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DNA Flow Cytometry in Pheochromocytoma and Paraganglioma

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Flow cytometric DNA analysis was performed on 19 adrenal pheochromocytomas and 6 extra-adrenal paragangliomas in parallel with clinical and histopathological review to determine the usefulness of this technique to predict biologic behavior of these tumors. In pheochromocytomas and paragangliomas, tetraploidy or near-tetraploidy occurred in 32% and 33% and aneuploidy in 10% and none respectively. A case of malignant pheochromocytoma had diploid DNA content. Occurrence of aneuploidy or tetraploidy is frequent in clinically benign tumors in conjunction with a marked degree of nuclear atypia and cannot be a predictor of malignancy.

Key Words: Adrenal gland, pheochromocytoma, paraganglioma, flow cytometry, DNA content, histopathology

Adrenal pheochromocytomas and extra-adrenal paragangliomas frequently show striking nuclear pleomorphism regardless of their biologic behavior. In addition, capsular invasion, vascular invasion within the tumor, atypical mitoses and necrosis may all be seen in clinically benign tumors. Therefore, the only absolute criterion of malignancy is distant metastasis of the tumor. The incidence of malignancy is rare (under 5 percent) especially in pheochromocytoma, when multifocal tumors are rigorously excluded.

Previous reports suggest that nuclear DNA aneuploidy may be a useful marker of malignancy in these tumors (Klein *et al.* 1985; Hosaka *et al.* 1986). However, one report indicates that aneuploid DNA content is not a specific marker of malignancy in adrenal pheochromocytomas (Amberson *et al.* 1985).

In our study, flow cytometric DNA analysis was performed retrospectively on 19 specimens of pheochromocytoma and 6 paraganglioma. We wished to characterize the pattern of flow cytometric DNA histograms in pheochromocytomas and paragangliomas and to find the relationships of nuclear DNA ploidy pattern (DNA index) and proliferative compartment (%SG2M) to age, sex, primary site, multiplicity, histopathologic findings, clinical or biochemical function of tumor and clinical behavior.

MATERIALS AND METHODS

Adrenal pheochromocytomas from 3 patients and extra-adrenal paragangliomas from 6 patients diagnosed between 1974 and 1989 were retrieved from the files of Department of Pathology, Yonsei University College of Medicine, Seoul, Korea. Six cases (case # 1~6) of pheochromocytoma provided by Department of Pathology. The Childrens Hospital, Boston, MA were added. The medical records, gross descriptions, and microscopic slides stained with hematoxyline-eosin and with special stains in selected cases, were reviewed. Follow-up information was obtained by review of medical records and personal communication with patients' primary physicians. The duration of clinical follow-up has ranged from 10 months to 8 years. One patient

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(case #20) diagnosed with malignant pheochromocytoma had histologically proven distant metastasis and has been alive with the disease for two years. One patient (case #2) with multicentric tumor was included in this study.

Age and Sex of Patients

Range: 11 to 65 years (median: 32 years)
15 males and 10 females

Number of Tumors by Primary Site

Adrenal: 19 (Right, 5; Left, 12; Bilateral, 2)
Neck: 3 Mediastinum: 1 Retroperitoneum: 2

Histologic Evaluation

The architectural and cytologic features of each tumor, i.e. histologic pattern, presence of brown fat within the periadrenal soft tissue, cell size and shape, çapsular and vascular invasion, necrosis, nuclear atypia, mitotic activity, and cytoplasmic hyaline globules were reviewed. Nuclear atypia was defined by hyperchromasia and enlargement of nuclei with polyploid form, and the degree of nuclear atypia was divided into 3 grades. If less than 10% of tumor cells showed nuclear atypia, it was called slight nuclear atypia and if more than 50% of tumor cells showed nuclear atypia, it was called marked. If it was in between, it was called moderate.

Tissue Specimens

Formalin fixed, paraffin embedded samples of tumor were selected for flow cytometric DNA analysis. The surgically removed adrenal glands obtained from patients with renal cell carcinoma or benign adrenal disease, e.g., hyperplasia or cysts were used as control tissue.

Flow Cytometric DNA Analysis

Flow cytometric DNA analysis (DNA FCM) was performed on isolated nuclei using a modification of the method of Headley *et al* (1985). Two or three 50 micron sections cut from the tissue blocks were deparaffinized in Histo-Clear (National Diagnostics, Manville, NJ) and rehydrated in a series of graded alchohols. Disaggregation of nuclei was accomplished by treatment with 2.5 mL of 0.5% pepsin (Sigma Chemical Corp., St. Louis, MO) at PH 1.5 at 37°C for 30 minutes with intermittent vortexing. Digestion was stopped by adding 1 mL of a 0.005% peptostatin solution (Sigma Chemical Corp. St. Louis, MO). The smaples were then centrifuged at 2,000 rpm, washed twice with Dulbecco's phosphate buffered saline (Sigma Chemical Corp., St. Louis, MO), and incubated with freshly prepared 0.5 mL of RNAse (2.50 mg/mL; Worthington Biochemical, Freehold, NJ) at 37°C for 30 minutes. The samples were filtered through a 50 micron nylon mesh filter (Small Parts Inc., Miami, FL), and stained with 0.025% propidium iodide (Sigma Chemical Corp. St. Louis, MO) at 50 uL/mL. The isolated nuclei were adjusted to a concentration of 1 to 3×10^6/mL by diluting with Dulbecco's phosphate buffered saline. Nuclei prepared and stained in parallel from formalin-fixed, paraffin embedded normal adrenal glands which included medulla served as controls.

Nuclei were analyzed in a Epics V FACS Analyser (Coulter, Hialeah, FL) with at least 10,000 nuclei read per sample. Data was analyzed using the Multiparameter Data Acquisition and Display System (MDADS Epics Division, Coulter Electronics, Hialeah, FL).

Single parameter histograms were utilized in the evaluation of the tumors. The first G0/G1 peak was assumed to be the diploid population and assigned a DNA index of 1.0 (ratio of nuclear DNA of sample to nuclear DNA of diploid control cells). DNA aneuploidy was defined by the presence of a distinct, separate second peak to the right of the first G0/G1 peak followed by a low G2M peak in the hexaploid to octaploid range. The half-peak coefficient of variance (CV)(a measure of quality control) as calculated for G0/G1 peaks (MDADS program) ranged from 5.36% to 15.88% (median 8.31%) with a mean of 8.93% and standard deviation 2.58 %. The DNA index was calculated for all non-aneuploid and aneuploid populations. The proportion of cells in the S and G2M phases of the cell cycle (%SG2M) was used as an estimate of the compartment of proliferative activity of the tumor. G2M peaks were identified as small humps to the right of the G0/G1 peaks. In the case of hyperdiploid tumors, only the second G0/G1 peak (greater DNA index) was used in these calculations, because it is assumed that only second peak consists entirely of abnormal cells.

Statistical Analysis

The chi-square test was used for statistical evaluation. A difference was regarded as statistically significant if p was less than 0.05.

Table 1. Clinical, histopathologic and flow cytometric characteristics of 25 patients

Case #	Age/Sex	Site	Catecholamine Function: Clinical	Biochemical	Periadrenal brown fat	Hyaline globule	Histologic & Flow Cytometric Findings: Pattern	Cell size cell shape	Capsule invasion	Vascular invasion	Necrosis	Nuclear Atypia	DNA Index	Mitosis	%SG_2M	Follow-up (yr.)
Pheochromocytomas (N=19)																
1	11/M	Lt. Adrenal	+	+	+	+	Alveolar	Large polygonal	−	−	Focal	+++	2.1	Frequent Atypical	25	A&W(6/12)
2	12/M	Lt. Adrenal +paraaortic	+	+	+	−	Alveolar	Large polygonal	+	−	−	+++	1.53	Frequent Atypical	42	A&W(8)
3	12/M	Lt. Adrnal	+	+	−	+	Trabecular	Large polygonal	+	+	Focal	+++	2.1	Rare Atypical	39	A&W(1)
4	13/M	Lt. Adrenal	NA	NA	−	−	Mixed	Small & large polygonal	+	+	−	++	2.1	−	51	NA
5	14/M	Bilateral	+	+	+	−	Diffuse	Samll & large polygonal	−	−	−	+++	2.0/2.0	−	42/34	A&W(18)
6	14/M	Rt. Adrenal	+	+	−	+	Mixed	Small round & spindle	−	−	Focal	+++	2.1	Rare Atypical	28	A&W(3)
7	14/F	Lt. Adrenal	+	+	+	−	Diffuse	Samll round	−	−	−	+	1.0	Frequent	24	A&W(6.6/12)
10	29/M	Rt. Adrenal	−	+	−	+	Diffuse	Large Polygonal	−	−	−	+++	1.86	−	35	A&W(4)
11	31/F	Rt. Adrenal	−	+	−	+	Mixed	Large polygonal	+	−	−	++	1.0	−	19	A&W(3.2/12)
13	34/M	Lt. Adrenal	+	+	−	+	Trabecular	Large polygonal	+	−	Focal	+++	1.0	−	41	A&W(2.6/12)
15	45/M	Lt. Adrenal	+	+	−	−	Diffuse	Small polygonal	−	−	−	+	1.0	−	22	A&W(3.1/12)
16	46/F	Lt. Adrenal	+	+	+	−	Diffuse	Large polygonal small spindle	+	−	−	++	1.0	−	28	A&W(4.6/12)
17	51/M	Lt. Adrenal	+	+	−	+	Trabecular	Samll & large polygonal	−	−	−	+/++	1.0	−	13	A&W(1.2/12)
18	52/F	Rt. Adrenal	+	+	−	+	Diffuse	Large polygonal small spindle	+	−	−	+++	1.0	−	28	A&W(2.2/12)
19	52/F	Lt. Adrenal	+	+	−	−	Diffuse	Small polygonal & spindle	+	−	−	+	1.0	−	20	A&W(3.9/12)
20	56/F	Bilateral	−	−	−	−	Trabecular	Small polygonal large spindle	+	+	Extensive	+	1.0/1.0	−	24/18	AWD(2)

Table 1. Continued

Case #	Age/Sex (yr.)	Site	Catecholamine Function				Histologic & Flow Cytometric Findings									Follow-up (yr.)
			Clinical	Biochemical	Periadrenal brown fat	Hyaline globule	Pattern	Cell size cell shape	Capsule invasion	Vascular invasion	Necrosis	Nuclear Atypia	DNA Index	Mitosis	%SG_2M	
21	58/F	Lt. Adrenal	+	+	−	−	Diffuse	Large polygonal & spindle	−	−	−	++	1.0	−	33	A&W(1.6/12)
22	58/F	Rt. Adrenal	−	+	−	+	Diffuse	Small round	−	+	Focal	++	1.0	−	20	A&W(4.4/12)
23	62/M	Lt. Adrenal	+	−	−	−	Trabecular	Large polygonal small spindle	+	−	Focal	+++	1.75	Rare	20	A&W(4.4/12)
Paragangliomas ($N=6$)																
8	20/M	Posterior mediastinum	−	−	NA	+	Mixed	Small round large polygonal	+	−	Focal	++	1.96	Frequent	36	A&W(3)
9	29/M	Neck	−	−	NA	−	Diffuse	Large polygonal	−	−	Focal	+++	1.9	−	31	A&W(1.7/12)
12	32/F	Neck	−	−	NA	−	Trabecular	Small round	−	−	−	+	1.0	−	27	A&W(4.2/12)
14	39/F	Neck	−	−	NA	−	Alveolar	Small round	+	+	−	++	1.0	−	16	A&W(1)
24	64/M	Retroperitoneum	+	+	NA	−	Diffuse	Large& small polygonal	+	+	−	++	1.0	−	32	A&W(2.10/12)
25	65/F	Retroperitoneum	+	−	NA	−	Trabecular	Large& small polygonal	+	−	Focal	+	1.0	−	25	A&W(10/12)

NA: Not Available A&W: Alve & Well AWD: Alive with Disease

RESULTS

The clinical, biochemical, histopathologic and flow cytometric data of each patient are summarized in Table 1. The cases are arranged by adrenal and non-adrenal origin and by increasing age at diagnosis.

DNA Index of Tumors

Nuclear DNA histogram patterns of 25 patients with pheochromocytomas or paragangliomas including normal control adrenal gland are depicted in Fig. 1. Fifteen (60%) of the tumors showed DNA histograms that resembled the DNA histograms observed for non-tumor control samples of normal human adrenal glands and were defined as diploidy with DNA index of 1.0. Two (8%) of the patients showed a distinct DNA aneuploid peak with DNA index of 1.5 and 1.8 respectively. Eight (32%) of the tumors showed substantial increases (quantitatively greater than 20% of total nuclei) in and around the 4C peak and were defined as DNA tetraploidy with DNA index of 1.9 to 2.1. All aneuploid and tetraploid tumors had diploid lines. There was no hypertetraploidy. The distribution of DNA index in pheochromocytomas and paragangliomas is depicted in Fig. 2. In pheochromocytomas and paragangliomas, tetraploidy or near-tetraploidy occurred in 32% and 33% and aneuploidy in 10% and none respectively. A case of malignant pheochromocytoma had diploid DNA content.

DNA Index Related to Age and Sex of Patients

The distribution of DNA index related to age and sex of patients is depicted in Fig. 3. Of eight patients less than 20 years of age at diagnosis, seven (88%) were males, six with tetraploid tumors and one with a triploid tumor. The tumor of the single

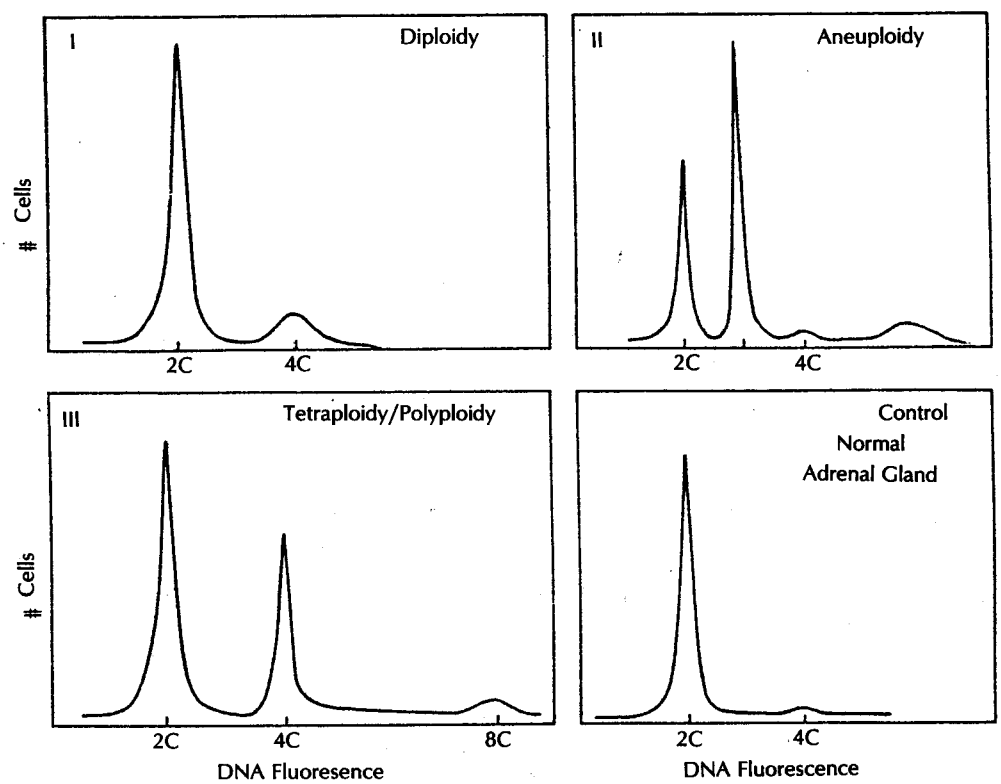

Fig. 1. Nuclear DNA histogram patterns of pheochromocytomas & paragangliomas.

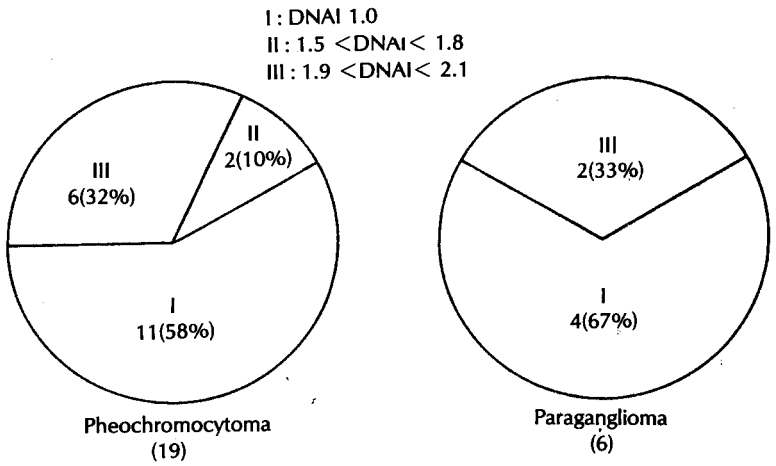

Fig. 2. *Distribution of DNA index (DNAI) in pheochromocytoma & paraganglioma.*

In pheochromocytomas and paragangliomas, tetraploidy or near-tetraploidy occurs in 32% and 33%, and aneuploidy in 10% and none respectively. A case of malignant pheochromocytoma has disloid DNA content.

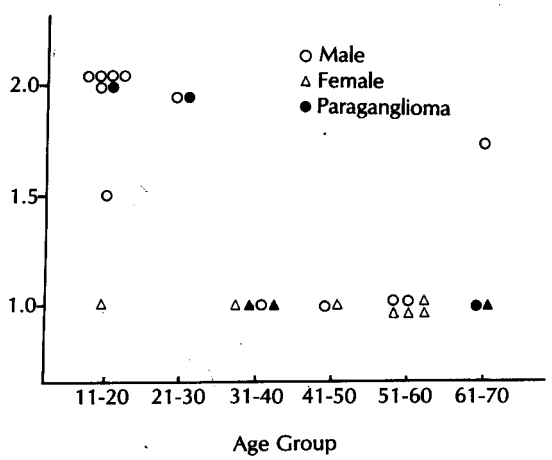

Fig. 3. *DNA index related to age & sex.*

All patients with hyperdiploid tumors are male and hyperdiploid tumors are significantly more common in patients less than 20 years of age ($p < 0.005$).

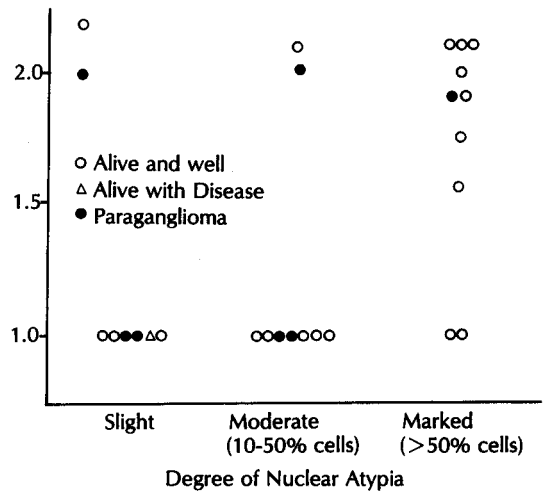

Fig. 4. *Degree of nuclear atypia related to DNA index of tumor.*

Marked nuclear atypia is significantly correlated with the hyperdiploid DNA content of the tumor ($p < 0.005$).

female in this group was diploid. Of ten patients with aneuploid or tetraploid tumors, nine (90%) were less than 30 years of age, and all were males. Hyperdiploid tumors were significantly more common in patients less than 20 years of age ($P < 0.005$) and in male patients ($P < 0.005$).

Degree of Nuclear Atypia Related to DNA Index of Tumor

Of ten tumors with marked nuclear atypia, eight (80%) had aneuploid or tetraploid DNA content,

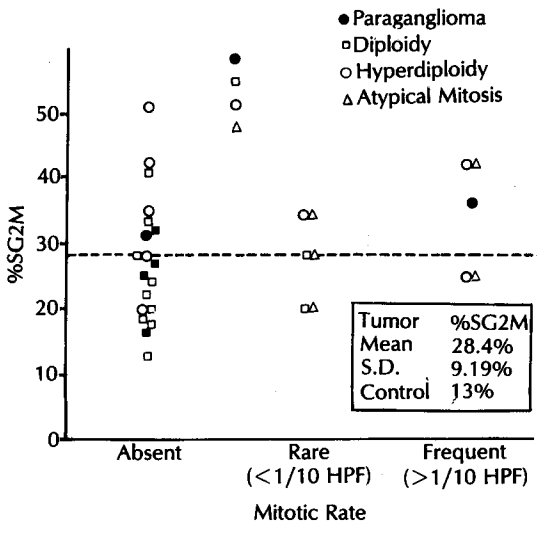

Fig. 5. *%SG2M related to mitotic rate and ploidy of tumor.*

Higher %SG2M is significantly correlated with hyperdiploid tumors ($p<0.01$) but not with mitotic rate of tumor.

and all six tumors with slight nuclear atypia had diploid DNA content (Fig. 4). Marked nuclear atypia is significantly correlated with the hyperdiploid DNA content of the tumor ($P<0.005$).

%SG2M Related to Mitotic Rate and Ploidy of Tumor

For the 25 tumors, the %SG2M ranged from 13 % to 51%, with mean and standard deviation (S.D.) of 28.4% and 9.19%, respectively. The mean value of %SG2M of control tissue was 13.0%. Of six tumors showing rare or frequent mitotic rate, five of which were combined with atypical mitosis, only three had more than the mean value (28.4%) of % SG2M cells. Of ten hyperdiploid tumors, seven contained more than the mean value (28.4%) of % SG2M cells. As indicated in Fig. 5, a higher %SG2M was significantly correlated with hyperdiploid tumors ($P<0.001$) but not with mitotic rate of tumor.

DISCUSSION

Most pheochromocytomas are benign tumors that can be treated by adrenalectomy. Approximately 10% of pheochromocytomas and as many as 40% of paragangliomas follow a malignant course demonstrated by gross local invasion or distant metastasis (van Heerden *et al.* 1982). However, the biologic behavior of adrenal pheochromocytomas and extra-adrenal paragangliomas cannot be predicted on the basis of histologic features.

Since flow cytometric techniques have been modified to estimate relative DNA content in cells including those of tumors and preneoplastic disorders of various organs, abnormal nuclear DNA content (aneuploidy) is an indicator of poor prognosis in a variety of neoplasms of adults (Barlogie *et al.* 1982; Dressler & Bartow, 1989; Jung *et al.* 1990). Pheochromocytomas and paragangliomas have not been previously studied extensively by flow cytometric nuclear DNA ploidy analysis. For the first time, Lewis (1971) studied the nuclear DNA content of pheochromocytomas by using Feulgen cytophotometry, demonstrating that patients having tumors with a diploid pattern followed a benign clinical course whereas aneuploid tumors had malignant behavior. Thereafter, Hosaka *et al* (1986) reported that DNA aneuploidy and tetraploidy/polyploidy were associated with 39% and 31% of malignancy respectively among pheochromocytomas, in contrast with one report (Amberson *et al.* 1987) indicating that aneuploid or tetraploid DNA content is a frequent occurrence in benign adrenal pheochromocytomas, which appears similar to ours.

In our small study, DNA tetraploidy or aneuploidy was frequently found in clinically benign pheochromocytomas and paragangliomas and the only one case of malignant pheochromocytoma with distant metastasis had diploid DNA content. It is not clear whether or not some tumors contained both diploid and aneuploid/tetraploid tumor cell clones. Since tissue blocks to be sampled were chosen for maximum purity of tumor cell populations, we believe that especially among aneuploid or tetraploid tumors, some may well have also included diploid tumor cell lines. For if a histogram contains two high diploid and aneuploid/tetraploid peaks in a sample composed of 90% tumor cells, both peaks must contain tumor cells. In addition, nuclear atypia defined as enlarged, hyperchromatic, sometimes bizarre nuclei was correlated significantly with a hyperdiploid DNA pattern that is believed to reflect abnormally increased DNA content. However, DNA content of tetraploid cells is theoretically equal to that of cells in the G2M phases of the cell cycle, which means that unlike aneuploid cells, tetraploid cells do not deviate from normal cells in

terms of cellular kinetics and are assumed to be metabolically less active to divide and proliferate than diploid or aneuploid cells. In conclusion, pheochromocytomas and paragangliomas share a propensity for tetraploidy with other endocrine tumors (Klein *et al.* 1985; Bronner *et al.* 1988) and the occurrence of aneuploidy or tetraploidy is frequent in clinically benign tumors in conjunction with a marked degree of nuclear atypia and cannot be a predictor of malignancy.

As shown in Fig. 3, hyperdiploid tumors occurred exclusively in males and younger patients. However, the association of young age and the male sex with hyperdiploidy might be a happenstance and further studies need to be established whether our findings are typical, even though there is a report that malignant paragangliomas were significantly more common in men (Linnoila *et al.* 1990).

Mitosis is very infrequently found in these tumors: 19 of 25 tumors show no mitosis. Three cases show rare mitotic activity, i.e. the mitotic count is less than one per ten high power fields, and three show frequent mitotic activity, i.e. more than one per ten high power fields. In various kinds of tumors (Gansler *et al.* 1986; Badalament *et al.* 1987; Bauer *et al.* 1987; Dressler *et al.* 1988; Kallioniemi *et al.* 1988), the percentage of cells in the S and G2 /M phases of cell cycle (% SG2M) showed a highly significant association with clinical outcome in conjunction with ploidy pattern. Whereas mitotic counting in histologic sections provides an imprecise estimate of the M compartment of cell cycle, flow cytometric DNA analysis rapidly and more precisely determines the percentage of cells in S and G2/M compartments, some of which cannot be discernible by light microscopy. In these tumors, mitoses are uncommon. This may be reflected by the fact that %SG2M is usually at or near "background" of control tissue levels and high %SG2M is not clearly a predictor of malignancy.

Histologically, alveolar (Zellballen) patterns similar to that of carotid body were infrequent (3 of 25 tumors), and diffuse patterns were most commonly observed in this series (11 of 25 tumors). It is possible that most adrenal or non-adrenal paragangliomas are originally of alveolar type but lose this pattern as they expand.

One report (Medeiros *et al.* 1985) indicated that necrosis, extensive and confluent in all malignant pheochromocytomas, was variable and focal in the benign ones. Additionally, all of the malignant tumors were composed of small cells. Only one case showing extensive tumor necrosis in our series was malignant, one which was composed exclusively of small polygonal cells. This confirms findings of other investigators (Hosada *et al.* 1976; Shapiro *et al.* 1984).

To evaluate the catecholamine function of the tumor, clinical symptoms and signs and laboratory data for biochemical function were investigated. Furthermore, the presence of periadrenal brown fat tissue was considered to be important, because it is suggested that catecholamine induced stress stimulates the appearance of brown fat (Melicow, 1957). Intracytoplasmic hyaline globules have been reported in normal and hyperplastic adrenal medulla as well as in pheochromocytomas and extradrenal paragangliomas. The significance of the hyaline globule is not known, but some investigators have associated them with secretory activity. In our study most of the tumors with hyaline globules turned out to have either clinicial or biochemical catecholamine function, and the hyaline globule is better than periadrenal fat in terms of predicting catecholamine function on a histologic basis.

In conclusion, neither ploidy pattern or %SG2M can be a predictor of malignancy in pheochromocytomas and paragangliomas, and the association of young age and the male sex with hyperdiploidy requires confirmation. This study provides new observations on the predictive value of certain flow cytometric and histologic parameters in clinically malignant pheochromocytomas and extra-adrenal paragangliomas.

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