Proteomic Approaches For Defining The Protein Profiles Of EWS/Fli1 In Ewing's Sarcomas

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Introduction: Ewing’s sarcoma is the second most frequent pediatric bone tumor. Chromosomal translocation t(11;22)(q24:q12) is detected in approximately 90% of tumors of the Ewing family. This translocation results in EWS/FLi1 gene fusion, which produces a EWS/FLi1 fusion protein that acts as an aberrant transcriptional activator. Several transcriptome studies have provided lists of gene associated with EWS/FLi1 expression. Functional studies focused on identifying gene lists are particularly attractive for clarifying the pathogenesis of Ewing’s sarcoma in a systematic manner, and the genes studied to date have been shown to silence EWS/FLi1, thus inducing cell cycle alteration, which ultimately leads to apoptosis. However, the protein expression profiles associated with EWS/FLi1 have yet to be elucidated, and the exact molecular mechanisms underlying this phenotype remain unclear. In this study, in order to identify regulated proteins associated with EWS/FLi1 and understand the function of EWS/FLi1 in protein levels, we conducted proteomic studies using both EWS/FLi1 knockdown in Ewing’s sarcoma cell lines and expressing EWS/FLi1 in human mesenchymal stem cell lines.

Methods: In order to identify the protein profiles associated with EWS/FLi1 proteins in Ewing’s sarcomas and understand the function of EWS/FLi1 in protein levels, we conducted proteomic analyses to identify proteins whose expression is regulated by EWS/FLi1. In particular, we employed siRNA assays and iTRAQ (Isobaric tags for relative and absolute quantitation) methods using EWS/FLi1 siRNA and four Ewing’s sarcoma cell lines. We also performed analyses expressing EWS/FLi1 in two human mesenchymal stem cell lines and analyzed the protein expression using iTRAQ to identify protein expression profiles associated with EWS/FLi1. In order to further understand these biological processes and networks, we applied the Ingenuity Pathways Analysis (IPA) system (Ingenuity Systems, Inc., CA, USA) using each silenced protein profile and expressed protein profile.

Results: In the EWS/FLi1 siRNA assays using the four Ewing’s sarcoma cell lines, the cell lines were transfected with either EWS/FLi1 type1 siRNA or EWS/FLi1 type2 siRNA and harvested after 72 hours. The expression of EWS/FLi1 in the two human mesenchymal stem cell lines was induced using the Retroviral Gene Transfer and Expression kit, according to the manufacturer’s recommendations. The suppression and expression of the fusion genes were confirmed using reverse transcription-PCR and/or a Western blot analysis. In the EWS/FLi1 siRNA assays, the growth of all Ewing’s sarcoma cell lines was inhibited by siRNA knockdown of EWS/FLi1, comparing to that observed in the control groups. Proteins extracted from the transfected cell lines were analyzed using the iTRAQ method. The analyses identified approximately 1,500-2,000 proteins that exhibited upregulation, downregulation or no changes, respectively. In the EWS/FLi1 siRNA cells, we analyzed the four profiles to identify proteins that were similarly altered in all four cell lines and found 65 consistently upregulated and 25 consistently downregulated proteins. In the network analyses using IPA, we performed network assessments using
each silenced protein profile and expressed protein profile. In both independent analyses using each set of data, the network assessments identified several common pathways which included CEBPA, E2F1, HIF1A, HSF1, JUN, MYC, MYCN, SMAD3, SP1, SRF, TP53 and TP63 playing a critical functional role as an upstream regulator in the setting of Ewing’s sarcomas.

Discussion: We conducted proteomic analyses of EWS/FLi1 associated with Ewing's sarcomas. Our data suggest that the growth of Ewing's sarcoma cells may be enhanced by the physical association between the identified proteins and EWS/FLi1. We believe that the findings obtained in the present functional analyses will help to improve our understanding of the relationship between EWS/FLi1 and malignant behavior in patients with Ewing's sarcomas and may lead to the development of novel therapeutic strategies.

Significance: To identify regulated proteins associated with EWS/FLi1 and clarify the function of EWS/FLi1 in protein levels, we conducted proteomic studies using both EWS/FLi1 knockdown in Ewing’s sarcoma cell lines and expressing EWS/FLi1 in human mesenchymal stem cells. We identified protein profiles which are associated with the EWS/FLi1 expression in Ewing's sarcomas and human mesenchymal stem cells. Our proteomic approach is the first study to reveal protein expression profiles of EWS/FLi1 in Ewing’s sarcomas.

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