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MINIREVIEW

Pathology of Peripheral Neuroblastic Tumors: Significance of Prominent Nucleoli in Undifferentiated/Poorly Differentiated Neuroblastoma

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The presence of large cells having simultaneously increased cytoplasmic and nuclear volume accompanied by prominent nucleoli; i.e., differentiating neuroblasts and ganglion cells, is well documented in peripheral neuroblastic tumors (pNTs), and considered as one of the signs of tumor maturation and an indication of a better prognosis of the patients. On the other hand, in 2004 it was reported that large-cell neuroblastoma composed of neuroblastic cells with only nuclear enlargement without recognizable cytoplasmic maturation behaved poorly clinically. Here we are proposing a new pNT subtype in the neuroblastoma category, in addition to the undifferentiated, poorly differentiated and differentiating subtypes: that is large nucleolar neuroblastoma (LNN) characterized by large prominent nucleoli and no or very little amount of discernible cytoplasm. LNN, whose neuroblastic cells are often large in size due to nuclear enlargement, includes those tumors previously categorized into the large-cell neuroblastoma group. LNN tumors, regardless of the size of nuclei, seem to behave aggressively with a very poor prognosis of the patients. It is speculated that nucleolar enlargement without cytoplasmic maturation in LNN tumor cells can be a sign of *MYCN* amplification. (Pathology Oncology Research Vol 13, No 4, 269–275)

Key words: LCN, LNN, neuroblastoma, nucleoli, INPC, *MYCN*, amplification, ganglion cell, maturation, Schwann cell

Introduction

Peripheral neuroblastic tumors (pNTs: neuroblastoma, ganglioneuroblastoma, ganglioneuroma) are far more heterogeneous than they are generally thought, not only morphologically, but also biologically. Some tumors are biologically favorable with a potential of regression or age-dependent tissue maturation, while others that are biologically unfavorable often demonstrate a lack of age-appropriate tumor maturation and/or are associated with higher mitotic and karyorrhectic activities.

It is generally considered that increasing number of differentiating neuroblasts; i.e. large cells, in a given tumor tissue is one of the signs of tumor maturation suggesting a good prognosis for the patients. The International Neuroblastoma Pathology Classification (INPC) uses both cytoplasmic and nuclear enlargement as morphologic indicators of neuroblastic differentiation. However, there is a group of rare tumors that are composed of large neuroblastic cells showing only nuclear enlargement but with inconspicuous cytoplasm. In 2004, we collected those cases under the name of large-cell neuroblastoma (LCN) and reported an extremely poor prognosis

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Abbreviations: LCN: large cell neuroblastoma; LNN: large nucleolar neuroblastoma; INPC: International Neuroblastoma Pathology Committee> pNT: peripheral neuroblastic tumors; NB: neuroblastoma; NUD: neuroblastoma, undifferentiated; NPD: neuroblastoma, poorly differentiated; NDF: neuroblastoma, differentiating; GNBi: ganglioneuroblastoma, intermixed type; GNBn: ganglioneuroblastoma, nodular type; GN: ganglioneuroma; MKI: mitosis-karyorrhexis index

of the patients. LCN tumors are not described as a separate entity, and are included in the undifferentiated/poorly differentiated subtypes of the neuroblastoma (Schwannian stroma-poor) category according to the INPC. In this paper, we outline the INPC, then illustrate the LCN in detail as well as the significance of prominent nucleoli whose occurrence is not restricted to LCN. Lastly, we propose a new entity of large nucleolar neuroblastoma (LNN) in pNTs which would represent a fourth subtype of the neuroblastoma category besides undifferentiated, poorly differentiated and differentiating subtypes. To identify this subtype of LNN by recognizing prominent nucleoli is important biologically and clinically.

Table 1. **INPC classification of neuroblastic tumors**

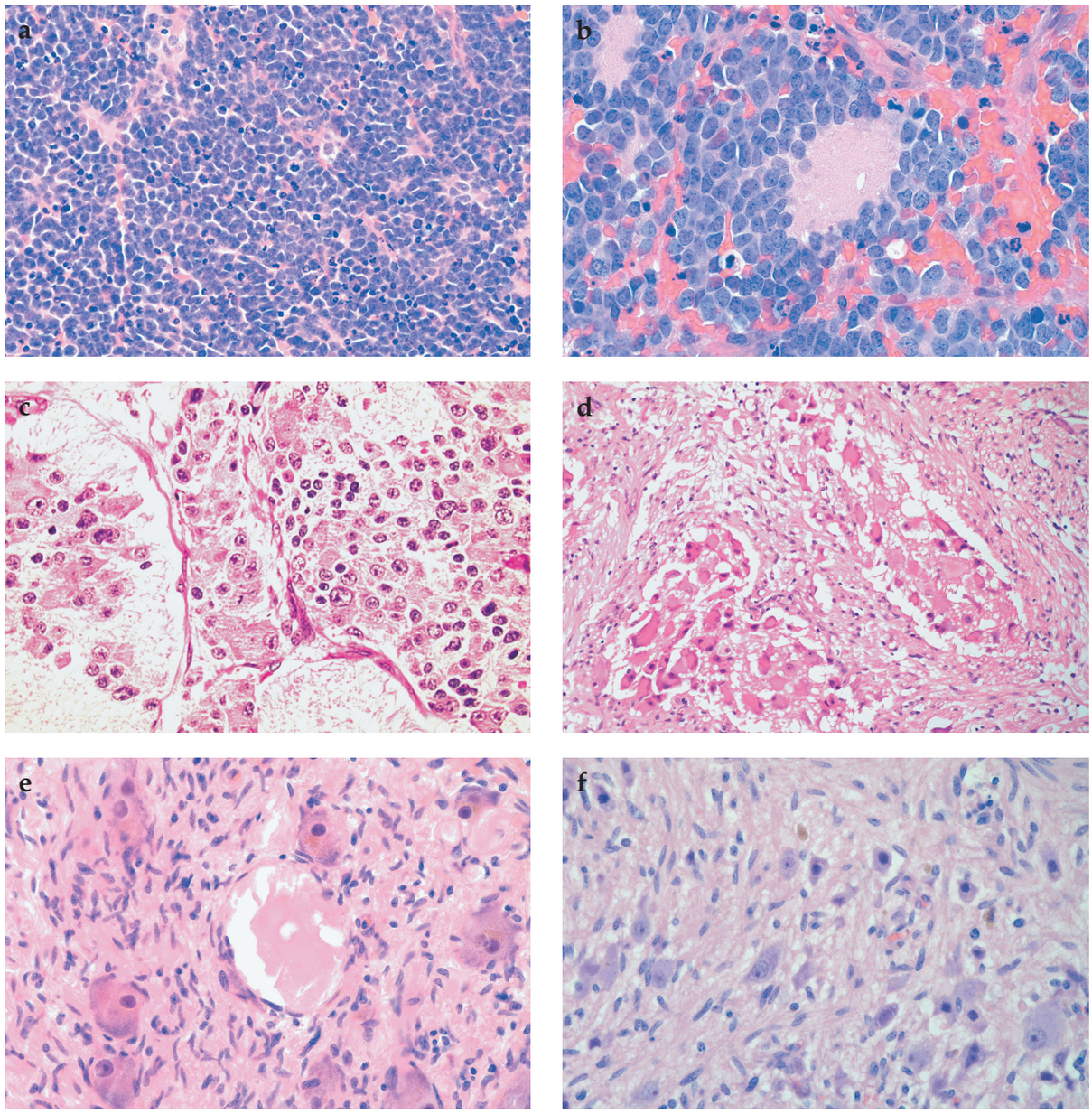
Main categories	*Subtypes*
Neuroblastoma (stroma-poor)	Neuroblastoma, undifferentiated Neuroblastoma, poorly differentiated Neuroblastoma, differentiating
Ganglioneuroblastoma intermixed (stroma-rich)	
Ganglioneuroma (stroma-dominant)	Maturing Mature
Ganglioneuroblastoma, nodular (composite)	

The International Neuroblastoma Pathology Classification (INPC)

The first and most complete, prognostically significant and biologically relevant "in depth" morphological classification of the pNTs was established in 1999 and modified in 2003 by the International Neuroblastoma Pathology Committee (*Table 1*).[1,2] The International Neuroblastoma Pathology Classification (INPC) distinguishes four basic categories: (1) neuroblastoma (Schwannian stroma-poor) – NB, (2) ganglioneuroblastoma, intermixed (Schwannian stroma-rich) – GNBi, (3) ganglioneuroma (Schwannian stroma-dominant) – GN, and (4) ganglioneuroblastoma, nodular (composite, Schwannian stroma-rich/stroma-dominant and stroma-poor) – GNBn. Tumors in the NB category are further classified into undifferentiated (NUD, totally undifferentiated with no recognizable neuropil by light microscopy), poorly differentiated (NPD, presence of clearly discernable neuropil, <5% of differentiating neuroblasts), and differentiating subtype (NDF, ≥5% of differentiating neuroblasts). The GNBi and GNBn categories are not subdivided further, while tumors in the GN category are classified into maturing and mature subtypes. These categories and subtypes are determined based on the light microscopic observation of two histologic markers; i.e., grade of neuroblastic differentiation towards ganglion cells, and degree of Schwannian stromal development (*Fig. 1a-f*) and macroscopically on the presence or absence of visible neuroblastomatous nodules (*Fig. 1g*).[1,2] The INPC includes an additional histologic marker in the NB and GNBn categories, mitosis-karyorrhexis index (MKI), for evaluating cellular kinetics, frequently, but not exclusively, affected by *MYCN* oncogene status.

The INPC, by adopting and slightly modifying the original Shimada system published in 1984, is an age-linked classification, since the prognostic effects of grade of neuroblastic differentiation and MKI are considered to be age-dependent.[3-5] As shown in *Table 2*, it clearly distinguishes a favorable histology (FH) and an unfavorable histology (UH) group. The FH group includes (1) NPD or NDF subtype with a low (<100/5,000 cells) or an intermediate (100-200/5,000 cells) MKI, diagnosed under 1.5 years of age; (2) NDF with a low MKI, diagnosed between 1.5 and 5 years of age (3) GNBi, at any age; (4) GN, at any age; and (5) GNBn of the favorable subset, at any age. Tumors in (3), (4) and (5) are usually diagnosed in older children. Cases in (5) are composite tumors of multiple clones of GNBi/GN and NB, whose neuroblastomatous nodule should have characteristics described in either (1) or (2). The UH group includes (6) NUD, at any age and with any class of MKI; (7) NB with a high

Figure 1. (a) *Small, undifferentiated neuroblasts, stromal connective tissue septae and blood vessels in the undifferentiated type of neuroblastoma.* ***(b)*** *Note the Homer Wright rosettes and the neuropil in the middle of them in a poorly differentiated neuroblastoma.* ***(c)*** *Neuroblastoma, differentiating type; more than 5% of the tumor cells are differentiating neuroblasts, while minimal or no Schwann cell component could be detected.* ***(d)*** *Ganglioneuroblastoma, intermixed; note the nesting arrangement of the ganglion cells and differentiating tumor cells surrounded by the Schwann cell stromal component.* ***(e, f)*** *Ganglioneuroma, mature and maturing, respectively; note the scant, large, mature ganglion cells surrounded by Schwann cell component in the mature variant. In the maturing type* ***(f)*** *some of the tumor cells are not completely matured, with smaller cytoplasm and nuclei. Tumor cells in GN do not form nests.* ***(g)*** *Gross aspect of a nodular ganglioneuroblastoma; note the sharp demarcation of the two components, the hemorrhagic stroma-poor (neuroblastomatous) and the white stroma-rich/dominant (mixed type of ganglioneuroblastomatous or ganglioneuromatous)*



(≥200/5,000 cells) MKI, at any age; (8) NPD subtype with a low or intermediate MKI, diagnosed between 1.5 and 5 years of age; (9) NB of any grade of differentiation and any class of MKI, diagnosed over 5 years of age; and (10) GNBn of unfavorable subset. Cases in (10) are composite tumors whose neuroblastomatous nodule has characteristics described in (6), (7), (8) or (9). The INPC has been used as one of the critical front-end prognostic factors for patient stratification and protocol assignment by the COG (Children's Oncology Group) Neuroblastoma Biology Study in North America, Australia and New Zealand.

Large primitive neuroblasts in large-cell neuroblastoma vs. large differentiating neuroblasts in biologically favorable neuroblastoma

Large-cell neuroblastoma (LCN), an entity not included in the INPC, makes a rare and unique subgroup in NB (Schwannian stroma-poor) category of pNTs. Patients with LCN have a worse prognosis than those with non-LCN tumors in general. It is also known that the LCN tumors have a significantly higher incidence of *MYCN* amplification, which alone is an indicator of poor clinical outcome.[6-8] The characteristic feature of LCN is the large size of tumor cell nuclei (12-17 mm in diameter) without, however, showing signs of differentiation.[9] Those large cells are primitive, and have a very high nucleus/cytoplasm ratio with scanty or often invisible cytoplasm. Our preliminary data show very low levels of TrkA expression, suggesting no potential of neuroblastic differentiation of LCN tumors. It is noted that neuroblasts of LCN have rather characteristic nuclei: they are sharply outlined, basophilic and not vacuolated, and contain 1 to 4 prominent nucleoli that are large and often eosinophilic. Many of the LCN tumors are uniformly composed of these large cells with a characteristic nucleus containing prominent nucleoli, and rare cases have a mixture of large cells and conventional neuroblasts with "salt and pepper" type of nuclei. LCN tumors are usually composed of densely packed neuroblastic cells with no or minimum neuropil, and are classified into either poorly differentiated subtype or undifferentiated subtype according to the INPC (*Fig. 2a, c-f*).[9]

On the other hand, neuroblastic cells in the biologically favorable neuroblastoma tumors, with a maturation potential associated with a higher TrkA expression, can also manifest cellular/nuclear enlargement as a sign of differentiation towards the ganglion cells in an age-dependent manner. The INPC defines those enlarged neuroblastic cells in a transitional stage of ganglionic maturation as "differentiating neuroblasts". Since both the primitive cells in clinically aggressive LCN tumors and differentiating neuroblasts in biologically favorable tumors are frequently large in size, clear distinction between them is critically important. According to the INPC definition, a "differentiating neuroblast" must show synchronous differentiation of the nucleus (an enlarged, eccentric nucleus with a vesicular chromatin pattern and usually a single prominent nucleolus) and of the conspicuous, eosinophilic or amphophilic cytoplasm. The greatest cytoplasmic dimension of differentiating neuroblasts should be more than twice as that of nucleus (*Fig. 2b*). It is noted here that many of the neuroblastoma tumors of differentiating subtype are often composed of a mixture of primitive, less differentiated and differentiating neuroblasts loosely or semi-compactly arranged in a neuropil background. These primitive or less differentiated neuroblasts in neuroblastomas with differentiation capacity typically have a nucleus of "salt-and-pepper" type. As already indicated, the cellularity of the tumors and the amount of neuropil should be assessed and are of help in the discrimination of neuroblastomas with signs of differentiation (decreasing cellularity, increasing amount of neuropil) from LCN (high cellularity, no or only sparse neuropil). Both features have been included when assessing prominent nucleoli in undifferentiated or poorly differentiated neuroblasts.[11]

Concept of large nucleolar neuroblastoma

Since we realized the distinct nucleolar morphology of LCN (see description above), we re-visited the descriptive term of "large cell" for this unique category of pNTs: Are those "large cells" in LCN really larger than those primitive/undifferentiated cells in conventional neuroblastoma? For this purpose, we conducted an image analysis and measured the mean nuclear area (MNA of at least 200 neuroblasts per tumor) from 10 tumors of the undifferentiated and poorly differentiated subtype and 4 LCN tumors. The results showed that MNAs of LCN tumors were significantly larger than those of the other tumor group. There was, however, a considerable overlap of MNA measurements between these two tumor groups (unpublished data).

Table 2. **Shimada system of neuroblastic tumors**

	Favorable	*Unfavorable*
NB		
<1.5 years	NPD or NDF with low or intermediate MKI	NUD or high MKI tumors
1.5-5 years	NDF with low MKI	NUD or NPD or intermediate or high MKI tumors
>5 years	none	all tumors
GNBi	all tumors	none
GN	all tumors	none
GNBn	depends on the neuroblastomatous component	

NB: neuroblastoma, GNBi: ganglioneuroblastoma, intermixed, GN: ganglioneuroma, GNBn: ganglioneuroblastoma, nodular, NPD: neuroblastoma, poorly differentiated, NDF: neuroblastoma, differentiating, MKI: mitosis-karyorrhexis index, NUD: neuroblastoma, undifferentiated

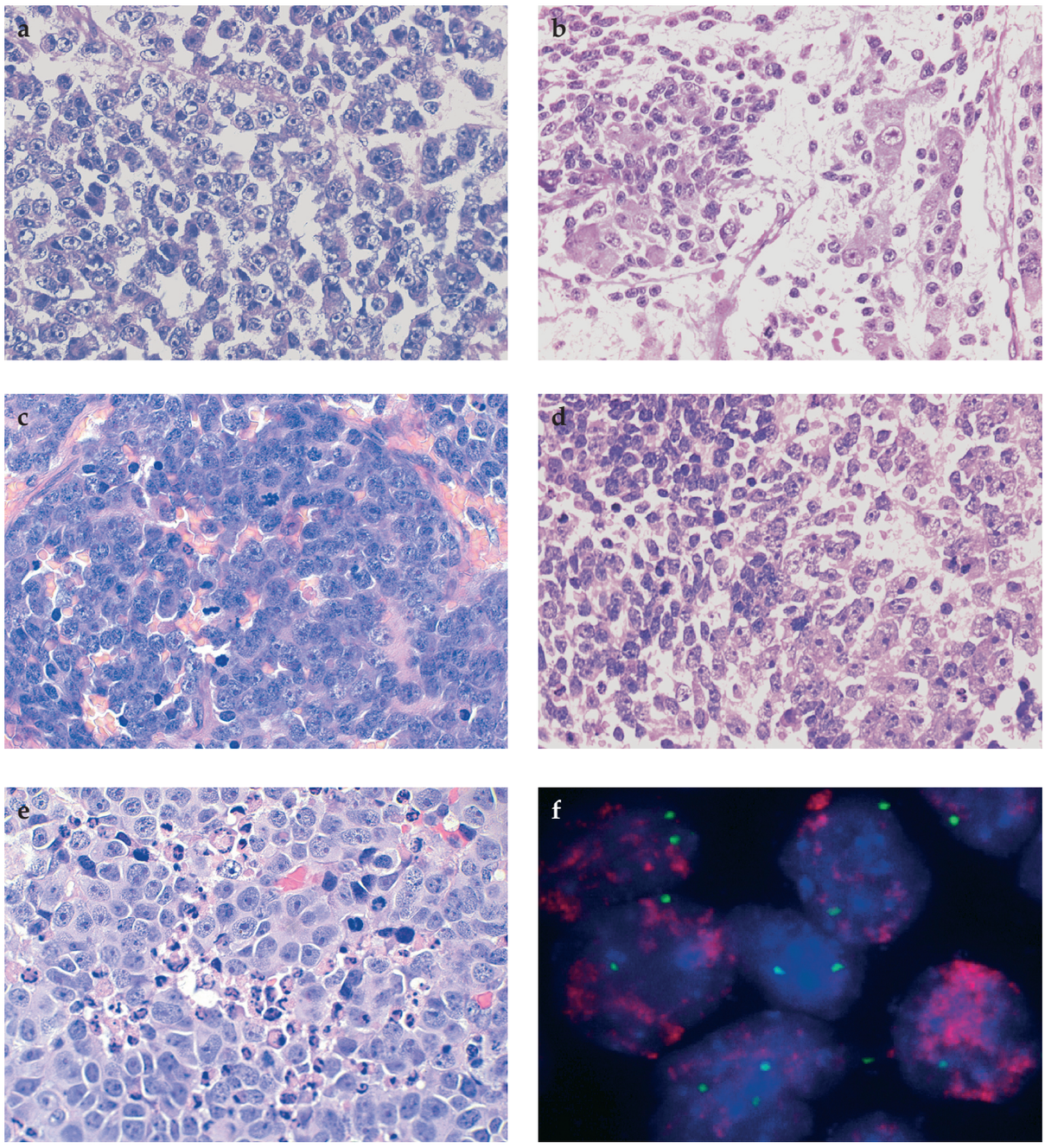


Figure 2. (a, c, e) *Different cases of large nucleolar (large cell) neuroblastomas. Note the undifferentiated cells with prominent nucleoli and rather large nuclei arranged in sheets surrounded by hemorrhagic connective tissue. No synchronous nucleoplasmic differentiation could be seen.* ***(b)*** *Neuroblastic differentiation; note the mature, large ganglion cells, the differentiating cells with enlarged, eosinophilic cytoplasm, large nucleus and prominent nucleoli. The cells are embedded in neuropil-rich background; see the small undifferentiated neuroblasts in between. Compare the large differentiated neuroblasts with variable size to the large, monomorphic population of undifferentiated cells in the LNN (LCN) without cytoplasmic enlargement seen in* ***Fig. 2 a, c, e.*** *Note the difference in cellularity as well.* ***(d)*** *Mixed case of conventional and large cell neuroblastoma. Note the size difference of the nuclei of the two components in the same tumor. The nuclear and cell size difference is frequently pronounced, but sometimes not so conspicuous. The nucleolar size (area) is always greater in the so-called large-cell cases compared to conventional neuroblastomas.* ***(f)*** *MYCN FISH in a large-cell (and also LNN) case (same case as in* ***Fig. 2 e)****. See the high number of red signals representing amplified MYCN status with 2 copies of centromeric signals of the reference chromosome 2*

In other words, although the nuclear area of LCN cases is frequently larger than that of the non-LCN cases, the cellular (eventually the nuclear) size alone may not be a reliable indicator for distinguishing LCN tumors from non-large cell and conventional neuroblastomas.

The next question was whether the presence of prominent nucleoli is specific for the LCN tumors. In 2002, Ambros et al. first reported that the presence of prominent nucleoli in the undifferentiated/poorly differentiated NB tumors is one of the indicators of unfavorable tumor biology.[11] Moreover, in the same study, the described kind of nucleolar enlargement turned out to be an additional hallmark of *MYCN* amplification (unpublished data). The recently published data by image analysis also supported their findings by demonstrating a frequent association of *MYCN* amplification and nucleolar enlargement (increased nucleolar area) in NB tumors.[10] These two reports seem to include both LCN and non-LCN in their series of cases without distinction. Now it becomes clear that prominent nucleoli are a very important cytological feature for predicting a poor clinical outcome of the disease, and it can be detected not only in the LCN, but also in non-LCN tumors of undifferentiated/poorly differentiated NB.

Effect of MYC gene amplification (MYCN and MYC) on the phenotype

The large nucleus and nucleoli are generally the signs of activated state of a cell. The possible explanation of these phenotypic changes in *MYC*-amplified cells was published in 2001.[12] Boon et al. using the SAGE technique proved that *MYCN*-transfected neuroblastoma cell lines and neuroblastoma tumor tissues with amplified *MYCN* status show upregulation of nucleolin, nucleophosmin and a series of ribosomal protein genes, all involved in either construction or processing/maturation of rRNA molecules and the ribosomal biosynthesis. Moreover, amplified *MYCN* also induces genes responsible for translation initiation and elongation. The activated RNA and protein synthesis machineries increase the nuclear, nucleolar and eventually the whole cell size. Other examples also support the above notion. Lamont et al. reported that *MYC* or *MYCN* amplification are significantly associated with large cell/anaplastic phenotype and poorer clinical outcome in medulloblastoma cases.[13] Later Stearns et al. demonstrated that *MYC*-transfected medulloblastoma cell lines with 10-30-fold *MYC* expression level compared to the non-transfected cell lines show "anaplastic" morphology including increased nuclear size and macronucleoli.[14] These findings indicate some homology between the two variants of malignancies of primitive neural crest origin: "anaplastic/large cell" medulloblastoma and "large cell/large nucleolar" neuroblastoma.

A new subtype of neuroblastoma: LNN (large nucleolar neuroblastoma)

Based on the reasons mentioned above, here we would like to propose a new subtype of large nucleolar neuroblastoma (LNN) in the NB category. Tumor cells in this entity have a characteristic nucleus containing large and prominent nucleoli, but do not show cytoplasmic enlargement/maturation. LCN can well be included in this group of LNN, as a large cell variant of undifferentiated/poorly differentiated NB.

LNN tumors are easy to recognize and diagnose by pathologists with routine review of H&E stained sections. Oil immersion lens (x1,000), if necessary, can assist us to identify the enlarged nucleoli. Because of its prognostic value and tight link to *MYCN* amplification, the presence of the prominent nucleoli should be described in the pathology report. We are also hoping to incorporate the LNN entity into the future INPC system.

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