

# Expression of Hedgehog Pathway Mediator *GLI* Represents a Negative Prognostic Marker in Human Acute Myeloid Leukemia and Its Inhibition Exerts Antileukemic Effects

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## Abstract

**Purpose:** The Hedgehog pathway plays an important role in stem-cell biology and malignant transformation. Therefore, we investigated the expression and prognostic impact of Hedgehog pathway members in acute myeloid leukemia (AML).

**Experimental Design:** Pretreatment samples from 104 newly diagnosed AML patients (AMLSC 07-04 trial) were analyzed by qPCR, and expression of Hedgehog family members was correlated with clinical outcome. Inhibition of *GLI* by GANT61 or shRNA was investigated in AML cells *in vitro* and *in vivo*.

**Results:** Expression of receptors Smoothed and Patched-1 and their downstream mediators, *GLI1*, *GLI2*, and *GLI3*, was found in AML patients in contrast to Hedgehog ligands. *GLI2* expression had a significant negative influence on event-free survival (EFS), relapse-free survival (RFS), and overall survival (OS;  $P = 0.037$ ,  $0.026$ , and  $0.013$ , respectively) and was correlated

with FLT3 mutational status ( $P < 0.001$ ). Analysis of a second, independent patient cohort confirmed the negative impact of *GLI2* on EFS and OS ( $P = 0.007$  and  $0.003$ , respectively;  $n = 290$ ). Within this cohort, *GLI1* had a negative prognostic impact ( $P < 0.001$  for both EFS and OS). Although AML cells did not express Hedgehog ligands by qPCR, AML patients had significantly increased Desert Hedgehog (DHH) plasma levels compared with healthy subjects ( $P = 0.002$ ), in whom DHH was presumably provided by bone marrow niche cells. Moreover, the *GLI* inhibitor GANT61 or knockdown of *GLI1/2* by shRNA caused antileukemic effects, including induction of apoptosis, reduced proliferation, and colony formation in AML cells, and a survival benefit in mice.

**Conclusions:** *GLI* expression is a negative prognostic factor and might represent a novel druggable target in AML. *Clin Cancer Res*; 21(10): 2388–98. ©2015 AACR.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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## Introduction

The cancer and leukemia stem cell (LSC) hypothesis suggests that malignant stem cells serve as reservoir for leukemia cell renewal (1). Stem cell activators such as Wnt, transforming growth factor  $\beta$  (TGF $\beta$ ), Notch, and Hedgehog (HH) signaling pathways have been implicated into stem cell self-renewal and resistance to chemotherapy (2). Furthermore, normal and LSC function is strictly dependent on extrinsic signals mediated by bone marrow niche cells, such as endothelial cells or osteoblasts, which regulate stem and progenitor cell homeostasis, mobilization, and regeneration (3).

Mammalian cells express three different Hedgehog ligands: Sonic Hedgehog (SHH), Desert Hedgehog (DHH), and Indian Hedgehog (IHH), which bind to the transmembrane receptor Patched-1 (PTCH1). Without the presence of ligands, PTCH1 represses activation of the transmembrane protein Smoothed (SMO). Upon HH ligand binding, PTCH1 is internalized, resulting in relief and thus activation of SMO that transduces the signal to the family of Glioma (GLI) zinc finger transcription factors GLI1, GLI2, and GLI3. GLI1 and GLI2 predominantly represent transcriptional activators while GLI3 acts as transcriptional repressor (4).

### Translational Relevance

The Hedgehog pathway is involved in stem cell proliferation, differentiation, and survival and may play an important role in leukemia biology. Therefore, we analyzed expression of members of the Hedgehog pathway in two independent cohorts of acute myeloid leukemia (AML) patients. We could show that the Hedgehog transcription factors *GLI2* and *GLI1* represent negative prognostic survival markers in AML. Interestingly, in both patient cohorts, a correlation between *GLI2* expression and *FLT3* mutational status was observed. As no expression of Hedgehog ligands was found in AML cells, we postulate a paracrine provision of Hedgehog ligands by bone marrow stromal cells, resulting in significantly elevated plasma levels of Desert Hedgehog as compared with healthy controls. Finally, inhibition of Hedgehog signaling using a specific *GLI* inhibitor or targeting *GLI* by shRNA showed effective antileukemic properties *in vitro* and *in vivo*, underlining that *GLI* inhibition might represent a promising target for drug development.

In this study, we analyzed the expression of Hedgehog family members by quantitative RT-PCR in two independent acute myeloid leukemia (AML) populations and correlated the gene expression to patients' baseline characteristics and clinical outcome. In addition, Hedgehog ligand expression was studied in bone marrow stromal cells and DHH plasma levels were measured in AML patients and controls. Finally, we investigated the effect of Hedgehog signaling inhibition on primary AML cells and AML cell lines.

## Materials and Methods

### Patients and samples

We analyzed bone marrow and peripheral blood mononuclear cells from 104 leukemic patients with newly diagnosed AML (referred to as cohort A). Patients were treated within the AMLSG 07-04 study of the Austro-German Study Group AMLSG (refer to the Supplementary Data for trial design). The trial was conducted between August 2004 and August 2009. The study (ClinicalTrials.gov Identifier: NCT00151242) was approved by the ethics committees of each study site and was conducted in accordance with the Austrian and German drug development regulations and the Declaration of Helsinki (5).

The second, independent patient cohort—referred to as cohort B—comprised 290 AML patients of whom microarray-based gene expression data were published by Verhaak and colleagues [data accessible at NCBI Gene Expression Omnibus (GEO) database, accession GSE6891; ref. 6]. Patients were derived from a clinical study by Lowenberg and colleagues (7). More information on the study is provided in the Supplementary Data.

### Cell culture and CD34 and endothelial cell enrichment

Primary AML cells for *in vitro* experiments were obtained after patient's informed consent and approval of the study by the ethics committee (PV3469, Ethik-Kommission der Ärztekammer Hamburg). Cells were isolated from bone marrow or peripheral blood using density gradient centrifugation. The AML cell line UKE-1 was derived from our laboratory (8). The AML cell lines KG-1,

MV4-11, and OCI-AML5 were authenticated by the Multiplex human Cell line Authentication Test (Multiplexion). Primary outgrowth endothelial cells (OEC) were isolated from the peripheral blood mononuclear cells (MNC) of healthy, anonymous donors obtained from buffy coats kindly provided by the blood bank of the University Medical Center Hamburg-Eppendorf (Hamburg, Germany) as described before (9).

CD34<sup>+</sup> progenitor cells were isolated from leukapheresis products of G-CSF-primed anonymous donors. Cell culture conditions and enrichment for CD34<sup>+</sup> and endothelial cells are described in the Supplementary Data.

### Lentiviral transduction of AML cell lines with *GLI1*- and *GLI2*-specific shRNA

pLKO.1-puro vector encoding *GLI1* (TRCN000020485, sequence 5'-CCGGCCTGATTATCTTCCTTCAGAACTCGAGTTCT-GAAGGAAGATAATCAGGTTTTT-3'), *GLI2* (TRCN0000238361, sequence 5'-CCGGCTGGACAGGGGATGACTGTAAGCTCGAGCT-TACAGTCATCCCTGTCCAGTTTTTG-3'), or scrambled shRNA were purchased from Sigma-Aldrich. Plasmids pMD2.G-VSV-G and psPAX2-Gag-Pol were purchased from Addgene. Cells were either transduced with nontargeting shRNA (negative control) or shRNA against *GLI1* or *GLI2*. The protocol is described in detail in the Supplementary Data.

### Cell growth, apoptosis, and colony formation assay

AML cells or CD34<sup>+</sup> progenitor cells were incubated with different concentrations of the *GLI* inhibitor GANT61 (2,20-[[dihydro-2-(4-pyridinyl)-1,3(2H,4H)-pyrimidinediyl]bis(methylene)]bis[N,Ndimethyl benzenamine]; Tocris) or DMSO as a solvent control. For shRNA experiments, AML cells with the *GLI1* or *GLI2* knockdown were compared with the negative control containing nontargeting shRNA.

Induction of apoptosis was measured by flow cytometry (FACS-Calibur; BD Biosciences) using Annexin-V and propidium iodide. Cell numbers for proliferation assays were determined using the Vi-Cell XR (Beckman Coulter). To determine the colony formation capacity, AML or CD34<sup>+</sup> progenitor cells were cultured in methylcellulose-based semi-solid media (MethoCult; STEMCELL Technologies). Assays are described in detail in the Supplementary Data.

### RNA isolation and cDNA synthesis

For samples of cohort A, total RNA of  $1 \times 10^7$  cells was extracted using the TRizol method (Invitrogen) and reversely transcribed into cDNA using MuLV-RT (Invitrogen). For all *in vitro* experiments, total RNA was extracted using the innuSPEED Tissue RNA Kit (Analytik Jena) and transcribed into cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific).

### Reverse transcription quantitative PCR, semi-quantitative PCR, and SMO mutation analysis

RT-qPCR analysis was carried out on the LightCycler 1.2 (Roche) using the FastStart DNA Master SYBR Green Kit (Roche). Relative amount of expressed cDNA was calculated using a standard curve obtained from log dilutions of plasmids containing the corresponding genes. Calculated cDNA amounts of the target genes were normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Data are represented as ratio of the target gene/*GAPDH*. Because of low expression and the

possible occurrence of primer dimers in primary AML samples, *GLI2* PCR products were separated in gel electrophoresis subsequent to LightCycler analysis, and *GLI2* positivity was evaluated semi-quantitatively on the basis of a distinct band in gel electrophoresis (refer to Supplementary Fig. S1 for representative gel electrophoresis pictures). Because mutations within exon 9 or 10 of the *SMO* gene represent a frequent genetic alteration in basal cell carcinoma (10), both *SMO* exon 9 and 10 were analyzed for mutations.

A detailed PCR and mutation analysis protocol is described in the Supplementary Data. Primers and data on PCR specificity and efficiency are listed in Supplementary Tables S1 and S2.

#### DHH immunofluorescence and immunohistochemistry

Primary endothelial cells, osteoblasts, MNCs from healthy donors, or AML bone marrow sections were investigated for the expression of DHH in immunofluorescence staining and immunohistochemistry. A detailed protocol is provided in the Supplementary Data.

#### Collection of human plasma samples and anti-human Desert Hedgehog ELISA analysis

For analysis of DHH plasma levels, peripheral blood samples of AML patients and healthy donors were collected. Plasma was separated and stored at  $-70^{\circ}\text{C}$  until use. DHH protein levels were measured using the enzyme-linked immunosorbent assay (ELISA) Kit for DHH (Cusabio Biotech) and quantified with the Sunrise ELISA plate reader and Magellan software (Tecan).

#### Xenograft model

MV4-11 cells ( $5 \times 10^5$ ) transduced with *GLI1* and *GLI2* shRNA (double knockdown cells) or with scrambled shRNA (control cells) were injected intravenously into NSG mice (NOD.Cg-*Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/Sz*). Mice were sacrificed when showing clear symptoms of leukemia. Leukemic infiltration of peripheral blood, bone marrow, and spleen was confirmed by flow cytometry. See the Supplementary Data for a more detailed protocol.

#### Statistical analysis

All statistical analyses were done with SPSS 17 (SPSS Inc.). Differences in gene expressions between demographic variables were accessed either by the Fisher test for categorical variables or by *t* test for continuous data. Overall survival (OS) was defined as time from study inclusion to death. Relapse-free survival (RFS) was defined as time from achieving a complete remission (CR) after the first therapy to relapse or death from any cause, while event-free survival (EFS) was defined as time from study inclusion to any predefined event (first therapy failure, relapse or death).

Classification-and-regression-tree (CART) analysis was used to estimate thresholds for each gene expression measurement that would discriminate between high and low overall mortality (11). If no convenient cutoff level could be determined, gene expressions were categorized into mRNA expressor and nonexpressor groups. The Kaplan–Meier survival curves were calculated for different categories and compared with the log-rank tests. To identify those gene expressions with independent significant predictive power, gene expressions were entered simultaneously into the same multivariable Cox model and a backwards selection was applied. Michael Friendly's 4-fold displays implemented in

R 2.15 were used to display contingency tables for gene expressions (12).

Differences in DHH plasma levels between AML patients and healthy controls and differences in GANT61 treatment or shRNA assays were accessed by the Welch *t* test. For all analyses, a  $P < 0.05$  was considered to be statistically significant.

## Results

### Expression of Hedgehog family members in study patients

To determine expression of Hedgehog signaling members, leukemic blasts from 104 newly diagnosed AML patients were studied (cohort A). Samples were generated from patients of the AMLSG 07-04 study of the Austro-German Study Group AMLSG who were treated according to a uniform chemotherapy protocol (5). Median follow-up time was 37 months. Sixty-three percent of analyzed patients achieved a CR after induction chemotherapy. Median EFS, RFS, and OS were 8, 15, and 27 months, respectively. Comprehensive patient's characteristics are listed in Table 1.

Expression levels of Hedgehog ligands, receptors *PTCH1* and *SMO* as well as downstream transcription factors *GLI1*, *GLI2*, and *GLI3*, were quantified by RT-qPCR. No appreciable expression of *SHH*, *IHH*, or *DHH* was found in 30 patients each, and thus no further samples were analyzed. mRNA expression of receptors *SMO* and *PTCH1* was found in 69% and 41% of cases, respectively. Measurable expression of *GLI1*, *GLI2*, and *GLI3* was detected in 73%, 20%, and 26% of cases, respectively (see Supplementary Fig. S2 for relative gene expression distribution).

### Mutation analysis of *SMO* exon 9 and 10

Mutations within exon 9 or 10 of the *SMO* gene represent a frequent genetic alteration in basal cell carcinoma (10). Therefore, both *SMO* exons have been amplified in an exon-9-and-10-spanning PCR analysis followed by direct sequencing in 65 AML samples. Sequence analysis revealed that none of the 65 AML patients carried a mutation in *SMO* exon 9 or 10.

**Table 1.** Patient characteristics

Patient characteristics	Patients, n (%) <sup>a</sup>	
	Cohort A (n = 104)	Cohort B (n = 290)
Median age in years (range)	51 (20–61)	44 (15–60)
Sex (male/female)	62 (60)/42 (40)	128 (44)/162 (56)
AML type		
<i>De novo</i> AML	92 (88)	n.a.
Secondary AML	5 (5)	
Therapy-related AML	7 (7)	
Unfavorable karyotype	39 (41)	85 (35)
Unknown cases	9	
<i>FLT3</i> mutated (including <i>FLT3</i> -ITD and <i>FLT3</i> -TKD)	26 (28)	119 (41)
Unknown cases	9	
<i>NPM1</i> mutated	18 (19)	92 (32)
Unknown cases	8	
<i>NPM1</i> mutated/ <i>FLT3</i> WT	9 (9)	28 (10)
Unknown cases	9	
<i>MLL</i> mutated	7 (7)	n.a.
Unknown cases	9	
Stem cell transplantation	71 (69)	n.a.
Unknown cases	1	

Abbreviations: ITD, internal tandem duplication; n.a., not available; TKD, tyrosine kinase domain.

<sup>a</sup>Because of incomplete data, the numbers might not add up to the number of patients in the cohort.

### Clinical significance

Clinical outcome of AML patients was correlated to Hedgehog pathway expression variables. The influence of the gene expression and baseline characteristics [sex, age, karyotype, and *FLT3*, *NPM1* mut/*FLT3* wild-type (WT) as well as *MLL* mutation status] on EFS, RFS, and OS was investigated in a Cox proportional hazards model. Because of the low number of cases displaying a favorable karyotype, patients with intermediate and favorable karyotype were combined. All analyzed genes were grouped into mRNA expressors and nonexpressors as no convenient cutoff levels could be found in CART analysis.

Univariate analysis revealed that mRNA expression of *PTCH1*, *SMO*, *GLI1*, and *GLI3* had no significant effect on survival. However, expression of downstream target gene *GLI2* had a significant impact on EFS, RFS, and OS ( $P = 0.037$ ,  $0.026$ , and  $0.013$ , respectively; see the Kaplan–Meier curves in Fig. 1; overview of hazard ratios in Table 2). As a control, *GLI2* expression was also detected in 5 of 11 cases of CD34<sup>+</sup> hematopoietic progenitor cells from healthy donors, while the peripheral blood MNCs from 20 healthy donors did not express *GLI2* in RT-qPCR (data not shown).

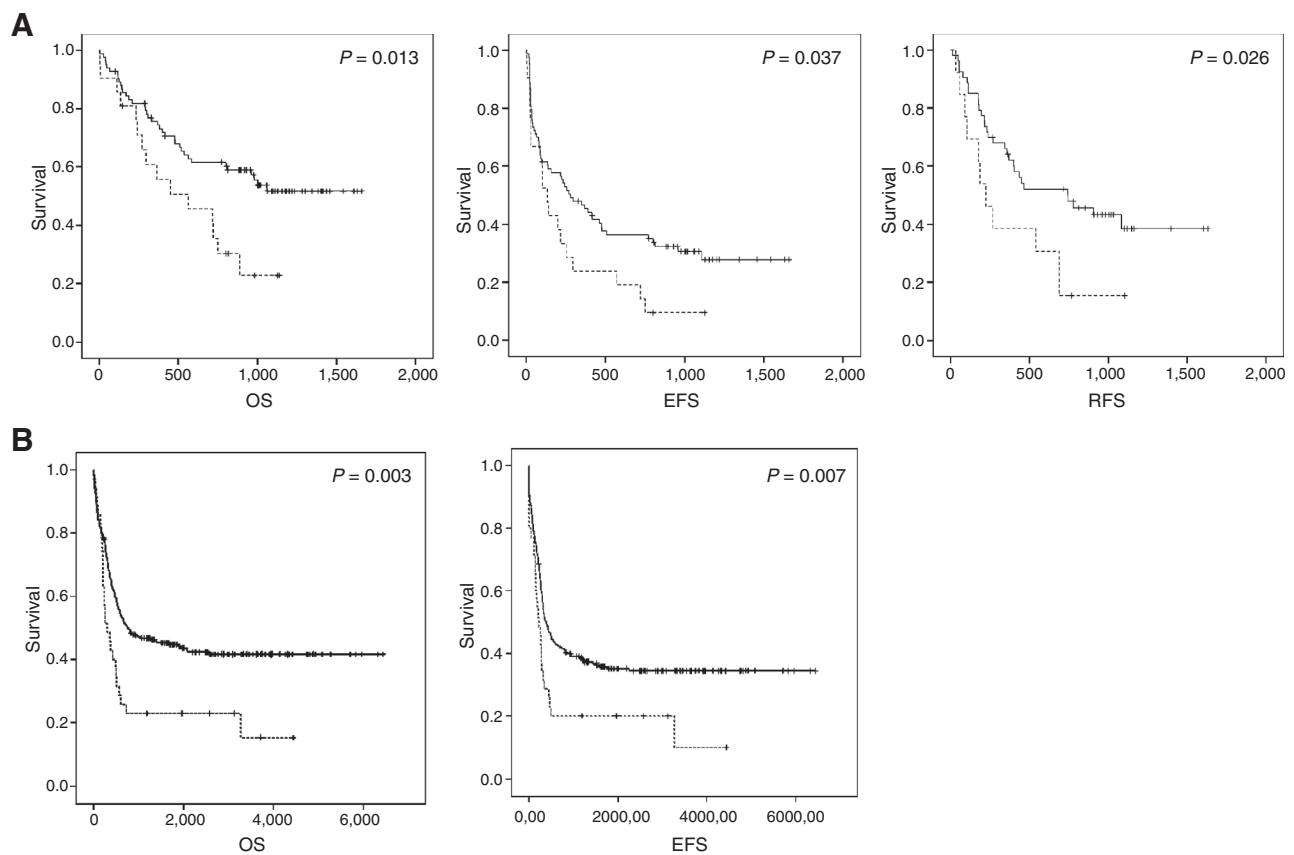
Of baseline characteristics, a *FLT3* mutation conferred a significant negative prognosis ( $P = 0.006$ ,  $0.001$ , and  $0.002$  for EFS, RFS, and OS, respectively; Table 2), whereas age was only a risk factor for the OS ( $P = 0.019$ ) and an unfavorable karyotype resulted only in reduced EFS ( $P = 0.023$ ; Table 2).

Coexpression of *GLI2* and the other Hedgehog family members was analyzed using Michael Friendly's 4-fold displays (12). The majority of *GLI2* expressors showed coexpression of *SMO* and *GLI1* (86% for *SMO*,  $P = 0.067$  and 100% for *GLI1*,  $P = 0.002$ ), which might be indicative for canonical activation of the Hedgehog pathway in AML. Additional correlations are shown in Supplementary Fig. S3.

To further explore our findings, patient characteristics including gender, age, karyotype, *FLT3* mutation, *NPM1* mut/*FLT3* WT, or *MLL* status were correlated with the expression of *GLI2*. In this analysis, a significant association between *FLT3* mutation and *GLI2* expression could be established. Interestingly, 80% of *GLI2* expressors carried a *FLT3* mutation compared with only 13% in nonexpressors ( $P < 0.001$ ; Table 3).

The effect of *GLI2* expression on EFS, RFS, and OS was also analyzed in a multivariate Cox proportional hazards model adjusted for baseline parameters karyotype, *FLT3*, and *NPM1* status implementing a stepwise removal of insignificant terms. Regarding the highly correlated markers *GLI2* and *FLT3*, the latter had a slightly higher discriminating power and therefore was maintained in the selection procedure. Including age and gender as confounders did not substantially alter the results (data not shown).

Because 70% of patients received allogeneic stem cell transplantation during the observation period, putative interactions



**Figure 1.**

Impact of *GLI2* expression on clinical outcome. A, a significant difference for *GLI2* expression (dashed line) versus no *GLI2* expression (continuous line) on OS, EFS, and RFS was determined in cohort A ( $n = 104$ ). B, in cohort B, a significantly different impact on OS and EFS was observed for high (dashed line) versus low *GLI2* expression (continuous line;  $n = 290$ ).



**Table 2.** Endpoint values of univariate analysis for EFS, RFS, and OS

Survival	Variable	Cohort A (n = 104)		Cohort B (n = 290)	
		HR (95% CI)	P	HR (95% CI)	P
EFS	<i>GLI2</i> expression	1.731 (1.025–2.923)	0.040	1.703 (1.146–2.532)	0.008
	<i>FLT3</i> mutation	2.031 (1.225–3.366)	0.006	1.076 (0.779–1.487)	0.657
	Karyotype	1.757 (1.083–2.851)	0.023	1.264 (0.996–1.605)	0.054
	Age	1.017 (0.995–1.040)	0.140	1.004 (0.993–1.016)	0.460
RFS	<i>GLI2</i> expression	2.165 (1.078–4.349)	0.030	n.a. (n.a.)	n.a.
	<i>FLT3</i> mutation	3.038 (1.563–5.903)	0.001	n.a. (n.a.)	n.a.
	Karyotype	1.224 (0.604–2.481)	0.575	n.a. (n.a.)	n.a.
	Age	1.027 (0.999–1.055)	0.056	n.a. (n.a.)	n.a.
OS	<i>GLI2</i> expression	2.108 (1.151–3.859)	0.016	1.849 (1.231–2.776)	0.003
	<i>FLT3</i> mutation	2.497 (1.382–4.513)	0.002	1.190 (0.845–1.677)	0.318
	Karyotype	1.719 (0.954–3.096)	0.071	1.344 (1.039–1.739)	0.024
	Age	1.035 (1.006–1.066)	0.019	1.012 (0.999–1.024)	0.072

Abbreviation: n.a., not available.

between *GLI2* expression and the transplantation status were analyzed using a time-dependent Cox regression model. This analysis showed that the negative impact of *GLI2* expression on survival of AML patients was independent of the transplantation status (data not shown).

#### Correlation of findings with an independent AML treatment group

To verify our findings, the microarray-based gene expression data published by Verhaak and colleagues (6) was used. Clinical data of 290 AML patients was available providing the possibility to analyze the impact of Hedgehog receptors *SMO* and *PTCH1* and transcription factors *GLI1*, *GLI2*, and *GLI3* on OS and EFS in a second independent cohort (cohort B; see Table 1 for patient characteristics). CART analysis was used to estimate the optimal cutoff levels for low versus high gene

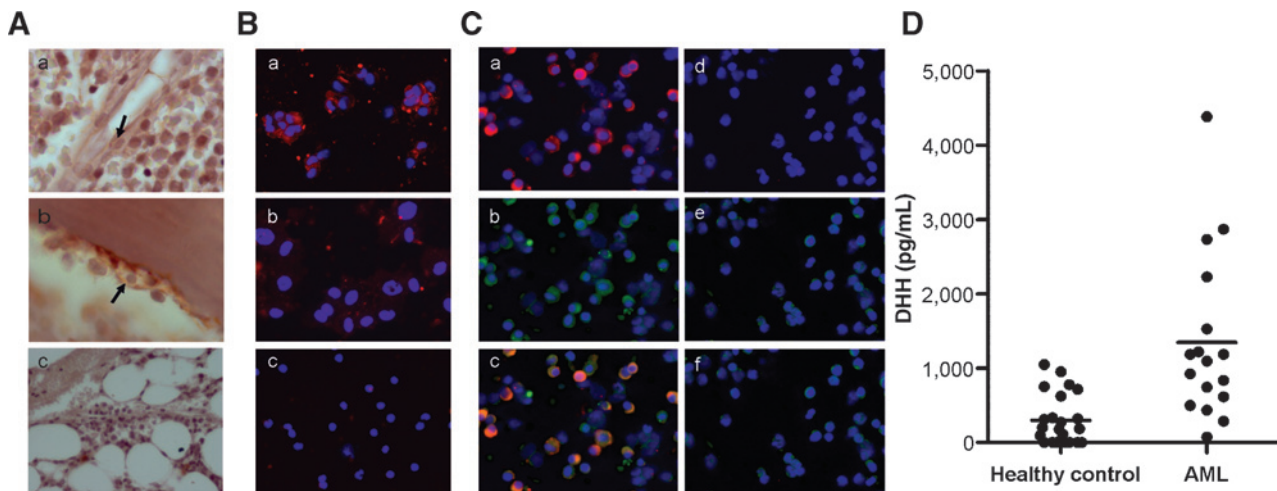
expression. Using this method, a significant negative impact on EFS and OS (data for RFS not available) could be observed for *GLI2* ( $P = 0.007$  and  $0.003$ , respectively; compare Fig. 1 for the Kaplan–Meier curves and Table 2 for hazard ratios). Interestingly, within this cohort, *GLI1* also had a negative impact on the EFS and OS ( $P < 0.001$  and  $P < 0.001$ , respectively; Supplementary Fig. S4 for the Kaplan–Meier curves and Supplementary Table S3 for hazard ratios). To test the effect of *GLI1* and *GLI2* adjusted for baseline parameters age, *FLT3* and *NPM1* mutational status, we entered all effects simultaneously in a multivariate Cox proportional hazards model. In addition to age ( $P = 0.012$ ), *FLT3* and *NPM1* mutational status ( $P = 0.004$  and  $0.002$ , respectively), also *GLI2* and *GLI1* remained significantly predictive after adjustment ( $P = 0.020$  and  $P < 0.001$ , respectively), therefore revealing that each parameter represents an independent risk factor for the survival of AML patients.

**Table 3.** Patient characteristics with respect to *GLI2* expression

Patient characteristics	Cohort A, patients, n (%) <sup>a</sup>			Cohort B, patients, n (%) <sup>a</sup>		
	<i>GLI2</i> expressors (n = 21)	<i>GLI2</i> nonexpressors (n = 83)	P <sup>b</sup>	<i>GLI2</i> high expressors (n = 35)	<i>GLI2</i> low expressors (n = 255)	P <sup>b</sup>
Median age in years (range)	54 (24–61)	50 (20–61)	0.868	47 (18–60)	44 (15–60)	0.698
Sex (male/female)	12 (57)/9 (43)	51 (61)/32 (39)	0.804	20 (57)/15 (43)	142 (56)/113 (44)	1.000
Karyotype						
Unfavorable	7 (33)	27 (36)	1.000	8 (29)	77 (35)	0.536
Favorable/intermediate	14 (67)	47 (64)		20 (71)	143 (65)	
Unknown	0	9		7	35	
<i>FLT3</i>						
Mutated	16 (80)	10 (13)	<0.001	22 (63)	91 (36)	0.003
WT	4 (20)	65 (87)		13 (37)	163 (64)	
Unknown	1	8		0	1	
<i>NPM1</i> mutated/ <i>FLT3</i> WT	0 (0)	9 (12)	0.197	1 (3)	27 (11)	0.222
Other <i>NPM1/FLT3</i> status	20 (100)	67 (88)		34 (97)	227 (89)	
Unknown	1	8		0	1	
<i>MLL</i>						
Mutated	1 (5)	6 (8)	0.881	n.a.	n.a.	
WT	19 (95)	69 (92)				
Unknown	1	8				
AML type						
<i>De novo</i> AML	20 (95)	72 (87)	0.866	n.a.	n.a.	
Secondary AML	0 (0)	5 (6)				
Therapy-related AML	1 (5)	6 (7)				
Stem cell transplantation	16 (80)	54 (65)	0.100	n.a.	n.a.	

Abbreviation: n.a., not available.

<sup>a</sup>n = 104 for cohort A and n = 290 for cohort B if not indicated otherwise.<sup>b</sup>The Fisher test for nominal variables and the Mann–Whitney U test for age.



**Figure 2.**

DHH secretion in AML. A, immunohistochemistry of bone marrow specimens revealed DHH-positive bone marrow niche cells, including endothelial cells (a, arrow) and osteoblasts (b, arrow; c, corresponding control without primary antibody; a and b, 1,000×; c, 400×). B, primary OECs (a) and osteoblasts (b) stained positively for DHH in immunofluorescent staining, whereas no DHH expression could be detected on mononuclear cells from healthy donors (c; a-c, 400×). C, immunofluorescent staining of the endothelial cell-enriched AML bone marrow (a-c) revealed that the majority of endothelial cells expressed DHH (a), as shown by double staining with CD146 (b); overlay of DHH and CD146 staining in c). On the contrary, virtually no DHH (d) or CD146 (e; see overlay in f) expression was detected within the whole leukemic mononuclear cell compartment before immunomagnetic separation (a-f, 400×). D, DHH plasma levels of AML patients ( $n = 17$ ) were analyzed in an ELISA. Compared with healthy donors ( $n = 23$ ), the DHH plasma levels of AML patients were significantly increased compared with 298 pg/mL in healthy donors (Welch  $t$  test,  $P = 0.002$ ; mean plasma levels are marked as horizontal line, each dot represents the DHH level of a single patient in the dot plot).

Although not included into the model due to many missing values, inclusion of the karyotype only resulted in minimal alterations of the hazard ratios (data not shown).

Furthermore, the correlation of *GLI2* expression to the *FLT3* mutational status could also be confirmed in cohort B ( $P = 0.003$ ; Table 3). Subgroup analysis revealed that the significant negative impact of *GLI2* expression on the survival was only present within the subgroup of *FLT3*-mutated patients [ $P = 0.005$  for EFS and  $P = 0.004$  for OS with hazard ratios, 2.1 (95% confidence interval (CI), 1.2–3.5) and 2.2 (95% CI, 1.3–3.8), respectively; Supplementary Fig. S5A and S5B], whereas no significant differences could be determined for the *FLT3* WT subgroup [ $P = 0.544$  for EFS and  $P = 0.353$  for OS with hazard ratios 1.2 (95% CI, 0.6–2.3) and 1.4 (95% CI, 0.7–2.6), respectively; Supplementary Fig. S5C and S5D]. Because of the low number of patients, no *FLT3* subgroup analysis could be performed in patient cohort A.

#### Expression of HH ligands in bone marrow stromal cells

Because no expression of *SHH*, *DHH*, or *IHH* could be detected in primary AML blasts, we wondered if the activation of the pathway might be due to paracrine interactions of AML blasts with the bone marrow microenvironment. Therefore, primary endothelial cells [OECs and human umbilical vein endothelial cells (HUVEC)] and osteoblasts were analyzed for expression of HH ligands. Twelve of 16 OEC clones, HUVECs, and osteoblasts showed clear mRNA expression of *DHH*, whereas *SHH* expression was detected in only five of 16 OEC clones and in HUVECs but not in osteoblasts. *IHH* mRNA expression could neither be detected in endothelial cells nor in osteoblasts (data not shown). Immunofluorescent staining confirmed DHH protein expression in OECs and osteoblasts, whereas no DHH expression was detected in MNCs from healthy donors (Fig. 2B). Furthermore,

bone marrow biopsies of 7 AML patients were stained for DHH in immunohistochemistry. Positive staining for DHH was observed in osteoblasts and endothelial cells of AML patients (representative images are shown in Fig. 2A).

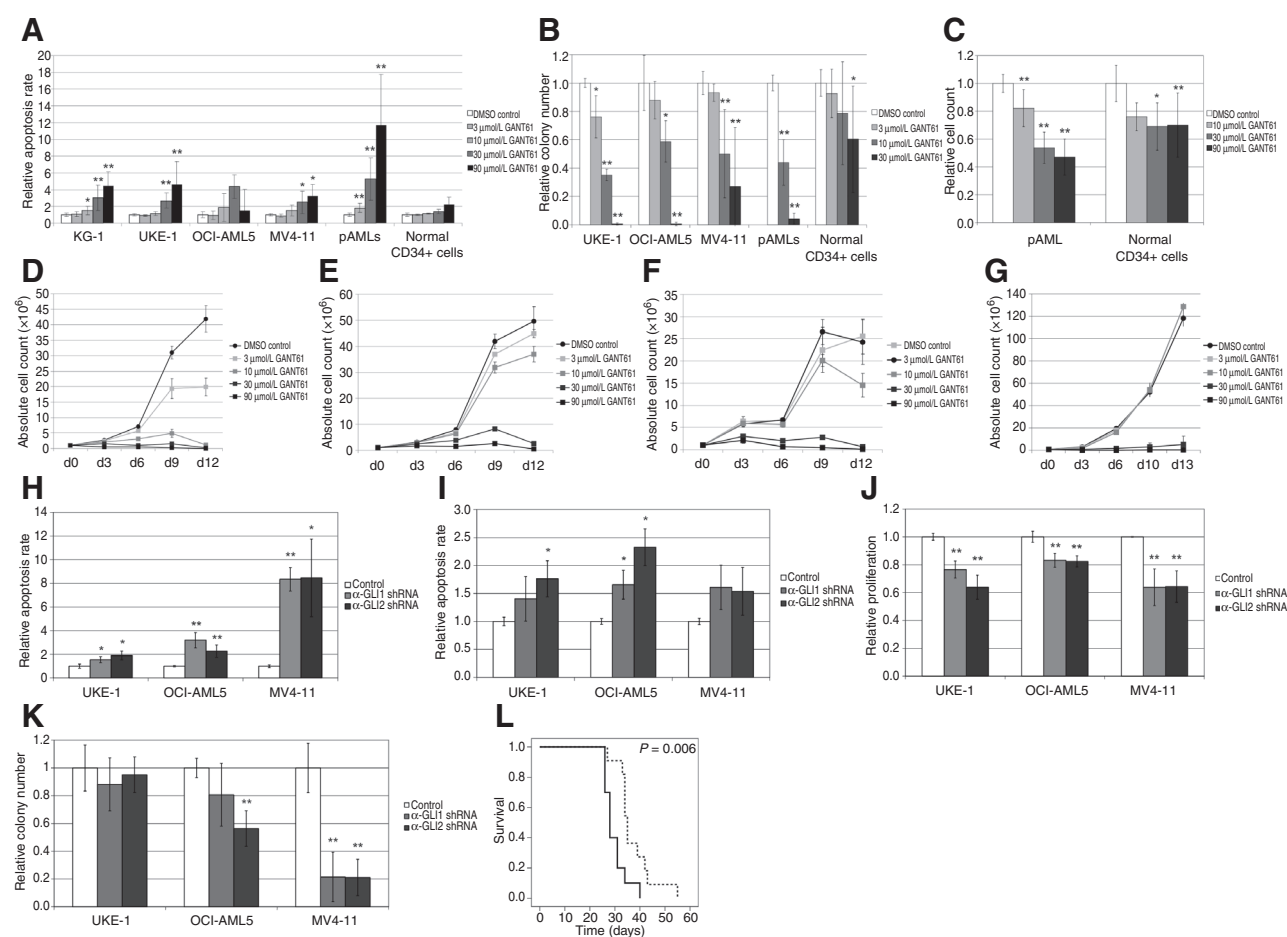
To provide evidence for the endothelial origin of Hedgehog ligands, the mononuclear cell fraction of six AML bone marrow aspirates was enriched for endothelial cells using immunomagnetic-based depletion of CD45<sup>+</sup> cells followed by enrichment of CD146<sup>+</sup> cells. The proportion of CD146<sup>+</sup> cells was determined by flow cytometry before and after immunomagnetic separation, demonstrating an accumulation of endothelial cells ranging from 240- to 1,120-fold (data not shown). The successful enrichment for endothelial cells was additionally confirmed in RT-qPCR analysis by increased expression of endothelial markers Cadherin-5 (CDH5) and CD146 and decreased expression of hematopoietic markers CD45 and CD33 (Supplementary Table S4).

Expression of Hedgehog ligands in the endothelial cell-enriched compartment was compared with expression levels in the whole leukemic MNC fraction before separation using RT-qPCR. DHH mRNA levels were clearly elevated in 4 of 6 AML patients with a 17-, 24-, 29-, and 64-fold relative expression rate, respectively. Expression of *SHH* or *IHH* could not be detected in the samples (Supplementary Table S4).

Furthermore, immunofluorescent staining of the endothelial cell-enriched fraction confirmed DHH protein expression on the endothelial cells (as shown by DHH and CD146 double staining) while virtually no DHH-positive cells were detected in the whole mononuclear cell fraction (Fig. 2C).

#### Elevated DHH plasma levels in AML patients

To investigate DHH protein expression in the circulation of AML patients, plasma samples of 17 AML patients and 23



**Figure 3.**

GANT61 and *GLI* shRNA induce apoptosis and growth inhibition in AML cells *in vitro* and *in vivo*. A, apoptosis induction was measured using Annexin-V and propidium iodide staining in flow cytometry 24 hours after GANT61 treatment in AML cell lines, primary AML samples ( $n = 10$ ), and CD34<sup>+</sup> hematopoietic progenitor cells from healthy donors ( $n = 3$ ). B, the clonogenicity of AML cell lines, primary AML cells ( $n = 4$ ), and CD34<sup>+</sup> progenitor cells isolated from healthy donors ( $n = 4$ ) under GANT61 treatment was investigated in colony formation assays using methylcellulose-based semi-solid medium. C, cell growth of primary AML cells ( $n = 10$  patients) and CD34<sup>+</sup> hematopoietic progenitor cells from healthy donors ( $n = 4$ ) was determined using the ViCell cell counter 3 days after GANT61 treatment. D–G, effects of GANT61 treatment on AML cell lines KG-1 (D), UKE-1 (E), OCI-AML5 (F), and MV4-11 (G) were analyzed for proliferation over 2 weeks. H–K, AML cells transduced with shRNA against *GLI1* and *GLI2* were analyzed in comparison with control cells transduced with a scrambled shRNA with regard to apoptosis induction measured by Annexin-V propidium iodide staining in flow cytometry on days 3 (H) and 7 (I) after transduction, cell growth over 3 days using the ViCell cell counter (J), and colony formation in semi-solid medium (K). L, the Kaplan-Meier survival curve of NSG mice transplanted with MV4-11 GLI1/2 double knockdown cells (dashed line) compared with mice transplanted with the MV4-11 control cells transduced with scrambled shRNA (continuous line). All data presented as mean  $\pm$  standard deviation; \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  in the Welch *t* test.

healthy controls were analyzed for DHH concentrations using a commercial human DHH ELISA. With a mean concentration of 1,342 pg/mL, the DHH plasma levels were significantly elevated compared with those of healthy donors with a mean DHH level of 298 pg/mL ( $P = 0.002$ ; Fig. 2D).

#### Inhibition of GLI by the small-molecule inhibitor GANT61

The AML cell lines KG-1, OCI-AML5, UKE-1, and MV4-11, as well as freshly isolated primary AML cells from 10 patients (bone marrow aspirates from 6 and peripheral blood from 4 patients), were investigated for effects of GANT61 treatment on cell viability and growth as well as colony formation. RT-qPCR analysis revealed that all analyzed cell lines and primary AML samples showed mRNA expression of *GLI1* while *GLI2* was detected in all

four cell lines and in five of 10 primary AML samples (data not shown).

GANT61 significantly and dose-dependently induced apoptosis in all four cell lines and primary AML cells investigated ( $n = 10$ ; six bone marrow and four peripheral blood samples; Fig. 3A). In proliferation assays of AML cell lines, the number of viable cells was decreased below 10% after 2 weeks of treatment with GANT61 at 10  $\mu$ mol/L (KG-1 cells), 30  $\mu$ mol/L (UKE-1, OCI-AML5, and MV4-11 cells) compared with the DMSO control (Fig. 3D–G). Cell counts of primary AML cells ( $n = 9$  patients, six bone marrow and three peripheral blood samples) were significantly and dose-dependently reduced upon treatment with GANT61 after 3 days (Fig. 3C).

Moreover, the impact of GANT61 on colony formation was investigated. The AML cell lines OCI-AML5, MV4-11, and UKE-1,

as well as primary AML cells, were treated with different concentrations of GANT61. Colony formation was observed in 4 of 9 AML patients (two bone marrow and peripheral blood samples, respectively; *GLI2* expression was detected in two of the samples). GANT61 mediated a strong and significant inhibitory impact on the colony formation capacity of AML cell lines and primary AML cells (Fig. 3B).

The effect of GANT61 on primary AML samples was independent of sample origin (bone marrow vs. peripheral blood).

To investigate GANT61's effect on normal hematopoiesis, apoptosis, proliferation, and colony formation of CD34<sup>+</sup> progenitor cells isolated from healthy donors was analyzed. Weak mRNA expression of *GLI1* and *GLI2* was detected in all CD34<sup>+</sup> samples using quantitative RT-PCR. Importantly, compared with AML cells, the effect of GANT61 on CD34<sup>+</sup> progenitor cells isolated from healthy donors was less pronounced in all assays (Fig. 3A–C).

#### Targeted inhibition of GLI using shRNA

To investigate whether the antileukemic effects mediated by GANT61 were due to specific inhibition of *GLI*, we performed targeted knockdown of *GLI* in the AML cell lines, UKE-1 and OCI-AML5, using shRNA against *GLI2* and *GLI1*. In parallel to functional assays, the knockdown efficiency was analyzed in RT-qPCR analysis. Compared with the control, the remaining *GLI2* and *GLI1* expression ranged from 13% to 50% and 39% to 55% in UKE-1 cells, from 9% to 47% and 31% to 53% in OCI-AML5 cells and from 18% to 62% and 31% to 38% in MV4-11 cells, respectively (data not shown). Furthermore, the mRNA expression of *GLI* target gene *BCL2* was also reduced in the *GLI* knockdown cells compared with their scrambled control counterparts (Supplementary Fig. S6).

Knockdown of *GLI2* as well as *GLI1* resulted in a significantly elevated induction of apoptosis and significantly reduced proliferation and colony formation in all three AML cell lines (Fig. 3H–K).

#### Targeted inhibition of GLI1/2 in a leukemic mouse model

To investigate *GLI*'s role *in vivo*, NSG mice were transplanted with *GLI1* and *GLI2* double knockdown MV4-11 cells. As a control, RT-qPCR analysis of the *in vitro* cultured cells was done to ensure a stable knockdown of *GLI*, *GLI2*, and *GLI* target gene *BCL2*, which was confirmed over a period of 28 days. Compared with mice transplanted with MV4-11 cells transduced with a scrambled shRNA, the *GLI1/2* knockdown mice survived significantly longer (median survival of 35 vs. 30 days, respectively;  $P = 0.006$ ; Fig. 3L).

## Discussion

In this study, samples from 104 patients enrolled into the AMLSG 07-04 study of the Austro-German AMLSG Study Group were analyzed for mRNA expression of Hedgehog family members by RT-qPCR. We show that HH receptors, *PTCH1* and *SMO*, as well as downstream transcription factors, *GLI1*, *GLI2*, and *GLI3*, are expressed in a part of AML patients, whereas no expression of HH ligands could be observed. To elucidate the prognostic value of the gene expression of Hedgehog family members, univariate analysis with baseline clinical variables was performed. For *GLI2* expression, a significant negative impact on EFS, RFS, as well as OS of AML patients, was

established. Moreover, investigating the microarray-based gene expression data of a second, independent AML patient cohort of 290 patients confirmed the prognostic impact of *GLI2*. These findings underline the robustness of our findings because they were confirmed not only in two independent cohorts but also across two platforms of gene expression analysis namely RT-qPCR and array-based techniques. Furthermore, within this second cohort, the expression of *GLI1* had also a negative impact on the survival of AML patients underlining *GLI*'s importance in AML biology for AML patients of younger age.

Interestingly, in both patient cohorts, a correlation between *GLI2* expression and *FLT3* mutational status was observed both conveying a negative prognosis to patients. Although much work has to be done to elucidate the interaction between both pathways, cooperation between the Hedgehog and receptor tyrosine kinase pathways, for example, for epidermal growth factor receptor, has been described in malignant transformation (13).

Because no expression of ligands *DHH*, *SHH*, or *IHH* could be detected in AML cells, HH activation in AML cells might be due to a paracrine interaction between leukemic and bone marrow stromal cells. In our hands, primary endothelial cells as well as osteoblasts showed mRNA and protein expression of HH ligands. Importantly, compared with the whole mononuclear leukemic cell fraction, the endothelial cell-enriched compartment of AML bone marrow samples showed markedly elevated *DHH* mRNA levels and protein expression supporting the concept of Hedgehog ligand provision by bone marrow niche cells. In addition, the *DHH* plasma levels of AML patients were significantly increased compared with healthy donors underlining the importance of HH ligands for activation of the pathway.

The contribution of paracrine Hedgehog signaling mediated by bone marrow stromal cells could be shown for several hematologic malignancies. Dierks and colleagues (14) revealed that the *in vitro* survival of lymphoma and multiple myeloma cells was, in part, dependent on secretion of HH ligands by stromal cells. Hedge and colleagues showed that stroma-induced HH signaling could increase the survival of B-cell chronic lymphocytic leukemia (B-CLL) cells, whereas treatment of the coculture with the HH inhibitor cyclopamine abrogated the stroma-induced prosurvival effects. Furthermore, gene expression profiling of primary B-CLL cells revealed that HH signaling molecules, including *GLI1* and *GLI2*, were increased and correlated with the patients' disease progression (15).

Dysregulated HH signaling plays a role in many cancers of which basal cell carcinoma is the most prominent because mutations in HH family members are found in virtually all patients with metastasized tumors (16, 17). As in our study, the dysregulated HH signaling is often mirrored by increased expression of downstream transcription factors *GLI* (18, 19). In line with our data, a negative prognostic impact of *GLI2* expression was very recently shown for gastric, hepatocellular and oral squamous cell cancer (20–22).

The efficacy of HH signaling inhibition, especially the inhibition of receptor *SMO*, was analyzed for a number of cancer entities including basal cell carcinoma, medulloblastoma, pancreatic cancer, and lung cancer in preclinical *in vitro* and *in vivo* models (20, 23–25). Interestingly, one mechanism responsible for resistance to *SMO* inhibitors was chromosomal amplification of *GLI2*, underlining the importance of this transcription



factor for tumor growth (26). The specific *GLI1* and *GLI2* inhibitor GANT61 mediated strong antitumor effects *in vitro* and in several murine xenograft models including neuroblastoma, pancreatic, prostate, and hepatocellular cancer (20, 27–29). Notably, several studies observed superior anticancer efficacy of GANT61 compared with Smo inhibitors cyclopamine or GDC-0449 (30–34). GANT61 is a highly selective inhibitor interfering with DNA binding of both *GLI1* and *GLI2* without having influence on pathways such as Ras–Raf–Mek–Mapk, TNF signaling/NF- $\kappa$ B, or C/EBP $\alpha$  (27, 35).

Our data on AML cells, including primary cells from 10 AML patients, reveal that GANT61 leads to significant induction of apoptosis, reduced growth, and more importantly, diminished colony formation. This is in line with the publication by Pan and colleagues (33) who observed increased apoptosis in several AML cell lines upon GANT61 treatment. Furthermore, the concentrations of GANT61 used in our experiments correspond to commonly used ranges published by others (27, 29, 32, 33).

Furthermore, we observed comparable effects through targeted knockdown of *GLI2* and *GLI1* using shRNA in AML cell lines. Both the knockdown of *GLI2* as well as *GLI1* led to a significant induction of apoptosis and significantly reduced cell growth and colony formation of AML cells. Moreover, the double knockdown of *GLI1/2* significantly prolonged the survival in a leukemic mouse model, underlining the importance of *GLI1* and *GLI2* for the survival of AML cells.

These data reveal that both *GLI2* and *GLI1* seem to be involved in the pathogenesis of AML and therefore the antileukemic effects of GANT61 are likely to be mediated by both *GLI2* and *GLI1* as both factors are frequently coexpressed. Concordantly, while all AML cells lines and half of primary AML samples that we treated with GANT61 showed expression of both *GLI1* and *GLI2*, the five primary AML samples only expressing *GLI1* but not *GLI2* responded also to GANT61 treatment.

Our observation that the effect of GANT61 on the colony formation capacity of normal hematopoietic progenitor cells was less pronounced than on AML cells, might open a therapeutic window for *GLI* inhibition in patients. The reduced sensitivity may be due to the fact that hematopoietic progenitor cells dispose lower *GLI1* and *GLI2* expression levels compared with AML cells as observed in our own data and also published by others (36). Furthermore, these observations are in line with data published on B-CLL where normal B lymphocytes showed less sensitivity toward GANT61 treatment than primary CLL cells (30).

Although the importance of HH signaling in adult hematopoiesis is discussed controversially, several studies indicate an essential role for HH signaling in leukemia stem-cell maintenance supporting our data. In a BCR-ABL–induced CML mouse model, combined treatment with the SMO inhibitor cyclopamine and the BCR-ABL inhibitor nilotinib resulted in a reduced number of LSCs and prolonged survival of mice (14). Another study on CML revealed that the dormant human blast crisis LSCs entered the cell cycle resulting in reduced blast crisis LSC survival upon treatment with the small-molecule inhibitor PF-04449913 in a xenograft mouse model (37). Studying AML, Kobune and colleagues (38) revealed that combination of cytarabine with cyclopamine significantly reduced the drug resistance of AML cell lines and primary CD34<sup>+</sup> AML cells to cytarabine. Very recently, Liu and colleagues (39) demonstrated autocrine activation of the HH pathway in myeloma and that combinational treatment with

chemotherapy and a HH signaling inhibitor displayed synergistic antimyeloma effects.

Because of the importance of dysregulated HH signaling in basal cell carcinomas, first clinical trials using HH inhibitors were conducted with basal cell carcinoma patients (40, 41). Furthermore, first signs of clinical efficacy have been observed in solid tumors such as medulloblastoma, pancreatic cancer, or sarcomas (42–44).

In a recently published phase I dose-escalation study, 35 patients with hematologic malignancies (including 20 AML patients) were treated with the small-molecule SMO inhibitor PF-04449913 as a single agent. The treatment was well tolerated and early signs of clinical benefit have been observed in patients with different hematologic diseases. Thirty percent of AML patients had more than a 50% reduction in bone marrow blast counts with 1 patient achieving a CR with incomplete blood count recovery (45). On the basis of these encouraging results, an international, multicenter, three-armed study of PF-04449913 in combination with chemotherapeutic agents was initiated in patients with AML or myelodysplastic syndrome (NCT01546038).

As already mentioned, in some studies, the side-by-side comparison of SMO inhibitors to GANT61 revealed that direct *GLI* inhibition mediated stronger antitumor effects than blocking the Hedgehog cascade at the receptor level (30–34). Most likely, this observation was due to the fact that *GLI* transcription factors can additionally be activated through several noncanonical pathways including TGF $\beta$ , Ras, or BET bromodomain proteins (46–48), emphasizing that the development of clinically applicable *GLI* inhibitors might represent a reasonable alternative to SMO inhibitors.

In conclusion, *GLI* expression is a negative prognostic factor in AML that mirrors the activation state of Hedgehog signaling in AML blasts that is presumably dependant on paracrine Hedgehog signals. Inhibition of Hedgehog signaling using a specific *GLI* inhibitor or targeting *GLI* by shRNA showed effective antileukemic properties *in vitro*, underlining that *GLI* inhibition might represent a promising target for future therapies. The observation that *GLI2* is preferentially expressed in patients with a mutated *FLT3* might help to preselect patients for upcoming clinical trials.

### Disclosure of Potential Conflicts of Interest

W. Fiedler is a consultant/advisory board member for and reports receiving commercial research grants from Novartis and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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