplasias (Sinn *et al.* 1989, Thickett *et al.* 1989). Accordingly, in several tissues, the increase in the area occupied by AgNORs has been shown to correlate positively with elevated Bromodeoxyuridine (BrdUrd) labelling index (Leek *et al.* 1991, Trere' *et al.* 1991). Recently Ruschoff *et al.* (1990) proposed that the mean area per silver-stained NOR particle is a good way to provide reproducibility of AgNOR value. The mean area of the NOR particles is inversely related with AgNOR number and higher NOR counts with smaller mean NOR particle areas characterize tumours with unfavourable outcome (Chomette & Auriol 1991).

In the present study we compared mean AgNOR values (number, area and area per silver-stained NOR particle) per nucleus of 40 colorectal cancers with two established parameters of cell proliferation, namely ^3H dT and BrdUrd labelling indices (LIs). To ensure the reliability and reproducibility of AgNOR evaluation we carried out a control study by independently repeating the same measurements (number, area and area per silver-stained NOR particle) in two centres with different operators and different computer-assisted image analysers. Then we investigated the relationship between AgNOR values, BrdUrd LI, ^3H dT LI and clinicopathological variables of the tumours. Our results indicate that AgNORs do not correlate with either ^3H dT or BrdUrd LIs, suggesting that the evaluation of AgNORs cannot be a reliable estimate of proliferative activity in colorectal cancers.

MATERIALS AND METHODS

Patients

We examined 40 volunteers (20 females and 20 males) with colorectal cancer. The mean age was 66.6 years, (range 41–83). 14 tumours were from the right colon (i.e. proximal to the splenic flexure: 11 from the caecum and ascending colon, one from hepatic flexure and two from the splenic flexure), 13 from the left colon (two from the descending and 11 from the sigmoid) and 13 from the rectum or rectosigmoid junction. Tumours were staged according to the Dukes stage (Dukes 1932) in stage A ($n = 2$), stage B ($n = 20$), stage C ($n = 14$), stage D ($n = 4$). Two patterns of tumour growth were defined: expanding ($n = 29$), when the tumour showed a well delineated and circumscribed border of growth, and infiltrating ($n = 11$), when single tumour cells or clusters of cells spread through and beyond the bowel wall (Jass *et al.* 1986, Ponz de Leon *et al.* 1992). Tumours were graded according to Dukes & Bussey (1958) as well ($n = 9$), moderately ($n = 28$) and poorly ($n = 3$) differentiated.

Bromodeoxyuridine labelling

40 patients were intravenously given 500 mg of 5'-bromo-2'-deoxyuridine (BrdUrd) (Sigma, St Louis MO, USA) sterilized by filtration, dissolved in 100 ml isotonic solution, 2–6 h before surgery. Immediately after surgery, wedge-shaped samples of colonic neoplasia were taken, fixed in 70% ethanol overnight and embedded in paraffin. Four μm sections were prepared. The immunohistochemical detection of nuclei that had incorporated BrdUrd was carried out using the avidin-biotin peroxidase complex method (Vectastain ABC Kit PK-4002, Vector, Burlingame, CA, USA) and anti-BrdUrd monoclonal antibody (Dakopatts, Glostrup, Denmark) diluted 1:50. One thousand or more cells were scored for each carcinoma. BrdUrd-positive cells were easily identified at histology because the nuclei stained dark-brown against a clear background. BrdUrd LI was defined as the fraction of BrdUrd labelled nuclei in the total scored tumour nuclei.

³[H]dT-thymidine labelling

In 20 of the 40 cases, small fragments of neoplastic tissue were incubated in a shakerbath for 60 min at 37°C immediately after surgery. The incubation medium was composed of 2 ml Eagle's minimal essential medium supplemented with 10% FCS and L-glutamine [GIBCO, Grand Island, NY, USA], and 222 kBq or 6 μ Ci/mmol of ³[H]dT (specific activity of 925 GBq or 25 Ci/mmol, Amersham, Aylesbury, UK). After fixation in Bouin's solution for 1 h and in 80% ethanol overnight, the fragments were embedded in paraffin. Four μ m sections were dewaxed, rehydrated and coated with Ilford F-5 photographic emulsion. The slides were exposed for 3–6 days at 4°C, developed in D19b Kodak solution and stained with haematoxylin. The cells accepted as positively labelled were those with at least 6 black grains per nucleus against a clear background. ³[H]dT LI was defined as the fraction of labelled nuclei in the total neoplastic nuclei in each microscopic field at $\times 1000$ magnification with an oil immersion lens.

AgNOR staining

Tumour specimens parallel to the wedge-shaped samples used for BrdUrd labelling were routinely fixed in 10% buffered formalin and embedded in paraffin. Sections, 2 μ m thick, were dewaxed and post-fixed for 30 min in a 3:1 ethanol acetic acid solution. Then, the sections were rehydrated and incubated in the dark for 20 min at 37°C in a medium made up of one volume of 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate. Quantitative evaluation of interphase AgNORs was performed using an automated image analyser. The slides were scanned by the operator under the microscope at $\times 400$ magnification. The selected image was captured into digital memory and visualized on the monitor. Here, the silver-stained NORs appeared as well-defined, dark grey structures on a lighter background. The operator defined the grey threshold which permitted the selective identification of dots corresponding to AgNORs, by interactively comparing the video-image with the original microscopic image. When the structures defined by the chosen grey threshold were superimposable on the silver-stained dots in the original field the measurement was performed. By means of this morphometric technique, the interphase AgNOR number and area (in square microns) were evaluated.

For each case a total of 100 randomly selected nuclei of neoplastic cells were evaluated and the mean AgNOR number and area per nucleus were calculated from the data stored on a file. The mean area per silver-stained NOR particle was also calculated by dividing AgNOR area per AgNOR number in each nucleus (Ruschoff *et al.* 1990).

To evaluate the reliability and the reproducibility of AgNOR measurements, number and area were independently assessed in two centres with two different computer-assisted image analysers—system 1: ASEM Pc IBM compatible with SISTEMA MONO (Immagini e Computer, Milano); system 2: COMMODORE-AMIGA 3000 with BIOIMAGE ANALYSIS (Eurosoft, International, Italy).

Student's *t*-test and linear regression were performed for statistical analysis.

RESULTS

The mean number of AgNOR per nucleus in the 40 cases of colorectal carcinomas ranged from a minimum of 1.35 (SD ± 0.33) to a maximum of 6.48 (SD ± 1.08) with a medium value of 2.31 in system 1 and from a minimum of 1.70 (SD ± 0.42) to a maximum of 5.60 (SD ± 0.99) with a median value of 2.7 in system 2.

Analogously, the mean area of AgNOR proteins per nucleus varied from a minimum of 2.33 μ m² (SD ± 0.78) to a maximum of 7.32 μ m² (SD ± 1.79) with a median of 4.65 μ m² in system 1 and from a minimum of 4.02 μ m² (SD ± 1.21) to a maximum of 8.73 μ m² (SD ± 1.65) with a median

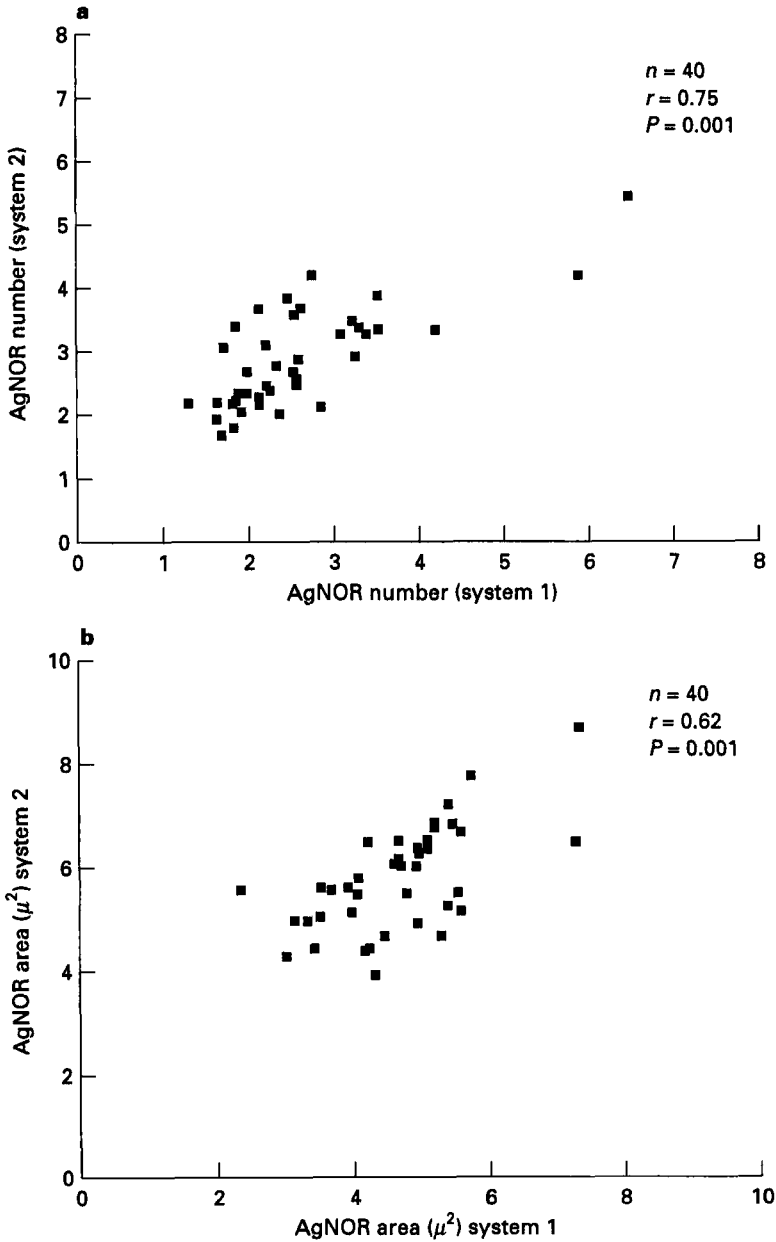


Figure 1. Correlation between AgNOR values (a number/nucleus and b area/nucleus) evaluated by two different computer systems.

of 5.15 in system 2. As shown in Figure 1 the results of AgNOR evaluation obtained with two analyzing systems were reproducible and comparable ($r = 0.75$ $P < 0.001$ for number; $r = 0.62$, $P < 0.001$ for area). Also, the range of mean area per NOR particle did not differ in the two systems (0.62 to 4.26 μm^2 with a median of 1.79 μm^2 for system 1 and 0.99 to 3.56 μm^2 with a median of 2.05 μm^2 in system 2) and the values were significantly correlated ($r = 0.63$, $P < 0.001$).

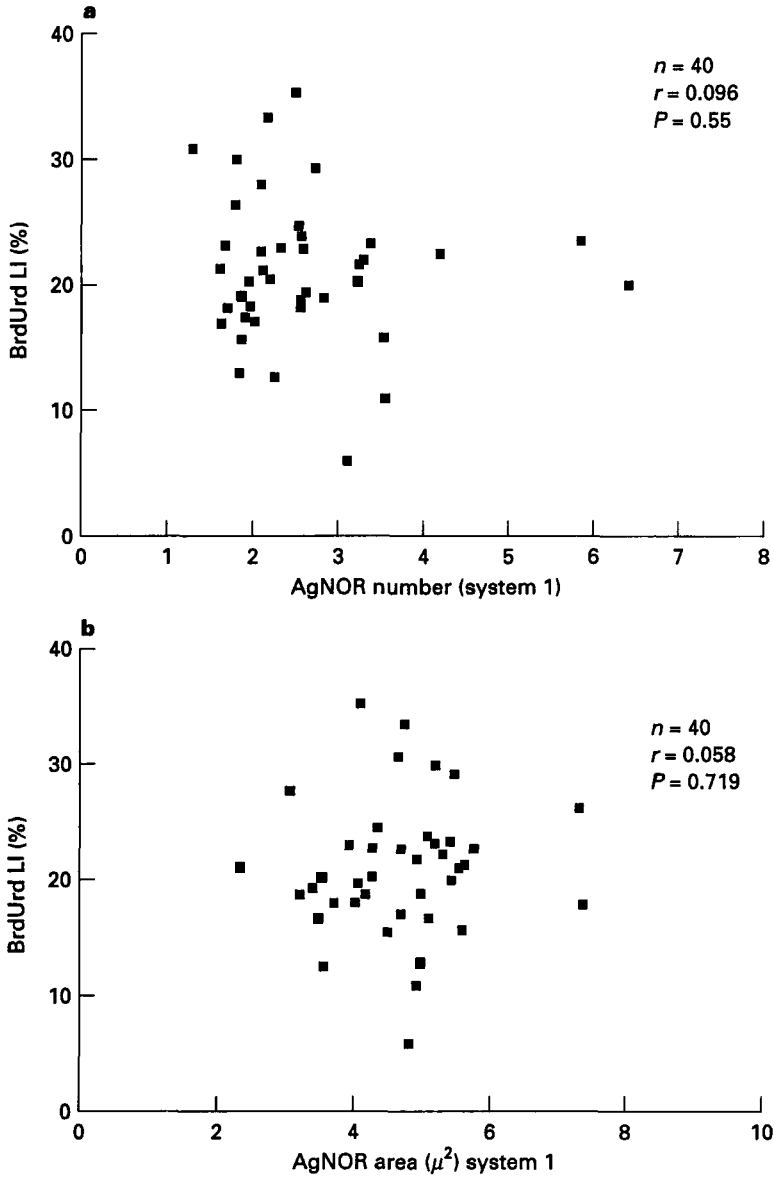


Figure 2. Correlation between AgNOR values (**a** number/nucleus and **b** area/nucleus) and BrdUrd Labelling Index (system 1).

In the 40 specimens analysed, the BrdUrd LI ranged from 6.0 to 35.4%. No significant differences were found in the values of BrdUrd LI in relation to the different time intervals which elapsed between BrdUrd administration and surgery. No significant relationships were detected between AgNOR values (number and area/nucleus) and BrdUrd LI (Figure 2). Analogously, no significant correlation could be found when AgNOR values were compared with ^3H dT LI (Figure 3), which ranged from 7.0 to 23.4 in the 20 cases studied. In addition, no relationships

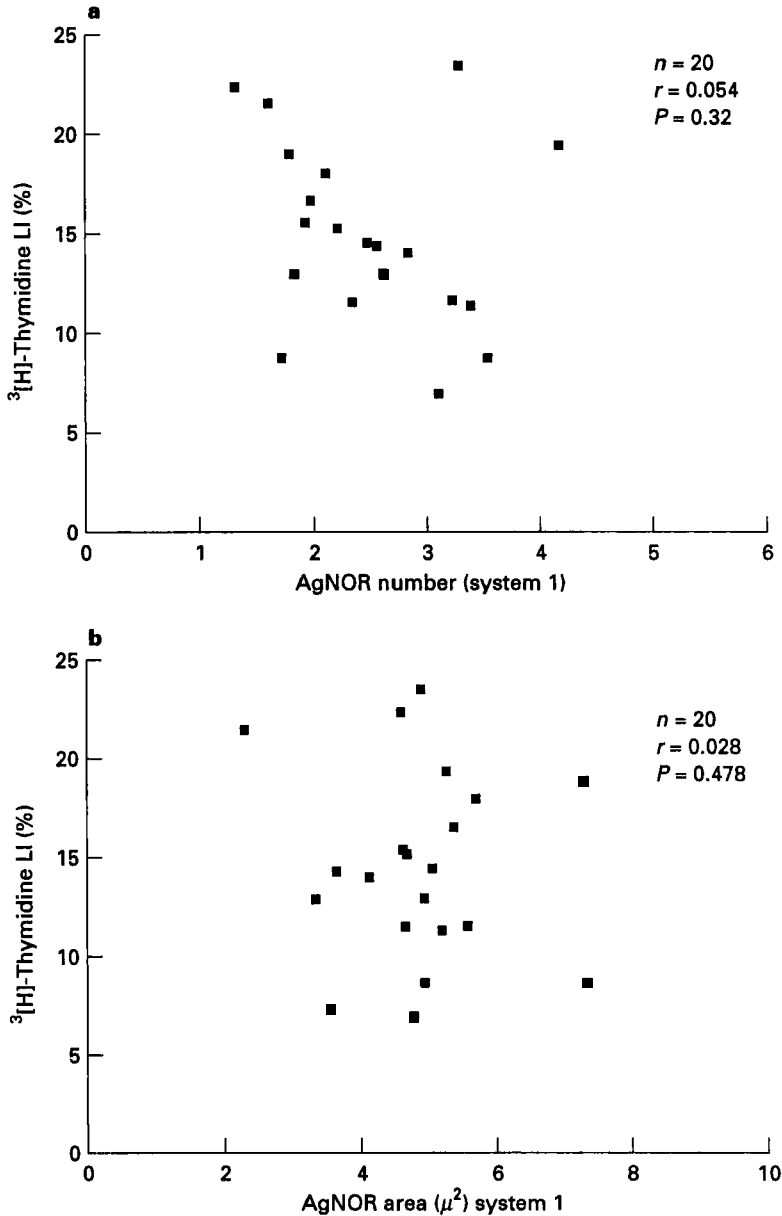


Figure 3. Correlation between AgNOR values (a number/nucleus and b area/nucleus) and ^3H Thymidine Labelling Index (system 1).

were observed when BrdUrd LI or ^3H dT LI were correlated with mean area per silver-stained NOR particle.

A significant correlation was instead found by comparing BrdUrd LI with ^3H dT LI ($r = 0.57$, $P = 0.007$) (Figure 4).

As previously reported (Roncucci *et al.* 1992), the two LIs did show a significant correlation with clinical and pathological parameters, like pattern of growth, grade and stage of the tumour. Analogously, no correlations were found between AgNOR values and the same parameters.

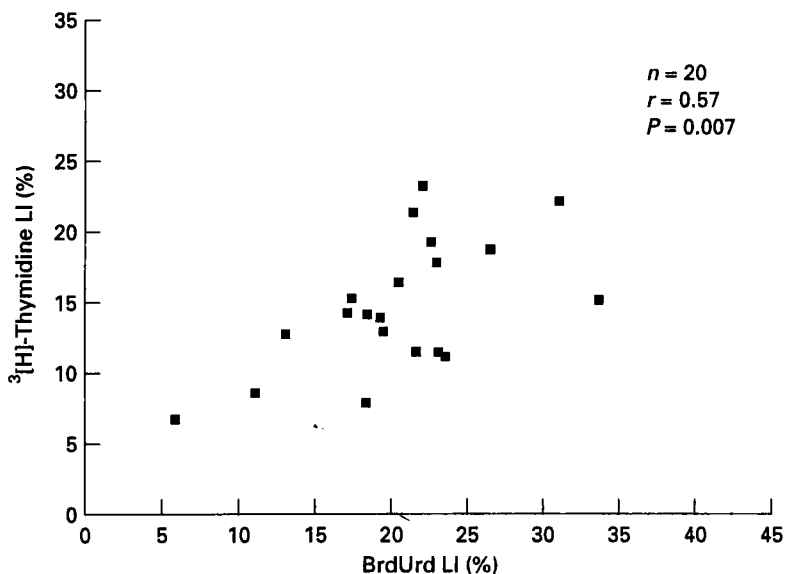


Figure 4. Correlation between BrdUrd and ³H-thymidine labelling indices.

DISCUSSION

The aim of this study was to establish whether AgNOR values could relate with parameters of cell proliferation like BrdUrd and ³H]dT LIs in colorectal carcinoma. The results failed to demonstrate significant correlations of AgNOR with the rate of cell proliferation. A significant correlation instead was seen between BrdUrd and ³H]dT LIs.

Thymidine and its analogue BrdUrd are incorporated into the nuclei during the S phase, thus providing reliable tools for evaluating tissue proliferative activity. However, both molecules require living cells to be incorporated and cannot be applied to tissues already processed for routine histology. On the contrary, AgNORs have the advantage of being visualized in paraffin-embedded material, but counting methods are tedious and time consuming. Furthermore, variations in section thickness and interobserver variability are known to influence the accuracy and reproducibility of counting.

For these reasons, the reliability of AgNOR values was assured in our study by repeating twice the measurements with two independent image-analyser systems. The results obtained were rather comparable, with coefficients of variation in the order of 0.6–0.7 ($P < 0.001$), and emphasized the lack of correlation with BrdUrd and ³H]dT LIs.

The quantity of AgNORs has been repeatedly shown to directly relate with cell replication rate (Trerè *et al.* 1989, Hittmair *et al.* 1994) and the amount of ³H]dT incorporation (Derenzini *et al.* 1989) either in cultured neoplastic cells or in tumour tissue samples. A positive correlation has been established between growth fraction assessed by Ki67 and AgNOR count in breast carcinomas (Raymond & Leong 1989, Dervan *et al.* 1989), non-Hodgkin-lymphomas (Hall *et al.* 1988), and gliomas (Hara *et al.* 1990).

Moreover, the AgNOR number correlated with DNA index and S phase determined by flow cytometry in breast lesions (Giri *et al.* 1989) and non-Hodgkin lymphoma (Crocker *et al.* 1988). Recently, AgNOR size has been shown to correlate closely with proliferative indices investigated by *in vivo* injection of BrdUrd in various normal and neoplastic rodent tissues (Leek *et al.* 1991)

and by *in vitro* BrdUrd incubation in several human malignant tumours of different origin (Trerè *et al.* 1991). In a study on 12 established cell lines derived from different tumour types including five colorectal carcinomas, a direct relationship has resulted between AgNOR protein quantity and growth rate of neoplastic cells evaluated by ^3H dT labelling independently of the cell line origin (Derenzini *et al.* 1990a).

However, other studies have failed to find positive correlations between the quantity of AgNORs proteins and the cell proliferation rate investigated by Ki-67 or flow cytometry in lung and breast carcinomas (Soomro & Whimster 1990, Hahn *et al.* 1990) and also in rectal carcinomas (Griffiths *et al.* 1989, Kram *et al.* 1989). Analogously, our results excluded significant correlation between values of AgNOR and parameters of cell proliferation in colorectal cancer. Moreover, in contrast to previous studies, the search for correlation was not only restricted to the study of AgNOR number, but also involved AgNOR area and area of single silver-stained NOR particle. In fact, as recently demonstrated by Hittmair *et al.* (1994), the distribution and quantity of AgNORs are strongly influenced by the phase of the cell cycle, the AgNOR number being strictly related to mitotic index and AgNOR area to S phase fraction.

However, in our study, none of the AgNOR parameters investigated showed correlation with proliferation indices. Therefore, AgNORs may be either related to other parameters of cell replication such as PCNA, Ki-67, Alpha Polymerase or, (probably better), to phases of increased ribosomal production and peptide synthesis not necessarily linked to cell proliferation as suggested by Griffiths *et al.* (1993). Indeed, Mourad *et al.* (1991, 1994) showed that the mean number of AgNORs is a reflection of the total chromosome count or ploidy rather than or proliferative activity. AgNOR counts are the result of the spatial arrangement of AgNOR-carrying chromosomes in different phases of the cell cycle.

In conclusion, as AgNOR values do not appear to relate to the more direct parameters of cell kinetics, it follows that their significance as a biomarker of cell proliferation remains questionable, at least in colorectal neoplasms.

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