

Protocol for the Examination of Specimens from Patients with Neuroblastoma

Protocol applies to neuroblastoma and related neuroblastic tumors.

No AJCC/UICC TNM Staging System

The International Neuroblastoma Staging System is recommended

Based on:

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None

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This protocol is revised to the current version of the CAP Cancer Protocol Neuroblastoma 3.1.0.3.

Procedures Covered in this Protocol:

- Resection
- Biopsy

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**AAPA Macroscopic Examination Guidelines:
Utilization of the *CAP Cancer Protocols* at the Surgical Gross Bench**

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The purpose of the Protocols is to support Laboratory Personnel engaged in the macroscopic examination of cancer resection specimens. The Protocols are based on specified relevant source documents, drafted by pathologists' assistant experts, and supported by information provided by the College of American Pathologists (CAP) and the American Joint Committee on Cancer (AJCC). These Protocols are intended to serve patients by ensuring that the macroscopic examination of cancer resection specimens is compliant with CAP Cancer Protocols, the AJCC Cancer Staging Manual, and provide optimization of the pre-analytic steps necessary to promote appropriate molecular studies.

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Molecular Considerations:

- Snap freeze at least 100 mg to 1 g of fresh tumor and store in a -70 degrees Celsius freezer for molecular studies.

First priority should always be given to formalin-fixed tissue for morphologic evaluation. Special studies (e.g., ploidy analysis, fluorescence in situ hybridization for *MYCN* status) are critical to the molecular workup of neuroblastoma and require at least 100 mg of viable, snap-frozen tumor as the second priority for workup.

MYCN Amplification

- Submit formalin-fixed, paraffin embedded sections or touch preparations for *MYCN*.
- Alpha MEM is also an accepted medium for *MYCN* if submitting fresh tissue.

The most prognostically relevant genetic alteration in neuroblastoma is *MYCN* amplification. *MYCN* gene amplification is associated with high-risk neuroblastic tumors and poor prognosis. *MYCN* is a proto-oncogene located on the short arm of chromosome 2. *MYCN* amplification is present in 20%-30% of primary tumors, most presenting as advanced-stage disease. The degree of amplification correlates with worse prognosis. *MYCN* status of a neuroblastic tumor can be determined by FISH within a relatively short period of time after the surgery or biopsy using touch preparations or formalin-fixed, paraffin-embedded sections.

Gene Sequencing

- ***ALK* Mutation and Amplification**
Recent studies have shown mutations in the anaplastic lymphoma kinase (*ALK*) gene in a subset of neuroblastic tumors and in the germline of patients with a familial predisposition to this disease. Although *ALK* immunohistochemistry does not always correlate with expression status, gene sequencing is sometimes performed in treatment-refractory patients who might be candidates for tyrosine kinase inhibitors.
- ***ATRX***
Mutations in the alpha-thalassemia/mental retardation X-linked syndrome (*ATRX*) gene are only found in 2% to 3% of all neuroblastic tumors. However, the vast majority of high-stage tumors in older children and adolescents have *ATRX* mutations. Congenital and infantile tumors only rarely have these mutations.

DNA Index

- Submit 100 mg to 1 g of fresh tumor in RPMI for flow cytometry.

Determination of DNA index by flow cytometry is also important; however, a minimum of 100 mg and preferably 1 g of fresh tumor is typically required for this purpose. A DNA index near diploid is unfavorable, while hyperdiploid tumors have a better prognosis. The prognostic effects of DNA index are reported to be limited to those patients diagnosed at younger than 2 years of age.

Others

Additional genetic abnormalities have clinicopathologic significance in neuroblastic tumors. Higher expression of TrkA (high-affinity nerve growth factor receptor) portends a good prognosis; *MYCN*-amplified tumors usually have a lower expression of TrkA.

Electron Microscopy:

- Submit 1 mm cubes of tumor in cold 2% glutaraldehyde or other suitable fixative for electron microscopy.

Ultrastructural studies are still of value in the diagnosis of relatively undifferentiated neuroblastoma, where the diagnosis is not readily evident by light microscopic study or urinary catecholamine study, especially given the variable specificity of immunostaining. Diagnostic criteria include dense core granules of neurosecretory type and cell processes (primitive neurites) containing typically arranged microtubules.

PROCEDURES AND GENERAL ANATOMIC CONSIDERATIONS:

■ Procedures Covered by this Protocol:

- Biopsy
- Resection

■ Specimen Size and Extent of Resection:

- Measure the specimen in three dimensions.
- Specify the specimen weight and tumor weight if separate from the total specimen.
- Identify and provide dimensions of attached structures.

■ Specimen Integrity, Adequacy, and Tumor Triage:

Submission of Tissue for Biologic Studies:

The International Neuroblastoma Staging System (INSS) is accepted as universally applicable and should always be recorded for new patients. Central to clinical staging is the size of the primary tumor, regional lymph node status, and the presence or absence of distant metastasis. The International Neuroblastoma Pathology Prognostic Classification (INPC) is used for risk assessment rather than staging and is based on age, neuroblastic maturation, Schwannian stromal content and MKI (Mitotic-Karyorrhectic Index).

For special biologic studies, a minimum of 2 samples (A and B, each 1 x 1 x 1 cm) should be taken, preferably from morphologically different areas. Samples A and B are each split into 4 pieces (A1,2,3, 4; B1,2,3, 4).

1	2
3	4

Submit tissue as follows:

A1, B1

Make at least 10 touch preparations from each (air-dried, unfixed, and, if necessary, stored at -20° C) for fluorescence in situ hybridization (FISH) (*MYCN*, chromosome 1p). Snap-freeze residuum of A1 and B1.

A2, B2

Submit tumor in sterile culture medium, such as alpha MEM or RPMI for *MYCN*, chromosome 1p, ploidy, cytogenetics, flow cytometry, and culture.

A3 A4, B3, B4

Snap-freeze two pieces in liquid nitrogen or at -70°C for molecular biology studies and immunohistochemistry.

The above recommendations are applicable when the entire or a large proportion of the tumor is resected, or when one or more large biopsy specimens are available. If the amount of tumor tissue is restricted, morphologic diagnosis is the prime consideration. Touch preparations (for FISH study of *MYCN*) should always be made from fresh tumor tissue.

If only core biopsies are performed, they should be multiple (2 to 4, for formalin fixation and snap-freezing), preferably concomitant with fine-needle aspiration specimens for FISH study of *MYCN*. A minimum of 100 mg snap-frozen tissue may be necessary for ploidy study by flow cytometry.

Procedures:

Core needle biopsies:

- Can obtain sufficient material for special studies and morphologic diagnosis, but sampling problems may limit tumor subtyping or grading, especially in tumors that are heterogeneous (i.e., ganglioneuroblastoma, nodular type).
- Grading can be performed on samples from metastatic sites provided that the specimen is large enough to be representative.

Resection:

- If oriented, differentially apply ink to the margins and section each margin.
- Sections should be obtained from central and peripheral areas of the tumor (at least 1 section per centimeter of the largest dimension and sections from all inked surgical margins).
- Note any extracapsular tumor extension into adjacent structures.
- All macroscopically visible nodules, cysts, or hemorrhagic foci should be sampled.
- Photographs should be taken, and if indicated, a section map can be made.

Definitive statements about specimen integrity should be provided, including positive (definitive statement that the specimen is intact) or negative (definitive statements that the specimen is not intact) statements. If not intact, statements should include, with as much clarity as can be provided, the anatomic location of the defects or disruptions, and should also incorporate statements which assess the relationship of any defects or disruptions to the tumor and final surgical margin. If defects or disruptions involve the tumor or margins and serve to hinder assessment of the final surgical margin this must be stated. Consultation with the surgeon for clarification should be considered, as well as differentially inking the margin in areas affected by defects or disruptions. Completely fragmented tumors, which cannot be reasonably measured, should be accompanied with a maximal tumor dimension on the requisition sheet or through consultation with the surgeon.

International Neuroblastoma Risk Group (INRG) Staging System (INRGSS)

A new clinical staging system, the INRGSS, has been proposed and increasingly adopted. The INRGSS relies only on pretreatment imaging, patient age, and clinical extent of disease. The INRGSS is summarized as localized disease (stage L1), regional disease (stage L2), metastatic disease (stage L3, and “special stage” (stage MS, similar the previous INSS stage 4S). This system relies heavily on image-defined risk factors. *

Stage L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
Stage L2	Locoregional tumor with presence of 1 or more image-defined risk factors
Stage M	Distant metastatic disease (except stage MS)
Stage MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow with minimal marrow involvement

***Image-Defined Risk Factors**

- Ipsilateral tumor extension within 2 body compartments
 - Neck-chest, chest-abdomen, abdomen-pelvis
- Neck
 - Tumor encasing carotid and/or vertebral artery and/or internal jugular vein
 - Tumor extending to base of skull
 - Tumor compressing the trachea
- Cervico-thoracic junction
 - Tumor encasing brachial plexus roots
 - Tumor encasing subclavian vessels and/or vertebral and/or carotid artery
 - Tumor compressing the trachea
- Thorax
 - Tumor encasing the aorta and/or major branches
 - Tumor compressing the trachea and/or principal bronchi
 - Lower mediastinal tumor, infiltrating the costo-vertebral junction between T9 and T12
- Thoraco-abdominal
 - Tumor encasing the aorta and/or vena cava
- Abdomen/pelvis
 - Tumor infiltrating the porta hepatis and/or the hepatoduodenal ligament
 - Tumor encasing branches of the superior mesenteric artery at the mesenteric root
 - Tumor encasing the origin of the celiac axis, and/or of the superior mesenteric artery
 - Tumor invading 1 or both renal pedicles
 - Tumor encasing the aorta and/or vena cava
 - Tumor encasing the iliac vessels
 - Pelvic tumor crossing the sciatic notch
 - Intraspinal tumor extension whatever the location provided that:
 - More than one-third of the spinal canal in the axial plane is invaded and/or the perimedullary leptomeningeal spaces are not visible and/or the spinal cord signal is abnormal
- Infiltration of adjacent organs/structures
 - Pericardium, diaphragm, kidney, liver, duodeno-pancreatic block, and mesentery
 - Conditions to be recorded, but not considered image-defined risk factors.
- Multifocal primary tumors
- Pleural effusion, with or without malignant cells
- Ascites, with or without malignant cells

TUMOR

■ **Tumor Size:**

- Measure tumor in three dimensions (solitary or multiple).
- Weight (as previously stated, specify the specimen weight and tumor weight if separate from the total specimen).

■ **Tumor Site(s):**

- Adrenal / peri-adrenal
- Retroperitoneal, non-adrenal
- Thoracic / paraspinal
- Cervical region
- Other (specify):

■ **Specimen Laterality and Contour:**

Ensure orientation of the specimen. Examine the contour if the specimen is the adrenal gland.

Specify laterality:

- Right
- Left
- Midline
- Other (specify):

Adrenal Contour:

- Pyramidal
- Compressed
- Irregular
- Bulging lobules
- Other (specify)

■ **Tumor Observations, and Relationship to Attached Organs / Structures:**

Adrenal gland: *

- Apply ink to the margins/periphery of the specimen.
- State whether the tumor is encapsulated.
- The cut surface of the tumor and its relationship to any identifiable normal tissue should be described including:
 - Color
 - Consistency
 - Necrosis
 - Hemorrhage
 - Fibrosis
 - Calcification
 - Cystic degeneration
 - Degree of circumscription
 - Degree of encapsulation
- If any portions of adjacent organs are attached, their appearance and relationship to the adrenal gland and tumor should be described.
- Demonstrate the relationship of the tumor to the associated soft tissue and adjacent organs.
- Demonstrate the relationship of the tumor to uninvolved adrenal gland.
- Include sections of peri-adrenal adipose tissue overlying a bulging mass.
- Include sections of uninvolved adrenal gland.

The macroscopic description should clearly document the sites of the sections taken.

*Tumor observations apply whether the tumor is an adrenal primary or other site.

■ **Explanatory Notes:**

Well-circumscribed, soft, variegated, predominantly gray-tan tumors, ranging from 2 cm to 10 cm in greatest dimension, with or without hemorrhage and calcifications is the common appearance of a poorly differentiated neuroblastoma, differentiating neuroblastoma, or a ganglioneuroblastoma. Some neuroblastomas are entirely hemorrhagic and have undergone near total cystic degeneration with or without calcifications.

■ **Margins: ***

- If oriented, differentially apply ink to the margins and section each margin.
- State whether the margins are involved or uninvolved by tumor.
- State the relationship of the tumor to the margin and the distance of the tumor to the surgical margin in cm.
- State if the tumor invades or extends through the surgical margin.

A large en bloc resection will require numerous sections from the peripheral margins.

*Margins are not actually applicable to the treatment and staging in neuroblastoma, and full margins need not be submitted. It is more important to sample variegated nodules than to emphasize margins, although representative sections of the closest approach of tumor to margin should be submitted.

LYMPH NODES

■ **Lymph Nodes:** (if applicable)

Regional lymph nodes for adrenalectomies include:

- Retroperitoneal
- Para-aortic
- Peri-aortic

Depending on the location of the specimen, lymph nodes may or may not accompany the specimen.

If lymph nodes are encountered:

- Identify and count all lymph nodes.
- Specify the site of the lymph nodes, if identifiable.
- Measure lymph nodes in three dimensions.
- Describe the cut surface of the identified lymph nodes.
- Submit all lymph nodes for microscopic examination.
 - Submit small lymph nodes in toto.
 - Serially section and entirely submit larger lymph nodes.
- If possible, submit lymph node sections so that the long axis of the lymph node is demonstrated.
- Take steps to ensure that an accurate lymph node count can be rendered.

METASTASIS

■ **Metastasis:**

- Distant metastasis (specify site, if known)

Neuroblastomas metastasize widely through blood and lymphatics, particularly to the liver, bones, and bone marrow. Proptosis and ecchymosis may also be present due to spread to the periorbital region, a classic metastatic site. In neonates, disseminated neuroblastomas may present with multiple cutaneous metastases that cause deep blue discoloration of the skin ("blueberry muffin baby").

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