Subgrouping of Unfavorable Histology Neuroblastomas With Immunohistochemistry Toward Precision Prognosis and Therapy Stratification

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INTRODUCTION

Neuroblastoma, as well defined by Willis,[1] is an embryonal tumor of neural crest origin. Tumors of the neuroblastoma group include neuroblastoma, ganglioneuroblastoma, and ganglioneuroma. We believe that all ganglioneuromas were once neuroblastomas in the early stage of tumor development.[1,2] They are collectively called peripheral neuroblastic tumors (pNTs) and are known to present with a wide range of clinical behavior, from spontaneous regression and tumor maturation to aggressive progression that is refractory to intensive treatment. Recent advances in research indicate that molecular and/or genomic properties of individual tumors are closely associated with their unique clinical behaviors.[3-5] During the past several decades, histopathologic analyses of pNTs have provided invaluable information for predicting prognosis and determining therapy stratification. The origin of International Neuroblastoma Pathology Classification (INPC) dates to the 1984 article by Shimada et al[6] that first introduced the age-linked pathology classification system. Since then, the Shimada system has laid the foundation for the current INPC.[2,7] The INPC, as a single prognostic parameter, clearly defines clinically aggressive and nonaggressive groups of pNTs.[7,8]

The INPC, distinguishing favorable histology (FH) and unfavorable histology (UH) groups, defines four categories of pNTs: neuroblastoma (Schwannian stroma poor); ganglioneuroblastoma, intermixed (Schwannian stroma rich); ganglioneuroma (Schwannian stroma dominant); and ganglioneuroblastoma, nodular (composite, Schwannian stroma rich/stroma dominant and stroma poor).[2] Cases in the ganglioneuroblastoma, intermixed and ganglioneuroma categories are always classified into the FH group, and the patients enjoy excellent clinical outcomes.[9] Patients with disease in the neuroblastoma and ganglioneuroblastoma, nodular categories are classified into either the FH group or UH group on the basis of age-linked evaluation (0 to 18 months, 18 to 60 months, and ≥ 60 months) of neuroblastic differentiation grade (undifferentiated, poorly differentiated, and differentiating) and Mitosis-Karyorrhexis index (low: < 100/5,000 cells; intermediate 100 to 200/5,000 cells; and high: ≥ 200/5,000 cells) of the individual neuroblastoma tumors or neuroblastoma component of ganglioneuroblastoma, nodular tumors.[2,7,8,10,11]

Whereas survival of patients with FH pNT is excellent (> 90%), survival of patients with UH pNT has only incrementally improved over the years and currently remains approximately 40% to 50%.[7,8,10] This seems to be mainly because we have had a limited idea of what molecular mechanism underlies the therapy-resistant behavior of UH tumors. These observations have led us to seek additional immunohistochemical biomarkers that are tightly associated with aggressive behaviors—that is, unresponsiveness or resistance to the current intensive multimodal therapy—of certain UH tumors. To address this problem, we and others have sought and identified the expression of potentially drug-targetable proteins that are responsible for their aggressive progression in the UH group. When we seek such biomarkers, they are preferably actionable/ druggable by existing pharmaceutical agents, US Food and Drug Administration approved, or currently tested in human clinical trials. If we could effectively neutralize the activity of such targets with specific drugs, then survival of UH patients as a whole would substantially improve.

POSSIBLE DRIVERS OF AGGRESSIVE UH NEUROBLASTOMA

Among UH neuroblastomas, approximately 50% of them carry *MYCN* amplification.[12] Approximately 80% to 90% of *MYCN*-amplified neuroblastomas show augmented expression of the MYCN protein.[12-14] This is associated with adverse clinical outcomes and considered a driver of clinically aggressive neuroblastoma.[15,16] What other mechanisms could account for driving aggressive neuroblastomas without *MYCN* amplification? The majority of neuroblastoma cell lines without *MYCN* amplification express high levels of MYC proteins, but the prognostic significance of MYC protein overexpression in patients

with neuroblastoma was defined only recently.[12] We have found that a considerable number of primary neuroblastomas without *MYCN* amplification—approximately 10% of the entire cohort or approximately 20% of UH cases—actually express high levels of MYC protein and their survival was similar to that of neuroblastoma with high MYCN protein expression (3-year event-free survival, 46.2 ± 12.0% for MYCN overexpressing neuroblastoma and 43.4 ± 23.1% for MYC overexpressing neuroblastoma, respectively).[12] On the basis of this finding, we immunohistochemically defined neuroblastomas with MYCN or MYC overexpression as MYC-driven neuroblastoma.[12]

Recent next-generation sequencing analyses of high-stage neuroblastomas have provided additional clues as to the possible drivers of non–MYC-driven aggressive neuroblastoma.[17-19] Those include *ATRX* (α-thalassemia/mental retardation syndrome X linked) mutations, leading to ATRX loss and the ALT (alternative lengthening of telomere) phenotype, and TERT overexpression as a result of *TERT* gene rearrangements. Both mechanisms prevent tumor cells from replication senescence/cell death as a result of telomere erosion and provide them with infinite proliferating capability. Of note, these three types of highly aggressive neuroblastomas—that is, MYC-driven neuroblastoma, ALT phenotype neuroblastoma because of ATRX loss, and TERT-overexpressing neuroblastoma with gene rearrangement—are mutually exclusive and seem to comprise the majority of therapy-resistant or refractory UH neuroblastomas. It should be noted that these three markers—that is, MYC family proteins, TERT, and ATRX—can be the subjects of immunohistochemical assay and potentially incorporated in future INPC.

Other than the above gene and expression alterations found in aggressive UH neuroblastomas, *ALK* mutations and amplification are found in approximately 10% of sporadic neuroblastoma cases.[20,21] It was also found that *ALK* gene amplification and F1174 mutations are associated with *MYCN* amplification.[22] Moreover, ALK overexpression as a result of *ALK* amplification/mutations seems to be responsible for its oncogenic activity[23]; however, the prognostic significance of *ALK* mutations/overexpression has been controversial, as some reports suggest an association of *ALK* mutations/overexpression with fatal outcomes of the disease, whereas others suggest otherwise.[22,23] In particular, Regairaz et al[24] showed immunohistochemically that expression of ALK and its active form pALK was observed in many neuroblastomas independent of *ALK* mutation/amplification. On the basis of these observations, future INPC will not likely incorporate the immunohistochemical detection of ALK and/or pALK expression.

PROPOSED SUBGROUPING OF UH NEUROBLASTOMAS

As indicated above and also detailed in our article,[25] we are gaining additional insight into the potential molecular targets that underlie the therapy-resistant and aggressive

behavior of UH neuroblastomas. Moreover, therapy resistance is often attributed to a protein functional level but it is difficult to defeat it because of the inherent plasticity/stemness of tumor cells. In this regard, MYC/MYCN and TERT overexpression[26-29] has been linked to the stemness of tumor cells, and direct and indirect therapeutic targeting of these alterations suppresses the stemness of aggressive and therapy-resistant UH neuroblastomas. Similarly, ALT is linked to tumor stem cells.[30,31] These observations allow us to incorporate the information into the INPC toward the goal of precision prognosis and therapy stratification.

Our plan is to refine the UH neuroblastoma stratification using immunohistopathologic analysis with anti–pan-MYC antibody (or anti-MYCN and anti-MYC), anti-TERT antibody, and anti-ATRX antibody. As shown in Table 1 and Appendix Figure A1 and summarized below, UH neuroblastomas are classified into four subgroups. Taken together, targeting TERT, MYCN/MYC, and ALT via ATRX would contain the stem-cell compartment of the UH neuroblastoma. It is expected that the nonresponder to current high-risk therapy—MYC, TERT, and ATL subgroups—could be separated from the possible responder—Null subgroup—with a high probability before the initiation of therapy:

MYC subgroup: MYC-driven neuroblastoma shows augmented expression of MYCN protein and/or MYC protein. As MYC family protein overexpression stimulates rRNA synthesis and protein translation, neuroblastoma cells in this subgroup often exhibit prominent nucleolar formation—nucleolar hypertrophy—and hypertrophic cell morphology.[13,14] Because of the presence of one to a few prominent nucleoli, MYC-driven neuroblastoma is cytologically distinguishable from the conventional small blue-cell neuroblastoma with salt-and-pepper nuclei. MYC-driven neuroblastomas, which comprise more than 50% of high-risk neuroblastomas, includes rare and unique tumor, named large-cell neuroblastoma, characterized by the bull's eye appearance of enlarged and uniquely open euchromatin-rich nuclei that contain highly conspicuous nucleoli.[33] Most large-cell neuroblastomas overexpress higher levels of MYCN or MYC protein than other MYC-driven neuroblastomas. Their euchromatin-rich open nuclei suggest the stem cell–like nature of the tumor cells.[29] A considerable number of MYC-driven neuroblastomas are also associated with TERT overexpression, as *TERT* is a direct transcriptional target of MYC family proteins. TERT can also stabilize MYC protein via its noncanonical enzymatic function, creating a positive feedback loop between MYCN/MYC and TERT expression.[34,35]

TERT subgroup: TERT is the protein component of telomerase, which also includes TERC, the telomerase RNA component. Higher levels of TERT expression are also observed in neuroblastomas without MYCN/MYC protein overexpression. TERT overexpression, in this case, is

TABLE 1. Proposed Subgroups of Unfavorable Histology Neuroblastoma

	Immunohistochemistry Marker*				Predicted Survival on the Basis of Current Therapy†
Subgroup	Pan-MYC or MYCN/MYC	TERT	ATRX	Histologic Marker	
MYC driven					
MYC	Overexpression	Overexpression or no overexpression	Retention	Nucleolar hypertrophy, including large-cell neuroblastoma	Dismal
Non-MYC driven					
TERT	No overexpression	Overexpression	Retention	Salt-and-pepper nuclei; conventional neuroblastoma	Dismal
ALT	No overexpression	No overexpression	Loss‡	Salt-and-pepper nuclei; conventional neuroblastoma	Dismal
Null	No overexpression	No overexpression	Retention	Salt-and-pepper nuclei; conventional neuroblastoma	Better response

Abbreviations: ALT, alternative lengthening of telomere; ATRX, α-thalassemia/mental retardation syndrome X linked; TERT, telomerase reverse transcriptase.

*Immunohistochemical grading of MYC family protein expression was performed according to Wang et al.[12] Evaluation of ATRX loss is done when tumor cell nuclei are negative and endothelial cell nuclei (built-in control) are positive in the same tumor tissue. Immunohistochemical grading of TERT is performed in essentially the same way as that of MYC family proteins.

†Estimated survival of each subgroup is based on Wang et al[12] and Valentijn et al.[18]

‡To exclude the possibility of overlooking ALT cases with *ATRX* point mutations with no ATRX loss and/or rare *DAXX* mutations, patients older than age 5 years in the Null group can be subjected to additional screening by either rDNA[32] or telomeric fluorescence in situ hybridization assay.[32a]

likely a result of long-range genomic rearrangements but not to promoter mutations in neuroblastoma.[36] *TERT* promoter hypermethylation may also be involved in its activating mechanism.[37,38]

ALT subgroup: ATRX loss results in the ALT phenotype. *ATRX* mutation is rarely observed in the MYC and TERT subgroups, and loss of ATRX is exclusively observed in patients with neuroblastoma older than age 5 years.[17] Because a normal ATRX function is to insert the variant histone H3, namely H3.3, in concert with DAXX, into chromatin to maintain transcriptionally active euchromatin,[39] ATRX loss may result in a more transcriptionally inactive heterochromatic state. Thus, the histologic/cytologic appearance of the ALT subgroup could be the conventional type with salt-and-pepper nuclei.

Null subgroup: There are still UH neuroblastomas without MYC family protein overexpression, TERT overexpression, and ATRX loss. As long as no other aggravated factors are found,[40] patients in the Null subgroup would, we hope, respond to current high-risk treatment regimens.

MOLECULAR TARGETING THERAPIES FOR UH NEUROBLASTOMA RESISTANT TO CURRENT THERAPY

To develop treatment strategies for MYC-driven neuroblastomas, new therapeutic agents should be clearly defined; however, direct targeting of MYC family proteins with small molecules has been a formidable task.[41] Hence, many have sought indirect approaches to downregulate MYC family protein expression in cancer cells. Strategies under consideration include, but are not limited to, transcriptional repression of *MYC* and *MYCN* genes by G-quadruplex (G4) stabilizers,[42,43] bromodomain and extraterminal protein inhibitors,[44,45] and inhibitors of a transcriptional cyclin-dependent kinase 7[46,47]; destabilization of MYCN by Aurora kinase A inhibitors[48] and of MYC and MYCN proteins by RAS signaling pathway inhibitors[49-51]; and translational blockade of *MYC* mRNA by eIF4F inhibition and stabilization of RNA G4 by RNA G4 ligands.[52,53] We recognize that UH tumors with high-level MYC family protein expression tend to exhibit hypertrophic nucleoli,[13,14] which indicates that they are highly active in rRNA synthesis and protein translation. Small-molecule inhibitors, including CX-5461, an RNA polymerase I inhibitor, and halofuginone, a protein translation inhibitor, could therefore effectively target these pathophysiologic features. As we have shown, these inhibitors in fact downregulate MYC family protein expression in neuroblastoma cells.[13]

For those patients with TERT-overexpressing neuroblastoma, telomerase inhibitors can be considered. Imetelstat (GRN163L)[54] is a potent and specific inhibitor of telomerase that binds with high affinity to TERC (telomerase RNA component). Of interest, sorafenib has been demonstrated to synergize with imetelstat to inhibit the growth of mouse xenografts of human cancer.[55] Sorafenib is a US Food and Drug Administration–approved kinase inhibitor,[56] but its synergetic effect with imetelstat seems to be a result of p21 attenuating activity.[55] On the basis of these observations, a combination of imetelstat and sorafenib may prove to be more efficacious than imetelstat alone to those neuroblastomas with elevated telomerase activity.

ALT inhibition could also be considered for those patients with neuroblastoma with ATRX loss; however, because ATRX loss is a result of structural alterations in the *ATRX*

gene,[57] it would be difficult to regain ATRX expression in neuroblastoma. If so, is there any other way by which the ALT phenotype can be suppressed? To address this question, we need to understand the mechanism of how ATRX loss leads to ALT. It has become evident that the acquisition of the ALT phenotype uses the DNA replication stress response,[30] which involves a cascade of events, including the obligatory activation of ATR (ataxia telangiectasia and Rad3 related) kinase. AZD6738 is a novel potent and selective inhibitor of ATR kinase with IC_{50} values of less than 1 μM in cell-based assays[58] and would effectively target ALT tumor cells.

CONCLUSION

Prognosis and therapy stratification of neuroblastomas have steadily improved since the introduction of the Evans staging system[59] with age used as a prognostic factor[60]; however, 5-year survival of patients with high-risk neuroblastoma, defined by the International Neuroblastoma Risk Group system (INRG), remains at approximately 40% or lower.[61,62] The same is true for the UH neuroblastoma group, which essentially overlaps high-risk neuroblastomas.[8,63] Unfortunately, no additional refinement for the INRG has been proposed since 2009.[61] The proposed histologic subgrouping of UH neuroblastomas represents a practical and immediately implementable approach with which to create the category leading to the most devastating clinical outcome, namely extremely unfavorable histology (EUH) neuroblastoma. We expect that conventional UH neuroblastoma tumors—that is, the Null subgroup—would, we hope, respond to the current multimodal high-intensity therapy designed for high-risk neuroblastoma, whereas EUH tumors—that is, those of the MYC, TERT, and ALT subgroups—would not. For those EUH tumors, appropriate therapy protocols will have to be designed and tested.

On the basis of our findings,[12] MYC family protein immunohistochemistry is already incorporated in the Children's Oncology Group pathology analysis as an integrated biomarker of neuroblastoma. Similar attempts are now underway for TERT and ATRX immunohistochemistry for precision prognosis and the stratification of neuroblastoma. Furthermore, we are planning to perform correlative studies between TERT protein expression and *TERT* gene rearrangements and between ATRX protein loss and *ATRX* gene mutations/deletions. We believe that comprehensive integration of improved INPC with immunohistochemical subgrouping into INRG will contribute significantly to the future management of patients with this devastating disease.

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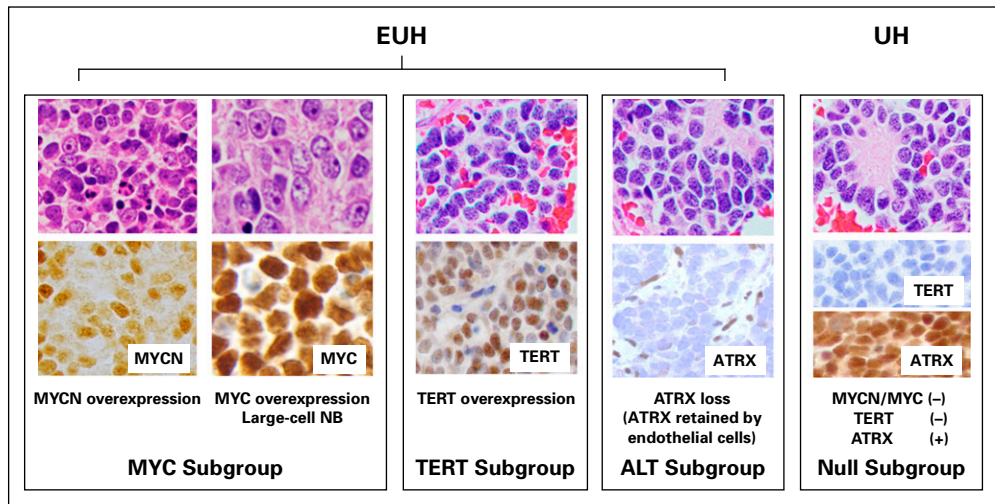
APPENDIX

FIG A1. Histological and immunohistochemical appearances of extremely unfavorable histology and unfavorable histology neuroblastomas. ALT, alternative lengthening of telomere; ATRX, α-thalassemia/mental retardation syndrome X linked; EUH, extremely unfavorable histology; NB, neuroblastoma; TERT, telomerase reverse transcriptase; UH, unfavorable histology.