Hedgehog-Induced Survival of B-Cell Chronic Lymphocytic Leukemia Cells in a Stromal Cell Microenvironment: A Potential New Therapeutic Target

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Abstract

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by an accumulation of neoplastic B cells due to their resistance to apoptosis and increased survival. Among various factors, the tumor microenvironment is known to play a role in the regulation of cell proliferation and survival of many cancers. However, it remains unclear how the tumor microenvironment contributes to the increased survival of B-CLL cells. Therefore, we studied the influence of bone marrow stromal cell–induced hedgehog (Hh) signaling on the survival of B-CLL cells. Our results show that a Hh signaling inhibitor, cyclopamine, inhibits bone marrow stromal cell–induced survival of B-CLL cells, suggesting a role for Hh signaling in the survival of B-CLL cells. Furthermore, gene expression profiling of primary B-CLL cells (n = 48) indicates that the expression of Hh signaling molecules, such as GLI1, GLI2, SUFU, and BCL2, is significantly increased and correlates with disease progression of B-CLL patients with clinical outcome. In addition, SUFU and GLI1 transcripts, as determined by real-time PCR, are significantly overexpressed and correlate with adverse indicators of clinical outcome in B-CLL patients, such as cytogenetics or CD38 expression. Furthermore, selective down-regulation of GLI1 by antisense oligodeoxynucleotides (GLI1-ASO) results in decreased BCL2 expression and cell survival, suggesting that GLI1 may regulate BCL2 and, thereby, modulate cell survival in B-CLL. In addition, there was a significantly increased apoptosis of B-CLL cells when cultured in the presence of GLI1-ASO and fludarabine. Together, these results reveal that Hh signaling is important in the pathogenesis of B-CLL and, hence, may be a potential therapeutic target. (Mol Cancer Res 2008;6(12):1928–36)

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia in the United States, with approximately 10,000 cases diagnosed every year (1). B-CLL is characterized by the accumulation of a monoclonal population of CD19+, CD5+, and CD23+ B cells in the blood, bone marrow, and peripheral lymphoid tissues (2). Because of its clinical heterogeneity and resistance to therapy, it is very difficult to treat patients with aggressive B-CLL effectively. However, several prognostic markers have been reported in the literature, such as Rai and Binet staging systems (3, 4), immunoglobulin VH gene mutational status (5), cytogenetic abnormalities (6), CD38 expression (7), ZAP-70 expression (8, 9), lymphocyte doubling time (10), and the serum β2-microglobulin level (11). These prognostic markers can be used to predict the survival outcome and also the need for treatment of B-CLL patients. However, in spite of an understanding of these prognostic markers, the mechanisms of disease progression are not clearly understood. Therefore, identification of molecules responsible for disease progression and increased resistance to apoptosis is warranted. These molecules could be then used for the development of effective targeted therapy for those with aggressive disease.

Emerging evidence suggests that the interaction of tumor cells with their microenvironment plays a pivotal role in the increased proliferation and survival, and thereby the pathogenesis, of B-CLL. Although the origin of B-CLL is still a matter of debate, it is known that B-CLL cells are constantly interacting with stromal cells in the bone marrow and/or lymph nodes, which contribute to their survival in vivo. Among the different cell survival molecules, stromally produced hedgehog (Hh) proteins such as sonic (Shh), indian (Ihh), and desert (Dhh) Hh have a role in the proliferation and survival of hematopoietic stem cells (12), B cells (13), T cells (14, 15), and malignant B cells (16-18). In addition, Hh proteins are expressed in stromal cells of the bone marrow (19) and lymph nodes (13) and either are secreted by these cells as soluble ligand or mediate interactions through direct cell contact. Typically, the signaling
pathway is activated by the binding of Hh ligand to the transmembrane receptor Patched (Ptc), which in turn decreases the repression of the receptor Smoothened (Smo), a seven-transmembrane protein. Smo then induces a series of intracellular events, resulting in the regulation of numerous downstream target molecules through a family of zinc finger transcription factors, such as the glioma-associated oncogenes GLI1, GLI2, and GLI3, as well as PTCH1, cyclin D1, and BCL2 (17, 20, 21). Constitutive activation of the Hh pathway by mutations in PTCH or SMO has been associated with basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma, and autocrine or paracrine activation of the pathway has been described for other solid tumors in the pancreas and liver (22).

However, the influence of stromally induced Hh signaling on the survival of B-CLL cells, as well as targeting GLI as a treatment of B-CLL, has not been studied. Therefore, we determined the influence of bone marrow–derived, stromal cell–induced Hh signaling on the survival of B-CLL cells and investigated the expression and functional modulation of GLI1 in the B-CLL cells. We found a smaller proportion of B-CLL cells undergoing apoptosis in the presence of stromal cells or exogenous Shh, and the effect was reversed in the presence of cyclopamine, a specific inhibitor of Hh signaling. Furthermore, there was a significantly higher expression of GLI1 transcripts in B-CLL cells from patients in poor clinical outcome subgroups, and this correlated with a shorter time to treatment compared with better clinical outcome subgroups. In addition, functional modulation of GLI1 by exogenous Shh, cyclopamine, and GLI-specific antisense oligonucleotides (ASO) clearly suggests a role of Hh signaling in the survival of B-CLL cells. Furthermore, down-regulation of GLI1 using specific ASO synergistically increased the susceptibility of B-CLL cells to fludarabine-mediated apoptosis. Together, our studies indicate that Hh signaling is active in aggressive B-CLL and may be a potential molecular target to improve the treatment of B-CLL.

**Results**

**Inhibition of Hh Signaling Prevents Stromal Cell–Induced B-CLL Cell Survival**

To determine the influence of Hh elaborated by stromal cells in the microenvironment, B-CLL cells were cultured on OMA-AD bone marrow stromal cells for 72 hours, and survival of the preapoptotic and apoptotic B-CLL populations was determined by Annexin V-FITC and propidium iodide (Annexin-Pi) staining using flow cytometry. There was a 6-fold decrease in preapoptotic (Annexin-Pi−; 4% versus 25%) and a 4-fold decrease in apoptotic (Annexin-Pi+; 5% versus 19%) B-CLL cell population when cultured on OMA-AD stromal cells compared with cells cultured in control medium alone (Fig. 1A-E). These results show the positive influence of a stromal microenvironment on the survival of B-CLL cells. Furthermore, to confirm the contribution of Hh signaling to stromal cell–induced B-CLL cell survival, B-CLL cells were similarly cultured on OMA-AD stromal cells in the presence of cyclopamine, an inhibitor of Hh signaling. There was a 4-fold increase in preapoptotic (Annexin-Pi−; 16% versus 4%) and a 7-fold increase in apoptotic (Annexin-Pi+; 35% versus 5%) B-CLL cell population when cultured in the presence of cyclopamine on OMA-AD cells compared with cells cultured on OMA-AD cells without cyclopamine (Fig. 1E and F), confirming the Hh-mediated increase in survival of B-CLL cells grown on stromal cells. In addition,
we have accessed the expression of Hh ligands such as Shh, Dhh, and Ihh in OMA-AD cells by reverse transcription-PCR (RT-PCR), and total RNA from mouse brain was used as a positive control. There was expression of Shh and Dhh in OMA-AD stromal cells (Fig. 1G), and the expression of Hh ligand in OMA-AD stromal cells was confirmed. In addition, to show the influence of Hh ligands secreted by OMA-AD cells on B-CLL cells, we collected the conditioned medium of OMA-AD cell culture (stromal-CM) and studied the influence of this stromal-CM on the expression of GLI1 transcripts, a specific target of Hh signaling, by real-time PCR and on CLL cell survival by Annexin-PI assay. There was a significantly increased expression of GLI1 transcripts and decreased apoptotic cell population when B-CLL cells were cultured in the presence of stromal-CM compared with control (Fig. 1H-K). Furthermore, the influence of stromal-CM was abrogated when we included cyclopamine along with stromal-CM (Fig. 1H-K). These results clearly show the positive influence of Hh signaling mediated by stromal cells on the survival of B-CLL cells. Together, these experiments show a role for the tumor microenvironment via stromal cell–mediated Hh signaling in the survival of B-CLL cells.

Modulation of Hh Signaling Influences the Survival of B-CLL Cells

To determine the specific influence of Hh signaling on the survival of B-CLL cells, Hh signaling of primary B-CLL cells was modulated in vitro. Hh signaling is increased in the presence of exogenous Hh ligand, including Shh, and the signaling is decreased in the presence of cyclopamine. To determine the influence of exogenous Shh or cyclopamine on the survival of B-CLL cells and the expression of the target molecule GLI1, the cells were cultured in the presence of medium alone, or medium containing exogenous Shh or cyclopamine for 72 hours, and the viability of the B-CLL cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), whereas the apoptotic cell population was determined by the Annexin-PI assay. There was significantly increased (P = 0.01) B-CLL cell survival in the presence of exogenous Shh (135 ± 3.4% versus 100%, P = 0.0005), and decreased survival in the presence of exogenous Shh plus cyclopamine (92 ± 9.4% versus 135 ± 3.4%, P = 0.003) or cyclopamine alone (84 ± 6.4% versus 100%, P = 0.05), compared with B-CLL cells cultured in medium alone (Fig. 2A). The survival of B-CLL cells was not changed when cultured in the presence of DMSO as a vehicle control. These results were also correlated with and supported by the results obtained using Annexin-PI staining to determine the proportion of preapoptotic and apoptotic cell populations (Fig. 2B). There was a significantly decreased preapoptotic and apoptotic B-CLL cell population in the presence of exogenous Shh compared with medium alone (Fig. 2B). Furthermore, addition of cyclopamine reversed the antiapoptotic effects of exogenous Shh on B-CLL cells in vitro (Fig. 2B). In addition, the results obtained by MTT and Annexin-PI assays were corroborated by using RT-PCR for GLI1, the Hh signaling target gene. There was increased expression of GLI1 in the presence of Shh, but decreased expression of GLI1 in presence of Shh and cyclopamine, compared with controls (Fig. 2C), thus supporting the results of the MTT and Annexin-PI assays. Together, these experiments clearly show that modulation of Hh signaling in B-CLL influenced the survival of B-CLL cells and, hence, suggest that Hh signaling may be involved in the pathogenesis of B-CLL.

**FIGURE 2.** Exogenous Shh increased the survival and cyclopamine decreased the survival of primary B-CLL cells in vitro. Primary B-CLL cells were cultured in the presence of medium, exogenous Shh (100 ng/mL), Shh + cyclopamine (5 μmol/L), and cyclopamine for 72 h, and cell survival was determined by the MTT and Annexin-PI assays. Target gene, GLI1, expression was determined by RT-PCR. There was significantly increased B-CLL cell survival in the presence of exogenous Shh and decreased B-CLL cell survival in the presence of cyclopamine as determined by the MTT assay (A) and the Annexin-PI assay (B). C. RT-PCR for the Hh signaling target gene, GL1, corroborated the results obtained by the MTT and Annexin-PI assays. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene control. Absorbance of B-CLL cells cultured in the presence of control medium was considered as 100% survival. B-CLL control was the same as from Fig. 1D as this assay was done together. Results are expressed as average ± SE of triplicates each from seven different patients.
Hedgehog Signaling Target Molecules Are Overexpressed in B-CLL Cells of Patients with Poor Clinical Outcome

To analyze the differential expression of Hh signaling components in B-CLL subgroups, B-CLL patients were divided into good and poor clinical outcome subgroups based on the cytogenetic abnormalities as prognostic markers. The poor clinical outcome subgroup included patients with 17p deletion, 11q deletion, and trisomy 12, whereas the good clinical outcome subgroup included those with 13q14 deletion or a normal karyotype. To validate the pattern of disease progression in our experimental subsets, good and poor clinical outcome subgroups were correlated with time to first treatment, which is the time from diagnosis to treatment, using Kaplan-Meier survival analysis. Patients in the poor clinical outcome subgroup had a significantly shorter time to treatment compared with the good clinical outcome subgroup (Fig. 3A), confirming the anticipated outcome in these patient subgroups.

To determine the expression of molecules associated with Hh signaling, such as PTCH, SMO, SUFU, GLI1, GLI2, GLI3, and BCL2, the normalized average ratio of the medians of these molecules obtained from microarray data of 48 B-CLL samples was compared between the good and poor clinical outcome subgroups (Fig. 3B-E). There was a significantly higher expression of GLI1 ($p = 0.03$), GLI2 ($p = 0.01$), SUFU ($p = 0.02$), and BCL2 ($p = 0.02$) in the poor clinical outcome subgroup compared with the good clinical outcome subgroup (Fig. 3B-E). Our results indicate that these transcripts are overexpressed by B-CLL cells from poor clinical outcome patients compared with B-CLL cells from good outcome patients. To validate the results obtained by microarray, expression levels of one of the mediators (SUFU) and the specific target gene of Hh signaling (GLI1) were further confirmed by real-time PCR. There were significantly lower normalized $C_t$ values for SUFU ($p = 0.002$) and GLI1 ($p = 0.003$) in the poor outcome subgroup compared with the good outcome subgroup, thus confirming the overexpression of SUFU and GLI1 in B-CLL cells from patients with poor-prognosis subgroup (Fig. 4A and B). To further strengthen this finding, expression of SUFU and GLI1 was correlated with CD38 expression levels on the B-CLL cells, another reliable prognostic marker of B-CLL (7). There were significantly decreased normalized $C_t$ values for SUFU ($p = 0.004$) and GLI1 ($p = 0.02$) in B-CLL cells from the high CD38-expressing subgroup (poor outcome) compared with the low CD38-expressing subgroup (good outcome; Fig. 4C and D). In addition, to determine the relationship of GLI1, GLI2, SUFU, and BCL2 expression with disease progression, expression of these molecules was correlated with the patient’s time to treatment using Kaplan-Meier analysis. Patients of B-CLL cells having higher expression of SUFU ($p = 0.01$), GLI1 ($p = 0.02$), GLI2 ($p = 0.04$), and BCL2 ($p = 0.04$) had a significantly shorter time to treatment compared with those with lower expressing B-CLL cells, indicating an association of these molecules with disease progression (Fig. 4E-H). Together, these results indicate that Hh signaling-associated molecules, such as SUFU, GLI1, GLI2, and BCL2, are overexpressed in...
poor-prognosis B-CLL, and the expression of these molecules correlates with the time to first treatment in B-CLL patients. These findings suggest a role for Hh signaling in the progression of B-CLL.

Targeting of GLI1 by ASO Synergistically Increased the Susceptibility of B-CLL Cells to Chemotherapy-Induced Apoptosis

To determine the influence of selective down-regulation of GLI1 on the survival of B-CLL cells, the cells were cultured in the presence of medium, or medium containing GLI1-ASO or control-ASO for 72 hours, and the viability was determined by the MTT assay. There was significantly decreased survival of B-CLL cells in the presence of GLI1-ASO compared with cells cultured in the presence of medium (100% versus 74 ± 4.4%, \( P = 0.004 \)) or control-ASO (Fig. 5A). In addition, this result was corroborated by RT-PCR. There was decreased expression of GLI1 transcripts in the presence of GLI1-ASO. In addition, there was lower expression of BCL2 transcripts when cells were cultured in the presence of GLI1-ASO (Fig. 5C), suggesting the regulation of BCL2 expression by GLI1 in B-CLL as reported in other tumors (23, 24). Together, these results indicate that Hh signaling target molecules, such as GLI1, regulate the survival of B-CLL cells by regulating BCL2.

Because the above results show the regulation of BCL2 by GLI1, we further hypothesized that down-regulation of GLI1 may increase the chemosusceptibility of B-CLL cells. To test this premise, B-CLL cells were cultured in the presence of GLI1-ASO with or without fludarabine, a commonly used chemotherapeutic agent for B-CLL. There was a significantly decreased survival of B-CLL cells in the presence of fludarabine compared with untreated cells in medium. The survival of B-CLL cells was significantly decreased much more in the presence of GLI1-ASO plus fludarabine compared with fludarabine alone (67 ± 4.6% versus 40 ± 6.1%, \( P = 0.008 \)) or fludarabine with control-ASO (Fig. 5A). In addition, the survival of B-CLL cells was significantly lower in the presence of GLI1-ASO and fludarabine compared with GLI1-ASO alone (74 ± 4.4% versus 40 ± 6.1%, \( P = 0.002 \); Fig. 5A). These results indicate that GLI-specific ASO increased B-CLL cell susceptibility to fludarabine-mediated cytotoxicity. Furthermore, the preapoptotic and postapoptotic populations in GLI-ASO plus fludarabine–treated B-CLL cells, Annexin-PI staining was used. The results showed a significant increase in apoptotic B-CLL cells following treatment with GLI1-specific ASO plus fludarabine (72 ± 8%) compared with either GLI1-ASO alone (45 ± 1%) or fludarabine alone (60 ± 6%; Fig. 5B), as observed in the MTT assay, and further strengthened the above findings. Together, these results show that inhibition of Hh signaling by down-regulating GLI1 decreased B-CLL survival and synergistically increased apoptosis when combined with fludarabine.

Discussion

Identification of the potential molecular targets in poor-prognosis patients is a key step in the drug discovery process. Emerging evidence suggests that both intrinsic defects in the presence of medium, or medium containing GLI1-ASO or control-ASO for 72 hours, and the viability was determined by the MTT assay. There was significantly decreased survival of B-CLL cells in the presence of GLI1-ASO compared with cells cultured in the presence of medium (100% versus 74 ± 4.4%, \( P = 0.004 \)) or control-ASO (Fig. 5A). In addition, this result was corroborated by RT-PCR. There was decreased expression of GLI1 transcripts in the presence of GLI1-ASO. In addition, there was lower expression of BCL2 transcripts when cells were cultured in the presence of GLI1-ASO (Fig. 5C), suggesting the regulation of BCL2 expression by GLI1 in B-CLL as reported in other tumors (23, 24). Together, these results indicate that Hh signaling target molecules, such as GLI1, regulate the survival of B-CLL cells by regulating BCL2.

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Discussion

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neoplastic cells and extrinsic survival-stimulating factors in the tumor microenvironment are major contributing factors in the pathogenesis of B-CLL. Because B-CLL cell proliferation and survival are regulated by stromal cells, stromal cell–associated regulatory molecules might be involved in this process.

Activation of the Hh signaling pathway, which is often associated with stromal cells, has been reported in various cancers, including B-cell malignancies (16-18). Thus, impaired Hh signaling in B-CLL may be either due to increased Hh ligand in the tumor microenvironment or due to defects in the intrinsic pathway. Despite many avenues for the activation of Hh signaling, there is ultimately manifested in the activation of GLI1 transcription factors, the key regulators of the Hh signaling pathway. Therefore, specifically down-regulating expression of the GLI1 transcription factor might be a viable approach to inhibit the Hh signaling pathway for therapeutic purposes.

Although B-CLL cells are resistant to apoptosis with increased survival in vivo, these cells die by spontaneous apoptosis in vitro when cultured in the complete medium (RF-10) over a few days. This suggests that factors provided by the microenvironment facilitate the survival of B-CLL cells in vivo. To show the involvement of stromal cells in the survival of B-CLL cells, we cocultured them on OMA-AD cells, a reliable bone marrow–derived stromal cell line that is known to promote the growth of hematopoietic stem cells (25). The increased survival and decreased apoptosis of B-CLL cells grown on OMA-AD stromal cells or stromal-CM (Fig. 1) showed the positive influence of stromal cell–mediated signaling on the survival of B-CLL cells. Therefore, this coculture system may mimic the in vivo microenvironment that is important in the pathogenesis of B-CLL. Our findings agree with the results of others who have used HS-5 stromal cells (26). We also found decreased survival and increased apoptosis of B-CLL cells in the presence of cyclopamine, confirming Hh-mediated influence of stromal cells on B-CLL cells. To further confirm these findings, we studied B-CLL cell survival in the presence of stromal-CM or exogenous Shh and cyclopamine. In spite of the expected heterogeneity of the primary clinical samples and the difficulty of growing B-CLL cells in vitro, B-CLL cells from the majority of patients responded to exogenous Shh and cyclopamine (Fig. 2). The survival of B-CLL cells was much higher when cultured on stromal cells compared with stromal-CM medium in the presence of Shh, suggesting a possible contribution of other signaling pathways on the survival of B-CLL in addition to Hh signaling. Such a contribution could be due to CD31-CD38 signaling or SDF1-CXCR4 signaling, as recently reported (27). These results further confirmed the ability of B-CLL cells to proliferate and survive in a microenvironment containing Hh ligand. This positive influence of Hh signaling from the stromal cells on different cancers, including lymphoma, has recently been reported by others (17, 18, 28-30).

Although the deregulation of Hh signaling leads to increased proliferation and/or decreased apoptosis in various cancers (22), including in B-cell malignancies (16, 17), the GLI1 transcription factor seems to be the most important overexpressed molecule. Among the GLI transcription factors, GLI1 and GLI2 have been extensively studied and are involved in the development of tumors. Therefore, we investigated the expression of Hh signaling components, particularly GLI1, and the correlation with disease progression in B-CLL patient subgroups with good or poor clinical outcome. The interpretation of our results obtained using primary B-CLL cells is
complicated by the heterogeneity of the patient population. In our study, B-CLL patients with chromosome 17p deletion or 11q deletion or trisomy 12 had a significantly shorter time to treatment compared with those with 13q14 or a normal karyotype (Fig. 3). Although we have previously reported a differential gene expression in B-CLL (7), we did not look at Hh signaling pathway-related molecules. Therefore, in an initial screening, we reanalyzed and compared the expression of these molecules in B-CLL. This approach has been used to study platelet-derived growth factor receptor and related molecule expression in hepatocellular cancer (31). Overexpression of GLI1, GLI2, SUFU, and BCL2 and their correlation with disease progression (Figs. 3 and 4) suggest a role for Hh signaling in the pathogenesis of poor clinical outcome B-CLL. This observation is also supported by the fact that GLI expression has not been observed in CD19+ normal B cells (12). In contrast, the expression of GLI1 has been reported in malignant B cells (16, 17, 32).

To develop an innovative strategy for the treatment of B-CLL, we attempted to target GLI, the key mediator of the Hh signaling. GLI1-ASO decreased not only GLI1 transcripts but also the viability of B-CLL cells and increased apoptotic B-CLL cells by decreasing BCL2 expression. These results confirm the presence of GLI-mediated regulation of BCL2 expression in B-CLL, as reported in other cancers (23, 24). In addition, selective down-regulation of GLI1 transcript by ASO in conjunction with fludarabine synergistically increased the apoptosis and decreased the survival of B-CLL cells (Fig. 5), indicating the potential of GLI targeting to increase the susceptibility of B-CLL cells to chemotherapy. Together, our studies suggest a hypothetical model of B-CLL pathogenesis (Fig. 6). As reported in the literature, Hh could be secreted by the bone marrow stromal cells (18, 19) or by T cells (14, 15) and increase the resistance to apoptosis and survival of B-CLL cells in the patients. If we target Hh signaling by cyclopamine, GLI-specific ASO, or other newer-generation Hh signaling inhibitors, increased susceptibility to chemotherapy may be achieved. Therefore, targeting of Hh signaling is a potential strategy to develop new therapies for B-CLL and will also open new avenues to better understand the mechanisms of Hh-mediated signaling in B-CLL.

Materials and Methods
Collection, Isolation, and Characterization of B-CLL Cells
Fresh peripheral blood samples from previously untreated patients or the patients who were not treated for prior 6 mo were collected from B-CLL patients using an Institutional Review Board–approved protocol and informed consent. Details of patient information are as previously described (7). Peripheral blood mononuclear cells from B-CLL patients were isolated using lymphocyte separation medium (Accurate Chemical and Scientific Corp.) as previously described (7, 33). The immuno-phenotypes of the peripheral blood mononuclear cells were determined by flow cytometry using phycoerythrin- or FITC-conjugated antibodies for CD3, CD5, CD19, and CD38 (BD Pharmingen). Cells were also double stained with CD5 and CD19, and CD38 and CD19. The proportion of cells positive for these markers was determined using a Becton Dickinson FACStar plus flow cytometer. B-CLL samples with >90% CD5+ and CD19+ cells were used in the study. Where necessary, we used CD3/CD56 depletion to reduce the contaminating T and natural killer cells using Miltenyi’s magnetic bead separation method (7). The purified B-CLL cells were reanalyzed using flow cytometry, and purity was confirmed.

B-CLL Cell Coculture with Stromal Cells or Stromal-CM
OMA-AD bone marrow–derived stromal cell line was used for this study (25). OMA-AD cells were maintained with RPMI 1640 containing 10% horse serum, 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 × 10−6 mol/L hydrocortisone. For coculture studies, OMA-AD cells were cultured in six-well plates. Once the cells reached about 50% to 60% confluence, these cells were washed with RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L l-glutamine, and 100 units/mL penicillin/streptomycin (RF-10). Freshly isolated primary B-CLL cells were cocultured on the OMA-AD stromal cell feeder layer in the presence of RF-10 medium or medium containing 5 μmol/L cyclopamn (Calbiochem) for 72 h in 37°C incubator with 5% CO2. Only B-CLL or OMA-AD cells cultured in the presence of RF-10 were used as controls. In addition, once OMA-AD cells reached 50% to 60% confluence, these cells were washed with RF-10 medium and continued to culture for 72 h. The conditioned medium (stromal-CM) was collected with centrifugation and stored at ~80°C for further use. Freshly isolated B-CLL cells were cultured in the presence of medium control, stromal-CM, and stromal-CM + cyclopamn for 72 h. The cells were washed and the preapoptotic and apoptotic cell populations were determined using the Annexin-PI assay by flow cytometry according to the

![FIGURE 6. A hypothetical model of Hh signaling in B-CLL tumor microenvironment. Hh ligand is secreted by bone marrow stromal cells or neighboring T cells, which may increase the survival of B-CLL cells. Inhibiting this signaling by molecular targeting of GLI increases not only apoptosis of B-CLL cells but also their susceptibility to chemotherapy and, thereby, suggests a means to improve the treatment of B-CLL.](https://example.com/figure6.png)
plates in the presence of RF-10 medium and medium containing analgesics was validated by doing real-time PCR for SUFU and Real-time PCR and Semiquantitative RT-PCR using GenePix 5.1 software. Fluorescence ratios for array elements were extracted and normalized using an Axon 4000B scanner (Axon Instruments). Fluorescence was measured at 570 nm using a plate reader (BioTek).

The cDNA was generated from the RNA obtained from B-CLL cells, and Stratagene reference RNA was labeled with Cy-5 and Cy-3 dye, respectively, and hybridized as previously described (7). Hybridized slides were scanned for microarray images using an Axon 4000B scanner (Axon Instruments). Fluorescence ratios for array elements were extracted and normalized using GenePix 5.1 software.

Real-time PCR and Semiquantitative RT-PCR

Differential expression of genes identified by microarray analyses was validated by doing real-time PCR for SUFU and GLI1. Complimentary DNA was synthesized from 5 μg of total RNA from each B-CLL samples according to the manufacturer’s instructions (Invitrogen). SYBR Green real-time PCR assay was done in 20 μL of PCR mixture volume consisting of 2 X SYBR Green PCR Master Mix (Roche), 200 nmol/L of primer mix, and 2 μL of cDNA template. Corresponding C\textsubscript{t} value of respective genes was normalized with C\textsubscript{t} value of housekeeping gene, HPRT. For RT-PCR, respective transcripts were amplified with gene-specific forward and reverse primers (Supplementary Table) using a step-cycle program using Taq polymerase (Invitrogen). PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Modulation of H\textsubscript{h} Signaling in B-CLL Cells

Freshly isolated B-CLL cells (10\textsuperscript{5}) were cultured in 96-well plates in the presence of RF-10 medium and medium containing exogenous Shh (100 ng/mL; Calbiochem), Shh and cyclopamine (5 μmol/L), and cyclopamine alone for 72 h in 37°C incubator with 5% CO\textsubscript{2}. Cells cultured in the presence of DMSO or only medium were used as control. After 72 h, cells were incubated with 25 μL of MTT (5 mg/mL in PBS) for 2 h and lysed using SDS-based lysis buffer. The developed color was measured at 570 nm using a plate reader (BioTek). The percentage of survival was determined by considering the absorbance of B-CLL cells cultured in medium only as 100%. In addition, these cells were harvested, and RNA was isolated and processed for RT-PCR.

RNA Extraction and DNA Microarray Analysis

Total RNA was extracted from B-CLL cells using Trizol reagent (Invitrogen) according to the manufacturer’s instructions, and RNA quality was determined by agarose gel electrophoresis and quantified by spectrophotometry. Expression of genes was determined by using a custom oligonucleotide microarray chip consisting of 50-mer oligos representing 9,986 different genes (Human 10Koligo set A; MWG Biotech). The cDNA was generated from the RNA obtained from B-CLL cells, and Stratagene reference RNA was labeled with Cy-5 and Cy-3 dye, respectively, and hybridized as previously described (7). Hybridized slides were scanned for microarray images using an Axon 4000B scanner (Axon Instruments). Fluorescence ratios for array elements were extracted and normalized using GenePix 5.1 software.

Table 1. List of Primers and Antisense Oligonucleotides (ASO) Used in this Study

<table>
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<tr>
<th>Gene name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5'-GAGGTGGAAGTGTCAGTCTC-3'</td>
</tr>
<tr>
<td>HPRT</td>
<td>F: 5'-GCAAGTCGAGGCTGTACGAC-3'</td>
</tr>
<tr>
<td>GLI1</td>
<td>F: 5'-CCCAATCACAAGTCCAGTTTCT-3'</td>
</tr>
<tr>
<td>BCL2</td>
<td>F: 5'-GTCATGGCCCTCTGTTGGAT-3'</td>
</tr>
<tr>
<td>SUFU</td>
<td>F: 5'-GGGAGGGAGACCATATT-3'</td>
</tr>
<tr>
<td>GLI1-ASO*</td>
<td>5'-ATGTTCAACTCGATGACCCC-3'</td>
</tr>
<tr>
<td>Control-ASO*</td>
<td>5'-GCCGAAGTGGTCAATGTCTCAGGC-3'</td>
</tr>
</tbody>
</table>

Abbreviations: F: Forward primer, R: Reverse primer.

*Phosphorothioate backbone for ASO.

Table 1. List of Primers and Antisense Oligonucleotides (ASO) Used in this Study

Differential expression of genes identified by microarray experiments, analyzed the data, and wrote the manuscript. K.J. Peterson

Acknowledgments

We thank Dr. John D. Jackson (Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE) for providing us OMA-AD bone marrow stromal cell line for this study.

Authorship Contribution: G.V. Hegde hypothesized the project, performed the experiments, analyzed the data, and wrote the manuscript. K.J. Peterson

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Statistical Analysis

The Kaplan-Meier method using log-rank test was used to estimate the time to treatment distribution among B-CLL patients. To determine the clinical outcome, time to treatment was used as a measure where the time to treatment was defined as the time in months for initiation of the first treatment cycle since diagnosis of B-CLL patients. The gene expression levels were dichotomized at their median value, and the log-rank test was used to compare the time to treatment between the below and above median groups. In addition, where appropriate, Student’s t test was used to determine the significance. \( P < 0.05 \) was considered as statistically significant.

Chemosusceptibility of GLI Down-Regulated B-CLL Cells

To determine the susceptibility of GLI down-regulated B-CLL cells to fludarabine, a standard chemotherapeutic drug for B-CLL, freshly isolated cells (10\textsuperscript{5}) were cultured in the presence of RF-10 medium or medium containing 3 μmol/L GLI1-ASO (Table 1) or control-ASO (antisense to human herpes simplex virus genome) for 72 h in 37°C incubator with 5% CO\textsubscript{2}. The viability of B-CLL cells was determined by MTT assay as described above. In addition, these cells were harvested and RNA was isolated and processed for RT-PCR as described above.

Down-Regulation of GLI1 in B-CLL Cells

To down-regulate GLI1 transcription factors, we have used ASO with phosphorothioate backbone. Freshly isolated B-CLL cells (10\textsuperscript{5}) were cultured in the presence of the RF-10 medium or medium containing 3 μmol/L GLI1-ASO (Table 1) or control-ASO (antisense to human herpes simplex virus genome) for 72 h in 37°C incubator with 5% CO\textsubscript{2}. The viability of B-CLL cells was determined by MTT assay as described above. In addition, these cells were harvested and RNA was isolated and processed for RT-PCR as described above.

GLI1. Complimentary DNA was synthesized from 5 μg of total RNA from each B-CLL samples according to the manufacturer’s instructions (Invitrogen). SYBR Green real-time PCR assay was done in 20 μL of PCR mixture volume consisting of 2 X SYBR Green PCR Master Mix (Roche), 200 nmol/L of primer mix, and 2 μL of cDNA template. Corresponding C\textsubscript{t} value of respective genes was normalized with C\textsubscript{t} value of housekeeping gene, HPRT. For RT-PCR, respective transcripts were amplified with gene-specific forward and reverse primers (Supplementary Table) using a step-cycle program using Taq polymerase (Invitrogen). PCR products were visualized on a 2% agarose gel stained with ethidium bromide.

Down-Regulation of GLI1 in B-CLL Cells

To down-regulate GLI1 transcription factors, we have used ASO with phosphorothioate backbone. Freshly isolated B-CLL cells (10\textsuperscript{5}) were cultured in the presence of the RF-10 medium or medium containing 3 μmol/L GLI1-ASO (Table 1) or control-ASO (antisense to human herpes simplex virus genome) for 72 h in 37°C incubator with 5% CO\textsubscript{2}. The viability of B-CLL cells was determined by MTT assay as described above. In addition, these cells were harvested and RNA was isolated and processed for RT-PCR as described above.

Chemosusceptibility of GLI Down-Regulated B-CLL Cells

To determine the susceptibility of GLI down-regulated B-CLL cells to fludarabine, a standard chemotherapeutic drug for B-CLL, freshly isolated cells (10\textsuperscript{5}) were cultured in the presence of RF-10 medium in triplicate wells with and without GLI1-ASO or control-ASO along with 0.5 μmol/L fludarabine (Sigma Chemicals) in 96-well plates. B-CLL cells were incubated at 37°C with 5% CO\textsubscript{2} for 72 h as described. The viability of both control and treated cells was determined using the MTT assay as described above. In addition, the cells were stained with Annexin-P1, and the proportion of apoptotic cells was determined by flow cytometry as described above.

Acknowledgments

We thank Dr. John D. Jackson (Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE) for providing us OMA-AD bone marrow stromal cell line for this study.

Authorship Contribution: G.V. Hegde hypothesized the project, performed the experiments, analyzed the data, and wrote the manuscript. K.J. Peterson

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References


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