Studies for Estimating the Biologic Behavior and Prognosis of Paragangliomas in the Head and Neck

Abstract—Despite a large number of histopathologic and immunohistochemical studies, the biologic behavior and prognosis of paragangliomas (glomus tumors) of the head and neck still remain uncertain. In the present study 36 specimens from 32 patients who underwent surgery for a paraganglioma were examined. The examinations included routine histology, quantitative DNA analysis based on image cytometry, immunohistochemical detection of the proliferating cell nuclear antigen (PCNA) along with visualization of nucleolar organizer regions (AgNOR). According to LeCompte, the paragangliomas were histologically divided into three subcategories: 16 patients had a paragangliomatous tumor, 14 patients had an adenomatous tumor, and 6 patients had an angiomatous tumor. Quantitative DNA analysis revealed three categories of tumors with characteristic DNA pattern: DNA type I tumors were pure diploid, DNA type II tumors had stemlines at 2c and 4c and were therefore recognized as diploid-tetraploid. Aneuploid cells were not apparent in these two groups. DNA type III tumors had stemline ploidies exceeding 2c and 4c. Aneuploid cells were present in all of these tumors. The biologic behavior of these lesions therefore must be recognized as suspicious. DNA type III tumors and adenomatous tumors showed the highest values for the PCNA scores, indicating a higher proliferation rate and a more rapid growth pattern in these lesions. Twenty patients could be followed over a period of up to 110 months. Five of these patients developed a recurrent tumor. All of them had DNA type III tumors. The DNA indices showed significantly higher values in the recurrent tumor group. The 2c deviation index (DI) and the entropy value had the highest prognostic significance. No correlation to clinical follow-up was found for the AgNOR score. Based on these results, prognostic indices for paragangliomas were developed: patients with a tumor having a 2c DI exceeding 2.0, entropy value of more than 4.0, 5c exceeding rate more than 8.0, and a PCNA score more than 20.0% can be recognized as being at high-risk for developing recurrent disease. (*Skull Base Surgery*, 5(3):149–156, 1995)

Paragangliomas (glomus tumors) of the head and neck are rare tumors with an overall incidence of 0.012%. They arise from paraganglionic precursor cells in the paragangliomatous tissue, which is widely distributed in

the head and neck and has some function in the regulation of blood pH and carbon dioxide concentration.¹

Paragangliomas are recognized as benign tumors. Nevertheless, some of these tumors show a biologic be-

havior similar to malignant lesions (development of recurrence, invasive growth pattern, and development of metastatic disease). Some authors found a recurrence rate of 10% in tumors of the carotid region, and of 30 to 50% in tumors involving the tympanic and jugular regions.² The criteria for determining the aggressive or malignant behavior of paragangliomas are: (1) central necrosis; (2) vascular invasion; (3) nuclear polymorphism; (4) number of mitosis per high-power field; and (5) percentage of sustentacular cells.²⁻⁵ On the other hand, many authors did not find a correlation between these morphologic features and the biologic behavior or recurrence rate in patients with paragangliomas.^{6,7} Despite a large number of histopathologic and immunohistochemical studies, there are currently no morphologic or cytologic markers available to estimate the biologic behavior or to predict the recurrence rate in patients with paragangliomas.^{3,7,8}

PATIENTS AND METHODS

Specimens of 32 patients who underwent surgery for a suspected paraganglioma were examined. The examinations included routine histology, quantitative DNA measurements, immunohistochemical assessment of the proliferating cell nuclear antigen (PCNA), and visualization of nucleolar organizer regions (AgNOR) in the tumor cell nuclei.

Routine Histology

Routine histologic examinations were performed on hematoxylin and eosin stained 4 μ m sections obtained from paraffin-embedded tissues. According to LeCompte,⁹ tumors are grouped into subcategories⁷: (1) paraganglio-

matous tumors show tumor cells in a strand-like pattern, with the cells separated by trabeculae of connective tissue with capillaries, imitating a paraganglioma (Fig. 1); (2) adenomatous subtype tumors are characterized by epitheloid-like cells with anisomorphic oval nuclei. Only a few blood vessels and capillaries are visible between the cell nests (Fig. 2); and, (3) angiomatic tumors show a fusicellular pattern of type I cells and numerous, large blood vessels (Fig. 3).

Quantitative DNA Measurements

Sections of 50 μ m were cut from the paraffin blocks, dewaxed with xylene, and rehydrated in a graded alcohol row. For cell separation, the material was incubated with 0.05% Pronase, washed in phosphate-buffered saline (PBS), filtered through a mesh (pore size of 105 μ m), and centrifuged onto glass slides (3000 rpm, 10 minutes) as described elsewhere.^{10,11} The nuclear DNA was stained with fuchsin sulfurous acid according to the technique of Feulgen.¹² Quantitative DNA analysis was then performed using a computerized image analysis system (Cytometer CM 1, Hund, Wetzlar, Germany). At least 300 tumor cells were measured at random after identification on a television screen. Additionally, a minimum of 30 lymphocytes were measured in the same smear to determine the individual 2c peak. The following indices were determined from the single cell measurements¹³⁻¹⁵: (1) The mean DNA content of the tumor cells; (2) the 2c deviation index (2c DI), defined as the mean square deviation from the diploid value; (3) the 5c exceeding rate (5c ER), defined as the percentage of tumor cells with an aneuploid DNA content exceeding 5c; (4) the entropy of the DNA distribution in the tumor; (5) the DNA malignancy grade (MG); and (6) stemline analysis, defined as the DNA content of the predominant tumor cell population.

Figure 1. Photomicrograph of a paragangliomatous subtype tumor. Tumor cells are located in strandlike pattern and separated by trabeculae of connective tissue with blood vessels. (H&E. $\times 400$.)

Figure 2. Adenomatous type of a paraganglioma showing epitheloid-like tumor cells with oval or round nuclei in strands and nestlike pattern. (H&E. $\times 200$.)

Immunohistochemical Identification of the Proliferating Cell Nuclear Antigen

PCNA is a nuclear protein with a molecular weight of 36 kDa. It is identical to the DNA polymerase delta auxiliary protein and therefore essential for DNA synthesis.^{16,17} This antigen is expressed in every active phase of the cell cycle with a clear peak in the S-phase. Therefore, it can be used as a proliferation marker.¹⁸ Recent cell biologic studies have shown that PCNA is also implicated in the DNA repair process.¹⁹

For immunostaining with the PCNA antibody, 4 μ m sections were cut from the paraffin blocks, placed on poly-L-lysine-coated glass slides, dewaxed in xylene, and then rehydrated in a graded alcohol row. The slides were then incubated with 1% hydrogen peroxide to block the endogenous peroxidase activity. After washing in

PBS, the slides were incubated with the PCNA antibody (Ab PC 10, Oncogene Science, Uniondale, NY) at room temperature for 60 minutes. The slides were then washed again with PBS and stained using the ABC method.²⁰ At least 1000 tumor cells were assessed. The PCNA score was determined as the percentage of positive tumor cells.

Nucleolar Organizer Regions

The AgNORs are large DNA loops located on the 5 acrocentric chromosomes. They play a role in coding ribosomal RNA and are therefore essential for the transcription process.^{21,22} For visualization of the AgNORs, 4 μ m sections were cut from the paraffin-embedded tissues, deparaffinized in xylol and a graded alcohol row, and then stained according to the technique of Ploton et

Figure 3. Angiomatous type of a paraganglioma: fuscicellular pattern of type I cells and numerous blood vessels along with vascular endothelial cells are predominant. (H&E. $\times 200$.)

al.²³ For this special silver-staining process, two staining solutions were required: (1) solution A was prepared by dissolving gelatine in 1 g/dL aqueous formic acid at a concentration of 2 g/dL; (2) solution B was prepared by dissolving 50 g silver nitrate in 100 ml distilled, deionized water. These two solutions were mixed (1:2 vol) to get the final staining solution. The deparaffinized slides were incubated with this silver-staining solution at room temperature in a dark room for 30 minutes. The silver colloid was then washed off by distilled, deionized water. Slides were dehydrated in a graded alcohol row and mounted with Kaiser's gelatine. The number of AgNORs were counted using a light microscope (Orthoplan, Leitz, Wetzlar, Germany) at a magnification of 1200 (oil immersion). At least 100 tumor cell nuclei were assessed. The AgNOR score was determined as the average number of AgNORs per nuclei.

RESULTS

Specimens of 32 patients (27 women, 5 men; average age, 50 years [range, 21 to 73]) were examined. Seventeen patients had a left-sided tumor and 11 patients had a right-sided tumor. Four patients had bilateral paragangliomas, yielding a total of 36 specimens examined. The tumor sites were as follows: 9 tumors of the carotid region, 17 tumors of the jugulare region, and 10 tumors of the tympanic region. Metastatic disease was not observed in any patient. All tumors were clinically completely resected. Pathohistologic examination of serial sections from the tumor margins were performed in 24 cases. All margins in these cases were histologically free of tumor.

The diameter of the resected tumors ranged between 0.9 and 5.1 cm. Pathohistologically, a paraganglioma was confirmed in all cases. According to morphologic characteristics, these tumors could be grouped into 16 paragangliomatous types, 14 adenomatous types, and 6 angiomatous types. No correlation was found between histologic subtype and tumor site (Table 1) or between tumor size and predominant histological subtype (data not shown).

Table 1. Predominant Histologic Subtype of Paragangliomas in Regard to Tumor Sites

Histologic Characterization	Jugular Region (n = 17)	Tympanic Region (n = 10)	Carotid Body (n = 9)
Paragangliomatous (n = 16)	7	5	4
Adenomatous (n = 14)	9	2	3
Angiomatous (n = 6)	1	3	2

Quantitative DNA measurements revealed three categories of paragangliomas with characteristic DNA patterns:

1. DNA type I tumors had a diploid DNA value with a stemline at 2c and a only few cells with a DNA content of 4c. No aneuploid tumor cells were apparent. The entropy value was below 3, indicating a low genetic variability. This type of paraganglioma could be characterized as a purely diploid lesion. Two tumors in our series had DNA type I (Fig. 4).
2. DNA type II tumors had two clear stemlines, one at 2c and the other at 4c. The existence of a second stemline at 4c, which represented the cells in the G2/M phase of the cell cycle, can be interpreted as increased proliferation in DNA type II lesions compared with DNA type I tumors. The entropy values were between 3 and 4. No aneuploid cells were apparent. These tumors could be characterized as diploid-tetraploid lesions. Eighteen tumors belonged to this group (Fig. 5).
3. DNA type III tumors also showed two stemlines but their ploidy was exceeding 2c and 4c. The entropy values were higher than 4 in all cases. Aneuploid tumor cells could be detected in all tumors of this group (Fig. 6). Therefore, these lesions could be characterized as the aneuploid type of paragangliomas and 16 tumors showed this pattern.

Figure 4. DNA type I pattern in a glomus tumor. There is only one clear-shaped stemline at 2c. No aneuploid cells can be detected. The entropy value is less than 3 in these cases. These tumors can be characterized as purely diploid.

Figure 5. Glomus tumor with DNA type II pattern. Two stemlines can be detected, one at 2c, the other at 4c. No tumor cells with an aneuploid DNA content are apparent. The entropy value is between 3 and 4 in these cases. These tumors can be characterized as diploid-tetraploid.

The correlation between PCNA scores and DNA types revealed lowest PCNA scores in DNA type I tumors, and highest scores in DNA type III lesions. The differences were statistically significant ($p < 0.05$; Mann-Whitney test). The results of DNA analysis and PCNA scores are summarized in Table 2. A correlation between DNA indices along with PCNA scores and histologic subtypes revealed the highest values for all indices tested in the adenomatous subtype tumors, and the lowest values in the paragangliomatous subtype lesions (Table 3). All DNA type I tumors were paragangliomatous lesions, and 9 of 14 adenomatous tumors were DNA type III (Table 4).

Adenomatous tumors showed significantly higher AgNOR scores (1.18 ± 1.01 ; range, 0 to 3.1) than paragangliomatous lesions (0.74 ± 0.937 ; range, 0.10 to 3.68; $p = 0.04$). Angiomatous tumors had an AgNOR score of 0.96 ± 0.536 with a range of 0.05 to 1.8. The differences between AgNOR scores in angiomatous tumors compared to those of adenomatous tumors and paragangliomatous tumors were not statistically significant. Likewise, the differences in AgNOR scores in the DNA types I to III tumors failed to achieve statistical significance.

Additionally, all biologic parameters examined were correlated with the clinical follow-up. Twenty patients

were followed for an average of 85 months (range, 24 to 110). Five of these patients (two patients with tumors of the jugular region, two patients with tumors of the carotid body, and one patient with a tumor of the tympanic region) developed a tumor recurrence with an average recurrence time of 64 months (range, 20 to 80 months). All five had DNA type III tumors, four of five had adenomatous tumors (two patients with tumors of the carotid region, one patient each with a tumor of the tympanic and jugular region), and one had an angiomatous lesion (patient with a tumor of the jugular region). The tumor size of the recurrent primary tumors ranged between 1.6 and 4.3 cm. The comparison of DNA indices in recurrent and nonrecurrent tumors revealed statistically significant differences, as detailed in Table 5. The highest significance was found for 2c DI, 5c ER, entropy, and DNA MG. No differences were found for AgNOR scores.

DISCUSSION

Despite a large number of reported histopathologic and immunohistochemical studies, the biologic behavior of paragangliomas in the head and neck region remains

Figure 6. Histogram of a glomus tumor with DNA type III pattern. There are two stemlines with a ploidy exceeding 2c and 4c. Many tumor cells with an aneuploid DNA content can be detected. The entropy value is more than 4 in these cases. These tumors can be characterized as the aneuploid type of paragangliomas.

Table 2. Results of DNA Indices and Proliferating Cell Nuclear Antigen Scores in 36 Paragangliomas According to DNA Pattern*

DNA Type	Mean DNA	2cDI [†]	5c ER [†]	DNA MG [†]	Entropy	Stemline	PCNA [†] Score
I (n = 2)	1.95 ± 0.21	0.27 ± 0.28	0.49 ± 0.70	0.18 ± 0.18	2.56 ± 0.25	1.95 ± 0.15	2.7 ± 3.8%
II (n = 18)	2.44 ± 0.23	1.17 ± 0.51	0.76 ± 0.71	0.57 ± 0.18	3.54 ± 0.28	1.98 ± 0.11	15.64 ± 13.9%
III (n = 16)	3.21 ± 0.68	4.09 ± 2.49	9.15 ± 7.52	1.14 ± 0.42	4.51 ± 0.29	2.35 ± 0.75	26.25 ± 18.9%

*Data are average ± 1 standard deviation.

[†]DI: deviation index; ER: exceeding rate; MG: malignancy grade; PCNA: proliferating cell nuclear antigen.

Table 3. Results of Quantitative DNA Analysis and Proliferating Cell Nuclear Antigen Scores in 36 Paragangliomas According to Different Histologic Subtypes**

Histologic Subtype	Mean DNA	2c DI	5c ER	DNA MG	Entropy	Stemline	PCNA Score
Paragangliomatous (n = 16)	2.36 ± 0.28	1.03 ± 0.68	2.56 ± 5.23	0.5 ± 0.24	3.56 ± 0.60	1.96 ± 0.15	11.69 ± 13.34%
Adenomatous (n = 14)	3.08 ± 0.76	3.64 ± 2.75	18.21 ± 18.93	1.04 ± 0.46	4.22 ± 0.56	2.38 ± 0.79	26.91 ± 13.55%
Angiomatous (n = 6)	3.05 ± 0.48	3.26 ± 1.95	9.33 ± 7.89	1.04 ± 0.34	4.16 ± 0.54	1.96 ± 0.18	23.87 ± 25.32%

*Data are average ± 1 standard deviation.

^{**}See Table 2 for abbreviations.

difficult to assess and is sometimes not possible by morphologic examination alone.^{4,5} In the present study we tried to define tumor biologic markers for determining the biologic behavior and for estimating the prognosis of these tumors.

Based on quantitative DNA analysis, paragangliomas could be grouped into three categories with significantly different DNA indices. DNA type I tumors could be identified as purely diploid. DNA type II tumors had two clear stemlines, one at 2c and the other at 4c. No aneuploid tumor cells were apparent in either type I or type II tumors. DNA type II tumors had an increased number of cells with a DNA content of 4c (representing the cells in the G2/M phase of the cell cycle), indicating a higher proliferation in these lesions when compared to DNA type I tumors. On the other hand, we could demonstrate a third group of paragangliomas with stemlines exceeding 2c or 4c and with aneuploid tumor cells in all cases. The entropy was exceeding 4 in these tumors. As aneuploidy is a strong cytogenetic marker for malignancy, one might expect that the DNA type III tumors should be considered as possibly premalignant. Although there are only a few reported studies on DNA measure-

ments in paragangliomas, our results are in accordance with the other reported findings.²⁴⁻²⁶ Von der May et al²⁴ reported flow cytometric DNA analysis in paragangliomas of the head and neck. They found aneuploid tumor cells in 39% of histologically benign tumors. Jovanovic et al²⁵ found aneuploid tumor cells in four of five metastatic paragangliomas by DNA measurements based on image analysis. The correlation of DNA analysis to the histopathologic subtypes revealed significantly higher indices in the adenomatous tumors. All DNA type I tumors were paragangliomatous lesions. These results are in accordance with other clinical and immunohistochemical observations that revealed that adenomatous tumors have the most aggressive behavior of all paragangliomas.^{7,27,28)}

Immunohistochemical identification of the PCNA revealed the highest scores in DNA type III tumors and in adenomatous tumors. Tumors of the paragangliomatous subtype showed the lowest scores. As PCNA can be used as a proliferation marker, it is obvious that DNA type III tumors had the highest proliferation rate and therefore should have the most rapid growth pattern.^{18,19}

Additionally, we correlated the results of tumor biologic examinations with clinical follow-up. Of 20 patients followed over a period of up to 110 months, five developed recurrent disease. All five recurrent tumors were DNA type III, four of these were adenomatous, and one was angiomatous. DNA analysis showed significant differences in the indices (mean DNA content, 2c DI, 5c ER, and entropy) between recurrent and nonrecurrent lesions. Highest prognostic significance was found for the 2c DI and entropy, and all recurrent tumors had a 2c DI of more than 2.0 and an entropy value of more than 4. Approx-

Table 4. Correlation Between Histologic Subtype and DNA Types I to III in 36 Paragangliomas

DNA Type	Paragangliomatous (n = 16)	Adenomatous (n = 14)	Angiomatous (n = 6)
I	2	0	0
II	10	5	3
III	4	9	3

Table 5. Results of Quantitative DNA Analysis, Proliferating Cell Nuclear Antigen, and Antigen Nucleolar Organizing Region Scores in 5 Recurrent and 75 Nonrecurrent Paragangliomas*†

	Mean DNA	2c DI	5c ER	DNA MG	Entropy	Stemline	PCNA Score	AgNOR Score
Recurrent (n = 5)	3.23 ± 0.46	4.1 ± 1.38	23.2 ± 20.62	1.22 ± 0.20	4.61 ± 0.26	2.32 ± 0.71	23.72 ± 16.7%	0.61 ± 0.38
Nonrecurrent (n = 15)	2.61 ± 0.50	1.77 ± 1.81	4.27 ± 7.46	0.68 ± 0.37	3.61 ± 0.49	2.00 ± 0.10	14.95 ± 20.0%	1.03 ± 1.06
p Value‡	0.029	0.011	0.017	0.011	0.0040	0.93	0.190	0.156

*Data are average ± 1 standard deviation.

†AgNOR: antigen nucleolar organizer region. For other abbreviations, see Table 2.

‡Mann Whitney test.

imately 80% of the nonrecurrent tumors had a 2c DI of less than 2.0. The 2c DI is a marker for tumor aggressiveness that has proven prognostic relevance in many human tumors.²⁹ The entropy is a marker for the genetic variability and instability of tumors.¹⁵ Recurrent tumors can therefore be recognized as being more aggressive while having a higher genetic variety.

The results of PCNA scores correlated also with the clinical follow-up. Recurrent tumors had an average PCNA score of 24% in contrast to 15% in the nonrecurrent group. No correlations were found for the AgNOR score in determining the biologic behavior or for estimating long-term prognosis.³⁰

In summary, we could define the likelihood for the development of tumor recurrences. Patients having a paraganglioma with a 2c DI more than 2.0, 5c ER more than 8.0%, entropy value more than 4, and a PCNA score of more than 20% can be recognized as being at high risk for developing tumor recurrences. DNA type III tumors contain aneuploid cells and their biologic behavior should be considered as possibly premalignant. Patients with these tumors need careful long-term follow-up to detect early tumor recurrences or to consider other treatment modalities.

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