Epigenetic Silencing of RIPK3 Augments Human Osteosarcoma Chemoresistance

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INTRODUCTION: Osteosarcoma (OS) is the most commonly diagnosed primary bone tumor affecting children and adolescents. OS presents with metaphases in lower limb long bones, which are treated with neoadjuvant/adjuvant chemotherapy and surgical resection. Aberrant epigenetic regulation, including DNA methylation, is known to contribute to the pathogenesis of OS: especially chemotherapeutic resistance that compromises therapeutic outcomes in a substantial portion of cases. Here, we performed CpG island methylation studies in primary tumors to identify silenced genes that play a role in chemo resistant OS. We identified necroptosis-associated receptor interacting protein kinase-3 (RIPK3), and our objective was to elucidate the role of this molecule in OS chemoresistance and it's as a potential pharmacologic target to improve chemotherapeutic efficacy in drug-resistant tumors

METHODS: We performed whole genome CpG methylation analysis on 16 human primary OS specimens, non-neoplastic controls, and tissue from human muscle, bone, liver, brain and adipose. (Samples were collected following informed consent under an IRB-approved protocol). We verified RIPK3 downregulation with pyrosequencing and NGS in the same tissues and validated these findings in OS cell lines. We performed forced-expression via transfection in cell line G292 (pcDNA3.1Neo-RIPK3 or pcDNA3.1NEO (control; TranOMIC)) with FuGENE 6 reagent (Promega). We also performed RIPK3 transduction in 143B (pLenti-C-mGFP-P2A-Puro-RIPKE ORF or pLenti-C-mGFP-P2A-Puro control vectors (Origene)), and DsiRNA-silencing of RIPK3 both lines. Forced expression and silencing of RIPK3 and MLKL-pathway activation was validated with both qPCR (PrimeTime qPCR probe assays, IDT) and Western Blot: RIPK3 (Origene), phospho-RIPK3 (Abcam), and phospho-MLKL(Origene). Secondary antibodies included goat anti-rabbit HRP and goat anti-mouse (both, Thermo Fisher). Survival following chemotherapy (cisplatin) was conducted with CellTiter 96 Aqueous One Solution (Promega).

RESULTS SECTION: Results demonstrated that dysregulated DNA methylation results in epigenetic silencing of RIPK3 in human OS. We validated this finding in OS cell lines and demonstrated enhanced chemoresistance with RIPK3 silencing. We developed RIPK3 expressing stable cell lines (143B and G292) and showed enhanced expression of RIPK3 results in significantly improved cisplatin sensitivity (**Fig1**). RIPK3 activates and regulates the necroptotic programmed cell death in OS and other solid cancers. Auto-phosphorylation of RIPK3 permits phosphorylation and activation of mixed lineage kinase domain protein (MLKL), which activated pathways leading to membrane permeabilization and cell death. We next explored whether increased necroptosis was seen following forced RIPK3 expression in our OS lines. Results showed that RIPK3 forced expression increases necroptotic cell death via phosphorylation and increased activation of mixed-lineage kinase domain-like (MLKL, **Fig2**). Data were analyzed using GraphPad Prism v10.4.1 software. Analysis used one-way Anova with Tukey's post hoc tests for multiple-group analyses. Two-group analyses used unpaired t-tests. Results were significant when $p<0.05$.

DISCUSSION: Patients with localized OS have an overall survival rate of 65% following traditional therapy. However, those with unresectable tumors, metastatic disease or poor chemotherapy response have very poor outcomes (survival <30%). Novel therapy development for OS is hampered by a lack of common underlying genetic mutations and biomarkers. Epigenetic dysregulation is known to affect the onset, progression, and therapy response in many pediatric solid tumors, including OS. Through examinations of CpG island methylation and we identified significant silencing of necroptosis-associated gene RIPK3 in primary tumors and examined its role in OS therapy resistance. Our data identify RIPK3 as a novel target where pharmacologic therapy to reverse RIPK3 methylation could significantly improve outcomes for pediatric patients with OS.

SIGNIFICANCE/CLINICAL RELEVANCE: OS lacks common risk factors and biomarkers, which complicates the development of novel therapies. Our findings are significant in that they identify RIPK3 as an important mediator of OS chemoresistance, and its role as a potential pharmacologic target to improve chemotherapy efficacy in OS and other drug-resistant tumors.

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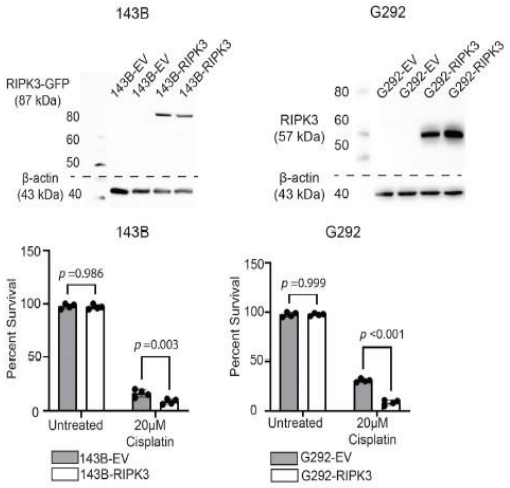

Fig1: Forced expression of RIPK3 in 143B and G292 cell lines improved response to cisplatin chemotherapy.

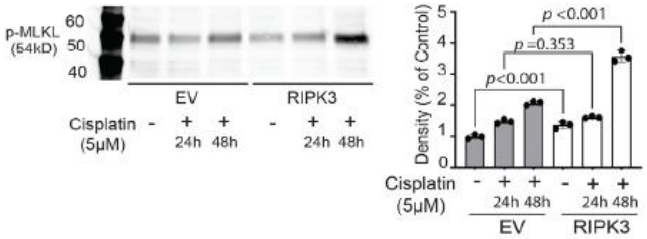

Fig2: RIPK3 overexpression improves cisplatin sensitivity and increases MLKL phosphorylation and activation.