Proteomic Approaches To Define The Functional Role Of Nucleophosmin In Ewing’s Sarcomas

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Disclosures:

Introduction: Nucleophosmin (NPM1) is a ubiquitously expressed protein belonging to the nucleoplasmin family of nuclear chaperones. Mutations in the nuclear localization signal of NPM1 occur in AML, where they lead to cytoplasmic retention and distinctive biological and clinical features. Recently, a proteomics study found that the expression of NPM1 is correlated with a poor prognosis in Ewing’s sarcoma (ES).1) To validate this finding, we evaluated the correlation between NPM1 expression and the clinicopathological features of ES in a cohort of patients from Memorial Sloan-Kettering Cancer Center. To evaluate the functions of NPM1 in ES, we also performed functional studies of NPM1 in ES.

Methods: Forty-seven ES cases were analyzed for NPM1 expression by immunohistochemistry (IHC) using two separate antibodies, and the correlation of NPM1 expression with various clinicopathological factors was analyzed. To evaluate the function of NPM1 in ES, we conducted proteomic analyses to identify proteins that bind NPM1 or whose expression is regulated by NPM1. To identify the proteins that interact with NPM1, we performed immunoprecipitation (IP) assays using two ES cell lines and NPM1 antibodies. To identify protein expression profiles regulated by NPM1, we employed siRNA assays and GelC-MS methods using NPM1 siRNA and four ES cell lines. To further understand the biological processes and networks, we employed the Ingenuity Pathways Analysis (IPA) system (Ingenuity Systems, Inc, CA, USA) using each interaction protein profile and regulated protein profile. And additionally, the identified networks were confirmed by siRNA assays and luciferase assay.

Results: NPM1 expression was seen in 28 of 47 cases and all NPM1 immunostaining in ES was nuclear. Nineteen of 28 NPM1 positive ES patients were dead of disease. Fourteen of 19 NPM1 negative ES patients were alive, either clinically disease-free or alive with disease. The 5-year overall survival rate was 28% and 73% for patients with NPM1 positive and negative tumors respectively (p=0.0130, Log-rank test). In immunoprecipitation of NPM1, proteins extracted from ES cell lines were immunoprecipitated using either NPM1 antibodies or IgG antibodies (control). The IP samples were separated using SDS-PAGE and the gel images were compared between the NPM1 IP samples and the control samples. We found 20 bands with significantly different densities between the two groups and the bands were found to consist of 63 proteins by MS spectrometry. In NPM1 siRNA assays using four ES cell lines, the cell lines were transfected with either NPM1 siRNA or control siRNA and harvested after 72 hours. In comparing to control groups, the growth of all ES cell lines was inhibited by siRNA knockdown of NPM1. Proteins extracted from these transfected cell lines were analyzed using GelC-MS based on the Protom method. We compared the resulting proteomic profiles between the control group and the NPM1 siRNA group to calculate the semiquantitative expressions. The comparisons identified approximately 1,500 proteins that exhibited upregulation, downregulation or no changes in each of the four cell lines. We analyzed the four profiles to identify proteins that were similarly altered in all four cell lines and found 36 consistently upregulated and 18 consistently downregulated proteins. In network analyses using IPA, we performed network analyses using each interaction protein profile and regulated protein profile. In both independent analyses using each set of data, the network analyses identified the MYC pathway as playing a critical functional role as an upstream regulator of NPM1 in ES. Additionally, in confirmation studies for the relationships between MYC and NPM1, we conducted siRNA assays and luciferase assay in the ES cell lines using MYC siRNA and luciferase reporter encoding NPM1 promoter. In siRNA assays, the Western-blotting assays revealed that silencing MYC reduced NPM1 expression. In the luciferase assay, siRNA to MYC decreased luciferase activity of a reporter plasmid driven by an NPM1 promoter in compared to control. These results support the hypothesis that MYC is a direct upstream regulator of NPM1 in ES.

Discussion: NPM1 expression correlates with poor prognosis of ES, and could potentially be useful in selecting the appropriate treatment intensity. These data suggest that the growth of ES cells may be enhanced by the physical association of NPM1 with MYC. We believe that the findings obtained in the functional analyses will contribute to improving our understanding of the relationship between NPM1 and malignant behavior in ES and could lead to the development of novel therapeutic strategies.

Significance: We found NPM1 expression correlates with poor prognosis of Ewing’s sarcomas and the growth of Ewing’s sarcoma cells may be enhanced by the physical association of NPM1 with MYC.

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