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AgNOR quantity as a prognostic tool in hyperplastic and neoplastic parathyroid glands

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Abstract Prediction of evolution of secondary hyperplasia and tumours of the parathyroid glands is still a problem in histopathology. To assess whether the quantity of silver-stained nucleolar organiser region (AgNOR) proteins might be used as a prognostic tool in parathyroid pathology, a standardised AgNOR analysis has been performed on 19 cases of parathyroid hyperplasia caused by secondary hyperparathyroidism (PH), 8 cases of adenoma (PA) and 10 cases of carcinoma (PC). Clinico-pathological data and follow-up information were available. On formalin-fixed and paraffin-embedded sections, the visualisation and quantification of AgNORs were achieved according to the 1995 guidelines of the Committee on AgNOR Quantification. Then, the mean area (square micrometres) of AgNORs per nucleus (NORA) was evaluated by means of an image analyser and specific softwares. After testing the normal distribution of NORA values, statistical parametric tests were utilised; Kaplan-Meier and Cox multivariate analyses were also performed. In parathyroid lesions, a progressive increase of mean NORA values was observed from PH (2.895 μm^2; SE 0.171) through PA (3.638 μm^2; SE 0.125) to PC (4.701 μm^2; SE 0.179); these differences were highly significant (P<0.001), although some degree of overlap was found among single NORA values. A significantly higher mean NORA value was revealed in PC with distant metastases than was noted in cases with no current clinical evidence of disease progression. Furthermore, a significantly (P<0.001) higher mean NORA value was encountered in the group of PH with recurrences (3.600 μm^2; SE 0.106) than in nonrecurrent PH (2.261 μm^2; SE 0.087). Multivariate analyses indicated that the NORA value was an independent prognostic parameter determining the risk of recurrence in PH. We suggest that AgNOR quantity may be a promising additional tool for predicting the biological behaviour of parathyroid lesions.

Keywords AgNORs · Standardised AgNOR analysis · Parathyroid tumour · Proliferation · Prognosis

Dedicated to my father on the occasion of his 80th birthday

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Introduction

The AgNOR technique allows visualisation at the light microscopic level of a set of argyrophilic nonhistone proteins localised in the nucleolar organiser region [9, 30]. These silver-stained nucleolar organiser region (AgNOR) proteins are associated with ribosomal genes, and their quantity has been demonstrated to be strictly related to the rate of cell proliferation [8, 11, 12, 24].

In histopathology, analysis of the interphase AgNOR proteins has been utilised largely to differentiate preneoplastic and neoplastic lesions [7, 10, 19, 20, 34], although the diagnostic utility of the AgNOR technique has been limited by the overlap of AgNOR values between benign and malignant neoplasms [10, 22]. In recent years, studies on this histochemical method have shown the prognostic value of AgNOR amount as an independent variable, able to predict the recurrences and/or the overall survival in various kinds of malignancies [3, 4, 8, 14, 16, 27, 28, 29, 31]. However, the AgNOR quantity has also been found to be increased in the active phase of nonneoplastic diseases [19, 34] and in regenerating cells [35]. Moreover, the introduction of international guidelines for AgNOR analysis allowed us to investigate the proliferation rate in routinely processed archival material in a reproducible manner [26].

In parathyroid pathology, few studies based on a nonstandardised AgNOR procedure have been performed exclusively for diagnostic purposes [6, 18]. The aim of

the present study was to perform a standardised AgNOR analysis in hyperplastic and neoplastic parathyroid lesions to assess its prognostic role, which has not so far been evaluated.

Materials and methods

Thirty-seven parathyroid samples were collected from the pathology file of the University of Turin, including 19 cases of secondary parathyroid hyperplasia (PH) caused by chronic renal failure (10 male, 9 female; mean age 50.1 years, ranging from 22 to 70 years), 8 cases of parathyroid adenoma (PA; 3 male, 5 female; mean age 50.6 years, ranging from 18 to 67 years) and 10 cases of parathyroid carcinoma (PC; 3 male, 7 female; mean age 47.2 years, ranging from 21 to 65 years). Diagnostic criteria were those currently accepted for hyperplasias (at least three glands enlarged and weighing more than 60 mg) and adenomas (a single nodule weighing more 60 mg, with a microscopic pattern of encapsulated growth consisting of monomorphous cells in the absence of fatty stroma). The classification proposed by Ellis [13] was used to distinguish the architecture of hyperplastic glands (nodular vs diffuse growth pattern). For carcinomas, clinical and morphological malignancy and morphological signs of malignancy only were considered separately; the presence of thick fibrous bands within the tumour, mitotic activity, extraparathyroid spread and capsular or vascular invasion were regarded as morphological markers of malignancy. The main clinico-pathological, laboratory and follow-up data recorded in the PH, PA and PC groups are summarised in Tables 1, 2, and 3, respectively. To identify cases with recurrent hyperparathyroidism, elevations over 10.5 mg/dl or 300 pmol/l of serum calcium and parathyroid hormone, respectively, were considered threshold levels.

All surgical samples were fixed in 10% neutral formalin for 12–24 h at room temperature and then embedded in paraffin at 56°C. From each tissue block, two consecutive 4-μm-thick sections were cut and mounted on silane-coated glasses, then dewaxed in xylene, rehydrated in graded ethanols and submitted to haematoxylin and eosin (H&E) staining and the AgNOR technique according to the guidelines of the Committee on AgNOR Quantification [26]. In detail, sections were immersed in sodium citrate buffer (pH 6) and incubated in a wet autoclave at 120°C (1.1–1.2 bar, at sea level) for 20 min and then allowed to cool down to 37°C. Subsequently, slides were immersed in a freshly prepared silver-staining solution containing one part by volume of 2% gelatin in 1% formic acid and two parts of 25% aqueous silver nitrate solution, at 37°C in a thermostatically controlled environment for 11 min. The reaction was then stopped by washing the slides with double-distilled deionised water to remove unwanted silver precipitates. Finally, all sections were dehydrated in ascending ethanols, clarified in xylene and mounted with a synthetic medium (Permount).

Table 1 Clinico-pathological data, serum levels of parathyroid hormone, phosphorus and calcium and corresponding mean NORA values in cases of parathyroid hyperplasia (*M* male, *F* female, *NED* no evidence of disease, *rHPT* recurrent hyperparathyroidism, *PTH* parathyroid hormone, *P* phosphorus, *Ca* calcium)

Case	Sex	Age	Clinical course	Growth pattern	Weight (mean g)	Serum PTH (pmol/l)	Serum P (mg/dl)	Serum Ca (mg/dl)	NORA (mμ²)
1	M	35	NED	Diffuse	2	1000	6	9.6	2.086
2	M	55	NED	Diffuse	1.2	1000	4.5	10.1	2.470
3	M	55	NED	Nodular	2.8	80	3.6	10.2	2.903
4	M	58	NED	Nodular	0.7	1000	7.5	10.4	2.093
5	M	70	NED	Nodular	3.7	1000	6.4	10.7	1.894
6	F	62	NED	Diffuse	0.7	90	3	10.5	2.150
7	F	59	NED	Nodular	6.2	658	6.6	11.6	2.249
8	F	56	NED	Nodular	1.7	80	3.5	10.4	2.374
9	F	38	NED	Diffuse	1.1	360	6	9	2.237
10	M	22	NED	Nodular	1.5	220	6	10.2	2.157
11	F	38	rHPT	Nodular	1.35	650	4.9	13	4.081
12	F	47	rHPT	Nodular	3.7	3500	5.4	11.7	3.090
13	M	55	rHPT	Nodular	1.9	480	7	9.5	4.044
14	F	52	rHPT	Nodular	2.6	260	5.3	11.8	3.423
15	M	50	rHPT	Nodular	4.1	1500	5.7	8.6	3.509
16	F	52	rHPT	Nodular	1.7	1000	6.5	11	3.722
17	M	35	rHPT	Diffuse	0.6	430	4.1	10.8	3.602
18	M	52	rHPT	Nodular	1	1000	5	10.4	3.575
19	F	60	rHPT	Nodular	7.6	200	6	11	3.354

Table 2 Clinico-pathological data, serum levels of parathyroid hormone, phosphorus and calcium and corresponding mean NORA values in cases of parathyroid adenoma (*na* not available)

Case	Sex	Age	Clinical course	Size (mean cm)	Weight (mean g)	Serum PTH (pmol/l)	Serum P (mg/dl)	Serum Ca (mg/dl)	NORA (mμ²)
1	F	65	NED	0.8	0.24	138	0.9	11	3.663
2	F	43	NED	2	2.6	335	1.6	12	4.204
3	M	37	NED	1.5	1	452	6.6	13	3.655
4	F	63	NED	1	0.4	63	2.1	11	3.924
5	F	57	NED	1	0.4	119	0.8	11	3.304
6	M	18	NED	1.5	4	690	2.2	15	3.842
7	F	55	NED	3	3	180	0.7	13	3.138
8	M	67	NED	4.5	na	3	2.1	11	3.371

Table 3 Clinico-pathological data, serum levels of parathyroid hormone, phosphorus and calcium and corresponding mean NORA values in cases of parathyroid carcinoma (*DOD* died of disease, *DOC* died of other cause)

Case	Sex	Age	Clinical course	Size (mean cm)	Weight (mean g)	Serum PTH (pmol/l)	Serum P (mg/dl)	Serum Ca (mg/dl)	NORA ($m\mu^2$)
1	M	43	NED	2	2.5	140	5.4	12	4.930
2	F	45	DOD	1.5	na	na	3.8	13.5	5.710
3	M	42	NED	1	1	637	1	13.4	5.207
4	M	40	DOC	4	10	300	1.5	16.6	5.084
5	F	64	NED	4	12	1000	1.6	12.4	4.061
6	F	21	NED	3	7	130	2.1	13.1	4.525
7	F	56	NED	3	7	554	2.1	14.5	4.683
8	F	55	DOC	1.5	2	360	3.9	9.6	4.795
9	F	41	NED	2	1.3	330	1.2	11.4	4.035
10	F	65	NED	3	5	95	1.8	12	3.978

The quantification of AgNORs was performed by an image analysis system consisting of an optical Leitz microscope fitted with a single chip colour CCD video camera (Ikegami ICD-840PDC, Ikegami Tsushinki, Tokyo, Japan) having a resolution of 460×420 (horizontal × vertical) TV lines, a colour monitor and an image processing unit installed in a 486/33 MHz processor-based personal computer. For each slide examined, microscopic fields representative of the lesions were assessed, excluding areas in which regressive changes, frank necrosis and technical artefacts were present, and compared with the corresponding H&E-stained section. Furthermore, the peripheral infiltrating portion of neoplasias was preferred. The mean area (square micrometres) of AgNORs per cell (NORA) was evaluated on chief cells on one focal plane with a ×40 objective lens in at least 100 nuclei per specimens (mean 123); specific softwares, IM 5200 (Microscience) and AgNOR (Immagini e Computer, Rho-Milan, Italy), were utilised to determine mean NORA values per cell and per case, respectively.

After we had tested the normal distribution of NORA values in all three groups of patients by Kolmogorov-Smirnov test, parametric tests were applied. A statistical descriptive analysis was performed for each clinico-pathological parameter (mean value and standard error of the mean). Differences among categories were assessed by analysis of variance and the Newman-Keuls' test, while correlations between continuous parameters were investigated by Pearson's *r* correlation coefficient. Recurrence and survival analyses were performed by the Kaplan-Meier method and for the comparison of the curves, the Mantel-Cox log-rank test was used. Finally, a multivariate analysis (Cox regression model) was utilised to determine the independent effect of each prognostic variable. A *P*-value less than 0.05 was considered statistically significant.

Results

All silver-stained specimens of parathyroid tissue showed that an adequate staining intensity was homogeneously present throughout the whole section. In nuclei of hyperplastic glands, and also within nucleoli, the AgNORs were clearly distinguishable as black dots (Fig. 1a); an increase of extranucleolar silver dots scattered throughout the nucleus was found in adenomatous (Fig. 1b) and carcinomatous (Fig. 1c) cells, where the AgNORs were often clustered in irregularly shaped collections. Mature lymphocytes, whenever present, exhibited a single round, centrally localised AgNOR.

Mean NORA values concerning hyperplastic, adenomatous and carcinomatous parathyroid samples are reported in Tables 1, 2 and 3, respectively. A progressive increase in the mean NORA value was noted from PH (2.895 μm^2; SE 0.171) through PA (3.638 μm^2; SE 0.125) to PC (4.701 μm^2; SE 0.179) (Fig. 2); these differences were highly significant (P=0.001).

A higher mean NORA value was found in the PH group showing a nodular pattern of growth (3.033 μm^2; SE 0.201) compared with the diffuse pattern (2.509 μm^2; SE 0.281), although this difference did not reach statistical significance. Moreover, no significant correlations were found among mean NORA values and other clinico-pathological parameters. The follow-up ranged from 24 to 96 months (mean 76.4); 9 patients who had recurrences showed significantly higher (P=0.001) mean NORA values (3.600 μm^2; SE 0.106) than 10 disease-free patients (2.261 μm^2; SE 0.087). Moreover, using the mean NORA value encountered in hyperplasias as a cut-off point, cases with NORA values greater than 2.895 μm^2 always developed recurrences, as shown by Kaplan-Meier curves (Fig. 3). In addition, according to Cox multivariate analysis, the NORA parameter represents the best independent predictor of recurrence in PH (Table 4).

In PA, no significant relationships were evident between mean NORA values and age, sex, size or weight of glands, serum PTH, calcium and phosphorus levels. No further evidence of disease was observed in any of these patients.

In PC, mean NORA values were unrelated to patient's age, sex, serum data and size or weight of tumour. The highest NORA values were encountered in 4 cases of PC with both clinical and morphological signs of malignancy; moreover, this group showed a significantly higher (P=0.005) mean NORA value (5.233 μm^2; SE 0.169) than was found in the group of patients with morphological signs of malignancy only (4.346 μm^2; SE 0.148). The follow-up ranged from 12 to 180 months (mean 80.4). Only 1 patient died of disease, and this patient's tumour had the highest NORA value. Seven patients have no evidence of disease and 2 have died of unrelated causes.

When all patients were subdivided into five groups, PH with recurrence, PH without recurrence, PA and PC with morphological signs of malignancy, and PC with clinically aggressive disease, a highly significant *P*-value (<0.001) was found, except when recurrent PH and PA were compared, since an evident overlap of single mean NORA values was encountered.

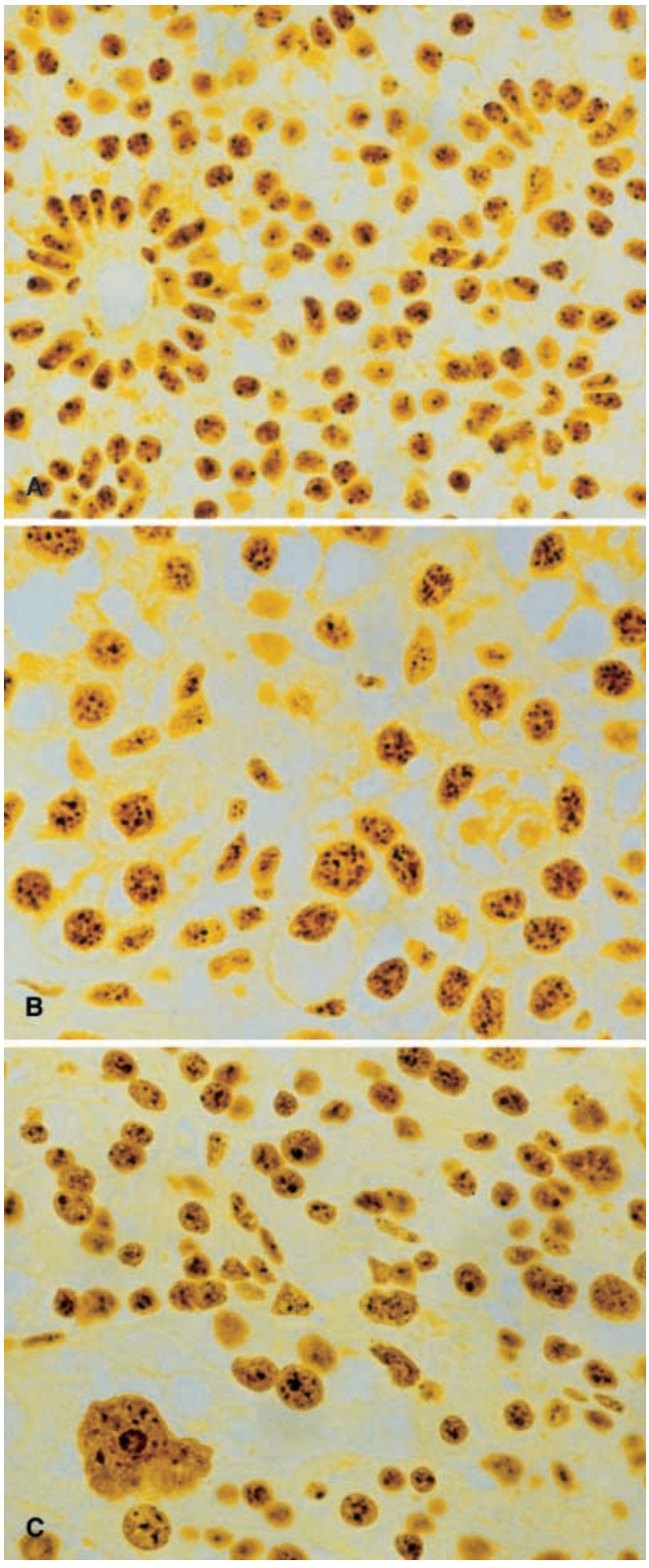

Fig. 1A–C AgNOR method. **a** Few single black dots are seen in the nucleus of hyperplastic parathyroid elements. ×200 **b** Scattered silver dots are increased in adenomas. ×200 **c** AgNORs frequently clustered in irregularly shaped collections inside carcinomatous cells. ×200

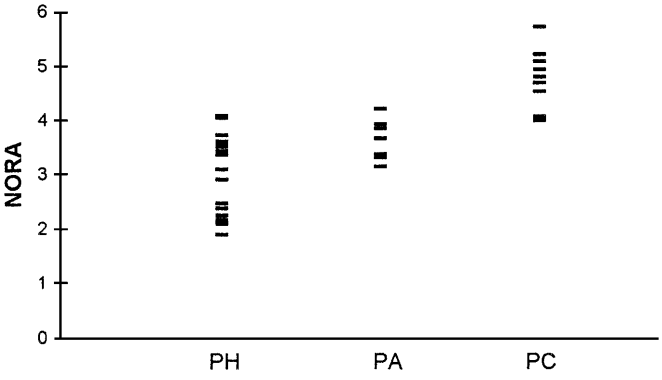

Fig. 2 The scattergram shows the distribution of mean NORA values of each case in hyperplastic, adenomatous and carcinomatous parathyroid samples

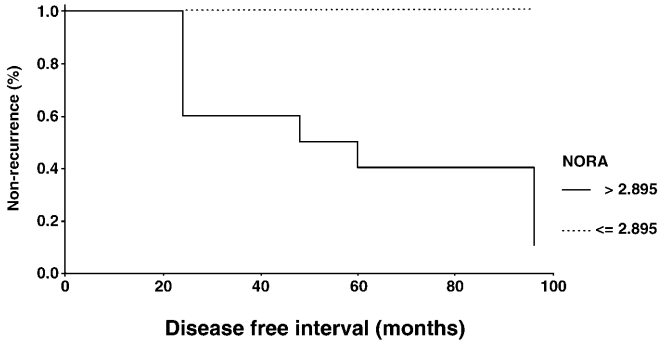

Fig. 3 Kaplan-Meier recurrence curves of PH patients with low and high NORA values

Table 4 Multivariate recurrence analysis by Cox regression model in cases of parathyroid hyperplasia (β regression coefficient, *SE* standard error, *Exp(β)* ratio of risk)

Variable	β	SE	Exp(β)	*P*-value
NORA	2.182	0.699	8.868	0.001
PTH	0.001	4.115	1.001	0.012

Discussion

In parathyroid pathology, the differential diagnosis between benign and malignant tumours and the prediction of recurrences in PH cases are still open questions. Many morphological parameters, such as presence of thick fibrous bands within the tumour, capsular penetration, blood vessel invasion, infiltration of surrounding tissues and mitotic count, have been proposed as hallmarks of malignancy [1], although only the presence of distant metastases allows a reliable diagnosis of parathyroid carcinoma [15]. Moreover, it is well known that recurrences of PH have been reported in spite of a radical and correct surgical approach. Therefore, many parameters have been claimed to be predictive of recurrence, such as total parenchymal mass [5], nodular pattern of growth [13, 21, 33], absence of fat cells [13, 21], and high mitotic index

[13, 17]. In recent years, we have shown that a high Ki-67 proliferative fraction is an useful indicator of malignant tumour growths [1] and of increased risk of recurrence in PH [2], even if the fixation procedures or the duration of block storage may interfere with the Ki-67 antigen preservation [2].

Few AgNOR studies have been carried out to discriminate between benign and malignant parathyroid tumours [6, 18], and the prognostic relevance of the AgNOR method in these conditions has not been considered; moreover, a nonstandardised AgNOR analysis based exclusively on a subjective counting procedure of the amount of intranuclear silver dots has been utilised [6, 18]. Nevertheless, in the above-mentioned reports, the AgNOR numbers in PC were significantly greater than those observed in either PA or PH [6, 18]. However, to our knowledge, the capability of AgNOR analysis to identify recurrent cases of PH has been not yet investigated. The present study is the first in which the parathyroid pathology has been examined by standardised AgNOR analysis in accordance with the guidelines of the Committee on AgNOR Quantification [26]; in particular, wet-autoclave pretreatment allowed us to obtain a consistently high quality of staining of single-interphase AgNORs, irrespective of the duration of formalin fixation and archival storage, similar to that reported elsewhere [23]. Moreover, the AgNOR area has been quantified by an image analyser system, which is more objective and free of observer bias than counting AgNORs by eye [23, 26, 32]. Furthermore, compared with previous studies [6, 18], ours involved a larger number of patients with available follow-up data; the additional cases of PC and PH were characterised and subdivided on the basis of the clinically aggressive behaviour or in relation to the event of recurrences, respectively.

In our case series, a highly significant difference in the AgNOR quantity among PH, PA and PC was found, suggesting a progressive increase in the proliferation rate from hyperplastic nodules to benign and malignant neoplasms. Nevertheless, the degree of overlap encountered among single NORA values strongly reduces the diagnostic impact of AgNOR analysis; this finding is in contrast with the results reported in an earlier paper [18], which did not show any overlap between AgNOR counts relative to PC and PA or PH, probably due to the low number of PC cases studied. Interestingly, we encountered higher NORA values in PC with both clinical and morphological signs of malignancy than in PC with morphological signs of malignancy only. Moreover, the highest NORA value was observed in the 1 patient who subsequently died of the disease.

In both neoplastic and hyperplastic parathyroid lesions, no significant relationships were found between mean NORA values and age, sex, size or weight of glands, and serum PTH, calcium and phosphorus levels. Nevertheless, a trend towards correlation between PH group showing a nodular pattern of growth and higher mean NORA values has been recognised. This is not surprising, since the nodular pattern of growth has been also associated with an elevated Ki-67 proliferative activity [2]. A wide range of NORA values has been found in PH, with higher values in patients developing recurrences than in disease-free patients. Moreover, in this latter group, the highest NORA value was 2.903 μm^2 without any overlap with other NORA values present in categories of recurrent PH, adenoma and carcinoma. On the contrary, an evident overlap between single NORA values for adenomas and recurrent hyperplasias has been noted, suggesting that only these last two parathyroid lesions have a similar proliferation activity. Therefore, the AgNOR analysis allows easy identification of cases of simple nonrecurrent PH on the basis of their low proliferation rate. Moreover, for better prediction of the risk of recurrence, we have used the mean NORA value encountered in cases of PH as a cut-off level; in this way, a significant difference became recognisable on comparison of the two different Kaplan-Meier curves. Finally, when clinico-pathological and laboratory data were utilised as covariates in Cox multivariate analysis as well as NORA, the NORA parameter emerged as the best independent predictor of recurrences in PH.

In conclusion, in light of the above considerations, we assert that the AgNOR quantity may be a promising additional tool for determination of the prognosis of parathyroid lesions.

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