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Original Article

Smo expression at diagnosis predicts prognosis in resected gastrointestinal stromal tumor patients and correlates to imatinib-resistance

Xiaosong Wang[1], Lin Tu[2], Yeqian Zhang[2], Yanying Shen[2], Hui Cao[2], Peiwu Yu[1]

[1]*Department of General Surgery, The First Hospital Affiliated to Army Medical University, Chongqing 400038, China;* [2]*Department of Gastrointestinal Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China*

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Abstract: Objective: The aim of this study was to investigate the prognostic value and role of imatinib-resistance due to the hedgehog signaling pathway in resected gastrointestinal stromal tumor (GIST) patients. Methods: The expression levels of Smoothened (Smo), Patched1 (Ptch1) and Gli1-3 were assessed in 12 resected GIST specimens by microarray, and were further verified in 24 Formalin-Fixed and Paraffin-Embedded (FFPE) tissues using RT-PCR. We also assessed the expression levels in 322 resected GIST specimens by using immunohistochemistry. The prognostic value of Smo, Ptch1 and Gli1-3 were investigated by overall survival (OS) and disease-free survival (DFS). The Smo, Ptch1 and Gli1-3 expression levels in GIST cells were evaluated in imatinib-resistant cell lines to verify their relationship with imatinib-resistance. Results: Higher expression levels of Ptch1 protein and lower expression levels Smo protein predict a better OS and DFS. Additionally, the Smo expression in imatinib-resistant cell lines was significantly higher than in imatinib-sensitive cell lines. The drug resistance decreased after interference of Smo in imatinib-resistant cell lines. Conclusions: Lower Smo expression and higher expression of Ptch1 at diagnosis predicts a better OS and DFS, and the high expression of Smo protein contributes to imatinib-resistance in GIST cells.

Keywords: Smo expression, gastrointestinal stromal tumor, imatinib-resistance

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor originating in the gastrointestinal tract, and the estimated incidence is 10-20/1,000,000 annually [1-3]. Small GISTs are usually detected during surgery for other conditions, gastroscopy or ultrasound, and GISTs may present with bleeding, perforation, obstruction or a combination of these symptoms; which are always considered high risk for relapse or metastases [4, 5]. Although the treatment of choice for localized GISTs is complete surgical excision, GISTs often relapse locally, spreads diffusely throughout the serosal surfaces of the abdomen, and/or metastasizes to the liver [6]. Targeted therapy with imatinib has been used for advanced or unresectable GISTs [7, 8]. However, there are still many patients with advanced or unresectable GISTs that are resistant to existing targeted medicines, and there is no biochemical index to evaluate the prognosis. Several recent studies reported a relationship between GIST and the hedgehog pathway [9-11], which may provide some help.

The Hedgehog (Hh) gene was first identified in *Drosophila melanogaster* in 1980. The encoded protein plays important roles in embryonic development, and its function is highly conserved from *Drosophila* to insects and mammals [12]. Hedgehog has three mammalian counterparts; Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). The Shh signaling pathway is activated by Shh binding to the Patched (Ptch)-Smoothened (Smo) membrane-receptor complex, and the pathway is regulated by kinesin-like protein Costal2 (Cos2), serine/threonine kinase Fused (Fu) and the suppressor of fused (Sufu). SMO promotes the nuclear translocation of the GLI family of transcription factors (Gli1, Gli2, and Gli3) that subsequently activate target gene expression, while Ptch plays the opposite role [13-15].

Many normal adult tissues exhibit little Hh activity. Hh signaling is usually activated in tissue development, repair, and tumorigenesis [16-18]. Hh signaling proteins are involved in cell growth, proliferation, invasion and metastases for many types of carcinomas [19, 20]. However, studies examining the relationship between Hh signaling and gastrointestinal stromal tumors are rare. In intestinal stromal tumor specimens, Ptch, Smo and Gli1 stain positive by immunohistochemistry, and Hh signaling may play an important role in the myogenic differentiation and malignant potential of human intestinal stromal tumors [11]. In another study, researchers created the GIST882 cell line by exposure to GANT61, which is a Gli small-molecule agonist, to assess the effect of hedgehog signaling inhibition. The mRNA levels of Gli1, Gli2, Gli3, and Ptch1 were significantly downregulated by treatment. After 72 hours of treatment with GANT61, the calculated dose response curve showed an important inhibition of cell viability, and this effect was accompanied by a significant downregulation of KIT [9]. It is known that a majority of GISTs originate from KIT gene mutations, which suggests that Hh signaling may be involved in cell transformation of GISTs. Moreover, the inactivation of Ptch results in the formation of Pdgfrα-positive Kit-negative GIST-like tumors in mice that develop due to cooperation between the Hh and Pdgfrα pathways from Kit-negative cells in the intestine [10]. The purpose of this study is to determine whether Gli1-3, Ptch and Smo expression within tumors is related to patient clinical outcome (prognostic value) and to assess the benefit of adjuvant therapy (predictive value) in patients with GISTs who underwent curative surgery.

Patients and methods

Patients and specimens

This study examined a cohort of 322 patients diagnosed with GIST. All subjects underwent curative surgery in a single center (Renji Hospital School of Medicine Shanghai Jiao Tong University) from January 1, 2003 to December 31, 2012. Patients who died of postoperative complications or other diseases following the surgery were excluded.

We chose 4 low-risk patients, 4 intermediate-risk patients and 4 high-risk patients (modified NIH classification system) [21] from all 322 patients to assess the expression of Smo, Ptch and Gli1-3 with microarray. Additionally, we selected 8 low-risk patients, 8 intermediate-risk patients, and 8 high-risk patients to verify the expression of Smo, Ptch1 and Gli1-3 with RT-PCR.

Immunohistochemistry scoring

TMA slide images were captured as high-resolution digital files. The evaluation of staining was performed independently by one specialized GIST pathologist (Yanying Shen) and 2 researchers experienced in GIST histopathology (Lin Tu and Xiaosong Wang). Discrepancies were resolved by consensus after conferencing. The percentage of stained cells, cellular staining localization (nuclear for Gli1-3, membranous for Smo and Ptch1) and staining intensities (0-2) were assigned (**Figure 1**). The scoring was conducted according to the ratio and intensity of positive-staining cells. Low staining was defined as staining intensity 0 if no staining was observed, and moderate staining was defined as staining intensity 1 if > 25% of the tumor cells had weak or moderate staining intensity. High staining was defined as staining intensity 2 if the tumor cells had strong staining intensity.

Ethics

The study protocol was approved by the Institutional Review Board of Renji Hospital, School of Medicine, Shanghai Jiao Tong University. Written informed consent was obtained from all chosen patients.

Statistical analysis

The statistical analyses were performed using SPSS (version 22.0) and GraphPad Prism 5 software. Overall survival (OS) was measured as the time from the date of surgery to the time of last follow-up or death of any cause. Disease-free survival (DFS) was defined as the time from surgery to the first non-disease-free event (local or distant disease relapse or death). The follow-up period was calculated using the reverse Kaplan-Meier method. A t-test was used to compare continuous variables. The median survival was estimated using the Kaplan-Meier method, and the difference was tested using the log-rank test. The analyzed clinicopathologic variables with a P value <

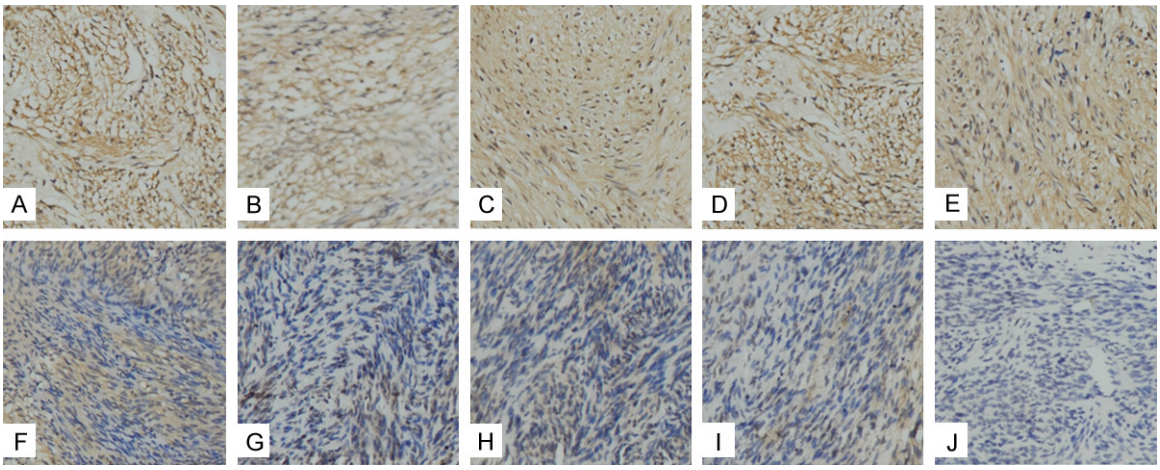


Figure 1. The high expression level (A-E) and low expression level (F-J) of Ptch1 (A, F), Smo (B, G), Gli1 (C, H), Gli2 (D, I) and Gli3 (E, J).

0.05 by log-rank test were entered into the Cox proportional hazards multivariate analysis.

The predictive effect of the immunohistochemical (IHC) factors was examined by assessing the interactions between targeted therapy and the factors Smo, Ptch1 and Gli1-3. This was done for each of these factors and included the respective factor and separate treatment effects for both values (low and high) of the interesting factor, respectively. The interaction tests between each IHC factor and the administration of targeted therapy were performed using the Cox model to calculate the HRs with 95% confidence intervals (CI).

Result

Case characteristics

There were 322 patients assessed for eligibility and considered for this study. These patients consisted of 172 men and 150 women. The median age at diagnosis was 58 years old. The median follow-up was 41 months (8-106 months). The median DFS and OS were not reached for the relatively good prognoses of GIST. The patients were not treated with the targeted imatinib therapy before surgery.

Expression level of hedgehog-related proteins were altered in different GIST risk groups

The results of the analysis of 12 cases of GIST (4 cases of low risk, 4 cases of intermediate risk, and 4 cases of high risk) showed that hedgehog-related proteins (Gli1-3, Smo and Ptch1) were in the GIST specimens (**Figure 2A1**). In addition, the expression levels of Ptch1, Smo and Gli3 in GIST patients were slightly higher than the levels of Gli1 and Gli2.

The expression of Gli1-3 and Smo in high risk GIST was significantly higher than in the low risk GIST group (**Figure 2A2-A4**, **2A6**) when compared with the expression of different hedgehog-related proteins in different risk groups for GIST. The expression of Ptch1 showed the opposite trend and in high-risk group for GIST the expression was significantly reduced (**Figure 2A5**).

Verification of the expression of hedgehog-related proteins in GIST specimens

The results showed that the expression of five hedgehog-related proteins in 12 different risk specimens were different. In this case, we expanded the sample size and extracted 24 cases (8 cases were low risk, 8 cases were at risk, and 8 cases were high risk) for GIST FFPE tissue to test the Smo, Ptch1, Gli1-3 expression. The results showed the expression of Ptch1 was similar to that of Gli1-3 and Smo. The expression level of Ptch1 was lower in the low risk, group and the difference was statistically significant (**Figure 2B1-B5**).

High Smo expression and low Ptch1 expression indicated worse prognosis in GIST

The immunohistochemical analysis of hedgehog-related proteins in 322 cases of GIST specimens showed the following were independent prognostic indicators: tumor local invasion, NIH classification, tumor site, tumor size, mitotic

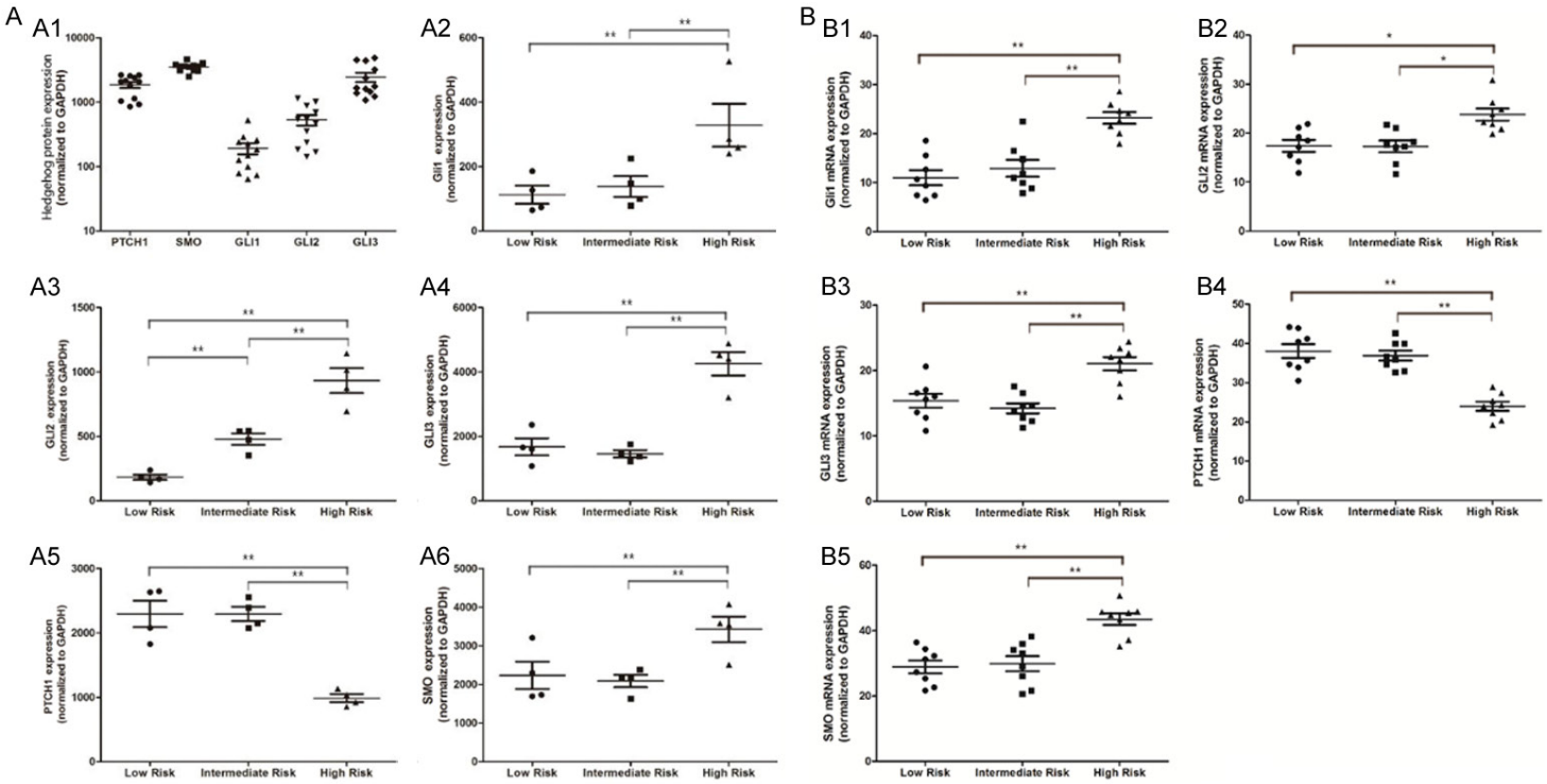
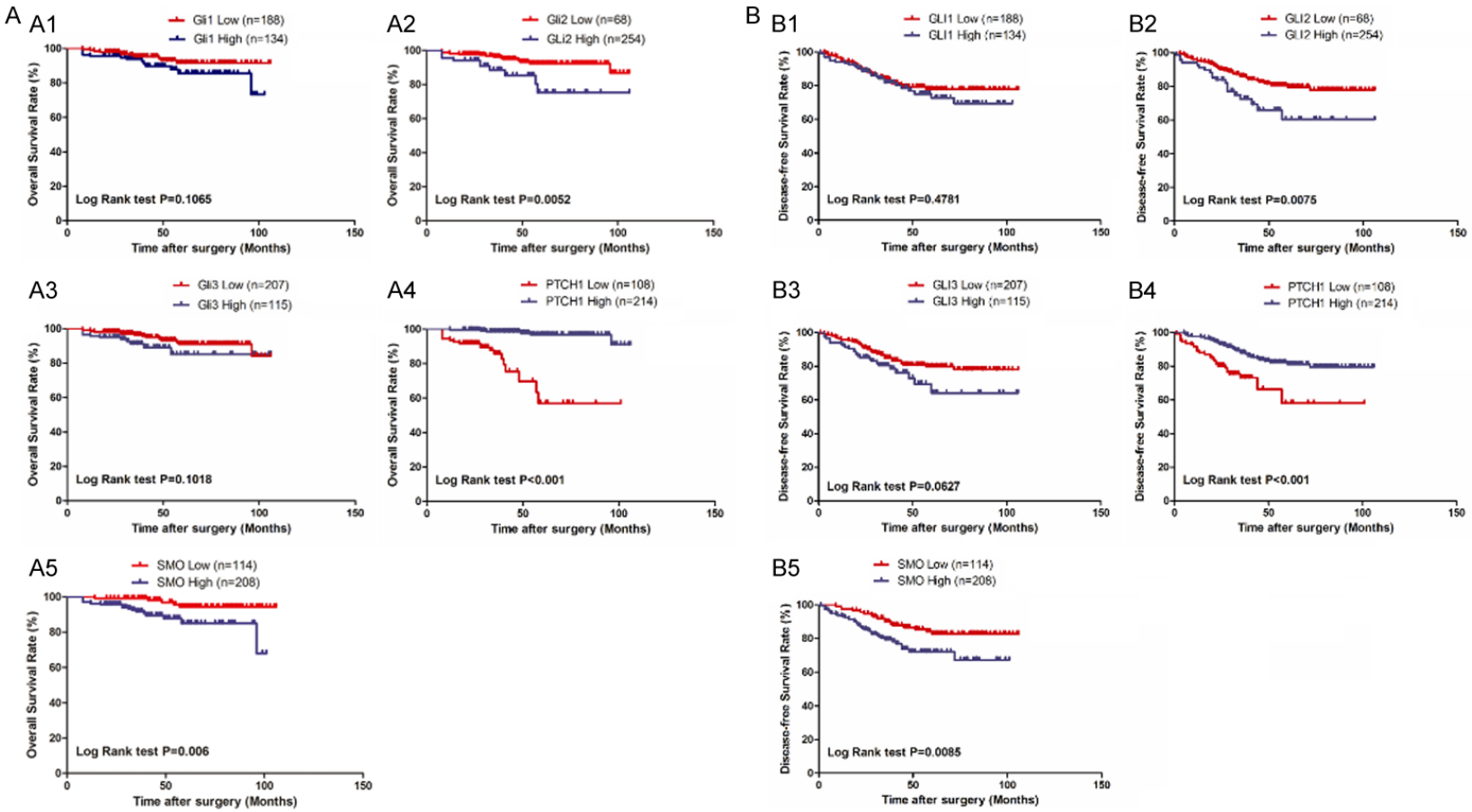


Figure 2. The result of TMA and PCR. A1: The expression level of hedgehog proteins in 12 GISTs. A2: The expression level of Gli1 in different risk of GISTs. A3: The expression level of Gli2 in different risk of GISTs. A4: The expression level of Gli3 in different risk of GISTs. A5: The expression level of Ptch1 in different risk of GISTs. A6. The expression level of Smo in different risk of GISTs. B1: The expression level of Gli1 in different risk of GISTs. B2: The expression level of Gli2 in different risk of GISTs. B3: The expression level of Gli3 in different risk of GISTs. B4: The expression level of Ptch1 in different risk of GISTs. B5: The expression level of Smo in different risk of GISTs (*P<0.05, **P<0.01).

Table 1. Patients characteristics and univariate analyses of survival prediction

Variable	n (%)	Median OS (months)	Log-rank *p* value	Median DFS (months)	Log-rank *p* value
Gender		Not reached	=0.056	Not reached	=0.016
Male	172 (53.4)				
Female	150 (46.6)				
Age		Not reached	=0.486	Not reached	=0.244
≤58	67 (20.81)				
>58	255 (79.19)				
Local invasion		Not reached	<0.001		<0.001
Yes	51 (15.84)			35.00	
No	271 (84.16)			Not reached	
NIH classification		Not reached	<0.001		<0.001
I	25 (7.76)				
II	110 (34.16)				
III	48 (14.91)				
IV	139 (43.17)			72.00	
Location		Not reached	=0.375		<0.001
Stomach	173 (53.73)				
Small bowel	106 (32.92)				
Colon	16 (4.97)				
Others	27 (8.38)			60.00	
Tumor size			<0.001		<0.001
≤2 cm	29 (9.01)				
2.1~5 cm	119 (36.96)				
5.1~10 cm	117 (36.34)				
>10 cm	57 (17.69)	96.00		48.00	
Mitotic count			<0.001		<0.001
<5/50 HPF	236 (73.29)				
6~10/50 HPF	46 (14.29)				
>10/50 HPF	40 (12.42)	58.00		28.00	
Gli1		Not reached	=0.106	Not reached	=0.478
Low	188 (58.39)				
High	134 (41.61)				
Gli2		Not reached	=0.005	Not reached	=0.008
Low	254 (78.88)				
High	68 (21.12)				
Gli3		Not reached	=0.108	Not reached	=0.063
Low	207 (64.29)				
High	155 (35.71)				
Smo		Not reached	=0.006	Not reached	=0.008
Low	114 (35.40)				
High	208 (64.60)				
Ptch1		Not reached	<0.001	Not reached	<0.001
Low	108 (33.54)				
High	214 (66.46)				

count, Gli2, Ptch1, and Smo expression (**Table 1**; **Figure 3A**, **3B**). We performed multivariate Cox regression tests (backwards) for the independent prognostic factors of the single factor analysis and the results showed the OS (P<0.001) and DFS (P=0.005) of the low Smo expression group and the high Ptch1 expression group were significantly higher than those


A
A1
Gli1 Low (n=188)
Gli1 High (n=134)
Overall Survival Rate (%)
Log Rank test P=0.1065
Time after surgery (Months)
A2
Gli2 Low (n=68)
Gli2 High (n=254)
Overall Survival Rate (%)
Log Rank test P=0.0052
Time after surgery (Months)
B
B1
GLI1 Low (n=188)
GLI1 High (n=134)
Disease-free Survival Rate (%)
Log Rank test P=0.4781
Time after surgery (Months)
B2
GLI2 Low (n=68)
GLI2 High (n=254)
Disease-free Survival Rate (%)
Log Rank test P=0.0075
Time after surgery (Months)
A3
Gli3 Low (n=207)
Gli3 High (n=115)
Overall Survival Rate (%)
Log Rank test P=0.1018
Time after surgery (Months)
A4
PTCH1 Low (n=108)
PTCH1 High (n=214)
Overall Survival Rate (%)
Log Rank test P<0.001
Time after surgery (Months)
B3
GLI3 Low (n=207)
GLI3 High (n=115)
Disease-free Survival Rate (%)
Log Rank test P=0.0627
Time after surgery (Months)
B4
PTCH1 Low (n=108)
PTCH1 High (n=214)
Disease-free Survival Rate (%)
Log Rank test P<0.001
Time after surgery (Months)
A5
SMO Low (n=114)
SMO High (n=208)
Overall Survival Rate (%)
Log Rank test P=0.006
Time after surgery (Months)
B5
SMO Low (n=114)
SMO High (n=208)
Disease-free Survival Rate (%)
Log Rank test P=0.0085
Time after surgery (Months)

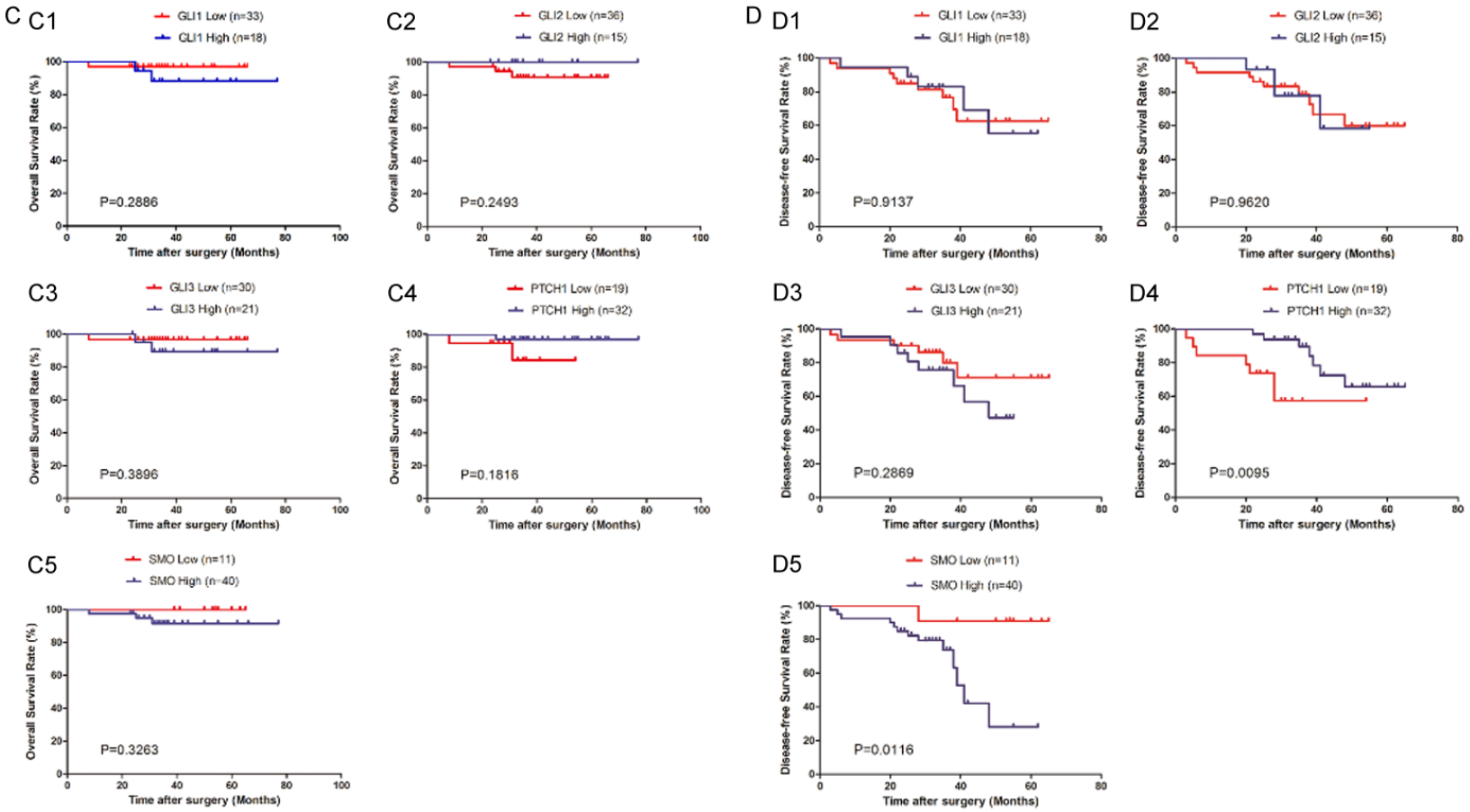


Figure 3. A. The relationship between expression level of Hedgehog proteins and OS. B. The relationship between expression level of Hedgehog proteins and DFS. C. The relationship between expression level of Hedgehog proteins and OS in patients received imatinib therapy. D. The relationship between expression level of Hedgehog proteins and DFS in patients received imatinib therapy.

Table 2. Multivariate Cox regression analyses of overall and disease-free survival

	OS		DFS	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Local invasion	1.775 (0.741-4.256)	0.198	1.880 (1.0846-3.260)	0.025
NIH classification	0.991 (0.231-4.245)	0.990	2.968 (1.447-6.088)	0.003
Tumor size	4.877 (1.870-12.72)	0.001	1.356 (0.866-2.123)	0.183
Mitotic count	2.448 (1.297-4.624)	0.006	1.907 (1.356-2.683)	<0.001
High Gli2	1.060 (0.426-2.636)	0.901	1.055 (0.597-1.864)	0.854
High Smo	4.455 (1.483-13.38)	0.008	2.464 (1.334-4.549)	0.004
High Ptch1	0.090 (0.030-0.272)	<0.001	0.511 (0.299-0.872)	0.014

of GIST (P<0.05). The results showed that Ptch1 and Smo were independent prognostic factors (P<0.05) (**Table 2**).

High Ptch1 expression and low Smo expression indicated better imatinib sensitivity for GIST patients

In the 322 patients with follow-up, 51 patients were treated with imatinib. Our previous experiments showed that Ptch1 and Smo were associated with the prognosis of GIST. Therefore, we next investigated whether they were associated with imatinib response. The results showed that there was no significant difference in Gli1-3, Smo, Ptch1 and total survival after surgery (**Figure 3C**). Gli 1-3 had no difference with disease-free survival. However, we found that the high Ptch1 expression and low Smo expression group showed a significant better DFS (P<0.05) (**Figure 3D**).

The expression of Smo was increased and Ptch1 expression was decreased in GIST-T1 resistant cell lines

Our data indicated Ptch1 and Smo levels impacted imatinib response in GIST patients with postoperative disease-free survival. Thus, we suspected that Ptch1, Smo and GIST imatinib efficacy are related. Therefore, we constructed the imatinib-resistant cell line (**Figure 4A**, **4B**) using GIST-T1 cells and detected the expression of Ptch1, Smo and Gli1-3 in GIST-T1 cells and GIST-T1R cells by PCR (**Figure 4C**). The expression levels were confirmed by Western blot (**Figure 4D**). The data showed the mRNA and protein expression of Smo were significantly increased, while the expression of Ptch1 in mRNA was decreased. However, the expression of Ptch1 was not significantly reduced in WB.

The imatinib resistance in GIST-T1R was reduced after interference with Smo in GIST-T1R cells

We assessed the mRNA and protein expression in GIST-T1 cells and GIST-T1R cells and found the expression of Smo mRNA and protein was significantly changed. Furthermore, the expression of Ptch1 mRNA was decreased but the overall expression was low. The WB data indicated Ptch1 expression was not significantly reduced. Thus, we examined resistance to imatinib by interfering with Smo in GIST-TIR cells and detecting changes in drug resistance to imatinib after cell interference (**Figure 5A**, **5B**). The results suggested GIST-T1R cells have reduced resistance to imatinib after Smo inhibition.

Discussion

The Hh signaling pathway is over-activated in liver cancer, pancreatic cancer, gastric cancer, colorectal cancer and other gastrointestinal malignancies and correlates to tumor progression [17, 22-25]. However, the relationship between Hh pathway and GIST is rarely reported.

GIST is one of the most common mesenchymal tumors of the digestive tract. The incidence of GIST is mainly caused by mutations in the KIT gene and the PDGFRA gene, which is also the molecular basis for the development of GIST. Although the pathogenesis of GIST has been gradually elucidated, there are currently no molecular prognostic indicators for the prognosis of GISTs. Penelope et al. [26] found downregulation of Ptch1 expression induced mouse cells to differentiate into GIST cells in animal experiments. The study by Yoshizaki et al. [11] found that activation of the Hh signaling pa-

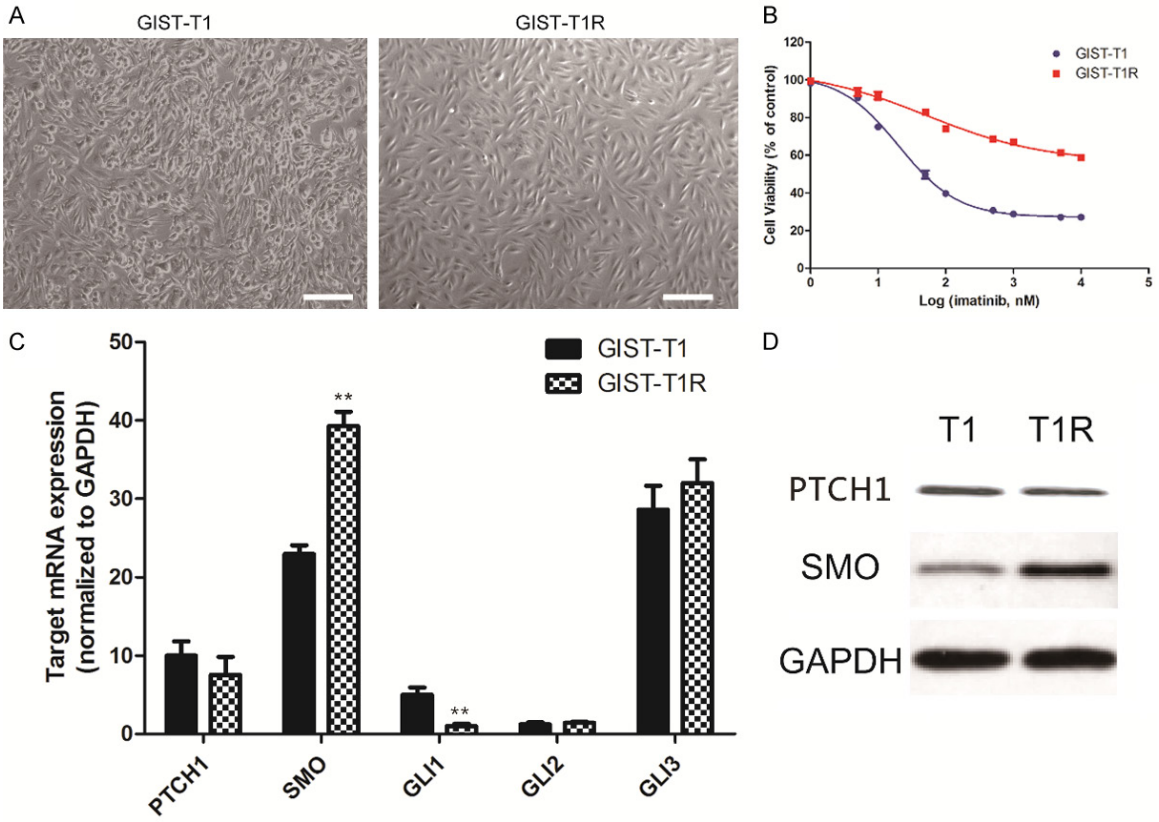


Figure 4. Cell viability and protein expression of GIST-T1 and GIST-T1R. A. GIST-T1 and GIST-T1R cells in Optical microscope. B. Cell viability of GIST-T1 and GIST-T1R in different imatinib concentration. C. The expression level of Ptch1, Smo and Gli1-3 in GIST-T1 and GIST-T1R by PCR. D. The expression of Ptch1 and Smo of GIST-T1 and GIST-T1R by western-blot (**P<0.01).

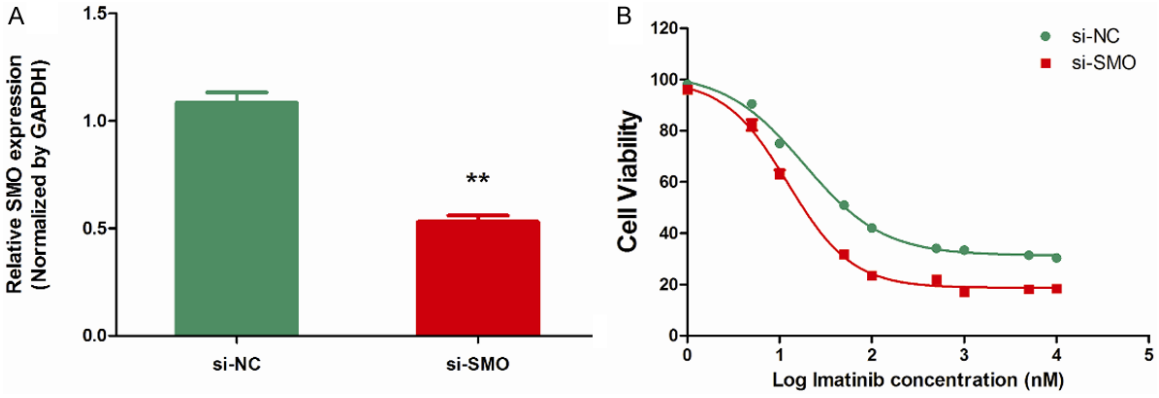


Figure 5. The Smo expression and cell viability of GIST-T1R and si-Smo GIST-T1R. A. Smo expression of GIST-T1R and si-Smo GIST-T1R. B. Cell viability of GIST-T1R and si-Smo GIST-T1R in different imatinib concentration (**P<0.01).

thway was associated with altered biological behavior of small intestinal stromal cells, which provides a theoretical basis for our research.

We assessed the expression of Hh signaling pathway proteins by microarray in 12 resected GIST specimens to identify a potential biochemical index that might correlate to GISTs. The data showed the expression of Smo, Ptch1 and Gli1-3 were different in different risk groups for GIST. Therefore, we assessed the expression of Hh related proteins in 24 FFPE tissues with RT-PCR. The PCR results showed similar outcomes. These two experiments suggested

the expression of Smo and Gli1-3 were relatively higher in high risk of GISTs than in low risk or intermediate risk GISTs. Additionally, the expression of Ptch1 showed the opposite result. Given that Smo is the activator of Hh signaling and Ptch1 is the inhibitor, we speculate that the aberrant activation of Hh signaling pathway may be associated with the progression of GIST.

The immunohistochemistry assessments revealed that the expression of Smo and Ptch1 provide significant and independent prognostic value in resected GIST patients. Higher expression of Ptch1 and lower of Smo in GIST tumor cells predicts a better OS and DFS. These findings indicate that Smo and Ptch1 expression at diagnosis could be used as predictors in resected GISTs.

Surgery can be curative for GIST patients and targeted therapy plays an important role for those unresectable patients. There are many GIST patients suffering from primary or acquired drug resistance to imatinib. Of the 322 patients in our study cohort, 51 were treated with imatinib and our stratified analysis found that Ptch1 and Smo were associated with the DFS of these patients. Alonsodominguez et al. [27] found that in patients with chronic myeloid leukemia the Ptch1 expression levels were associated with imatinib-resistance. Thus, the PTCH1 expression level might be used as a direct indicator of imatinib medication. We hypothesized that Ptch1 and Smo may play a role in imatinib-resistance in high-risk GISTs. Therefore, we investigated the expression of Ptch1, Smo and Gli1-3 in GIST-T1R (imatinib-resistance GIST-T1 cell lines) and GIST-T1 cells by PCR and found that the expression of Smo was relatively higher in the GIST-T1R cells. This result suggests the function of SMO in GIST cells plays the main role. In the imatinib-resistant cells the Hh signaling pathway was excessively activated, which indicates the Hh signaling pathway is associated with imatinib-resistance in GIST cells. The expression of Ptch1 and Smo proteins were then detected in both cell types by Western blot. The data showed a similar result and Smo expression was significantly higher in GIST-T1R cells. Finally, in order to further validate the role of Smo in imatinib-resistance, we blocked the function of Smo in GIST-T1R cells. We found that when Smo function was inhibited in GIST-T1R cells, the viability of the cells decreased, which suggests the SMO-mediated Hh signaling pathway plays a key role in imatinib-resistance in GIST cells. Cumulatively, we find the Smo-mediated Hh signaling pathway plays an important role in prognosis and imatinib-resistance. Further studies are needed to investigate the mechanisms linking the Hh signaling pathway and the imatinib-resistance of GIST.

Disclosure of conflict of interest

None.

Address correspondence to: Peiwu Yu, Department of General Surgery, The First Hospital Affiliated to Army Medical University, Chongqing 400038, China. E-mail: yupeiwu01@sina.com; Hui Cao, Department of Gastrointestinal Surgery, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China. E-mail: caohui@renji.com

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