FEBS Letters 589 (2015) 2248–2256

journal homepage: www.FEBSLetters.org

ILT4 drives B7-H3 expression via PI3K/AKT/mTOR signalling and ILT4/B7-H3 co-expression correlates with poor prognosis in non-small cell lung cancer

Pei Zhang [a], Shuwen Yu [b], Hongyu Li [c], Chuanyong Liu [a], Juan Li [a], Wenli Lin [a], Aiqin Gao [a], Linlin Wang [a], Wei Gao [d], Yuping Sun [a,*]

[a] Department of Oncology, Jinan Central Hospital, Shandong University, 105 Jie Fang Road, Jinan, Shandong 250013, PR China
[b] Department of Pharmacy, Jinan Central Hospital, Shandong University, 105 Jie Fang Road, Jinan, Shandong 250013, PR China
[c] Department of Geratology, Jinan Central Hospital, Shandong University, 105 Jie Fang Road, Jinan, Shandong 250013, PR China
[d] Department of Pathology, Jinan Central Hospital, Shandong University, 105 Jie Fang Road, Jinan, Shandong 250013, PR China

ARTICLE INFO

Article history:
Received 2 November 2014
Revised 23 June 2015
Accepted 24 June 2015
Available online 3 July 2015

Edited by W. Ellmeier

Keywords:
ILT4
B7-H3
PI3K/AKT/mTOR signalling
Non-small cell lung cancer

ABSTRACT

Immunoglobulin-like transcript (ILT) 4 is critical for the inhibitory function of certain immune cells. We previously demonstrated that ILT4 is over-expressed in human non-small cell lung cancer (NSCLC) cells and is involved in tumour evasion via an unknown mechanism. In this report, we demonstrate that ILT4 increases the expression of the co-inhibitory molecule B7-H3 through PI3K/AKT/mTOR signalling. In primary human NSCLC tissues, a significant positive relationship is observed between ILT4 and B7-H3 expression. ILT4/B7-H3 co-expression is significantly associated with a reduction in T infiltrating lymphoid cells and lower overall survival. In summary, ILT4 increases B7-H3 expression and ILT4/B7-H3 co-expression may be involved in NSCLC progression.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) accounts for 80–85% of all lung cancers [2]. Despite notable efforts to develop novel therapeutic strategies, overall 5-year survival among NSCLC patients is approximately 17%. This survival rate is significantly lower than those of the vast majority of malignant carcinomas, due in part to diagnosis at an advanced stage of metastasis [3,4]. Therefore, a better understanding of the molecular mechanisms that contribute to NSCLC progression and metastasis is critical for conquering this disease.

Immunoglobulin-like transcript (ILT) 4 belongs to the family of immunoglobulin-like transcripts and is mainly expressed in monocytes, dendritic cells (DCs) and endothelial cells [5]. ILT4 induces a tolerogenic phenotype of human DCs, consequently inducting immunosuppressive T cells and inhibiting T cell activation and proliferation [6,7]. ILT4 is over-expressed in leukaemia, breast cancer and NSCLC cells [8–11]. In mouse acute myeloid leukaemia (AML) transplantation models, the ILT4 mouse orthologue, paired immunoglobulin-like receptor (PIRB), supports AML development by inhibiting the differentiation of leukaemia cells [11]. We have previously demonstrated that ILT4 is significantly correlated with a reduction in T infiltrating lymphoid cells (TILs) in breast cancer and NSCLC and with IL-10 levels in breast cancer [9,10], suggesting an inhibitory function of ILT4 in tumour immunity. However, the molecular mechanisms underlying the involvement of ILT4 in the tumour immune response remain poorly understood.

Inhibitory members of the B7 family are over-expressed in multiple cancers and contribute to tumour immune evasion by inhibiting T cell activation [12–14]. Among them, B7-H3 is an adverse prognostic biomarker and has been implicated in NSCLC progression by inhibiting T cell function and inducing the transformation of monocytes into tumour-associated macrophages [15–18]. Tumour immune evasion plays an important role in promoting tumour progression and metastasis, and this tumourigenic function may require interactions among tumour-derived immune molecules [19]. Considering the similar functions of ILT4 and B7-H3 in

Author contributions: Y.S. conceived and designed the experiments. P.Z., S.Y., J.L., W.L., A.G. and W.G. performed experiments. Y.S., P.Z., H.L., C.L. and L.W. analyzed data and wrote the manuscript.

* Corresponding author.
E-mail address: 13370582181@163.com (Y. Sun).

http://dx.doi.org/10.1016/j.febslet.2015.06.037

tumour evasion, we hypothesized that there may be a significant connection between ILT4 and B7-H3 in tumour progression.

There is no evidence of a cause-effect relationship between ILT4 and B7-H3 in NSCLC. In the present study, B7-H3 expression was analyzed upon over-expression or silencing of ILT4 in vitro, and the potential signalling affecting ILT4-induced B7-H3 expression was investigated. Furthermore, the co-expression of ILT4 and B7-H3 was evaluated in human NSCLC tissues, and the relationship with clinicopathological features and survival was analyzed. Our results provide a new perspective on the underlying mechanism of NSCLC progression.

2. Materials and methods

2.1. Cell culture

NSCLC cell lines H1650, H226, H1299, H1975 and A549 (Type Culture Collection of the Chinese Academy of Sciences, Beijing,

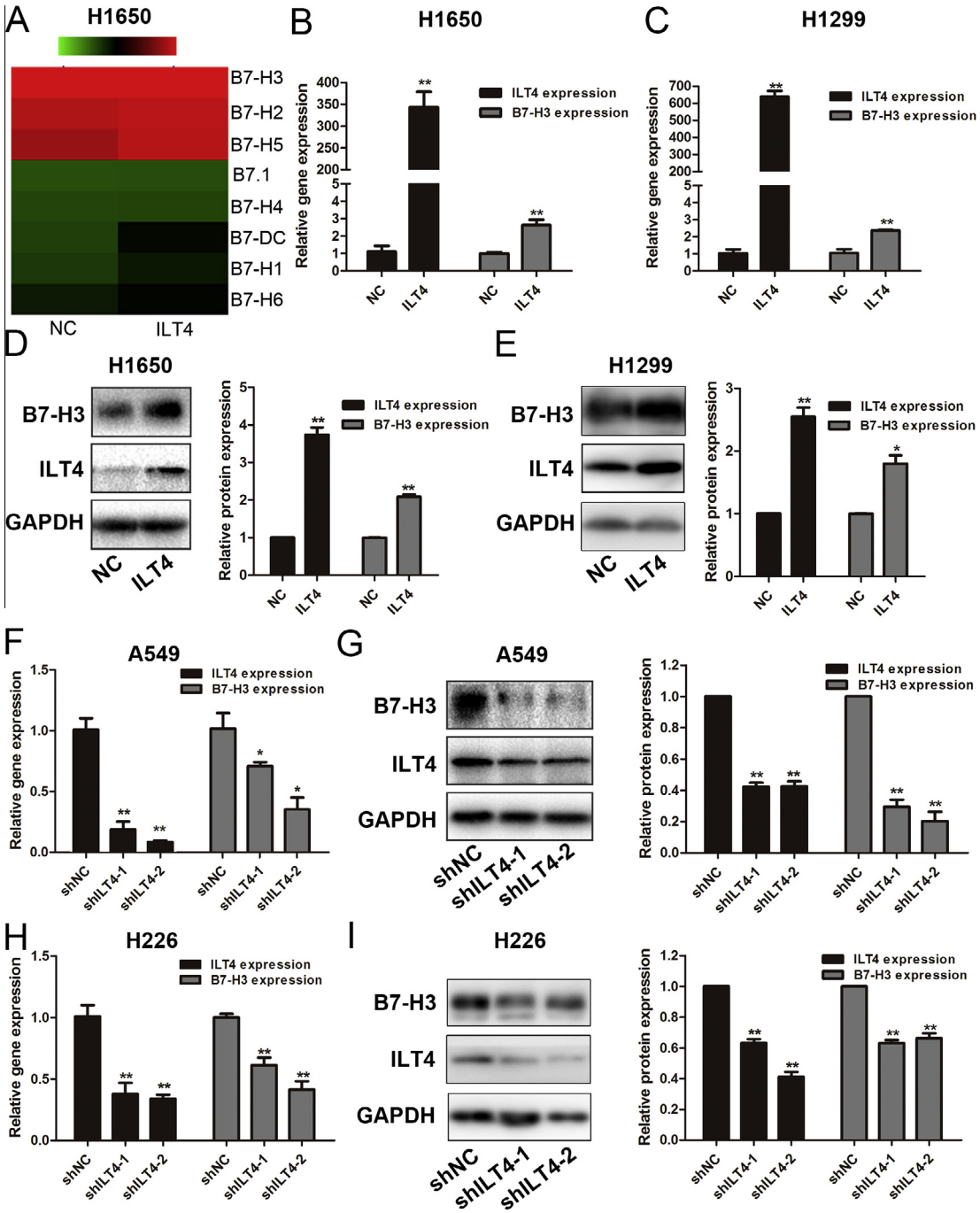

Fig. 1. Effect of ILT4 on the regulation of B7-H3 expression in NSCLC cells. (A) Gene expression levels of the B7 family in H1650 cells transfected with ILT4 or control plasmids. Red: high expression; green: low expression. (B)–(E) ILT4 was over-expressed using ILT4 expression plasmids (ILT4 vector), and the expression of B7-H3 in H1650 and H1299 cells was assayed at both the mRNA and protein levels. NC vector was used as a negative control. (F)–(I) ILT4 was knocked down using ILT4 shRNA (shILT4-1 and shILT4-2) in A549 and H226 cells, and the expression of B7-H3 was assayed at both the mRNA and protein levels. NC shRNA was used as a negative control. These data are representative of three independent experiments. The error bars indicate ±S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test.

China) were obtained and cultured in RPMI-1640 Medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco).

2.2. Patients and samples

A total of 118 primary NSCLC specimens obtained by surgical resection were collected from Jinan Central Hospital Affiliated to Shandong University, China from 2008 to 2013. This study was approved by the review board and ethics committee, and all patients provided written informed consent. Patients (90 men and 28 women with a mean age at diagnosis of 61 years old) without any preoperative therapy were included in the study. Patients from whom samples were collected from 2008 to 2010 were contacted by phone to confirm their health status. The last censor date was September 30, 2014.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using the RNA isolation reagent TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNA was synthesized from total RNA using a reverse transcription kit (Fermentas, Ontario, Canada). Real-time PCR was performed using the ABI7500 sequence detector (Applied Bio systems, Foster City, CA, USA) and ILT4 primers (forward, 5′-GCATCT TGGATTACACGGATACG-3′; reverse, 5′-CTGACAGCCATATCGCCCT G-3′), B7-H3 primers (forward, 5′-GGCAGCCTATGACATTCCCC-3′; reverse, 5′-GTCTTGGAGCCTTCTCCCTC-3′), and GAPDH primers (forward, 5′-AGAAGGCTGGGGCTCATTTG-3′, reverse, 5′-AGGGGCC ATCCACAGTCTTC-3′). ILT4 and B7-H3 mRNA expression were normalized to GAPDH expression. Gene expression for real-time PCR was calculated as the change relative to the control ($2^{-\Delta\Delta Ct}$).

2.4. Western blot analysis

Total protein was separated and then transferred onto polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membranes were incubated with the following primary antibodies: anti-ILT4 mAb (1:400; Abgent, San Diego, CA, USA), anti-B7-H3 pAb (1:500; Epitomics, Burlingame, CA, USA), anti-phospho-ERK pAb (1:1000; Epitomics), anti-ERK pAb (1:1000; Cell Signalling Technology, Danvers, MA, USA), anti-phospho-AKT pAb (1:1000; Epitomics), anti-AKT pAb (1:1000; Epitomics), anti-phospho-mTOR pAb (1:1000; Cell Signalling Technology), anti-mTOR pAb (1:1000; Cell Signalling Technology) and anti-GAPDH pAb (1:10000; Proteintech Group,

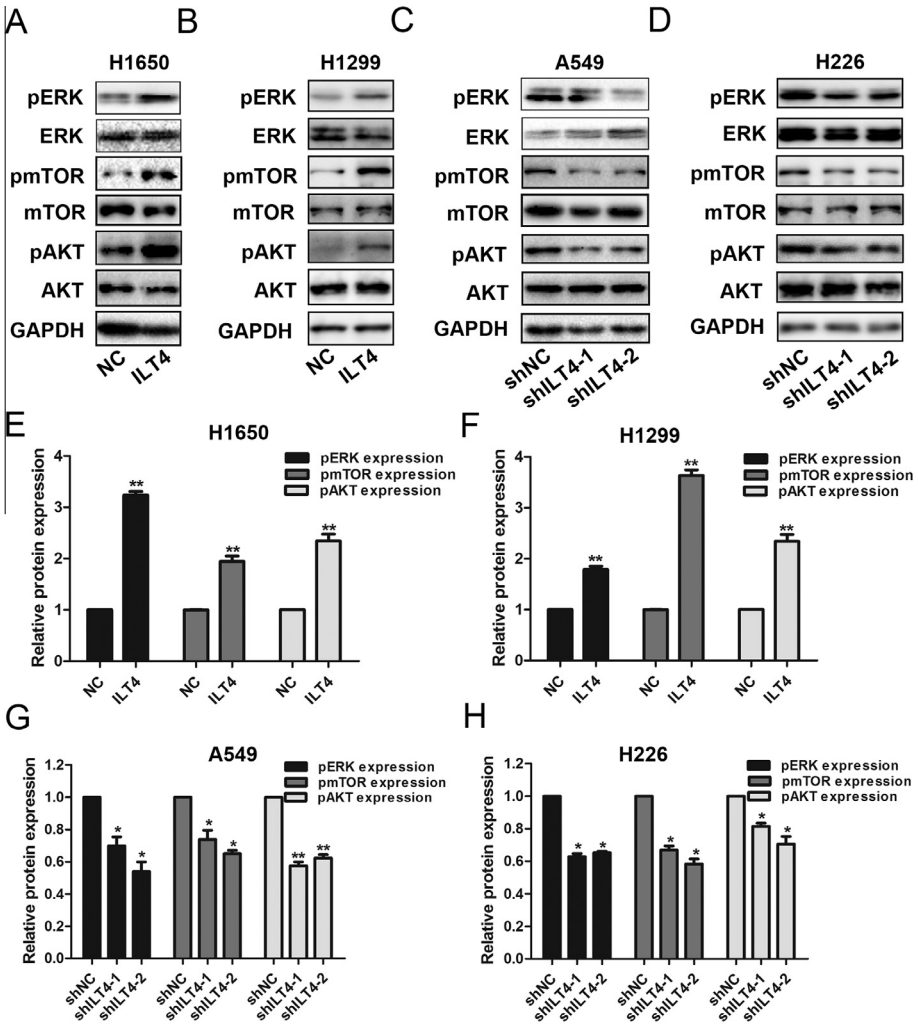

Fig. 2. ILT4 activates the PI3K/AKT/mTOR signalling pathway. Increased protein levels of pERK, pAKT and pmTOR were observed when ILT4 was up-regulated in H1650 (A and E) and H1299 cells (B and F) compared with negative control cells. Decreased protein levels of pERK, pAKT and pmTOR were observed when ILT4 was knocked down in A549 (C and G) and H226 (D and H) cells compared with negative control cells. These data are representative of three independent experiments. The error bars indicate ±S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test.

Inc., Wuhan, China). Blots were developed in ECL reagent (Beyotime Institute Biotechnology, Nantong, China) and imaged using a ChemiDoc™ XRS$^+$ system (Bio-RAD, Hercules, CA, USA).

2.5. Cell transfection

An expression plasmid containing ILT4, Pez-lv105-ILT4 (ILT4), and a negative control plasmid, Pez-lv105 (NC) (GeneCopoeia, Rockville, MD, USA), were transfected into H1650 and H1299 cells. Knockdown of ILT4 expression was achieved by transfecting pGPU6/GFP/Neo-shILT4-1 (shILT4-1) or pGPU6/GFP/Neo-shILT4-2 (shILT4-2) (Genechem Co. Shanghai, China) into A549 and H226 cells. A plasmid containing a non-targeting hairpin (shNC) was used as the negative control. The following shRNA sequences were used: shILT4-1, 5′-GAAGAAGAACACCCACAATGC-3′; shILT4-2, 5′-GCTATGGTTATGACTTGAACT-3′; and shNC, 5′-GTTCTCCGAACGTGTCACGT-3′. A total of 2×10^5 cells were transfected with 2 μg of plasmid using X-treme GENE HP Reagent (Roche, Basel, Switzerland) in 6-well plates. The cells were collected for RT-PCR and Western blot 36 h and 48 h after transfection, respectively. The transfection efficiency was determined by detecting ILT4 expression using RT-PCR and Western blot.

2.6. Signal transduction inhibitor treatment

Cells were incubated in 6-well plates for 24 h after transfection and then cultured with medium containing the ERK inhibitor U0126 (Selleck, Houston, TX, USA), the AKT inhibitor LY294002 (Selleck) or the mTOR inhibitor rapamycin (Alexis, Lausen, Switzerland) for 24 h. Cellular proteins were extracted and subjected to Western blot analysis.

2.7. Gene expression profiling

RNA from the NSCLC cell line H1650 transfected with ILT4 and control plasmids was harvested using TRIzol reagent 40 h after transfection. Microarray analysis was performed to visualize differences in gene expression between the H1650 cells transfected with ILT4 or the control plasmid. The mRNA was amplified and labelled using an Agilent Quick Amp labelling kit (Agilent Technologies, Palo Alto, CA, USA), and the probes were hybridized using a Whole Human Genome Oligo Microarray (Agilent Technologies). The processed slides were scanned with an Agilent DNA microarray scanner, and the data were collected and analyzed using the Agilent Feature Extraction software (version 11.0.1.1) and the GeneSpring GX software (version 11.5.1).

2.8. Immunohistochemistry analysis

Sections with a thickness of 4 μm were prepared for immunohistological staining. The sections were antigen retrieved using citric acid buffer with heating in a microwave oven for 10 min. The sections were then incubated overnight at 4 °C with anti-ILT4 mAb (1:400; Abgent), anti-B7-H3 pAb (1:200; Epitomics), anti-pAKT pAb (1:150; Epitomics) or anti-CD45RO mAb (1:100; Abcam, Cambridge, MA, USA). The sections were incubated with Enhanced primer and HRP goat anti-mouse/rabbit IgG polymer using the Elivision™ plus Polymer HRP (Mouse/Rabbit) IHC Kit (MXB) and visualized with 3,3′-diaminobenzidine solution (MXB).

Immunohistochemical assays were analyzed simultaneously by two independent investigators. The sections were scored as positive when the tumour cells exhibited positive staining in the membrane, cytoplasm, and/or nucleus. The proportion score represented the estimated fraction of positive tumour cells (0 = none; 1 = less than 25%; 2 = 25–75%; 3 = greater than 75%). The intensity score represented the estimated average staining intensity of positive tumour cells (0 = none; 1 = weak; 2 = intermediate, 3 = strong). The overall amount of protein present was then expressed as the total score of tumour cell (ranges = 0–9, respectively). Positive scores were defined as $\geqslant 4$, and negative scores were defined as <4. To evaluate the infiltration of TILs, we counted the number of CD45RO$^+$ cells per 1000 total nuclei as described previously [20].

2.9. Statistical analysis

SPSS version 19.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data were represented as means ± S.E.M. (standard error of the mean). The associations between the expression of

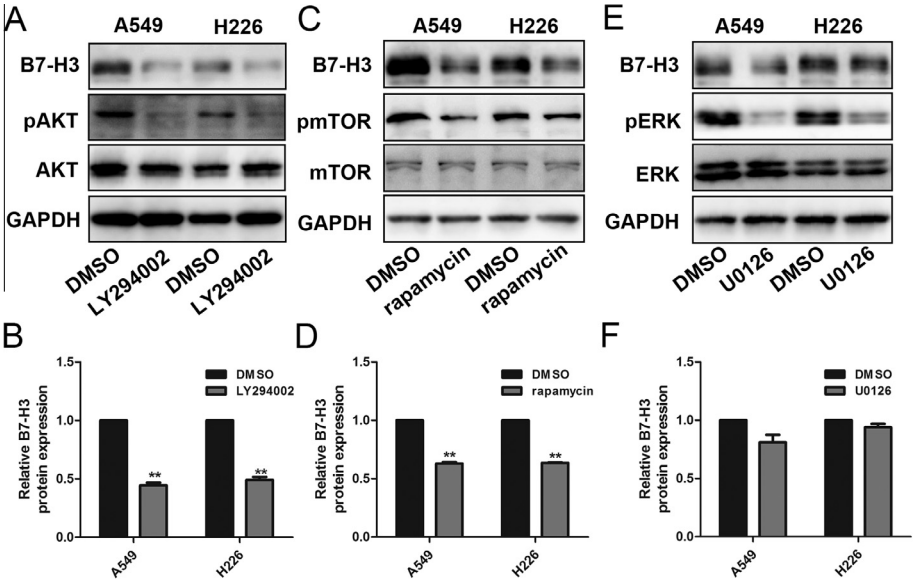

Fig. 3. PI3K/AKT/mTOR signalling influences B7-H3 expression in NSCLC cell lines. (A) and (B) Protein expression of B7-H3 in A549 and H226 cells after treatment with the AKT inhibitor LY294002 compared with DMSO-treated cells. (C) and (D) Protein expression of B7-H3 in A549 and H226 cells after treatment with the mTOR inhibitor rapamycin compared with DMSO-treated cells. (E) and (F) Protein expression of B7-H3 in A549 and H226 cells after treatment with the ERK inhibitor U0126 compared with DMSO-treated cells. These data are representative of three independent experiments. The error bars indicate ±S.E.M. of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test.

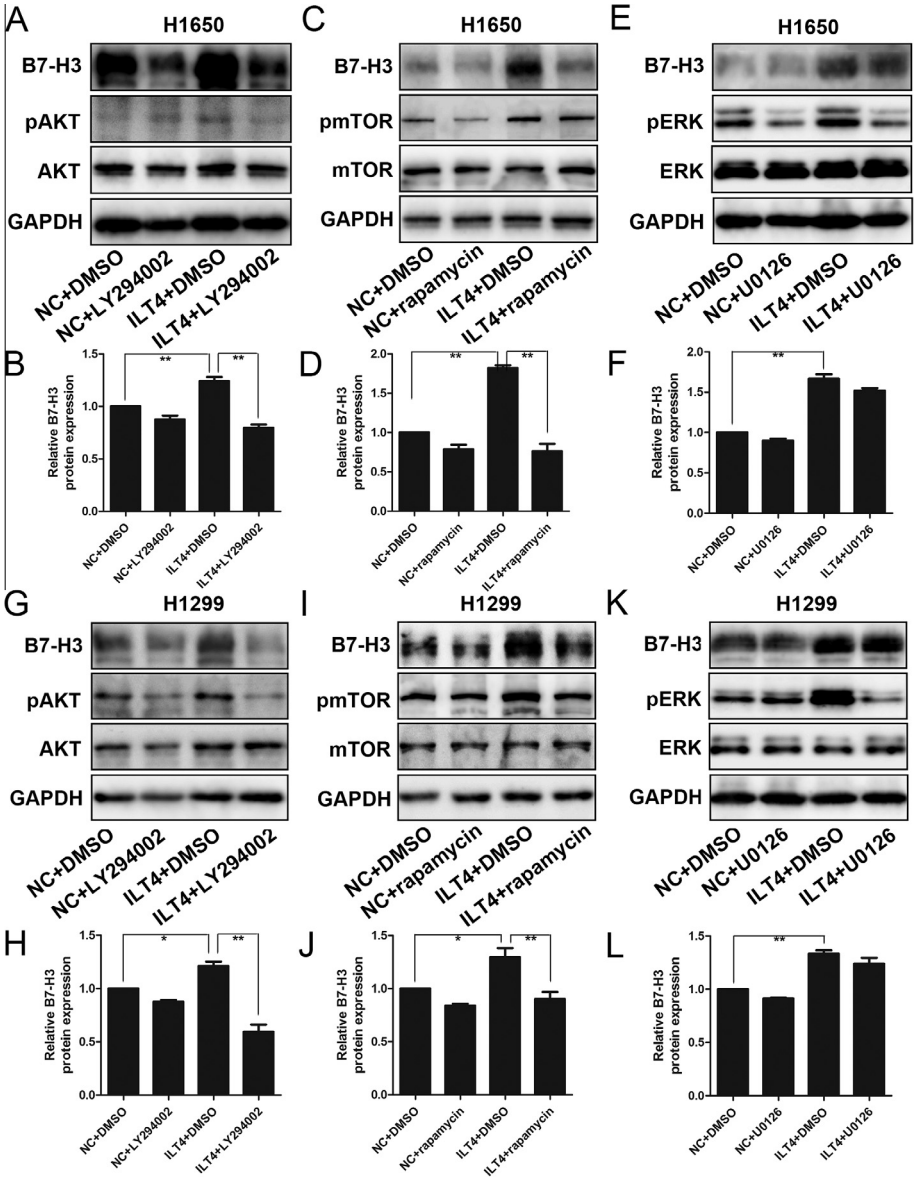

Fig. 4. ILT4 over-expression up-regulates B7-H3 expression via PI3K/AKT/mTOR signalling in NSCLC cells. Protein expression of B7-H3 in ILT4 over-expressing H1650 (A and B) and H1299 cells (G and H) after treatment with the AKT inhibitor LY294002 compared with control cells. Protein expression of B7-H3 in ILT4 over-expressing H1650 (C and D) and H1299 cells (I and J) after treatment with the mTOR inhibitor rapamycin compared with control cells. Protein expression of B7-H3 in ILT4 over-expressing H1650 (E and F) and H1299 (K and L) cells after treatment with the ERK inhibitor U0126, compared with control cells. These data are representative of three independent experiments. The error bars indicate ±S.E.M. of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test.

ILT4/B7-H3 or ILT4/pAKT and clinicopathological variables were analyzed using the χ^2 test and the two-tailed Student's *t* test. Survival curves were drawn using the Kaplan–Meier method and compared using the log-rank test. *P* values were considered to indicate significant differences at $P < 0.05$.

3. Results

3.1. ILT4 increases B7-H3 expression in NSCLC cells

To clarify the function and mechanism of tumour-associated ILT4, we performed gene expression profiling and identified several molecules related to the immune response that were altered in response to ILT4 over-expression. Most of the inhibitory members of the B7 family, including B7-H3, were deregulated in ILT4 over-expressing NSCLC cells (Fig. 1A and Supplementary Fig. 1). Thus, we hypothesized that there may be a significant causal relationship between ILT4 and B7-H3.

To confirm this result, we over-expressed ILT4 in H1650 and H1299 cells and knocked down ILT4 in A549 and H226 cells. B7-H3 expression was significantly increased in ILT4 over-expressing H1650 and H1299 cells (Fig. 1B–E and Supplementary Fig. 2) and down-regulated when ILT4 expression was silenced in A549 and H226 cells (Fig. 1F–I).

3.2. ILT4 significantly promoted the phosphorylation of signaling transduction molecules AKT and mTOR

Hyper-activation of PI3K/AKT/mTOR and ERK signalling are the main drivers of tumour progression in the majority of NSCLC. Thus, we evaluated whether ILT4 can activate these signalling cascades. We previously demonstrated that ILT4 can activate ERK signalling.

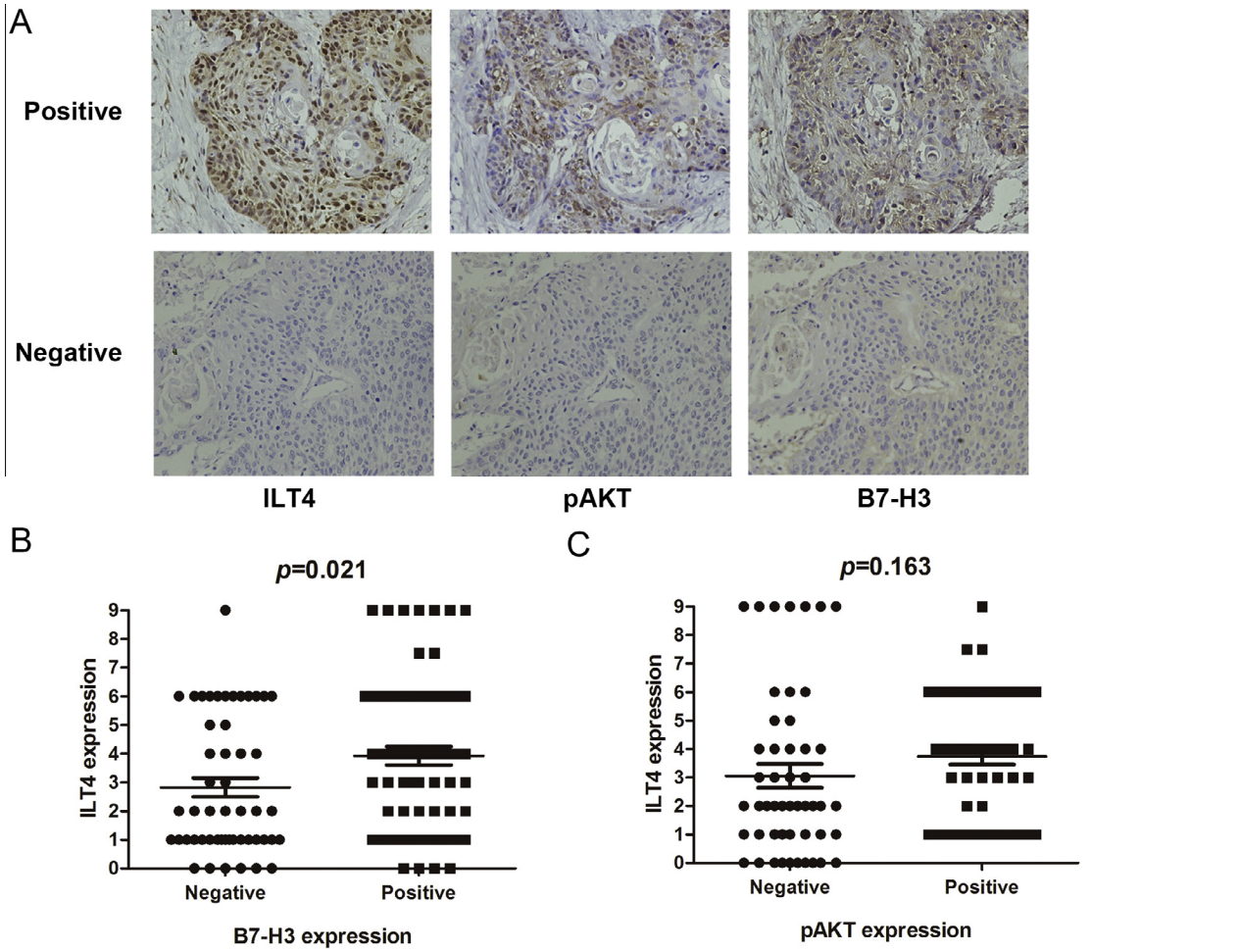

Fig. 5. Co-expression of ILT4 and B7-H3 in primary human NSCLC tissues. (A) Positive co-expression of ILT4, p-AKT and B7-H3 in tumour cells of representative NSCLC specimens (magnification, ×200). (B) ILT4 expression was detected in B7-H3-positive specimens compared with B7-H3-negative specimens. (C) ILT4 expression was detected in pAKT-positive specimens compared with pAKT-negative specimens.

Here, we observed that AKT and mTOR were phosphorylated in response to ILT4 over-expression in H1650 and H1299 cells (Fig. 2A, B, E and F) and un-phosphorylated in response to ILT4 knockdown in A549 and H226 cells (Fig. 2C, D, G and H), suggesting that ILT4 can activate not only ERK signalling but also PI3K/AKT/mTOR signalling.

3.3. Effects of PI3K/AKT/mTOR signaling on B7-H3 expression in NSCLC cells

To investigate the potential effects of PI3K/AKT/mTOR and ERK signalling on B7-H3 expression, A549 and H226 cells with endogenous high B7-H3 expression were treated with inhibitors of AKT (LY294002), mTOR (rapamycin) and ERK (U0126). Compared with the control group, treatment with LY294002 or rapamycin led to a significant decrease in B7-H3 expression in A549 and H226 cells (Fig. 3A–D). However, treatment with U0126 could not significantly influence B7-H3 expression (Fig. 3E and F).

3.4. ILT4 increases B7-H3 expression through PI3K/AKT/mTOR signalling

Next, we assessed the signalling cascade linking ILT4 to B7-H3 up-regulation using inhibitors. Compared with the control, treatment with LY294002 or rapamycin led to a significant decrease in B7-H3 expression in ILT4 over-expressing H1650 and H1299 cells (Fig. 4A–D and G–J). However, treatment with U0126 did not significantly influence B7-H3 expression (Fig. 4E, F, K and L).

3.5. Co-expression of ILT4 and B7-H3 in primary human NSCLC tissues

A total of 118 primary human NSCLC specimens were collected to examine ILT4 and B7-H3 expression by immunohistochemical analysis. ILT4 expression was detected in the nucleus, cytoplasm and membrane of cancer cells (Fig. 5A). B7-H3 expression was detected in the cell cytoplasm or/and membrane (Fig. 5A). ILT4 and B7-H3 expression were occasionally observed in stromal cells. ILT4 and B7-H3 expression was observed in 46.6% (55/118) and 60.2% (71/118) of the tissue samples, respectively. There was a significant positive correlation between ILT4 and B7-H3 expression; higher levels of ILT4 were observed in B7-H3-positive tissues compared with the corresponding B7-H3-negative tissues ($P = 0.021$) (Fig. 5B).

Patients were classified into 4 groups according to ILT4 and B7-H3 expression. As shown in Table 1, 31.4% patients (37/118) exhibited positive expression of both ILT4 and B7-H3 (I+B+), 15.3% (18/118) exhibited positive expression of ILT4 but negative expression of B7-H3 (I+B−), 26.2% (31/118) exhibited positive expression of B7-H3 but negative expression of ILT4 (I−B+), and 27.1% (32/118) exhibited negative expression of both ILT4 and B7-H3 (I−B−). The I+B+ group was significantly associated with increased regional lymph node involvement ($P = 0.001$) and advanced stage ($P = 0.001$) compared with the I−B− group. Also,

Table 1
Correlations of ILT4/B7-H3 co-expression with clinicopathological parameters in primary NSCLC tissues.

Variables	ILT4+/B7-H3+	ILT4+/B7-H3-		ILT4-/B7-H3+		ILT4-/B7-H3-	
	n	*n*	*p* 1	*n*	*p* 2	*n*	*p* 3
Age (yr)							
<60	12	5	0.259	16	0.586	16	0.138
⩾60	15	13		15		16	
Gender							
Male	27	17	0.131	25	0.458	21	0.508
Female	10	1		6		11	
Smoking history (yr)							
<30	10	1	0.131	6	0.458	15	0.087
⩾30	27	17		25		17	
Histology							
Non-squamous NSCLC	17	10	0.504	17	0.465	22	0.057
Squamous NSCLC	20	8		14		10	
Cellular differentiation							
Well	14	6	0.745	16	0.255	22	0.01
Worse	23	12		15		10	
Primary tumor size (cm)							
<5	24	13	0.585	17	0.4	17	0.322
⩾5	13	5		14		15	
Regional lymph node involvement							
N0–N1	20	14	0.16	28	0.003	31	0.001
N2–N3	17	4		3		1	
2009 TNM stage groupings							
I–II	15	13	0.027	24	0.002	28	0.001
III	22	5		7		4	

Note: p 1, *P* value between ILT4+/B7-H3+ and ILT4+/B7-H3-; *p* 2, *P* value between ILT4+/B7-H3+ and ILT4-/B7-H3+; *p* 3, *P* value between ILT4+/B7-H3+ and ILT4-/B7-H3-.
Abbreviations: NSCLC, non-small cell lung cancer; TNM, tumor node metastasis.

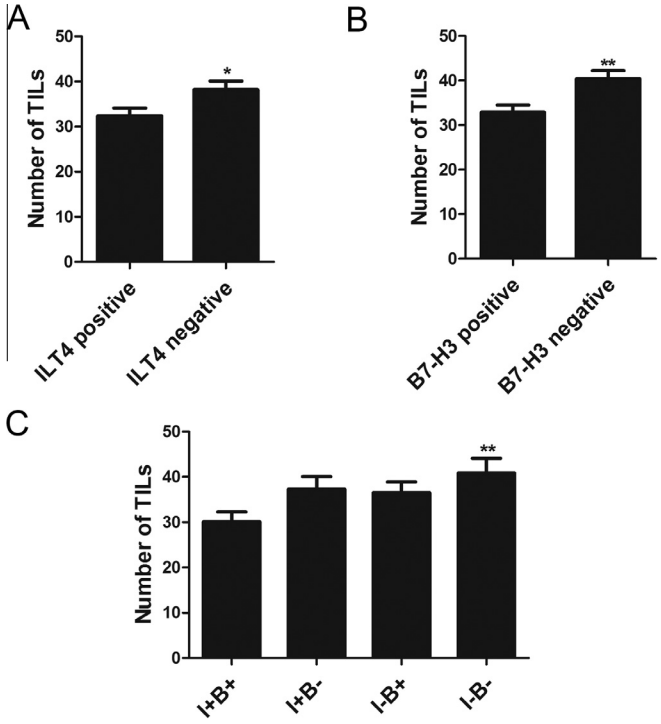

Fig. 6. The relationship between ILT4/B7-H3 co-expression and the number of TILs in primary human NSCLC tissues. Comparison of TIL intensity between patients with negative vs. positive ILT4 (A) or B7-H3 (B) expression. (C) Comparison of TIL intensity in I+/B+, I+B−, I−B+ and I−B− tissues. I+/B+: positive expression of both ILT4 and B7-H3; I−B−: negative expression of both ILT4 and B7-H3; I−B+: positive expression of B7-H3 but negative expression of ILT4; I+B−: positive expression of ILT4 but negative expression of B7-H3. The error bars indicate ±S.E.M. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's *t*-test.

ILT4 and B7-H3 co-expression were correlated with increased regional lymph node involvement ($P = 0.003$) and advanced stage ($P = 0.002$) compared with the I−B+ group, and advanced stage ($P = 0.027$) when compared with the I+B− group.

In addition, we detected expression of pAKT in primary human NSCLC tissues. pAKT expression was observed in the cytoplasm of primary NSCLC cells in 57.6% (60/118) of the tissue samples (Fig. 5A). ILT4 levels were also higher in pAKT-positive samples compared with pAKT-negative tissues ($P = 0.163$); however, this correlation was not significant (Fig. 5C).

3.6. Correlation between ILT4/B7-H3 co-expression and the number of TILs

As previously reported, ILT4 and B7-H3 were correlated with fewer TILs. The mean number of TILs was 32.35 ± 13.69 in the ILT4-positive group and 38.20 ± 13.76 in the ILT4-negative group ($P = 0.026$; Fig. 6A). In addition, the mean number of TILs was 32.34 ± 13.33 in the B7-H3-positive group, which was significantly lower than that in the B7-H3-negative group (39.15 ± 13.17, $P = 0.002$; Fig. 6B).

Furthermore, the relationship between ILT4/B7-H3 co-expression and TILs was examined in clinical specimens. The mean number of TILs was 30.09 ± 13.99 in the I+B+ group, which was much lower than that in the I−B− group (40.87 ± 14.38, $P = 0.007$). The number of TILs was also smaller in the I+B+ group compared to the I+B− group (37.22 ± 11.87, $P = 0.055$) or the I−B+ group (36.51 ± 13.31, $P = 0.104$), but the difference was not significant (Fig. 6C).

3.7. Relationship between ILT4/B7-H3 co-expression and overall survival of patients

Importantly, we examined the significance of ILT4/B7-H3 co-expression for prognosis in NSCLC patients. Kaplan–Meier

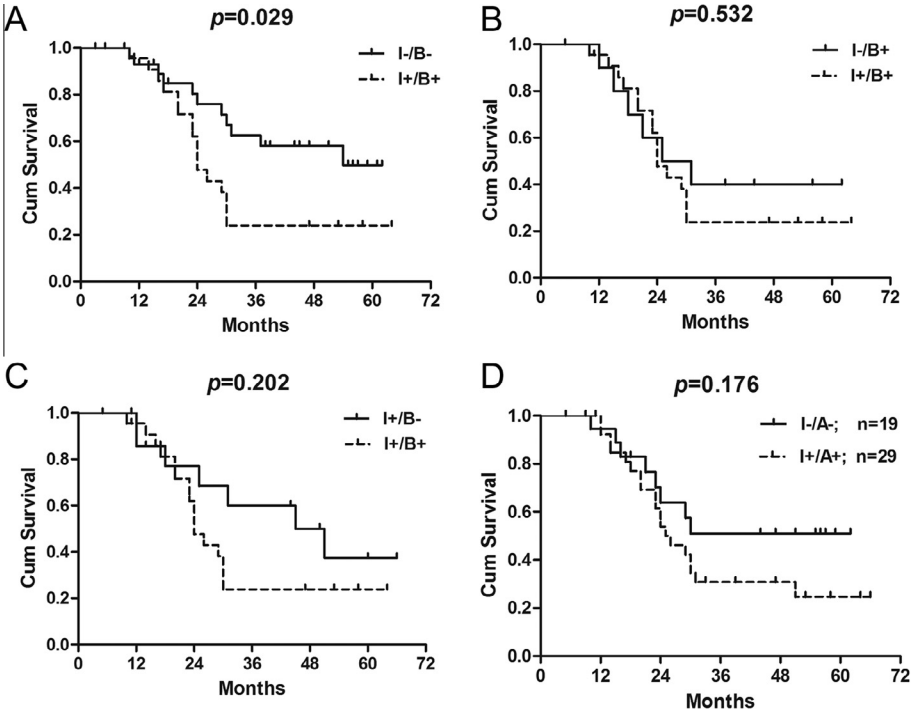

Fig. 7. Relationship between ILT4/B7-H3 co-expression and patient survival. (A) Survival analysis of I+/B+ and I−/B− NSCLC patients was performed by Kaplan–Meier survival analysis. (B) Survival analysis of I+/B+ and I−/B+ NSCLC patients. (C) Survival analysis of I+/B+ and I+/B− NSCLC patients. (D) Survival analysis of I+/A+ and I−/A− NSCLC patients. I+A+: positive expression of both ILT4 and pAKT; I−A−: negative expression of both ILT4 and pAKT.

analysis indicated that the overall survival (OS) of patients with I+B+ status was much lower than that of patients with I−/B− status (Fig. 7A, $P = 0.029$). However, no significant difference in OS was observed between I+B+ and I−B+ or I+B− patients (Fig. 7B and C, I−B+, $P = 0.532$; I+B−, $P = 0.202$).

In addition, the OS of patients with ILT4/pAKT co-expression was also lower than patients in the other three groups, but this difference was not significant (Fig. 7D, I−A−, $P = 0.176$; Supplementary Fig. 3, I−A+, $P = 0.121$; I+A−, $P = 0.179$).

4. Discussion

ILT4 expression is up-regulated in NSCLC cells and is associated with a lower number of TILs [10]. Prior to this study, ILT4 had been established as a primary molecule responsible for inhibiting immune function in the immune response [6,7,21]; however, the role of ILT4 expression in NSCLC progression remains unexplored.

Here, we demonstrated that B7-H3 is up-regulated in ILT4 over-expressing NSCLC cells and that there is a significant correlation between ILT4 and B7-H3 expression in primary NSCLC tissues. B7-H3, an inhibitor of the B7 family, is expressed in various cancers including breast cancer, colorectal cancer and NSCLC [22,23]. Although conflicting effects of B7-H3 have been observed in some tumours, B7-H3 is considered an inhibitory molecule and represents a promising target for immune-based antitumour therapies [24–27]. We first established that B7-H3 is expressed in NSCLC cells and correlates with a reduction in the number of TILs in tumour tissues [28]. Other groups have demonstrated that B7-H3 expression clearly inhibits T cell-mediated antitumour immunity in NSCLC [15,16]. Interestingly, we also previously demonstrated that ILT4 expression in NSCLC cells is negatively correlated with the number of TILs, indicating a potential role of ILT4 in the inhibition of T cell function in the tumour environment similar to that of B7-H3 [10]. In this study, we observed that ILT4/B7-H3 co-expression in patients was associated with a much lower number of TILs as well as other adverse clinicopathological features, including lymph node metastases and advanced tumour stage compared with other patient groups. Importantly, ILT4/B7-H3 co-expression was correlated with poor overall survival. Given the significant function of TILs in NSCLC progression [29,30], we conclude that ILT4 may inhibit the T cell-mediated antitumour immune response by up-regulating B7-H3 expression in NSCLC tissues, leading to NSCLC progression. Additionally, ILT4 and B7-H3 may synergistically act as biomarkers of negative clinical outcome in NSCLC patients.

As one of the most important signalling pathways in NSCLC, the PI3K/AKT/mTOR pathway participates in nearly all inspects of NSCLC development by regulating the expression of various cancer-related genes [31–33]. Recent studies have demonstrated that PI3K/AKT/mTOR signalling influences B7-H1 expression in melanoma and glioma cells [34–36]. Our results indicated that ILT4 activated the PI3K/AKT/mTOR signalling pathway, and blocking this pathway led to a down-regulation in B7-H3 expression. However, we did not detect a correlation between ILT4 and pAKT in NSCLC tissues. Additionally, ILT4/pAKT co-expression was not correlated with overall survival, which may be due to complex factors in human NSCLC tissues and an insufficient number of samples.

Collectively, ILT4 may exert its inhibitory function on the immune response toward the tumour by up-regulating B7-H3 via PI3K/AKT/mTOR signalling. Moreover, based on the non-immunological functions of PI3K/AKT/mTOR signalling and B7-H3 [37–41], ILT4 may also have a non-immunological role in tumour progression. Further studies are needed to gain a better understanding of the role of ILT4 in individual features of NSCLC.

In summary, our results demonstrate that ILT4 up-regulates B7-H3 expression via PI3K/AKT/mTOR signalling. Activation of the ILT4-PI3K/AKT/mTOR-B7-H3 axis may be involved in tumour immune evasion, leading to NSCLC progression. These data suggest a critical role of ILT4 in NSCLC progression and pave the way for novel antitumour treatment.

Conflict of interest

No potential conflicts of interest were disclosed.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81372334), the Department of Science and Technology of Shandong Province (Grant No. 2013GSF12107), the Project of Jinan Youth Team for Technological Innovation (Grant No. 2010-1) and the Department of Science and Technology of Jinan City (Grant No. 201201061). We thank Dong Zhao and Huiping Liu of Department of Pathology, Jinan Central Hospital, Shandong University for performing the immunohistochemical assays.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.06.037.

References

[1] Siegel, R., Ma, J., Zou, Z. and Jemal, A. (2014) Cancer statistics, 2014. CA Cancer J. Clin. 64, 9–29.
[2] Devesa, S.S., Bray, F., Vizcaino, A.P. and Parkin, D.M. (2005) International lung cancer trends by histologic type: male:female differences diminishing and adenocarcinoma rates rising. Int. J. Cancer 117, 294–299.
[3] Siegel, R. et al. (2012) Cancer treatment and survivorship statistics, 2012. CA Cancer J. Clin. 62, 220–241.
[4] Vineis, P. and Wild, C.P. (2014) Global cancer patterns: causes and prevention. Lancet 383, 549–557.
[5] Colonna, M., Nakajima, H. and Cella, M. (2000) A family of inhibitory and activating Ig-like receptors that modulate function of lymphoid and myeloid cells. Semin. Immunol. 12, 121–127.
[6] Ristich, V., Zhang, W., Liang, S. and Horuzsko, A. (2007) Mechanisms of prolongation of allograft survival by HLA-G/ILT4-modified dendritic cells. Hum. Immunol. 68, 264–271.
[7] Shiroishi, M. et al. (2003) Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. Proc. Natl. Acad. Sci. U.S.A. 100, 8856–8861.
[8] Colovai, A.I. et al. (2007) Expression of inhibitory receptor ILT3 on neoplastic B cells is associated with lymphoid tissue involvement in chronic lymphocytic leukemia. Cytometry B Clin. Cytometry 72, 354–362.
[9] Liu, J. et al. (2014) Inhibitory receptor immunoglobulin-like transcript 4 was highly expressed in primary ductal and lobular breast cancer and significantly correlated with IL-10. Diagn. Pathol. 9, 85.
[10] Sun, Y., Liu, J., Gao, P., Wang, Y. and Liu, C. (2008) Expression of Ig-like transcript 4 inhibitory receptor in human non-small cell lung cancer. Chest 134, 783–788.
[11] Zheng, J. et al. (2012) Inhibitory receptors bind ANGPTLs and support blood stem cells and leukaemia development. Nature 485, 656–660.
[12] Ceeraz, S., Nowak, E.C. and Noelle, R.J. (2013) B7 family checkpoint regulators in immune regulation and disease. Trends Immunol. 34, 556–563.
[13] Seliger, B. and Quandt, D. (2012) The expression, function, and clinical relevance of B7 family members in cancer. Cancer Immunol. Immunother. 61, 1327–1341.
[14] Lee, S.J. et al. (2006) Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274). FEBS Lett. 580, 755–762.
[15] Chen, C., Shen, Y., Qu, Q.X., Chen, X.Q., Zhang, X.G. and Huang, J.A. (2013) Induced expression of B7–H3 on the lung cancer cells and macrophages suppresses T-cell mediating anti-tumor immune response. Exp. Cell Res. 319, 96–102.
[16] Sun, J., Mao, Y., Zhang, Y.Q., Guo, Y.D., Mu, C.Y., Fu, F.Q. and Zhang, X.G. (2013) Clinical significance of the induction of macrophage differentiation by the costimulatory molecule B7-H3 in human non-small cell lung cancer. Oncol. Lett. 6, 1253–1260.
[17] Xu YH, Z.G., Wang JM, Hu HC. (2010 Sep). B7-H3 and CD133 expression in non-small cell lung cancer and correlation with clinicopathologic factors and prognosis. Saudi Med J 31, 980-6.
[18] Zhang, G., Xu, Y., Lu, X., Huang, H., Zhou, Y., Lu, B. and Zhang, X. (2009) Diagnosis value of serum B7–H3 expression in non-small cell lung cancer. Lung Cancer 66, 245–249.
[19] Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. Cell 144, 646–674.
[20] Jun Konishi, K.Y., Azuma, Miyuki., Kinoshita, Ichiro., Dosaka-Akita, Hirotoshi. and Nishimura1, Masaharu (2004) B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. Clin. Cancer Res. 10, 7.
[21] Castellaneta, A., Mazariegos, G.V., Nayyar, N., Zeevi, A. and Thomson, A.W. (2011) HLA-G level on monocytoid dendritic cells correlates with regulatory T-cell Foxp3 expression in liver transplant tolerance. Transplantation 91, 1132–1140.
[22] Loos, M., Hedderich, D.M., Friess, H. and Kleeff, J. (2010) B7-H3 and its role in antitumor immunity. Clin. Dev. Immunol. 2010, 683875.
[23] Wang, L., Kang, F.B. and Shan, B.E. (2014) B7-H3-mediated tumor immunology: friend or foe? Int. J. Cancer 134, 2764–2771.
[24] Loo, D. et al. (2012) Development of an Fc-enhanced anti-B7-H3 monoclonal antibody with potent antitumor activity. Clin. Cancer Res. 18, 3834–3845.
[25] Roth, T.J. et al. (2007) B7-H3 ligand expression by prostate cancer: a novel marker of prognosis and potential target for therapy. Cancer Res. 67, 7893–7900.
[26] Yang, H.Y., Chu, M., Zheng, L.W., Zwahlen, R.A., Luo, J., Zou, D.H. and Sun, S.T. (2008) Transgenic B7-H3 therapy induces tumor-specific immune response in human oral squamous cell cancer: an in vitro study. Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endodontol. 106, 721–728.
[27] Zang, X. and Allison, J.P. (2007) The B7 family and cancer therapy: costimulation and coinhibition. Clin. Cancer Res. 13, 5271–5279.
[28] Sun, Y., Wang, Y., Zhao, J., Gu, M., Giscombe, R., Lefvert, A.K. and Wang, X. (2006) B7-H3 and B7-H4 expression in non-small-cell lung cancer. Lung Cancer 53, 143–151.
[29] Kataki, A., Scheid, P., Piet, M., Marie, B., Martinet, N., Martinet, Y. and Vignaud, J.M. (2002) Tumor infiltrating lymphocytes and macrophages have a potential dual role in lung cancer by supporting both host-defense and tumor progression. J. Lab. Clin. Med. 140, 320–328.
[30] Kilic, A., Landreneau, R.J., Luketich, J.D., Pennathur, A. and Schuchert, M.J. (2011) Density of tumor-infiltrating lymphocytes correlates with disease recurrence and survival in patients with large non-small-cell lung cancer tumors. J. Surg. Res. 167, 207–210.
[31] Engelman, J.A. (2009) Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer 9, 550–562.
[32] Fumarola, C., Bonelli, M.A., Petronini, P.G. and Alfieri, R.R. (2014) Targeting PI3K/AKT/mTOR pathway in non small cell lung cancer. Biochem. Pharmacol.
[33] Kim, T.R., Cho, E.W., Paik, S.G. and Kim, I.G. (2012) Hypoxia-induced SM22alpha in A549 cells activates the IGF1R/PI3K/Akt pathway, conferring cellular resistance against chemo- and radiation therapy. FEBS Lett. 586, 303–309.
[34] Atefi, M. et al. (2014) Effects of MAPK and PI3K pathways on PD-L1 expression in melanoma. Clin. Cancer Res. 20, 3446–3457.
[35] Jiang, X., Zhou, J., Giobbie-Hurder, A., Wargo, J. and Hodi, F.S. (2013) The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. Clin. Cancer Res. 19, 598–609.
[36] Parsa, A.T. et al. (2007) Loss of tumor suppressor PTEN function increases B7–H1 expression and immunoresistance in glioma. Nat. Med. 13, 84–88.
[37] Cheng, Y., Li, Y., Liu, D., Zhang, R. and Zhang, J. (2014) MiR-137 effects on gastric carcinogenesis are mediated by targeting Cox-2-activated PI3K/AKT signaling pathway. FEBS Lett. 588, 3274–3281.
[38] Nygren, M.K. et al. (2014) Identifying microRNAs regulating B7-H3 in breast cancer: the clinical impact of microRNA-29c. Br. J. Cancer 110, 2072–2080.
[39] Tekle, C., Nygren, M.K., Chen, Y.W., Dybsjord, I., Nesland, J.M., Maelandsmo, G.M. and Fodstad, O. (2012) B7-H3 contributes to the metastatic capacity of melanoma cells by modulation of known metastasis-associated genes. Int. J. Cancer 130, 2282–2290.
[40] Wang, J. et al. (2013) B7-H3 associated with tumor progression and epigenetic regulatory activity in cutaneous melanoma. J. Invest. Dermatol. 133, 2050–2058.
[41] Zhao, X. et al. (2013) B7-H3 overexpression in pancreatic cancer promotes tumor progression. Int. J. Mol. Med. 31, 283–291.