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RESEARCH

Activation of Hedgehog Signaling Pathway in Human Non-small Cell Lung Cancers

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Abstract The activation of the hedgehog pathway, which is an important signaling mechanism crucial in embryogenesis, has strong links to carcinogenesis. Aberrant regulation of this pathway can result in the development of tumors. The present study was designed to investigate Hh related protein expression in non-small cell lung cancers. Fifty five non-small cell lung cancers samples were used in the study. By reverse transcription-polymerase chain reaction (RT-PCR), the expression of Shh, Ptch-1, and Gli-1 in tumor and adjacent normal tissues was examined and associated to clinical pathologic features. The expression levels of Shh, Ptch-1, Gli-1 in non-small cell lung cancer tissues were 63.64, 69.09, 43.64 %, respectively, higher than that in the adjacent normal tissues. Survival analysis showed that both Ptch-1 and Gli-1 expression were associated with poor survival (both $P<0.05$, log-rank test). Shh and Ptch-1 expression were correlated with lymph node metastasis. These results suggest that dysregulation of Hh signaling pathway plays an important role in the development of human NSCLCs. The expression of Ptch-1 and Gli-1 is possibly involved in NSCLCs progression, which may be a useful prognostic indicator of NSCLCs.

Keywords Shh · Ptch-1 · Gli-1 · Non-small cell lung cancer · Survival

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Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. Non-small cell lung cancers (NSCLCs), primarily adenocarcinoma (AC) and squamous cell carcinoma (SCC), represent approximately 85 % of all lung cancers [2]. Despite various treatments used in the clinical cases, patients with NSCLCs have poor overall survival, with a 5 year survival rate of only 15 % for all stages combined [3].

The Hedgehog (Hh) signaling pathway plays a critical role in the embryonic development, tissue polarity, and carcinogenesis [4, 5]. In mammals, the Hh family consists of three different members, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) [6]. In activated Shh signaling pathway, Shh protein binds to the transmembrane receptor Patched protein (Ptch), which relieves its inhibition on the transmembrane protein Smoothened (Smo) to initiate a series of intracellular cascades that affect the translocation of the transcription factor glioma-associated oncogene homolog 1 (Gli-1) into the nucleus [5, 7], resulting in the transcription of target genes related to cell proliferation including Gli-1, Ptch-1, WNT-1, transforming growth factor β family members, and oncogene B-cell leukemia 2 (BCL2) [8, 9]. Various cancers, including brain, lung, breast, gastrointestinal, and prostate cancers, have been found to show activation of this pathway [10]. Although the activation of the Hh pathway is

involved in several types of cancers, its role in NSCLCs is not well known. In this study, we analyzed the expression of Shh-related protein in 55 NSCLCs patients to determine the correlation between Shh signaling activation and clinical outcome including survival. By investigating the expression of the Shh pathway proteins Shh, Ptch-1, and Gli-1, we hoped to evaluate if Shh signaling activation can be utilized for diagnosis and treatment of non-small cell lung cancer.

Materials and methods

Patients and tumor samples

The tumor tissues from 55 patients with NSCLCs and information about clinicopathologic features were collected at the Jiangsu Cancer Hospital, Nanjing, China between 2001 and 2011. The clinical and pathologic data including demography (age, gender), tumor size, and TNM (tumor-node-metastasis) stage were obtained through in-person interviews and the pathologic and medical reports. The samples of 55 NSCLC (55 adenocarcinomas) were identified by pathological examination. All patients were ethnic Han Chinese and received surgical treatment between October 2001 and August 2004. Both the samples of tumor and adjacent non-tumor tissues were freshly obtained from surgery and then snap-frozen in liquid nitrogen with 30 min and stored at −80 °C. The procedures were approved by the ethics committee of Human Experimentation of Jiangsu Cancer Hospital & Institute.

RNA extraction and cDNA preparation

Total RNA was isolated from 100 mg of each tissue sample using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions, and stored at −80 °C for further used. The reverse transcription of the total RNA (1 μL) was done using SuperScript First-Strand Synthesis System (Invitrogen). The internal control was glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer: 5'~TCA ACG GAT TTG GTC GTA TT~3', reverse primer: 5'~AGT CTT CTG GGT GGG AGT GAT~3', 540 bp).

Polymerase chain reaction (PCR) amplification

To analyze the expression of individual Hh genes, 1 μL cDNA was amplified with 6.25 units AmpliTaq Gold (Roche, Basel, Switzerland), in a 25 μL reaction solution containing 0.5 mmol/L dNTPs and 1.5 mmol/L $MgCl_2$. The primer sequences for PCR for each genes were designed using PrimerExpress, version 3.0 (Applied Biosystems), based on Genbank (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/sites/entrez) sequences (Table 1). All reactions were carried out in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA). Electrophoresis was performed by loading 8 μL of each sample on a 1 % agarose gel. The reaction result was visualized by ethidium bromide staining using the Bio-imaging System (Ultra-Violet Products, UVP, Cambridge, UK).

Table 1 Primers and fragment sizes of Hh signaling genes

Genes	Primers	Fragment sizes (bp)
Shh	F: 5'-CCA CTG CTC GGT GAA AGC AG-3 R: 5'-GGA AAG TGA GGA AGT CGC TG-3'	181 (nt 694–875)
Ptch-1	F: 5'-CGC-CTA TGC CTG TCT AAC CAT GC-3' R: 5'-TAA ATC CAT GCT GAG AAT TGC A-3'	450 (nt 1,338–1,788)
Gli-1	F: 5' CTC AAC AGG AGC TAC TGT GG-3' R: 5'-GGG TTA CAT ACC TGT CCT TC-3'	396 (nt 2,789–3,185)

Abbreviation: F=forward primer; R=reverse primer

DNA sequencing

The representative PCR products of each gene were measured by Beijing AuGCT Biotechnology Co. Ltd. and were screened using Chromas 2.3 shareware (Technelysium, Australia).

Statistical analysis

The overall survival (OS) is defined as the time from initial diagnosis to death due to any cause or the date of last follow up. Survival curves were calculated using the Kaplan-Meier method and compared using the log-rank test. The exact $\chi 2$ tests were used to evaluate the statistical significance of Shh-related protein between the tumor and the normal tissues. The Statistical significance was accepted at $P<0.05$ (two tailed). The statistical data were analyzed using SPSS 17.0 (SPSS Inc, Chicago, IL, USA).

Results

Expression of individual Shh gene

We detected the expression of Hh signaling molecules in 55 NSCLCs tissues and the corresponding adjacent normal tissues. The positive rate of Shh expression was 35/55 (63.64 %) in the NSCLCs, while the corresponding adjacent normal tissues expressed Shh, 11/55 (20.00 %), significantly less frequently than the positive rate ($P<0.001$). In the expression of Ptch-1, positive rate was 38/55 (69.09 %) in the tumor

tissues, 27/55 (49.09 %) in the corresponding adjacent normal tissues ($P<0.05$). The expression of Gli-1 in the NSCLCs 24/55 (43.64 %) was significantly higher than the expression in the corresponding adjacent normal tissues 7/55 (12.73 %) ($P<0.001$) (Table 2).

Correlation between expression of Hh signaling genes and clinical prognosis of NSCLCs

The patients' characteristics are listed in Table 3. The correlation between the three gene expression and metastasis and prognosis was analyzed. We found no significant relationship between the levels of Shh expression in tumor tissues and clinical prognosis in the 55 enrolled lung NSCLCs patients (Fig. 1a). In contrast, the expression of Ptch-1 and Gli-1 in tumor tissues showed a significant relationship with OS (Fig. 1b; Fig. 1c). The Shh, Ptch-1 and Gli-1 expression in adjacent non-tumor lung tissues did not show any significant relationship with the clinical prognosis (Fig. 1d; Fig. 1e; Fig. 1f). Furthermore, of the 55 cases, 29 patients had lymph node metastasis (Table 3; N0, N1 stage). We analyzed the correlation between the three gene expression and lymph node metastasis, and found that Shh and Ptch-1 expression was linked to lymph node metastasis (Table 4). Adjuvant chemotherapy in patients showed no significant improvement of OS (Fig. 2).

Discussion

Hh pathway is activated significantly during embryogenesis, and aberrant activation of this pathway is involved in the development of a variety of human cancers, including breast, gastrointestinal, prostate cancers, intestinal adenocarcinoma and small cell lung cancer [10–12]. However, in NSCLCs, gene expression in the Hh pathway has not been well studied.

In the present study, we investigated the expression level of Hh signaling pathway proteins in patients with NSCLCs. We found that the three Hh genes, Shh, Ptch-1 and Gli-1, expressed more frequently in the tumor tissues than in the corresponding adjacent lung tissues. These observations indicate that Hh signaling pathway may play an important role in the pathogenesis of NSCLCs. Other studies reported that Hh signaling pathway, as an essential pathway for embryonic development, is inactive in adult tissues [11]. The pathway is frequently oncogenic while activated aberrantly in adult tissues [13–15]. Previous study has shown that the amounts of injected Gli mRNA influence the development of squamous cell carcinoma and basal cell carcinoma [16]. Consistent with these studies, our findings that tumor tissues expressed higher Hh gene than the normal tissues suggest that the development of the NSCLCs has a close relationship with these three Hh genes. An interesting finding of our study was that patients express Shh didn't always express Ptch-1 and

Table 2 Summary of Shh, Ptch-1, and Gli-1 expression in NSCLC and adjacent normal tissues by RT-PCR

Variable	Rate (%, $N=55$)	P value[a]
Shh		
Cancer tissue	63.64 (35/55)	<0.001
Adjacent normal tissue	20.00 (11/55)	
Ptch-1		
Cancer tissue	69.09 (38/55)	0.033
Adjacent normal tissue	49.09 (27/55)	
Gli-1		
Cancer tissue	43.64 (24/55)	<0.001
Adjacent normal tissue	12.73 (7/55)	

[a] Exact $\chi 2$ test

Table 3 Patient characteristics

Patients	Number ($N=55$)
Gender	
Female	21
Male	34
Age (year)	
Mean	61.91±8.09
Median	64
Range	40–73
Invasion[a]	
T1	19
T2	24
T3	10
T4	2
Lymph node meta[a]	
N0	26
N1	14
N2	15
TNM stage[a]	
Stage IA	8
Stage IB	10
Stage IIA	4
Stage IIB	11
Stage IIIA	16
Stage IIIB	3
Stage IV	3
Treatment	
Non-chemotherapy	10
Cisplatinum with paclitaxel	34
Cisplatinum with gemcitabine	4
Cisplatinum with navelbine	7

Abbreviation: TNM=tumor-node-metastasis

[a] According to the AJCC Cancer Staging Handbook 6th Edition

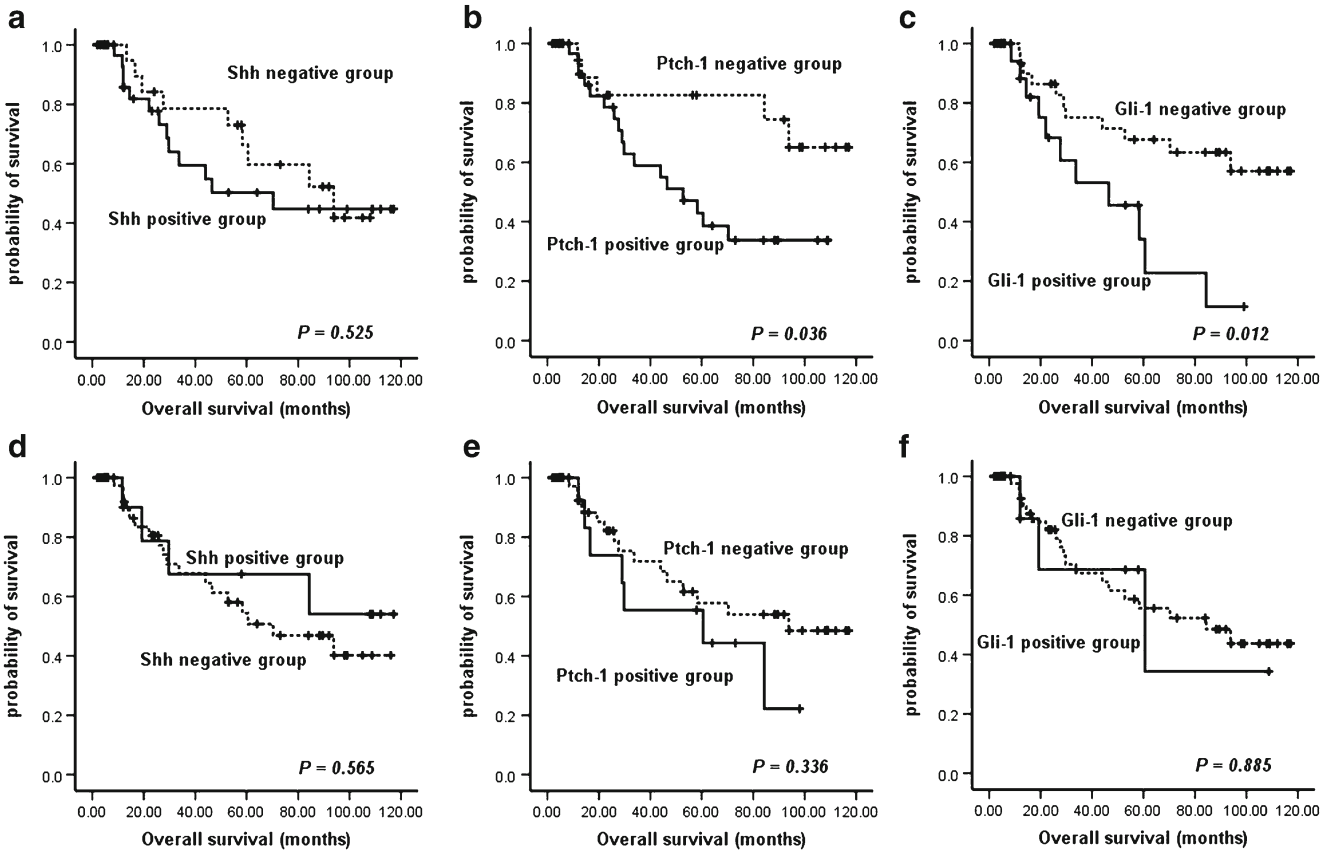

Fig. 1 The Kaplan-Meier survival curves of patients with NSCLCs based on Shh, Ptch-1, and Gli-1 expression. Prognosis was not related with Shh expression in tumor tissues (**a**). The expression of Ptch-1 (**b**) and Gli-1 (**c**) in tumor tissues was correlated with OS. Prognosis was not related with Shh (**d**), Ptch-1 (**e**) and Gli-1 (**f**) expression in adjacent non-tumor tissues

Gli-1. This observation suggests that some ligand-independent mechanisms including overexpression of Gli transcription factors and mutations of Ptch-1, Smo, and SuFu are involved in the activation of the pathway, as it has been reported in lung cancer and other malignancies [17–20].

Since the Hh signaling pathway is frequently activated in NSCLCs, the markers for the activation, Shh, Ptch-1 and Gli-1 may be useful for prognosis. It has been reported that patients with positive expression of Shh, Ptch-1 and Gli-1 proteins showed poor OS than those with negative expression, and these proteins were independent, unfavorable prognostic

Table 4 Association of Shh, Ptch-1, and Gli-1 expression with lymph node metastasis in NSCLCs

Variable	Lymph node metastasis rate (%)	*P* Value[a]
Shh		
Positive group	62.86 (22/35)	0.047
Negative group	35.00 (7/20)	
Ptch-1		
Positive group	63.16 (24/38)	0.021
Negative group	29.41 (5/17)	
Gli-1		
Positive group	41.67 (10/24)	0.148
Negative group	61.29 (19/31)	

[a] Exact $\chi 2$ test

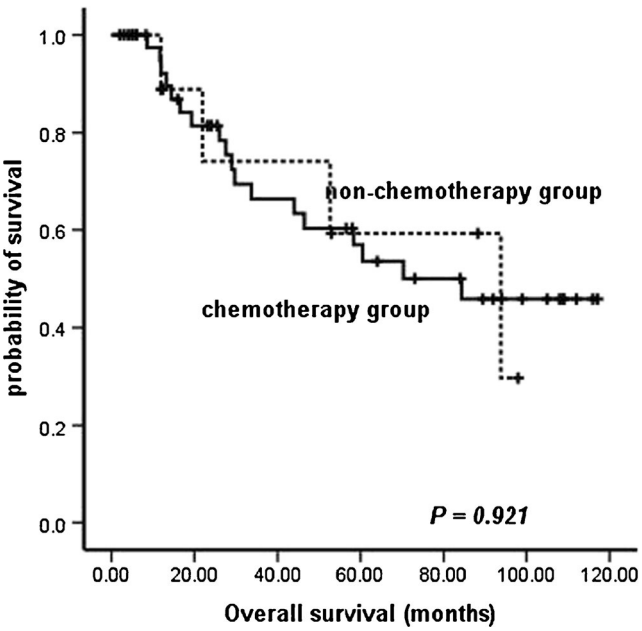

Fig. 2 The Kaplan-Meier survival curves of patients with NSCLCSs with chemotherapy effect. Chemotherapy effect on OS showed no significance

factors [21]. In breast cancer and hepatocellular carcinoma, increased expression of Gli-1 protein is significantly correlated with poor OS [22, 23]. A recent study found that elevated levels of Gli-1 and Ptch-1 expression significantly correlated with poor overall survival of ovarian cancer patients [24]. Patients with ovarian cancers that expressed high level Gli-1 proteins had a decreased survival (37.3±8.7 months) compared with patients with lower Gli-1 expression (128.2± 14 months). Similarly, patients with high level Ptch-1 expression also had poorer survival (38.7±7.4 months) than those having lower Ptch-1 expression (130.3±14.3 months). In our study, we found that Ptch-1 and Gli-1 expression in NSCLCs tissues showed a significant relationship with OS, as the same tendency was observed in ovarian cancers [24]. The enhanced Hh pathway activation leads to downstream expression of target genes, including Ptch-1 and Gli-1. Despite that the tumor suppressor Ptch-1 is a negative regulator of the Hh signaling pathway, its expression in the tumor tissues implicates that this pathway is active, because Ptch-1 is also a target gene of the transcription factor Gli-1 [17, 25, 26]. The phenomenon that Ptch-1 and Gli-1 over- expressed in the tumors indicates aberrant activation of Shh signaling pathway [27]. These data suggest that the activation of Shh signaling pathway is potential prognostic indicator in human NSCLCs. The markers for Shh signaling activation, especially Gli-1 and Ptch-1, may be useful for the judgment of clinical prognosis.

It has been reported that Shh expression regulates metastasis and lymphangiogenesis by paracrine signaling in pancreatic cancer [28]. In prostate cancer, inhibition of Shh signaling has been shown to reduce tumor metastasis [29]. In gastric cancer, Shh pathway promotes metastasis and lymphangiogenesis via activation of Akt, EMT, and MMP-9 pathway [30]. In our study, expression of Shh and Ptch-1 was significantly associated with metastasis, and the metastasis rate of Gli-1 positive group was higher than that of the negative group. Preliminary studies show that complex networks rather than an individual gene are involved in metastasis [31]. We may hypothesize that Hh pathway is involved in the metastasis of NSCLCs. However, further analysis of this hypothesis is necessary. In our studies, patients with NSCLCs didn't benefit from adjuvant chemotherapy in OS. It is reported that the effect of chemotherapy is mainly related with tumor staging [32]. The chemotherapy effect showed no significance when the numbers of stage IA patients were excluded, implying that stage IA patients benefits more from chemotherapy. The number of stage IA patients in our studies is too small (8/55), explaining why there was no significant variation on the OS.

The results suggest that the Hh signaling pathway is overexpressed in human NSCLCs, and the expression of Ptch-1 and Gli-1 are related to patients' survival. Although the functional mechanism of the pathway remains unknown, it might play a central role in NSCLCs tumorigenesis. The Hh signaling pathway may be a potential therapeutic target in human NSCLCs. Because of the limited sample size in our analysis, more studies are needed to prove the prognostic value of the pathway.

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