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Targeting of sonic hedgehog-GLI signaling: a potential strategy to improve therapy for mantle cell lymphoma

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Abstract

Mantle cell lymphoma (MCL) has one of the worst clinical outcomes among the B-cell lymphomas, with a median survival of only 3 to 4 years. Therefore, a better understanding of the underlying mechanisms that regulate MCL proliferation/survival is needed to develop an effective therapy. Because sonic hedgehog (Shh)-GLI signaling has been shown to be important in the proliferation and survival of several cancers, and no such information is available for MCL, this study was undertaken. Our results show that the molecules associated with Shh-GLI signaling, such as PTCH and SMO receptors, and GLI1 and GLI2 target transcription factors were expressed in the human MCL cell lines and primary MCL cells from patients. Perturbation of this signaling in the presence of exogenous Shh/cyclopamine significantly ($P < 0.001$) influenced the proliferation of JVM2 MCL cells. Furthermore, down-regulation of GLI transcription factors using antisense oligonucleotides not only resulted in significantly ($P < 0.001$) decreased proliferation of the MCL cells but also significantly ($P < 0.05$) increased their susceptibility to chemotherapeutic drug, doxorubicin. Also, down-regulation of GLI decreased cyclin D1 and BCL2 transcript levels, which suggests that these key molecules might be regulated by GLI in MCL. Thus, our results indicate a significant role for Shh-GLI signaling in the proliferation of MCL, and molecular targeting of GLI is a potential therapeutic approach to improve the treatment for MCL. [Mol Cancer Ther 2008;7(6):1450–60]

Introduction

Mantle cell lymphoma (MCL) accounts for 6% to 10% of non-Hodgkin's lymphoma and has the worst clinical outcome of all B-cell lymphomas, with a median survival of only 3 to 4 years (1). MCL patients usually present with widespread disease including generalized lymphadenopathy and extranodal involvement of the bone marrow, spleen, and gastrointestinal tract. MCL cells have a characteristic surface phenotype including IgM^+, IgD^+, $CD5^+$, $CD10^-$, $CD19^+$, $CD20^+$, $Bcl2^+$, $CD23^-$, and $CD24^+$. Also, MCL has the characteristic chromosomal translocation (11:14) (q13:q32) involving the Bcl-1 locus and IgH joining region, resulting in up-regulation of the *cyclin D1* gene with increased cyclin D1 expression (2). Although high-dose therapy followed by stem cell transplantation is effective in reducing tumor burden, graft-versus-host disease due to allogeneic stem cell transplantation and recurrence due to residual tumor make the clinical management of MCL challenging (3). Therefore, new and effective modalities are needed to treat MCL. To develop better therapies, however, we need to understand the molecular basis of key underlying signaling pathways, which regulate disease progression in MCL. Targeting key signaling molecules involved in MCL pathogenesis may provide new treatments for MCL (4). In this regard, dissecting the role of sonic hedgehog (Shh)-GLI signaling in the regulation of MCL cell proliferation and survival may reveal a new disease mechanism that has not been reported in MCL.

Shh belongs to the hedgehog family of proteins that include desert hedgehog and Indian hedgehog. Initially, Shh was first discovered in *Drosophila*, but later it was found to be highly conserved in different species including the human, mouse, rat, frog, fish, and chicken. In the absence of Shh, patched (PTCH), the receptor protein, suppresses the signaling activity of another membrane protein, smoothened (SMO). When Shh binds to PTCH, it allows SMO to transduce a signal into the cytoplasm (5). This signal leads to the breakdown of a large protein complex formed by Fused, Sufu, and GLI in the cytoplasm and releases the GLI transcription factors. The released GLI transcription factors translocate into the nucleus resulting in transcriptional activation of specific target genes (Fig. 1A). This signaling is involved in diverse functions in mammalian development such as patterning in the central nervous system, cell fate determination, proliferation, differentiation, and survival (5). The role of

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Shh-GLI signaling is known in the proliferation of hematopoietic stem cells (6) and T cells (7, 8) and the survival of B cells (9), and impaired Shh-GLI signaling has been reported to lead to cancer (10, 11). The role of Shh-GLI signaling as a mediator in proliferation and survival of various cancers including basal cell carcinoma, pancreatic, prostate, gastric, and breast cancers is well known (5, 10, 12–17). Impaired signaling in the tumor development could be due to either mutations in any molecules associated with this signaling such as PTCH, SMO, and Sufu, as reported in basal cell carcinoma, or due to increased Shh ligand in the microenvironment as reported in prostate, lung, and pancreatic cancers (10, 11). In addition, studies in a medulloblastoma mouse model (Ptc neo67/+ mice) showed that irradiation-induced PTCH mutations lead to the development of lymphoma (18), suggesting a role of impaired Shh-GLI signaling in lymphomagenesis. Very recently, stromally induced hedgehog signaling has been reported in B-cell malignancies (19, 20). However, the role of Shh-GLI signaling has not been reported in MCL. Therefore, we investigated the role of Shh-GLI-mediated signaling in MCL and targeted GLI in MCL cells. Our results show that Shh-GLI signaling molecules such as PTCH, SMO, GLI1, and GLI2 are expressed in human MCL cell lines as well as in primary MCL cells from patients. In addition, perturbation of this signaling by exogenous Shh/cyclopamine significantly influenced the proliferation of JVM2 MCL cells. Down-regulation of GLI transcription factors by antisense oligonucleotides (ASO) significantly decreased the proliferation of MCL cells and resulted in decreased expression of BCL2 and cyclin D1. In addition, down-regulation of GLI significantly increased the susceptibility of MCL cells to chemotherapy. This is the first report on the role of Shh-GLI signaling in the proliferation of MCL, and our results suggest that molecular targeting of GLI is a potential therapeutic approach that may improve the treatment for MCL.

Materials and Methods

Cells and Cell Culture

Human MCL cell lines JVM2 (21) and Granta 519 and Jeko1 (22) were obtained from DSMZ, and Z138 cell line (21) was a gift from Dr. Michael Williams (University of Virginia). JVM2, Z138, and Jeko1 were maintained in RPMI containing 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin (RF10). Granta 519 was maintained in similar medium with DMEM instead of RPMI. Primary MCL cells were obtained from patients using an institutional review board–approved protocol and informed consent. Primary MCL cells from patients with leukemia phase of the disease were isolated from peripheral blood using lymphocyte separation medium (Accurate Chemical and Scientific) as described previously. B lymphocytes were isolated from normal donor peripheral blood using B-cell isolation kit (Miltenyi Biotec) with the negative selection method according to

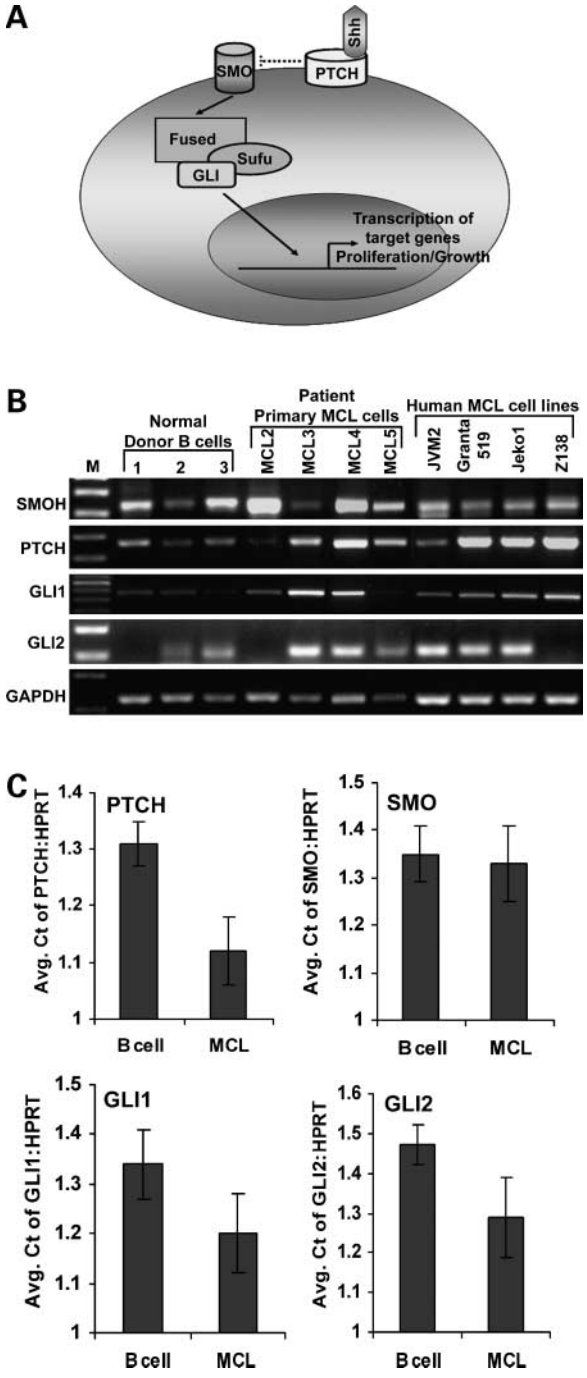

Figure 1. Expression of Shh-GLI signaling components in MCL. **A,** schematic representation of Shh-GLI signaling in a cell. **B,** expression of Shh-GLI signaling molecules in MCL. The expression of transcripts associated with Shh-GLI signaling was determined in B cells from normal donors, primary MCL cells from patients, and JVM2, Granta 519, Jeko1, and Z138 human MCL cell lines by RT-PCR for PTCH, SMO, GLI1, and GLI2. Glyceraldehyde-3-phosphate dehydrogenase expression was used for normalization. PTCH, SMO, GLI1, and GLI2 genes were expressed in MCL cells, suggesting active Shh-GLI signaling in MCL. *M,* base pair standard. **C,** real-time PCR for Shh-GLI signaling molecules in MCL. Increased expression (lower Ct value) of PTCH, GLI1, and GLI2 in MCL cells compared with normal donor B cells suggests a role for Shh-GLI signaling in MCL. HPRT expression was used for normalization.

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the manufacturer's instruction. The purity of isolated B lymphocytes was determined by flow cytometry using PE-anti-CD19 antibodies. B lymphocytes with >90% purity were used for these studies.

Reverse Transcription-PCR and Real-time PCR Analysis

Total RNA was isolated from the respective MCL cell lines/patients using Trizol reagent or Qiagen RNA isolation kit according to the manufacturer's instructions. RNA (~10 μg) was further treated with DNase to remove any genomic DNA contamination using Ambion DNA removal kit. In addition, the integrity of RNA was confirmed using gel electrophoresis. cDNA was prepared from 5 μg DNase-treated RNA using Superscript Reverse Transcriptase (Invitrogen). PCR for the respective genes were done using Taq polymerase (Invitrogen) with gene-specific primers (Supplementary Table)[5] in a 20 μL total reaction using a Bio-Rad MyCycler. PCR products were separated on 2% agarose gel and visualized using ethidium bromide staining. Furthermore, the expression of respective transcripts was confirmed by real-time PCR in a 20 μL total reaction with 2× Power SYBR Green PCR Master Mix (Applied Biosystems) using ABI Prism 7000 according to the manufacturer's instructions. PCR for HPRT was used as control. The ratio of Ct value of respective gene to Ct value of respective HPRT was compared for the analysis. The primers used for real-time PCR are provided in Supplementary Table.[5]

Proliferation Assay

The influence of Shh/cyclopamine/ASO/chemotherapeutic drug on the proliferation of MCL cells was determined *in vitro* by the [^{3}H]thymidine uptake assay (23). To perturb the Shh-GLI signaling in MCL, a fresh passage of JVM2, Granta 519, Jeko1, and Z138 MCL cells (20,000) were cultured in the presence of control medium with reduced (2%) fetal bovine serum (RF2), medium containing exogenous Shh (200 ng/mL; R&D Systems), and medium containing exogenous Shh along with cyclopamine (5 μmol/L; Calbiochem) in 96-well plates in humidified 5% CO_2 incubator for 24, 48, and 72 h. [^{3}H]thymidine (1 μCi/well) was added 15 h before each time cell harvest (PHD; Cambridge Technology), and the incorporated radioactivity was measured using a scintillation counter (Packard). The count per minute for the MCL cells cultured in the presence of control medium was considered as 100% proliferation for the comparison with other experimental groups. The data were taken from triplicates of at least three independent experiments and the significance was calculated using Student's *t* test. The respective MCL cells were harvested for RNA isolation at 24 h, and reverse transcription-PCR (RT-PCR) and real-time PCR was done as explained above.

Down-regulation of GLI by ASO in MCL *In vitro*

Down-regulation of GLI *in vitro* was achieved by using gene specific ASO with phosphorothioate backbone to GLI1 (GLI1-ASO) and GLI2 (GLI2-ASO). ASO to human herpes simplex virus genome was used as an irrelevant target (control-ASO). JVM2, Granta 519, Jeko1, and Z138 MCL cells (10,000) were cultured in the presence of control medium (RF10), medium containing 2.5 μmol/L GLI1-ASO, or GLI2-ASO, or control-ASO in 96-well plates in humidified 5% CO_2 incubator for 48, 72, and 96 h. The proliferation was assessed by the [^{3}H]thymidine uptake assay as described above. The respective MCL cells were harvested for RNA isolation at 24 h, and RT-PCR and real-time PCR were done as explained above.

Susceptibility of GLI-Down-regulated MCL to Doxorubicin *In vitro*

The effect of down-regulation of GLI transcription factors on the susceptibility of MCL cells to doxorubicin, a standard chemotherapeutic drug used for MCL patient in the CHOP regimen, was determined as carried for diffuse large cell lymphoma (24, 25). MCL cells (1×10^6) were cultured in the presence of control medium (RF10), 2.5 μmol/L ASO specific to *GLI1* or *GLI2* genes, or an irrelevant target in six-well plates. After 24 h, the cells were washed with the RF10 medium, and 20,000 MCL cells per well were cultured in 96-well plates in the presence of control medium (RF10), 2.5 μmol/L respective ASO, or 50 nmol/L doxorubicin for 24 h. The proliferation was accessed by the [^{3}H]thymidine uptake assay as described above.

Results

Expression of Shh-GLI Signaling Molecules in MCL

The expression levels of components of Shh-GLI signaling were determined in the primary MCL cells from patients, and JVM2, Granta 519, Jeko1, and Z138 human MCL cell lines using RT-PCR and real-time PCR. The expression of transcripts revealed that the Shh-GLI signaling receptors PTCH and SMO, and the target transcription factors GLI1 and GLI2, were expressed in these MCL cells (Fig. 1B and C). Although the molecules of the Shh-GLI pathway were expressed in primary cells from patients and human MCL cell lines to different extents, target molecules of the pathways, PTCH, GLI1, and GLI2 transcript levels were much higher as represented by the lower Ct value in majority of MCL samples relative to normal B cells as determined by real-time PCR (Fig. 1B and C). The expression levels of SMO and GLI3 were heterogeneous (data for GLI3 not shown). Together, these results indicate that the molecules associated with Shh-GLI signaling are expressed in MCL, and the target transcripts of this signaling (PTCH, GLI1, and GLI2) are overexpressed in MCL. These results show that Shh-GLI signaling is active in MCL and suggest that the extent of signaling may vary in the subset of MCL.

Influence of Shh-GLI Signaling Perturbation on the Proliferation of MCL

As reported in other cancers, Shh-GLI signaling could be autocrine or paracrine in nature (26). In either case, MCL cells would proliferate in response to exogenous Shh

[5] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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stimulation. Furthermore, Shh-induced proliferation would be attenuated in the presence of cyclopamine, a specific inhibitor of Shh-GLI signaling (27). To determine the autocrine or paracrine mode of Shh-GLI signaling in MCL, and the influence of exogenous Shh/cyclopamine on MCL cell proliferation, JVM2, Granta 519, Jeko1, and Z138 MCL cells were cultured in the presence of medium alone, exogenous Shh, and exogenous Shh with cyclopamine. The extent of proliferation was analyzed by the [^{3}H]thymidine uptake assay at 24, 48, and 72 h. Also, expression of the target transcripts, GLI1, was determined by RT-PCR and real-time PCR. There was a significant increase in [^{3}H]thymidine uptake when JVM2 MCL cells were cultured in the presence of exogenous Shh compared with cells in medium alone at 24, 48, and 72 h (Fig. 2A; 111 ± 3.6% versus 100%, P = 0.03 at 24 h; 132 ± 6.5% versus 100%, P = 0.002 at 48 h; 146 ± 3% versus 100%, P = 0.001 at 72 h), suggesting that exogenous Shh increased the proliferation of JVM2 cells. In addition, the extent of JVM2 cell proliferation in the presence of exogenous Shh at 48 h was significantly more compared with 24 h (Fig. 2A; 132 ± 6.5% versus 111 ± 3.6%, P = 0.003), and proliferation at 72 h were significantly more compared with either 48 h (Fig. 2A; 146 ± 3% versus 132 ± 6.5%, P = 0.01) or 24 h (Fig. 2A; 146 ± 3% versus 111 ± 3.6%, P = 0.0003), further suggesting that Shh-GLI signaling mediates MCL cell proliferation. Furthermore, there was a significant decrease in [^{3}H]thymidine uptake when JVM2 cells were cultured in the presence of Shh and cyclopamine compared with Shh alone (Fig. 2A; 106 ± 2% versus 132 ± 6.5%, P = 0.002 at 48 h; 118 ± 3% versus 146 ± 3%, P = 0.0003 at 72 h), confirming the specificity of Shh-mediated cell proliferation. Furthermore, there was a Shh dose-dependent increase in the proliferation of JVM2 cells (data not shown). In addition, the results obtained by [^{3}H]thymidine uptake were corroborated by RT-PCR and real-time PCR analyses. There was a remarkable increase in the transcript level of the Shh-GLI signaling target transcription factor GLI1 in the presence of exogenous Shh (lower Ct value) as determined by real-time PCR compared with medium controls (Fig. 2A). The level of GLI1 transcripts was decreased (higher Ct value) in the presence of exogenous Shh and cyclopamine compared with Shh alone (Fig. 2A). Surprisingly, exogenous Shh or Shh with cyclopamine did not significantly influence the proliferation of Granta 519, Jeko1, or Z138 human MCL cell lines as shown in Fig. 2B to D. Also, there was no significant change in GLI1 transcripts in response to perturbation of Shh-GLI signaling (Fig. 2B-D). Together, our results indicate that exogenous Shh promotes the proliferation of JVM2 MCL cells and this was inhibited by the specific inhibitor cyclopamine, suggesting a role for Shh-specific induction of proliferation in JVM2 MCL cells. However, the proliferation of Granta 519, Jeko1, and Z138 MCL cells were not much affected with this perturbation. These results were correlated with the higher expression of Shh-GLI signaling target transcripts such as PTCH, GLI1, and GLI2 in MCL cells compared with normal B cells (Fig. 1B and C), suggesting the constitutive activation of Shh-GLI signaling in these subset of MCL cells.

Influence of GLI-Down-regulation in MCL on the Proliferation *In vitro*

Whether ligand-dependent or ligand-independent signaling in MCL, the ultimate overexpressed transcription factor would be GLI, which may further regulate the proliferation of MCL cells. If this is true, down-regulation of GLI transcription factors should lead to decreased proliferation of MCL cells, as GLI is an important mediator in Shh-GLI-mediated signaling. To determine the influence of GLI transcription factors on the proliferation of MCL cells, MCL cells were cultured in the presence of serial dilutions of GLI1-ASO or GLI2-ASO or control-ASO, and the proliferation was ascertained by the [^{3}H]thymidine uptake assay. There was a dose-dependent decrease in the proliferation of MCL cells following treatment with GLI1-ASO or GLI2-ASO oligonucleotides (data not shown). To determine the influence of down-regulation of GLI on MCL cell proliferation, Jeko1, Z138, Granta 519, and JVM2 MCL cells were cultured in control medium or medium containing 2.5 μmol/L GLI1-ASO or GLI2-ASO oligonucleotides in 96-well plates. MCL cells were cultured only in the medium or in medium with control-ASO as controls. The extent of proliferation was again determined by the [^{3}H]thymidine uptake assay at 48, 72, and 96 h. There was a significant (P < 0.001) decrease in the proliferation (~40-50%) of all four MCL cell lines in the presence of GLI1-ASO or GLI2-ASO compared with controls at all three time points as shown in Fig. 3A to D. Furthermore, there was not any inhibition of MCL cell proliferation in the presence of control-ASO. Also, there was ~75% to 85% decrease in MCL cell proliferation in the presence of 5 to 10 μmol/L GLI1-ASO or GLI2-ASO (data not shown). Interestingly, down-regulation of GLI2 by ASO leads to a significantly greater decrease in the proliferation of all four MCL cell lines compared with down-regulation of GLI1 by ASO (Fig. 3). In addition, there was an increased frequency of cells undergoing apoptosis when MCL cells were treated with GLI-ASO compared with controls as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell viability and Giemsa staining for cytomorphology (data not shown). Together, these results show that down-regulation of GLI transcription factors by ASO decreased the proliferation of MCL cells as has been reported in other cancers (28, 29).

Influence of GLI-Targeting on the Expression of BCL2 and Cyclin D1 in MCL

To determine the influence of GLI on the regulation of BCL2 and cyclin D1, the key genes involved in the pathogenesis of MCL, RT-PCR and real-time PCR were done for these genes in GLI-down-regulated JVM2, Granta 519, and Jeko1 MCL cells. The expression of GLI1 and GLI2 transcripts was remarkably reduced (higher or no Ct value) when MCL cells were cultured in the presence of GLI1-ASO and GLI2-ASO, respectively, compared with control (Fig. 4A-C). This finding indicates that down-regulation of

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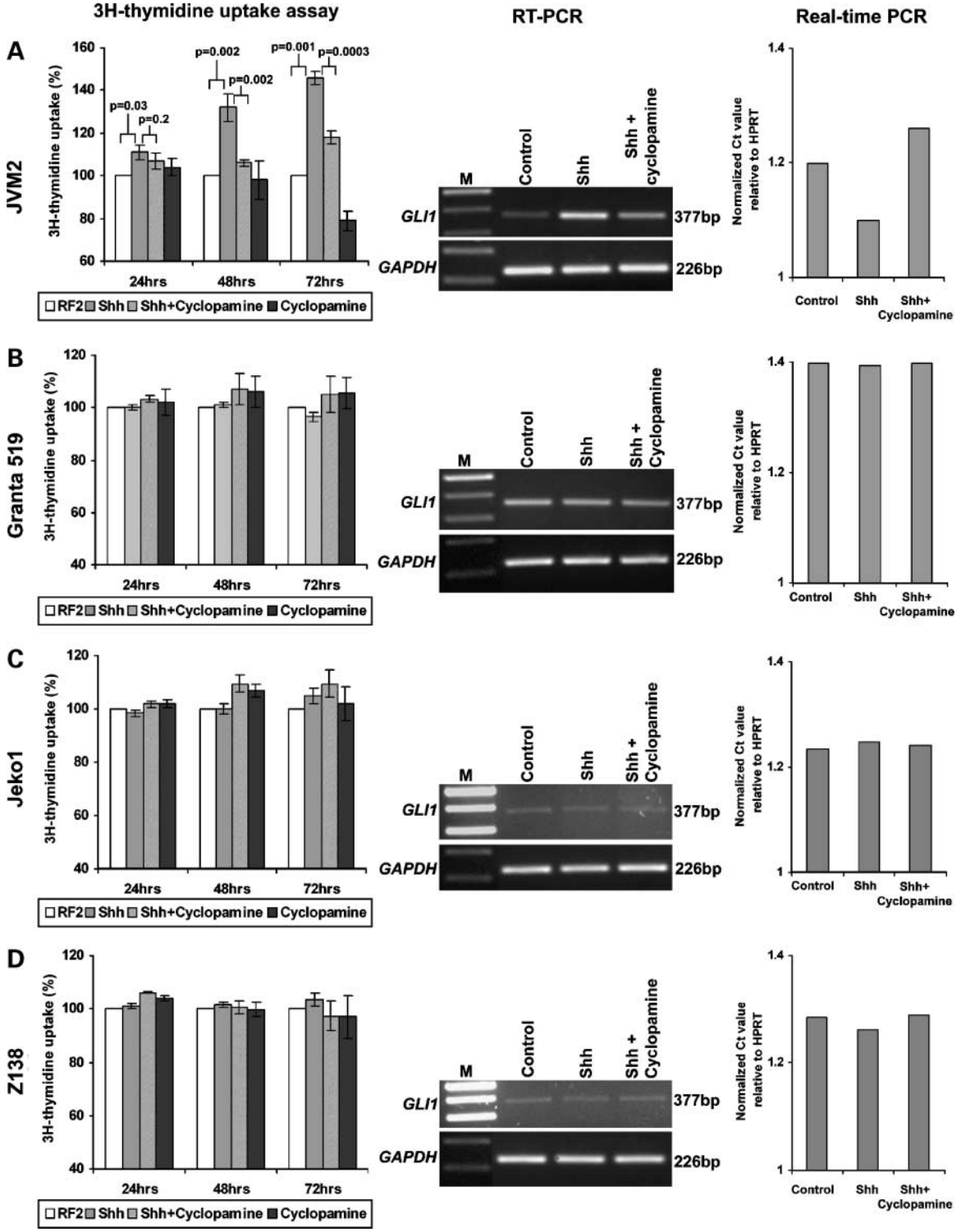

Figure 2. Perturbation of Shh-GLI signaling in MCL cells *in vitro.* The influence of exogenous Shh, with and without cyclopamine, was determined in the JVM2, Granta 519, Jeko1, and Z138 MCL cell lines at 24, 48, and 72 h by the [^{3}H]thymidine uptake assay. The result was also corroborated by RT-PCR and real-time PCR for Shh-GLI signaling target transcript, GLI1, expression as shown in respective panels. **A,** JVM2. **B,** Granta 519. **C,** Jeko1. **D,** Z138. Perturbation of Shh-GLI signaling significantly influenced the proliferation of JVM2 MCL cells but did not influence the Granta 519, Jeko1, and Z138 MCL cells. Data for the experimental groups were calculated by considering the radioactive count obtained in cells cultured in control medium as 100%. Mean ± SD from triplicates of at least three different experiments. Statistical analysis was done by the Student's *t* test. *RF2,* RPMI containing 2% fetal bovine serum. Expression of glyceraldehyde-3-phosphate dehydrogenase and HPRT was used as housekeeping genes for RT-PCR and real-time PCR, respectively. Lower normalized Ct value represents the increased expression.

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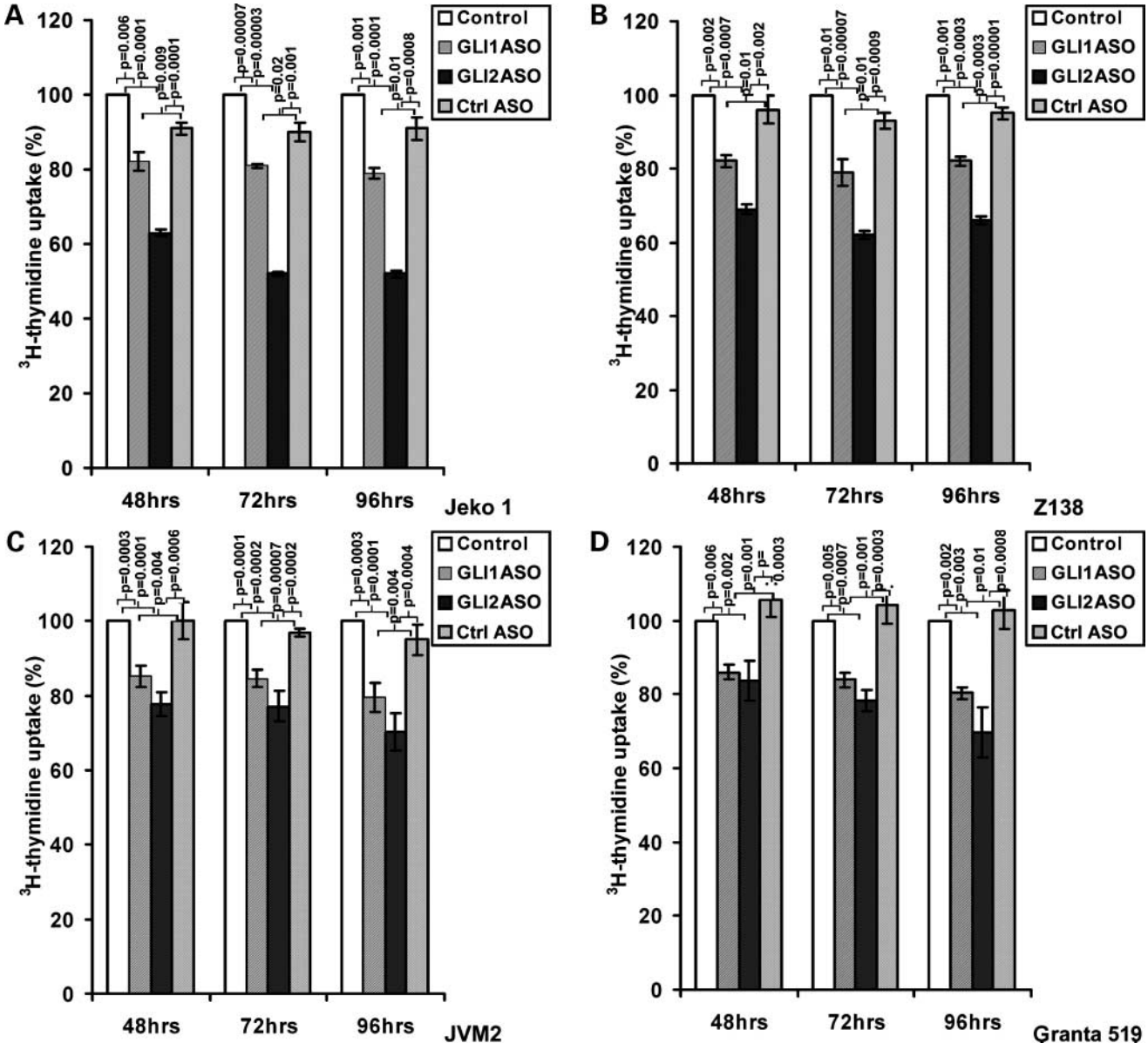

Figure 3. Influence of down-regulation of GLI in MCL cells *in vitro* on proliferation. Proliferation of MCL cells was determined in the presence of control medium, GLI1-ASO, GLI2-ASO, and control-ASO at 48, 72, and 96 h by the [^{3}H]thymidine uptake assay. **A,** Jeko1. **B,** Z138. **C,** JVM2. **D,** Granta 519. There was a significant decrease in [^{3}H]thymidine uptake in the presence of GLI1-ASO or GLI2-ASO compared with control cells or control-ASO, indicating that targeting of GLI will decrease MCL proliferation. Data for the experimental groups were calculated by considering the radioactive count in the cells cultured in control medium as 100%. Mean ± SD from triplicates of at least three different experiments. Statistical analysis was done by the Student's *t* test.

GLI1 or GLI2 was efficient using these ASO. Furthermore, reduced levels of GLI1 transcripts in the presence of GLI2-ASO compared with control validate the specificity of GLI2 knockdown, as GLI1 is a known downstream signaling target of GLI2. Surprisingly, there was a remarkably reduced expression (higher Ct value) of BCL2 and cyclin D1 when MCL cells were cultured in the presence of GLI-ASO (Fig. 4A-C). These results suggest that GLI may regulate both BCL2 and cyclin D1 in MCL; hence, there was a more significant decrease in proliferation in the presence of GLI-ASO compared with control in the above results (Fig. 3). Together, these results indicated that overexpressed GLI transcription factors regulate cyclin D1 and BCL2 expression and thereby may regulate the proliferation and survival of MCL.

Influence of GLI Targeting on the Susceptibility of MCL Cells to Doxorubicin Chemotherapy *In vitro*

Because down-regulation of GLI attenuates BCL2 expression in MCL, we hypothesized that targeting of GLI may influence the susceptibility of MCL cells to chemotherapy

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as reported for other cancers (30, 31). To test this premise, MCL cells were cultured in the presence of GLI-ASO for 24 h, which down-regulated GLI and its target genes such as BCL2 and cyclin D1 (Fig. 4A and B). These GLI-down-regulated MCL cells were then cultured in the presence of doxorubicin chemotherapeutic drug as has been used for diffuse large cell lymphoma along with BCL2 antisense (24, 25). The extent of proliferation was determined by [^{3}H]thymidine uptake at 24 h. As expected, there was a greater inhibition of MCL cells when cultured in the presence of doxorubicin compared with medium. Interestingly, there was further significant decreased (~2-fold) MCL cell proliferation in the presence of doxorubicin followed by MCL cells treated with either GLI1-ASO or GLI2-ASO compared with MCL cells treated with control-ASO or doxorubicin alone as shown in Fig. 5A to D. In addition, there was a significant decrease in the proliferation of these MCL cells in the presence of ASO followed by

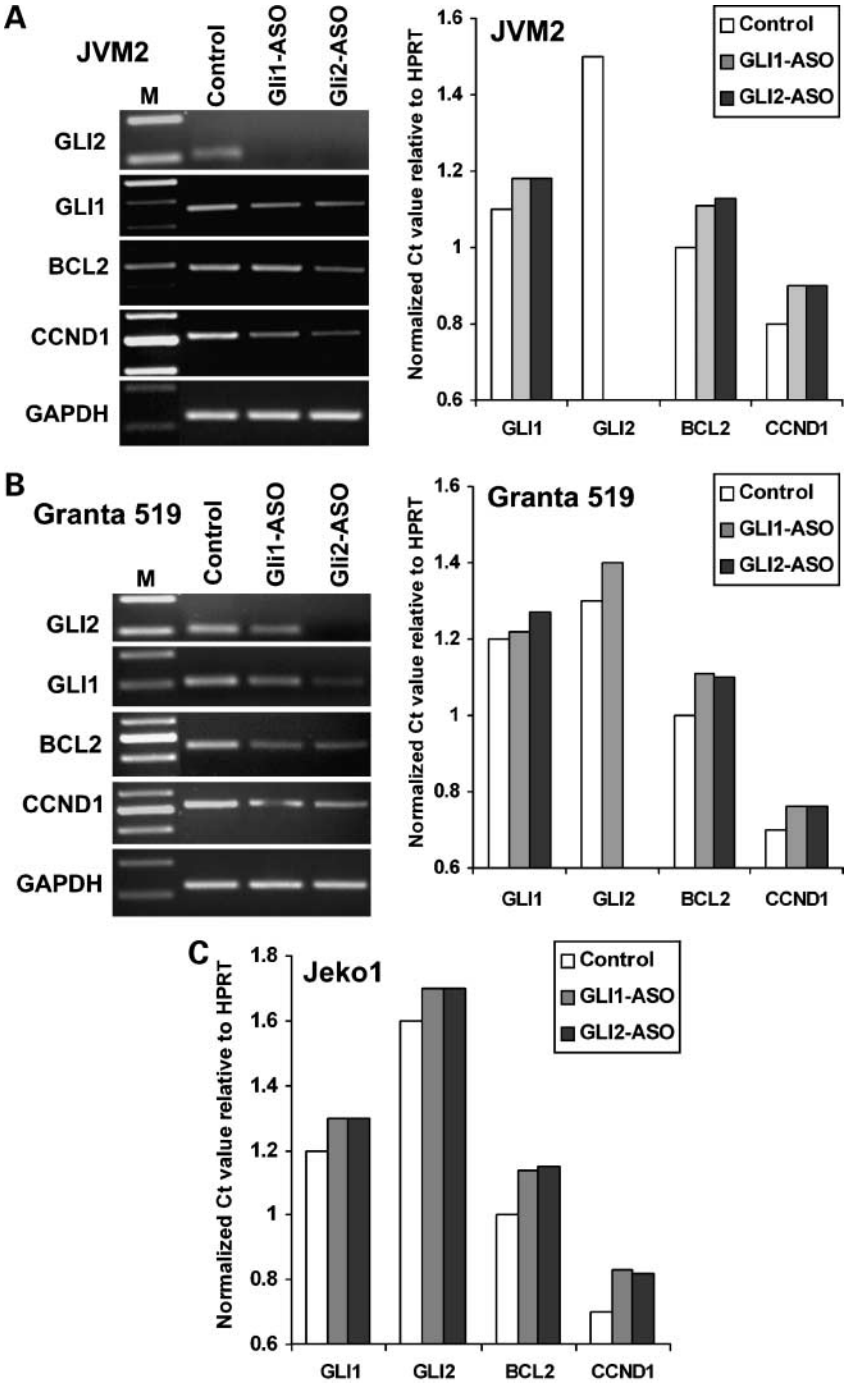

Figure 4. Influence of GLI-down-regulation on BCL2 and CCND1 expression in MCL. RT-PCR and real-time PCR for GLI1, GLI2, BCL2, and cyclin D1 were done using gene-specific primers, and glyceraldehyde-3-phosphate dehydrogenase and HPRT were used for normalization in RT-PCR and real-time PCR, respectively. The levels of transcripts were decreased (higher Ct value) for cyclin D1 and BCL2 in the presence of both GLI1-ASO and GLI2-ASO compared with controls. **A,** JVM2. **B,** Granta 519. **C,** Jeko1.

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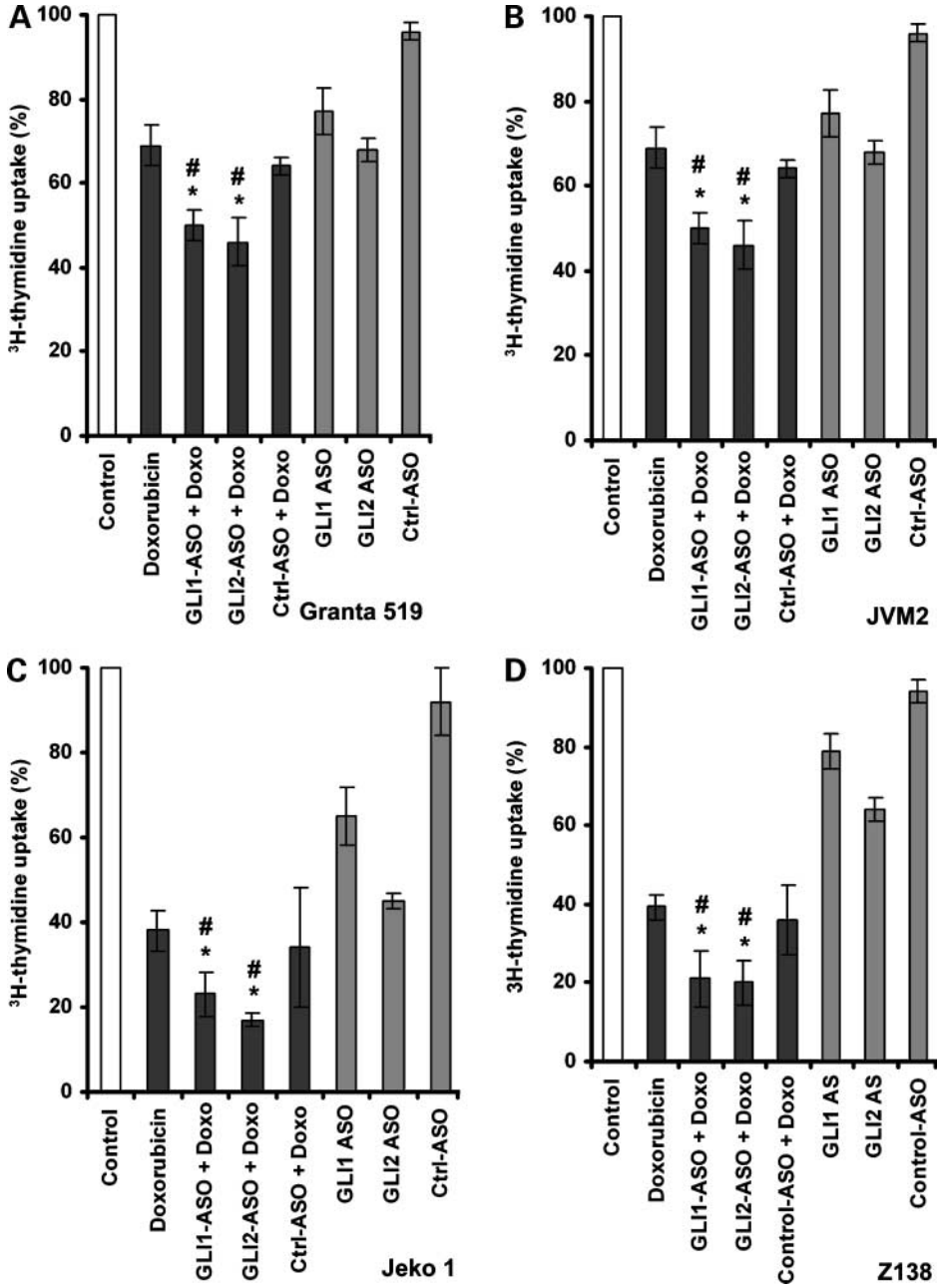

Figure 5. Influence of GLI down-regulation on the susceptibility of MCL cells to chemotherapy *in vitro*. GLI-down-regulated MCL cells were cultured in the presence of doxorubicin for 24 h, and proliferation was determined by the [^{3}H]thymidine uptake assay. Significant decreases in [^{3}H]thymidine uptake in the presence of doxorubicin were noted when MCL cells were treated GLI1-ASO or GLI2-ASO compared with control doxorubicin or control-ASO, indicating GLI-mediated susceptibility of MCL to chemotherapy. **A,** Granta 519. **B,** JVM2. **C,** Jeko1. **D,** Z138. Data for the experimental groups were calculated by considering the radioactive count in the cells cultured in control medium as 100%. Mean ± SD from triplicates of at least three different experiments. Statistical analysis was done by the Student's *t* test. *, $P < 0.05$, compared with doxorubicin only; #, $P < 0.05$, compared with respective ASO only.

doxorubicin treatment when compared with respective ASO alone also (Fig. 5A-D). Taken together, these results indicated that GLI transcription factors may be responsible for the increased proliferation and survival of MCL, and down-regulation of GLI renders MCL cells more susceptible to chemotherapy. Thus, targeting of GLI shows potential to improve the treatment of MCL.

Discussion

The results of this study represent the first demonstration of a role for Shh-GLI signaling in MCL. The potential role of Shh-GLI signaling in the disease progression of several cancers prompted us to investigate this signaling in MCL. Although the role of Shh signaling has been extensively studied in the central nervous system, its role in the proliferation of hematopoietic stem cells and T cells has only been reported (6–8). Shh is reported to promote cell cycle progression in activated peripheral $CD4^+$ T lymphocytes (7). Furthermore, recent reports indicate that Shh is produced by follicular dendritic cells and protects germinal center B cells from apoptosis (9). In addition, the role of Shh-GLI signaling in the proliferation and/or apoptosis of various cancers is well known (10, 11). Very recently, the

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role of Shh signaling in B-cell and plasma-cell malignancies has been also reported (19, 20). Therefore, we investigated the role of Shh-GLI signaling in the proliferation of MCL, and targeted GLI, a key signaling mediator as well as target of the signaling, as a basis to potentially improve the treatment of MCL.

In normal tissue, Shh-GLI signaling is regulated by the availability of Shh ligand and negative feedback mechanisms such as increased PTCH expression in response to signaling activation. Consequently, a precise balance between GLI activation and repression regulates numerous functions governing proper development and differentiation (Fig. 1A). However, impaired Shh-GLI signaling in tissues leads to cancer (10, 11). To determine whether the molecules associated with this signaling are expressed in MCL, we screened for transcripts related to Shh-GLI signaling, such as PTCH and SMO receptors, GLI1 and GLI2 transcription factors in Granta 519, Jeko1, Z138, and JVM2 human MCL cell lines, and primary MCL cells from patients. Our results indicate that transcripts for PTCH, SMO, GLI1, and GLI2 were expressed in these MCL cell lines as well as primary MCL cells from patients (Fig. 1B and C). Furthermore, Shh-GLI signaling target genes such as GLI1, GLI2, and PTCH were significantly overexpressed in majority of MCL cells compared with normal donor B cells (Fig. 1B and C). Also, the absence of transcripts of GLI transcription factors in $CD19^+$ B cells has been reported (6), which indicates increased Shh-GLI signaling in MCL compared with normal B cells. It is anticipated that there could be some discrepancy in the gene expression while working using human samples, as it could depend on various patients' clinical situations as reported in other cancers (29). This discrepancy might be also due to classic (JVM2) and blastoid (Granta 519, Jeko1, and Z138) MCL subsets. However, it is premature to conclude the comparison between classic and blastoid MCL as we had limited number of classic and blastoid MCL samples. Additional studies focused on the characterization of blastoid versus classic MCL are essential with adequate number of samples representing each type of MCL subsets. Nevertheless, we have an adequate number of samples to conclude that Shh-GLI signaling is active in MCL and likely involved in the regulation of proliferation.

As reported in other cancers, Shh-GLI signaling may be active due to an autocrine or paracrine mechanism or constitutive activation due to a mutation in any one of the signaling components (26). Whether it is autocrine- or paracrine-mediated Shh signaling in MCL as reported in other tumors (10), MCL cells need to respond to exogenous Shh stimulation. Also, Shh-mediated influence should be attenuated by its specific inhibitor, cyclopamine. To confirm this, we perturbed Shh-GLI signaling using exogenous Shh and cyclopamine in four human MCL cell lines and their proliferation was determined. These human MCL cell lines are derived from different MCL patients and hence provide a robust model system to study the mechanisms of MCL pathogenesis (21, 22). The proliferation of JVM2 cells were significantly influenced by the perturbation of this signaling (Fig. 2A). However, the proliferation of Granta 519, Jeko1, and Z138 MCL cell lines were not much influenced by this perturbation (Fig. 2B-D). This discrepancy may be due to different subset of MCL such as classic and blastoid. These results are well correlated with the expression of signaling molecules in Fig. 1B and C. Together, these results suggest that the different mode of impairment of Shh-GLI signaling might be involved in different subsets of MCL such as classic versus blastoid MCL. Such discrepancies have been reported in other cancers also (12, 13, 29).

Other mechanisms underlying Shh-GLI-mediated proliferation have been reported in cancers such as basal cell carcinoma, including a ligand-independent mechanism due to constitutive activation of the signaling. Ligand-independent tumor growth is known to arise from loss of PTCH or Sufu repressor function due to mutation, or activating mutations in the Shh effector SMO (12, 13), or due to increased expression of other cross-talking signaling pathways such as transforming growth factor-β signaling (32, 33), which can increase the expression of GLI transcription factors independent of hedgehog ligand (34). Our results suggest that subset of MCL such as blastoid MCL could arise due to mutation in genes associated with Shh-GLI signaling or other cross-talking signaling, which would constitutively activate the signaling target genes such as GLI that will further regulate the growth of MCL. This premise in MCL remains unknown and yet to be discovered in our ongoing studies. Because the perturbation was not active in all MCL, but the target molecules, GLI was expressed in all MCL cell lines (Fig. 1B and C) and GLI is known to be involved in the regulation of proliferation of tumor cells, we tested the effects of targeting of GLI in all MCL cell lines using ASO to GLI1 and GLI2. There was a significant decrease in the proliferation of all MCL cell lines in the presence of both GLI-ASO compared with controls or control-ASO, suggesting a direct role of GLI in the proliferation of MCL (Fig. 3A-D). The difference in the extent of decreased proliferation among different MCL cell lines could be due to difference in GLI expression or GLI-down regulation; such studies need to be further explored. Although this finding is reported in other cancers (29), this is the first ever report in MCL. However, targeting *BCL2* gene using ASO as a treatment for hematologic malignancies has been reported (35), suggesting the feasibility of GLI-ASO for further ongoing *in vivo* studies. In addition, the extent of inhibition of proliferation of MCL by GLI2-ASO was significantly greater compared with GLI1-ASO (Fig. 3A-D) as reported in hepatocellular carcinoma (29), suggesting different modes of regulation in MCL. Studies in cancer cells suggest that activation of GLI proteins leads to up-regulation of BCL2 expression. Furthermore, recent findings in basal cell carcinoma reveal that GLI transcription factors bind to the BCL2 promoter and thereby regulate the expression of BCL2 transcripts (36, 37). Also, the levels of BCL2 transcripts were increased in the presence of exogenous Shh in $CD4^+$ T cells. Also, cyclin D1, c-myc, and

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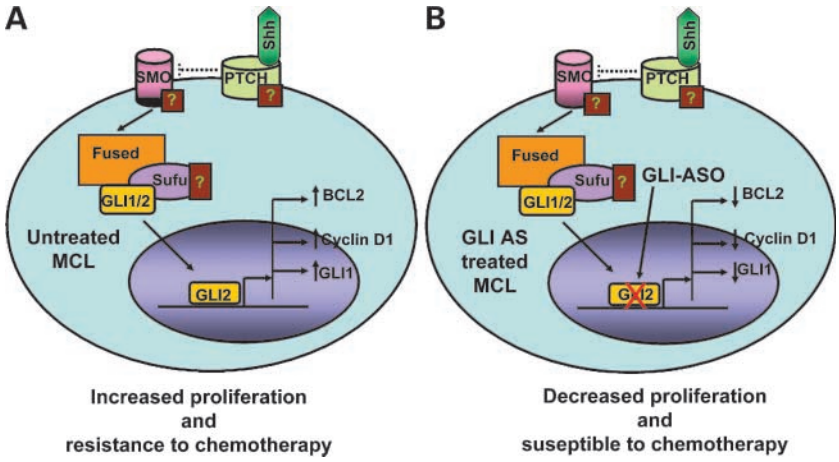

Figure 6. Proposed model of impaired Shh-GLI signaling in MCL and targeting of GLI to improve the treatment of patients with MCL. **A,** impaired Shh-GLI signaling in MCL cells leads to overexpression of GLI transcription factors. These overexpressed GLI molecules in turn induce the expression of cyclin D1 and BCL2, which thereby increase proliferation and resistance to therapy in MCL. **B,** when overexpressed GLI is down-regulated by ASO, GLI-mediated cyclin D1 and BCL2 expression is decreased and leads to decreased proliferation and increased susceptibility of MCL to therapy. ?, molecules that may have mutations that may constitutively activate the signaling.

N-myc are known to be important mediators of Shh-induced proliferation and tumorigenesis (29, 38–40). Together, these reports suggest that constitutively overexpressed GLI transcription factors in MCL may increase the BCL2, N-myc, c-myc, and cyclin D1 expression and thereby decrease apoptosis and increase proliferation in MCL. To test this possibility, we determined the expression of BCL2 and cyclin D1 in GLI-down-regulated MCL and compared it with untreated MCL. We found decreased levels of BCL2 and cyclin D1 expression in the presence of GLI-ASO (Fig. 4A-C). This suggests that GLI regulates the expression of BCL2 and cyclin D1 and thereby may regulate cell survival and proliferation in MCL. Because MCL cells are resistant to chemotherapy that might be due to overexpressed BCL2, and GLI regulates BCL2 in MCL as observed above, we further hypothesized that targeting GLI may render MCL cells more susceptible to chemotherapy. We have used doxorubicin, one of the active components of CHOP regimen. As expected, doxorubicin significantly decreased the proliferation of GLI-down-regulated MCL compared with untreated MCL, Furthermore, MCL cells treated with GLI-ASO followed by doxorubicin treatment had significantly (~2 fold) reduced proliferation compared with control-ASO or treated with only doxorubicin, suggesting that down-regulation of GLI has the potential to increase the susceptibility of MCL cells to chemotherapy (Fig. 5A-D). This kind of strategy using chemotherapy along with BCL2 antisense has been used earlier for diffuse large cell lymphoma (24, 25, 41).

In summary, our results clearly show Shh-GLI signaling in both human MCL cell lines and primary MCL cells from patients. Based on our results in MCL, and known mechanisms of Shh-GLI signaling induced tumor progression in other cancers, we propose a hypothetical model for Shh-GLI-mediated disease progression in MCL (Fig. 6). In this model, GLI is constitutively overexpressed in MCL due to impaired Shh-GLI signaling by mechanisms yet to be discovered, which regulates the expression of key molecules (BCL2 and cyclin D1) involved in the pathogenesis of MCL (Fig. 6A). Therefore, targeting GLI by ASO and thereby down-regulating BCL2 and cyclin D1 expression would decrease cellular proliferation and increase the susceptibility to chemotherapy (Fig. 6B). Thus, our studies lay a foundation for further preclinical studies in MCL to determine the feasibility and efficacy of approaches targeting Shh-GLI signaling molecules to improve the treatment for MCL. Also, this study opens an avenue to test this strategy in other hematologic malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Targeting of sonic hedgehog-GLI signaling: a potential strategy to improve therapy for mantle cell lymphoma

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