Immunohistochemical Analyses of α1 and α3 Na^+/K^+-ATPase Subunit Expression in Medulloblastomas

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Abstract. *Background: The levels of expression of the α1 and α3 subunits of the Na^+/K^+-ATPase (the NaK sodium pump) in medulloblastomas are unclear. Patients and Methods: This study investigated the expression of the NaK subunits using immunohistochemical methods in 29 medulloblastomas including 23 classic, three large-cell/anaplastic and three nodular/desmoplastic medulloblastomas, as well as in three atypical teratoid/rhabdoid tumors (AT/RTs). Results: There was overexpression of the α1 or α3 NaK subunits in more than half of the medulloblastomas and atypical AT/RTs, with about one-third of these tumours displaying overexpression of both subunits. Conclusion: These preliminary data suggest that targeting these subunits in AT/RTs and medulloblastomas that overexpress these proteins may lead to therapeutic benefit. These findings warrant confirmation in larger numbers of patients than those used in this study. Moreover, it should be determined whether inhibition of the α1/α3 NaK subunits can be integrated into the risk stratification schemes already in use for medulloblastoma patients.*

Medulloblastoma is the most common malignant tumor of the central nervous system in children (1-4). Current treatment strategies are relatively ineffective in that nearly 50% of medulloblastoma patients die from tumor progression (5). Targeted therapeutic strategies based on novel biomarkers are, therefore, warranted in the treatment of this disease. Several risk stratification systems for pediatric medulloblastoma have been proposed based on a combination of histopathological evaluation and targeted molecular analysis (6, 7). Four medulloblastoma variants have been recognized by the 2007 World Health Organization (WHO) classification; patients with desmoplastic medulloblastoma and medulloblastoma with extensive nodularity have significantly better survival than patients with classic medulloblastoma, whereas patients with large-cell and anaplastic medulloblastoma have a poorer prognosis (7). Moreover, 17p loss, *c-MYC* amplification/overexpression, and 1q gain are associated with poor prognosis, whereas monosomy 6, mutations in *CTNNB1*, and *TRKC* overexpression are associated with more favorable outcomes (7). A risk stratification model has therefore been proposed based on *c-MYC*, LDHB and CCNB1 expression combined with clinical variables (6). Moreover, a very recent and comprehensive review of medulloblastoma describes the clinical landscape, the current WHO classification system, the status of molecular subgroups and how potential stratification schemes may impact pathologists and their practice (2).

The sodium pump, or Na^+/K^+-ATPase (*i.e.*, NaK), acts as a versatile signal transducer and is a key player in cell adhesion. Furthermore, its aberrant expression and activity have been implicated in the development and progression of different types of biologically aggressive cancer types (8, 9), suggesting that it may be an important target for the development of anticancer drugs. Targeting the α1 and/or α3 subunits of NaK has been shown to induce non-apoptosis-related cell death in cancer cells that are intrinsically resistant to proapoptotic stimuli (10-12) and/or in cells with the multidrug-resistant (MDR) phenotype (13). Non-small cell lung cancer (NSCLC) (14), glioblastomas (12,15) and melanomas (16) have been found to overexpress the α1 NaK subunit, and all three tumor types have shown marked resistance to proapoptotic stimuli (17-19). In addition, the α3 NaK subunit is overexpressed in colon cancer (20).

Atypical teratoid/rhabdoid tumors (AT/RT) are a type of malignant primary brain tumor that occurs in early childhood and that is frequently misdiagnosed as primitive neuro-ectodermal tumor/medulloblastoma (4). However, the biological features and clinical outcomes of AT/RT tumors and medulloblastomas are notably different and AT/RT tumors may present embryonic stem-like gene expression patterns (4).

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The level of expression of the α1 and α3 subunits of NaK has not yet been investigated in medulloblastomas and AT/RTs. In this study, therefore, the expression of these two proteins was assayed, using immunohistochemical methods in 29 medulloblastomas, including 23 classic, 3 large-cell/anaplastic and 3 nodular/desmoplastic medulloblastomas, as well as in 3 atypical AT/RTs.

Patients and Methods

Clinical samples. The 29 medulloblastomas and 3 AT/RT tumors analyzed in the present study were obtained from patients who underwent surgery between 1982 and 2005 at the Department of Anatomic Pathology of the University Hospital of Saint Joan de Deu in Barcelona, Spain. Average patient age at diagnosis was 5.6 years, while the patient age range was 1 to 14 years. Use of these samples was approved by the Ethics Committee of the University Hospital of Saint Joan de Deu.

Immunohistochemistry. Two-μm-thick sections from each sample were deparaffinized, rehydrated and incubated with antibody to the α1 (Bio-Connect BV, Huissen, the Netherlands) or α3 (Sigma-Aldrich, Bornem, Belgium) subunit of NaK, using an immunohistochemical procedure identical to that previously described (14). Kidney and brain tissues were used as positive controls for the α1 and α3 NaK subunits, respectively. Tissue samples processed in the absence of primary antibody were used as negative controls. The results were assessed by two pathologists (M.S. and V.C.) and the clinical dates were assessed by an oncologist (O.C.). The percentages of α1- and α3-positive cells were determined on a 10×10 grid. Since immunohistochemical staining for α1 and α3 NaK may have been nuclear as well as cytoplasmic (Figure 1), nuclear and cytoplasmic staining were analyzed independently and the percentages of nuclear *versus* cytoplasmic positive cells were determined. Staining that appeared rather homogeneous and similar among all samples was ignored.

Results

No significant ($p>0.05$: Mann-Whitney test) differences in the percentages of tumor cells positive for α1 (Figure 2A) and α3 (Figure 2B) were observed among the four histopathological groups under investigation, regardless of whether the immunohistochemical staining was nuclear or cytoplasmic.

The percentage of classic and nodular/desmoplastic (NodDesm) medulloblastoma samples positive for the α3 NaK subunit was slightly higher than the percentage of positive samples for the α1 NaK subunit, using >5% positivity as a cutoff value, whereas none of the three large-cell/anaplastic medulloblastomas were immunohistochemically positive for the α1 NaK subunit (Figure 3A). In contrast, all three AT/RTs were immunohistochemically positive for both α1 and α3 (Figure 3A). These findings suggest that the α1/α3 profiles may differ between medulloblastomas and AT/RTs as well as among the various medulloblastoma subgroups.

Of the 32 samples studied, including 23 classic, 3 nodular/desmoplastic and 3 large-cell/anaplastic medulloblastomas and 3 AT/RTs, 19 (59%) were immunohistochemically positive for either α1 or α3 while 9 (28%) were positive for both (Figure 3B).

Discussion

This study showed that more than half of the medulloblastoma and AT/RT samples studied overexpressed the α1 or α3 subunit of NaK, with around one-third of these samples overexpressing both subunits. These preliminary findings suggest that targeting the α1 and/or α3 NaK subunits in AT/RTs and medulloblastomas that overexpress these proteins may lead to therapeutic benefits. Selective α1 and/or α3 NaK subunit ligands have been found to markedly inhibit the growth of cancer cells that overexpress these biomarkers, while displaying much weaker growth inhibitory activity in normal cells (12, 14, 21). Although these findings are preliminary, they warrant confirmation in a notably larger population of patients than that of the present study.

Targeting the α1 and α3 subunits of the sodium pump may eliminate *c-MYC* activation in cancer cells that overexpress these subunits. Compounds that target the α1 and α3 subunits may inhibit the activity of cyclin-dependent kinase and suppress *c-MYC* expression and related signaling pathways, activities paralleled by the disorganization of cancer cell-specific perinucleolar bodies, as revealed by the disruption of Sam68 (21). *c-MYC* expression is dysregulated in a wide range of human cancer types and is often associated with aggressive, poorly-differentiated tumors (22). *c-MYC* is also overexpressed and/or overactivated in medulloblastomas (4, 23-25). The *c-Myc* protein is a transcription factor that regulates a variety of cellular processes, including cell growth and proliferation, cell-cycle progression, transcription, differentiation, apoptosis and cell motility (22). *c-MYC* overexpression in medulloblastoma has been shown to cause anaplasia and to correlate with an unfavorable prognosis (23-25).

Combinations of mechanistically different cytotoxic drugs targeting the *c-MYC* protein have been designed to eradicate *c-MYC*-activated tumor cells (22). A recent review described the potential strategies currently being developed to inhibit the proliferation-promoting effect of *c-MYC versus* activating its pro-apoptotic functions (26). Moreover, targeting *c-MYC* may represent a novel therapeutic strategy for the treatment of medulloblastomas (27).

Apart from overexpressing *c-MYC*, which is associated with a dismal prognosis in patients with medulloblastoma, these tumors are intrinsically resistant to pro-apoptotic stimuli, making them particularly aggressive. For example, the expression of the proto-oncogene BCL-2, encoding an anti-apoptotic protein, correlates with poor outcome in

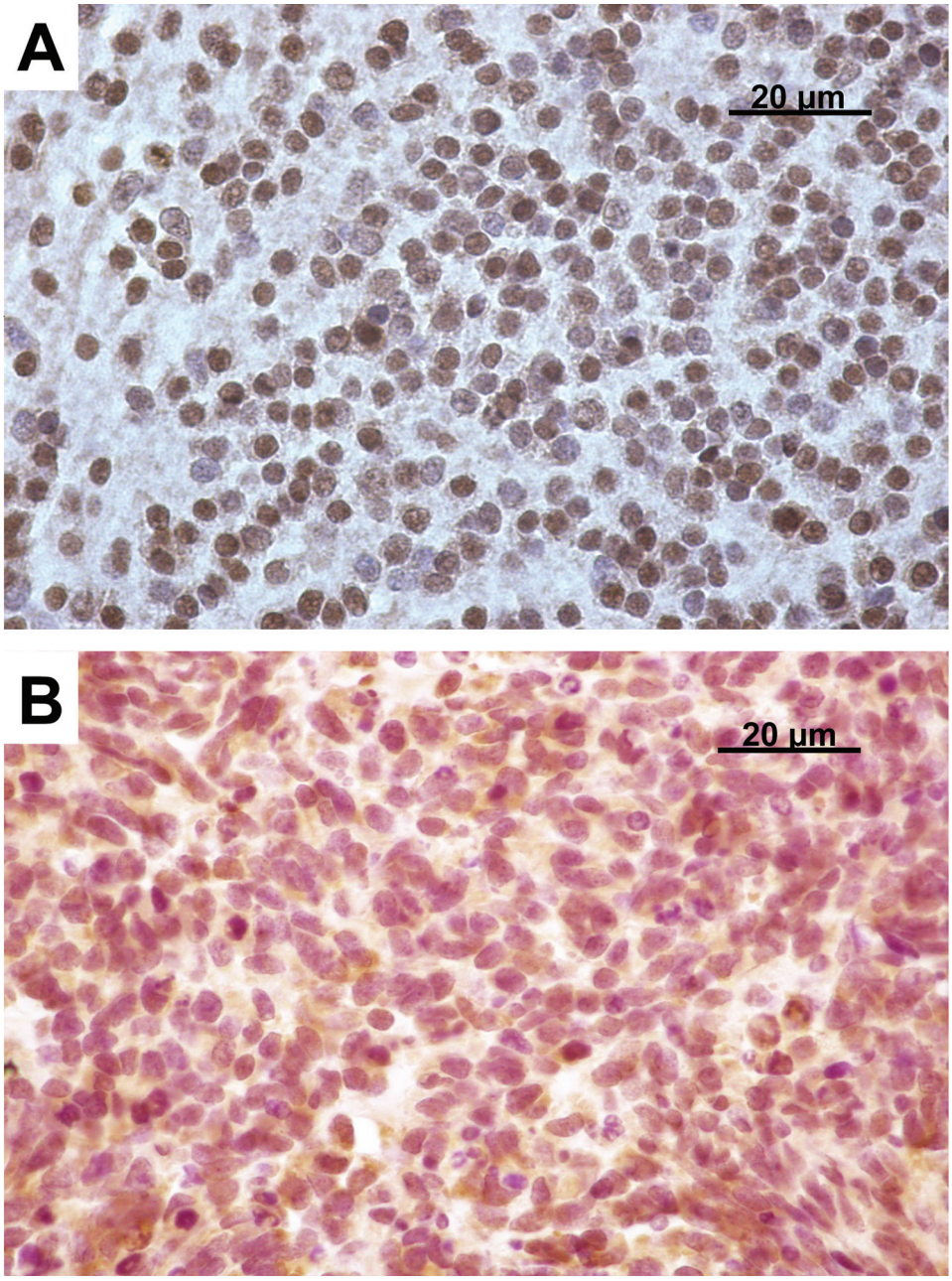

Figure 1. *Morphological illustrations of immunohistochemical patterns of (A) nuclear (α3 Na^+/K^+-ATPase (NaK) subunit) and (B) cytoplasmic (α1 NaK subunit) staining. Magnification: ×60.*

classical medulloblastoma (28). Survivin, a member of a family of proteins that inhibit apoptosis, has been implicated in the dysregulation of apoptosis in human cancer and is frequently overexpressed in medulloblastomas (29). Many other proteins and/or genes participate in the intrinsic resistance of medulloblastomas to pro-apoptotic stimuli and therefore correlate with poor prognoses; these include, for example, caspase-8 (30), *TP53* (31), the MAGE and GAGE genes (32) and the PI3K p110α isoform (33). Targeting the NaK subunits may overcome, at least in part, the intrinsic resistance of cancer cells to pro-apoptotic stimuli because such targeting induces non-apoptotic-related death in cancer cells. These pathways may include lysosomal membrane permeabilization processes in NSCLC cells (10) and sustained pro-autophagic effects, leading to cell death in glioblastoma cells (12).

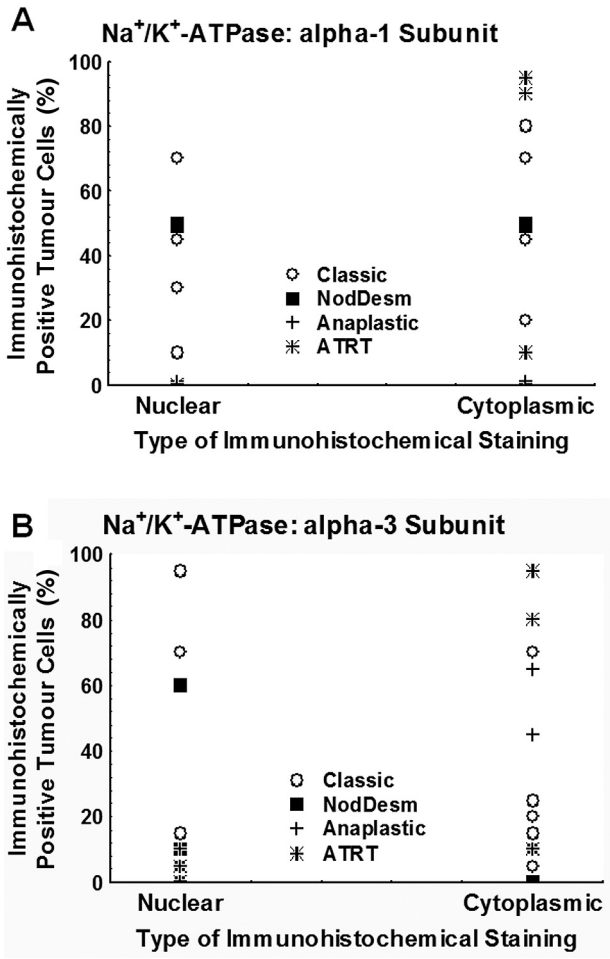

Figure 2. *Percentage of nuclear- versus cytoplasmically immunohistochemically positive tumor cells for the α1 (A) and α3 (B) NaK subunits in 29 medulloblastomas, including 23 classic, 3 nodular/desmoplastic (Noddesm) and 3 large-cell/anaplastic medulloblastomas, as well as 3 atypical teratoid/rhabdoid tumors (ATRTs).*

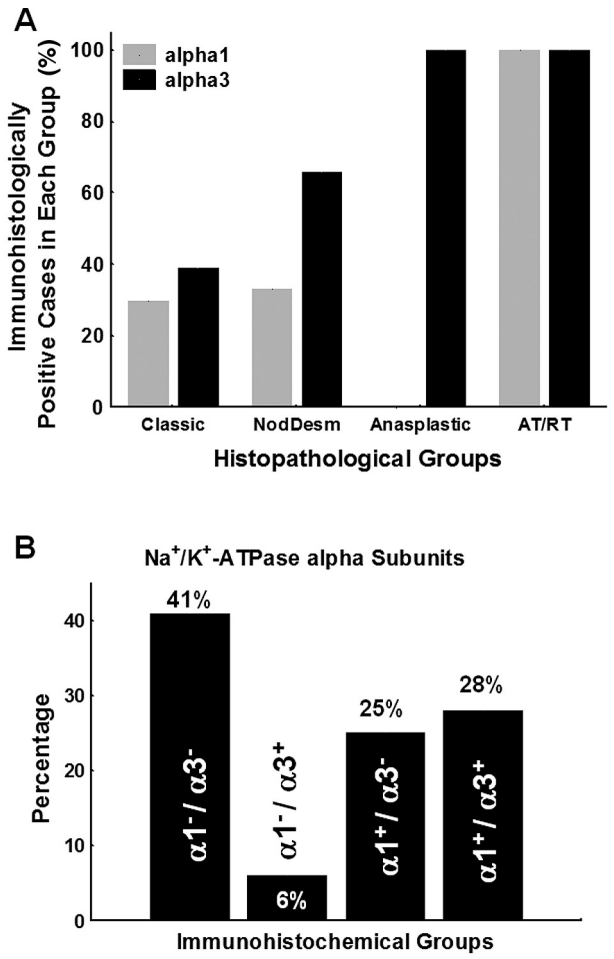

Figure 3. *A: Percentages of medulloblastoma and ATRT samples positive for the α1 and α3 NaK subunits. The samples assayed were identical to those described in Figure 2. B: Percentages of α1−/α3−, α1−/α3+ α1+/α3− and α1+/α3+ NaK subunit patterns of immunohistochemical staining in these samples.*

Medulloblastoma cells can also resist pro-apoptotic stimuli through overactivation of NF-κB (34, 35), a transcription factor that participates in the resistance of many cancer types to chemotherapeutic agents (36, 37). Targeting the NaK α1 and/or α3 subunits can overcome such NF-κB-mediated resistance to pro-apoptotic stimuli because NaK ligands are able to markedly reduce NF-κB activation (11).

In conclusion, the results of the present study suggest that the α1 and α3 subunits of NaK warrant further in-depth analyses to determine whether their levels of expression translate into distinct clinical outcomes for medulloblastoma patients. Current clinical indices for the prediction of outcomes in medulloblastoma patients are imprecise, even after the identification of a series of molecular and histopathological biomarkers (38). The present findings suggest that the prognostic value of the α1 and α3 NaK subunits should be determined, in particular, because novel anti-α NaK subunit compounds have recently entered clinical trials.

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