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Proteomic Analysis of Neuroblastoma Subtypes in Response to Mitogen-Activated Protein Kinase Inhibition: Profiling Multiple Targets of Cancer Kinase Signaling[1]

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Introduction. **Survival for high-risk neuroblastoma (NB) remains poor despite aggressive therapy. Novel therapies are vital for improving prognosis. We previously showed differential NB subtype sensitivity to p42/44 mitogen-activated protein kinase (ERK/MAPK) pathway inhibition. In this study, we investigated proteomic changes associated with resistance or sensitivity to MAPK kinase (MEK) inhibition in NB subtypes.**

Materials and Methods. **SH-SY5Y (N-type), BE(2)-C (I-type), and SK-N-AS (S-type) were treated with MEK inhibitor U0126 (10 μM) for 1 and 24 h. Proteins were extracted from untreated and treated cells and analyzed for differential expression by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Selected polypeptides were extracted from the gel and identified by liquid chromatography-linked tandem mass spectrometry (LC-MS/MS).**

Results. **We identified 15 proteins that were decreased by 2.5-fold between untreated and 1 h treated cells and subsequently up-regulated 5-fold after 24 h drug treatment. N-type NB (MEK-resistant) showed the least altered proteomic profile whereas the I-type (MEK-sensitive) and S-type NB (MEK-intermediate) generated significant protein changes. The majority of proteins identified were induced by stress.**

Conclusions. **Protein differences exist between MEK inhibitor-treated NB subtypes. Identified polypeptides all have roles in mediating cellular stress. These data suggest that inhibition of the ERK/MAPK in NB subtypes leads to an intracellular stress response. The most resistant NB cell line to MEK inhibitor treatment generated the least protective protein profile, whereas the intermediate and most sensitive NB cells produced the most stress response. These findings suggest stress related protein expression may be targeted in assessing a response to ERK/MAPK therapeutics.**

Key Words: **neuroblastoma; I-type; S-type; N-type; proteomics; ERK/MAPK; MEK inhibition; U0126; drug sentivity/resistance; cell stress.**

INTRODUCTION

Neuroblastoma (NB), a malignant childhood neural crest-derived tumor, frequently presents as advanced stage disease and, despite multimodal therapy, remains largely incurable [1]. A reason for the dismal outlook for high-risk NB is the acquisition by the patient of multidrug resistance (MDR) upon treatment with anticancer drugs. Although several mechanisms are responsible for contributing to NB MDR [2–6], heterogeneous cell populations constituting these solid tumors have been shown to play a major role in the emergence of drug resistance [7]. For these reasons, a better understanding of tumor cell diversity may aid in the development of novel therapeutic treatments needed to improve survival in high-risk NB. Our laboratory has been

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actively evaluating the role of tumor heterogeneity and alternative therapies for NB. For instance, the mitogen-activated protein kinase (MAPK) signal transduction pathway is a well-characterized biochemical cascade mediating cell survival and death. Reports indicate the MAPK pathway is dysregulated in a significant proportion of tumors, and several components of this pathway present strategic targets for cancer therapeutic development [8]. With regards to NB, Misawa and coworkers supported the rationale for focused MAPK mechanism-based therapeutic approaches in the management of N-*myc*-amplified NB [6].

Moreover, because current therapies for NB do not use MAPK-directed treatments, we are investigating whether inhibition of one of the key kinases in this pathway, known as MAPK kinase (MEK), represents a viable treatment option for NB. Our laboratory targeted the p44/p42 MAPK signaling pathway (also known as the ERK/MAPK pathway) in three distinct NB cell phenotypes (N-, S-, and I-NB subtypes). After selective inhibition of this pathway by the MEK inhibitor, U0126, differential expression and activation of p44/p42 MAPK among the three cell types between NB subtypes was shown [9]. I-type NB demonstrated the greatest antiproliferative effect to MEK inhibitor treatment, whereas S-type and N type NB showed intermediate and the least sensitivity to U0126 treatment, respectively. These data are consistent with the concept of heterogeneity among solid tumors and, thus, infers that NB may have inherent differences in the ability to respond to MAPK therapeutics based on distinct tumor subpopulations.

An important question related to these data are whether the mechanisms that confer NB cell subtype sensitivity or resistance to MEK inhibition are related to distinct proteins that are potentially associated with the drug-resistant phenotype. In this study, using proteomic profiling we investigated polypeptides that may be implicated in inducing MEK inhibition sensitivity or resistance in three distinct NB subtypes. The ultimate goal of these investigations is to identify a set of chemosensitivity and/or resistance proteins for MAPK drug therapies that are predictive of treatment response. Expression of a distinct chemoresistant panel of proteins could serve as a clinical predictor of cellular response to MAPK-mediated resistance, yielding potential targets for increasing chemosensitivity to these alternative drugs.

MATERIALS AND METHODS

Cell Culture and MEK Inhibitor (U0126) Treatment

Human NB cell lines (SK-N-AS (S-type), BE(2)-C (I-type), and SH-SY5Y (N-type)) were cultured as described previously [9]. Briefly, cells were grown in 100-mm × 20-mm plates and treated with 10 μM of U0126 (Promega, Madison, WI) when cultures reached approximately 80% confluence. Duration of drug incubation was 1 h and 24 h at 37°C, 5% CO_2.

Two-Dimensional Gel Electrophoresis and Image Analysis

After three washes in ice-cold phosphate-buffered saline (PBS), cells were lysed and prepared according to Eppstein *et al* [9]. Total protein (500 μg) was rehydrated into 11-cm pH 3-10 ReadyStrips (Bio-Rad, Hercules, CA) for 12 h at 20°C in a PROTEAN IEF Cell (Bio-Rad) before focusing. Focusing was performed at 8,000 V for 30,000 h at 20°C. Reduction and alkylation were accomplished by following the manufacturer's instructions (Bio-Rad) and the second dimension SDS-PAGE gels (8–16%) were fixed and stained with Colloidal Coomassie Blue (GelCode Blue Stain Reagent). Imaging and analysis for spot detection, quantitation, and matching were accomplished by previously described methods [10].

In-Gel Enzymatic Digestion and Mass Spectrometry

Differentially expressed protein spots were excised from the gels, in-gel enzymatic digestion was performed with trypsin, and protein spots were processed for mass spectrometric analysis as described in Sandoval *et al* [10].

RESULTS

Proteomic Profile Induced by MEK Inhibition in 3 NB Subtypes

Treatment of NB cell subtypes with 10 μM U0126 produced detectable absence of phosphorylated ERK (p-ERK) for 1 h and varied recovery of p-ERK among the cell types at 24 h; I-type demonstrated persistent suppression, S-type had intermediate recovery, and N-type regained baseline levels of p-ERK activity [9]. Because of these observations, we used this concentration of U0126 in NB cell types for 1 h and 24 h to analyze and identify MEK inhibitor target-specific proteins that contributed to ERK/MAPK sensitivity or resistance. Using high-resolution two-dimensional gel electrophoresis, we determined the protein expression profiles of NB I-, S-, and N-types treated with U0126 at 1 and 24 h. An expression profile map from control-treated cells (dimethyl sulfoxide) was used to generate an untreated profile map of each cell type. Using these protein expression profile gels from untreated (control) and treated (1 and 24 h) cell types, we analyzed and identified differentially expressed proteins using computer-assisted spot detection (Phoretix 2D Evolution software) between Coomassie blue-stained control and treated cell type gels. Fig. 1 shows proteomic profiles from control (top panel), 1 h U0126-treated (middle panel), and 24 h U0126-treated (lower panel) NB subtypes (I-type, Fig. 1a; S-type, Fig. 1b; and N-type, Fig. 1c). To enhance the understanding of the mechanisms underlying sensitivity or resistance to MEK inhibition, we focused on the identification of spots that were down-regulated >2.5-fold after 1 h of U0126 and subsequently up-regulated >5.0-fold after 24 h of MEK inhibitor. By finding protein spots whose