Histopathological and Immunohistochemical Studies on the Adrenal Medullary Tumors in Egyptian Patients

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Abstract: The present study provides guide lines for the diagnosis of adrenal medullary tumors in Egyptian patients. This retrospective study included, 73 cases of adrenal medullary tumors (39 pheochromocytoma, 13 neuroblastoma, 12 ganglioneuroblastoma and 9 ganglioneuroma) admitted to Mansoura Urology and Nephrology Center, Egypt.. All tumors were studied histologically and immunohistochemically. In pheochromocytomas, 33 patients became normal after 24 hours, the other 6 died from distant metastases. 6 patients with neuroblastoma and ganglioneuroblastoma were still living after adrenalectomy, while the other 19 patients received chemotherapy and were non-living after 24 months. Nine patients with ganglioneuroma were still living after adrenalectomy. All prepared slides were stained with periodic-acid Schiff' reaction (PAS) and reticulin stains. Hyaline globules which were (PAS) positive were pheochromocytomas, while, they were not detected in neuroblastoma groups. All tumors were positive for reticulin stain. All cases of adrenal medulalry tumors were examined immunohistochemically using antibodies against chromogranin A, S-100 protein and neuron-specific enolase . Chromogranin A was expressed in all cases (39/39) pheochromocytoma, 5/13 neuroblastoma, 7/12 ganglioneuroblastoma and 7/9 ganglioneuroma. S-100 protein was expressed in 32/39 pheochromocytoma, 9/13 neuroblastomas, and all cases of ganglioneuroblastoma and ganglioneuroma. Neuron-specific enolase was expressed in all cases of pheochromocytoma, neuroblastoma, ganglioneuroblastoma and ganglioneuroma. The neuroendocrine tumors were stained with high specificity and sensetivity for the neuroendocrine markers; chromogranin A and neuron-specific enolase. Histomorphological features of benign and malignant pheochromocytomas may be similar. Neuroendocrine markers (chromogranin A, neuron-specific enolase) are useful in diagnosis of pheochromocytoma. Frequency of S-100 protein positive sustentacular cells is high in benign pheochromocytomas and low in malignant pheochromocytoma (our results suggest that, S-100 immunostaining is a useful marker to predict malignant behavior in pheochromocytoma. Intensity of neuron-specific enolase may be similar in both benign and malignant pheochromocytoma). No significant correlation was observed between expression of chromogranin A and neuron-specific enolase in pheochromocytoma and survival. The features of histopathological changes are the most important basis to make diagnosis for neuroblastomas group. Immunohistochemical staining can verify it further and play an important role in its differential diagnosis.

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1. Introduction:

Tumors in the adrenals originate from the adrenal cortex and medulla or as metastases from-extra adrenal primaries (Tatic *et al.* 2002). Differentiation between these three groups is the first task a pathologist to tackle when dealing with specimens from the adrenal region. The second great problem is the dignity of adrenal tumors, which cannot be determined in many adrenomedullary and some adrenocortical tumors. Immunostaining is helpful but the basic methods remain the histopathological examination of paraffin sections (Saeger, 2000). The adrenal medullary tumors include pheochromocytoma and neuroblastoma groups. They arise from chromaffin cells and neuroblast, respectively (Tatic *et al.*, 2002).

With the development of immunohistochemical techniques a greater understanding of abnormal neural and neuroectodermal differentiation was achieved (Achilles *et al.*, 1991). The developments of monoclonal antibodies have permitted the localization of single peptides and proteins that are produced by the cells of the tumor. However, it is a difficult to predict clinical outcome for patients with paraganglioma on morphologic backgrounds only. So, the use of monoclonal antibodies directed against S-100, NSE and chromogranin A can help in their localization and consequently their expression is

noticed in benign and malignant lesions (Feng *et al.*, 2005). As regard neuroblastoma group that are derived from primitive neuroblasts,these tumors can be conceptualized as three different maturational manifestation of a common neoplasm (Shimada *et al.*, 2004). The expression of S-100 protein is associated with good prognosis.

The present study has been carried out on the adrenal medullary tumors obtained from patients in Urology and Nephrology centre, Mansoura University, Egypt. The morphologic distiniction of adrenal medullary tumors is still difficult. Therefore, this study was conducted to:

1-Threw light on the origin of the different adrenal medulary tumors.

2-Study the clinical approachs of the patients infected by these tumors.

3-Differentiate between the different types of adrenal tumors using thehistological and immunohistochemical methods.

4- Investigate the expression of chief cell markers and sustentacular cell markers in the adrenal medullary tumors as pheochromocytoma and ganglioneuroblastoma groups.

5-Examine the diagnostic usefulness of immunohistochemical techniques in determining the presence of differentiation and maturation in case of neuroblastoma.

2. Patients and Methods

This retrospective study was carried out on 73 cases of adrenal medullary tumors (39 pheochromocytoma, 13 neuroblastoma, 12 ganglioneuroblastoma and 9 ganglioneuroma). These cases were obtained from Urology and Nephrology Center, Mansoura University, Egypt during the period from 1985-2002.

All patients were routinely investigated after hospitalization to evaluate the extent of tumor. The study included clinical examination, conventional laboratory investigation, intravenous urography, abdominal ultrasonography, chest x-ray to rule out pulmonary metastases and radio isotopic bone scan to exclude skeletal metastases. Also, computerized axial and tomography and radical adrenalectomy in all patients were performed. Clinical data included in this study were obtained from surgical requests including patients' age, sex and the size of each tumor.

Histopathological examination:

After radical of the adrenal medullary tumor, the resected tumor was fixed in 10% buffered formalin for 24 hours and processed for the preparation of paraffin wax blocks. These blocks were sectioned at 4-5 µm and were stained by the following methods:

1.Routine hematoxylin and eosin stain for general histological examination (Harris, 1900).

2.Periodic acid-Schiff (PAS) reaction for the detection of intracytoplasmic hyaline globules (Hotchkiss, 1948).

3.Reticulin stain by Gordon method for demonstrating the distribution of reticular fibers in the tumor tissues (Gordon and Sweets, 1936).

4.Moreover, immunohistochemical stainings were carried out using different monoclonal and polyclonal primary antibodies.

Immunohistochemical examination:

Immunohistochemical staining of 4-6 µm paraffin sections was performed on the Dako Autostainer (Dako corporation, Carpentaria, Calif) using streptavidin-biotin peroxidase method of Hsu et al. (1981). Histostain plus Kits (Zymed USA) which contain 10% non-immune serum biotinylated secondary antibody used in this study. These kits contain 10% non-immune serum, biotinylated secondary antibody and streptavidin-perxidase for antibodies against chromogranin A and Neuron specific enolase (monoclonal, 1:50, Zymed) and S-100 protein (polyclonal, 1:50; Zymed) for adrenal medullary tumor .

Immunohistochemical reagents:

Immunohistochemistry was performed by using a panel of three monoclonal primary antibodies and 2 polyclonal primary antibodies. A panel of 5 reagents (3 monoclonal and 2 polyclonal) was also used in this study. All reagents were mouse antibodies.

1- Chromogranin A:

This is a second generation of polyclonal mouse antibody. This antibody is especially designed for sensitive detection of chromogranin A. It is purchased from Zymed laboratories INC., So. San Francisco. It is a cytoplasmic marker as it presents in cytoplasm.

2- Neuron -specific Enolase:

This is a monoclonal mouse anti-NSE protein. It reacts predominantly with the gamma subunits of NSE of neuronal origin but not with alpha subunits of glial origin. It may cross react with beta subunits from muscle. It is purchased from zymed lab., INC., So. San Francisco. It is a cytoplasmic marker.

3- S-100 protein:

This S-100 protein (21-24Kda) is a mouse polyclonal antibody. S-100 stains almost all benign and malignant melanomas and their metastases. It is purchased from Zymed laboratories, INC, South San Francisco, Calif. It is a nuclear and cytoplasmic marker as it is present in both cytoplasm and nuclei.

Immunohistochemical procedure (Elias et al., 1989):

Semi serial sections were cut at 4-6 µm and deparaffinized with xylene, rehydrated with

descending grades of alcohols and immersed in deionized water.

Deparaffinized sections were incubated with 0.3% hydrogen peroxide in methyl alcohol to block the endogenous peroxidase activity for 30 minutes. Then, washed in phosphate-buffered saline (PBS) at PH 7.4 and incubated with non-immune serum. After application of the primary antibody, a biotinylated secondary antibody was added followed by the avidin-biotin-peroxidase complex. After each immunostaining procedure, sections were incubated with 3, 3´ diaminobenzidine-hydrogen peroxide substrate and counterstained with hematoxylin. Appropriate positive and negative control sections were also used.

Result of stain:

Immunohistochemical reactions revealed cytoplasmic brown stain in positive cases with the used antibodies. Accumulation of Cg A and NSE in tumor cells which take a brown color. Whereas these reactions revealed cytoplasmic and nuclear accumulation of staining with a brown color in case of S-100 protein especially in the sustentacular cells.

Follow up of patients:

The patients were followed postoperatively in the outpatient clinic for available period (mean = 47.46 month). This included, clinical examination, body weight and hypertensive state. Moreover, radiological follow up for local recurrence and distant metastasis in malignant cases was carried out.

3. Results

Histopathological examination of the excised adrenal medullary tumors revealed that these tumors could be divided into two main types, pheochromocytoma and neuroblastoma groups. The last group was subdivided, on some histopathological criteria, into neuroblastoma, ganglioneuroblastoma and ganglioneuroma.

Meanwhile, the pheochromocytoma was represented by 39 patients (32 benign and 7 malignant). Of the 39 patients, 21 were males and 18 females. A total number of 34 patients represented the neuroblastoma group. The number of patients was 13, 12, and 9 for NB, GNB and GN, respectively .

It could be observed that, the pheochromocytoma represents the high frequency, while ganglioneuroma represent the lowest one. In view of the fact that these tumors represent different clinical entities, their results will be individually displayed.

I- Clinical findings:

1- Pheochromocytoma:

The clinical data of the patients with pheochromocytoma is outlined in table (1). Thirty-nine cases of pheochromocytoma were examined clinically to illustrate the sex and age of patients. In addition, the size of the tumors was recorded. The patients were followed for a definite period. Most of them were died after a follow up period reaching 58 months.

Age distribution:

The age distribution among the patients with pheochromocytoma is outlined in table (1a). The age of patients ranged from 19 up to 61 years with an average age of 36.4 years. Most of the cases are in the 4^{th} and 5^{th} decade of life.

Sex distribution:

Sex distribution in patients is represented in table (1b). 39 cases of pheochromocytoma were 15 males with a percentage 38.46% and 24 were females with a percentage 61.54%. The male to female ratio was 1:1.6.

Tumor size

The tumor size in patients with pheochromocytoma is outlined in table (1c). The sizes ranged from 4 -14 cm. with an average size 8.543 cm.

2- Neuroblastoma and ganglioneuroblastoma:

The clinical data in patients with neuroblastoma represented by 13 patients and ganglioneuroblastoma represented by 12 patients is outlined table (2).

Age distribution:

The age distribution among the patients with neuroblastoma and ganglioneuroblastoma is outlined in Table (2a). The age of patients ranged from 4 months up to 44 years (average age 6 years). Most cases are in the first year of life.

Sex distribution:

Sex distribution among the patients with neuroblastoma and ganglioneuroblastoma are recorded in table (2b). There were 13 male with percentage 52% and 12 females with a percentage 48%. The male to female ratio is 1.08:1.

Tumor Size:

The size of tumor in 25 patients with neuroblastoma and ganglioneuroblastoma is recorded in table (2c). The size of tumors ranged from 9.5 to 20 cm. with the average size of 14.56 cm.

3- Ganglioneuroma:

Nine patients had ganglioneuroma; there were five males and four females. The male to female ratio was 1.25:1. The clinical data are shown in table (3).

Age distribution:

Age distribution in patients with ganglioneuroma is recorded in table (3). The age of the patients ranged from 15 up to 35 years with average value (23.2) years. Most of the cases are in the 2^{nd} decade of life.

Tumor size:

Tumor size was recorded in nine patients with ganglioneuroma with an average diameter of 11.7

cm. The size of tumors ranged from eight to 14cm (Table 3).

The outcome of patients:

All patients were followed postoperatively in the out patient clinic for available periods ranged from 11 to 192 months in case of pheochromocytoma (Table 1), from 16 to 52 months for NB and from 10 to 45 months for GNB patients (Table 2). The GN patients were followed in the outpatient clinic for a period ranged from 23 to 121 months (Table 3).

All patients with adenoma and GN are alive but in case of ACC 3 patients from 12 were alive while nine patients with a percentage of 66.7 % were died from distant metastases. In case of pheochromocytoma, 32/39 patients were alive during the follow up period while 7 cases were died. Moreover, 2/13 patients with NB and 4/12 cases with GNB are still alive after adrenalectomy. The patients of this study were examined clinically to determine the body weight, hypertension, monitoring blood pressure. Moreover, radiological investigations were carried out for local or distant metastases in malignant cases.

II- Histological Observations:

1- Pheochromocytoma:

The pheochromocytoma showed the typical cell nests (Zellballen pattern) of the chief parenchymal cells in 35 cases of the studied 39 cases (Fig. 1). Whereas, four cases only of the studied tumors showed spindling of the chief parenchyma cells (Fig. 2). Generally, the cytoplasm of the tumor cells is often lightly eosinophillic and finely granular. The nuclei were also lightly basophilic and possessed spherical shape with prominent nucleoli. Two cases of composite pheochromocytoma were studied (Fig. 3), spindle cell schwannian stroma admixed with pheochromocytoma. The sections of the tumor were stained with PAS and reticulin stains. The results of staining are recorded in table (4). This table showed that, the PAS positive intracytoplasmic hyaline globules were detected in most cases (28/39) of pheochromocytoma (Fig. 4). No hyaline globules were demonstrated in 11 cases of pheochromocytoma. Most cases of tumor were rich in reticular fibers (Fig. 5). Moreover, the results of reticulin staining showed highly rich stain ability (33/39), moderately rich (4/39) and weak stain ability (2/39) for the reticular fibers.

2- Neuroblastoma and ganglioneuroblastoma:

Neuroblastoma showed accentuation of fibro vascular stroma and tumor nodules formed of monotonous primitive cells (Fig. 6). Some areas suggest the formation of homer Wight pseudo rosettes. These rosettes appear as pale zones with fibrillar matrix corresponding to neuritic cell processes (Fig. 7). Sometimes, there are areas with a more diffuse or solid pattern with patches of calcification (Fig. 8).

Ganglioneuroblastoma showed patchy nodules of immature neuroblasts set within a full-grown Ganglioneuromatous stroma (Fig. 9).

From table (4), it can be observed that, no intracytoplasmic hyaline globules were detected in all cases of neuroblastoma and ganglioneuroblastoma (Figs. 10 and 11). Moreover, both NB and GNB were rich in reticular fibers (Figs. 12 and 13). These figures showed that, the reticular fibers are more abundant in case of Neuroblastoma.

3-Ganglioneuroma:

Microscopically, it can be observed that, two distinct cell groups were identified. The first is ganglionic cells and the second is Schwannian cells placed in an eosinophillic matrix (Fig. 14). From table (4), it can be observed that, no intracytoplasmic hyaline globules were detected in all cases of tumor (Fig. 15). Positive PAS reaction is observed in the stroma of ganglioneuroma. All cases of tumors are rich in reticular fibers (Fig. 16). The abundant reticular fibers are observed in between the tumor cells of GN.

III- Immunohistochemical observations:

Table (5) shows the immunoreactivity of the adrenal medullary tumors with Cg A, NSE and S-100 protein.

1- Expression of Cg A :

a- Pheochromocytoma:

Chromogranin A showed diffuse cytoplasmic staining in all chromaffine cells (Fig. 17). Cg A staining was consistently intense in most tumors, 34 cases of tumor showing intense staining pattern and 5 cases of tumor show moderate staining pattern. The two cases of composite pheochromocytoma (pheochromocytoma and ganglioneuroma) showed intense immunoreactivity for Cg A in pheochromocyte components (Fig. 18), whereas the other components for ganglioneuroma were negative for Cg A immunoreactivity (Table 5).

b- Neuroblastoma (NB):

In five cases of 13 Neuroblastoma Cg A expression was observed. It revealed intense immunoreactivity in one case of 13 neuroblastoma and the other four cases being moderately immunoreactive for Cg A. The other eight cases were completely negative for Cg A. The immunoreactivity of Cg A is detected as brown deposits in the fibrillar areas with overlapping inter twisting neuritic processes (Fig. 19).

c- Ganglioneuroblastoma (GNB):

The immunoreactivity of Cg A revealed intense cytoplasm staining in the large neuroblasts and

immunostaining in neuritic processes in five cases of tumors (Fig. 20). Two cases only gave weak immunoreactivity. The other five cases were completely negative for Cg A (Table 5).

d- Ganglioneuroma (GN):

The immunoreactivity of Cg A is noticed in the cytoplasm of the ganglionic cells that gave an intense reaction in five cases of tumors (Fig. 21). The other four cases were not expressed for Cg A giving negative results.

2- Expression of NSE:

a- Pheochromocytoma:

All pheochromocytoma were immunoreactive for NSE with different degree of staining as follow; 37 cases of tumors being intense, 2 cases showed moderate staining. The other two cases were negative for NSE immunoreactivity. The reactions appear as brown color and diffuse in the cytoplasm of the chief cells of tumor (Fig. 22).

b- Neuroblastoma (NB):

Expression of NSE has been observed in almost all the cell of NB. An intense reaction is seen in the cytoplasm of theses cells, whereas the stroma is unstained. It also can be observed in abundance in the neuritic process (Fig. 23). The staining was intense in 5 cases, moderate in also five cases and the other three cases being slight immunoreactivite for NSE.

c- Ganglioneuroblastoma (GNB):

Expression of NSE immunostaining was observed in abundant stromal septa. In addition, the cytoplasm of cell bodies is intensely positive. All cases of ganglioneuroblastoma (GNB) were intensely reactive for NSE (Fig. 24).

d- Ganglioneuroma (GN):

The immunoreactivity of NSE is observed in the ganglionic cells and Schwann cells of GN. These cells showed a wide range of immuno- reactivity. In addition, the stroma of GN was stained in a blue colour by haematoxylene as a counter stain (Fig. 25). Six cases of tumor showed intense immunostaining reactivity and the other three cases gave negative immunoreactions (Table 5).

3- Expression of S-100 protein:

a- Pheochromocytoma:

S-100 protein was absent in chromaffin cells but was present in the cytoplasm and nuclei of sustentacular cells surrounding chromaffin cells (Fig. 26). S-100 protein, was stained moderately to intensely in all cases. 27 cases were intensely stained and five cases showed moderate staining. The other seven malignant pheochromocytoma were negative for the immunoreactivity of S-100 protein.

b- Neuroblastoma:

The immunoreactions of S-100 protein are seen adjacent to the vascular septa. The cells of NB are consistent with Schwann or sustentacular cells that gave intense reaction. However, S-100 protein expression was observed in all cases of Neuroblastoma (Fig. 27). The staining was generally strong in 7 cases and the other 6 cases of tumor showed moderate immunoreactivity cells.

c- Ganglioneuroblastoma (GNB):

In GNB, the cells are in contact with ganglionic cells. These cells showed intense immunoreactive S-100 protein. They are in the typical location of satellite cells. All 12 GNB were positively stained for S-100 protein (Fig. 28) and the other three cases of tumor showed moderate immunoreactivity for S-100 protein.

d- Ganglioneuroma (GN):

S-100 protein expression revealed a brown staining in the cytoplasm of the ganglionic cells. In addition, an intense S-100 protein immuno reactivity was observed in the Schwann cells as well as in the matrix of GN. In the GN stained positively with S-100 protein, immunoreactivity was consistently present in nerve fibrils. All cases of tumors demonstrated a strong staining pattern for S-100 protein (Fig. 29).

Table (1): The clinical data of patients with pheochromocytoma

Case	Sex	Age (year)	Size (cm)	Follow up (months)	Status
1	F	36	5	192	S
2	F	43	7	120	S
3	M	35	8	96	S
4	F	58	8	82	S
5	F	21	10	94	S
6	M	19	8	86	S
7	F	30	10	81	S
8	F	36	11	78	S
9	F	43	13	72	S
10	F	40	14	66	S
11	M	28	7	58	S
12	F	60	5	58	D
13	M	53	4	55	D
14	F	45	4	71	S
15	F	52	7	51	S
16	M	41	6	49	S
17	F	35	6	47	S
18	M	46	7	44	S
19	M	46	5	42	S
20	F	54	13	38	D
21	M	36	10	37	S
22	F	24	11	32	S
23	F	34	8	30	S
24	M	61	7	28	D
25	M	20	11	26	S
26	F	25	13	24	S
27	F	35	7	24	S
28	F	24	6	21	S
29	F	19	9	20	S
30	F	42	11	19	S
31	M	44	4	17	D
32	F	19	7	15	S
33	M	44	6	14	S
34	F	20	5	13	S
35	M	42	12	11	S
36	F	34	14	24	S
37	F	54	12	58	D
38	F	30	11	36	S
39	F	37	12	48	S

Table (1a): Age distribution in patients with pheochromocytoma.

Age group	No. of patients	Percentage %
1-10	0	0%
11-20	5	12.8%
21-30	8	20.51%
31-40	10	25.64%
41-50	9	23.07%
51-60	6	15.38
Over 60	1	2.5%
Total	39	100.0

Table (1b): Sex distribution in the patients with Pheochromocytoma.

Sex	No. of patients	%
Male	15	38.46%
Female	24	61.54%
Total	39	100.0

Table (1c): Tumor sizes in 39 patients with pheochromocytoma.

Tumor size (cm)	No. of patients	%
0-5	6	17.1%
6-10	17	48.6%
11-15	16	34.3%
Total	39	100.0

Table (2): Clinical data of patients with neuroblastoma and ganglioneuroblastoma (25 cases)

Tumors	Case	Sex	Age	Size	Follow up	Status
I-Neuroblastoma	1	F	4 m	10	30	D
	2	M	3 y	9.5	23	D
	3	M	4 y	17	19	D
	4	M	7 m	17	20	D
	5	M	10 m	10	18	D
	6	M	3 y	13	24	S
	7	M	7 m	17	26	D
	8	M	3 y	12	52	D
	9	F	6 m	17	16	S
	10	F	11 m	20	22	D
	11	F	1 y	13	60	D
	12	F	9 m	10	96	D
	13	F	5 y	12.5	20	D
Ganglioneuroblastoma	14	F	4 y	14	23	D
	15	M	44 y	16	20	D
	16	M	3 y	10	30	D
	17	M	10 m	11	16	S
	18	F	8 m	18	24	D
	19	M	43 y	12	45	D
	20	M	5 y	13	24	D
	21	F	2 y	15	12	S
	22	F	16 y	10	10	S
	23	F	3 y	17	17	D
	24	M	1 y	20	24	D
	25	F	4 y	18	17	S

y= Year m=Months, S= survival, D= died

Table (2a): Age distribution in patients with neuroblastoma and ganglioneuroblastoma.

Age group	No. of patients	%
1 month- 11 m	9	36%
1 y-10 y	13	52%
11 y-20 y	1	4%
21 y-30 y	0	0
31 y-40 y	0	0
41 y-50 y	2	8%
Total	25	100%

Table (2b): Sex distribution in (25) patients with neuroblastoma and ganglioneuroblastoma

Age sex	No. of patients	%
Male	13	52%
Female	12	48%
Total	25	100.0%

Table (2c): Tumor size in (25) patients with neuroblastoma and ganglioneuroblastoma.

Tumor size	No. of patients	%
0.5	0	0
6-10	6	20%
11-15	9	36%
16-20	10	40%
Total	25	100.0

Table (3): Clinical data of 10 patients with ganglioneuroma

Case	Sex	Age (years)	Size (Cm)	Follow up (Months)	Status
1	F	20	13	30	S
2	M	19	14	23	S
3	M	31	8	23	S
4	M	35	13	31	S
5	M	17	10	18	S
6	F	24	12	121	S
7	F	28	15	46	S
8	M	15	11	52	S
9	M	20	10	36	S

Table (4): Results of PAS and Reticulin stains for adrenal medullary tumors (AMT).

Stain / Tumor	PAS		Reticulin stain		
	+ve	-ve	high +2	low +1	-ve -
Adrenal medullary tumor					
- Pheo.	28/39	11/39	33/39	6/39	-
- NB	-	13/13	13/13	-	-
- GNB	-	12/12	12/12	-	-
- GN	-	9/9	9/9	-	-

+ve Positive **-ve Negative**

Table (5): The immunoreactivity of AMT with Chromogranin A (Cg A), Neuron-specific enolase (NSE) and S-100 protein.

Stain / AMT	Intensity for Cg A +3 +2 +1 -ve	Intensity for NSE +3 +2 +1 -ve	Intensity for S-100 +3 +2 +1 -v
- Pheo.	34/39 5/39 -	37/39 2/39 -	27/39 5/39 -
- NB	-	-	7/39
- GNB	1/13 4/13 -	5/13 5/13	7/13 6/13 -
- GN	8/13	3/13 -12/12	-
	5/12 - 2/12	- - -	9/12 3/12 -
	5/12	6/6 3/9 -	-
	5/9 - -	-	9/9 - -
	4/9		-

+ 3 intense immunoreactivity. + 2 moderate immunoreactivity. + 1 weak immunoreactivity. - ve negative immunoreactivity.

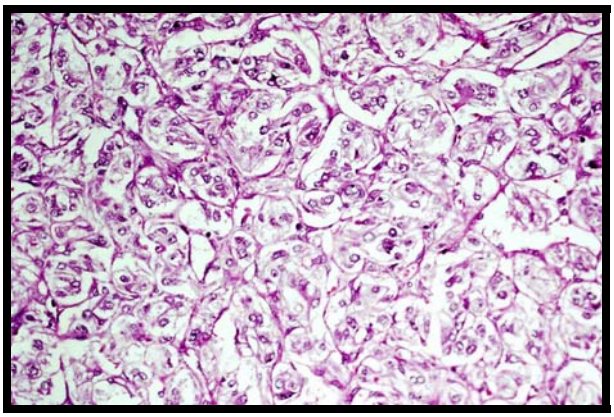

Fig. (1): Photomicrograph of adrenal pheochromocytoma showing the typical nesting pattern (Zellballen appearance). (Hx & E X 200).

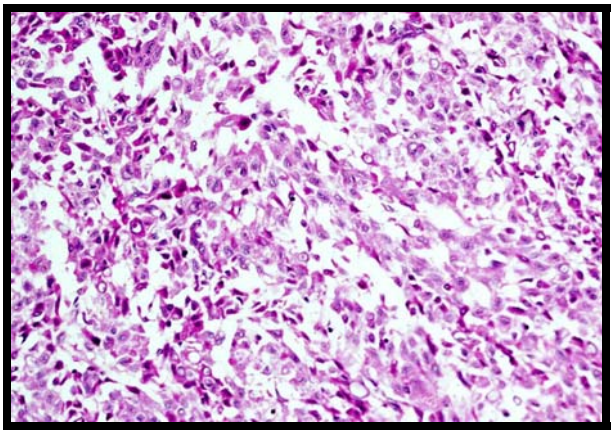

Fig. (2): Photomicrograph of adrenal pheochromocytoma, spindle cell pattern. (Hx & E X200).

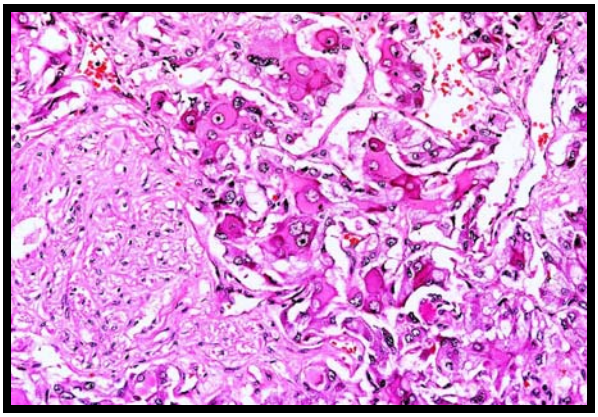

Fig. (3): Photomicrograph of composite pheochromocytoma showing spindle cell schwannian stroma admixed with pheochromocytoma. (Hx & E X 200).

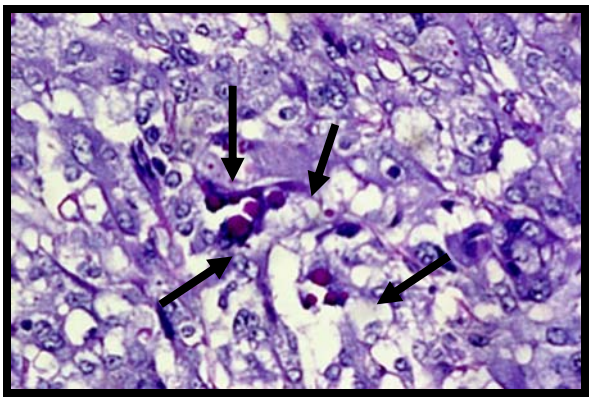

Fig. (4): Photomicrograph of pheochromocytoma showing intra-cytoplasmic hyaline globules (arrows). (PAS x400).

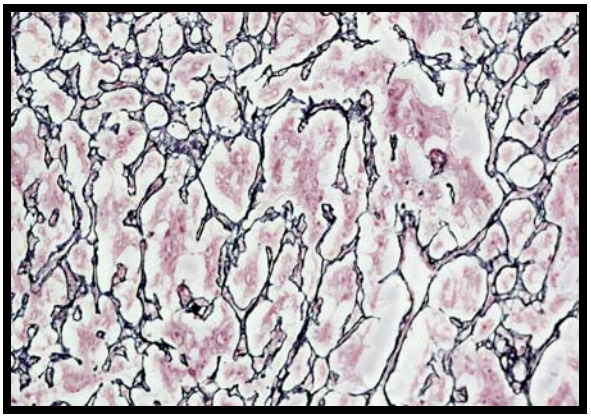

Fig. (5): Photomicrograph of pheochromocytoma showing accentuated alveolar pattern bounded by condensation of reticular fibers.(Gordon stain X 200).

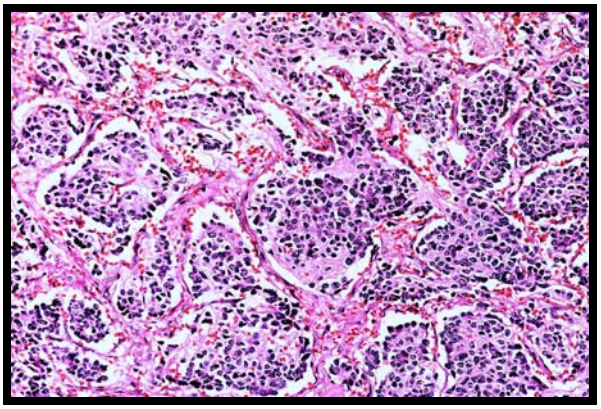

Fig. (6): Photomicrograph of neuroblastoma showing accentuation of fobrovascular stroma and tumour nodules formed of monotonous primitive cells. (H x & E X 200).

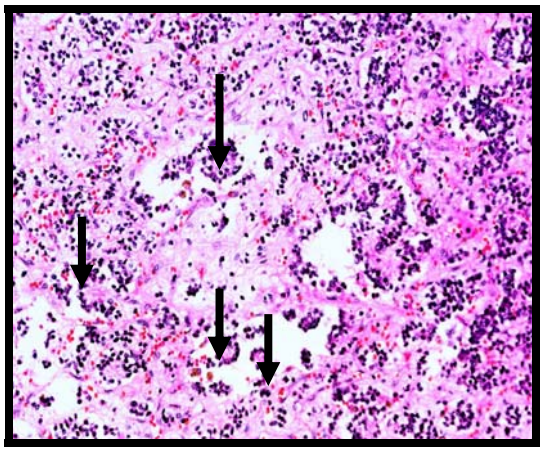

Fig. (7): Photomicrograph of neuroblastoma showing numerous Homer Wright rosettes. (H x & E X 200).

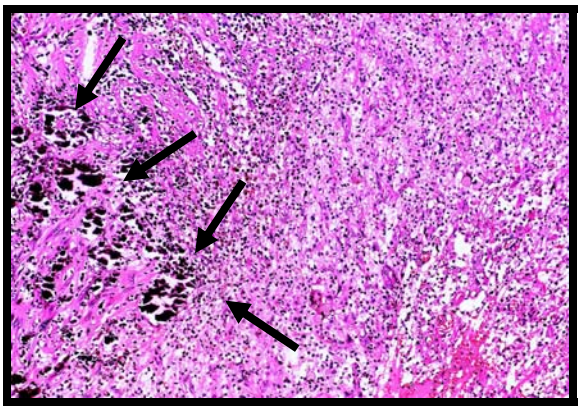

Fig. (8): Photomicrograph of neuroblastoma showing patches of calcification. (Hx & E X 100).

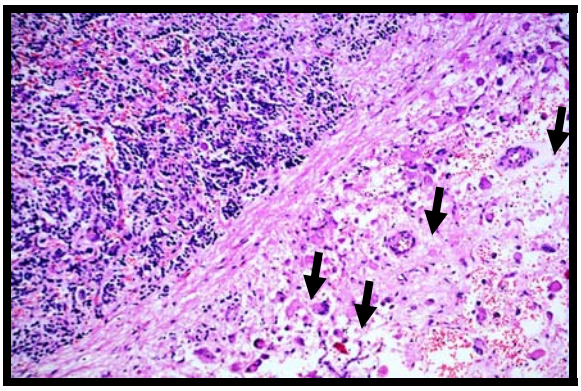

Fig. (9): Photomicrograph of ganglioneuroblastoma showing patchy nodules of immature neuroblasts set within a mature ganglioneuromatous stroma arrows.(Hx& E X100).

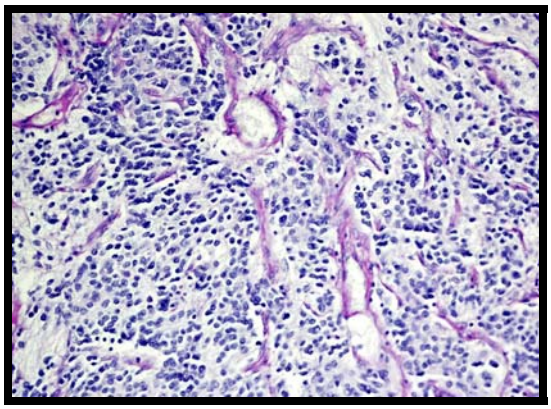

Fig. (10): Photomicrograph of neuroblastoma showing no intracytoplasmic hyaline globules. (PAS stain X 200).

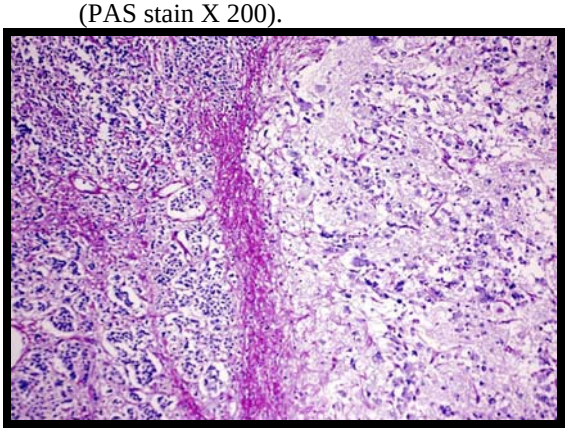

Fig. (11): Photomicrograph of ganglioneuroblastoma showing no intracytoplasmic hyaline globules. (PAS stain X 100).

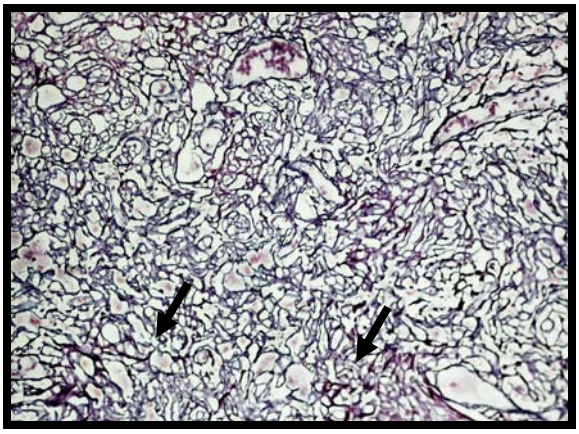

Fig. (12): Photomicrograph of neuroblastoma showing abundant reticular fibers in tumour cells. (Gordon stain X 200).

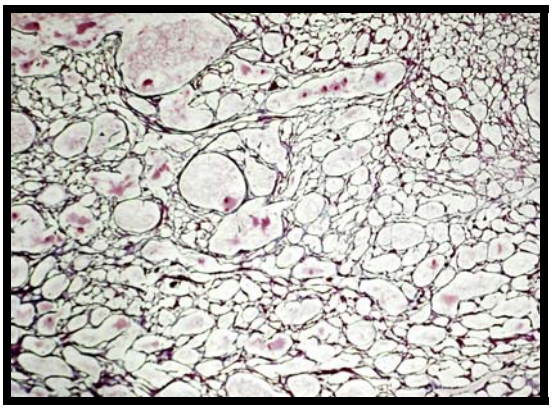

Fig. (13): Photomicrograph of ganglioneuroblastoma showing abundant reticular fibers in tumour cells. (Gordon stain X 200).

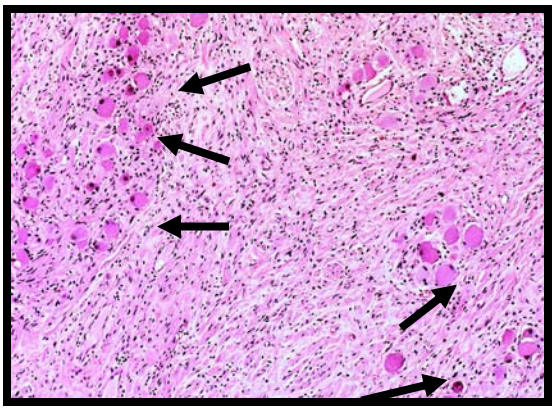

Fig. (14): Photomicrograph of ganglioneuroma showing mature ganglion cells (arrows) in Schwannian cell dominant stroma.(H & E X200).

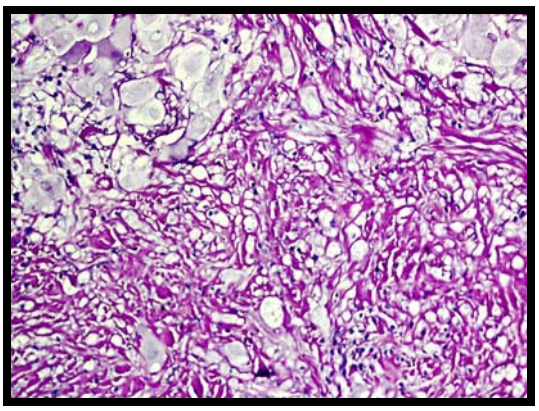

Fig. (15): Photomicrograph of ganglioneuroma. Note that the intracytoplasmic hyaline globules are not detected in tumour cells.(PAS stain X 200).

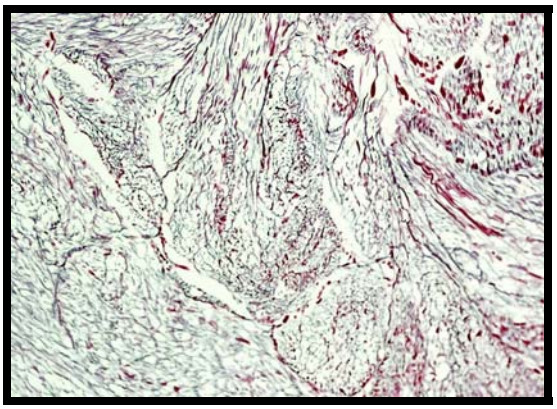

Fig. (16): Photomicrograph of ganglioneuroma showing abundant reticular fibers in tumour cells. (Gordon stain X 100).

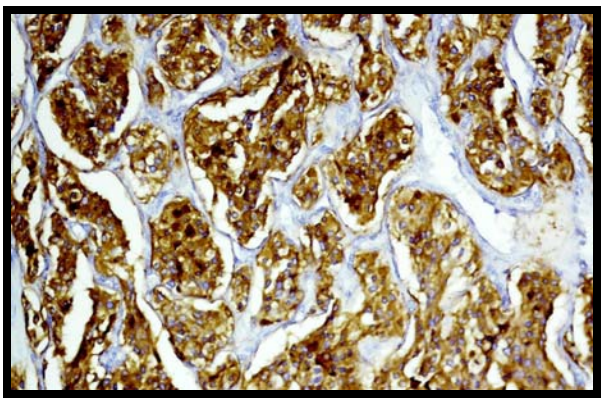

Fig. (17): Photomicrograph of pheochromocytoma showing intense cytoplasmic immunoreactivity of chromaffin cells for Chromogranine (The brown color) (Immunoperoxidase X 200).

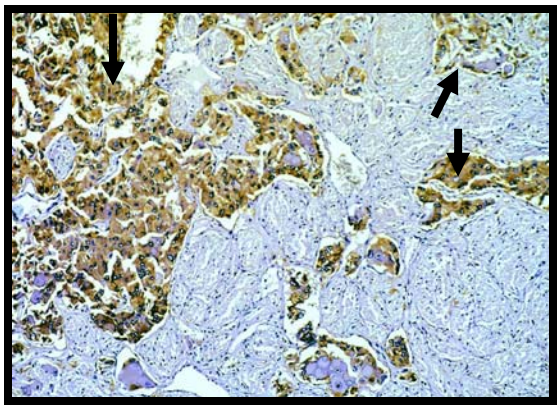

Fig.(18):Photomicrograph of composite pheochromocytoma showing intense cytoplasmic immunoreactivity for Chromogranine within the chromaffin cells (arrows). (Immunoperoxidase X 200).

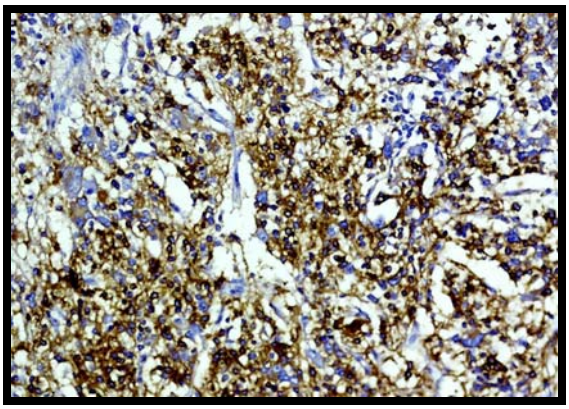

Fig.(19): Photomicrograph of neuroblastoma . immunoreactivity for chromogranin A was seen in brown fibrillar areas with overlapping interwining neuritic processes (brown color) (Immunoperoxidase X 200).

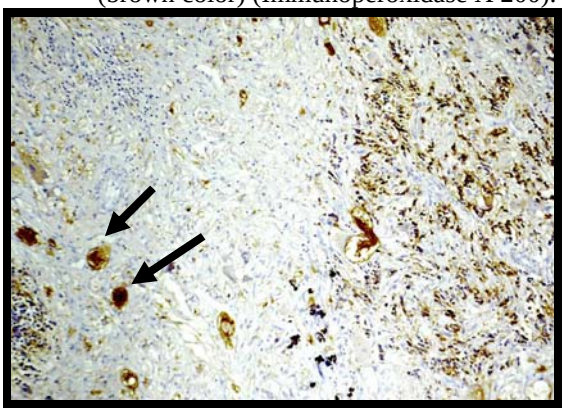

Fig. (20): Photomicrograph of ganglioneuroblastoma, Immunohistochemical analysis directed to chromogranin A. Note the large neuroblasts with intense cytoplasmic staining also staining in processes. (Immunoperoxidase X 100).

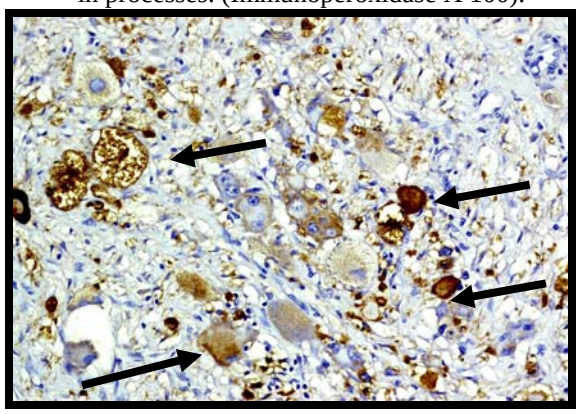

Fig. (21): Photomicrograph of ganglioneuroma showing immunoreactivity for chromogranin A. Note ganglion cells(arrows) are strongly stained. (Immunoperoxidase X 200).

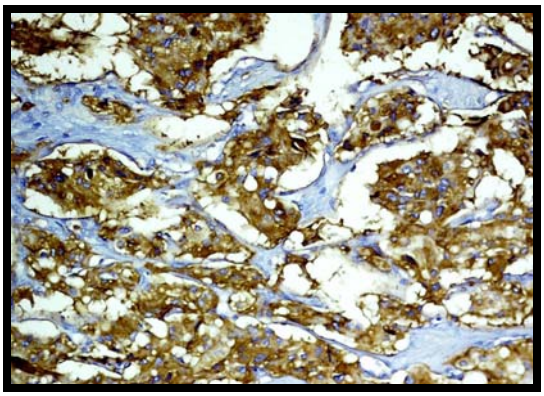

Fig. (22): Photomicrograph of pheochromocytoma showing immunoreactivity for neuron specific enulase, Note the diffusely strong staining within the cytoplasm of Chief cells. (Immunoperoxidase X 200).

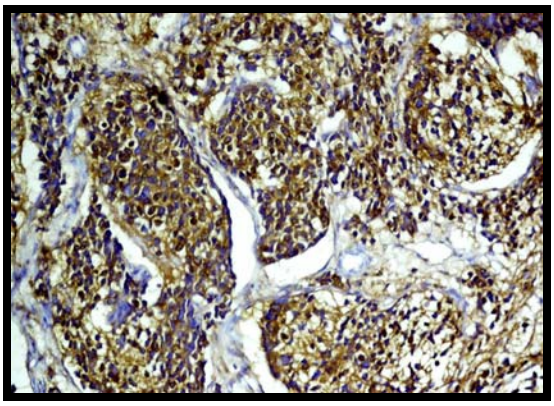

Fig. (23): Photomicrograph of neuroblastoma stained for neuron specific enulase. Almost all of the cells show a positive cytoplasmic reaction, whereas the stroma is unstained. (Immunoperoxidase X 200).

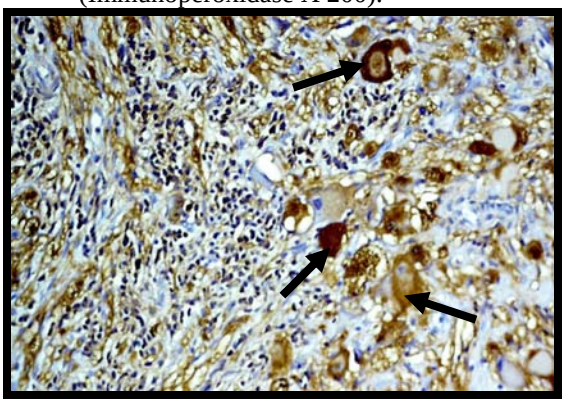

Fig. (24): Photomicrograph of ganglioneuroblastoma stained for neuron specific enolase, highlights abundant stromal septa. The cytoplasm of ganglion cells (arrows) is also intensely positive.(Immunoperoxidase X 200).

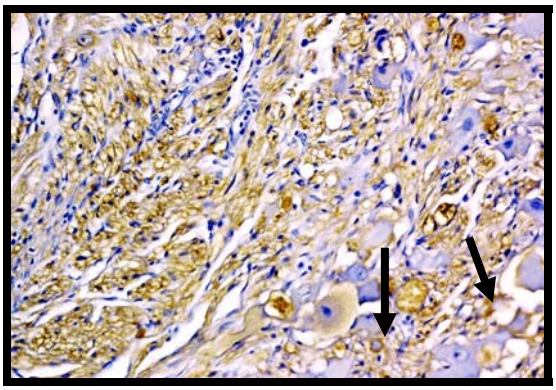

Fig. (25): Photomicrograph of ganglioneuroma showing immunoreactivity for NSE. Ganglion cells are strongly stained (arrows). Schwann cells are also stained positively. (Immunoperoxidase X 200).

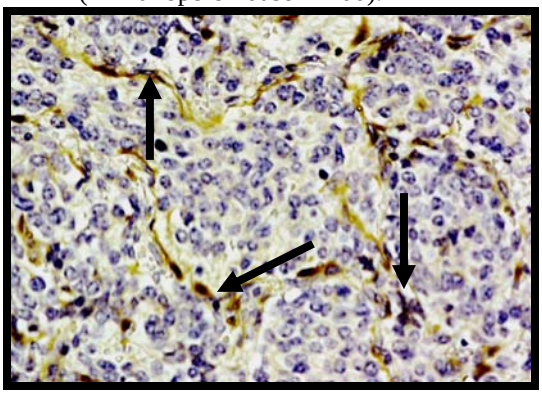

Fig. (26): Photomicrograph of pheochromocytoma showing immunoreactivity for S-100 protein in sustentacular cells (arrows) surrounding the chromaffin cells. (Immunoperoxidase X 200).

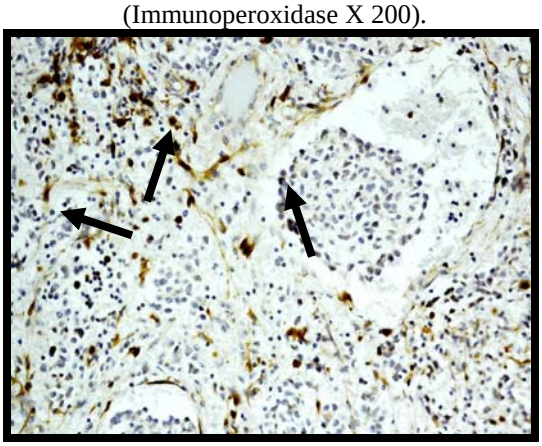

Fig. (27): Photomicrograph of neuroblastoma showing satellite to dendritic cells immunoreactive for S-100 protien are adjacent to vascular septa (arrows). Cells are consistent with Schwann or sustenticular cells. (Immunoperoxidase X 100).

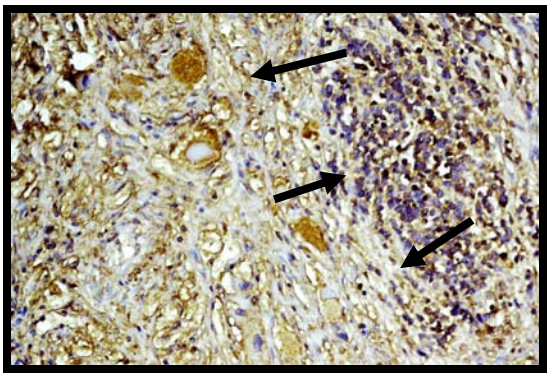

Fig. (28): Photomicrograph of ganglioneuroblastoma, immunostained for S-100 protein. Cells in intimate contact with ganglion cells show intense staining (arrows), and are in the typical location of satellite cells. (Immunoperoxidase X 100).

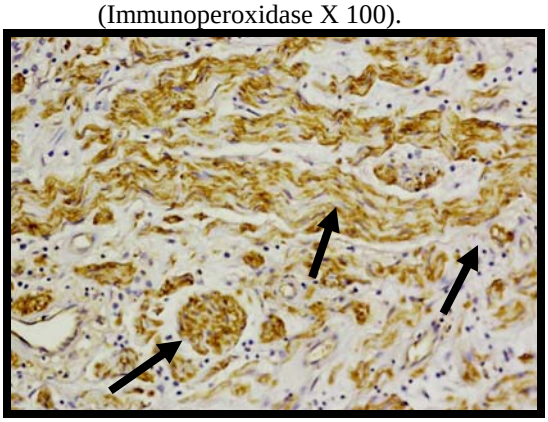

Fig. (29): Photomicrograph of ganglioneuroma showing immunoreactivity for S-100 protein, revealed cytoplasmic stain of the ganglion cells (arrows). Matrix and schwann cells were also strongly positive. (Immunoperoxidase X 100).

4. Discussion

a- Pheochromocytoma:

Pheochromocytomas are rare tumors of the adrenal medulla or the paraganglion system. There are no histological or immunohistochemical markers available that define the malignant behavior of these tumors, so for only the discovery of metastases reveals malignancy (Salmenkivi *et al.*, 2001 and David *et al.*, 2004). Thus, 39 pheochromocytomas (32 benign and 7 malignant) were analyzed histomorphologically and immunohistochemically. The patients were between 19 and 61 years of age (average age 36.4 years). Most of the cases are in 4th and 5th decade of life and there were 24 females (61.5%) and 15 males (38.4%). The largest pheochromocytoma had the diameter of 14cm. In this respect, Lack (1994) documented that, the peak age at diagnosis is in the 5th decade of life, but

pheochromocytomas can affect any age group. Most progressive information revealed a roughly equal sex incidence, but some show a slight predilection for both male and female (Tatic *et al.,* 2002). However, they reported that, the incidence of pheochromocytoma is similar in both sexes and most frequent between the ages of thirty and fifty. The largest pheochromocytoma had a diameter of 12 cm. In this study, histological examination of pheochromocytomas showed that the cells appeared with significant irregularities in shapes , dimensions and their patterns. Pheochromocytes cells were mostly of polygonal shape (35 cases of 39); whereas in the other four cases spindling cells were evident. This result is in agreement with other international published studies reported by Saeger *et al.* (2003). They reported that, histological criteria for aggressive biological behavior of pheochromocytoma include limited pattern of growth, 3-5 mitotic figures on 10 microscopic high power fields and invasion of a capsular lymphatic and blood vessels.

In the present study, the intracytoplasmic hyaline globules which are periodic acid-Schiff positive were detected in 26 of 32 clinically benign and only 2 of 7 malignant pheochromocytoma. Dekker and Oehrle (1971) in their study reported that there might be intracytoplasmic hyaline globules that are periodic acid-Schiff (PAS) positive and resistant to diastase pre- digestion. These globules are identical to those observed in the normal and hyperplastic adrenal medulla. In the meantime, hyaline globules were detected in 38 of 64 clinically benign and only eight of 34 malignant sympathoadrenal paragangliomas (Linnoila *et al.,* 1990). It has been found that these globules are related to secretory activity in some way, but their functional significance is not clear (Mendlesohn *et al.,* 1978). All cases of pheochromocytoma in the present study were positive for reticulin stain. This stain for reticulin clearly accentuates the alveolar anatomizing trabecullar patterns with distinct nests of cells (Zellballen appearance).

All the studied pheochromocytomas (39 benign and 7 malignant) showed chromogranin A and neuron specific enolase immuno-positive reaction in nearly all tumor cells. Similarly, Pace *et al.* (2002) and Feng *et al.* (2005) reported that all pheochromocytomas were strongly immunoreactive for chromogranin A and neuron-specific enolase. In our study, there is no differences between expressions of Cg A in malignant or in benign pheochromocytoma. Whereas, Feng *et al.,* (2005) concluded that, there was statistically significant difference of Cg A expression between ACT and AMT and also between benign and malignant pheochromocytoma.

The results of the present study revealed that, the intensity of staining for neuron specific-enolase is supreme in cases comprising benign and all malignant pheochromocytoma. This result is different with Kliewer *et al.* (1987). They showed that, the intensity of staining of neuron-specific enolase is furthermost (strong) in benign paragangliomas and weak (faint) in malignant tumors. Tatic *et al.* (2002) reported that, immunohistochemical analysis has confirmed the importance of pan-neuron endocrine markers (Cg A and NSE) in pheochromocytoma identification. Pheochromocytomas have a multiple synthetic activity as chief neuroendocrine feature.

Giovanella (2005) reported that, chromogranin A is a member of the granin family contained in secretory vesicles of chromaffin adrenal cells. Despite pheochromocytoma tumor cells heterogeneity chromogranin A and neuron-specific enolase are the most common neuoropeptides synthesized. They are associated with the presence of neuroendocrine storage granules. Report of Moreno *et al.* (1999) showing the presence of neuron-specific enolase in all pheochromocytomas extended our initial observation on the localization of neuron-specific enolase in pheochromocytomas and other neuroendocrine tumors.

The detection of neuron-specific enolase in pheochromocytomas can be helpful in cases in which the differential diagnosis between pheochromocytomas and other tumors, such as ACC, may be a diagnostic problem although the histological features of pheochromocytomas occurring in patients with malignant and benign tumors are variable. These neuroendocrine tumors all possess a distinguishing histochemical marker that is the neuron-specific enolase.

Giovanella (2005) and Feng et al. (2005) showed that, the expression of chromogranin A was strongly positive in chief cells that contained granins in their vesicles. These results correspond to our results in two cases of composite pheochromocytoma in which the component of pheochromocytes are strongly positive for chromogranin A and neuron-specific enolase. The other components of ganglioneuroma were positive for chromogranin A in some ganglion cells and were positive for neuron-specific enolase. Since, in this material, staining for S-100 protein was restricted to dendritic cells which are located at the periphery of the trabecullar or solid main cell clusters, all such S-100 immunoreactive elements were assumed to represent sustentacular cells. S-100 positively of sustentacular cells in paragangliomas has been reported in several studies covering adrenal and extra-adrenal sympathetic neoplasms (Guido Belleza *et al.,* 2004). The present work showed that, S-100 protein positive

sustentacular cells were found in 32 of 39 benign pheochromocytomas in immunohistochemical assessment. It can be observed that, complete loss of sustentacular cells was in malignant pheochromocytoma (7 of 39 cases). Achilles *et al.* (1991) discovered that, S-100 protein staining of sustentacular cells was seen in both adrenal and extra-adrenal lesions. The 10 malignant tumors are entirely devoid of S-100 protein positive cells. Therefore, the presence of S-100 protein positive cells may help to exclude malignancy in individual pheochromocytoma cases. Achilles *et al.* (1991) stated also that, the immunohistochemical staining for S- 100 protein has been designated on appropriate tool to further subclassify paragangliomas, at least in the case of adrenal pheochromocytoma.

b- Neuroblastoma and ganglioneuroblastoma:

The age of patients was between 4 months and 44 years of age (average age 6 years). Most of the patients occur in the first 4 years of life and they were 12 females (48%) and 13 males (52%). Size of tumors ranged from 9.5-20 cm with the average diameter of 14.5cm. In this respect, Young *et al.* (1986) reported that, 85% of all neuroblastoma and ganglioneuroblastoma cases occur in the first 4 years of life and there are no sex-related differences in incidence rate. Koike *et al.* (2003) reported that, adrenal ganglioneuroblastoma is extremely rare in adults. The present study revealed that 19 patients with neuroblastoma and ganglioneuroblastoma are still living after adrenalectomy, while six of the cases received chemotherapy and none was living after 24 months. Evans *et al.* (1996) reported that, neuroblastoma at different stages has an 80% of survival rate and no evidence indicates that chemotherapy improves survival.

Torrisi *et al.* (2003) reported that, there are obvious difficulties in determining the incidence of neuroblastoma giving its capacity for regression, maturation and early death. Treatment complications prohibit reproduction and prevent multigenerational pedigrees for evaluation. Bundy *et al.* (1982) indicated essentially no risk of offspring of surviving patients with neuroblastoma or ganglioneuroblastoma developing similar tumors. Koike *et al.* (2003) studied a case of adult type ganglioneuroblastoma and they observed that, there has was no evidence of recurrence for 2.5 years after the operation.

In the current results, histological appearance of neuroblastoma showed accentuation of fibrovascular stroma and tumor nodules formed of monotonous primitive cells. Sometimes, there are areas with a more diffuse or solid pattern, while ganglioneuroblastoma showed a patchy nodules of immature neuroblasts set within a mature ganglioneuromatous stroma. Shimada *et al.* (1984) and Lack *et al.* (1990), reported that, neuroblastoma and ganglioneuroblastoma often have a lobular growth pattern with delicate, often incomplete fibrovascular septa. Sometimes there are areas with a more diffuse or solid pattern. Hachitanda *et al. (1994)* showed that, a distinct organoid pattern has been recently described in which a thin fibrovascular meshwork isolates regular nest of neuroblastoma cells. This is reported to be an indicator of good prognosis in younger children.

c- Ganglioneuroma:

The patients were between 15-35 years of age (average age 23.2 years) and there were four female and five males. Size of tumors range from eight to 15cm with the average diameter of 11.7 cm. In general, survival in our population was excellent with no deaths seen at a mean follow up of 42.5 months (range 18-121 months*).*

Bove and Mcadams (1981) reported that, ganglioneuroma usually occur in an older age group than neuroblastoma and showed that many patients are 7 years. Nelims *et al.* (2004) showed in some cases, ganglioneuroma was the end stage of maturation of less differentiation tumors such as NB and GNB. It was based on the age at diagnosis over 10 years of ages and the anatomic location of these tumors appears to arise de novo.

The present work revealed that, two distinct cell groups were identified, ganglion cells and Schwann cells placed in a loose myxoid stroma. In this respect, Torrisi *et al.* (2003)found that, the tumor of ganglioneuroma consisted of proliferation of well differentiated Schwann cells and ganglion cells in the lamina properia.

In the present study, special stains such as periodic acid Schiff reaction (PAS) and reticulin stain were applied on all cases of neuroblastoma groups (Neuroblastoma, ganglioneuroblastoma, and ganglioneuroma). The results showed that, most of cases were rich in reticular fibers in tumor cells but there was no hyaline globules in neuroblastoma group. Triche *et al.* (1987) were be able to support this result. They reported that, neuroblastomas are usually glycogen-poor and PAS negative. This property is classically used to distinguish neuroblastoma from Ewing's tumor.

In this study, it can be concluded that, the tumor cells, in a large number, of neuroblastoma groups (NB, GNB and GN) were positively stained by several neuroendocrine antibodies as well as by antibodies specific for chromogranin A, neuron-specific enolase (NSE) and S-100 protein.

Examination of chromogranin A showed that, 19 of 30 neuroblastoma groups were chromogranin A-positive (5 cases NB, 5 cases GNB and out of 9 cases GN). This result can be compared to those of

Douglas *et al.* (1994) who reported that, five of six pure neuroblastoma were reacted positively with chromogranin A. In addition, Molenar *et al.* (1990) showed that, immunostaining for chromogranin A has been demonstrated in neuroblastomas, ganglioneuroblastomas and ganglioneuromas. Positive results are expected where the concentration of neurosecretory granules is greatest such as ganglionic cells with active synthesis or areas with overlapping or intertwining of numerous neuritic processes (e.g., Homer Wright rosettes) or matted neuropil. Eder *et al.* (1998) studied several cases for neuroblastomas and ganglioneuroblastomas and they reported that chromogranins are a class of acidic proteins found in large secretory granules of neuroendocrine tissues and tumors derived from them.

Tornoczky *et al.* (2004) reported that, both neuronal cells expressed immunoreactivity for chromogranin A and neuron-specific enolase. Small neuronal cells showed more intense chromogranin A immunoreactivity, indicating an earlier stage of neuronal differentiation. Liu *et al.* (2003) showed neuron specific enolase and chromogranin A were expressed in small cells in neuroblastomas. Other authors often showed positive immunoreactivity for multi-neural markers such as chromogranin A, synaptophysin and neuron-specific enolase but were negative for S-100 protein, CD34 Desmin and CD45 in 8 cases of neuroblastomas (Hasegawa *et al.*, 2001). The results of the present study revealed that, four cases of ganglioneuroma stained with chromogranin A were negative and 5 cases were positive in cortical tissues. Kazantesva *et al.* (2002) confirm our results when they observed chromogranin A in cells of cortical adenoma and in 20-75% of carcinoma cells. These ultramicroscopically findings were confirmed by the observation of typical neuroendocrine granule. In this study, it can be concluded that, the tumor cells of a large number of neuroblastomas are positively stained by antibodies specific for neuron- specific enolase. All neuroblastomas irrespective of their degree of differentiation stained positively with the neuron-specific enolase antibody. NSE stained both cytoplasm in precursor cells and nerve processes in neuroblastomas.

Thomas *et al.* (1987) studied the immunostaining of neuron-specific enolase in neuroblastomas. They found that, immunostaining for neuron- specific enolase is usually apparent in neuroblastoma and ganglioneuroblastomas, with highlighting of neuritic extensions in the form of rosettes, matted neuropil or sparse internuclear fibrillar matrix.

In the present study, the immunohistochemical analysis of the adrenal neuroblastomas directed to S-100 protein revealed cytoplasmic staining of the ganglion cells in ganglioneuroma. The matrix and Schwann cells were also positive for S-100 protein. While in ganglioneuroblastoma, the cytoplasm of some cell bodies was also intensely positive for S-100 protein. Immunostaining for S-100 protein may demonstrate small to elongate dendritic-shaped cells in NB and GNB, particularly in fibro- vascular septa separating the tumor cells. These have been regarded as Schwann cells or precursor cells, suggesting possibility of differentiation within the tumor. Pace *et al.* (2002) in his study found that, matrix and schwann cell were positive for S-100 and for glial fibrillary acidic protein. Aoyama *et al.* (1990) suggested that, immunoreactivity for S-100 protein has been used to evaluate differentiation of neuroblastoma cells into Schwann cells, and may provide some information regarding prognosis, even though it is not entirely specific for Schwann cells. Liu *et* al. (2003) reported that, S-100 protein was positive in the area of bunch of neurofibrils. The present results also keep in toutch with Wallace *et al.* (2003) who found the spindle cell component stained positively for S-100 protein in ganglioneuroma. The ganglion cells in the studied tumors were stained positively with S-100 protein. On the contrary, this result is dissimilar with the last authors who reported that, the ganglion cells were unstained positively with S-100 protein but were stained positively for glial fibrillar acidic protein by routine immunohistochemical staining.

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