Relationship of AgNOR counts and nuclear DNA content to survival in patients with parathyroid carcinoma

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Abstract

The aim of this study was to evaluate the usefulness of DNA flow cytometry to determine tumor nuclear DNA index (DI), and nucleolar organizer region protein counts visualized by the argyrophil (AgNOR) technique, in confirming diagnosis and predicting clinical outcome of patients with parathyroid carcinoma (PC). We reviewed paraffin-embedded tissue sections, from 15 patients (median age 63 years, range 30–68 years) with PC who died of the disease, which were randomly compared with tissue sections from 15 age- and sex-matched patients with parathyroid adenoma (PA). The proliferative activity in parathyroid tumours as detected by DI and AgNOR counts was evaluated in all specimens. Both DI (1.37 ± 0.33 vs 1.0 ± 0.1) and AgNOR (3.01 ± 0.31 vs 1.54 ± 0.35) counts were higher ($P < 0.001$) (Student's *t*-test) in patients with PC than in those with PA. Diploid (DI=1), aneuploid (DI > 1) and hypoploid (DI < 1) neoplasms were found in 11 (PC=4, PA=7), 14 (PC=11, PA=3) and five (PC=0, PA=5) patients respectively. The average postoperative survival in patients with PC was 46.9 ± 37.4 months (range 21–146 months). The survivals of patients with aneuploid ($n = 11$) and diploid ($n = 4$) PC were 74.0 ± 58.1 and 34.1 ± 18.4 months ($P=0.21$) respectively. There was a significant relationship between DI and AgNOR counts ($R=0.69$, $P < 0.01$), but no correlation was found between survival and both DI ($Rho=0.17$, $P = 0.55$) and AgNOR counts ($Rho=0.26$, $P = 0.35$). Moreover, there was no correlation ($P = NS$) between the main preoperative biochemical parameters and survival. In conclusion, DI and AgNOR are useful in confirming the diagnosis of PC, but they are of little value in predicting the clinical outcome of patients with PC.

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Introduction

Primary hyperparathyroidism is a common disease. Its estimated prevalence is up to 4 per 1000 in women aged over 60 years, and its incidence is approximately 42 per 100 000 (Shoback *et al.* 2001). In patients with primary hyperparathyroidism, abnormal parathyroid glands have been characterized as being hyperplastic, adenomatous or malignant, but parathyroid carcinoma (PC) is an uncommon finding, accounting for only 1–2% of cases (Arnaud 1994, Shane 2001, Shoback *et al.* 2001). The National Cancer Data Base (NCDB) reported that the mean age at

diagnosis of patients with PC was 55 years (range 14–88 years), and the median tumour size was 3.3 cm (Hundal *et al.* 1999). Unfortunately, the histopathological distinction between adenomas and carcinomas, as well as between adenomas and hyperplasia, on frozen-section examination is difficult or impossible, and often no reliable signs of malignancy are detectable even on final pathology (Arnaud 1994, Shane 2001). None of the classical criteria suggested in 1973 by Shantz and Castleman are pathognomonic of PC, since several of such features may be found in parathyroid adenomas (Shantz & Castleman 1973,

Levin *et al.* 1987, Shoback *et al.* 2001). Thus, further histological techniques have been proposed with the aim of diagnosing malignancy and suggesting the biological behaviour of patients with PC.

The aim of this study was to evaluate the usefulness of DNA flow cytometry to determine tumour nuclear DNA index (DI), and nucleolar organizer region (NOR) protein counts visualized by the argyrophil (AgNOR) technique, in confirming diagnosis and predicting clinical outcome of patients with PC.

Materials and methods

Paraffin-wax-embedded tissue sections from 15 patients (11 men and four women, median age 63 years, range 30–68 years) with confirmed PC were randomly compared with tissue sections from 15 age- and sex-matched patients with parathyroid adenoma (PA). Overall, the 15 patients with PC had undergone 32 operations. The greatest diameter of the tumour (size) was measured by the pathologist in each patient. The histopathological characteristics of all carcinomas, according to Bondeson *et al.* (1993), are reported in Table 1. A low (5–25 mitoses per high-power fields [HPF]) mitotic activity ($\times 40$ objective, $\times 10$ oculars) was found in seven cases, while five tumours showed high (> 25 mitoses per 50 HPF) mitotic activity.

Fourteen patients developed local recurrence, and the tumour caused lung metastasis in nine patients and multiple metastases (lung, bone and mediastinum) in the others. The proliferative activity in parathyroid tumours, as detected by AgNOR counts and DI, was evaluated in all specimens.

All patients died of the disease, 21–146 months (median 29 months) after the first operation.

NOR silver staining and AgNOR measurement

Sections of 3 μ m, cut from blocks routinely processed in paraffin wax, were dewaxed in xylene and ethanol, post-fixed for 30 min in 3:1 absolute ethanol–acetic acid solution and rehydrated (Ploton *et al.* 1986, Trerè *et al.* 1991). AgNOR staining was performed with a solution obtained by dissolving gelatine in aqueous formic acid (final concentration 2 g/dl) mixed with a 50 g/dl aqueous silver nitrate solution (Ploton *et al.* 1986). Silver staining was done at room temperature. The numbers of AgNOR, visualized as distinct black dots, were counted by means of an image-analysis system, using a graphic monitor (Crocker & Nar 1987). AgNOR counting was performed on 100 randomly selected nuclei of parathyroid cells, using a $\times 100$ oil-immersion objective lens (Kanematsu *et al.* 1997). The mean number of AgNORs per nucleus in each specimen was recorded in a database.

Table 1 Histopathological characteristics of all parathyroid carcinomas

Parameter	Cases
Trabecular growth pattern	12 (80.0%)
Mitotic figures	12 (80.0%)
Areas of fibrosis	11 (73.3%)
Spindle cells	10 (66.7%)
Nuclear atypia	10 (66.7%)
Macronucleoli	7 (46.7%)
Pleomorphism	6 (40.0%)
Large nucleoli	4 (26.7%)
Coagulation necrosis	5 (41.7%)
Blood vessels invasion	2 (13.3%)

Nuclear DNA content

Sections of 30 μ m were deparaffinized as described above. As suggested by Obara *et al.* (1990), disaggregation was obtained by using 0.5% pepsin solution at pH 1.5. The sample was filtered (40 μ m nylon mesh), and the single-cell nucleus suspension was resuspended in a solution containing propidium iodide and ribonuclease A. Flow cytometer measurement was used, and 20 000 nuclei were read for each sample (Obara *et al.* 1990, Bosci *et al.* 1998). The DNA content was interpreted as diploid if only one G0/G1 peak was present (DI=1.0), and the DI was calculated as the ratio of the peak channel number of the aneuploid G0/G1 peak to the peak channel number of the diploid G0/G1 peak (August *et al.* 1993).

Statistical analysis

Differences between means (that is, biochemical parameters) were tested by unpaired Student's *t*-test, while the Fisher exact test was used for fractions. The Spearman rho calculation was used to evaluate the linear relationship between age, biochemical parameters and size of the tumour and survival. The logistic regression model was used to identify factors independently predicting the clinical outcome. In the multivariate analysis, only the variables found to be significant in the univariate analysis were used.

Median survival time (MST) was calculated by the Kaplan–Meier method, while differences in MST were tested for statistical significance by the log-rank test. The differences were considered significant at a *P* value of < 0.01 .

Results

Table 2 reports the main clinical, biochemical, and pathological data from patients with either PC or PA. In patients with PC, the average survival was 46.9 ± 37.4

Table 2 Main clinical, biochemical, and pathological data

Sex	Parathyroid carcinomas							Parathyroid adenomas							
	Age (years)	Ca (mmol/l)	PTH (ng/l)	ALP (U/l)	Size (mm)	AgNOR	DI	Sex	Age (years)	Ca (mmol/l)	PTH (ng/l)	ALP (U/l)	Size (mm)	AgNOR	DI
M	30	3.43	NA	156	25	3.12	1.5	M	30	3.03	121	125	30	1.23	1.0
M	41	4.05	518	210	45	3.11	1.8	M	41	3.05	125	88	18	1.25	1.0
F	43	3.09	312	210	25	3.01	1.8	F	43	2.97	221	210	25	1.95	1.1
M	51	3.88	990	450	20	3.29	1.9	M	51	3.11	210	200	15	2.05	1.0
M	56	4.12	313	411	40	3.21	1.7	M	56	3.57	198	112	20	1.97	1.1
F	59	3.33	124	107	20	2.85	1.5	F	59	2.79	191	116	15	1.26	1.1
M	66	3.02	107	146	15	3.11	1.0	M	63	2.89	102	107	23	1.48	1.0
M	65	2.65	NA	96	15	3.14	1.2	M	65	2.74	98	83	17	1.26	0.9
M	68	3.32	95	106	35	3.10	1.0	M	68	3.00	129	106	30	1.23	1.0
M	68	3.21	NA	155	25	3.12	1.1	M	67	2.79	142	142	25	1.86	1.0
F	70	3.09	NA	188	25	1.98	1.0	F	70	3.12	96	236	15	1.48	0.9
M	72	4.25	NA	352	30	3.25	1.2	M	72	2.75	212	98	25	2.18	0.9
M	74	2.62	282	133	30	3.10	1.3	M	73	2.70	78	110	20	1.24	0.8
M	76	2.92	291	115	40	2.90	1.5	M	75	2.75	71	119	18	1.28	0.9
F	78	2.90	126	107	40	2.86	1.0	F	78	2.85	137	127	15	1.43	1.0

M=male, F=female, NA=not available, Ca=preoperative serum calcium (mmol/l; normal values: 2.10–2.55), PTH=preoperative serum parathyroid hormone (ng/l; normal values: 10–65), ALP=preoperative serum alkaline phosphatase (U/l; normal values: 53–141); size=greatest diameter of the parathyroid tumour (mm) at first operation; AgNOR=AgNOR counts; DI=DNA index.

Figure 1 Cumulated proportions of survival according to the Kaplan–Meier method in male and female patients with parathyroid carcinoma.

months (range 21–146, median 29 months), and the cumulated proportions of survival, according to the Kaplan–Meier method, was different between men and women (Fig. 1), but the difference was not significant (log-rank test, $P = 0.09$).

In the univariate analysis, the mean serum calcium levels and the mean size of the removed parathyroid tumours were significantly ($P < 0.01$) higher in patients with PC, while the mean serum alkaline phosphatase (ALP) and parathyroid hormone (PTH) levels did not differ ($P = \text{NS}$) between patients with PC and those with PA (Table 3). Both AgNOR counts and DI were significantly higher ($P < 0.01$) in patients with PC. The logistic regression showed that only AgNOR was the true

independent variable, and could correctly identify 29 out of 30 (96.7%) cases.

Diploid ($\text{DI}=1$), aneuploid ($\text{DI}>1$) and hypoploid ($\text{DI}<1$) tumours were found in 11 (PC = 4, PA = 7; $P = 0.34$), 14 (PC = 11, PA = 3; $P = 0.07$), and five (PC = 0, PA = 5; $P = 0.05$) patients respectively.

The survivals of patients with aneuploid ($n = 11$) and diploid ($n = 4$) PC were 74.0 ± 58.1 and 34.1 ± 18.4 months ($P = 0.21$) respectively. No correlation was found between survival and the main preoperative clinical and biochemical parameters (Table 4). There was a significant relationship between DI and AgNOR counts ($\rho = 0.69$, $P < 0.01$), but no correlation ($P = \text{NS}$) was found between survival and both AgNOR counts and DI (Fig. 2).

Table 3 Univariate analysis between patients with parathyroid carcinomas and adenomas. Values are expressed as mean $\pm$ standard deviation

Parameters	Carcinomas	Adenomas	P value
Age (years)	60.9 $\pm$ 14.2	60.7 $\pm$ 14.0	0.97
Size (mm)	28.7 $\pm$ 9.5	20.7 $\pm$ 5.3	0.009
Ca (mmol/l)	3.33 $\pm$ 0.52	2.94 $\pm$ 0.22	0.01
PTH (ng/l)	315.8 $\pm$ 271.3	142.1 $\pm$ 51.4	0.02
ALP (U/l)	190.5 $\pm$ 116.3	131.9 $\pm$ 46.1	0.08
AgNOR counts	3.01 $\pm$ 0.31	1.54 $\pm$ 0.35	<0.001
DNA index	1.37 $\pm$ 0.33	1.0 $\pm$ 0.1	<0.001

Table 4 Linear relationship (Spearman's rho) between survival (months) and age, main biochemical parameters, AgNOR counts and DNA index in patients with parathyroid carcinomas

Parameter	Rho	t (N–2)	P level
Age (years)	0.08	0.28	0.78
Size (mm)	0.01	0.03	0.97
Serum calcium (mmol/l)	0.09	0.03	0.97
PTH (ng/l)	0.13	0.36	0.73
ALP (U/l)	0.02	0.06	0.95
AgNOR counts	0.26	0.97	0.35
DNA index	0.17	0.61	0.55

Figure 2 Relationship between survival, AgNOR counts and DNA index in patients with parathyroid carcinomas.

Discussion

PC causes 0.6–5.2% of all cases of primary hyperparathyroidism (August *et al.* 1993, Favia *et al.* 1998). The NCDB reported 286 cases of PC between 1985 and 1995 (0.005% of the total NCBD cases), and approximately 390 cases were reported in the English literature from 1930 to 2000 (Hundhal *et al.* 1999, Shane 2001). The preoperative localization of such tumours is usually not difficult (Lumachi *et al.* 2001).

Neither tumour size nor lymph node status appear to predict survival in patients with PC, but the reported 5- and 10-year survival rates are approximately 85% and 50% (Shane 2001). The classic pathological features are not always present in all patients with PC, although 70% of patients may exhibit local invasion (Clayman *et al.* 2004). Moreover, recurrence is common, and persistent or recurrent disease may be observed at 1–20 years (Kebebew *et al.* 2001). Patients with PC should have lifelong follow-up, and different parameters should be considered to predict the biological behaviour of the tumour. Bondeson *et al.* (1993) showed a positive correlation between morphological variables such as fibrosis, necrosis, nuclear atypia and mitotic figures, and an aberrant DNA pattern demonstrated by image cytometry. August *et al.* (1993), reviewing nine patients with PC, found that four of the five patients with evidence of tumour aneuploidy died of metastatic disease, while the four patients with diploid

tumours were disease free at 1–8 years after the first operation. They concluded that DNA cytometry may help differentiate patients whose PC is likely to be indolent (diploid tumours) from those with a tumour (aneuploid) more likely to be aggressive. Unfortunately, the aneuploid DNA pattern occurs in too large a fraction of benign parathyroid lesions to be useful for differentiating between PA and PC (Mallette 1992, Obara *et al.* 1997). However, the DNA ploidy pattern may be a valuable prognostic factor to predict recurrence (Obara *et al.* 1990, 1997). We found aneuploid tumours in 11 of 15 patients with PC, and in three of 15 patients with PA. Overall, DI (mean $\pm$ S.D.) was significantly ($P < 0.01$) higher in patients with PC than those with PA, but no correlation ($P = \text{NS}$) was found between DI and survival.

It is difficult to determine the prognosis for patients with PC solely from the results of tumour DNA cytometry, and some studies considered the DI to be of no value in deciding the benign or malignant character of a parathyroid tumour (Obara *et al.* 1990, Kameyama *et al.* 1999). Thus, parameters other than DI were investigated, such as proliferating cell nuclear antigen (PCNA), the cell cycle-associated antigen Ki-67 and AgNOR counts. Few studies consider the usefulness of labelling indices of PCNA and Ki-67 positivity as markers of the biological behaviour of PC (Abbona *et al.* 1995, Kameyama *et al.* 2000). Kanematsu *et al.* (1997), studying

25 parathyroid lesions of which three were PC, found that the AgNOR numbers were significantly higher in PC than in PA. The authors concluded that AgNOR may be useful as an adjunct in discriminating PC from benign parathyroid disease. More recently, the multivariate analysis of Tuccari *et al.* (2000) showed that the mean area of AgNORs per cell (NORA) was unrelated to age, sex, main biochemical parameters and tumour size in a group of 10 patients with PC. A significantly higher mean NORA value was observed in patients with distant metastasis than in those with no clinical evidence of disease progression. However, surprisingly, only one patient died of disease at a mean follow-up of more than 80 months. We have not found any correlation ($P = \text{NS}$) between survival and AgNOR counts in our patients.

We conclude that DI and AgNOR are useful in confirming diagnosis of PC; however, they are of little value in predicting the clinical outcome of patients with PC.

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