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Targeting DDX3 in Medulloblastoma Using the Small Molecule Inhibitor RK-33

Saritha Tantravedi[*], Farhad Vesuna[*], Paul T. Winnard Jr.[*], Allison Martin[†], Michael Lim[‡], Charles G. Eberhart[§], Cynthia Berlinicke[¶], Eric Raabe[#], Paul J. van Diest[†,] and Venu Raman[*,†,**]**

[*]Division of Cancer Imaging Research, Department of Radiology and Radiology Science, Johns Hopkins University, School of Medicine, Baltimore, MD; [†]Department of Oncology, Johns Hopkins University, School of Medicine, Baltimore, MD; [‡]Department of Neurosurgery, Johns Hopkins Hospital, Baltimore, MD; [§]Department of Pathology and Oncology, Johns Hopkins Hospital, Baltimore, MD; [¶]Wilmer Eye Institute, Johns Hopkins University, School of Medicine, MD; [#]Department of Pediatric Oncology and Pathology, Johns Hopkins Hospital, Baltimore, MD; [**]Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract

Medulloblastoma is the most common malignant tumor that arises from the cerebellum of the central nervous system. Clinically, medulloblastomas are treated by surgery, radiation, and chemotherapy, all of which result in toxicity and morbidity. Recent reports have identified that DDX3, a member of the RNA helicase family, is mutated in medulloblastoma. In this study, we demonstrate the role of DDX3 in driving medulloblastoma. With the use of a small molecule inhibitor of DDX3, RK-33, we could inhibit growth and promote cell death in two medulloblastoma cell lines, DAOY and UW228, with IC50 values of 2.5 μM and 3.5 μM, respectively. Treatment of DAOY and UW228 cells with RK-33 caused a G1 arrest, resulted in reduced TCF reporter activity, and reduced mRNA expression levels of downstream target genes of the WNT pathway, such as Axin2, CCND1, MYC, and Survivin. In addition, treatment of DAOY and UW228 cells with a combination of RK-33 and radiation exhibited a synergistic effect. Importantly, the combination of RK-33 and 5 Gy radiation caused tumor regression in a mouse xenograft model of medulloblastoma. Using immunohistochemistry, we observed DDX3 expression in both pediatric (55%) and adult (66%) medulloblastoma patients. Based on these results, we conclude that RK-33 is a promising radiosensitizing agent that inhibits DDX3 activity and down-regulates WNT/β-catenin signaling and could be used as a frontline therapeutic strategy for DDX3-expressing medulloblastomas in combination with radiation.

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Introduction

Medulloblastoma is the most common malignant form of pediatric brain tumor that occurs in the cerebellum of the central nervous system [1]. Despite treatment advances in recent years, current treatment strategies are associated with long-term toxicities. About 40% of patients experience recurrence of the disease and 30% of patients will eventually die [2]. Current standard treatments include surgical resection and craniospinal irradiation, followed by chemotherapy. Although these strategies have the potential to increase the

Address all correspondence to: Venu Raman, Division of Cancer Imaging Research, Russell H. Morgan Department of Radiology and Radiological Science, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Traylor 340, Baltimore, MD 21205.
E-mail: vraman2@jhmi.edu
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survival of 70–80% of patients with medulloblastoma, they are associated with serious treatment-induced morbidity. Histologically, medulloblastoma is defined into four subtypes: classic, desmoplastic/nodular; medulloblastoma with extensive nodularity; and large cell/anaplastic [3]. Genetically, medulloblastoma is subtyped into five subgroups: WNT-activated; Sonic hedgehog (SHH)-activated and p53-mutant; SHH-activated and p53-wildtype; non-WNT/non-SHH Group 3; and non-WNT/non-SHH Group 4 [4]. More recently, medulloblastoma has been subtyped into 7 distinct groups [5,6]. Approximately 10% of medulloblastoma show up-regulation of the WNT pathway with mutations mainly in β-catenin (*CTNNB1*), which is the principal effector of the WNT pathway [7–10]. Less common mutations, including adenomatous polyposis coli (APC), Axin1, and Axin2, which are the key factors in the WNT signaling pathway, are also found in medulloblastoma [7,11]. Somatic mutations in Smoothened (SMO), Suppressor of Fused (SUFU), and Patched-1 (PTCH-1) are the molecular abnormalities found in SHH-type medulloblastoma [12–14]. Group 3 and group 4 tumors are associated with genomic instability [11] and MYC amplification [15,16].

DDX3, an RNA helicase, is involved in many biological activities that regulate multiple steps in gene expression, such as transcription [17,18], mRNA translation [18,19], splicing [20], and nuclear export [21,22]. However, mutations in DDX3 have been reported in various cancers, such as chronic lymphocytic leukemia [23], head and neck squamous cell carcinomas [24], and T-cell acute lymphoblastic leukemia [25]. About 8% of medulloblastoma cases involve mutations in DDX3, of which 11% involve the pediatric WNT- and -SHH subtypes [26]. Mutations in DDX3 lead to the activation of mutated β-catenin in TCF/LEF reporter assays, suggesting the oncogenic role of DDX3 in medulloblastoma [27]. DDX3 is also involved in the potentiation of β-catenin signaling through translational regulation of Rac1 in a helicase-dependent manner [28]. In colorectal cancer, DDX3, acting as a mediator in the activation of β-catenin through the CK1ε/Dvl2 axis, is associated with tumor invasion and a worse prognosis [29]. In recent studies, we have shown that RK-33 impaired WNT signaling through the disruption of the DDX3-β-catenin pathway in both lung [30] and colorectal cancers [31]. We have also shown that RK-33, in combination with radiation, induced tumor regression in mouse models of lung cancer [30]. Moreover, RK-33 specifically promoted radiation sensitization in DDX3 over-expressing cells [30,32].

To validate the functional utility of inhibiting DDX3 activity for tumor ablation, a small molecule that targets DDX3 has been synthesized by our laboratory, referred to as RK-33 [30]. RK-33 binds to the ATP-binding domain of DDX3 and inhibits its RNA-helicase activity [30]. Here, we show the growth inhibition of medulloblastoma cell lines by RK-33 which was associated with reduced transcript levels of WNT-regulated genes. We also demonstrate synergy between RK-33 treatment and radiation both *in vitro* and in a mouse model of medulloblastoma. Lastly, we also studied DDX3 expression in human medulloblastoma samples.

Materials and Methods

DAOY and UW228 cells were a kind gift from Dr. Michael Lim (Johns Hopkins University, Baltimore, MD, USA). The DAOY cell line (SHH subtype with a mutated p53 gene) was grown in DMEM/F-12 50/50, 1x (Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 mix) with 10% fetal bovine serum, L-glutamine and 15 nM HEPES. The UW228 cell line (SHH subtype with a mutated p53 gene) was grown in MEM, 1x (Minimum Essential Medium Eagle) with Earle's salts and L-glutamine supplemented with 10% fetal bovine serum. The cell lines were maintained under sterile conditions in a humidified incubator with 5% CO_2 at 37°C. Transfection was performed using the JetPrime transfection reagent (Polyplus, New York, NY, USA) and TransLT1 (Mirus, Madison, WI, USA). For the DDX3 knockdown experiments, siControl (non-targeting pool) and siDDX3 sequences were purchased from Dharmacon, Lafayette, CO, USA.

Patient Samples

A medulloblastoma tissue microarray with 80 cases was previously assembled at the Johns Hopkins Hospital Department of Pathology. Survival data are available for these de-identified patient specimens under IRB approved protocol, NA_00015113. These cases were previously assigned to a molecular subgroup by the German Cancer Research Center (DKFZ), Heidelberg, Germany, using an immunohistochemical panel to identify 4 distinct subgroups; WNT, SHH, Group 3, and Group 4 using antibodies as previously described [16,33]. Missing cases were attributable to damaged or detached cores during cutting or staining, or to cores not containing tumor. Pathology was reviewed according to the 2007 WHO classification for central nervous system tumors [3]. Clinicopathological data were retrieved from the pathology report and patient files.

Immunohistochemistry

Four μm-thick sections were cut, mounted on Super Frost slides (Menzel & Glaeser, Brunswick, Germany), deparaffinized in xylene, and rehydrated in decreasing ethanol dilutions. Endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide buffer for 15 minutes and was followed by antigen retrieval through boiling for 20 minutes in 10 mM citrate buffer (pH 6.0) for DDX3. Slides were subsequently incubated in a humidified chamber for 1 hour with anti-DDX3 (1:1000, pAb r647) [34]. After washing with PBS, slides were incubated with poly-HRP-anti-mouse/rabbit/rat IgG (BrightVision Immunologic, Duiven, The Netherlands) as a secondary antibody for 30 minutes at room temperature. Peroxidase activity was developed with diaminobenzidine and hydrogen peroxide substrate solution for 10 minutes. The slides were lightly counterstained with hematoxylin and mounted. Appropriate positive and negative controls were used throughout. Scoring of DDX3 was performed by P.V.D. The intensity of cytoplasmic DDX3 expression was scored semi-quantitatively as absent (0), low (1), moderate (2), or strong (3). Cases with a score of 0 to 1 were classified as having low DDX3 expression and evaluated against cases with strong expression.

Immunoblotting

DAOY and UW228 cells were harvested at 60–70% confluence for protein expression analysis. Standard SDS-PAGE and immunoblotting protocols were followed. The primary antibody used for DDX3 and actin was mouse monoclonal antibody and the secondary antibody used was anti-mouse antibody for both DDX3 and actin.

DDX3 Knockdown in Medulloblastoma Cell Lines

DAOY and UW228 cells were plated at 7.5×10^4 cells per well in six-well plates and incubated overnight. Cells were transfected with siDDX3 (25 nM) using the JetPrime transfection reagent. Media was refreshed 24 h after transfection. The cells were harvested after 72 h of transfection and protein concentration was estimated before Western blotting.

Cytotoxicity Assay

Cytotoxicity was determined using an MTS assay. DAOY and UW228 cells were plated in duplicates at 1×10^3 cells per well in a 96-well plate. After overnight incubation, cells were treated with