Brief Communication:
Morphoproteomic Confirmation of Constitutively Activated mTOR, ERK, and NF-kappaB Pathways in High Risk Neuroblastoma, with Cell Cycle and Protein Analyte Correlates

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Abstract. Morphoproteomic analysis reveals the constitutive activation of the mTOR, ERK, and NF-kappaB pathways in high risk neuroblastoma (HRN) cases as evidenced by (a) collective commonalities of: phosphorylated (p)-mTOR, p70S6K, ERK 1/2, and NF-kappaBp65 protein analytes using phosphospecific probes directed against sites of activation; (b) nuclear translocation of p-p70S6K, p-ERK 1/2, and p-NF-kappaBp65; and (c) correlative expression of the S phase-associated kinase Skp-2 (at a relatively high percentage in tumoral nuclei) and of the anti-apoptotic protein bcl-2. Based on a review of the literature, these preliminary observations appear to be the first morphoproteomic study on primary neuroblastomas.

Keywords: neuroblastoma, mTOR, ERK, NF-kappaB, cell cycle, morphoproteomics

Introduction

High-risk neuroblastoma (HRN) portends a poor prognosis for patients in terms of relapse rates and survival [1]. Rapamycin, U0126, and bortezomib (inhibitors of the mammalian target of rapamycin [mTOR], extracellular signal-regulated kinase [ERK], and nuclear factor-kappaB [NF-kappaB] pathways, respectively) have been shown to inhibit the in vitro proliferation of human neuroblastoma cells [2-5]. Based on these observations, we investigated: (a) the state of activation of components of the mTOR, ERK, and NF-kappaB signal transduction pathways in HRN cases; and (b) cell cycle and protein analyte correlates that are associated with these pathways.

Materials and Methods

Archival biopsy materials from 3 patients with HRN were studied with approval of the appropriate Institutional Review Board. Each of these cases was classified as high risk according to the Children’s Oncology Group (COG) protocol; all 3 were stage IV with metastatic dissemination, and 2 had N-Myc gene amplification. Immunohistochemical probes were utilized for detection of the following antigens in these tissues: phosphorylated (p)-mTOR (Ser 2448) and one of its downstream effectors p-p70S6K (Thr 389), p-ERK 1/2 (Thr 202/Tyr 204), and p-NF-kappaBp65 (Ser 536) (all obtained from Cell Signaling Technology, Beverly, MA), Skp-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and bcl-2 (Dako Corp., Carpinteria, CA). The intensity of chromogenic signal and cellular compartmentalization (i.e., plasmalemmal, cytoplasmic, nuclear) were assessed by bright-field microscopy on a scale of 0 to 3+, except for the S phase-associated protein kinase Skp-2 [6], which was quantified by an automated cellular imaging system (ACIS [7]).
Results and Discussion

Bright-field microscopy revealed moderate to strong expression (up to 2+ or 3+ intensity on a scale of 0 to 3+) of p-mTOR (Ser 2448), and one of its downstream effectors, p-p70S6K (Thr 389) in these 3 cases (Fig. 1). Similarly, p-ERK 1/2 (Thr 202/Tyr 204) and p-NF-kappaBp65 (Ser 536) expressions were evident at comparable intensities. Nuclear translocation of p-p70S6K, p-ERK 1/2, and p-NF-kappaBp65, respectively, was also noted in each case (Fig. 1). Moderate to strong cytoplasmic expression of the anti-apoptotic protein bcl-2 was present in all cases (Fig. 1). Analysis of the cell cycle-related parameter Skp-2 revealed relatively high percentages of nuclear expression for this protein analyte at 45%, 19%, and 40%, respectively (Fig. 2).

Morphoproteomics utilizes phosphospecific probes and cellular compartmentalization to assess...
directly the state of activation of protein analytes in lesional tissues [8]. In addition, this approach allows for functional grouping with correlative expression of protein analytes to paint a portrait of the protein circuitry in tumor cells. In the process, we gain insight into the biology of the tumor and expose potential targets for therapeutic intervention. In this context, the HRN cases in our study show constitutive activation of: (a) the mTOR signal transduction pathway, based on the expression of mTOR phosphorylated at serine 2448 and concomitant and correlative nuclear translocation of p70S6K, phosphorylated at threonine 389 [9-16]; (b) the ras/Raf kinase/ERK pathway as evidenced by the phosphorylation of ERK 1/2 at threonine 202/tyrosine 204 and its nuclear translocation [8,17-20]; and (c) the phosphorylation of NF-kappaBp65 at serine 536 and its nuclear translocation thereby creating an opportunity for the tumor cell to form p-NF-kappaBp65·DNA complexes leading to transcriptional activation [8,21]. Moreover, concurrent cytoplasmic expression of bcl-2, one of the anti-apoptotic protein products of transcriptional activation of its gene by NF-kappaB in a neuroblastoma cell line [22], provides supportive evidence for the genomic impact of p-NF-kappaBp65 (Ser536) in HRN. Similarly, the relatively high percentage of nuclear immunoreactivity for S phase-associated protein kinase Skp-2 in these 3 cases accords with flow cytometric data in HRN showing S phase percentages at 9 to 41% in advanced stage disease (Stages III and IV [23]). Huddart and colleagues [24] reported a poor prognosis in children with neuroblastoma whose S phase was >14%. Finally, functional grouping of these constitutively activated protein analytes reveals internal consistency of signaling in HRN. That is to say, both the mTOR and ERK 1/2 pathways effect activation of the NF-kappaB pathway [8,25-26]; the NF-kappaB pathway transcriptionally activates the synthesis of bcl-2 [22,27]; and all 3 pathways converge to promote G1 cycle progression with entry into the S phase [25,28]. This is depicted in Fig. 3.

Fig. 2. Nuclear expression with variable brown DAB chromogenic signal of the S phase-associated protein kinase Skp-2 in a case of high risk neuroblastoma (original magnification X 400). Quantification using an automated cellular imaging system (ChromaVision ACIS) reveals a percent positive nuclear average of 40% (inset).
Fig. 3. Diagrammatic representation of the signal transduction pathways identified by the authors and others in high risk neuroblastoma and in neuroblastoma cell lines (analytes with *). Specifically, downstream signaling by IGF-1R and its ligand in neuroblastoma proceeds through the PI3′-K/Akt and ras/Raf kinase / ERK 1/2 pathways. The former leads to phosphorylative activation of mTOR and its effector p70S6K and the latter (p-ERK 1/2) converges on p-p70S6K promoting G1 cell cycle progression, as evidenced by increased Skp-2 protein expression. Bi-pathway signal transduction also converges on phosphorylative activation of NF-kappaBp65 with nuclear translocation contribution to tumoral proliferation, chemoresistance, and anti-apoptosis including the synthesis of bcl-2. N-Myc gene amplification, when present, could contribute to propagation of this signaling by upregulating the insulin-like growth factor pathway and, in turn, to its own upregulation in an autocrine loop via ERK signaling. Opportunities for therapeutic intervention include sorafenib as a Raf kinase inhibitor, rapamycin or one of its analogues, bortezomib as a proteosome inhibitor to reduce p-NF-kappaBp65 formation and bcl-2 production, and an S phase-active cytotoxic agent to take advantage of the G1 cell cycle progression in high risk neuroblastoma.

Abbreviations: IGR-1R: insulin-like growth factor type I receptor; PI3′-K: phosphatidylinositol 3′-kinase; mTOR: mammalian target of rapamycin; ERK: extracellular signal-regulated kinase; IKK: inhibitor kappaB kinase; I-kappaB: inhibitor-kappaB; NF- kappaB: nuclear factor-kappaB; Skp-2: S phase-associated protein kinase.
A computer-assisted review of the National Library of Medicine’s MEDLINE database provides observations from preclinical studies to support our findings. Specifically, these include: (a) a report by Misawa and co-authors [2] on the role of the insulin-like growth factor (IGF) system, which signals through both the ERK and mTOR pathways [2,8] to promote G1 to S phase progression in human neuroblastoma cells, and the ability of rapamycin, an mTOR inhibitor, to inhibit both cell cycle progression and proliferation in these same cells; (b) an observation by Eppstein and co-workers [3] linking growth inhibition in a chemoresistant neuroblastoma sub-type following MAPK-targeted treatment with the MEK inhibitor U0126 to elevated constitutive total ERK and p53 expressions; (c) the findings by Brignole et al [4] and Michealis et al [5] concerning the ability of the proteosome inhibitor bortezomib, which inhibits the NF-kappaB pathway, also to inhibit proliferation of human neuroblastoma cells in vitro; and (d) a report by Ogawa and co-workers [29] on the high sensitivity of neuroblastoma, both in vitro and in xenograft models, to gemcitabine, an S phase active agent [30].

Additionally, the genomic correlate of poor prognosis in neuroblastoma, namely \( N-Myc \) gene amplification [31], when present in high risk patients could serve to perpetuate the constitutive activation of the mTOR, ERK, and NF-kappaB pathways and thereby the cell cycle progression in HRN. That is to say, \( N-Myc \) acts to upregulate signaling in neuroblastoma through the insulin-like growth factor pathway [32-33] and, in turn, \( N-Myc \) is upregulated by activation of the downstream effectors of this signal transduction pathway to include ERK [34-36]. These events are depicted in Fig. 3. Although 1 of our 3 patients did not show \( N-Myc \) amplification in his neuroblastoma, karyotypic analysis of the tumor revealed a complex cytogenetic pattern that included deletions of chromosome 11q22q24. In general, such loss in chromosome 11q identifies tumors with increased risk for metastatic relapses in neuroblastoma [37]. Because one of the genes determining tuberous sclerosis has been mapped to human chromosome 11q14-11q23 with a positive lod score at 11q22-11q23 [38] and because the tuberous sclerosis complex locus for TSC2 maps in the region of the anonymous DNA marker Lam L7 and the dopamine D2 receptor gene at 11q23 [39], it is possible that deletions at 11q22q24 may be affecting this gene and locus and, thereby, be contributing to constitutive activation of the mTOR pathway in this patient [40].

The therapeutic implications of these aforementioned observations include consideration of the incorporation of one or more small molecule inhibitors into clinical trials in HRN patients who have failed to respond to conventional therapy. Combinational strategies could include the use of rapamycin or one of its analogs, sorafenib and/or bortezomib, as inhibitors of the mTOR, Raf kinase/ERK, and NF-kappaB pathways, respectively [8]. Alternatively, the use of an S phase-active cytotoxic agent and bortezomib to counter chemoresistant pathways such as bcl-2 might also be a consideration [41]. These are depicted graphically in Fig. 3. Most importantly, as we design combinatorial therapies, consideration of their impact upon and interaction with potential antitumoral aspects of these signal transduction pathways in HRN will be essential. Specifically, rapamycin enhances bcl-2 protein expression in human neuroblastoma cells [42] and this could contribute to an anti-apoptotic effect by the neuroblastoma [43]. Similarly, the apoptotic effect of the S phase-active agent gemcitabine is associated with ERK activation and bcl-2 down regulation [44]. In short, successful combinational therapies may require the strategic application of several agents, such as rapamycin, bortezomib, and gemcitabine.

In summary, our morphoproteomic studies reveal constitutive activation of mTOR, ERK, and NF-kappaB pathways in high risk neuroblastoma and the correllative expression of S phase-associated kinase Skp-2 and the anti-apoptotic protein bcl-2. Although these findings are preliminary, based on a review of the literature these observations appear to be the first on primary neuroblastoma specimens. Moreover, they concur with the preclinical data on the efficacy of small molecule inhibitors directed against these pathways in neuroblastoma cell lines and represent a starting point for studies on a larger series of cases and the design of clinical trials utilizing small molecule inhibitors and S phase
active agents in a combinational strategy against high risk neuroblastoma.

Acknowledgments

The authors thank Laurie Kneller-Walter, HT (ASCP), for technical assistance and Bheravi Patel for secretarial support and help with the graphics.

References


