Virchows Archiv A Pathol Anat (1991) 419:455–461

Virchows Archiv A
Pathological Anatomy and Histopathology

Paragangliomas: neuroendocrine features and cytometric DNA distribution patterns

A clinico-pathological study of 22 cases*

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Received May 7, 1991 / Received after revision July 15, 1991 / Accepted July 16, 1991

Summary. Paragangliomas from 22 patients with extra-adrenal tumours of this type were studied. Neuroendocrine features were examined using immunohistochemical techniques. Twenty-two antisera raised against neuroendocrine "markers", regulatory peptides, serotonin and intermediate filament proteins were studied in this group and cytometric DNA assessments were made by means of image cytometry. One normal and 5 hyperplastic carotid bodies were used as controls in the DNA cytometric investigations. Clinical and/or histopathological evidence of "malignancy" was present in 5 cases. The tumour cells showed heterogeneity with regard to their expression of different peptides, and the immunohistochemical analyses did not permit differentiation between benign and malignant paragangliomas. An euploid nuclear DNA distribution pattern was found in all controls and in 17 of the tumours; all except 1 were clinico-pathologically benign. An aneuploid DNA pattern was observed in 5 of the cases and some malignant features were present in 4 of these cases. DNA data may give further information apart from that obtained from the histopathological findings which may be of value in predicting the biological behaviour of this tumour type.

Key words: Paragangliomas – Immunohistochemistry – Image cytometry – DNA ploidy pattern – Malignancy grading

* A preliminary report was given at the XIIth European Congress of Pathology, Porto, Portugal, 3–9 September 1989

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Introduction

Extra-adrenal paragangliomas are usually considered to be benign tumours, but some of these can show malignant behaviour clinically with metastasis to regional lymph nodes and even to distant organs (Glenner and Grimley 1974). This metastatic potential is difficult, but not impossible, to recognise histopathologically (Linnoila et al. 1990), as most malignant paragangliomas are devoid of the conventional structural features of a malignant neoplasm. Attempts that have been made to predict the malignancy of paragangliomas by means of ultrastructural and immunohistochemical investigations have recently been reviewed (Kliewer et al. 1989).

The cytometric DNA ploidy pattern of the parenchymal cells of these tumours has previously been studied, both by means of flow cytometry (Granger and Houn 1990; Sauter et al. 1991; van der Mey et al. 1991) and image cytometry (Barnes and Taylor 1990). The results are controversial. In some other tumours, however, the nuclear DNA ploidy pattern is a useful tool in assessing the degrees of malignancy. The nuclei of the neoplastic cells of benign tumours usually have a euploid DNA distribution pattern, whereas those of malignant neoplasms often have a DNA ploidy pattern of the aneuploid type (Auer et al. 1989). When analysing tumours with a heterogenous cell population and an admixture of normal cells, it is important to use image cytometric techniques, rather than the flow cytometric procedure alone, to analyse the DNA ploidy pattern of the neoplastic nuclei (Falkmer 1989). In our opinion, the paragangliomas belong to this group of tumours.

The aim of our study was to investigate the immunohistochemical features and the image cytometric DNA

distribution patterns of the neoplastic cells of paragangliomas of various degree of clinico-pathologically malignant neoplastic features. It was made in order to assess the value of these variables in the prediction of the biological behaviour of these neuroendocrine neoplasms.

Patients and methods

A total of 22 patients, 13 women and 9 men, ranging in age from 24 to 82 years (median age 53 years), were included (Table 1). Data from follow-up examinations were available for 19 of the 22 patients. The observation times varied from 6 months to 26 years. Carotid body tumours were observed in 10 patients, and glomus jugulare tumours in 9 patients. Other sites were the glomus vagale (2 patients) and the retroperitoneum (1 patient). As shown in Table 1, 3 patients (cases 2, 6 and 22) with symptoms of excessive catecholamine secretion had significantly increased urinary secretion of noradrenaline or its metabolites (Table 1). Two patients (cases 2 and 6) had, at the time of operation, clinically malignant tumours with metastases to regional lymph nodes. Cases 4, 18, and 22 displayed histopathologically malignant features at the time of operation. Case 18 developed local recurrence and brain invasion 5 years after operation. No patient had multiple paragangliomas; there was no family history of paraganglioma; none of the patients were chronically hypoxic or lived at high altitudes. Additional co-existing tumours were not observed at the time of operation.

As controls for the image cytometrical DNA assessments of the DNA ploidy pattern, carotid bodies from 6 male autopsy cases (age range 9 days to 3 years) were used. Five of these patients had hypoxaemia due to different congenital heart diseases. The 6th died of encephalitis. When differential cell counts were performed, the carotid bodies from the 5 children with hypoxaemia were found to be hyperplastic (differential sustentacular cell count in excess of 47%), whereas that from the 6th child was normal (33% sustentacular cells) (Smith and Heath 1982).

All specimens were fixed in 10% buffered, neutral formaldehyde and routinely processed to paraffin; the sections, 4 μm thick, were stained with haematoxylin and eosin and with the modified Grimelius silver nitrate technique (Grimelius and Wilander 1980).

Either a newly developed modification of the immunogold-silver staining method (Hacker et al. 1988), or the avidin-biotin-peroxidase complex method (Hsu et al. 1981) was applied. Information concerning the primary antisera used and their dilutions is given in Table 2. Positive controls were obtained by using human

Table 1. Survey of the cytometric nuclear DNA ploidy pattern of the chief cells of 22 paragangliomas and the course of the neoplastic disease

Case no.	Sex	Age (years)	Location[a]	Function	DNA[b] ploidy	Follow-up (years)	Clinical data
1.	F	71	CB	No	A	15	No recurrence
2.	F	43	CB	Yes	T	5	Malignant, metastasis, no recurrence after operation
3.	F	61	CB	No.	D	4	No recurrence
4.	F	34	CB	No	A	1.3	No recurrence[c]
5.	F	24	CB	No	D	0.6	No recurrence
6.	M	65	CB	Yes	A	0	Malignant, metastasis. Died 4 days after operation (cerebral infarction)
7.	M	42	CB	No	D	18	No recurrence
8.	M	68	CB	No	T	3	No recurrence
9.	M	38	CB	No	D	–	(No follow-up)
10.	M	63	CB	No	T	–	(No follow-up)
11.	F	82	GJ	No	D	5	No recurrence
12.	F	78	GJ	No	D	1	No recurrence
13.	F	55	GJ	No	D	4	No recurrence
14.	F	73	GJ	No	T	9	No recurrence
15.	F	51	GJ	No	D	5	No recurrence
16.	M	21	GJ	No	D	26	No recurrence
17.	M	40	GJ	No	D	–	(No follow-up)
18.	M	57	GJ	No	A	5	Not radically operated[c]. After 5 years, recurrence with brain invasion
19.	M	58	GJ	No	T	6	No recurrence
20.	F	44	GV	No	D	15	Single recurrence after 7 years
21.	F	47	GV	No	T	7	No recurrence
22.	F	41	R	Yes	A	0.7	No recurrence[c]

[a] CB, Carotid body; GJ, glomus jugulare; GV, glomus vagale; R, retroperitoneum
[b] D, "Diploid"; T, "tetraploid"; A = "aneuploid"
[c] Histopathologically, these tumours were found to display malignant features, such as capsular invasion, cellular pleomorphism and/or vascular invasion

Table 2. Survey of the primary antibodies used; the dilution shown gave maximum antigen detection at the lowest rate of background

Antigen	Type	Dilution	Code	Source
a) General neuroendocrine markers				
Protein gene product (PGP) 9.5	p	1/1600	RA95101	Ultraclone, Cambridge, UK
Synapsin/SYN)	p	1/100	R4	H. Holländer, Munich, FRG
Anterior pituitary of pig (APPG/7B2)	p	1/200	1986	J.M. Polak, London, UK
Neuron-specific enolase (NSE)	m	1/100	M-002h	Innogenetics, Ghent, Belgium
Chromogranin A (CgA)	m	1/600	LK2H10	Boehringer, Mannheim, FRG
Synaptophysin (SYPT)	m	1/10	M 776	Dakopatts, Glostrup, Denmark
PHE-5 (chromogranin B and C)	m	1/2000	EAB 932	Enzo, New York, USA
b) Regulatory peptides and amines				
Pancreastatin (PANCR)	p	1/1000	CA08-312	CRB, Cambridge, UK
Gastrin-flanking peptide (PSN	p	1/600	CA-260	CRB
VIP/PHM precursor (111–122) (pre-VIP)	p	1/400	CA-345	CRB
Neuropeptide tyrosine (NPY)	p	1/500	CA08-295	CRB
C-flanking peptide of NPY (CPON)	p	1/500	CA-300	CRB
Substance P (SP)	p	1/200	SP-8	P.C. Emson, Cambridge, UK
Glycentin (GLYC), N-terminal	p	1/2000	R-64	A.J. Moody, Bagsvaerd, Denmark
Glucagon (GLUC)	p	1/3000	E-7	G. Lundqvist, Uppsala, Sweden
Somatostatin (SOM)	p	1/400	A-566	Dakopatts
Pancreatic polypeptide (PP)	p	1/1800	A-619	Dakopatts
Bombesin (BOM)	p	1/1000	B-43	Dakopatts
Serotonin (5HT, SER)	m	1/250	MAS055	Sera-Lab, Sussex, UK
c) Other markers				
S-100 proteins (S-100)	p	1/400	PUO58UP	GioGenex, Dublin, Calif., USA
Vimentin (VIM)	m	1/25	M 725	Dakopatts
Cytokeratin (PKK1)	m	1/600	GF-205	Labsystems, Helsinki, Finland

p, Polyclonal; m, monoclonal; CRB, Cambridge Research Biochemicals

tissue specimens that contain the substance in question. Pre-absorption controls with antibodies to regulatory peptides were also performed. Negative controls were obtained by omitting the primary antiserum and replacing it by a non-immune serum or antibody diluent alone.

The frequency of occurrence of immunoreactive cells – as well as of argyrophil cells – was graded semi-quantitatively from " + " to " + + + ", where " + " indicated the presence of less than 30% immunoreactive (or argyrophil) cells, " + + " from 30% to 60%, and " + + + " over 60%.

The cytometric DNA assessments were made by means of two parallel image cytometric techniques, both based on the Feulgen-staining procedure. One consisted in assessments on 4-μm-thick paraffin sections, and the other in measurements on the nuclei of the tumour cells obtained after deparaffinization and disintegration (van Driel-Kulker et al. 1985). The actual cytometric determinations were performed in a rapid scanning and integrating microspectrophotometer (Caspersson and Kudynowski 1980). The absorption was measured at 546 nm and was used as an expression of the total amount of DNA in the cell nuclei. At least 100 identified neoplastic cells were analysed in each specimen. In the disintegrated material structural identification was based on conventional cytodiagnostic criteria of the nuclei of the neoplastic chief parenchymal cells. To determine the modal value of the nuclear DNA content of the tumour cells, granulocytes or endothelial cells were used as internal diploid DNA standard cells. The DNA histograms obtained were interpreted according to the descriptions given in previous reports from one of our laboratories (Auer et al. 1980, 1984; Fallenius et al. 1987; Falkmer 1989).

Results

Each tumour consisted of chief and sustentacular cells together with a prominent fibrovascular stroma. The chief cells, which were polygonal and had eosinophilic cytoplasm, were arranged in nests (*Zellballen*), and thus showed histopathological features characteristic of paraganglioma. Occasionally, prominent nucleoli were present in the chief cells. The sustentacular cells showed no obvious abnormalities. Significant pleomorphism, mitotic figures, areas of necrosis, an invasive growth pattern and vascular invasion were observed in 3 cases (nos. 4, 18 and 22). These 3 tumours had different locations, namely the carotid body, glomus jugulare and retroperitoneum, respectively. The latter lesion was a functional tumour (see below). All tumours, except those from 2 patients (cases 13 and 14) contained argyrophil cells (Table 3).

The immunohistochemical results are summarized in Table 3. The chief cells reacted with antibodies to markers for neuroendocrine differentiation. All the tumours displayed immunoreactivity to at least 4 out of the 8 neuroendocrine "markers" used. Neuron-specific enolase (NSE) and protein-gene-product-9.5-immunoreactive cells were present in all 22 tumours. Synaptophysin and PHE-5 (chromogranin-B and C) immunoreactivities (Fig. 1a) were identified in 21 and chromogranin-A in 20 tumours. Immunoreactivity for synapsin was observed in 17 and for 7B2 in 13 tumours.

Regarding the general neuroendocrine markers, the frequency of occurrence of immunoreactive cells did not show any correlation with the clinical or histopathological features of malignancy.

Neuropeptide hormone immunoreactivity was ob-

Table 3. Summary of the argyrophil reaction and the immunohistochemical results from use of antibodies against general neuroendocrine, regulatory peptides, neuronal, and glial mesenchymal markers in chief and sustentacular cells of the 22 paragangliomas

Case no.	Loc.[a]	No. of chief cells immunoreactive with antisera[c] to:															No of sustencular cells immunoreactive with antisera[c] to:	
		Grim.[b]	NSE	PGP	SYPT	PHE-5	CgA	SYN	7B2	SP	PANCR	GLYC	Pre-VIP	NPY	CPON	S-100	VIM[d]
1	CB	++	+	++	++	+	++	++	+	−	−	+	+	−	−	−	++
2	CB	++	++	++	+	++	++	+	+++	−	+	+	+	+	+	+	−
3	CB	++	+++	+++	+	++	++	+	++	−	−	+	−	+	+	+++	+++[d]
4	CB	+	++	++	++	+	+	++	+	−	+	+	−	−	++	−	++
5	CB	+	+++	+++	+++	+	++	++	++	+	+	++	−	++	+	++	+++
6	CB	+++	+	++	++	+	+++	++	+	−	−	−	+++	−	++	+	++
7	CB	++	++	++	++	++	++	++	−	++	+	+	++	−	+	−	++[d]
8	CB	+++	++	+++	+	+	+++	−	−	−	−	−	−	−	−	+++	+
9	CB	+	+	++	+	++	+	++	+	+	+	−	+	+	+	+	++
10	CB	++	+++	+++	+	++	++	+	++	−	++	−	−	+	++	+	++
11	GJ	+	+	+	++	+	+	+	−	−	−	−	+	−	−	+	−
12	GJ	++	++	++	++	++	++	++	++	−	−	−	−	−	−	++	+
13	GJ	−	+++	+++	+	−	−	−	+	+	−	−	+	−	−	++	+
14	GJ	−	++	+	−	++	−	++	−	−	−	−	+	−	−	+	++
15	GJ	+++	+++	+++	+	++	+++	−	−	−	+	−	−	−	−	++	−
16	GJ	+++	+++	+	++	+++	+++	−	+	+	+	−	−	−	−	+	+++[d]
17	GJ	++	+	++	++	++	+++	++	−	++	+	−	+	−	−	−	−
18	GJ	+++	+	+	+	+	+++	++	−	+	+	+	−	−	−	+	+
19	GJ	++	++	++	+	++	++	+	−	−	−	−	−	−	+	++	++[d]
20	GV	++	++	++	++	++	++	++	+	−	+	+	++	−	+	++	++
21	GV	++	++	++	+	+++	+++	−	−	−	++	−	−	−	−	+	++
22	R	++	++	+++	+	++	++	++	+	−	+	−	−	++	−	++	−
Total no. and		20	22	22	21	21	20	17	13	7	13	8	10	6	10	18	17
% of immunoreactive cases		(91)	(100)	(100)	(95)	(95)	(91)	(77)	(59)	(32)	(59)	(36)	(45)	(27)	(45)	(81)	(77)

−, No immunoreactive cells; +, less than 30% immunoreactive cells; ++, 30–60% immunoreactive cells; +++, more than 60% immunoreactive cells

[a] Location; for abbreviations see Table 1
[b] The Grimelius technique for argyrophilia
[c] For abbreviations see Table 2
[d] Present also in chief cells

served only in chief cells of the paragangliomas (Table 3). Pancreastatin immunoreactivity (Fig. 1b) was most often demonstrated (13 out of 22 tumours), followed by immunoreactivity against pre-vasoactive intestinal polypeptide cryptic fragment 111–122 and C-flanking peptide of neuropeptide tyrosine (CPON), glycentin, substance P and neuropeptide tyrosine (NPY) (Fig. 1c). The NPY immunoreactivity was related to the tumour location; it occurred in carotid body but not in glomus jugulare tumours. CPON immunoreactivity was also present in the carotid body neoplasms, with one exception (case 19). Two tumours were found to contain serotonin-immunoreactive cells. Tumour cells immunoreactive with antisera against somatostatin, glucagon, pancreatic polypeptide, gastrin-flanking peptide, or bombesin, were not observed.

S-100 protein immunoreactivity was identified in 18 tumours. Immunoreactivity against the "mesenchymal" intermediate filament protein vimentin was detected in sustentacular cells in 17 cases (Table 3). In 4 of 17 cases vimentin immunoreactivity was found also in chief cells. Cytokeratin immunoreactivity was not observed in any of the tumours.

The nuclear DNA distribution pattern of the 6 control carotid bodies was of the diploid type, irrespective of whether the carotid bodies were hyperplastic or normal.

The nuclear DNA distribution pattern of the neoplastic cells was found to be of the diploid type in 11 cases, tetraploid in 6 cases and aneuploid in the remaining 5 cases (Table 1). These results were the same irrespective of which of the two variants of the cytometric technique was used (Fig. 2).

The 17 paragangliomas in which the nuclear DNA distribution pattern of the tumour cells was found to be of the euploid (diploid or tetraploid) type were all benign with one exception (case 2). Only 1 patient (case 20) had a recurrence of the tumour, 7 years after the operation. Since re-operation the patient has been tumour-free for the last 7 years.

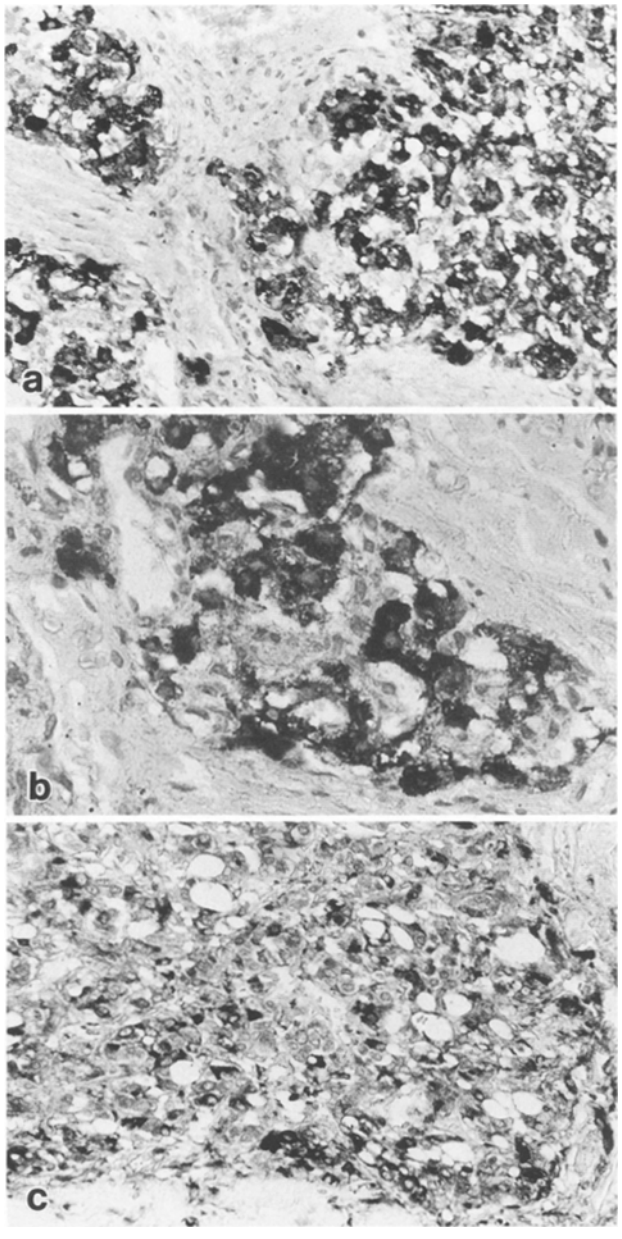

Fig. 1. Photomicrographs of paragangliomas, immunostained with monoclonal mouse antibody "PHE-5" (**a**), with polyclonal rabbit antibodies to pancreastatin (**b**), and with polyclonal rabbit antibodies to neuropeptide tyrosine (NPY) (**c**). As seen in all photomicrographs, the chief cells showed a marked variation in their degree of immunoreactive intensity. Immunogold-silver staining with silver acetate autometallography, counterstained with haematoxylin. **a** ×230; **b** ×460; **c** ×230

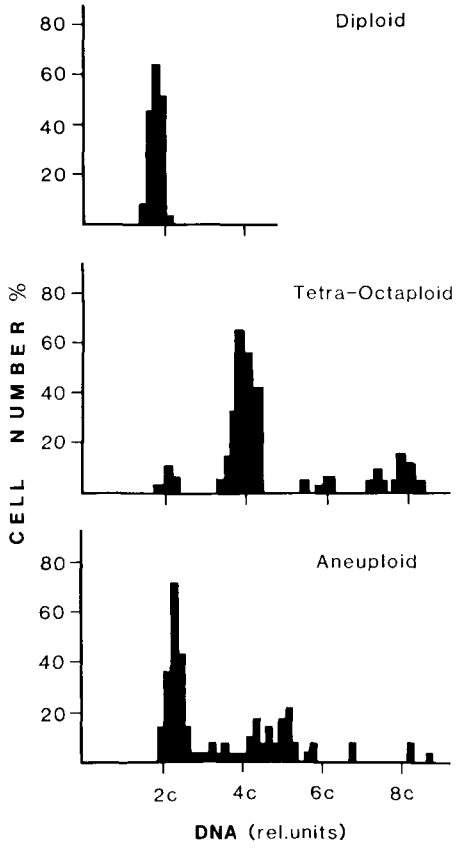

Fig. 2. DNA histograms, obtained by means of an image cytometric technique, showing the nuclear DNA distribution pattern of the neoplastic chief parenchymal cells of two clinically benign paragangliomas (diploid and tetraploid histograms) and of one malignant paraganglioma (aneuploid histogram). In the tetraploid histogram, there is also a small octaploid peak; this is a common finding in the nuclei of normal neuroendocrine parenchymal cells and in those of benign neuroendocrine neoplasms and hyperplastic lesions

Of the 5 patients with paragangliomas in which the DNA distribution pattern was aneuploid, 4 displayed histopathological and/or clinical features of malignancy. Of the tumours in these 4 patients (cases 4, 6, 18 and 22), 2 originated from the carotid body, 1 from the glomus jugulare and 1 from the retroperitoneal region. The tumours in the 5th patient (case 1), from the carotid body, showed no signs of malignancy.

No relationship was found between the DNA ploidy patterns of the nuclei of the neoplastic cells and either the functional status or the anatomical location of the paragangliomas.

With regard to the relationship between immunohistochemical and cytometric nuclear DNA distribution patterns, the staining intensity with use of NSE antiserum was less intensive in tumours in which the nuclear DNA distribution pattern was of the aneuploid type than in those with a diploid pattern, except in 1 case (case 22). The frequency of sustentacular cells, identified by immunostaining with S-100-protein antiserum, did not show any correlation to the DNA ploidy pattern of the tumours (Table 3). No relationship between the neuropeptide hormone content, on the one hand, and the tumour-biological behaviour and DNA ploidy, on the other, was found.

Discussion

There was marked heterogeneity in our series of paragangliomas regarding their immunoreactivity against regulatory peptides. The panel of antisera we used was to some extent selected from the results published from

other laboratories in investigations of this kind, and our observations were in good agreement with theirs (Warren et al. 1985; Hamid et al. 1987; Bishop et al. 1988). In addition, we applied some antisera against neuroendocrine "markers" that had not been used previously in this tumour entity.

We were unable to confirm previous statements from other laboratories (Linnoila et al. 1988; Kliewer and Cochran 1989; Kliewer et al. 1989) that the results of immunohistochemical investigations allow a distinction between benign and malignant extra-adrenal paragangliomas. Thus, neither a decreased frequency of the S-100-protein-immunoreactive sustentacular cells (Kliewer and Cochran 1989; Kliewer et al. 1989; Achilles et al. 1991) nor a diminished expression of neurohormonal peptides (Linnoila et al. 1988) characterized the 5 paragangliomas of our series that showed certain malignant features. However, the claim that a decrease in the intensity of the immunoreactivity against NSE characterizes malignant paraganglioma (Kliewer and Cochran 1989; Kliewer et al. 1989) received some support from our observations.

Differences in the cytometric DNA distribution patterns were noted between benign and malignant paragangliomas. Here, our observations conform to those of a recent flow cytometric study (Sauter et al. 1991). Tumours in which the nuclear DNA distribution pattern of the neoplastic cells was of the euploid type, had – with the exception mentioned above – benign characteristics, despite the fact that 9 of these 17 paragangliomas were from the glomus jugulare. Epithelial tumours – benign or malignant – in which the nuclear DNA distribution pattern of the neoplastic cells was of the tetraploid type, have sometimes been classified as aneuploid tumours (Stuart-Harris et al. 1985; Masters et al. 1987; Owainati et al. 1987). It would seem, however, that the prognosis is significantly better in patients suffering from tumours in which the nuclear DNA distribution pattern of the neoplastic cells is of the tetraploid type than in those whose tumours are of the aneuploid type (Auer et al. 1989). This is in accordance with the present findings in our case 2. Depsite the fact that at the time of operation the patient had a metastasis in a regional lymph node, no recurrence or further metastases occurred during the post-operative follow-up period of 5 years. Concerning the exceptional case among those paragangliomas in which the nuclear DNA distribution pattern of the tumour cells was of the aneuploid type (case 1), it might be added that carotid body paragangliomas belong to those tumours in which signs of malignancy may appear even more than 20 years after the operation (Glenner and Grimley 1974).

In some of the previous reports of flow cytometric DNA assessments of the nuclei of paraganglioma cells (Granger and Houn 1990; van der Mey et al. 1991), it was found that DNA ploidy of these tumours could not serve as a predictor for an expected growth pattern, because unequivocal evidence of DNA aneuploidy was found in almost 40% of clinically and histologically benign tumours. With the present results of our image cytometric DNA technique at hand, we agree with the conclusion of a recent flow cytometric report (Sauter et al. 1991).

In conclusion, the cytological heterogeneity of paragangliomas found in the present study is in agreement with observations made in previous investigations. Our immunohistochemical data do not permit distinction between malignant and benign paragangliomas. However, some differences in the image cytometric DNA distribution patterns observed indicate that these analyses may be of some help in predicting the biological behaviour of this tumour type. As the malignant features of many paragangliomas appear late, a longer time than the present follow-up period is necessary in order to draw more certain conclusions about the implication of the DNA results.

Acknowledgements. This study was supported by the Swedish Medical Research Council (project nos. 102 and 718), the Cancer Society of Stockholm and the Research Funds of the Faculty of Medicine at the Karolinska Institute, Stockholm. Some antibodies were kindly provided by Drs. P. Emson (Neurochemical Pharmacology Unit, Medical Research Council Centre, Cambridge, UK), H. Holländer (Max Planck Institute of Psychiatry, Munich-Martinsried, FRG), L. DeGennaro, (Department of Neurology, University of Massachusets Medical School, Worchester, Mass., USA), G. Lundqvist (Department of Clinical Chemistry, University Hospital, Uppsala, Sweden), A.J. Moody (Novo Research Laboratories, Bagsverd, Denmark) and J.M. Polak (Hitochemistry Department, Hammersmith Hospital, London, UK).

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