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Hedgehog signaling drives medulloblastoma growth via CDK6

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Medulloblastoma, an aggressive cancer of the cerebellum, is among the most common pediatric brain tumors. Approximately one-third of medulloblastomas are associated with misactivation of the Hedgehog (Hh) pathway. GLI family zinc finger 2 (GLI2) coordinates the Hh transcriptional program; however, the GLI2 targets that promote cancer cell proliferation are unknown. Here, we incorporated a Gli2-EGFP allele into 2 different genetic mouse models of Hh-associated medulloblastoma. Hh signaling induced GLI2 binding to the Cdk6 promoter and activated Cdk6 expression, thereby promoting uncontrolled cell proliferation. Genetic or pharmacological inhibition of Cdk6 in mice repressed the growth of Hh-associated medulloblastoma and prolonged survival through inhibition of cell proliferation. In human medulloblastoma, misactivation of Hh signaling was associated with high levels of Cdk6, pointing to Cdk6 as a direct transcriptional target of the Hh pathway. These results suggest that Cdk6 antagonists may be a promising therapeutic approach for Hh-associated medulloblastoma in humans.

Introduction

Medulloblastoma, an aggressive cancer of the cerebellum, is among the most common pediatric brain tumors (1). Transcriptional profiling studies reveal that medulloblastomas exist as 4 main molecular subgroups (2). Approximately one-third of medulloblastomas are associated with misactivation of the Hedgehog (Hh) pathway, a signal transduction pathway that is essential for development (3). Vertebrate Hh signals are transduced through the primary cilium, an antenna that projects from the surface of most cells. Cells of the cerebellar external granule layer (EGL) give rise to Hh-associated medulloblastoma and are ciliated (4, 5). Other Hh-related cancer cells, such as basal cell carcinoma cells, are also ciliated, and disrupting either cilia or ciliary Hh signaling blocks cancer growth in both basal cell carcinoma and medulloblastoma (5, 6).

Hh ligands relieve Patched1 (PTCH1) repression of Smothened (SMO), allowing SMO to localize to cilia and activate GLI family zinc finger 2 (GLI2), the principle effector of the Hh transcriptional program (3). The targets of GLI2 that drive uncontrolled cell proliferation in cancer are poorly understood. Here, we demonstrate that GLI2 binds to the Cdk6 promoter to induce cell proliferation in response to Hh signals. Inhibiting Cdk6 blocks the growth of Hh-associated medulloblastoma in vivo, suggesting that pharmacologic inhibition of Cdk6 may be an effective strategy for patients with Hh-associated cancers.

Results and Discussion

To study how misactivation of GLI2 causes cancer, we used the Floxin system to generate a Gli2-knockin allele that encodes a fusion of GLI2 to EGFP and FLAG tags (Gli2-EGFP) (7). Mice homozygous for the Gli2-EGFP allele are viable and morphologically indistinguishable from WT, revealing that this fusion protein is functional (8). To investigate the function of GLI2 in medulloblastoma, we incorporated the Gli2-EGFP allele into 2 established mouse models of Hh-associated medulloblastoma. These models make use of Cre recombinase under the control of Math1 regulatory sequences to express a constitutively active, oncogenic point mutant of Smo (SmoM2) or to inactivate Ptc1 (Ptc1t) in the EGL (9, 10). In these mouse models, Gli2-EGFP (i) was expressed under endogenous regulatory control, (ii) recapitulated GLI2 activity, interactions, and localization, and (iii) allowed us to immunoprecipitate GLI2 and identify target genes (8).

RNA sequencing of Math1-Cre SmoM2 Gli2-EGFP medulloblastomas demonstrated that, as expected, general Hh target genes, such as Ptc1, Ptc2, and Gli1, and recognized markers of Hh-associated medulloblastoma, including Sfrp1, Ptlim3, and Met, were increased relative to control cerebellums (Figure 1A, Supplemental Figure 1, A and B, and Supplemental Table 1). Other genes not involved in Hh signaling itself were also upregulated, including Cdk6, the expression of which was 167 ± 42-fold higher with that in controls (Figure 1A, A and B). Cdk6 encodes cell division kinase 6 (Cdk6), which, when bound to cyclin D, phosphorylates retinoblastoma protein (RB) and activates E2F transcription factors to stimulate cell-cycle progression. Like Cdk6 transcript, Cdk6 protein was strongly increased in Hh-associated medulloblastoma (Figure 1C). As (i) Cdk6 is a target of Hh signaling during limb-bud development, (ii) expression of Cdk6 is an independent negative prognostic factor in human medulloblastoma, and (iii) inhibition of Cdk6 in vitro suppresses medulloblastoma cell proliferation, we hypothesized that Cdk6 could be functionally important in Hh pathway–associated medulloblastoma (11–13).

Therefore, we compared Cdk6 expression in diverse adult and pediatric human brain tumors and found that Cdk6 was particularly elevated in medulloblastoma (Supplemental Figure 1, C and D). Interestingly, Cdk6 expression was equivalent in all 4 classes of medulloblastoma, suggesting that it may be a common effector
of uncontrolled cell proliferation in medulloblastoma regardless of genetic etiology (Supplemental Figure 1E). In support of this hypothesis, small molecule inhibition of CDK6 confers a survival benefit in mice bearing patient-derived xenographs of group 3 medulloblastomas (14).

The levels of the CDK6-interacting cyclin cyclin D1 were also elevated in Hh pathway–associated medulloblastoma (Figure 1C, Supplemental Table 1, and Supplemental Figure 2A). We therefore assessed phosphorylated RB levels and found them to be dramatically increased in Hh pathway–associated medulloblastoma (Figure 1C). Consistently, medulloblastomas displayed markedly elevated expression of E2F target genes (Supplemental Figure 2A), further suggesting that misactivation of Hh signaling may drive cell cycle progression via CDK6 (15). Of note, the read count of the related mitogenic kinase Cdk4 was higher than that of Cdk6 in Hh-associated medulloblastoma, but the differential expression of Cdk4 in Hh-associated medulloblastoma relative to normal cerebellum was 13.9-fold less than that of Cdk6 (Supplemental Figure 2, A and B, and Supplemental Table 1).

In human medulloblastoma, CDK6 expression was elevated even relative to that in highly proliferative neural progenitors (Supplemental Figure 2C), further raising the possibility that misactivation of Hh signaling induces super-physiological levels of Cdk6 expression. To test whether Hh signals are sufficient to induce Cdk6 expression, we activated NIH/3T3 cells, a Hh-responsive cell type, with SmoM2c Gli2-EGFP to inhibit E2F DNA binding (black). E2F antagonism inhibits induction of all cell cycle effectors in response to Hh stimulation except for Cdk6 and Ccnd1. Concurrent inhibition of translation blocked the induction of Ccnd1 in response to Hh stimulation, but did not affect Cdk6 induction (Figure 2A). These data suggest that, whereas Hh signaling affects the expression of diverse cell cycle effectors, Cdk6 is exceptional in that it is a direct transcriptional target of the pathway.

The Cdk6 promoter binds GLI3, the principle repressor of the Hh transcriptional program, during limb-bud development (11, 17). ChIP analysis of GLI2-EGFP–binding sites in medulloblastomas from Math1-Cre SmoM2c Gli2-EGFP mice revealed that, compared with cerebella from control GLI2-EGFP–expressing mice, GLI2-EGFP is selectively enhanced at a previously identified cis-regulatory element at the Cdk6 promoter that is involved in Hh signaling–mediated limb development (site 4, Figure 2, F and G). Further-
more, multimerized site 4 without surrounding Cdk6 sequences was sufficient to confer transcriptional responsiveness to GLI2-CLEG (Figure 2H and Supplemental Table 2).

Cdk6-null mice have no overt developmental phenotypes, and Cdk6 is not required for cerebellar development (Supplemental Figure 4, A and B) (18). To test whether Cdk6 is functionally important for Hh-associated cancer growth, we genetically removed Cdk6 from the Math1-Cre SmoM2/ mouse model. Homozygous genetic deletion of Cdk6 (Math1-Cre SmoM2/ Cdk6−/−) reduced the weight (32% ± 2%) and size of tumors relative to those with either 1 or 2 copies of the Cdk6 allele (Figure 3A). Homozygous genetic deletion of Cdk6 also reduced the prevalence of small round blue cells that are characteristic of medulloblastoma and partially restored cerebellar architecture (Figure 3B and Supplemental Figure 4, C and D). Moreover, genetically removing Cdk6 function prolonged median survival in Math1-Cre SmoM2/ mice (97 days versus 52 days) (Figure 3C). To confirm the involvement of Cdk6 in Hh-associated medulloblastoma, we genetically removed Cdk6 in a second tumor model, one that relies on the loss of the negative regulator of the pathway, PTCH1, rather than activation of SMO. As with the SMO misactivation tumors, homozygous genetic deletion of Cdk6 in the Math1-Cre Ptc1−/− tumors prolonged median survival relative to animals with 2 copies of the Cdk6 allele (135 versus 68 days, Figure 3D).

We hypothesized that, like genetic deletion of Cdk6, pharmacological inhibition of Cdk6 would inhibit the growth of medulloblastoma. To test this hypothesis, we treated Math1-Cre SmoM2/ and Math1-Cre Ptc1−/− mice with a small molecule inhibitor of CDK4/6, palbociclib. A positive control, the SMO inhibitor vismodegib, reduced medulloblastoma weight (32% ± 2%, Figure 3A) (19). Pharmacologic inhibition of CDK4/6 with palbociclib reduced tumor weight in both Math1-Cre SmoM2/ (31% ± 3%, Figure 3A and E) and Math1-Cre Ptc1−/− (41% ± 5%, Figure 3F) mice to an extent similar to that seen with vismodegib. Much like genetic deletion of Cdk6, palbociclib also reduced the size of tumors, decreased the prevalence of small round blue cells, partially restored cerebellar architecture, and prolonged survival (Figure 3D and Supplemental Figure 4, C and D).

To confirm the efficacy of Cdk6 inhibition for medulloblastoma, we treated Math1-Cre Ptc1−/− mice with a different small molecule CDK4/6 antagonist abemaciclib, which also reduced the size of tumors (14% ± 2%, Figure 3F). As both palbociclib and abemaciclib inhibit CDK4 in addition to CDK6, we treated Math1-Cre SmoM2/ Cdk6−/− mice with palbociclib to test whether inhibition of CDK4 contributes to their effect on medulloblastoma. We did not detect a difference in Math1-Cre SmoM2/ Cdk6−/− tumor weight with and without palbociclib, suggesting that CDK4 is not a significant driver of Hh-associated medulloblastoma growth (Figure 3A).

Medulloblastoma acquires resistance to single-agent molecular therapy with vismodegib (19, 20). Further suggesting a possible role for CDK4/6 inhibition in medulloblastoma treat-
gest that simultaneous molecular inhibition of SMO and CDK6 may be an effective strategy for inhibiting the growth of Hh-associated medulloblastoma.

To understand the mechanism by which CDK4/6 inhibition attenuates the growth of Hh-associated medulloblastoma, we quantified tumor cell apoptosis and proliferation after palbociclib treatment in Math1-Cre SmoM2 c mice. Pharmacologic inhibition of CDK6 had no effect on tumor apoptosis (Supplemental Figure 4, F and G). In contrast, palbociclib reduced the amount of BrdU-positive cells by 35% ± 2%, indicating that CDK4/6 inhibition diminished cell proliferation (Figure 3G and Supplemental Figure 5A). As inhibiting CDK6 induces G1 arrest and cellular senescence (22, 23), we hypothesized that the effect of CDK4/6 inhibition on Hh-associated medulloblas-
Concise Communication

Activating Smoothened mutations in sporadic basal-cell carcinoma.


