Histol Histopathol (2004) 19: 785-792

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Multiparameter analysis of AgNOR in thyroid lesions: comparison with PCNA expression

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Summary. The aim of the study was to examine numerous features of argyrophilic proteins related to nucleolar organizer regions (AgNORs) in thyroid tumors, relate them to PCNA expression and evaluate which of these features might be useful in the diagnosis of thyroid lesions. Paraffin sections of 100 thyroid tumors were silver-stained and divided into 9 groups: nodular goiter (NG), simple adenoma (SA), microfollicular adenoma (MFA), follicular carcinoma (FC), follicular variant of papillary carcinoma (PC-F), classical variant of papillary carcinoma (PC-C), Hürthle cell adenoma (HA), Hürthle cell carcinoma (HC), and anaplastic carcinoma (AC). The slides were analyzed with the computerized system for image analysis. A weak correlation was found between PCNA expression and AgNOR size. AC differed significantly from all other examined groups in many features of AgNOR dots. Hürthle cell neoplasms were characterized by the presence of a usually single and relatively large dot. With respect to diagnosing follicular lesions, we found that the evaluation of the total area of dots in the nucleus seemed to be the most useful for discrimination: the assumption of 4.9 μm^2, as a cut-off value, allowed a correct classification of 77% of FC cases. Computer-aided morphometric analysis of AgNORs may be useful in the diagnostics of thyroid lesions.

Key words: Thyroid, AgNOR, Computer-aided analysis, Neoplasm, Follicular lesions

Introduction

Nucleolar organizer regions (NORs) are genomic DNA segments, encoding for ribosomal RNA. They appear on the short arms of the five acrocentric chromosomes (numbers: 13, 14, 15, 21 and 22) (Howell, 1982). NORs are associated with argyrophilic proteins, involved in ribosomal gene transcription. These are mainly nucleolin, a phosphoprotein of 105 kDa, which plays an important role in the transcription of rRNA molecules, and nucleophosmin (or B23 protein), a phosphoprotein of 38-39 kDa, which is engaged in late steps of preribosomal particle organization (Derenzini, 2000; Sirri et al., 2000). These proteins can be localized through silver staining. The result of staining is dots of silver (AgNOR dots or AgNORs). It was found that the mean number of AgNORs was related to the proliferation rate of tumor cell population, since it is proportional to the rapidity of cell duplication (Öfner et al., 1992). It has been suggested that the number of AgNORs may have some potential diagnostic and prognostic value in different neoplasms (Bankfalvi et al., 1998; Pich et al., 2000). However, only in a limited number of tumors was, the diagnosis of malignancy possible on the basis of higher AgNOR quantity, while – in the majority of tumor types – a variable overlap was found between AgNOR values of benign and malignant cells (Derenzini, 2000). There are many other morphological features of AgNORs which exhibit some potential diagnostic or prognostic value. These features mainly include the variables, which describe the AgNOR dot area, distribution, and location within the nucleus. Derenzini and Trere (1994) demonstrated a linear relationship between the interphase AgNOR area, as evaluated by a computer-assisted image analysis system, and the cellular doubling time. Computerized image analysis enables not only measurements of the above mentioned features, but it is also highly reproducible, objective, and markedly less dependent on possible imperfectness of the staining technique (Derenzini and Trere, 1991; Trere, 2000). Nowadays, the determination of features describing AgNOR area per nucleus by means of computerized image analysis is considered to be the state-of-the-art method of AgNOR evaluation (Aubele et al., 1994).

With respect to thyroid tumors, the role of silver staining in diagnosis has not been clearly evaluated. In only a few papers has computerized morphometric

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analysis of AgNORs been used (Rüschoff et al., 1993; Solymosi et al., 1996; Zaczek et al., 1996). Thus, the aim of the present study was to examine numerous features of AgNORs in thyroid tumors, relate them to proliferating cell nuclear antigen (PCNA) expression and to evaluate which of these features could be useful in the diagnosis of thyroid lesions.

Materials and methods

Paraffin sections (4 μm thick) of 100 thyroid tumors were silver-stained and divided into 9 groups: nodular goiter (NG; n=22), simple adenoma (SA; n=11), microfollicular adenoma (MFA; n=16), follicular carcinoma (FC; n=13), follicular variant of papillary carcinoma (PC-F; n=13), classical variant of papillary carcinoma (PC-C; n=13), Hürthle cell adenoma (HA; n=5), Hürthle cell carcinoma (HC; n=4) and anaplastic carcinoma (AC; n=3). In 3 cases of NG, features of chronic thyroiditis were found. In all the cases, the specimens were fixed in 10% buffered formaldehyde solution and embedded in paraffin, according to standard procedures. The applied silver staining protocol was adapted from the method described by Ploton et al. (1986), according to current recommendations (Aubele et al., 1994). Histopathological slides were post-fixed for 30 min in Clarke's solution to reduce non-specific staining, then hydrated through graded alcohols to distilled water and stained in the dark at a constant temperature of 37 °C for 15 minutes, using a pre-warmed 33% solution of silver nitrate in gelatine – formic acid. An optimal staining protocol was established after a series of test stainings (with the time of reaction being modified). AgNOR dot area increased progressively by increasing the staining period and if the reaction was prolonged for above 15 min, silver dots merged.

PCNA expression was assessed using the monoclonal antibody anti-PCNA and EnVision System AP (DAKO). The number of immunopositive cells was evaluated in 1000 cells and expressed in percent values as the PCNA index. Human tonsils were employed as the positive control.

Only nuclei with clearly defined borders and without signs of degenerative changes were eligible for the measurements.

Quantification of the AgNORs

The microscopic slides were analyzed by a computerized system for image analysis, including the computer program called "AgNORmeter95", developed with Visual C++ 5.0 (Microsoft, USA) (Klencki et al., 2001a). This program has been designed to provide quick and straightforward quantitative AgNOR measurements. It can automatically localize the contours of nuclei and AgNOR dots, and gives the possibility of adjusting contour searching criteria. The influence of these criteria on search results is instantly visualized, which greatly facilitates the adjustments. The program processes nearly 20 variables (listed in Table 1), which can be divided into these 3 groups: describing the area of dots; their intranuclear localization; and the number of dots (including the percentage of nuclei with various numbers of dots). The choice of variables, as well as their precise definitions, was applied as proposed by

Table 1. AgNOR dot features measured with the "*AgNORmeter*" program.

VARIABLE	DESCRIPTION
Size-related variables	
total area of dots in nucleus	The sum of the area of all dots in the nucleus
mean area of dots in nucleus	The total area of dots in the nucleus divided by the number of dots in the nucleus
SD of dot area in nucleus	The standard deviation of areas of dots in a single nucleus
absolute area of the biggest dot in nucleus	The area of the biggest dot in the nucleus
relative area of the biggest dot in nucleus	The area of the biggest dot in the nucleus divided by the area of the nucleus
Location-related variables	
location index	Index, calculated as the distance between the dot center and the center of the nucleus, divided by the nuclear radius less the dot radius. It is 0 for a dot in the center of the nucleus and 1 for a dot at the nuclear border.
percentage of central dots	The percentage of dots, the location index of which is lower than 0.2
percentage of marginal dots	The percentage of dots, the location index of which is above 0.8
minimal distance between dots in nucleus	The distance between the two closest dots in the nucleus
relative mean distance between dots in nucleus	The mean distance between dots in the nucleus (distances between every two dots in the nucleus are considered) divided by the maximal diameter of the nucleus
Number-related variables	
number of dots in nucleus	The number of AgNOR dots in the nucleus
percentage of nuclei with 1 dot	The percentage of nuclei with a certain number of dots differ from all other variables, as they are undefined for a single measured nucleus. These variables are only calculated for the whole evaluated microscopic slide.
percentage of nuclei with 2 dots	
percentage of nuclei with 3 dots	
percentage of nuclei with 4 dots	
percentage of nuclei with 5 and more dots	

Hufnagl et al. (1994). The segmentation of nuclei and AgNORs was automatically performed by the program and, in some cases, manually corrected. The measurements were performed on 100 nuclei per slide. In order to gain objective and reproducible values, we did not attempt to resolve smaller dots within large clusters (Howat et al., 1989; Ahiskali et al., 1995). All the slides were examined under the same magnification – about 0.12 μm/pixel. The operator of the morphometric analysis was not aware of the real histopathological results obtained.

A statistical analysis of the obtained data was performed by using ANOVA and the Neuman-Keuls' test. As ANOVA was not applicable for the groups of HA, HC and AC, because of the small size of those groups, non-parametric tests (Kruskal-Wallis' ANOVA test and Wald-Wolfowitz runs test) were used in those cases. Multiple regression analysis (forward stepwise model) was performed to evaluate the correlation between the PCNA index and AgNOR variables, as well as to assess the usefulness of measured variables in discrimination between analyzed pathological lesions. All the tests were performed with the use of 'Statistica for Windows' ver. 5.5 software (Statsoft, USA).

Results

The PCNA indexes observed in the examined groups of lesions are shown in Table 2 and the values of variables, describing AgNOR dots, are presented in Tables 3-5. We found that AC differed significantly from all the other examined groups with regard to the sizes of the nuclei (the biggest mean nuclear area), the number of dots in the nucleus (the highest in AC), and the percentage of both central and marginal dots (also the highest in AC). Significant differences between AC and other examined lesions were also found in the total area of dots in the nucleus (with the exception of HC) and in the relative mean distance between dots (with the exception of SA). In the AC group, more than 50% of the examined nuclei contained 4, 5 or more AgNOR dots, while in the groups of differentiated carcinomas, there were only 5 – 10% of such nuclei, and in benign lesions there were less than 5%: AC vs. well-differentiated carcinomas (FC and PC-F and PC-C), $p<0.0005$; AC vs. benign lesions (NG and SA and MFA), $p<0.0001$; well-differentiated carcinomas vs. benign lesions – $p<0.005$. On the other hand, the percentage of nuclei with 1 or 2 dots was low in AC – about 15% in total, while in all the other groups, it was above 65% (AC vs. other groups – $p<0.0002$).

Hürthle cell neoplasms (Fig. 1) differed from the other studied lesions with respect to many examined AgNOR features (Tables 3-5). HC revealed the highest values of the mean area of dots in the nucleus and the absolute and the relative area of the biggest dot in nucleus, and the second highest value of the total area of dots in the nucleus (after AC). Similarly, the

Table 2. PCNA indexes (percentages of immunopositive cells) in the examined groups of lesions.

GROUP	NG	SA	MFA	FC	PC-F	PC-C	HA	HC	AC
mean	44.5	41.9	47.4	60.9	57.1	74.2	45.1	73.2	83.9
SD	31.1	28.6	30.0	23.2	26.9	23.1	25.6	37.9	8.8
median	46.2	37.4	45.5	59.5	65.2	82.6	44.3	73.2	86.2

Table 3. Values of size-describing features of AgNOR dots and the nuclear area in examined lesions (mean ± SD).

GROUP	AREA OF NUCLEUS (μm^2)	MEAN AREA OF DOTS IN THE NUCLEUS (μm^2)	TOTAL AREA OF DOTS IN THE NUCLEUS (μm^2)	SD OF DOT AREA IN THE NUCLEUS (μm^2)	ABSOLUTE AREA OF THE BIGGEST DOT IN THE NUCLEUS (μm^2)	RELATIVE AREA OF THE BIGGEST DOT IN THE NUCLEUS
NG	34.14±8.35	2.60±0.55	4.29±1.06	0.33±1.19	2.86±0.65	0.091±0.021
SA	33.83±6.39	2.10±0.24[c]	4.09±0.33	0.38±0.04	2.40±0.23[i]	0.076±0.020
MFA	34.52±6.05	2.77±0.78	4.10±0.56	0.30±0.14	2.99±0.71	0.093±0.030
FC	38.83±5.02	2.79±0.62	5.10±0.87[a]	0.47±0.13[a]	3.18±0.62	0.088±0.025
PC-F	42.86±9.84[a]	2.61±0.54	4.94±0.89[a]	0.46±0.14[h]	3.01±0.50	0.076±0.024
PC-C	39.78±11.92	2.70±0.46	4.26±0.56[e]	0.40±0.23	3.03±0.46	0.087±0.022
HA	34.20±9.65	3.59±1.27[d]	4.40±0.96	0.16±0.21	3.72±1.16[d]	0.119±0.040
HC	39.12±5.19	5.30±0.81[b]	6.43±0.23[f]	0.32±0.32	5.55±0.56[b]	0.149±0.008[j]
AC	56.76±15.57[b]	1.77±0.41	7.12±1.87[g]	0.50±0.23	2.36±0.88	0.049±0.031

Letters denote statistical significance (some levels of significance have been rounded up for the sake of clarity): a, $p<0.05$ vs. NG, SA and MFA; b, $p<0.05$ vs. others; c, $p<0.05$ vs. NG, MFA, FC and PC-C; d, $p<0.05$ vs. SA and AC; e, $p<0.05$ vs. FC; f, $p<0.02$ vs. others except AC; g, $p<0.01$ vs. others except HC; h, $p<0.05$ vs. MFA; i, $p<0.05$ vs. NG, MFA, FC, PC-F and PC-C; j, $p<0.05$ vs. others except HA.

corresponding values in the HA group were higher than those in other benign lesions. Hürthle cell neoplasms also showed the highest percentage of nuclei with a single dot (nearly twice as high vs. the other examined lesions and several times higher than that in the AC group). HA showed the second highest percentage of central dots (after AC).

For the evaluation of correlation between the PCNA index and AgNOR dots features, multiple regression analysis was performed. The AC cases were excluded from that analysis as they highly differed from all the other groups in values of numerous AgNOR variables and thus, they could have introduced a significant bias. Because the number of predictors (evaluated AgNOR dot features) was high in relation to the number of examined cases (and some of the predictors were strongly correlated with one another), the forward-stepwise model of regression analysis was used. The correlation coefficient R was 0.42 and the only significant predictor was the mean nuclear area (partial correlation 0.31; $p<0.01$), which actually is not a AgNOR dot feature. Among AgNOR variables, the highest correlation with the PCNA index was observed for the total area of dots in the nucleus (Pearson's coefficient r=0.28) (Fig 2). The analysis of the PCNA indexes in particular groups of lesions showed significant differences only between PC-C and NG, PC-C and SA, PC-C and MFA ($p<0.005$, $p<0.01$, $p<0.02$, respectively).

With respect to diagnosing follicular lesions – nodular goiter, follicular adenomas, and carcinomas (Fig. 3), as well as the follicular variant of papillary carcinoma – we found that PC-F showed a significantly higher mean area of nuclei than benign lesions (PC-F vs. NG, SA, MFA – $p<0.01$, $p<0.01$, $p<0.02$, respectively). Moreover, the total areas of dots in the nucleus in FC and PC-F were significantly higher than those in benign lesions (FC vs. NG, SA, MFA – $p<0.05$, $p<0.005$, $p<0.001$, respectively; PC-F vs. NG, SA, MFA – $p<0.05$, $p<0.01$, $p<0.005$, respectively). Standard deviation of

Table 4. Values of location-related features of AgNOR dots in examined lesions (mean ± SD).

GROUP	LOCATION INDEX	PERCENTAGE OF CENTRAL DOTS	PERCENTAGE OF MARGINAL DOTS	MINIMAL DISTANCE BETWEEN DOTS (μm)	RELATIVE MEAN DISTANCE BETWEEN DOTS
NG	0.69±0.51	0.05± 0.03	0.88±0.39	2.54±0.97	0.08±0.04
SA	0.85±0.12[a]	0.03±0.02[b]	1.14±0.23	2.86± 0.55	0.18±0.08[d]
MFA	0.70±0.08	0.04±0.03	0.81±0.34	2.50±1.18	0.07±0.04
FC	0.75±0.06	0.04±0.03	1.17±0.29	3.48±0.77[c]	0.10±0.03[e]
PC-F	0.68±0.05	0.07±0.03	0.95±0.38	2.89±0.96	0.10±0.05
PC-C	0.67±0.05	0.06±0.02	0.83±0.28	2.33±1.07	0.07±0.03
HA	0.53±0.08[a]	0.11±0.03[a]	0.45±0.55	1.28±1.85	0.03±0.05
HC	0.69±0.07	0.02±0.01	0.67±0.35	1.08±1.05	0.03±0.03
AC	0.73±0.14	0.16±0.08[a]	2.21±0.83[a]	2.68±1.37	0.18±0.08[f]

Letters denote statistical significance (some levels of significance have been rounded up for the sake of clarity): a, $p<0.05$ vs. others; b, $p<0.05$ vs. PC-F and PC-C; c, $p<0.05$ vs. others except PC-F and AC; d, $p<0.01$ vs. others except AC; e, $p< 0.05$ vs. MFA and PC-C; f, $p<0.05$ vs. others except SA and PC-F.

Table 5. Values of number-related features of AgNOR dots and nuclear area in examined lesions (mean ± SD).

GROUP	NUMBER OF DOTS IN THE NUCLEUS	PERCENTAGE OF NUCLEI WITH 1 DOT	PERCENTAGE OF NUCLE WITH 2 DOTS	PERCENTAGE OF NUCLEI WITH 3 DOTS	PERCENTAGE OF NUCLEI WITH 4 DOTS	PERCENTAGE OF NUCLEI WITH 5 OR MORE DOTS
NG	1.77±0.39	43.38±22.78	39.38±11.78	14.17±11.89	2.59±2.76	0.47±0.94
SA	2.06±0.31	22.29±11.89[b]	54.44±12.85[c]	19.25±12.96	3.21±4.42	0.79±0.96
MFA	1.65±0.41	48.46±26.07	38.97±15.56	11.24±12.26	1.22±1.86	0.11± 0.32
FC	1.99±0.39	31.01±17.91	44.92±6.92	18.69±11.68	4.48±4.04	0.89± 1.65
PC-F	2.12±0.58	31.84±23.73	35.61±7.67	23.29±15.43	6.99±6.68[e]	2.26±3.60[f]
PC-C	1.79±0.37	47.01±23.32	33.92±16.01	13.56±7.02	4.44±3.35	1.08±1.32
HA	1.39±0.56	72.46±36.91	18.62±21.29	7.18±13.07	1.23±2.48	0.49±0.57
HC	1.39±0.29	72.79±22.32	18.36±17.89	6.88±2.99	0.98±0.036	1.00±1.41
AC	3.77±1.23[a]	4.25±2.38[a]	10.56±8.61[d]	28.01±17.44	21.42±19.78[a]	36.03±32.56[a]

Letters denote statistical significance (some levels of significance have been rounded up for the sake of clarity): a, $p<0.01$ vs. others; b, $p<0.05$ vs. MFA, PC-C, HA and HC; c, $p< 0.05$ vs. others except FC; d, $p<0.05$ vs. NG, SA, MFA, FC, PC-F and PC-C; e, $p<0.05$ vs. NG and MFA; f, $p<0.05$ vs. MFA.

dot sizes in the nucleus, as observed in FC and PC-F, was similar to that found in AC and significantly higher than that found in benign lesions (FC vs. NG, SA, MFA – $p<0.05$, $p<0.05$, $p<0.01$, respectively; PC-F vs. MFA – $p<0.01$). It was also noted that the minimal distance between dots in the nucleus was significantly higher in the FC group than that in benign lesions (FC vs. NG, SA, MFA – $p<0.02$, $p<0.05$, $p<0.05$, respectively). Unfortunately, the ranges of values of the dot features, observed in the groups of follicular lesions were partially overlapping.

The power of analyzed AgNOR features and of the PCNA index to discriminate between malignant and benign lesions was assessed by multiple regression analysis including all the cases (except for AC) classified as either malignant (FC, PC, PC-F, and HC) or

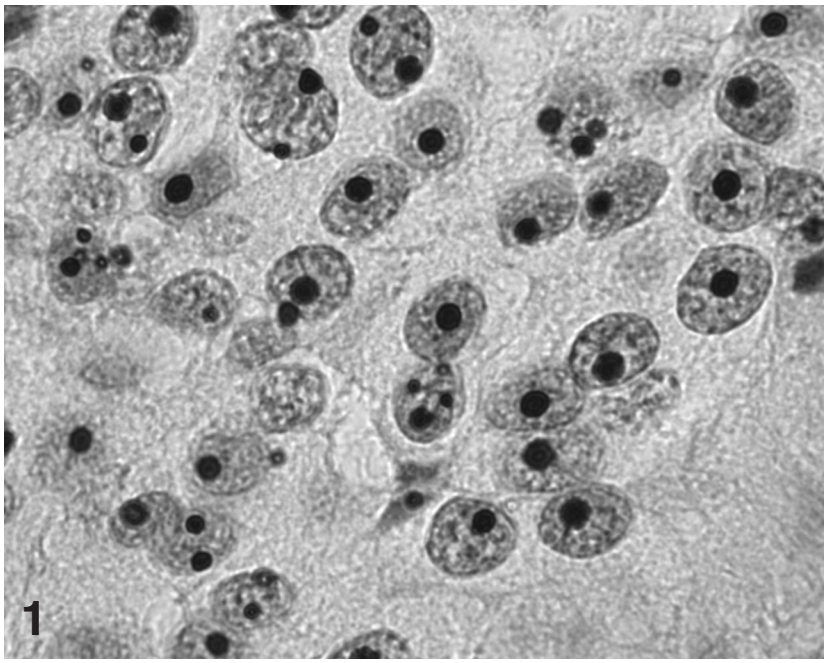

Fig. 1. Hürthle cell carcinoma, AgNOR staining. x 1,073

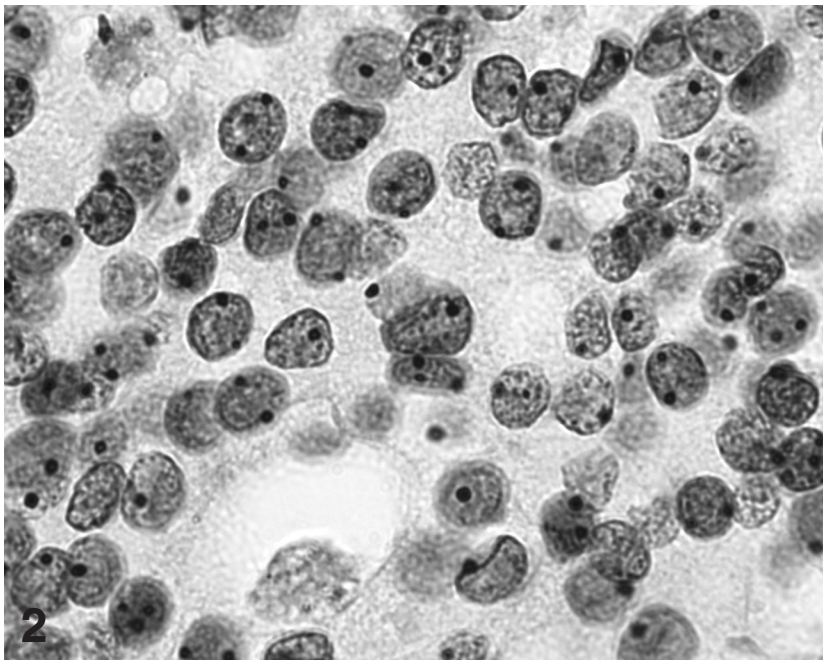

Fig. 2. Follicular carcinoma, AgNOR staining. x 1,073

benign lesions (SN, SA, MFA, HA). The correlation coefficient R was 0.56 and there were the following significant predictors: the mean nuclear area (partial correlation 0.29; $p<0.001$); the relative area of the biggest dot in the nucleus (partial correlation 0.28; $p<0.001$), and the PCNA index (partial correlation 0.27; $p<0.02$). All 3 significant predictors correlated positively with malignancy. When the assessment of AgNOR quantification usefulness was limited to diagnosing follicular neoplasms only - and a similar multiple regression analysis with cases included from only 2 groups: MFA and FC was performed - then the correlation coefficient R increased to 0.82 and 4 predictors significantly participated in the regression equation: total area of dots in nucleus (partial correlation 0.64; $p<0.001$); the mean area of dots in the nucleus (partial correlation -0.55; $p<0.01$); the percentage of nuclei with 4 dots (partial correlation 0.60; $p<0.01$); and the number of dots in the nucleus (partial correlation -0.48; $p<0.02$). The evaluation of the total area of dots in the nucleus seemed to be the most useful for discrimination between follicular adenomas and carcinomas; the assumption of 4.9 μm^2 as a cut-off value for that feature allowed a correct classification of 100% of MFA and 77% (10 of 13) of the cases of FC (Fig. 4). Also, all the cases of SA showed the mean total area of dots in the nucleus to be smaller than 4.9 μm^2. However, 18% (4 of 22) of NG cases would have been falsely classified as malignant lesions if such a cut-off value had been used. A detailed analysis of those 4 NG cases showed features of chronic thyroiditis in the nodular goiter in 2 of them. Unfortunately, 5 out of 13 PC-F cases would also have been misclassified as benign lesions.

Discussion

AgNOR analysis is a method for routine assessments of cell proliferation rapidity (Öfner et al., 1992; Trere, 2000; Canet et al., 2001). However, the results of AgNOR measurement are significantly influenced by several factors. First of all by the staining procedure (incubation time, temperature) and the method of evaluation (manual counting vs. image analysis) (Trere, 2000). In the past years, the silver-staining technique has been applied using varying conditions of staining, and quantitative analysis has been limited to easy-for-assessment features, evaluated without image analysis, namely to those describing the number of AgNOR dots in the nucleus. Such an assessment is relatively less objective and more susceptible to differences in staining protocol. This could have been the cause of the observed discrepancies between investigation results in assessing the usefulness of AgNOR analysis in thyroid diagnosis. Some investigators indicate a limited application for AgNOR counting in the diagnosis of thyroid follicular lesions because of score overlapping observed in adenomas and carcinomas (Nairn et al., 1988; Khan and Pandey, 1996; Zaczek et al., 1996). Others consider this method to be useful only as one of some additional techniques, facilitating the final diagnosis (Shem-Tov et al., 1994; Karmakar and Dey, 1995; Solymosi et al., 1996, Shechtman et al., 1998; Cor, 1999, Mehrotra et al., 1998, 2002; Lewy-Trenda and Bienkiewicz, 1999). Only Rüschoff et al. (1993) have demonstrated that the determination of AgNOR score (the percentage of cells with at least five AgNORs within one nucleolus) allowed for differentiation between malignant and benign follicular lesions. However, they applied a

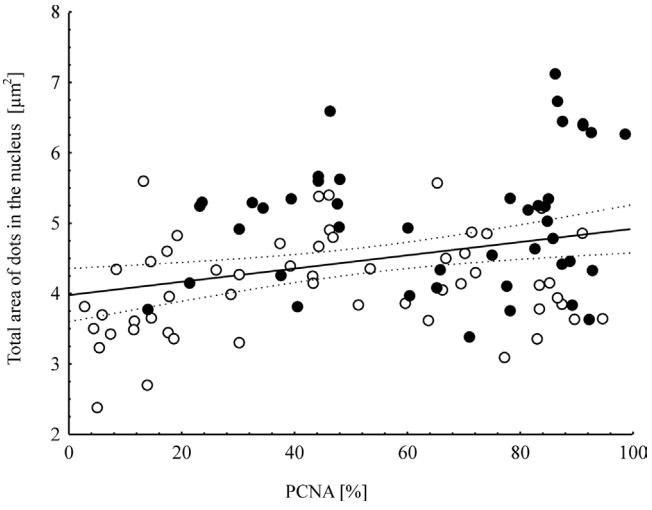

Fig. 3. Correlation between the PCNA index and the total area of AgNOR dots in the nucleus; Pearson's coefficient r=0.28 (all the examined lesions except for AC), black dots denote malignant lesions, white dots denote benign lesions.

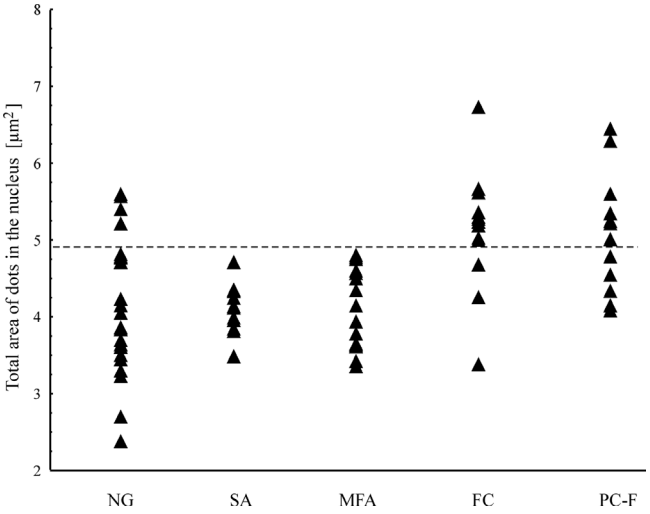

Fig. 4. Scattergram of the total area of AgNOR dots in the nucleus in the examined follicular lesions.

multistep procedure of preselecting cells for examination. As Solymosi et al. (1996), we believe that this may result in reproducibility failure. Rüschoff et al. (1993) did not find any statistically significant differences in AgNOR dot size between malignant and benign lesions. So far, apart from the investigations by Rüschoff et al. (1993) only in a few other papers on AgNOR dot analysis in thyroid lesions has computerized morphometric analysis been applied (Solymosi et al., 1996; Zaczek et al., 1996). In one of those investigations, Zaczek et al. (1996) did not find any statistically significant differences between adenomas and carcinomas of the thyroid in the mean area of dots. They did demonstrate, however, that the number of AgNOR dots correlated with the histological type of the thyroid lesion, e.g., single clusters of dots were observed in oxyphilic tumors, both malignant and benign. In the present study, these observations has been confirmed, and it has been demonstrated that Hürthle cell neoplasms differed from other follicular lesions in the variables describing AgNOR dot sizes (these lesions were usually characterized by the presence of a single and relatively large dot). On the contrary to Rüschoff et al. (1993) and Zaczek et al. (1996), and similarly to Solymosi et al. (1996), we have demonstrated the usefulness of features describing the area of AgNOR dots in diagnosing follicular lesions. The results obtained are particularly reliable, as the examined groups were homogenous in respect to the histological type of neoplasm. Solymosi et al. (1996) evaluated a group of adenomas in which there were above 40% of Hürthle cell adenomas; there was also an anaplastic carcinoma in the group with differentiated carcinomas. Moreover, they used a markedly longer time of staining (45 min), which is now not recommended.

Our present results suggest the relevance of the total area of dots in the nucleus for distinguishing between malignant and benign thyroid follicular neoplasm. This is concordant with our earlier report on AgNOR dot assessment in cytological specimens obtained by means of FNAB, in which an arbitrary borderline value of the total AgNOR area allowed separating between follicular adenoma and follicular carcinoma (Slowińska-Klencka et al., 2004). Unfortunately, in the case of the follicular variant of papillary carcinoma, the evaluation of the total area of dots in nucleus seems to be less useful.

The influence of coexisting chronic thyroiditis on the results of AgNOR dots quantification should be further investigated. In the present data, features of chronic thyroiditis were observed in 2 out of 4 cases of nodular goitre with a relatively big total area of dots in the nucleus. In our simultaneous investigation on the material from patients with chronic thyroiditis, increased values of features describing the area of AgNOR dots were observed (Slowińska-Klencka et al., 2003). Similar observations were reported by Solymosi et al. (1996).

The standard deviation of dot area in the nucleus was characterized by broader overlapping, when compared with the total area of dots in the nucleus. Similarly, the evaluation of the area of the biggest dot in the nucleus (reflecting the tendency of AgNOR dots to form clusters or large aggregates), has been shown to be less useful in the present data.

In our opinion, a single evaluation of the mean number of AgNORs per nucleus does not allow one to distinguish between follicular carcinoma, follicular adenoma, and nodular goiter, which is in agreement with the observations of Solymosi et al (1996) and of Zaczek et al. (1996). However, an evaluation of the percentage of nuclei with 4 or more dots may be helpful in certain cases of thyroid neoplasms. We observed significantly higher percentages of nuclei with 4 and 5 or more dots in the PC-F group, when compared with benign lesions.

In the present examination we found that the PCNA index was higher in malignant lesions than benign, but the correlation between the PCNA index and the variables, describing AgNORs was rather weak, if there was any. There have been discrepancies on the existence of such a correlation among published reports (Derenzini and Trere, 1994; Irazusta et al., 1998). Interestingly enough, we observed that the correlation of the PCNA index with the variables describing the area of AgNOR dots, when even very low, was higher than its correlation with the number of dots, which is in agreement with our previous findings in pituitary tumors (Klencki et al., 2001b). The evaluation of PCNA and other markers of proliferating cells were shown to be not very useful for the discrimination between follicular lesions, which is the main problem of the thyroid pathology (Vassko et. al., 1999). It seems that the qualitative computerized evaluation of AgNOR dots may turn out to be more promising in that area of investigation. It should be kept in mind that AgNOR staining is not an indicator for the number of growing cells but that it reflects the process of protein synthesis and the rapidity of the cell cycle. Moreover, the NOR silver-staining technique encompasses several advantages with respect to other methods aimed at improving the diagnostic ability of FNAB (Aogi et al., 1998; Gasbarri et al., 1999). This staining, being relatively simple and economical, can be performed on routinely-fixed cytological and histological samples. It is also possible to silver-stain destained slides (e.g. previously stained with hematoxylin and eosin), which eliminates the need for additional punctures when this method is applied for FNAB (Shechtman et al., 1998).

In conclusion, AgNOR assessment with computer-aided analysis of microscopic images is a promising diagnostic method, which may possibly facilitate the differentiation between malignant and benign follicular tumors. The results, as obtained by us, encourage further continuation of investigations on AgNOR quantitative analysis in thyroid diagnosis.

Acknowledgements. This work was supported in part by the Ministry of Scientific Research and Information Technology, grant Nr 4 P05B 030 18, in the years: 2000-2004.

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Accepted March 2, 2004