

Significance of Cytological Findings of Neuroblastomas: Rosette Arrangement and Neuropil Structure

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Abstract

Objectives: Improve treatment outcomes and clarify the biological characteristics of neuroblastoma, development of an international histological classification started several years ago. Aiming at the establishment of a cytology criteria corresponding to the new histological classification, we investigated a criteria comparing lesions related to neuroblastoma on referring to the morphological indices of neuroblastoma reported in the international classification.

Methods: Several tumor specimens were investigated: 37 cases of neuroblastoma (undifferentiated type: 3, poorly differentiated type: 34), 3 cases of ganglioneuroblastoma (mixed type: 2, nodular type: 1), and one case of ganglioneuroma. Stamp cytology samples were prepared from cut surfaces of the tumors and then stained to the Papanicolaou method.

Results: In neuroblastoma of the undifferentiated type, tumor cells contained a small oval nucleus with a high N/C ratio, showing a bare nucleus, and the nucleolus was distinct: no rosette formation or neutrophil was observed. In the poorly differentiated type, tumor cells showed a round-oval bare nucleus were scattered: rosette arrangement was observed in the background neuroblasts containing a bare nucleus. In ganglioneuroblastoma, immature neuroblasts showed a round-oval nucleus and large ganglion-like cells possessed a distinct nucleolus similar to poorly differentiated-type epithelial adenocarcinoma.

Conclusion: In neuroblastoma, neutrophils were stained light green, and a partial Homer-Wright-type rosette arrangement was observed in the background. In the poorly differentiated type, tumor cells were generally large compared to those observed in the undifferentiated type. In ganglioneuroblastoma, cytological diagnosis can be relatively easily made when differentiated mature ganglion like cells are observed. In the case of surgery, a histological diagnosis of nervous system tumors is often performed using frozen sections, however tissue is usually damaged during freezing. Thus, cytology is more advantageous for diagnosis. The diagnostic accuracy can be improved utilizing the cytological characteristics of neuroblastic tumors.

Keywords: Neuroblastic tumors; INPC; Cytological findings; Morphological image analysis; Rosette arrangement; Neuropil structure

Introduction

Neuroblastic tumors are embryonal tumors originating from the neural crest upon neural tube formation and developing in the adrenal medulla or sympathetic nerve tissue. The most frequently develop among solid tumors arising in regions other than the central nervous system, accounting for 10% of childhood malignant tumor cases. The tumor development sites are the adrenal medulla and retroperitoneum, the posterior mediastinal space, the pelvis, and the cervical paravertebral region innervated by sympathetic nerves, and development in the adrenal gland and retroperitoneum account for about 80% of all cases [1]. Neuroblastoma often spreads to the bone marrow, lymph nodes, liver, orbit, skull, and femur.

The neural crest has the ability to differentiate into ganglion, chromaffin cells and melanocytes. Neuroblastoma, ganglioneuroblastoma, ganglioneuroma, and pheochromocytoma may occur in undifferentiated sympathetic ganglions and the adrenal medulla. Neuroblastic tumors originate due to abnormalities in this differentiation/maturation process. Transformed cells have the characteristics of spontaneously regressing while retaining differentiation and maturation capabilities. These characteristics may be incidentally discovered in the morphology of in-situ neuroblastoma upon autopsy of infants [1,2]. Neuroblastoma may change to benign

ganglioneuroma, resulting in a favorable outcome. Tumors to be differentiated from neuroblastoma include small round tumors, such as rhabdomyosarcoma, Ewing sarcoma, malignant lymphoma and Wilms tumor. About 200 patients are diagnosed with neuroblastic tumors every year in Japan and about 20% of all registered cases were discovered by mass screening [1]. Neuroblastic tumors have been classified into neuroblastoma, ganglioneuroblastoma, and ganglioneuroma based on the grade of tumor tissue differentiation. Some cases of neuroblastoma resist to all treatments and result in a poor outcome, whereas other neuroblastic tumors spontaneously regress or differentiate/mature to ganglioneuroblastoma and benign ganglioneuroma, showing the presence of specific types [3].

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In Japan, a qualitative test of urinary vanillyl mandelic acid (VMA) had been performed in neonates (6 months after birth) by administrative organs (health care centers and medical institutions) as nationwide neuroblastoma mass screening since 1984. However the outcomes of patients examined by the VMA qualitative test were mostly favorable and no recurrence has occurred in any case. On the basis of this situation and health economics, the administrative organs stopped the VMA qualitative test in 2003. Careful analysis of its influence on the therapeutic results of this tumor may be necessary.

After the introduction of neuroblastoma mass screening, biological genetic analysis was also actively performed. The MYCN gene is present on the short arm of chromosome 2, and it is considered to be involved in the development of the central nervous system in the fetal period. It has been reported that MYCN gene amplification is frequently noted in patients with poor outcomes [4,5]. The nerve growth factor receptor TrK-A is involved in the differentiation of the sympathetic nerve-adrenal gland system in the embryonic period. In neuroblastoma patients with a favorable outcome, the TrK-A expression rate is high, whereas the expression is lost in poor outcome cases, suggesting that TrK-A is a prognostic factor [6]. The blood ferritin level may be high when tissue destruction is present, such as in inflammation, trauma, and tumor. It has been reported that ferritin level may also be high in neuroblastoma [7]. Chromosome 1p deletion is observed in oligodendroglioma, is also observed in neuroblastoma patients with a poor outcome [8]. In addition, ras gene mutation [9,10], src gene [10], and CD44 overexpression [11] have been reported as prognostic factors.

Distinctive tumors not observed in adults arise in various organs during the neonatal period through childhood. Incidence of embryonal tumors, teratoma, and hamartoma are high in the neonatal period and infancy, and leukemia, malignant lymphoma, brain tumor, and musculoskeletal tumor develop in childhood and adolescence. Accurate differential diagnosis of these tumors is very important to decide on a treatment strategy and predict its outcome. Incidence of neuroblastoma is the highest among childhood solid tumors, and it is an embryonal tumor with biological specificity including histological and molecular-genetic characteristics. We investigated the cytological characteristics of neuroblastic tumors, which may be particularly important among the many types of childhood tumor. Cytological investigation of neuroblastic tumors has not previously been performed. We investigated the cytological characteristics by comparing the cytomorphology of different histologic types of neuroblastoma and ganglioneuroblastoma, for which differentiation is important, referring to the cytomorphological indices of neuroblastoma included in the international classification.

Materials and Methods

Case selection

The subjects were patients who were admitted to the Tokyo Metropolitan Hachioji Children's Hospital between 1991 and 1993 and to the Division of Surgical Pathology, Chiba Cancer Center, between 1990 and 2009 that underwent surgical excision of tumors. We examined 37 neuroblastomas (undifferentiated type: 3, poorly differentiated type: 34), 3 ganglioneuroblastomas (mixed type: 2, nodular type: 1), and one ganglioneuroma. Age ranged from 23 days to 7.5 years old (mean age: neuroblastoma, undifferentiated type, 4.3 years old; poorly differentiated type, 2.1 years old; ganglioneuroblastoma, 3.9 years old; ganglioneuroma, 3 years old). There were one female and 2 males with neuroblastoma, undifferentiated type, 20 females and 14

males with the poorly differentiated type, one female and 2 males with ganglioneuroblastoma, and one female with ganglioneuroma. Tumors were resected from the excised specimens in cooperation with pediatric surgeons, and cytology samples were prepared from unfixed tumors. Small tissue fragments were collected from the tumor cut surface, directly stamped on a slide glass and immediately fixed with 95% ethyl alcohol. The fixed sample was stained to the Papanicolaou method. The remaining tumor sample was fixed with 15% neutral buffered formalin, and a paraffin-embedded pathological block was prepared. Specimens (3 μ m thickness) were prepared from the paraffin-embedded block using a microtome and stained with hematoxylin-eosin. In addition to Papanicolaou staining, Giemsa staining was applied because important findings were readily observed. Giemsa staining was performed as follows: Tumor-stamped slide glasses were rapidly dried with cold air using a dryer. After complete drying, the sample was fixed with methanol for 5 minutes followed by staining with 10% Giemsa solution for 10 minutes. After staining, the sample was lightly rinsed with water, completely dried, dipped in xylene, and mounted with a water-insoluble mounting agent. The cytoplasm is stained blue by Giemsa staining in proportion to the RNA amount, and the nucleus is stained purplish red. If azurophil granules are present in the cytoplasm, they are clearly stained. This study was performed after approval by the Ethics Committees of the Tokyo Metropolitan Hachioji Children's Hospital and the Chiba Cancer Center.

Cytomorphological analysis

Cytomorphological analysis of neuroblastoma and ganglioneuroblastoma was performed by 2 cytotechnologists and one pathologist. The condition (bare nucleus or not) and differences in the shape and size of the nucleus, cytoplasmic condition and shape, pattern of appearance of cells, the presence or absence of neurofibril and rosette structures, and the presence or absence of ganglion-like cells and Schwannian cells in ganglioneuroblastoma were investigated.

Photographs were taken with a digital camera (DP-71, Olympus, Tokyo, Japan) attached to a microscope. The radius of the long axis, area, perimeter, roundness and linear factor of the nuclei in neuroblastoma tumor cells and the ganglion-like cells of ganglion neuroblastoma were calculated from the microscope image. The radius of long axis, area, perimeter and linear factor of the tumor nuclei were calculated by Matlab. The radius of the long axis is maximum length of the nucleus when scanned from 360 degrees. The degree of roundness is an index that indicates how closely (the nucleus) matches the shape of a circle. The linear factor is an index that indicates an object's slenderness.

Classification of neuroblastic tumors

The incidence of neuroblastoma is the highest among pediatric solid tumors, and it is an embryonal tumor with biological specificity including histological and molecular-genetic characteristics. In the old histological classification¹⁾, neuroblastic tumors were classified into 3 groups: neuroblastoma (rosette-fibrillary and round cell types), ganglioneuroblastoma (well-differentiated, composite, and poorly differentiated types), and ganglioneuroma (Table 1). In the International Neuroblastoma Pathology Classification (INPC), the rosette-fibrillary type is included in the poorly differentiated or differentiating type of neuroblastoma, and the round cell type is included in the undifferentiated or poorly differentiated type of neuroblastoma (Table 2). The well-differentiated type of ganglioneuroblastoma is classified into diverse types: the differentiating type of neuroblastoma, intermixed type of ganglioneuroblastoma, or maturing type of ganglioneuroma. The composite type is included in the nodular type of ganglioneuroblastoma,

Ganglioneuroma
Ganglioneuroma
A. Well differentiated type
B. Composite type
C. Poorly differentiated type
Neuroblastoma
Rosette-fibrillary type
Round cell type

Neuroblastoma is classified to rosette-fibrillary type and round cell type, Ganglioneuroblastoma is classified to well differentiated type, composite type and poorly differentiated type. Benign lesion of ganglioneuroma is not classified in histological classification of childhood neuronal tumors in the Japanese society of pathology.

Table 1: Histological classification of childhood neuronal tumors in the Japanese society of pathology (1992)

1. Neuroblastoma, Subtype
a) Undifferentiated
b) Poorly differentiated
c) Differentiating
2. Ganglioneuroblastoma
a) Intermixed
b) Nodular
3. Ganglioneuroma, Subtype
a) Maturing
b) Mature

The neuroblastoma category in the INPC was revised to two subtypes of conventional classification to three subtype's classification. Ganglioneuroblastoma are divided three to two subtypes. Ganglioneuroma was classified to two subsets.

Table 2: The International neuroblastoma pathology classification (INPC).

Neuroblastoma (Schwannian stroma-poor)
Definition: Neuroblastoma (Schwannian stroma-poor) composed of undifferentiated neuroblasts in the most part and interstitial components consist of Schwannian cells and fibroblasts are less than 50% of tumor tissue.
1) Subtype
(1) Undifferentiated (The neuropil in the tumor are not clearly recognized.)
(2) Poorly differentiated (The tumor consists of less than 5% of ganglion like cells differentiation.)
(3) Differentiation (The tumor consists of more than 5% of ganglion like cells differentiation.)
The definition of ganglion like cells differentiation: Size of cytoplasm is twice that of nucleus. The things of eosinophilic cytoplasm and eccentric nuclei in the cytoplasm.
2) Mitosis karyorrhexis-index (MKI)
(1) low MKI, < 2% (less than 100 / 5000 cells)
(2) intermediate MKI, 2-4% (100-200/5000 cells)
(3) high MKI, >4% (more than 200/5000 cells)
The mitosis karyorrhexis index (MKI) is defined as the number of tumor cells with karyorrhexis and mitosis in the tumor tissues. MKI is classified with 3 categories according to the number of tumor cells in mitosis.

Table 3: Morphological indication of neuroblastoma and subtypes (INPC).

and the histology of the poorly differentiated type is considered to correspond to that of the differentiating type of neuroblastoma. Regarding the definition of each type and its correspondence to INPC, the rosette-fibrillary type represents tumors comprised of immature oval tumorous neuroblasts and neurofibrils (neuropils) present between tumor cells, and this corresponds to the histology of the poorly differentiated or differentiating type of neuroblastoma in INPC. In the round cell type, immature oval neuroblasts densely proliferate, and no or very few neuropils are formed between tumor cells. INPC strictly specifies the absence of neuropils on light microscopy in the definition of neuroblastoma, undifferentiated type.

The well-differentiated type of ganglioneuroblastoma is mainly comprised of mature ganglion cells with abundant cell components, and a few neuroblasts are also contained. Since the interstitium was not taken into consideration in the old classification, this type included diverse INPC types, such as neuroblastoma differentiating type, ganglioneuroblastoma intermixed type, and ganglioneuroma maturing type. The composite-type tumors contain nodules with macroscopically different properties and show the histology of highly differentiated/matured ganglioneuroma and ganglioneuroblastoma. Other nodules are comprised of immature neuroblastoma components. This type corresponds to ganglioneuroblastoma nodular type in INPC. In the poorly differentiated type, many tumor cells showing maturation to ganglion cells are mixed with neuroblasts, corresponding to the histology of neuroblastoma differentiating type in INPC. In ganglioneuroma, interstitium formed by Schwannian cells occupies the majority of the tumor, and differentiated ganglion cells are also present. This tumor progresses from ganglioneuroblastoma intermixed type to ganglioneuroma maturing and mature types. These tumors are defined based on the differentiation/maturation process. The characteristics of INPC are complete incorporation of the Shimada classification, which is a system to predict the outcome based on the correlation between the age and histology (Table 3). The Shimada classification is based on the grades of neuroblast differentiation and interstitial Schwannian cells. The outcome prediction system in INPC was designed based on the concept of the Shimada classification (Table 4). The mitosis karyorrhexis index (MKI) was proposed in the Shimada classification in 1984. Mitosis and karyorrhexis of tumor cells in tumor tissue are quantified and classified into mild, moderate, and severe, and these are used as a scale to judge the prognosis of neuroblastoma. MKI of 5,000 tumor cells observed under a microscope is determined, and when it is high, the prognosis is judged as poor regardless of the grade of tumor cell differentiation and age. The prognosis of the undifferentiated type is poor regardless of the age. The prognosis of neuroblastoma poorly differentiated type developing before 1.5-5 years old is also judged as poor.

Results

Histological and cytological findings of the neuroblastic tumors

Neuroblastoma undifferentiated type: The undifferentiated type in INPC corresponds to the round cell type in the old classification. Histologically, cell components were abundant, and the tumor was comprised of small to medium-sized immature undifferentiated neuroblasts containing a small cytoplasmic volume and a round-oval

Neuroblastoma (stroma-poor tumors)	
Undifferentiated type	This type includes undifferentiated type and poorly differentiated type in INPC, and neuropils are not found at all or scanty.
Differentiating type	This type is equivalent to the differentiating type in INPC. Neuropil is present.
Ganglioneuroblastoma (stroma-rich tumors)	This tumor occupies more than 50% stroma consist of mainly Schwannian cells.
Intermixed type	This tumor is equivalent to the GNB, intermixed in INPC.
Nodular type	This tumor is equivalent to the GNB, nodular in INPC.
Well differentiated type	This tumor is equivalent to the GN, maturing in INPC.
Ganglioneuroma (stroma-rich tumors)	This tumor is equivalent to the GN, mature in INPC.

GNB; Ganglioneuroblastoma, INPC; The International neuroblastoma pathology classification.

Table 4: Shimada classification.

bare nucleus. No neuropil was microscopically observed between tumor cells (Figure 1a). The prognosis of this tumor is considered poor regardless of the age, but this histologic type of neuroblastoma is rare. In stamped tumor cytology specimen (Figure 1b), cells contained a small round-oval nucleus and a distinct nucleolus, and the N/C ratio (nucleus/cytoplasm volume ratio) was high, showing a bare nucleus. No tumor cell rosette formation or neuropil was observed. The resected tumor shows Figure 1c.

Neuroblastoma poorly differentiated type: The poorly differentiated type in INPC corresponds to the rosette-fibrillary type in the old classification. Histologically, neuropils were present between proliferating tumor cells, and tumor cells were small and round containing a bare nucleus. The cytoplasm of tumor cells were scanty (Figure 2a). Rosette arrangement termed the Homer-Wright type was often noted [1]. Neurofibrils stained pale red were present in the central region (Figure 2b). Calcification was often observed in neuroblastoma (Figure 2c). In stamped tumor cytology specimen, red blood cells and round-oval neuroblasts containing a bare nucleus were scattered in the background (Figure 2d), and rosette arrangement was noted (Figure 2e). Calcification was noted around tumor cells. Even though no rosette formation is observed in a cytological specimen, this

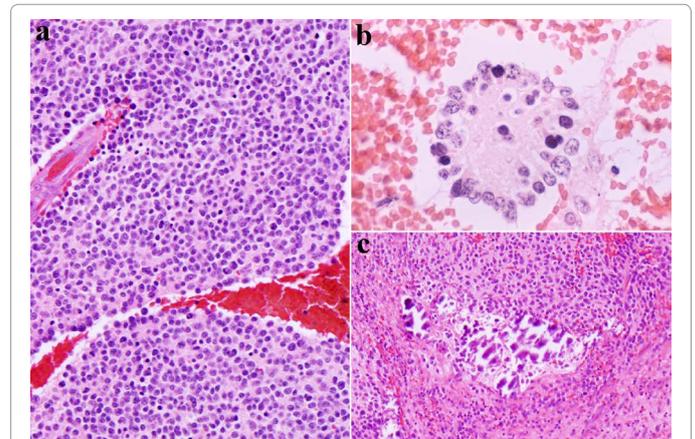


Figure 2: a) Poorly differentiated neuroblastoma, HE, 40X. Neuroblastic cells have round to oval nuclei in shape. The neuropils are barely seen. b) Poorly differentiated neuroblastoma, HE, 40X. Tumor cells are possessed bare nuclei with round and oval in shape. Homer-Wright rosette arrangements are formed by tumor cells that are radially arranged in circle. The neuropil are stained pink with eosin dye. c) Poorly differentiated neuroblastoma, HE, 40X. Neuroblastic cells are seen in the background. Psammoma bodies are seen occasionally.

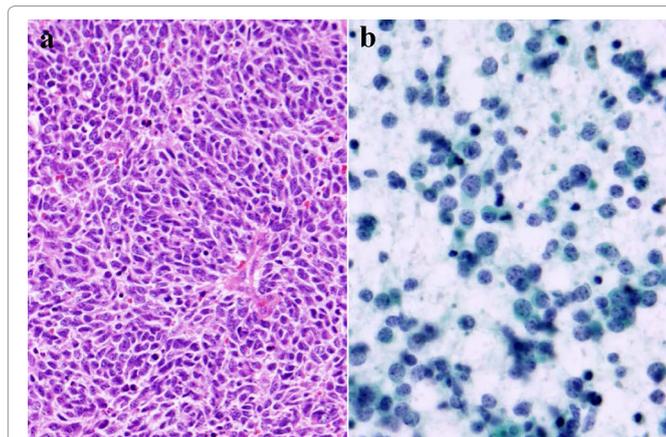


Figure 1: a) Undifferentiated neuroblastoma, HE, 20X. Neuroblastic cells have large prominent nucleoli. The neuropils are not seen. b) Undifferentiated neuroblastoma, Pap, 40X. Tumor cells possessed bare nuclei with round and oval in shape with anisokaryosis. Small nucleoli are seen. The debris that stained light green and picnotic karyorrhexis are seen in the background.

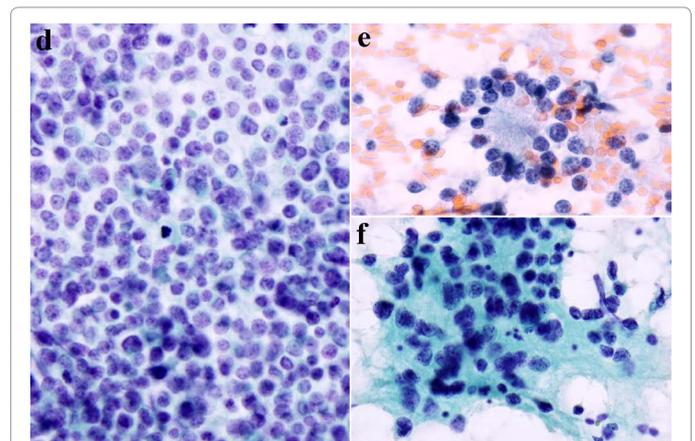


Figure 2: d) Poorly differentiated neuroblastoma, Pap, 60X. Scattered neuroblastic cells are seen. These cells have round to oval nuclei in shape. Nuclear chromatin shows coarse granular pattern. The neuropils are barely seen. e) Poorly differentiated neuroblastoma, Pap, 60X. Tumor cells are possessed bare nuclei with round and oval in shape. Homer-Wright rosette arrangements are formed by tumor cells that are radially arranged in circle. The neuropils stained eosinophilic are seen in the center of rosette arrangement. Red blood cells are seen around tumor cells. f) Poorly differentiated neuroblastoma, Pap, 40X. Tumor cells show high N/C ratio and these nuclei are round to oval in shape with anisokaryosis. The nuclear chromatin expressed a granular pattern. The neuropils are stained light green.



Figure 1c: Macro image of poorly differentiated neuroblastoma. The resected tumor shows small hemorrhage and degenerative change.

tumor can be easily diagnosed when neuropils stained light green are present between cells (Figure 2f).

Neuroblastoma differentiating type: INPC defines tumors in which neuroblasts account for more than 5% of all cells as the differentiating subtype. The differentiating type is mixed with the poorly differentiated type in many cases. The analyzed cases were the poorly differentiated and undifferentiated types, and no patient with purely differentiated-type neuroblastoma was encountered, but tumor cells enlarged, separately from many small neuroblasts, in some cases of the poorly differentiated type, in which the cytoplasm was wide, the nucleus was eccentric, and the nucleolus was clearly observed (Figure 3a). The nuclear shape was irregular, being likely to be misjudged as strongly atypical cells. These

had differentiated into ganglion cells, termed ganglion-like cells (Figure 3b). Large cells containing the cytoplasm and distinct nucleolus were also observed in the stamped tumor cytology specimen (Figure 3c and 3d). The cytological findings of neuroblastoma undifferentiated, poorly differentiated, and differentiating types are shown in Table 5.

(4) Ganglioneuroblastoma: In the old classification, ganglioneuroblastoma was classified into the differentiated, composite, and poorly differentiated types based on the grade of differentiation to ganglion cells, but it was classified into the intermixed and nodular types in the new classification (Table 2). The definition of ganglioneuroblastoma was previously unclear,

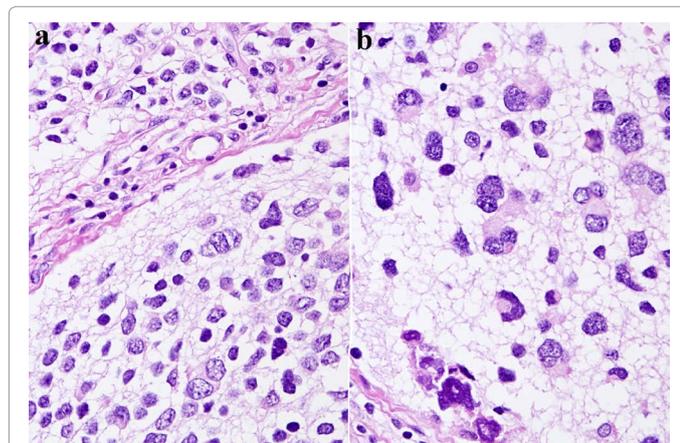


Figure 3: a) Differentiating neuroblastic cells, HE, 20X. Differentiated large ganglion like cells are seen. Ganglion like cells are seen at the top of the photomicrograph. b) Differentiating neuroblastic cells, HE, 20X. Differentiated large ganglion like cells with eccentric nuclei are seen.

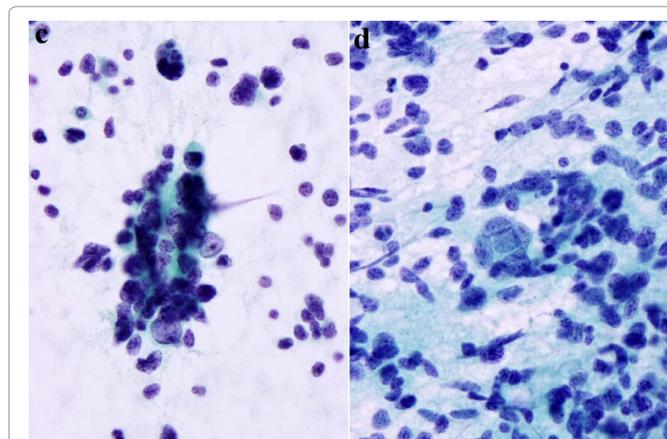


Figure 3: c) Differentiating neuroblastic cells, Pap, 60X. Tumor cells are differentiated into large cells with obvious nucleoli than neuroblastic cells presence in the background. d) Differentiating neuroblastic cells, Pap, 60X. Multiple nuclei tumor cells are seen. These cells are bigger than neuroblastic cells are observed in the background.

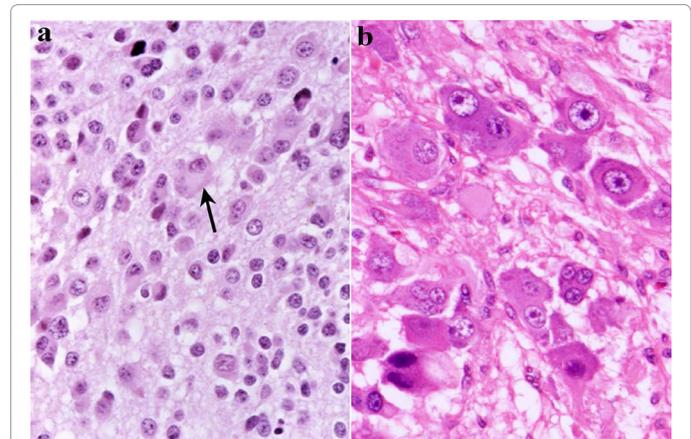


Figure 4: a) Ganglioneuroblastoma, intermixed, HE, 40X. Neuroblastic cells are seen in the background. Ganglion like cells appeared in these neuroblastic cells. b) Ganglioneuroblastoma, intermixed, HE, 60X. It is differentiated into large ganglion like cells with the prominent nucleoli.

but the introduction of INPC facilitated histological distinction between neuroblastoma and ganglioneuroblastoma and between ganglioneuroblastoma and ganglioneuroma. Tumors in which interstitium formed by Schwannian cells occupies more than 50% of the tissue in the process of differentiation/maturation from neuroblastoma to ganglioneuroblastoma and then ganglioneuroma was defined as ganglioneuroblastoma, resolving the lack of clarity of the previous definition. Histologically, mature Schwannian cells forming interstitium account for 50% of tumor cells in ganglioneuroblastoma, and large ganglion-like cells and small immature neuroblasts are mixed and form an alveolar lesion. In Figure 4a, neuroblasts are present in the background, in which ganglion-like cells (arrow) are also present. Ganglion-like cells are thick and large containing a wide cytoplasm and distinct nucleus (Figure 4b).

In the stamped tumor cytology specimen, immature neuroblasts and large ganglion-like cells were mixed (Figure 4c and 4d). Neuroblastic cells (arrow) contained a round-oval nucleus with a high N/C ratio and were scattered. No cytoplasm was observed, the nuclear chromatin showed a fine-coarse granular pattern, and the nuclear size was irregular. Ganglion-like cells (arrowhead) were single cells cytoplasm of rhomboid shapes containing an eccentric nucleus and a distinct nucleolus, showing a morphology suggesting poorly differentiated adenocarcinoma. Neuropils similar to those observed in poorly differentiated-type neuroblastoma were also present. These large cells were immature and atypia and may show the transitional features to differentiated ganglion cells, making the differentiation of these cells difficult in some cases. Small background neuroblasts were assumed to be malignant. Schwannian cell outgrowth was partial. This tumor may have the potential to differentiate into ganglioneuroma. Schwannian cells were firmly connected and rarely observable on stamp cytology. Comparison of cytological findings between neuroblastoma and ganglioneuroblastoma is shown in Table 6. The results of

Differentiation	Nucleus			N/C ratio	Cytoplasm	Neurophils	Rosette forming	MKI
	Shape	Size	Chromatin					
Undifferentiated	Round	Small	Prominent hyperchromatism	Very high	Scant	Seldom seen	Absence	high
Poorly differentiated	Round to oval	Small to midium	Hyperchromatism	High	Little	Seen in background	Presence	low
Differentiating	Round to oval	Small, large	Hyperchromatism	Low	Abundant	Relatively abundant	Occasionally presence	-

Table 5: Comparison of cytological findings of neuroblastomas.

morphological image analysis of the nuclei in each histological type are show in Table 7.

Discussion

Neuroblastoma cells contain a round-oval bare nucleus. The N/C ratio is high and the cytoplasm cannot be confirmed. Intercellular connection is weak and cells appear isolated and scattered. Typical cases show Homer-Wright-type pseudo-rosette arrangement and neuropils stained light green by Papanicolaou staining are present in the lumen. The neuropil islands of fibril components in the central region are large and irregular and the fiber region shows positive reaction for synaptophysin. These have recently been termed neurocytic rosette [12,13]. In rare cases, early central neurocytoma represented Homer-Wright-type-like structure, not limiting to neuroblastoma [13,14]. A Homer-Wright rosette is simply formed by tumor cells that are radially arranged with fibrillated material in the central luminal area.

This arrangement may be observed in the histology of neuroblastoma and ependymoma and the cytology of poorly differentiated-type neuroblastoma, and termed pseudo-rosette, in contrast to ependymal rosette present in the central region of the lumen [12,15]. The structure termed neurocytic rosette is the same as the pineocytomatous rosette observed in pineocytoma. Regarding the neuroblastoma rosette formation, small homogeneous tumor cells are present around the rosette and project nerve fiber processes inward, forming an assembled structure. In cytological specimens other than neuroblastoma, adenocarcinoma cells, which are from a malignant epithelial tumor, may show this cell arrangement and are termed rosette-like in the broad sense. Embryologically, neuroblastic tumors originate from the neural crest appearing on the lateral side of the neural tube, suggesting that the morphology formed in the process of differentiation from neural stem cells is neurocytic rosette. A rosette formation is considered to represent the grade of tumor cell differentiation. Two-dimensional observation of the structure of rosette arrangement of poorly differentiated neuroblastoma is limited. For close observation, analysis by 3-dimensional reconstruction may be necessary [16].

While no significant difference was observed in the morphological analysis of the radius of the long axis, area and perimeter of the nuclei of poorly differentiated neuroblastoma and undifferentiated neuroblastoma, there was a tendency for poorly differentiated neuroblastoma to be larger. The degree of roundness of the nuclei in poorly differentiated neuroblastoma is lower than that of undifferentiated neuroblastoma ($p < 0.0001$). On the other hand, the linear factor of the nuclei in poorly differentiated neuroblastoma was higher than that of undifferentiated neuroblastoma ($p < 0.05$). The radius of the long axis and perimeter of ganglion-like cells in the well differentiated sites of neuroblastoma were higher than that of ganglioneuroblastoma ($p < 0.05$). Moreover, the cytoplasm of ganglion-like cells in the well differentiated sites of neuroblastoma was scarcely found, and it was also observed that the cell that holds the form of neuroblastoma tumor cells (Table 7). Because poorly differentiated neuroblastoma can be differentiated into ganglioneuroblastoma and ganglioneuroma through the well-differentiated neuroblastoma, it is thought that the size and morphology of these cells is indicated by the morphological

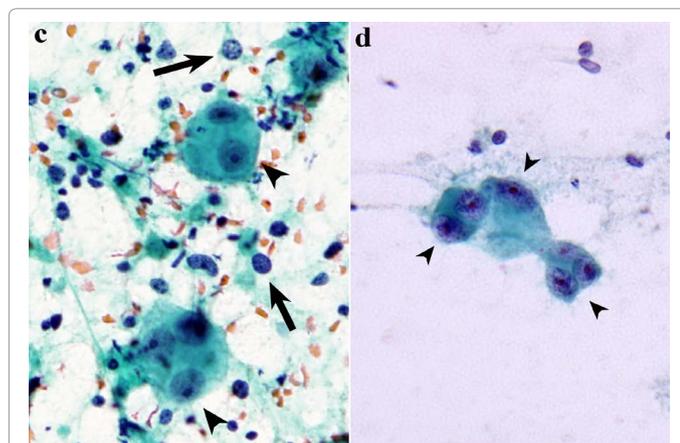


Figure 4: C) Ganglioneuroblastoma, intermixed, Pap, 60X. Neuroblastic cells show high N/C ratio and these nuclei are round to oval in shape are seen in the background (arrow). d) Ganglioneuroblastoma, intermixed, Pap, 60X. Ganglion like cell with eccentric nuclei have a prominent nucleoli are observed (arrow head).

Histological types	Nucleus		Nucleolus	Appearance	Cytoplasm	Neuropils	Rosette forming	Schwannian cells
	Situation	Shape						
Neuroblastoma	Bare nucleus	Round to oval	Discreet to prominent	Scattered	Scanty	Presence in tumor cells background	Presence	Absence
Ganglioneuroblastoma	Ganglion like cells Cytoplasm present	Oval	Prominent	Isolated	Abundance	Absence	Absence	Presence (More than 50%)

Table 6: Comparison of cytological findings between neuroblastoma and ganglioneuroblastoma.

(INPC)

Histological type		Radius of long axis (μm)			Area (μm)			Perimeter (μm)			Roundness			Linear factor		
		Average	±	SD	Average	±	SD	Average	±	SD	Average	±	SD	Average	±	SD
Neuroblastic cells	UNB	4.34	±	0.75	327.07	±	110.39	26.64	±	4.49	0.903	±	0.02	1.162	±	0.11
	PNB	4.59	±	1.18	349.62	±	181.55	27.62	±	6.62	0.881	±	0.04	1.243	±	0.20
Ganglion like cells	DNB	8.54	±	1.16	916.23	±	138.62	47.82	±	3.99	0.804	±	0.07	1.603	±	0.41
	GNB	7.42	±	0.95	791.66	±	247.52	42.69	±	5.88	0.851	±	0.05	1.432	±	0.29

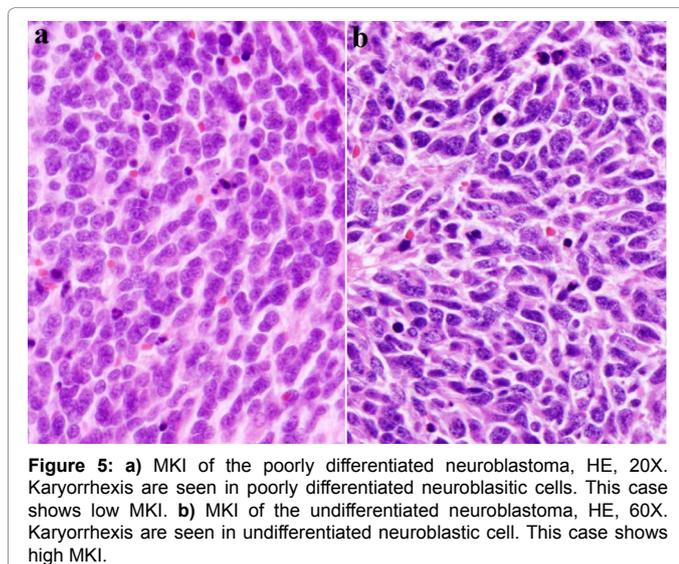
UNB: Neuroblastoma undifferentiated, PNB: Neuroblastoma poorly differentiated, DNB: Neuroblastoma differentiating, GNB: Ganglioneuroblastoma. * $p < 0.0001$, † $p < 0.05$; significant, Mann-Whitney U-test.

Table 7: The Correlation between Nuclear Morphological image analysis in Neuroblastic tumor.

features of the differentiation process from neuroblastoma cells to ganglioneuroblastoma. In addition, it is interesting to note that the cell nuclei of the ganglion-like cells of the ganglion neuroblastoma become slightly smaller as they differentiate.

Cells present in neuroblastoma poorly differentiated type show generally anisokaryosis than those in the undifferentiated type and increased nuclear chromatin are observed. In the background, neuropils stained light green are present and the Homer-Wright rosette arrangement is partially noted. The cytological characteristics of the undifferentiated type are small round nucleus, a high N/C ratio showing a bare nucleus, increased nuclear chromatin, and clearly observable nucleolus. Cells are not connected, similarly to the poorly differentiated type, and tumor develops in a solitary scattered or loosely connected pattern. The absence of rosette-forming cells in cytological specimens is another characteristic. In the undifferentiated type, which rarely develops, cells are small and contain a distinct nucleolus compared with the poorly differentiated type, and these are marked differences. No neuropil is present in the background, and rosette arrangement is not observed in many cases. INPC defines tumors in which differentiated-type neuroblasts account for 5% of all cells as the differentiating subtype. In differentiated-type neuroblastoma, tumor cells become large, the size of the cytoplasm is 2 times the nuclear size or larger, the nucleus is eccentric, and a distinct nucleolus is contained [17]. Since neuroblasts actively form neuropils, increased neuropils stained light green is another characteristic [18]. Differentiated-type neuroblastoma is mixed with the poorly differentiated type in many cases. Although the analyzed cases were the poorly differentiated and undifferentiated types, and no patient with purely differentiated-type neuroblastoma was encountered, the features of differentiated neuroblasts were noted in some cases of the poorly differentiated type. Regarding differentiation between the differentiated-type neuroblastoma and ganglioneuroblastoma, when neuroblasts showing a differentiation tendency account for 5% or more, the tumor is considered differentiated-type neuroblastoma, but practically, neuroblastoma mostly comprised of differentiated neuroblasts is impossible. Neuroblastoma and ganglioneuroblastoma are not distinguished based on the frequency of differentiated neuroblasts, but distinguished based on whether or not Schwannian cells account for 50% or more of interstitial cells.

The cytological features of ganglioneuroblastoma mostly reflect

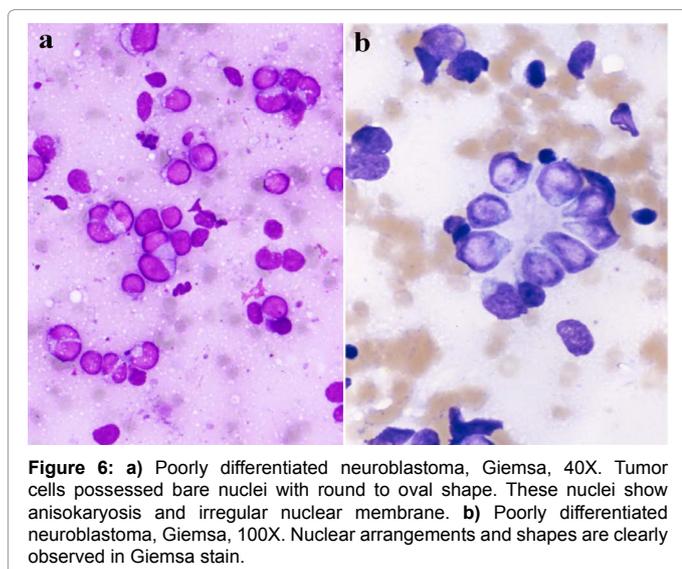


the histology. Neuroblasts were present in the background, and differentiated/maturated ganglion cells, which are large and contain a distinct nucleolus, were scattered. If cells showing these cytological characteristics are observed, it is relatively easy to make a cytological diagnosis. The cytological features of ganglioneuroblastoma are the presence of a distinct nucleolus and appearance similar to adenocarcinoma cells, which is a malignant epithelial tumor. It should be noted that these cells are differentiated/maturated ganglion cells, not malignant cells, which may be a characteristic of this tumor, whereas cells containing a round-oval nucleus and increased chromatin observed in the background are malignant.

MKI was investigated in histological specimens from neuroblastoma patients. MKI was \pm to +(low) in the poorly differentiated type and 2+ (high) in the undifferentiated type (Table 5). Marked mitosis and karyorrhexis were noted in the undifferentiated type, the prognosis of which is poor (Figure 5a and 5b). A patient is included in the neuroblastoma high-risk group when the stage is 3 and 4 on the international neuroblastoma staging system classification, the age is 1 year old or older, chromosome 1p deletion and MYCN gene amplification are observed, the ferritin level is high, and the H-ras and Trk-A expression levels are low. In the Shimada classification, the age is divided to below 1 year old and 1 year old or older, and the former and latter are regarded as having favorable and poor prognoses, respectively. In addition, the prognosis is judged as poor regardless of the grade of tumor cell differentiation and age when MKI is high. The prognosis of undifferentiated-type neuroblastoma is judged as poor, and that of the poorly differentiated type developing at 1.5 years old is also judged as poor. In the favorable outcome group, tumor spontaneously regressed or tumor cells differentiated/maturated to ganglion cells and ganglioneuroma. Outgrowth of interstitial Schwannian cells, which cannot be observed in cytological specimens, can be confirmed in histological specimens. It is assumed that differentiation induction of tumor cells from poorly differentiated neuroblastoma to benign ganglioneuroma progressed through the differentiation and maturation of tumor cells in the favorable outcome group.

Tumor cells in neuroblastoma are undifferentiated and show severe atypia, based on which the tumor can be diagnosed as malignant; that is, the cell morphology is within the category of malignancy because cells show poorly differentiated immature morphological characteristics. In terms of their clinical outcomes, tumor spontaneously regresses and differentiation/maturation to benign ganglioneuroma and ganglion cells are observed, leading to a favorable outcome in many cases. Generally, the prognosis of other malignant epithelial tumors becomes poor as the cell differentiation grade decreases, which is contradictory to the cell morphology. It has been reported that the differentiation of neuroblastoma tumor cells is inhibited by MYCN gene amplification, resulting in a poor outcome [19,20]. MYCN gene amplification is absent in many neuroblastoma cases with favorable outcomes. Differentiation induction is retained due to the absence of MYCN gene amplification, suggesting that the development and differentiation from neural stem cells and apoptosis system are conserved. Embryologically, the outcome was assumed to be favorable although cells formed in the process of neuronal stem cell differentiation are poorly differentiated because neuroblastic tumors originate from the neural crest appearing on the lateral side of the neural tube [21].

For nerve system tumors, intraoperative examination of frozen sections is performed before histological diagnosis in many cases, but damage of the tissue by freezing is severer in rapid than in permanent samples. In contrast, stamped cell samples retain fresh



cells, and judgment can be more readily made than for HE-stained tissue samples. In nerve tumors, a small volume of blood components containing tumor cells is adhered to gauze submitted with the specimen to histological examination, and cytology samples can be prepared from it. For cytology, it is desirable to prepare not only Papanicolaou-stained but also Giemsa-stained samples simultaneously. Giemsa staining emphasizes the nuclear and cytoplasmic morphology compared with Papanicolaou staining (Figure 6a and 6b), which is useful to differentiate nerve system tumors. We believe that the diagnostic accuracy will be improved by utilizing the cytological characteristics of neuroblastic tumors identified in this study for the differentiation of tumors on intraoperative cytology.

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