



STAT3 activation and aberrant ligand-dependent sonic hedgehog signaling in human pulmonary adenocarcinoma

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ABSTRACT

The Sonic hedgehog (SHH) signaling and STAT3 pathways play important roles during carcinogenesis with possible interaction. To determine the association of the activation of SHH signaling pathway and STAT3 pathway in carcinogenesis of human non-small cell lung carcinomas (NSCLC), firstly we examined the expression of SHH signaling molecules including SHH, Gli1 (glioma-associated oncogene homolog 1) and HHIP (Hh interacting protein), as well as p-STAT3 (phosphorylation at Tyr705) by immunohistochemistry in 87 cases of NSCLC, 12 atypical adenomatous hyperplasia (AAH) and 20 adjacent normal lung tissues. **The expression of SHH, Gli1, HHIP and p-STAT3 was detected respectively in 87/87(100%), 74/87(85.1%), 75/87(86.2%) and 54/87(62.1%) of the NSCLC cases, but not in the adjacent normal lung parenchyma.** Ligand-dependent SHH pathway activation and STAT3 signaling activation were observed in most cases of NSCLC, and the high SHH pathway activation rate and STAT3 activation rate were significantly higher in adenocarcinoma compared with squamous cell carcinomas and large cell carcinomas ($P < 0.05$). Both SHH and STAT3 pathway activation level correlated with histological grade in adenocarcinoma, being higher in well-differentiated types ($P < 0.05$). Furthermore, high SHH pathway activation and p-STAT3 expression were also detected in 10/12(83.3%) of AAH cases as well as in most cases of early-stage adenocarcinoma – adenocarcinoma in situ (AIS) and minimally invasive adenocarcinomas (MIA). Correlation analysis showed that p-STAT3 protein expression level was correlated positively with ligand-dependent activation level of SHH signaling in adenocarcinoma ($r_s = 0.585$, $P = 0.000$) and AAH ($r_s = 0.996$, $P = 0.000$). We speculated that activation of STAT3 could up-regulate SHH gene expression directly or indirectly, and thereby activated SHH signaling resulting in lung tumor cell ontogeny. To explore the interactional mechanism, we then performed serial transient co-transfection assay in human pulmonary adenocarcinoma cell line H441 cells, and the results confirmed STAT3 activation can up-regulate SHH gene expression indirectly. In conclusion, aberrant ligand-dependent SHH signaling activation occurs frequently in NSCLC. The signaling plays a more active role in adenocarcinoma, and is an early event in carcinogenesis of lung adenocarcinoma. The involvement of STAT3 pathway activation might function in inducing the process.

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Introduction

Lung cancer is the leading cause of cancer death for man and women worldwide. It is difficult to detect in its early stages. In most cases, the tumors are detected at advanced stages. Thus, it is essential to explore the molecular mechanism in early events of carcinogenesis of lung cancer.

It has been suggested that lung tumor cell ontogeny is determined by consequences of gene expression that recapitulate events important in embryonic lung development (Borczuk et al., 2003). The tightly regulated sonic hedgehog (SHH) signaling is such a pathway that is crucial for vertebrate development including lung organogenesis (van Tuyl and Post, 2000). With SHH signaling activation, the downstream glioma-associated oncogene homolog (Gli) family members are stabilized and translocated into the nucleus and lead to the transcriptional activation of target genes involved in a variety of processes, such as feedback regulation, cellular proliferation, maintenance of stemness, cellular survival and epithelial-to-mesenchymal transition (Katoh and Katoh, 2009), which constitute the basis of normal embryogenesis and cell differentiation. Among the numerous transcripts of target genes, Gli1, which is also a vital transcriptional factor of SHH signaling and can be considered a perfect indicator for SHH pathway activation, plays important role in positive feedback loop. Whereas hedgehog-

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interacting protein (HHIP), an important upstream inhibitor of SHH signaling, which can bind directly to SHH with an affinity similar to its membranous receptor but lacks signal transduction capacity and acts to internalize and degrade the SHH ligand, involves in negative feedback loop (Chuang and McMahon, 1999). Based on these fine-tuned feedback loops, SHH signaling ensures its powerful physiological functions. However, dysregulation of this signaling can lead to aberrant reactivation during adult homeostasis. The dysregulation can occur from either ligand-dependent mechanisms such as overexpression of SHH ligand or ligand-independent mechanisms including mutations in some SHH-pathway genes (Ehteshami et al., 2007; Villavicencio et al., 2000). Several studies have demonstrated that aberrant SHH signaling reactivation, resulting from dysregulation of this pathway, appears to play an integral role in the initiation and propagation of many cancers including the skin (Von Hoff et al., 2009), prostate (Karhadkar et al., 2004), pancreas (Morton et al., 2007; Thayer et al., 2003), brain (Berman et al., 2002), endometrium (Liao et al., 2009), gastrointestinal tract (Berman et al., 2003; Yoo et al., 2011) and lung (Watkins et al., 2003). Although the involvement of aberrant SHH pathway in small-cell lung carcinoma (SCLC) is well established (Watkins et al., 2003), its role in the carcinogenesis of non-small cell lung carcinomas (NSCLC) is still not fully understood (Chi et al., 2006; Yuan et al., 2007).

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor encoded in humans and is an oncogene (Bromberg et al., 1999). In response to cytokines and growth factors, phosphorylation at Tyr705 by Janus-activated kinases (JAK) causes STAT3 form homo- or heterodimers that translocate to the cell nucleus, where they act as transcription activators and activate downstream target genes. Lots of studies in tumors and cell lines indicated that inappropriate activation of STAT3 occurs with a surprisingly high frequency (50–90%) in a wide variety of human cancers, including lung cancers (Haura et al., 2005; Song et al., 2003; Yeh et al., 2006). Our previous research firstly showed persistent activation of the STAT3 signaling leads to inflammation and spontaneous tumorigenesis in the mice lung (Li et al., 2007). In addition, we have observed that some developmental genes such as SHH and TTF-1 were up-regulated in the STAT3-overexpressing transgenic mice lung by Affymetrix GeneChip microarray analysis. Thus, we hypothesized that SHH gene expression may be directly and indirectly regulated at the level of transcription by STAT3, and SHH signaling could cooperate with STAT3 in the lung tumorigenesis. Thus, we investigated the expressions of SHH signaling molecules including SHH, Gli1 and HHIP, as well as STAT3 with phosphorylation at Tyr705 (abbreviation as p-STAT3 in next content), in a series of human surgically excised NSCLC tissues. The correlation between STAT3 and ligand-dependent SHH pathway activation and in vitro interactional molecular mechanism between SHH and STAT3 were further analyzed.

Materials and methods

Clinical samples

Formalin-fixed, paraffin-embedded tissues of 87 surgically resected NSCLC (20 samples of them with adjacent normal lung tissues), between September 2009 and March 2011, were retrieved

from the archives of the Pathology Department of two large hospitals in China — Tongji Hospital of Huazhong University of Science and Technology, Wuhan and Cancer Hospital of Shanghai Medical College of Fudan University, Shanghai. Histological classification and tumor grade were assessed according to the established criteria by World Health Organization (WHO) in 2004 and by the International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS) in 2011. Among the 87 NSCLC, there were 54 adenocarcinomas including 4 cases of adenocarcinoma in situ (AIS), 5 minimally invasive adenocarcinomas (MIA) and 45 invasive adenocarcinomas (12 cases showed accompanied precursor lesion of atypical adenomatous hyperplasia in the adjacent lung tissue), 25 squamous cell carcinomas and 8 large cell carcinomas. Fifty-eight patients were male and 29 were female with a mean age 58 years (range: 37 to 78 years). Twenty-eight of 87 tumors (32.2%) were grade 1, 31 (35.6%) were grade 2 and 28 (32.2%) were grade 3. Lymph node metastasis was present in 44 of 87 (50.6%) cases.

Cell line

Human pulmonary adenocarcinoma cell line H441 was obtained from American Type Culture Collection (ATCC). H441 cells were cultured in RPMI 1640 medium (Thermo Scientific HyClone, Logan, Utah, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin for cotransfection assay.

Immunohistochemistry

A standard immunohistochemical protocol for all markers is followed as described herein. Briefly, 5 μ m thick tissue sections were deparaffinized. Antigen retrieval was performed by heating for 5 min in a pressure cooker using 10 mM citrate buffer (pH 6.0). After treated with 3% hydrogen peroxide and 10% blocking serum (DAKO, Glostrup, Denmark), the sections were incubated with primary antibodies. Optimal, reproducible results were attained employing dilutions/incubation time and pre-treatment conditions as shown in Table 1. The Envision Detection System (DAKO, Glostrup, Denmark) was used for visualization according to manufacturer's instructions. Sections were then counterstained with hematoxylin. Negative controls were performed in all cases by omitting the primary antibodies.

Assessment of immunohistochemical staining

Cases with staining in >10% of cells were considered positive. Immunohistochemical reactivity was graded on a scale of 0–3 according to intensity of staining and percentage of immunopositive cells as follows: 0 (negative expression): no staining or <10% positive cells, 1 (low expression): weak staining in >10% of tumor cells or moderate staining in 10–40% of tumor cells, 2 (moderate expression): moderate staining in >40% of tumor cells or strong staining in 10–40% of tumor cells, 3 (high expression): strong staining in >40% of tumor cells (Gialmanidis et al., 2009). In mammalian cells the subcellular localization of signaling proteins is vital to signal transduction. The cellular localization of related signaling proteins and the defining criteria for active protein were summarized in

Table 1
Antibody characteristics.

Antibody	Source	Catalogue number	Dilution	Antigen retrieval	Incubation time
SHH	Abcam	ab73958	1:800	0.01 M citrate buffer pH 6.0	Overnight 4 °C
HHIP	Novus	H00064399-M01	1:300	0.01 M citrate buffer pH 6.0	Overnight 4 °C
Gli1	Santa Cruz	sc20687	1:450	0.01 M citrate buffer pH 6.0	Overnight 4 °C
p-STAT3	CST	4113s	1:50	0.01 M citrate buffer pH 6.0	Overnight 4 °C

CST: cell signaling technology.

Table 2

The subcellular location of related signaling proteins and the defining criteria for active protein detected by immunohistochemical staining.

	Possible localization	Functional localization	Standard to judge protein with activity
SHH	Cytoplasm and membrane	Membrane	Positive expression in both cytoplasm and membrane or just in membrane
Gli1	Cytoplasm and nucleus	Nucleus	Positive expression in both cytoplasm and nucleus or just in nucleus
HHIP	Cytoplasm and membrane	Membrane	Positive expression in both cytoplasm and membrane or just in membrane
p-STAT3	Cytoplasm and nucleus	Nucleus	Positive expression in nucleus

Table 2. Ligand-dependent SHH pathway activation was considered positive in cases with staining of both active SHH and active Gli1 in >10% of tumor cells (score>0). For statistical analysis the pathway activation level was graded as follows: 0(no activation): score 0 for either active SHH or active Gli1, 1 (low activation): positive cases with $0 < \text{score} < 2$ for either SHH or Gli1, and 2 (high activation): score ≥ 2 for both SHH and Gli1. Nuclear expression of p-STAT3 indicates the activation of STAT3 pathway.

Plasmid construction

The SHH 2.0 kb promoter was amplified from mouse tail genomic DNAs using an upstream primer with the MluI site (5'-CTAGACGCGTTGAGCCTG ACCACAGGA-3') and a downstream primer with the BglII site (5'-GAAGATCTCTCGTCGCGGAACCTGA-3') by PCR. The TTF-1 1.5 kb promoter was amplified from mouse tail genomic DNAs using an upstream primer with the MluI site (5'-CGACGCGTCTGGCTTACTCTTGTTGGATGTC-3') and a downstream primer with the XhoI site (5'-CCGCTCGAGGCTTAGGAG GAAGTGGGTTTC-3') by PCR. The italicized sequences are restriction enzyme sites. The PCR

products were digested with restriction enzymes and subcloned into the pGL2-B luciferase reporter gene vector to generate SHH 2.0 kb or TTF-1 1.5 kb luciferase reporter gene construct. The TTF-1/PCR3.0 was made as previously described (Yan et al., 2002), and STAT3C/PCR3.0 expression plasmid were gifted by Dr CongYan (Indiana University School of Medicine, IN, USA).

Transient cotransfection assays

For transient cotransfection assay, H441 cells were seeded at a density of 2×10^5 cells per well in six-well plates 1 day before transfection. The SHH 2.0 kb luciferase reporter gene construct (0.25 μg) or TTF-1 1.5 kb luciferase reporter construct (0.25 μg) and pCMV- β gal plasmid (0.5 μg) were co-transfected with various concentrations of TTF-1/PCR3.0 or STAT3C/PCR3.0 constructs into H441 cells. The CMV- β -galactosidase plasmid was included for normalization. Luciferase assays were performed using the luciferase assay system (Promega, Madison, WI). The light units were assayed by luminometry (Mololight 3010; BD Pharmingen, San Diego, CA). Triplicate transfections

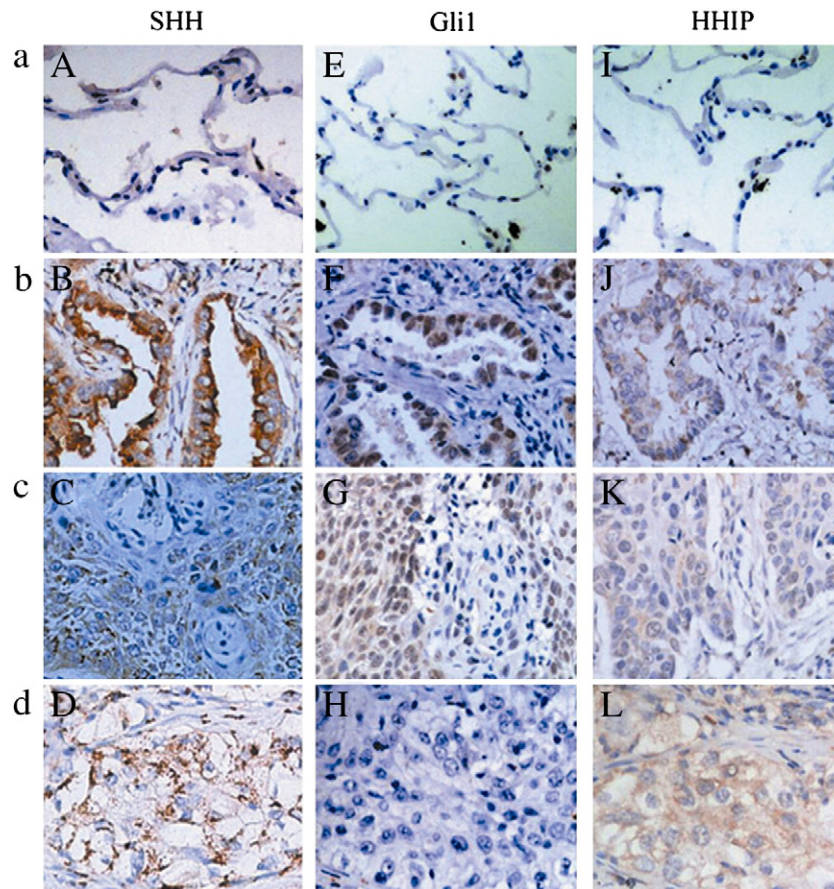


Fig. 1. Aberrant expression of SHH signaling molecules in different histological types of NSCLC (200 $\times$). Compared to negative staining in alveolar epithelium of adjacent normal lung tissue (a), adenocarcinoma (b), squamous cell (c) and large cell carcinoma (d) present differently aberrant expression of SHH signaling molecules. Serial sections in b, c and d show the co-expression of SHH, Gli1 and HHIP respectively, which reflect the positively correlation between SHH and Gli1 expression, and adenocarcinoma (b) presents higher ligand-dependent SHH pathway activation level than squamous cell (c) and large cell carcinoma (d).

Table 3
Correlation of the expression of SHH signaling molecules SHH, Gli1 and HHIP with clinicopathological features in NSCLC.

	N	SHH expression				P-value	Gli1 expression				P-value	HHIP expression				P-value
		0	1	2	3		0	1	2	3		0	1	2	3	
		n (%)	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	n (%)	
NSCLC total cases	87	0 (0)	14 (16.1)	24 (27.6)	49 (56.3)	0.000 ^a	13 (14.9)	28 (32.2)	33 (38)	13 (14.9)	0.000 ^b	12 (13.8)	49 (56.3)	22 (25.3)	4 (4.6)	0.159 ^c
Histological type																
Adenocarcinoma	54	0 (0)	2 (3.7)	8 (14.8)	44 (81.5)		2 (3.7)	14 (25.9)	26 (48.1)	12 (22.2)		5 (9.3)	32 (59.3)	14 (25.9)	3 (5.6)	
Squamous	25	0 (0)	8 (32)	12 (48)	5 (20)	0.000 ^d	5 (20)	12 (48)	7 (28)	1 (4)	0.000 ^d	6 (24)	12 (48)	6 (24)	1 (4)	0.123 ^d
Large cell	8	0 (0)	4 (50)	4 (50)	0 (0)		6 (75)	2 (25)	0 (0)	0 (0)		1 (12.5)	5 (62.5)	2 (25)	0 (0)	
Grade																
Grade 1	28	0 (0)	1 (3.6)	1 (3.6)	26 (92.8)	0.218 ^d	0 (0)	3 (10.7)	13 (46.4)	12 (42.9)	0.602 ^d	3 (10.7)	21 (75)	3 (10.7)	1 (3.6)	0.588 ^d
Grade 2	31	0 (0)	5 (16.1)	10 (32.3)	16 (51.6)		1 (3.2)	14 (45.2)	15 (48.4)	1 (3.2)		6 (19.4)	14 (45.2)	10 (32.3)	1 (3.2)	
Grade 3	28	0 (0)	8 (28.6)	13 (46.4)	7 (25)		12 (42.9)	11 (39.3)	5 (17.8)	0 (0)		3 (10.7)	14 (50)	9 (32.1)	2 (7.1)	
Stage						0.102 ^a					0.910 ^b					0.230 ^c
0	4	0 (0)	0 (0)	0 (0)	4 (100)		0 (0)	1 (25)	2 (50)	1 (25)		0 (0)	4 (100)	0 (0)	0 (0)	
I	38	0 (0)	8 (21.1)	6 (15.8)	24 (63.2)		8 (21.1)	10 (26.3)	14 (36.8)	6 (15.8)		10 (26.3)	17 (44.7)	11 (28.9)	0 (0)	
II–IV	45	0 (0)	6 (13.3)	18 (40)	21 (46.7)	0.873 ^a	5 (11.1)	17 (37.8)	17 (37.8)	6 (13.3)	0.890 ^b	8 (17.8)	22 (48.9)	11 (24.4)	4 (8.9)	0.683 ^c
LN metastases																
Yes	44	0 (0)	5 (11.4)	18 (40.9)	21 (47.7)		5 (11.4)	16 (36.4)	17 (38.6)	6 (13.6)		8 (18.2)	22 (50)	10 (22.7)	4 (9.1)	
No	43	0 (0)	9 (20.9)	6 (14)	28 (65.1)	0.873 ^a	8 (18.6)	12 (27.9)	16 (37.2)	7 (16.3)	0.890 ^b	4 (9.3)	27 (62.8)	12 (27.9)	0 (0)	
Age																
<58	41	0 (0)	6 (14.6)	11 (26.8)	24 (58.5)		6 (14.6)	13 (31.7)	17 (41.5)	5 (12.2)		5 (12.2)	23 (56.1)	10 (24.4)	3 (7.3)	
≥58	46	0 (0)	8 (17.4)	13 (23.9)	25 (54.3)		7 (15.2)	15 (32.6)	16 (34.8)	8 (17.4)		7 (15.2)	26 (56.5)	12 (26.1)	1 (2.2)	

^a Compared the high SHH expression (3+) rate by chi-square test.

^b Compared the strong and moderate Gli1 expression (both 2+ and 3+) rate in different groups by chi-square test.

^c Compared the positive HHIP expression rate in different groups by chi-square test.

^d Analyzed by Spearman's test.

were performed for each experiment, and the mean values were obtained for analysis.

Statistical analysis

All analyses were carried out by the use of SPSS 17.0 for windows (SPSS Inc., Chicago, IL, USA). Correlations among the protein expression were evaluated by Spearman's test as well as correlations between the protein expression and pathway activation and the clinicopathological parameters such as tumor grade. The difference of protein expression in categories of the clinical parameters including histological type, gender and age was analyzed by chi-square or Fisher's exact tests. All statistical tests were two-sided. Differences were considered significant if $P < 0.05$.

Results

Aberrant expression of SHH signaling pathway molecules in NSCLC

By immunohistochemistry, the expression of SHH, Gli1, HHIP was detected in 87/87 (100%), 74/87 (85.1%), and 75/87 (86.2%) of the NSCLC cases respectively, but not in the adjacent normal lung tissue (Fig. 1; Table 3). The positive staining of SHH and HHIP was localized in both cytoplasm and membrane of cancer cells, and the Gli1 staining was observed in either cytoplasm alone (4/74, 5.4%) or both cytoplasm and nucleus (70/74, 94.6%) (Fig. 1). Forty-nine NSCLC cases (49/87, 56.3%) showed SHH high expression, and 46 cases (46/87, 52.9%) presented Gli1 moderate to high expression

(overexpression). Either the SHH or Gli1 expression was closely associated with histological type since the SHH high expression rate and Gli1 overexpression rate were both significantly higher in adenocarcinoma than that in squamous cell carcinomas and large cell carcinomas ($\chi^2 = 36.633$, $P = 0.000$; $\chi^2 = 17.491$, $P = 0.000$). Besides, SHH and Gli1 expression correlated significantly with histological grade, being higher in well-differentiated types ($r_s = 0.458$, $P = 0.000$; $r_s = 0.679$, $P = 0.000$). Of all the HHIP positive cases, just 4 cases showed high expression while 49 cases (65.3%) were found low expression. When all the NSCLC samples were included in statistical analysis, HHIP expression was not correlated with any of the clinicopathological parameters (Table 3). Still, there was a statistically significantly positive correlation between expression of SHH and Gli1 in all the NSCLC samples ($r_s = 0.592$, $P = 0.000$; Figs. 1 and 2), whereas no correlation was found between the expression of SHH or Gli1 and HHIP (Figs. 1 and 2).

Aberrant activation of SHH signaling pathway in NSCLC

Ligand-dependent activation of the SHH pathway was observed in 74 (85.1%) cases of NSCLC (Table 4). Among them, the activation level in 29 (39.2%) cases was low, while 45 (60.8%) cases showed high SHH pathway activation. Different histological types of NSCLC showed significantly different activation level. For adenocarcinoma, either the activation rate or the high level activation rate, was highest in all NSCLC types ($\chi^2 = 11.913$, $P = 0.001$; $\chi^2 = 19.822$, $P = 0.000$), while no high activation of SHH pathway was detected in large cell carcinomas, and most of them presented inactivation level (Fig. 1; Table 4). Besides, a significant positive correlation was observed between the

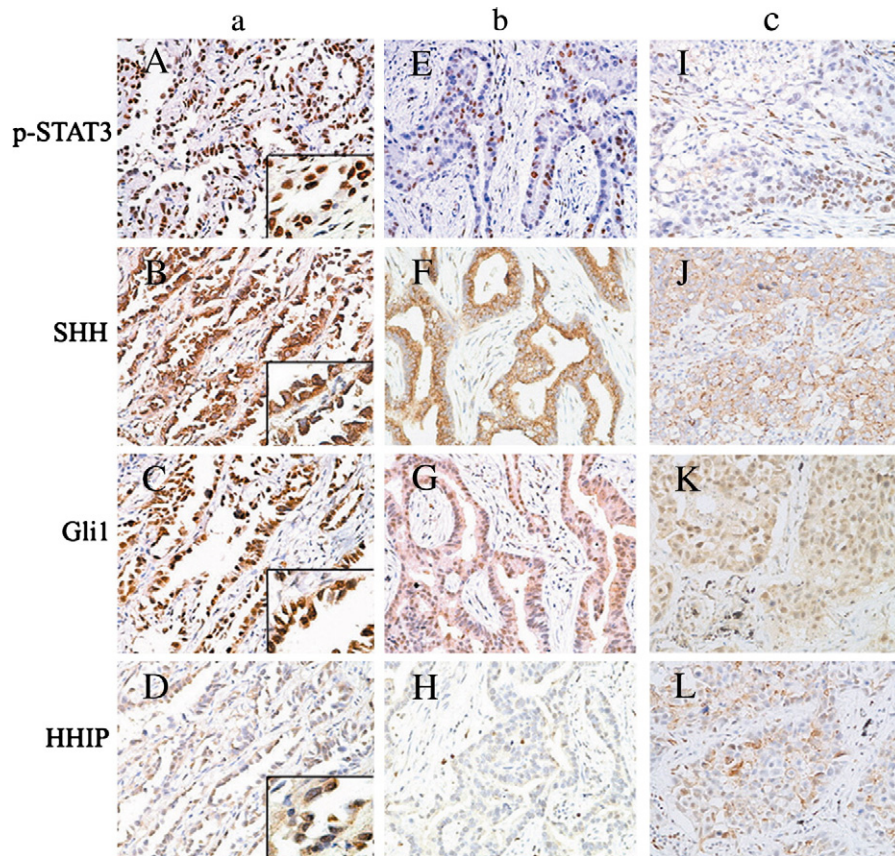


Fig. 2. Immunohistochemical features in differently differentiated adenocarcinoma tissues (200 $\times$): (a) well-differentiated adenocarcinoma (consecutive sections from A to D) with high nuclear p-STAT3 expression, high cytoplasmic and membranous SHH expression, high cytoplasmic and nuclear Gli1 expression, and low cytoplasmic and membranous HHIP expression. Insets in lower right corner show the subcellular localization of immunostaining at higher magnification (2 $\times$ original objects); (b) moderately differentiated adenocarcinoma (consecutive sections from E to H) with moderate p-STAT3, SHH and Gli1 expression respectively, and negative HHIP expression; (c) poorly differentiated adenocarcinoma with low p-STAT3, SHH and Gli1 expression respectively, and moderate HHIP expression.

Table 4

Correlation of the expression of p-STAT3 and SHH signaling with clinicopathological features in NSCLC.

	N	p-STAT3 expression				P-value	SHH pathway activation			P-value
		0	1	2	3		0	1	2	
		n (%)	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	
NSCLC total cases	87	33 (37.9)	20 (30)	19 (21.8)	15 (17.2)		13 (14.9)	29 (33.3)	45 (51.7)	
Histological type						0.033 ^a				0.000 ^b
Adenocarcinoma	54	13 (24.1)	9 (16.7)	17 (31.5)	15 (27.8)	0.009 ^c	2 (3.7)	14 (25.9)	38 (70.4)	
Squamous	25	12 (48)	11 (44)	2 (8)	0 (0)		5 (20)	13 (52)	7 (28)	
Large cell	8	8 (100)	0 (0)	0 (0)	0 (0)		6 (75)	2 (25)	0 (0)	
Grade						0.000 ^d				0.000 ^d
Grade 1	28	5 (17.9)	3 (10.7)	6 (21.4)	14 (50)		0 (0)	3 (10.7)	25 (89.3)	
Grade 2	31	10 (32.3)	9 (29.0)	11 (35.5)	1 (3.2)		1 (3.2)	15 (48.4)	15 (48.4)	
Grade 3	28	18 (64.3)	8 (28.6)	2 (7.1)	0 (0)		12 (42.9)	11 (39.3)	5 (17.9)	
Stage						0.132 ^d				0.630 ^d
0	4	1 (25)	0 (0)	0 (0)	3 (75)		0 (0)	1 (25)	3 (75)	
I	38	14 (36.8)	7 (18.4)	9 (23.7)	8 (21.1)		8 (21.1)	10 (26.3)	20 (52.6)	
II–IV	45	18 (40)	13 (28.9)	10 (22.2)	4 (8.9)		5 (11.1)	18 (40)	22 (48.9)	
LN metastases						0.563 ^a				0.745 ^b
Yes	44	18 (40.9)	12 (27.3)	10 (22.7)	4 (9.1)		5 (11.4)	17 (38.6)	22 (50)	
No	43	15 (34.9)	8 (18.6)	9 (20.9)	11 (25.6)		8 (18.6)	12 (27.9)	23 (53.5)	
Gender						0.160 ^a				0.224 ^b
Male	58	25 (43.1)	16 (27.6)	9 (15.5)	8 (13.8)		10 (17.2)	20 (34.5)	28 (48.3)	
Female	29	8 (27.6)	4 (13.8)	10 (34.5)	7 (24.1)		3 (10.3)	8 (27.6)	18 (62.1)	
Age						0.807 ^a				0.733 ^b
<58	41	15 (36.6)	11 (26.8)	8 (19.5)	7 (17.1)		6 (14.6)	13 (31.7)	22 (53.6)	
≥58	46	18 (39.1)	9 (19.6)	11 (23.9)	8 (17.4)		7 (15.2)	16 (34.8)	23 (50)	
Pathway activation						0.000 ^d				
No	13	10 (76.9)	3 (23.1)	0 (0)	0 (0)		–	–	–	–
Low	29	14 (48.3)	12 (41.4)	3 (10.3)	0 (0)		–	–	–	
High	45	9 (20)	5 (11.1)	16 (35.6)	15 (33.3)		–	–	–	

^a Compared the positive p-STAT3 expression rate by chi-square test.^b Compared the high SHH pathway activation (2+) rate by chi-square test.^c Compared the high p-STAT3 expression rate in different groups by chi-square test.^d Analyzed by Spearman's test.**Table 5**

Correlation of the expression of p-STAT3 and ligand-dependent activation of SHH Pathway with clinicopathological features of lung adenocarcinoma and the precursor lesion AAH in resection specimens.

	N	p-STAT3 expression*				P-value	SHH pathway activation*			P-value
		0	1	2	3		0	1	2	
		n (%)	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	
Normal lung tissues	20	20 (100)	0 (0)	0 (0)	0 (0)	0.000 ^{a1}	20 (100)	0 (0)	0 (0)	0.000 ^{a3}
AAH	12	1 (8.3)	1 (8.3)	0 (0)	10 (83.3)	0.001 ^{a2*}	0 (0)	2 (16.7)	10 (83.3)	0.580 ^{a4*}
Adenocarcinoma	54	13 (24)	9 (16.7)	17 (31.5)	15 (27.8)		2 (3.7)	14 (25.9)	38 (70.3)	
Histological type										
AIS	4	1 (25)	0 (0)	0 (0)	3 (75)	0.611 ^{a2**}	0 (0)	1 (25)	3 (75)	0.586 ^{a4**}
MIA	5	1 (20)	0 (0)	1 (20)	3 (60)	0.585 ^{a2#}	0 (0)	1 (20)	4 (80)	0.011 ^{a4#}
Lepidic pred.	7	1 (14.3)	0 (0)	0 (0)	6 (85.7)	0.000 ^{a2##}	0 (0)	0 (0)	7 (100)	
Acinar pred.	16	0 (0)	4 (25)	11 (68.8)	1 (6.2)		0 (0)	3 (18.8)	13 (81.2)	
Papillary pred.	9	3 (33.3)	1 (11.1)	3 (33.3)	2 (22.2)		0 (0)	3 (33.3)	6 (66.7)	
Micropapillary pred.	3	3 (100)	0 (0)	0 (0)	0 (0)		1 (33.3)	2 (66.7)	0 (0)	
Solid pred.	10	4 (40)	4 (40)	2 (20)	0 (0)		1 (10)	4 (40)	5 (50)	
Grade						0.000 ^b				0.000 ^b
Grade1	25	3 (12)	2 (8)	6 (24)	14 (56)		0 (0)	2 (8)	23 (92)	
Grade 2	17	3 (17.6)	4 (23.5)	9 (52.9)	1 (5.9)		0 (0)	6 (35.3)	11 (64.7)	
Grade3	12	7 (58.3)	3 (25)	2 (16.7)	0 (0)		2 (16.7)	6 (50)	4 (33.3)	
Stage						0.039 ^b				0.629 ^b
0	4	1 (25)	0 (0)	0 (0)	3 (75)		0 (0)	1 (25)	3 (75)	
I	23	4 (17.4)	4 (17.4)	7 (30.4)	8 (34.8)		1 (4.3)	5 (21.7)	17 (73.9)	
II–IV	27	8 (29.6)	5 (18.5)	10 (37)	4 (14.8)		1 (3.7)	8 (29.6)	18 (66.7)	
LN metastases						0.05 ^{a2}				0.860 ^{a4}
No	28	5 (17.9)	5 (17.9)	7 (25)	11 (39.2)		1 (3.6)	7 (25)	20 (71.4)	
Yes	26	8 (30.8)	4 (15.4)	10 (38.5)	4 (15.4)		1 (3.8)	7 (26.9)	18 (69.2)	
Gender						0.761 ^{a2}				0.551 ^{a4}
Male	27	7 (25.9)	5 (18.5)	7 (25.9)	8 (29.6)		1 (3.7)	6 (22.2)	20 (74.1)	
Female	27	6 (22.2)	4 (14.8)	10 (37)	7 (25.9)		1 (20.7)	8 (51.7)	18 (20.7)	

Abbreviations: AAH, atypical adenomatous hyperplasia; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; Pred., predominant.

^{a1}, ^{a3} Compared the p-STAT3 expression rate and SHH pathway activation rate between normal lung tissue, AAH and adenocarcinoma.^{a2} Compared the high p-STAT3 expression rate in different groups by chi-square test (^{a2*} compared AAH with adenocarcinoma; ^{a2**} compared AIS&MIA with AAH; ^{a2#} compared AIS&MIA with lepidic predominant type of invasive adenocarcinoma; ^{a2##} compared lepidic predominant type with other invasive adenocarcinoma types).^{a4} Compared the high activation rate in different groups by chi-square test (^{a4*} compared AAH with adenocarcinoma; ^{a4**} compared AIS&MIA with AAH; ^{a4#} compared micropapillary predominant and solid predominant types with other adenocarcinoma types); ^b Analyzed by Spearman's test.* Correlation analysis showed that p-STAT3 protein expression level was correlated positively with ligand-dependent activation level of SHH signaling in AAH ($r_s = 0.996$, $P = 0.000$) and adenocarcinoma ($r_s = 0.585$, $P = 0.000$).

pathway activation level and tumor differentiation ($r_s=0.624$, $P=0.000$; Table 4).

Aberrant reactivation of ligand-dependent SHH signaling is an early event in carcinogenesis of lung adenocarcinoma

Since our results showed SHH signaling played a more active role in lung adenocarcinoma than in other histological types of NSCLC, we further studied the SHH pathway activation in adenocarcinomas. In the different histological subtypes (IASLC/ATS/ERS classification of lung adenocarcinoma in 2011), we detected high SHH pathway activation in most AIS and MIA cases, which did not show significantly difference in the high activation rate from some subtypes of invasive adenocarcinomas such as lepidic predominant type, but higher than micropapillary predominant and solid predominant types, and the high activation rates in the latter two types were lower than that in the other adenocarcinoma subtypes ($\chi^2=6.467$, $P=0.011$; Table 5). Moreover, the high SHH pathway activation level correlated significantly with well-differentiated histological grade ($r_s=0.513$, $P=0.000$). We next examined the expression of SHH signaling molecules in 12 atypical adenomatous hyperplasia (AAH) foci adjacent to the corresponding adenocarcinomas. Similar SHH, Gli1 and HHIP protein expression pattern was observed in these precursor samples when compared with adenocarcinomas. Most AAH samples showed high SHH and Gli1 protein expression and weak HHIP staining in the atypical hyperplastic epithelia (Figs. 3D,E,F), and the high

pathway activation rate in AAH showed no significant difference from that in AIS and MIA ($P=0.586$; Table 5). These findings indicated that aberrant activation of SHH signaling pathway was an early event in carcinogenesis of lung adenocarcinoma.

Abnormal expression of p-STAT3 in NSCLC and AAH

Nuclear staining of p-STAT3 was present in 54 (62.1%) cases in all NSCLC samples (Fig. 2; Table 4), but not in the adjacent normal lung tissues. The p-STAT3 expression was associated with histological type, no positive p-STAT3 expression was observed in large cell carcinoma cases, and both p-STAT3 positive expression rate and high expression rate were higher in adenocarcinoma than those in squamous cell carcinomas ($\chi^2=4.522$, $P=0.033$; $\chi^2=6.861$, $P=0.009$; Table 4). The other immunohistochemical stain results of NSCLC tissues were detailed in Table 4. The expression of p-STAT3 in adenocarcinoma and AAH was further investigated. Most cases of AAH, AIS and MIA showed high expression of p-STAT3 with the rate of 83.3%, 75% and 60% respectively, and there were no significant difference between them. In invasive adenocarcinomas, the cases of lepidic predominant type presented higher p-STAT3 high expression rate than the other subtypes ($\chi^2=17.774$, $P=0.000$); Whereas no significant difference in high expression rate was found between this subtype and AIS, MIA (Figs. 4A,C). In addition, the high expression of p-STAT3 in adenocarcinoma was found to be correlated significantly with low-grade ($r_s=0.587$, $P=0.000$; Figs. 2A,E,I) and early-stage cancers ($r_s=0.282$, $P=0.039$; Table 5; Fig. 4A). There

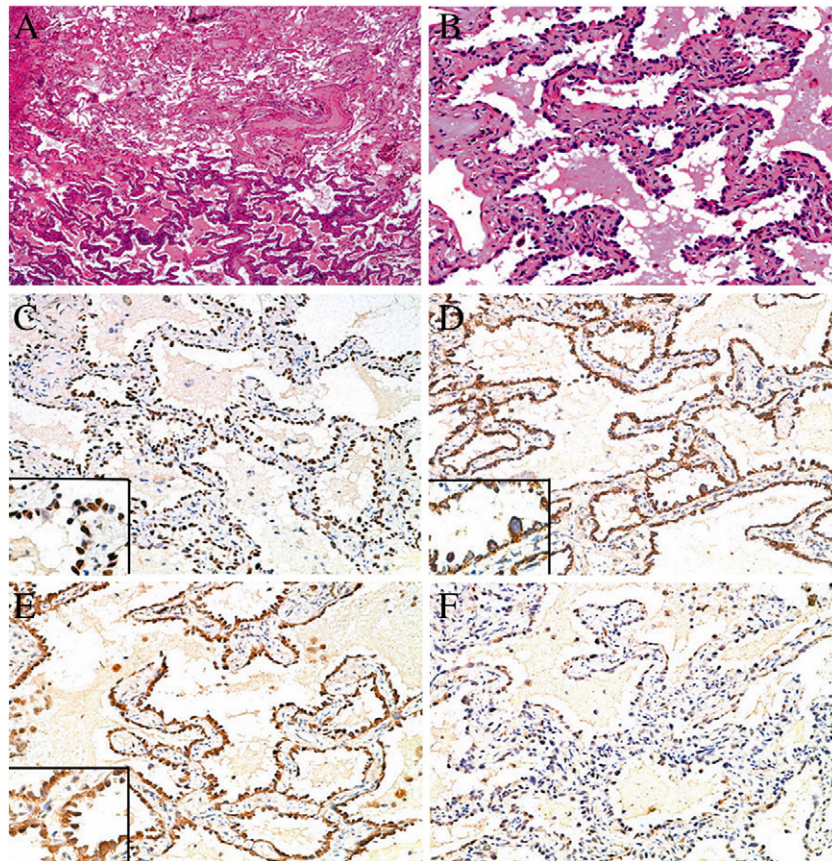


Fig. 3. Histologic and immunohistochemical features of atypical adenomatous hyperplasia (AAH, consecutive sections). (A) Hematoxylin and Eosin stain section of AAH (40 $\times$); (B) Hematoxylin and Eosin stain section of AAH (200 $\times$); (C) AAH showing strong nuclear positive stain for p-STAT₃ (200 $\times$); (D) AAH showing strong cytoplasmic and membranous positive stain for SHH (200 $\times$) in the atypical hyperplastic epithelia; (E) AAH showing strong cytoplasmic and nuclear positive stain for Gli1 (200 $\times$) in the atypical hyperplastic epithelia; (F) AAH showing weak cytoplasmic and membranous positive stain for HHIP (200 $\times$) in the atypical hyperplastic epithelia. For C, D, and E, insets in lower left corner show the subcellular localization of immunostaining at higher magnification (2 $\times$ original objects).

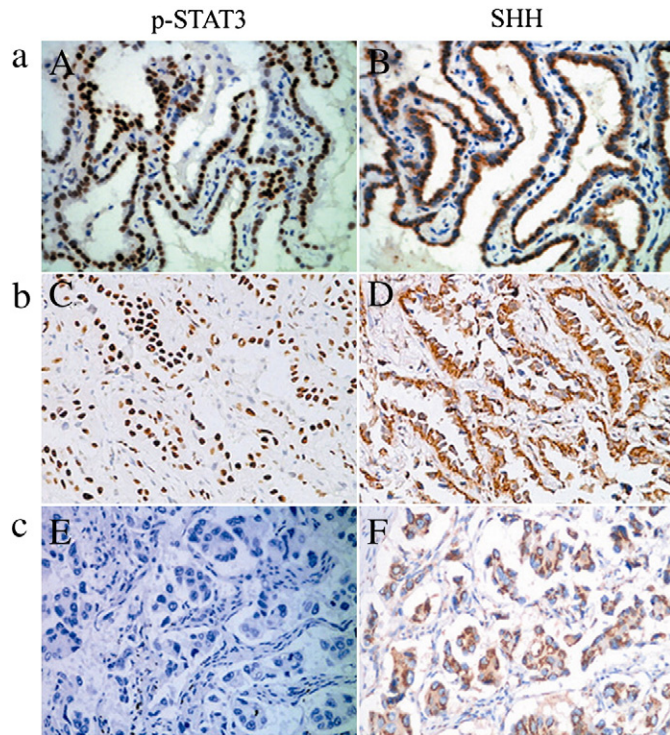


Fig. 4. The correlative expression between p-STAT3 and SHH in several subtypes of lung adenocarcinoma. (a) adenocarcinoma in situ (AIS, 400 $\times$); (b) lepidic predominant adenocarcinoma (200 $\times$); (c) Micropapillary predominant adenocarcinoma (200 $\times$). Serial sections in a and b show high p-STAT3 and SHH co-expression, and negative p-STAT3 and low SHH expression are presented in serial section c.

was no significant correlation between p-STAT3 expression and the other clinicopathological parameters (Table 5).

The correlation between STAT3 activation and aberrant SHH signaling pathway in lung adenocarcinoma and AAH

The significant positive correlation between p-STAT3 and SHH expression was found in adenocarcinoma ($r_s = 0.279$, $P = 0.041$; Figs. 2,4), not in squamous and large cell carcinoma respectively. Furthermore, the expression level of p-STAT3 protein presented a positive correlation with activation level of ligand-dependent SHH signaling ($r_s = 0.585$, $P = 0.000$) in adenocarcinoma. In AAH, we also demonstrated such markedly similar correlation between p-STAT3 expression and SHH pathway activation ($r_s = 0.996$, $P = 0.000$). The detailed datum else were summarized in Table 5.

Activation of STAT3 upregulates SHH gene expression through TTF-1

To further explore the mechanisms underlying the positive correlation between STAT3 and SHH, transient cotransfection was performed on transcriptional stimulation of SHH promoters by transcriptional factor STAT3 in H441 cells. But we found that STAT3 failed to up-regulate SHH gene expression directly (Fig. 5A). In our previous studies, we detected up-regulation of both SHH and TTF-1 genes in STAT3-overexpressing transgenic mice. In addition, STAT3 is co-localized in alveolar type II cells with both TTF-1 and SHH. To further assess if the SHH or TTF-1 promoter is under the control of STAT3, transient transfection experiments were performed on transcriptional stimulation of TTF-1 promoters by transcriptional factor STAT3. Interestingly, we found transcriptional factor STAT3 showed direct stimulation on the TTF-1 promoter (Fig. 5A). Furthermore, TTF-1 transcriptional factor showed direct stimulation on the SHH promoter (Fig. 5B). Therefore, we conclude

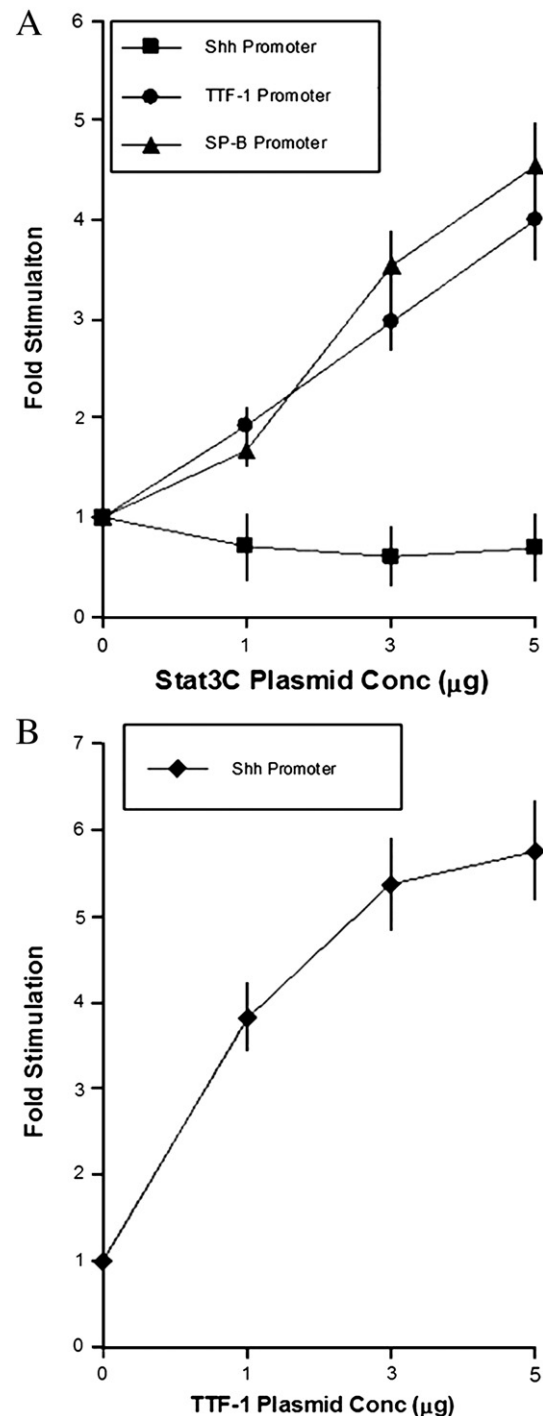


Fig. 5. (A) Respiratory adenocarcinoma epithelial H441 cells were transfected with the Shh or TTF-1 luciferase reporter gene construct (0.25 µg/well) and Stat3C mammalian expression plasmid at concentrations as indicated. Luciferase activity was measured 72 h after transfection. Luciferase activity of the Shh or TTF-1 luciferase reporter gene alone was defined as 1. Values were determined and expressed as means $\pm$ SD ($n = 3$). The SP-B 500 bp luciferase reporter gene was used as a positive control for Stat3C as previously reported by our group. In this experiment, Stat3C stimulated the TTF-1 promoter, but failed to stimulate the Shh promoter. (B) In a separate experiment, H441 cells were transfected with the Shh luciferase reporter gene construct (0.25 µg/well) and the TTF-1 mammalian expression plasmid at concentrations as indicated. TTF-1 showed direct stimulation on the Shh promoter. At least we can conclude that Stat3C is able to up-regulate Shh gene expression through TTF-1 indirectly. In both experiments (A) and (B), the CMV-galactosidase plasmid was included for normalization. Triplicate transfections were carried out for each experiment.

that STAT3 can up-regulate SHH gene expression through TTF-1 indirectly (Fig. 5).

Discussion

As a highly conserved developmental signal pathway, the SHH signaling is critical for lung development and its aberrant reactivation has been implicated in injury responses and cancer formation (van Tuyl and Post, 2000) (Watkins et al., 2003). It is important to clarify the role of reactivation of this pathway in the process of carcinogenesis and progression in NSCLC. In this study we showed that the active SHH ligand expression was increased in all NSCLCs, particularly in adenocarcinoma. It is known that Gli1 is a target gene transcript of SHH signaling as well as the key transcriptional effector of this pathway, which can be used as a powerful indicator of SHH signaling activity (Kubo et al., 2004; Liao et al., 2009). The previous reports regarding the role of this pathway reactivation in NSCLC have been controversial (Chi et al., 2006; Yuan et al., 2007), in the present study we confirmed that SHH signaling was activated in most NSCLC cases, and more than half of them presented high level activation indicating dysregulation of SHH signaling may play an important role in NSCLC carcinogenesis. In addition, we demonstrated that Gli1 expression was positively correlated with SHH expression, which is in keeping with a recent report by Raz et al. (2011), suggesting the aberrant SHH signaling reactivation was to a large extent due to ligand-dependent mechanism in NSCLC.

HHIP is a natural antagonist of SHH signaling, and the availability of the SHH ligand for signaling is regulated by the expression of HHIP on the cell surface of SHH responsive cell. HHIP itself is also a general transcriptional target of hedgehog signaling. Thus a negative regulatory feedback loop could be established in this way modulating the SHH signaling. Some studies have reported a down-regulation of HHIP in hepatocellular carcinoma (Tada et al., 2008). In our study we detected frequent HHIP expression in NSCLC but most cases presented widely reduced HHIP expression contrary to extensively high Gli1 expression, reflecting collapsed negative feedback of SHH signaling as well as reduced inhibition of SHH ligand, which could cause the signaling dysregulation and lead to carcinogenesis. Such frequent low HHIP expression in NSCLC could be due to epigenetic or genetic alterations and the underlying mechanisms need further exploration (Tada et al., 2008).

Although frequent reactivation of SHH pathway was observed in the NSCLC sample, different histological types of NSCLC showed significantly different activation level. We noted that SHH signaling obviously played more active role in adenocarcinoma. As our primary results in this study showed that either the activation rate or the high level activation rate was highest in lung adenocarcinoma, then we investigated this pathway more detailedly in adenocarcinoma. Besides ligand-dependent SHH signaling high level activation correlated with well-differentiated tumor histology and early-stage subtypes in adenocarcinoma, we demonstrated that AAH, a putative precancerous lesion, also exhibit reactivation of SHH signaling. Such findings presented here indicated that aberrant ligand-dependent SHH signaling activation was an early event in carcinogenesis of lung adenocarcinoma, as it has been previously reported in other tumors (Liao et al., 2009).

We also detected the STAT3 pathway by the protein expression of p-STAT3 which can be considered as the indicator for this pathway. In our current study, STAT3 pathway showed to be most active in adenocarcinoma since both p-STAT3 positive expression rate and high expression rate were highest in adenocarcinoma of all the NSCLC types. We also found the high expression of p-STAT3 in adenocarcinoma was correlated significantly with low-grade and early-stage cancers. Moreover, increased expression of p-STAT3 was still observed in AAH, but not in normal lung parenchyma, indicating the STAT3

pathway activation also acts as an early event of carcinogenesis of lung adenocarcinoma, just as SHH signaling does.

We then correlated the protein expression of p-STAT3 and activation of SHH signaling in lung adenocarcinoma and AAH. In our immunostaining study, p-STAT3 protein expression level was correlated positively with both SHH expression and ligand-dependent activation level of SHH signaling in adenocarcinoma and AAH, which suggested the internal relationship between the two pathways. The interesting interaction between them has been reported by Sengupta et al. in chronic myeloid leukemia (Sengupta et al., 2007), but we know little about that in lung cancer before. Based on our previous study showing the up-regulation of SHH gene in STAT3C transgenic mice (Li et al., 2007), in this study we speculated that the activation of transcriptional factor STAT3 could upregulate SHH gene expression and thereby activate ligand-dependent SHH pathway resulting in lung tumor cell ontogeny. However, our first cotransfection of STAT3c and SHH construct did not find the direct up-regulation as we expected, which suggests that an indirect regulation mechanism may be responsible. As our previous research also showed the up-regulation of TTF-1 gene besides SHH gene in STAT3C transgenic mice, our following cotransfection assays demonstrate that transcriptional factor TTF-1 acted as a bridge mediating the STAT3-induced up-regulation of SHH gene expression. Combining the current above-mentioned findings, we thought this regulation mechanism might particularly function in early-stage carcinogenesis, and more research on interaction pattern between the two pathways is worth doing in future.

Regarding the squamous and large cell carcinoma, both STAT3 and SHH pathway showed low and even no activation in most cases, which indicated diverse mechanisms involved in carcinogenesis of different NSCLC subtypes with distinct genetic characteristics, and the more unknown mechanisms need further investigation.

In summary, our study confirmed that aberrant ligand-dependent SHH signaling reactivation occurred frequently in NSCLC. The signaling showed more active in adenocarcinoma, and was an early event in carcinogenesis of lung adenocarcinoma. Furthermore, we revealed that STAT3 activation functioned in inducing the process. The findings may provide rationale to antagonize the interaction of SHH with STAT3 signaling pathway in the prevention and treatment of pulmonary adenocarcinoma.

Conflict of interest statement

This study has no conflicts of interest (both financial and personal).

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