5-5-2022

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Background and Significance: Ewing Sarcoma (EWS) is the second most common malignant bone tumor of children and adolescents. Patients with metastatic or recurrent disease have very poor outcomes. The receptor tyrosine kinase(RTK) insulin-like-growth-factor-1-receptor (IGF-1R) is upregulated in 93% of EWS patients with anti-IGF-1R antibodies and kinase inhibitors in clinical studies. However, with only ~10% of patients achieving objective responses, delineation of novel pathways that facilitate IGF-1R-driven oncogenesis in EWS could provide avenues for more effective therapy. The RTK levels and compartmentalization at the cell surface determine their access to growth factors, thus dictating the downstream oncogenic signaling. Our lab has demonstrated that EPS15-homology-domain-containing-protein-1 (EHD1) regulates traffic of cell surface receptors, including RTKs. We observed high frequency (67%) of EHD1 overexpression in 266 primary EWS patient tumor tissues, and Kaplan-Meier survival analysis of publicly available mRNA expression data showed that high EHD1 expression was associated with shorter patient survival.

Objective/Question: This study aims to comprehend the underlying role of EHD1 in EWS oncogenesis.

Experimental design and Results: In both dox-inducible EHD1-shRNA knockdown and EHD1-CRISPR-Cas9-knockout (KO) EWS cell line models(TC71, A673, and SKES1), we observed a significant impairment of *in vitro* oncogenic properties namely, cell proliferation, migration, invasion, soft-agar colony formation, and tumor-sphere formation, and the phenotypes were restored upon mouse-EHD1 rescue. Furthermore, by orthotopically implanting TC71 cells in the tibia of nude mice(xenograft model), we demonstrated a significant reduction in tumor size upon EHD1-depletion. Using a phospho-RTK profiling antibody array, we found reduced phospho-IGF-1R levels upon EHD1-KD, identifying IGF-1R as a potential target of regulation by EHD1. EHD1-KO reduced surface IGF-1R levels under steady-state and ligand-free conditions in EWS cells. IGF-1R and EHD1 were also found to colocalize intracellularly and co-immunoprecipitate after IGF-1 stimulation. Notably, EHD1-KO impaired the IGF-1R-mediated activation of downstream AKT and MAPK pathways. Mechanistically, EHD1 was shown to regulate traffic of newly synthesized IGF-1R and recycled pools from the Golgi to the cell surface, and in absence of EHD1, intracellular IGF-1R was shunted to the lysosome resulting in degradation. Finally, by dual targeting of EHD1 (genetic depletion) and IGF-1R (small-molecule-inhibitor Linsitinib), we observed an additive effect on inhibition of EWS cell proliferation and migration and upregulation of apoptosis.

Conclusions: Our studies indicate a novel regulatory pathway of EHD1 requirement in IGF-1R cell surface display and sustaining IGF-1R-mediated oncogenesis in EWS. This highlights the prospects of therapeutic co-targeting of EHD1 and IGF-1R, thus enhancing IGF-1R targeted therapies in EWS.