

## Original Article

# Activation of hedgehog pathway in acute myeloid leukemia patients

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**Abstract:** Hedgehog (Hh) signaling pathway relates with a variety of tumors-related diseases. To examine whether Hh signaling has a role in acute myeloid leukemia (AML), twenty cases of AML patients were chosen, and the transcript levels of Hh and its receptors between untreated group and minimal residual disease (MRD)(-) group in patients with AML were compared. We also compared the transcript levels of Hh and its receptors in patients with AML between MRD(-) and relapse group. We found that relative expression levels of Shh, Smo, and Gli1 mRNA in untreated group and relapse group were significantly higher than those in normal control group and MRD(-) group, while the level of Ptch mRNA did not show significant difference. Our results suggested that there was inappropriate activation of the Hh signaling pathway in AML patients, Hh signaling could be an essential requirement in AML, and inhibition of Hh signaling maybe a new treatment for AML patients.

**Keywords:** Hedgehog, AML, Shh, Ptch, Smo, Gli1

### Introduction

As the most common acute leukemia in adults, AML is often resistant to conventional therapies. While numerous gene mutations and the differential expression of leukemia-associated genes have been identified [1], and new molecularly targeted therapies are needed to improve the prognosis and treatment of AML more generally. Of these pathways, Hh signaling is considered as a therapeutic target for myeloid malignancies [2].

In hematopoiesis, it is shown that Hh family members play an important role in regulation of stem/progenitor cell expansion in vitro and in vivo [3]. Three hh proteins of humans and mice, including Sonic (Shh), Indian (Ihh) and Desert (Dhh), are both secreted and membrane anchored, and can act on both nearby and distant cells [4]. In mammals, Shh ligand mediates a pathway through the dual lipid-modified signaling cascade. Shh proteins bind to the receptor patched (Ptch), thereby releasing the latent inhibition of smoothened (Smo), and leading to the activation of Gli1 to Gli3 and downstream

target genes such as Ptch, cyclin D1, and Bcl2 [5], then regulate cell survival, metastasis, and proliferation [6]. Recent studies suggested that Hh signaling contributed to tumor maintenance, growth and resistance to chemotherapy in myeloid leukemia [7]. Inhibition of Hh signaling induced apoptosis and reduced drug-resistance in AML cells [3].

The aim of this study was to show and compare the expression and significance of Hh signaling pathway target genes Shh, Ptch, Smo and Gli1 between untreated AML patients and MRD(-) complete remission (CR) AML patients.

### Materials and methods

#### Samples

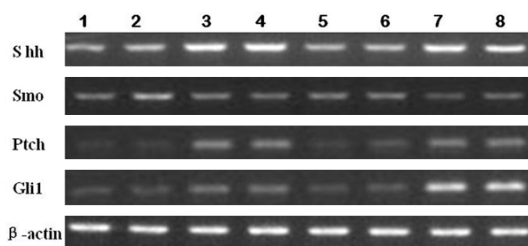
Twenty cases of AML patients treated at the first affiliated hospital of Jinzhou medical university were included in this study from January 2014 to January 2015. The diagnosis of AML was established on the basis of WHO guideline. According to the FAB classification, all patients were divided into five groups: AML-M2, AML-M3,

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**Table 1.** Patients characteristics

Patients characteristic	20
Sex	
Male	9
Female	11
FAB classification	
AML-M2	8
AML-M3	6
AML-M4	2
AML-M5	3
AML-M7	1

Abbreviations: AML-M2: acute myelogenous leukemia with maturation; AML-M3: acute promyelocytic leukemia; AML-M4: acute myelomonocytic leukemia; AML-M5: acute monocytic leukemia; AML-M7: acute megakaryoblastic leukemia.



**Figure 1.** Expression of Hh and its receptors in AML patients and normal control. Lane 1: normal control 1; Lane 2: normal control 2; Lane 3: AML-untreated case 1; Lane 4: AML-untreated case 2; Lane 5: AML-MRD(-) case 1; Lane 6: AML-MRD(-) case 2; Lane 7: AML-Relapse case 1; Lane 8: AML-Relapse case 2.

AML-M4, AML-M5, AML-M7 (see **Table 1**). All patients were received inducing chemotherapy and consolidation chemotherapy. After chemotherapy, all patients' bone marrow reached CR, and MRD were negative (for acute promyeloid leukemia patients were PML/RARa gene negative). This study also included 3 iron-deficiency anemia patients for contraction. Mononuclear cells were obtained by bone marrow aspiration after obtaining informed consent.

### RT-PCR and real-time PCR

Total RNA was extracted using RNeasy mini kit from mononuclear cells according to the manufacturer's instruction (Qiagen). RNA quality was determined by agarose gel electrophoresis and quantified spectroscopically (260 nm) using a Biophotometer (Eppendorf, Hamburg, Germany). RNA (1 µg) was reverse-transcribed by SuperScript™ II (Invitrogen, Takara, Japan).

Reverse transcription (Invitrogen) was done on genomic DNA-free RNA using Random primer (as a primer). The RT-PCR reactions were carried out in GeneAmp Pcr System 9700. The expression of the housekeeping gene  $\beta$ -actin was used as a control.

Real-time PCR was subsequently performed using SYBR Green core PCR reagents (Rotor-Gene 6000) to quantify Shh, Ptch, Smo, Gli1 mRNA steady-state levels. The expression of the housekeeping gene ABL was used as a control. Every sample was repeated three times. The final results were compared by Comparative Delta-delta Ct method. The primer pairs for Shh (5'-CCTCGTGCTGGTATGCTCGGACT-3' and 5'-CTCTGAGTCATCAGCCTGTCGCTC-3'), Ptch (5'-CTGTTGGCATAGGAGTGGAGTTCACC-3' and 5'-CTGCTGGGCCTCGTAGGCCGAAGC-3'), Smo (5'-CAGAACATCAAGTTCAACAGTTCAGGC-3' and 5'-ATAGGTGAGGACCACAACCAAACCACACC-3'), Gli1 (5'-CTCCGAAGGACAGGTATGTAAC-3' and 5'-CCCTACTCTTAGGCACTAGAGTTG-3') and ABL (5'-CGAGAGCCTGGCCTACAACAA-3' and 5'-CTAGCAGCTCATACACTGGGACA-3') were designed and synthesized by Takara company. PCR amplification was carried out using 45 cycles of 95°C for 60 s, 95°C for 10 s and 60°C for 30 s.

### Statistical analysis

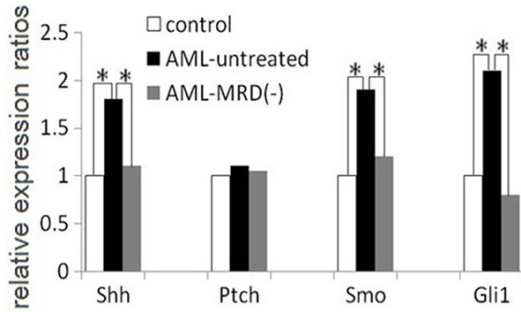
The data are presented as means  $\pm$  SEM. For comparison of three groups (expression of Hh and its receptors between untreated, control and MRD(-) groups), we used one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparison. The differences between the mean values of two groups (comparison of Hh and its receptors expression between MRD(-) groups and relapse groups.) were evaluated by using the Student's t-test (paired comparison). *P* values of 0.05 were considered statistically significant.

## Results

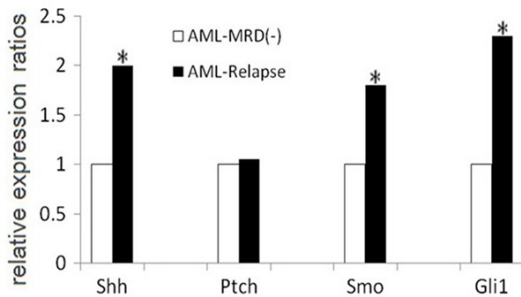
### Hh target gene expression in AML patients and normal controls

We examined expression of Hh and its receptors in AML patients and normal controls by semiquantitative PCR. Shh, Ptch, Smo, Gli1 mRNA can be detected in both AML group and normal control group (see **Figure 1**).

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**Figure 2.** Expression of Hh and its receptors between different groups. \* $P < 0.05$ .



**Figure 3.** Comparison of Hh and its receptors expression between MRD(-) groups and relapse groups. \* $P < 0.05$ .

### Expression of Hh and its receptors between different groups

We compared the transcript levels of Hh and its receptors in patients with AML between untreated group and MRD(-) group. The levels of Shh mRNA in patients of untreated group were obviously higher than normal control and MRD(-) group ( $P < 0.05$ ), but there were no significant differences between normal control and MRD(-) group. Our results also demonstrated elevated Smo expression in patients of untreated group. The relative expression levels of Smo mRNA in untreated group were much higher than normal control and MRD(-) group, but no significant differences were found between normal control and MRD(-) group. Moreover, in all of the cases, increased levels of Shh were consistent with elevated levels of Smo expression. We also found high Gli1 transcripts in patients of untreated group compared with normal control and MRD(-) group, but there were no significant differences of Ptch between these three groups ( $P > 0.05$ ) (see **Figure 2**).

### Comparison of Hh and its receptors expression between MRD(-) groups and relapse groups

We also compared the transcript levels of Hh and its receptors in patients of AML between MRD(-) and relapse group. The levels of Shh mRNA in patients of MRD(-) group were obviously lower than that of relapse group ( $P < 0.05$ ). The relative expression levels of Smo mRNA in relapse group were much higher than in MRD(-) group. Moreover, increased levels of Shh were consistent with elevated levels of Smo expression. We also found high Gli1 transcripts in patients of relapse group compared with the MRD(-) group, but there were no significant differences of Ptch between these two groups ( $P > 0.05$ ) (see **Figure 3**).

### Discussion

As one of the most examined signal pathways, Hh signaling is critical during embryogenesis, cell proliferation, apoptosis, carcinogenesis and acquirement of drug resistance [8]. The Hh signaling leads to activation of Gli, which transcriptionally regulates various target genes that determine the Hh-dependent survival. Gli1 and Gli2 are the activators of Hh signaling, and constitutive activation of at least one of them is critical for cancer development [9]. While inappropriate activated Hh signaling contributes to the development of various cancers [10]. Several mechanisms, such as activating point mutations of Smo or inactivating point mutations in Ptch have been described that lead to the activation of the Hh signaling pathway in tumor cells [11]. But the contribution of Hh signaling pathway in hematologic malignancies has not been thoroughly examined. In consideration of Hh signaling between regulation of hematologic malignancies and proliferation of primitive human hematopoietic cells [12], we examined whether Hh signaling also had a role in AML patients.

Here, we showed that the Hh signaling components Shh, Ptch, Smo and Gli1 expressed in all untreated AML patients with the use of real-time PCR analysis. And the relative expression levels of Shh, Smo, and Gli1 mRNA in untreated AML group were significantly higher than those in normal control group and MRD(-) group; It meant that activation of the Hh pathway was fairly common in untreated AML. But the level

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of Ptch mRNA in untreated AML, normal control group and MRD(-) group did not show significant difference. We repeated this process for several times, but still did not find significant differences. And we also found that the level of Shh, Ptch, Smo and Gli1 between normal control group and MRD(-) group have no significant difference. Furthermore, we tested Shh, Ptch, Smo, Gli1 transcripts in relapse AML patients, we found that the levels of Shh, Smo, Gli1 expression increased significantly comparing with MRD(-) group. But the level of Ptch mRNA in relapse AML group and MRD(-) group also did not show significant difference. From upon results, we could find inappropriate activation of the Hh signaling pathway in untreated and relapse AML group. The expression of Hh signaling components was closely related to the pathogenesis of leukemia. These findings implicated that Hh signaling could be a basal, yet essential requirement in AML, and Hh signaling pathways could provide new targets for the treatment of leukemia.

Since activation of Hh signaling pathway has been shown to have a potential role in leukemia maintenance [13], Toxicity of Hh signaling inhibitors should be our concern. Because of the potential important role of Hh signaling in leukemia, the development of strategies targeting the Hh signaling has attracted a lot of attentions. The major targeting points for Hh signaling inhibitors are Gli protein inhibitors (such as GANT61 and HPI-1), SMO protein inhibitors (such as GDC-0449 and cyclopamine) and Shh neutralizing antibodies [14]. Recent studies reported a correlation between expression levels of Gli1 in tumor samples with disease progression and poor clinical outcome [15]. Higher Gli1 expression also means a worse survival in AML [16]. Hence, Gli1 might be an alternative target for treatment of AML.

In conclusion, our results showed that the Hh signaling pathway was a potential therapeutic target for patients with AML. Inhibition of Hh signaling could represent a new treatment possibility for AML patients.

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### Disclosure of conflict of interest

None.

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