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Cellular Proliferation in Cancer

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The AgNOR protein quantity is a parameter of tumor growth rate

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The quantitative distribution of AgNOR proteins represents a reliable index for predicting the clinical outcome of neoplastic patients. The rationale for the utilization of the AgNOR parameter in tumor pathology is based on the following observations: (1) the total quantity of AgNOR proteins evaluated *in situ* in human cancer cell lines by morphometric analysis is related to the rapidity of cell proliferation; (2) the quantity of Western-blotted nucleolin and protein B23 revealed by specific antibodies followed by reaction for chemoluminescence and measured by densitometric analysis of autoradiographic signals was also clearly related to cell doubling time; (3) in 30 human carcinoma xenografts growing in nude mice a highly significant correlation was demonstrated between AgNOR protein quantity and the tumor mass doubling time; and (4) in 18 untreated nodules of human hepatocellular carcinoma the tumor growth measured by "real time" ultrasonography was significantly related to the AgNOR protein quantity.

These results demonstrate that the predictive power of the AgNOR protein parameter in tumor pathology is due to the fact that the AgNOR protein quantity is strictly related to the tumor mass growth rate.

Relationship between p120 antigen expression and cell proliferation rate in cancer cells

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The p120 antigen is a nucleolar protein which has been identified after the development of a monoclonal antibody (FB-2) to nucleoli isolated from human cancer cells [1]. The present study was aimed to evaluate the relationship between p120 antigen expression and cell proliferation rate in cancer

cells. In a first experiment, the quantitative expression of protein p120 was evaluated in 6 human cancer cell lines characterized by various DTs (range 20–82 h). P120 antigen was evaluated on Western blots of SDS-polyacrylamide gel-separated nuclear proteins immunolabeled with FB-2 mAb. The results of the computerized densitometric analysis demonstrated a highly significant inverse correlation between the integrated optical density values of the bands at 120 kDa and the DT scores ($r = -0.91$, $p < 0.001$). In a second experiment, p120 antigen expression was evaluated in 16 infiltrating ductal carcinomas of the breast immunostained with FB-2 mAb, and correlated with the tumor cell proliferation rate determined by AgNOR protein quantitative analysis. p120 immunolabelling and AgNOR staining were quantified on histological sections by image cytometry. When p120 and AgNOR scores were compared by linear regression analysis, a highly significant correlation was demonstrated ($r = 0.98$, $p < 0.001$). Our results demonstrate that p120 expression represents a reliable parameter for defining the rapidity of cell duplication, which can be easily assessed on formalin-fixed and paraffin-embedded samples by routine immunohistochemistry.

Reference

[1] Freeman et al., *Cancer Res.* **48** (1988), 1244–1251.

p120 immunopositivity in formalin-fixed, paraffin wax-embedded tissues

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The monoclonal antibody nominated FB-2 (Clone FB-2 by Biogenex, USA) recognizes the antigen p120 kDa protein (p120) associated with the nucleolar matrix. p120 quantitative expression was found to be correlated with cell proliferation and patient survival in breast carcinomas. By indirect immunofluorescence on histological sections, p120 antigen was localized diffusely throughout interphase nucleoli of rapidly proliferating cells, while it was not detectable in most normal resting cells or in many benign and slowly growing malignant tumors. In the present study p120 expression has been evaluated in routinely formalin-fixed and paraffin-embedded tissues. Tissue samples from neoplastic and non-neoplastic lesions, of different organs (brain, breast, colon, lung, prostate, bladder, lymph nodes, skin, tongue and liver) were evaluated. By applying a specific retrieval protocol, based on 6 consecutive cycles of microwave oven heating, a clear and strictly nucleoli-confined immunopositivity could be demonstrated in stromal as well as in normal, hyperplastic and malignant cells. Moreover, this new method offers several advantages: the possibility to perform retrospective studies on filed materials; an easy applicability and reproducibility, in several different pathologic conditions, using an automatic immunocolorimeter (Ventana); the possibility to objectively detect p120 expression by automated image analysis, taking into account both the number and the area of FB-2 positive dots. If a role as diagnostic and prognostic tool is demonstrated for p120, it will be useful to compare p120 expression with that of other well-known cell cycle-associated proteins, such as AgNOR proteins, given their topographical relationship.

p120 immunoexpression in formalin-fixed, paraffin-embedded neoplastic tissues: Technical aspects and relationships with AgNOR quantity

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p120 is a 120 kDa nucleolar matrix protein which has been proposed as closely related to cell proliferation in some *in vitro* and *in vivo* human cancer studies. Until now the p120 immunoexpression in formalin-fixed, paraffin-embedded tissues has been documented by antigen retrieval (AR) procedures in few reports, although the detectability of p120 has not been completely achieved.

In the present study, we have analysed the p120 immunoexpression by different AR techniques in 30 surgical samples taken from malignant neoplasms of breast, endometrium, skin, colon, stomach, pancreas and liver; on corresponding 3 μm sections, heating AR protocols utilising microwave oven or wet autoclave have been performed with different application times, combined or not to protease digestion made by trypsin. In addition, 25 unfixed frozen specimens of above mentioned neoplasms were also available and immunohistochemically studied. The p120 primary antibody was commercially purchased (BioGenex, Menarini; w.d. 1 : 20). Better results were obtained with a combined trypsin predigestion (0.1% for 8–10 min, at 37°C) and microwave oven (5 min $\times$ 3 times) or wet autoclave (120°C, 20 min) heating; by these procedures, a clearly discernible immunoreaction and a good preservation of the morphology were achieved, like to those usually encountered in frozen sections.

After this step, we have investigated the p120 immunoexpression in 20 early or advanced gastric carcinomas, utilising trypsin plus microwave AR treatment; the mean area (μm^2) of p120 immunostaining was evaluated at one focal plane with a $\times$40 objective lens in 200 nuclei per specimen by an image analyser. When p120 values were compared to corresponding AgNOR quantity (μm^2), previously determined in same cases, a significant linear correlation was found (Pearson's $r = 0.835$, $p < 0.001$). Finally, by the Kaplan–Meier method, the p120 parameter allows to distinguish two groups of patients with a different overall survival; in particular, the prognosis was worse for patients with p120 values >5 μm^2 ($\chi^2 = 9.376$, $p = 0.002$).

Immunohistochemical detection of p120 on needle biopsies of prostate cancer: Comparative study with AgNOR, PCNA and Ki67/MIB1

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The aim of this study was to evaluate the reliability of p120 detection in prostate cancer (PC) with low and high Gleason histological scores (GHS), in comparison with AgNOR, PCNA and Ki67/MIB1 indices. Formalin-fixed and paraffin-embedded needle biopsies were selected from 20 patients with PC, equally divided into two groups: *Group* 1, having low GHS ($\leqslant$6), and *Group* 2, with high GHS ($\geqslant$7). The labelling index of all markers were evaluated by an optical microscope as the numbers of nuclei (40$\times$) or nucleolar granules or dots (1,000$\times$, oil immersion) out of 100 cells. The AgNOR

labelling rate appeared to be increased from low to high GHS. The p120 nucleolar protein could be detected particularly in PC with high GHS. No differences were observed between PCNA and Ki67/MIB1 immunoreactivity between the groups, whereas PCNA showed higher positivity in Group 1, thus appearing to be more sensitive for cases presenting differentiated features. Proliferative nuclear and nucleolar markers in PC with high GHS were significantly greater (ANOVA – two-tailed $p < 0.0001$) than that of low GHS. In conclusion, the present result, obtained from fixed and paraffin-embedded needle biopsies of PC indicates that p120 detection seems particularly suitable in cell kinetic analysis and points out that p120 expression can be considered a reliable marker of poorer outcome of PC.

The distribution of B23 genes in malignant and normal human tissues

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Protein B23 or Nucleophosmin (NPM) is a major nucleolar phosphoprotein involved in ribosomal biogenesis and shuttling proteins between the nucleolus and nucleoplasm [1,2]. NPM is also a member of the family of silver staining proteins found in the nucleolus. Recently, a breakpoint at the t(2;5)(p23;q35) was found in a large number of anaplastic large cell (ALC, K1, CD30+) lymphomas as well as Hodgkin's disease. The translocation joins the NPM gene located on 5q35 with the ALK gene at 2p23, yielding a hybrid protein with tyrosine kinase activity. NPM/ALK was not detected in normal tissues. In the present study, we have performed *in situ* hybridization studies using a 448 bp PCR fragment of NPM cDNA to generate the probe [3]. Of a large variety of human tumors and normal tissues investigated, positive staining was noted primarily in cases of Hodgkin's and ALC lymphoma. The staining was present in large lymphocytes and interstitial cells but not in RS or atypical RS cells. No staining was noted in normal lymph nodes, although rare staining was seen in normal spleen. In addition, some infrequent nuclear staining was seen in lung carcinoma, mesothelioma and astrocytoma. No other normal tissue showed nuclear staining. These results will be interpreted in view of the potential role of NPM in human malignancy and the possible relationship between ALCL and Hodgkin's lymphoma.

References

[1] Olson, 1991.
[2] Valdez, 1995.
[3] Chan et al., 1989.

Standardized AgNOR analysis in radically resected prostatic cancer: Correlation with PSA values and survival

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Background: recent methodological advances in AgNOR staining and analysis offer the possibility to investigate retrospectively the prognostic value of this method in large scaled studies.

Patients and methods: standardized AgNOR staining and analysis was performed on a series of 126 radically resected adenocarcinomas of the prostate with a patient follow-up period of at least 8 years. Morphometric AgNOR parameters have been correlated with common staging and grading classifications, serum prostatic specific antigen (PSA) values and with the clinical outcome.

Results: standardized AgNOR parameters were shown to be statistically significantly (mean AgNOR number: $p = 0.03$; mean AgNOR area: $p = 0.002$) correlated with the clinical course of patients after surgical resection of prostatic carcinoma but not with other staging and grading classifications investigated. A multivariate analysis showed that AgNOR quantity (mean AgNOR area: $p = 0.0001$), tumor grade ($p = 0.02$), and R-stage ($p = 0.04$), were statistically significantly and independently associated with survival. Furthermore AgNOR analysis of the primary prostatic carcinoma was statistically significantly correlated ($p = 0.001$) with elevated PSA levels during follow-up.

Conclusion: standardized analysis of AgNORs is proposed as an important independent prognostic factor in surgical resected prostatic carcinoma. The method is suitable for routine use on paraffin-embedded archival material.

The effect of *Helicobacter pylori* eradication on antral epithelium proliferation – AgNORs evaluation

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Introduction: the *Helicobacter pylori* (Hp) infection may alter the replication cycle of the antral mucosa epithelium cells.

Aim: the purpose of this prospective study was to evaluate the impact of Hp eradication on antral epithelial proliferation.

Methods: we studied 18 patients with peptic ulcer (6 patients with gastric ulcer and 12 patients with duodenal ulcer). Ten patients were submitted to successful Hp eradication treatment and eradication was confirmed by urease test and histology in all of them. Eight patients were only treated with omeprazole and persistence of Hp after treatment was confirmed in all of them.

Brush cytology was obtained by endoscopy before and 1 month after treatment. Silver-stained slides were prepared for image cytometry analysis. AgNORs studies were carried out using a video-camera–computer system with commercial software (Visilog® – Microptic, Barcelona, Spain).

Results: before treatment, the mean number and mean area (no. $\pm$ standard error) of AgNORs per nucleus were not significantly different between:

(a) gastric and duodenal ulcer patients (no. per nucleus: 2.87 ± 0.21 vs. 2.96 ± 14; area: 1.72 ± 0.51 μ vs. 1.49 ± 0.39 μ);
(b) eradicated patients and not-eradicated patients (no. per nucleus: 2.74 ± 0.16 vs. 3.17 ± 0.13; area: 1.59 ± 0.20 μ vs. 1.86 ± 0.13 μ).

After treatment, patients who cleared Hp showed a significant decrease in the mean number of AgNORs per nucleus and mean area per nucleus (Student's t-test, $p < 0.003$).

AgNORs values after treatment in patients that remained Hp-positive were significantly higher than values in cleared patients (no. per nucleus: 2.84 ± 0.29 vs. 1.81 ± 0.21, $p < 0.01$; area: 1.74 ± 0.97 μ vs. 1.11 ± 0.16 μ, $p < 0.03$).

Conclusion: these data suggest that Hp eradication reduce the antral epithelial cells proliferation rate, as evaluated by the AgNORs staining before and short-time after therapy.

Proliferation in the lining and stromal cells of synovia in inflamatory disease with tissue remodelling

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Proliferation is a hallmark of controlled or uncontrolled tissue neoformation. Knowledge of one may enrich the other. There are many touchpoints in proliferations of different quality. Because of tumor like proliferation (TLP) of synoviocytes the NOR morphology of synovial cell was analysed for 8 variables: the number of clusters per nucleus (CN), the number of AgNOR dots per cluster (DC) and per nucleus (DN), cluster area (CA), cluster area per nucleus (CAN), dot area (DA), dot area per cluster (DAC) and dot area per nucleus (DAN), in 13 synovial biopsies from clinically diagnosed rheumatoid arthritis (RA) and osteoarthritis (OA). For almost all the variables the RA exceeded OA (CN 1.54 vs. 1.44, $p < 0.33$; DC 4.79 vs. 3.38, $p < 0.001$; DN 7.15 vs. 5.15, $p < 0.001$; DA 0.17 vs. 0.14, $p < 0.001$; DAN 1.24 vs. 0.73, $p < 0.001$). Some relations between the variables were underlined. The results obtained are considered to be of importance for understanding the pathogenesis of diseases and are in agreement with the proliferative properties of RA tissue destruction and remodelling.

Quantitative analysis of AgNORs in the study of the regenerative capacity in normal, dystrophin-deficient and poliomyositic muscles

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By AgNOR technique, we have investigated *vastus lateralis* muscle samples from 13 patients with Duchenne muscle dystrophy (DMD) (6 months–12 years), 9 with Becker muscle dystrophy (BMD) (13 months–36 years), 5 with poliomyositis (PM) (8–77 years) and 10 normal subjects (5 months–32 years) that underwent to orthopaedic surgery. Specimens had been frozen in isopentane cooled in liquid nitrogen, stored at -60°C and from each sample, sections (4 μm) were cut on cryostat, mounted on xylane-coated slides and submitted to the AgNOR technique according to guidelines of the Committee on AgNOR Quantification, omitting the wet autoclave pretreatment recommended only for formalin-fixed and paraffin-embedded tissues. The mean area (μm^2) of AgNORs per nucleus (NORA) was evaluated at one focal plane with a $\times 40$ objective lens in at least 200 nuclei per specimen; specific softwares were utilized to determine NORA values. The results were expressed as mean $\pm$ SD. Differences of NORA values among muscle specimens taken from DMD, BMD, PM and control subjects were assessed by analysis of variance and the Newman–Keuls' test.

The mean NORA values encountered in DMD (4.327 ± 0.791 μm^2), BMD (3.534 ± 0.312 μm^2) and PM (3.781 ± 0.499 μm^2) samples were significantly higher than those of normal muscle ($1.682 \pm$

0.288 μm^2); a $p < 0.001$ was achieved when NORA values concerning DMD and BMD were compared. Again, a $p < 0.001$ was found when the NORA values were calculated in DMD, BMD and PM regenerating myofibers with reference to normal muscle, while no difference was appreciable between DMD and BMD. On the other hand, in non-regenerating myofibers, the NORA values were significantly ($p < 0.001$) different between DMD and BMD or PM and also from controls. Our study documents that muscle pathologic conditions, in which the regeneration of myofibers is a constant finding, have a high proliferation rate as indicated by NORA values; in particular, DMD affected muscles showed the highest AgNOR quantity, independently of the functional (regenerating or quiescent) status of myofibers.

Application of the standardized AgNOR analysis and Ki67 immunohistochemistry double-labeling in feline epithelial skin tumors

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Epithelial skin tumors, especially papillomas, basal cell tumors and squamous cell carcinomas are common in all domestic animals, with preference of dogs, cats and horses. Pathogenesis, classification and prognostic estimation of these tumors are therefore of high practical relevance. The term "basal cell tumor" is used in veterinary dermatopathology to classify a large group of neoplasms of small animals presumed to be derived from epithelial cells of both epidermal and adnexal origin. Mitotic figures are frequently encountered in these tumors. Despite their often histologically anaplastic appearance, in contrast to man, most basal cell tumors are benign and usually do not metastasize or recur after surgical removal. Only a rare locally invasive variant, the so-called feline basal cell carcinoma differs in its clinical appearance from the previously described benign feline basal cell tumors and is therefore considered as a separate entity.

This prompted us to analyze 25 basal cell tumors and 5 feline basal cell carcinomas for their differential proliferation state using the dual staining standardized AgNOR method followed by the Ki67 immunohistochemistry. The results obtained were compared with those of 10 feline papillomas and 26 squamous cell carcinomas. The Ki67 index increased from papillomas (6.15 ± 1.25), basal cell tumors (16.38 ± 1.51) to squamous cell carcinomas (29.64 ± 5.93). In squamous cell carcinomas expressing the p53-regulated key-cell-cycle controller p21-WAF-1, lower Ki67 indices could be encountered, which reflects the p21-WAF-1-mediated cell cycle arrest. Basal cell tumors showed smaller values concerning all relevant AgNOR parameters (nucleus area: 22.31 ± 3.7 μm^2, AgNOR cluster/cell: 1.15 ± 0.14, AgNOR area/cell: 0.68 ± 0.29 μm^2, AgNOR ratio: 0.03 ± 0.01) than squamous cell carcinomas (nucleus area: 47.4 ± 13.41 μm^2, AgNOR cluster/cell: 1.85 ± 0.34, AgNOR area/cell: 2.08 ± 0.68, AgNOR ratio: 0.04 ± 0.01). The investigation of the feline basal cell carcinomas resulted in intermediate values concerning the AgNOR parameter nucleus area (31.99 μm^2) and AgNOR area/cell (1.12 μm^2). Significant differences of the AgNOR parameters in p21-positive and p21-negative squamous cell carcinomas could not be encountered.

AgNOR quantity and MIB1 score in ocular melanomas

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By AgNOR technique, we have studied 12 surgical samples obtained from an equal number of patients (M/F = 4/8; age range 30–78 years) subjected to the enucleation of the eye for malignant melanoma. The histopathological diagnosis, made according to the criteria of Zimmerman, was the following: spindle cell variety (7 cases), epithelioid variety (4 cases), necrotic variety (1 case); for all cases survival data were available (range 6–169 months, mean 57.5 months). From the corresponding formalin-fixed paraffin-embedded tissue blocks, sections (4 μm) were cut, mounted on xylane-coated slides and submitted to the AgNOR technique according to guidelines of the Committee on AgNOR Quantification. The mean area (μm^2) of AgNORs per nucleus (NORA) was evaluated at one focal plane with a $\times 40$ objective lens in at least 100 nuclei per specimen; specific softwares were utilized to determine NORA values. The results were expressed as mean $\pm$ SD. In addition, on parallel sections, after microwave oven heating, the ABC technique with the utilization of MIB1 monoclonal antibody (Immunotech, DBA, Italy – w.d. 1:100) was made; the MIB1 score was achieved calculating the percentage of stained neoplastic elements in 1,000 nuclei. In order to compare AgNOR and MIB1 data, a regression linear test was utilized; finally, survival analysis was performed by the Kaplan–Meier method and for the comparison of the survival curves, the Mantel–Haenszel log-rank test was applied.

The mean NORA value encountered in ocular melanomas was 4.159 μm^2 (range 2.548–6.104 μm^2), while the MIB1 score ranged from 3 to 27% (mean 10.75%); when these values were compared, a significant linear correlation was found ($r = 0.766$, $p < 0.004$). By the Kaplan–Meier method, both parameters allow to distinguish two groups of patients with a different overall survival; in particular, the prognosis was worse for patients with NORA values >4.2 μm^2 ($\chi^2 = 5.978$, $p = 0.015$) and MIB1 score $>10\%$ ($\chi^2 = 5.164$, $p = 0.023$).

Proliferative (AgNOR/Ki67/mitosis) and apoptotic activity of non-invasive and invasive lobular breast cancer

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Aims: within the different forms of breast cancer, lobular carcinomas are characterized by tumor-specific biological and clinical features. Until now, no studies dealing with the proliferative and apoptotic activity of lobular breast carcinomas have been published. In the present study, kinetic parameters including AgNOR count, Ki67 and mitotic index as well as apoptotic rate were analyzed in lobular *in situ* carcinomas (LCIS) and in invasive lobular carcinomas (ILC) of the breast.

Methods: paraffin sections from 25 ILC (all classic type, grade 2) and from 12 LCIS were silver-stained to visualize AgNOR. Ki67 antigen was stained immunohistochemically with the MIB1 antibody (Dianova, Hamburg). Apoptotic cells were detected with the "TUNEL" method. On H&E-stained slides, mitotic index was counted within 1,000 tumor cells. Additionally, 12 samples from normal breast tissue and 31 invasive ductal breast carcinomas (grade 2) were included in the study.

Results: all parameters showed increasing values from control tissue (MIB1 index (MIB1-I): 1.9%; mitotic index (M-I): 0%; mean AgNOR count (NOR): 1.7; apoptotic index (APO-I): 0.03%) to LCIS (MIB1-I: 3.2%; M-I: 0.4%; NOR: 2.2; APO-I: 0.29%) and eventually to ILC (MIB1-I: 12.1%; M-I: 1.0%; NOR: 2.5; APO-I: 0.61%). NOR and MIB1-I correlated positively with APO-I in ILC. All values of ILC were significantly lower than those of IDC. On the contrary, the MIB1-I : APO-I ratio was significantly higher in ILC (19.8) compared to IDC (12.8).

Conclusions: invasive growth in lobular breast carcinoma is accompanied by increased proliferation. The lower proliferative activity of ILC (compared to IDC) correlates with their known tendency to grow more slowly. On the other hand, the high MIB1-I : APO-I ratio of ILC may provide a possible explanation for why the clinical prognosis of ILC and IDC is known to be similar.

Standardized AgNOR analysis in lymph node negative breast cancer

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Background: a multicenter trial on lymph node negative breast cancer specimens has been proposed with the consent of all participants at the last AgNOR workshop in Taormina. The present pilot study has been in order to evaluate whether standardized AgNOR assessment may reflect clinical differences in this highly selected group of patients.

Patients and methods: 56 consecutive cases of stage pN0 breast cancer specimens of patients operated at the Department of Surgery I, University Hospital, Innsbruck, Austria, between 1985 and 1990 were retrieved from the files of the Department of Pathology, University of Innsbruck. Standardized AgNOR analysis was performed as described recently in detail.

Results: five patients out of 56 developed distant metastases during follow-up, one patient had a local recurrent tumor. CV of AgNOR number in these tumors ranged from 0.51 to 0.72. From the remaining 50 patients with an uneventful clinical course of at least 7 years only 10 tumors showed a CV number of AgNORs higher than 0.51. Furthermore, 3 out of the 6 patients with a poor clinical course responded to adequate therapy and are still alive. These tumors showed lower CV of AgNOR number values (0.51 and twice 0.53) when compared with those who died so far (0.59, 0.66 and 0.72, respectively).

Conclusion: in lymph node negative breast cancer patients standardized AgNOR analysis provides an easy to use tool in order to define a group of patients with an increased risk for tumor recurrence. Additionally, chemotherapy response seems to be predictable, which underlines the clinical significance of standardized AgNOR analysis. Therefore a multicenter AgNOR study on stage pN0 breast cancer specimens should be initiated.

AgNOR quantity in endometrial adenocarcinomas: A reliable tool for the nuclear grading

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The histologic grade of endometrial adenocarcinomas (EA) has been related to the aggressiveness of the disease, but the ideal system to assign it still remains controversial; in 1995 a Gynecologic Oncology Group Study revised the FIGO recommendations about nuclear grading, utilising the nuclear shape, chromatin distribution and nucleolar size. However, these nuclear criteria applied to EA maintain a largely subjective rate; in fact, in a selected casuistry obtained from files of the Institute

of Pathological Anatomy of Genoa University, the determination of nuclear grade showed only a moderate strength of agreement ($k = 0.434$) between two observers (M.G. and E.F.) according to Landis and Koch "benchmarks", making a new assessment necessary based upon a consensus of the two pathologists, achieved by the use of a double-headed microscope.

In order to identify a more reliable objective parameter, we have investigated the AgNOR quantity in 38 formalin-fixed paraffin-embedded surgical samples of EA obtained from an equal number of patients (age range 48–84 years, mean age 62.2), of which survival data were available (range 8–119 months, mean 81.5). From the corresponding tissue blocks, sections (4 μm) were cut, mounted on xylane-coated slides and submitted to the AgNOR technique according to guidelines of the Committee on AgNOR Quantification. The mean area (μm^2) of AgNORs per nucleus (NORA) was evaluated at one focal plane with a $\times 40$ objective lens in at least 100 nuclei per specimen (mean 132); specific softwares were utilised to determine NORA values. The results were expressed as mean $\pm$ SD; a regression linear test was applied in order to compare the nuclear grade and NORA values. Finally, survival analysis was made by the Kaplan–Meier method and the Mantel–Haenszel log-rank test.

The NORA values encountered in EA ranged from 2.273 to 9.004 μm^2 (mean 4.339 μm^2); when NORA values were compared to the different nuclear grade, the most significant linear correlation ($r = 0.7565$, $p < 0.001$) was found for the nuclear assessment obtained by a consensus basis. In addition, by the Kaplan–Meier method, the prognosis was worse for patients with NORA values >4.456 μm^2 ($\chi^2 = 5.680$, $p = 0.018$).

Standardized AgNOR analysis in primary colorectal adenocarcinomas and corresponding recurrences and lymph node metastases

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Background: it is generally accepted that clonal selection is an underlying mechanism in tumor cell spread. In this context only little is known about the significance of the cellular proliferative activity, in particular regarding the AgNOR content. For this purpose we have performed standardized AgNOR analysis in primary as well as corresponding lymph node metastases and recurrent tumor tissues.

Material and methods: standardized AgNOR analysis has been carried out on 17 lymph node metastases of colorectal and 11 local recurrent tumors of rectal adenocarcinomas and their respective primary tumors.

Results: none of the lymph node metastases showed markedly different AgNOR parameters when compared with their primary tumors. In the local recurrent tumors only two out of 11 cases showed slightly elevated CV of AgNOR numbers and a further 2 cases showed additionally higher CV of AgNOR area.

Conclusion: cellular proliferative activity with regard to the AgNOR content is not significantly different between primary colorectal adenocarcinomas and corresponding lymph node metastases or local recurrences. This phenomenon delineates that other mechanisms than selection of a highly proliferating tumor cell clone play the leading role in tumor spread.

DNA and AgNOR assessment by image analysis for biparametric analysis and bidimensional study of AgNOR granules as marker of heterogeneity

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DNA and AgNOR content are usually employed as different biological indicators of ploidy and kinetics and are separately assessed: the simultaneous acquirement of DNA and AgNOR content was the aim of this experience by image analysis for better defining normal and neoplastic entities, diploid and non-diploid, and assessing the relationship between ploidy and kinetics. Biparametric analysis (area and diameter) of AgNOR granules was also carried out, with reference to granule's heterogeneity and nuclear AgNOR content.

Material and methods: cytological samples (by touch) of 30 breast cancer (15 diploid and 15 non-diploid), activated and non-activated lymphoid tissue and normal liver, were stained with both Feulgen and AgNOR double technique. Image analysis was carried out with Imago Pro Plus and custom developed software. DNA content was carried out by densitometric procedure, AgNOR content was expressed as area and number of granules for each nucleus. Bidimensional (area–diameter) evaluation of each granule was carried out for heterogeneity assessment.

Results: biparametric scattergram for DNA/AgNOR was slightly concentrated in diploid entities and expanded in non-diploid ones. Within the diploid entities (i.e., lymphoid tissue), the activated areas expressed a wider scattergram compared to resting ones. Diploid carcinomas had an increasingly wider scattergram compared to normal diploid tissues; non-diploid entities had a more variable distribution. Biparametric analysis of AgNOR granule was represented by a progressively more extended scattergram, related to nuclear AgNOR content, as activation of diploid versus non-diploid population increased. In the DNA/NOR scattergram of activated diploid and non-diploid entities, growing levels of AgNOR were moreover identified, and corresponded to identical values of DNA: the finding was pointed out better with statistical analysis.

Conclusions: in diploid entities, biparametric DNA/AgNOR analysis allowed the distinction of different scattergram according to activation or kinetics of nuclear population: this was evident for healthy and neoplastic tissues. Bidimensional evaluation of granules expressed heterogeneity as wider scattergram also had a more elevated SD and related to DNA/NOR biparametric evaluation. Both analyses confirmed the existence of high kinetics diploid lesions with scattergram similar to non-diploid entities. Mathematical analysis also pointed out better, within the same population tested, events with growing activation levels in the same DNA content area, confirming the possibility of ploidy/kinetics dissociation.

Cell doubling time and N-myc amplification in neuroblastoma cell lines

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In neuroblastoma N-myc amplification has been found to be strikingly associated with rapid tumor progression and poor prognosis. In a previous investigation carried out on 48 neuroblastoma tumors we failed to demonstrate any significant correlation between N-myc amplification and AgNOR protein

amount [1,2]. In the present study the relationship between N-myc amplification and AgNOR protein quantity was assessed in 7 established human neuroblastoma cell lines characterised by different doubling times (DTs). Four cell lines (CHP 212, SJNKB, SKNBE and NB 100) had low DTs (range 20–28 h) and three cell lines (HTB 10, SY5Y and IMR 32) had high DTs (range 52–72 h). N-myc amplification was evaluated by Southern-blot analysis using the NB 19-12 probe, and the AgNOR protein quantity was defined by image analysis (VIDAS System, Kontron Elektroniks, Germany) on cytological preparations selectively stained with silver. N-myc amplification was found to be totally independent of population DT. Indeed, the rate of N-myc amplification was 25% (1 out of 4) in the group of cell lines with low DTs and 33.3% (1 out of 3) in the group of cell lines with high DTs ($\chi^2 = 0.058$, $p = 0.70$). The N-myc copy number was also found to be totally independent of AgNOR protein quantity ($r = 0.18$, p – NS) which, on the contrary, was strictly related to the population DT ($r = -0.947$, $p < 0.001$).

Our results have confirmed that N-myc amplification and cell proliferation rate are not interrelated in neuroblastoma, each representing independent biological parameters of cancer cells.

References

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AgNOR quantitation by image analysis in medulloblastomas

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Introduction: medulloblastoma (MB) is a small cell tumor which arises in the cerebellum of patients in the first two decades of life. A different evolution has been found without an association with histological subtypes, cellular differentiation and ploidy. By contrast, high mitotic index and high percentage of proliferating cells have been associated with a poorer prognosis. Although survival is a complex variable influenced by many factors, we aim in this paper to know if cellular proliferation by itself, as it is measured by AgNOR quantitation, allows us an automatic classification with prognostic value.

Methods: this is a partial analysis of a larger multicentric study. Sixty-three cases aged between 2 and 30 years old ($m = 10$) at diagnosis were statistically analyzed (join and k-means cluster analyses) in order to obtain at least two clusters of different proliferation degree by using a combination of two or more AgNOR variables. Cases with a minimum follow-up of 24 months or death as a consequence of the tumor before this time were statistically analyzed (Mann–Whitney test, dep. var.: Months Survival (MSV)) to look for an association between clusters and MSV. Paraffin cuts were AgNOR-stained following the consensus rules for standardization. AgNOR number (NUNOR), AgNOR area (ARENOR) per nucleus, AgNOR area relative to nuclear area (NORREL) and AgNOR individual particle area (PARAREA) were quantified (software: ARGENTA, Barcelona, Spain).

Results: a cluster analysis by a combination of ARENOR and PARAREA provided two clusters. One of them ($n = 10$) with higher (H) values in AgNOR variables than the other (L) ($n = 53$). These differences had an statistical significance (see Table 1) not only in AgNOR variables but also in MSV.

Table 1

	H	L	p
NUNOR	2.1 ($n = 10$)	1.4 ($n = 53$)	0.000
ARENOR	1.2 ($n = 10$)	0.5 ($n = 53$)	0.000
NORREL	3.6 ($n = 10$)	2.2 ($n = 53$)	0.000
PARAREA	0.6 ($n = 10$)	0.3 ($n = 53$)	0.000
MSV	26.6 ($n = 5$)	69.4 ($n = 32$)	0.039

Conclusions: a combination of AgNOR variables (ARENOR and PARAREA) allows to differentiate MB with a different proliferation level and they seem to be associated with a different prognosis. This should be confirmed in future analyses with a larger number of cases.