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**RESEARCH ARTICLE**

Xiangli Meng, et al.: Phosphorylation of ITGB4 at Y1510 promotes pancreatic tumorigenesis via the MEK1-ERK1/2 pathway

Integrin beta 4 (ITGB4) and its tyrosine-1510 phosphorylation promote pancreatic tumorigenesis and regulate the MEK1-ERK1/2 signaling pathway

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ABSTRACT
Pancreatic cancer is the fourth leading cause of cancer death, with a 5-year survival rate of only 1–4%. Integrin-mediated cell adhesion is critical for the initiation, progression, and metastasis of cancer. In this study we investigated the role of integrin β4 (ITGB4) and its phosphorylation at tyrosine Y1510 (p-ITGB4-Y1510) in the tumorigenesis of pancreatic cancer. We analyzed the expression of ITGB4 and p-ITGB4-Y1510 in pancreatic cancer tissue and cell lines using immunohistochemistry, Western blot, or semi-quantitative reverse transcription PCR. ITGB4 and p-ITGB4-Y1510 were highly expressed in pancreatic cancer (n = 176) compared with normal pancreatic tissue (n = 171). High p-ITGB4-Y1510 expression correlated with local invasion and distant metastasis of pancreatic cancer, and high ITGB4 was significantly associated with poor survival of patients. Inhibition of ITGB4 by siRNA significantly reduced migration and invasion of PC-1.0 and ASPC-1 cells. Overexpression of the mutant ITGB4-Y1510A (a mutation of tyrosine to alanine at 1510 position) in PC-1.0 and ASPC-1 cells not only blocked the ITGB4 phosphorylation at Y1510 but also suppressed the expression of ITGB4 (p < 0.05 vs. wild-type ITGB4). The transfection of PC-1.0 and ASPC-1 cells with ITGB4-Y1510A significantly decreased the level of p-mitogen-activated protein kinase kinase (MEK)1 (T292) and p-extracellular signal-regulated kinase (ERK)1/2 but did not affect the level of p-MEK1 (T386) and p-MEK2 (T394). Overall, our study showed that ITGB4 and its phosphorylated form promote cell migration and invasion in pancreatic cancer and that p-ITGB4-Y1510 regulates the downstream MEK1-ERK1/2 signaling cascades. Targeting ITGB4 or its phosphorylation at Y1510 may be a novel therapeutic option for pancreatic cancer.

KEYWORDS: ITGB4; integrin; MEK1 (T292); invasion; migration; pancreatic cancer
INTRODUCTION

Pancreatic cancer is one of the severe malignant tumors in digestive system. Although pancreatic cancer accounts for only 1-3% of newly diagnosed cancer patients every year, it ranks the fourth among cancer death rates and is one of serious threats to human health [1, 2]. Currently, surgery is a relatively effective treatment for pancreatic cancer, in spite of most patients are in the advanced stage of treatment and tumor cells have different degrees of distant metastasis. However, even if the patient has undergone surgical resection, 70% of patients will relapse and metastasize within one year [3]. In recent years, more new understandings have been made in the study of the occurrence, development, invasion and metastasis of pancreatic cancer [4-6]. However, effective molecular targets for pancreatic cancer have not been fully revealed. Therefore, identification of potential targets and a better understanding of the molecular mechanism driving pancreatic tumorigenesis are crucial to archive new strategies for early diagnosis and novel target therapy [7-9].

Integrin-mediated interaction between pancreatic tumor cells and extracellular matrix (ECM) were recently suggested to play an important role in promoting the malignancy of pancreatic cancer [10, 11]. As a member of the integrin family, integrin β4 (ITGB4) generally forms heterodimers with integrin α6 to achieve its biological functions, and integrin molecules can also coordinate with receptor tyrosine kinases (RTKs) to regulate downstream signaling pathways. Recent studies demonstrated that ITGB4 play an important role in promoting tumorigenesis in prostate cancer [12], breast cancer [13], gastric cancer [14] and lung squamous cell carcinoma [15]. Several studies found that ITGB4 was involved in the invasion and metastasis of breast cancer and prostate cancer, but the role of ITGB4 in pancreatic cell invasion and metastasis as well as pancreatic tumorigenesis remains unclear. In addition, although ITGB4 was able to activate ERK signaling pathway [12, 16], the
molecular mechanism of ITGB4-mediated signaling cascade for pancreatic cancer cells remains unclear.

The MAPK/MEK/ERK signaling pathway plays an important role in regulation of gene expression, cell proliferation, differentiation and apoptosis [17, 18]. In pancreatic cancer patients, the frequency of RAS mutations occurs in 70-90% patients [19], which result in abnormal activation of MEK1 and MEK2, and subsequent hyperproliferation of pancreatic cancer cells. Activation of MEK1 and MEK2 can rapidly activate downstream ERK1/2 and lead to the activation of hundreds of target genes, including transcription factors, kinases and cytoskeletal proteins to affect tumor invasion, metastasis and angiogenesis [20-23].

Furthermore, targeting MEK can effectively inhibit the growth of pancreatic cancer cells, block cell cycle changes, and reduce DNA methylase activity [24].

We previously conducted a high-throughput phosphorylation array to identify a series of candidate proteins involved in the tumorigenesis of pancreatic cancer. The level of phosphorylated ITGB4 at tyrosine site 1510 (p-ITGB4-Y1510) was upregulated more than 2-fold in higher invasive pancreatic cancer cells, suggesting that phosphorylated ITGB4 may be involved in the migration and invasion of pancreatic cancer as well as pancreatic tumorigenesis. Thus, in this study, we further demonstrated that ITGB4 and its phosphorylation are not only responsible for the regulation of pancreatic cancer migration and invasion, but also associated with the tumorigenesis and poor survival of pancreatic cancer patients. Furthermore, abolished the tyrosine phosphorylation of ITGB4 at Tyr1510 inhibited phosphorylation of MEK1 at T292, but did not affect MEK1 phosphorylation at T386 and MEK2 phosphorylation at T394. Moreover, inhibition of ITGB4 phosphorylation at Tyr1510 significantly reduced ERK1/2 activation. Hence, our study provides a pivotal role of ITGB4 and its phosphorylation at Y1510 in the tumorigenesis of pancreatic cancer, and provides an innovative link between ITGB4 phosphorylation and the downstream MEK1
(T292)-ERK1/2 signaling cascades. In addition, our study sheds new light on further targeted therapy against malignant pancreatic tumors with ITGB4 overexpression.
MATERIALS AND METHODS

Human tissue specimens
A total of 176 cases of pancreatic tumor tissues and 171 cases of normal control pancreatic specimens were collected from general surgery of Shengjing Hospital affiliated to China Medical University from January 2011 to January 2018. The pancreatic tumors were diagnosed by postoperative pathological diagnosis. All specimens were collected according to the human specimen collection procedure, approved by the Chinese Ethics Review Committee, and written informed consent was obtained from all patients. This study was conducted in accordance with the guidelines of the Declaration of Helsinki.

Cell culture and transfection
The pancreatic cancer cell PC-1.0 and AsPC-1 were established from a BOP-induced Syrian hamster pancreatic cancer model [25, 26]. The cells were seeded in polylysine-coated flasks with a density of $5 \times 10^5$/mL in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 95% air and 5% CO$_2$. For transfection, cells grown to 70% confluence were transfected WT-ITGB4 or ITGB4-Y1510A plasmid using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 6 h of transfection, culture medium was replaced with fresh medium, and the cells were continued cultured for additional 48h before the following experiments. For gene silencing by siRNA oligonucleotides, cells were transfected with scramble control siRNA or ITGB4 siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) using an RNAiMax reagent (Invitrogen) by following the manufacturer’s instructions. In brief, the siRNA oligonucleotides were mixed with RNAiMAX reagent in a Opti-MEM medium (Invitrogen). After 20 min of incubation at room temperature, the cells were incubated with the well-optimized transfection mixture and incubated for 24 h at 37 °C.
Migration assay

Migration ability was analyzed by cell scratch assay. Cells were seeded in the 6-well plates at a density of $5 \times 10^5$ per well, and allowed to grow overnight to reach an approximate confluence of 85%. A 200 μL sterile pipette tip was used to draw several straight lines across the cell layer. The cells were washed twice with PBS, and cultured in a complete medium containing 10% FBS. After 6 and 12 h incubation, the cells were imaged under a microscope.

Invasion assay

PC-1.0 and AsPC-1 cells were suspended in a FBS-free medium with cell density of $1 \times 10^5$ /mL. For invasion assay, 200 μL of cell suspension was added into the upper chamber of the transwell, and 600 μL of complete medium containing 10% FBS was placed in the lower chamber. After 24 h of incubation, the medium in the lower chamber was aspirated and washed 3 times with PBS. Invasive cells in the surface of lower chamber were fixed with 4% paraformaldehyde for 30 min, and stained with 0.1% crystal violet for 30 min. After the upper chamber cells were wiped off, the cells were imaged under a microscope. A total of six fields were randomly selected, and total invasive cells were counted.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

The total RNA were extracted from pancreatic normal and tumor tissues by TRzol reagent (Gibco Invitrogen). For 100 mg sample, 1 mL of TRIzol was used to extract the total RNA. The quality and integrity of the RNAs were examined by agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from 1 mg of total RNA using M-MLV reverse transcriptase (Ambion) as previously described [27]. After incubation at 42°C for 2 h, the reaction mixtures were heat inactivated at 70°C for 10 min and then applied as templates to amplify cDNAs specific for ITGB4 (amplified with the forward primer 5'
CTGGAGGTGTTTGAGCCACT-3’ and the reverse primer 5’-TTACAACAGCATTGGTACTTGGAT-3’). The GADPH served as internal control was amplified using the primer set 5’-TGACTTCAACAGCGACACCCA-3’ and 5’-CACCCTGTTGCTGCTAGCCAAA-3’. The relative expression changes of genes were calculated by 2 − △ △ CT method. The reaction conditions were as follows: pre-denaturation at 95 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, 30 cycles, and 72 °C for 1 min.

**SDS-PAGE and Western blotting analysis**

Western blotting were performed as previously described [28]. Samples were lysed in a lysis buffer containing 0.5 mM PMSF, phosphatase inhibitors, and protease inhibitor cocktail. After removing the cell debris, protein concentration was analysed using a BCA protein assay and subsequently subjected to electrophoresis in polyacrylamide gels. After transferred the protein onto PVDF membranes, the membranes were blocked with TBS containing 5% w/v nonfat milk, and probed with specific primary antibodies. Primary antibody against ITGB4, p-ITGB4 (Tyr1510), MEK-1, p-MEK-1 (T292), p-MEK-1 (T386), MEK-2, p-MEK-2 (T394), ERK1/2, p-ERK1/2 (T202/T204) were purchased from ImmunoWay (1:1000 dilution). Control antibody GAPDH was obtained from Santa Cruz Biotechnology (1:1000 dilution). After incubation for 2h at room temperature, the membranes were washed with TBST for 3 times and probed with secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA, 1:1000) for 1 h at room temperature. The chemiluminescent signal was visualized using a Western Lightning Plus ECL reagent (Perkin Elmer), and the signals were imaged by gel imaging system.

**Immunohistochemistry**
After routine dewaxing of the tissue sections, the tissue sections were placed in a citrate buffer solution, and the antigen was repaired by microwave method, heated in microwave oven, and cooled down to room temperature; 3% H$_2$O$_2$ was added dropwise, and incubated at room temperature for 10 min to block endogenous Oxidase, washed in PBS for 5 min for 3 times; added ITGB4 (Tyr1510) primary antibody (1:300), wash in PBS for 5 min for 3 times, add HRP labeled secondary antibody (1:500, Santa Cruz, USA). Incubated it for 1 h at room temperature, washed for 5 min for 3 times with PBS; DAB was exposed to light for 5 min at room temperature, counterstained for 3 min with hematoxylin, washed for 5 min for 3 times with PBS. Dehydrated, transparented and neutral gum seals were prepared. Six sections of specimens were selected from each group, and the expression of each group was observed under light microscope. The optical density values were analyzed and measured using Image-proplus 6.0 software.

**Statistical analysis**

All experiments were performed at least three independent replicates. The experimental data were presented as mean ± standard deviation (Mean ± SD). The significance test of the differences between the experimental groups was analyzed by one-way ANOVA by using the SPSS 17.0 statistical software. The $P$ values are denoted with asterisks: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. In this study, $P < 0.05$ was considered statistically significant.
RESULTS

ITGB4 is highly expressed in pancreatic cancer tissues and is associated with poor survival in patients with pancreatic cancer

In our previous study, we found ITGB4 was highly expressed in high invasive metastatic pancreatic cell line PC-1.0 compared to low invasive pancreatic cell line PC-1 [29], implying that ITGB4 may be functionally related to tumorigenicity of pancreatic cancer. Thus, we first examined whether ITGB4 was highly expressed in pancreatic cancer tissues. A total of 176 cases of pancreatic tumor tissues and 171 cases of normal pancreatic specimens was used to examine the expression of ITGB4. As shown in Figure 1A, immunohistochemistry (IHC) analysis showed that the expression ITGB4 was highly increased in pancreatic cancer tissues compared to normal tissues. The expression of ITGB4 was further confirmed by quantitative RT-PCR. The results confirmed that the expression of ITGB4 was significantly increased in pancreatic cancer tissues (Figure 1B, p<0.05). We then estimated the prognostic implications of ITGB4 in pancreatic patients. The estimated five-year overall survival rates among the 176 patients were 55% and 22% for the low ITGB4 and high ITGB4 expression, respectively. The expression of ITGB4 was significantly correlated with poorer overall survival (Figure 1C, p<0.001).

The pivotal role of ITGB4 in migration and invasion of pancreatic cancer cells

To further explore the biological function of ITGB4 in pancreatic cancer, we analyzed the cell migration by using scratch assay in pancreatic cell lines PC-1.0 and ASPC-1. Cells were transfected scramble siRNA (si-NC) or ITGB4 siRNA (si-ITGB4) for 24 h, and the knockdown effect was examined by Western blot analysis. Compared to scramble control, ITGB4 siRNA significantly inhibited the ITGB4 expression to 27% and 33% in PC-1.0 and
AsPC-1 cells, respectively (Figure 2A, p<0.05). To analyze the role of ITGB4 in the pancreatic cell migration, cell monolayers with scramble siRNA or ITGB4 siRNA were scratched with pipette tip. Analysis of the wound-healing assay showed a significant reduction of the migration ability in PC-1.0 and ASPC-1 cells after transfected with ITGB4 siRNA (Figure 2B). The control cells of PC-1.0 and ASPC-1 almost healed the wounds after 12 h. However, the healed areas of ITGB4 siRNA transfected cells were less than half the wounds. Compared to scramble siRNA control, the migration ability was significantly inhibited in si-ITGB4 PC-1.0 and AsPC-1 cells (Figure 2C, p<0.05). Next, transwell chamber experiments were conducted to examine the potential association between ITGB4 and the invasion abilities of pancreatic cells. In consistent with the wound healing assay, the transwell chamber assay showed that PC-1.0 and AsPC-1 cells transfected with si-ITGB4 exhibited much less invasive cells (Figure 3A). There was significant difference between si-NC and si-ITGB4 transfected PC-1.0 and AsPC-1 pancreatic cells (Figure 3B, p<0.05). In summary, these results indicated that ITGB4 play a crucial role in the migration and invasion abilities in pancreatic cancers.

**Phosphorylation ITGB4 at Tyr1510 was associated with tumorigenicity of pancreatic cancer**

Since integrin phosphorylation is of pivotal importance for activity, we next examine whether the phosphorylation level of ITGB4 at tyrosine 1510 (p-ITGB4-Y1510) was increased in pancreatic cancer tissues. The level of p-ITGB4-Y1510 was examined in 176 cases of pancreatic tumor tissues and 171 cases of normal control pancreatic specimens. The results showed that the level of p-ITGB4-Y1510 was significantly increased in pancreatic cancer tissues compared to normal tissues (Figure 4A, p<0.05). To further confirm whether
phosphorylation of ITGB4 was associated with tumorigenicity of pancreatic cancer, tissues from local invasion and distant metastasis was used to examine the expression of p-ITGB4-Y1510. Consistent with the above results, the level of p-ITGB4-Y1510 was significantly increased in local invasion and distant metastasis pancreatic tissues (Figure 4B, p<0.05).

To further confirm the role of phosphorylation site Y1510 of ITGB4 (pITGB4-Y1510) in the ITGB4-mediated cell signaling in pancreatic cancer, we carried out a mutation at Tyrosine residue at position 1510 of ITGB4. This tyrosine residue was mutated to alanine (Y1510A) to abolish the tyrosine phosphorylation of ITGB4. Next, PC-1.0 and AsPC-1 pancreatic cells were transfected with wild-type ITGB4 (ITGB4-WT) or mutant ITGB4 (ITGB4-Y1510A), and the expression level of ITGB4 and p-ITGB4-Y1510 was determined by Western blotting analysis. Compared to ITGB4-WT, ITGB4-Y1510A not only reduced the level of p-ITGB4-Y1510, but also inhibited the expression of ITGB4 in both PC-1.0 and AsPC-1 pancreatic cells (Figure 5A). The intensities of ITGB4 was normalized to GADPH and intensities of p-ITGB4 was normalized to ITGB4. As shown in Figure 5B, the expression level of p-ITGB4 was significantly reduced to 24% and 28% in PC-1.0 and AsPC-1 cells, respectively (p<0.05). Interestingly, the expression of ITGB4 in PC-1.0 and AsPC-1 cells was also reduced when ITGB4-Y1510A was transfected (Figure 5C), suggesting that inhibition of ITGB4 phosphorylation at tyrosine 1510 may negatively regulate the expression of ITGB4.

**Phosphorylation of ITGB4 at tyrosine 1510 regulates MEK1-ERK1/2 pathway**

Since activation of Mitogen-activated protein kinase (MAPK) cascades have shown to play a key role in the pancreatic cell proliferation, differentiation, migration, senescence and apoptosis [30, 31] as well as tumorigenesis of pancreatic cancer, we next examine whether phosphorylation of ITGB4 at tyrosine 1510 is functionally involved in the regulation of
MAPK signaling cascades. PC-1.0 and AsPC-1 cells were transfected with ITGB4-WT or ITGB4-Y1510A plasmid, and the expression of MEK1, MEK2, ERK1/2, p-MEK1 (T292), p-MEK1 (T386), p-MEK2 (T394), p-ERK1/2 and GADPH were examined by Western blot analysis (Figure 6A). Compared with ITGB4-WT, ITGB4-Y1510A transfection did not affect the expression of MEK1 and MEK2, but downregulated the expression of ERK1/2 (Figure 6B, p<0.05). Interestingly, the expression level of p-MEK1 (T292) and p-ERK1/2 was significantly decreased in ITGB4-Y1510A transfected PC-1.0 and AsPC-1 cells (Figure 6C-D, p<0.05). However, ITGB4-Y1510A transfection did not affect the level of p-MEK1 (T386) and p-MEK2 (T394). Taken together, these results indicate that ITGB4 not only functionally participates in the migration and invasion of pancreatic cancer cells, but also regulates the MEK1-ERK1/2 signaling cascade through its phosphorylation at Y1510.
DISCUSSION

In our previous study, the Phospho Explorer antibody chip was used to identify potential candidates for regulating the invasive abilities of pancreatic cancer [29]. As a result, at least 32 phosphorylated proteins were highly increased in highly invasive metastatic pancreatic cells. In particular, phosphorylated ITGB4, member of the integrin family, was increased at least 2-fold. ITGB4 was reported to be involved in cell death, autophagy, angiogenesis, senescence and differentiation, and has a regulatory effect on the development of tumor and nervous system diseases [32, 33]. However, the role of ITGB4 in the tumorigenesis of pancreatic cancer remains unclear. On the other hand, although ITGB4 contains multiple tyrosine phosphorylation sites, its biological role of tyrosine phosphorylation at 1510 site is still unclear. Thus, this study aims to clarify whether ITGB4 is involved in the pancreatic tumorigenesis and invasion/migration of pancreatic cancer cells, and elucidate the molecular mechanism of p-ITGB4-Y1510 in pancreatic cancer cells. Our results found that both ITGB4 and p-ITGB4-Y1510 were highly expressed in pancreatic cancer tissues, and higher ITGB4 expression was significantly associated with poor survival in patients with pancreatic cancer (p<0.05). Inhibition of ITGB4 expression by siRNA indeed suppressed pancreatic cancer migration and invasion, indicating that ITGB4 plays a pivotal role in the malignancy of pancreatic cancer. Of interest, ITGB4 generally associates with integrin α6 to form α6β4 heterodimers to achieve its biological functions. Meanwhile, integrin α6 has been shown to be associated with carcinoma progression [34-36], including pancreatic cancer [37]. This suggested that ITGB4 may cooperate with integrin α6 or function independently to trigger downstream signaling cascade for pancreatic tumor development, invasion, and metastasis. This is because that recent study demonstrated that ANXA7 can trigger ITGB4 phosphorylation at Y1494 and then promote ITGB4 nuclear translocation to activate the
expression of ATF3 and a series of downstream genes [38, 39]. It also suggests that phosphorylation of ITGB4 may play an important role in its functional independence. The phosphorylation of ITGB4 could affect the biological function of ITGB4 and cellular localization. ITGB4 phosphorylation on residues S1356 and S1364 plays important roles in the formation and/or stability of hemidesmosomes [40]. T1736 phosphorylation leads to the disruption of the binding site of the plakin domain of plectin [40-42]. Tyrosine phosphorylation of ITGB4 at Y1440, Y526, Y1640 and Y1422 were associated with the expression of inflammatory cytokines [43]. Ionizing radiation (IR) triggered the phosphorylation of ITGB4 at Y1510 and subsequently lead to the activation of integrin α6β4-Src-AKT signaling pathway [44]. However, it remains unclear whether phosphorylation of ITGB4 at Y1510 is involved in the tumorigenicity of pancreatic cancer. Here, we further revealed that the level of ITGB4 phosphorylation at Y1510 was significantly increased in pancreatic tumor tissues (Figure 4A). A similar observation of increased expression was noted in pancreatic cancer with local or distinct invasion (Figure 4B). To understand whether this p-ITGB4-Y1510 medicated cell signaling in pancreatic cancer cells, competition assay was performed by using ITGB4-Y1510A plasmids containing a tyrosine mutation at position 1510 of ITGB4. Interestingly, ITGB4-Y1510 transfection not only inhibited the level of p-ITGB4-Y1510, but also reduced the expression of ITGB4. This implies that there was a feedback regulatory mechanism at Y1510 sites of ITGB4, or phosphorylation of ITGB4 at tyrosine residue 1510 may activate ITGB4. Similar observation was observed in ionizing radiation (IR)-treated A549 cells [44]. In A549 cells exposed to 6 Gr IR, the expression of ITGB4 was decreased, but the level of p-ITGB4-Y1510 was increased. Whether p-ITGB4-Y1510 is involved in a feedback regulation in the integrin expression warrants further investigation.
ITGB4-containing α6β4 heterodimers can function as a laminin receptor [45], an important component of extracellular matrix ECM, affecting the adhesion, spreading, migration, invasion, proliferation and apoptosis of the cells. In addition, the interaction of ITGB4 with plectin is necessary to establish a connection between ECM and intermediate filament system. ITGB4 inactivation resulted in pyloric atresia associated with junctional epidermolysis bullosa [46]. During adhesion, the interaction of ECM with ITGB4 allows the cell to simultaneously initiate the MAPK signal transduction pathway, playing an important role in the evolution of pancreatic cancer. Inhibition MAPK signaling pathway suppress the proliferation and invasion in choriocarcinoma [47], hepatocellular carcinoma [48] and breast cancer [49] as well as pancreatic cancer [50, 51]. Remarkably, our study identified that the phosphorylation of ITGB4 at tyrosine 1510 is involved in the regulation of MAPK-MEK1-ERK1/2 signaling pathway (Figure 6). Mutation of ITGB4-Y1510 reduced the level of p-MEK1 (T292) and p-ERK1/2, but the level of p-MEK1 (T386) and p-MEK2 (394) were not changed. T292 is located in the proline-rich segment of the protein kinase domain of MEK1, and T386 is located in the C-terminal tail of MEK1 [52]. T292 phosphorylation of MEK1 decrease S298 phosphorylation as catalyzed by p21-activated kinase-1 (PAK1). On the other hand, Cdk5 and ERK1 has been shown to phosphorylate MEK1 at T386 and contribute to regulate the kinase activity [52, 53]. However, Eblen’s study demonstrated that ERK-mediated feedback T386 phosphorylation have only minor effect in the inhibition of S298 phosphorylation by ERK, and that excess PAK may overcome the inhibition of S298 phosphorylation which caused by ERK-mediated T292 phosphorylation [54]. Taken together, these studies and our study suggest that T292 and T386 phosphorylation in MEK1 may play different roles in downstream signaling cascades. In addition, phosphorylation of ITGB4 at Y1510 plays an important role in pancreatic tumorigenesis by regulating the T292 phosphorylation of MEK1 rather than T386 phosphorylation.
MAPKs are widely used in many physiological and pathological processes during evolution, including coordinating gene expression, promoting mitosis, regulating metabolism, regulating cell motility and promoting cell apoptosis and differentiation. MEK1/2 and its downstream kinase ERK1/2 are important kinases in the MAPK/MEK/ERK signaling pathway, which can rapidly activate its unique target protein ERK1/2 after activation by its upstream kinase. ERK1/2 is mainly responsible for mediation. The functions of cell proliferation, differentiation and survival are downstream proteins of various growth factors [55, 56]. In tumor cells, ERK overactivation can act on downstream transcription factors, regulate the expression of certain oncogenes, and thus play a role in tumor invasion and metastasis [24, 57]. The results of this study revealed inhibition of ITGB4 phosphorylation at Y1510, the level of p-ERK1/2 was significantly decreased (P<0.05, Figure 6D), implying that ERK1/2 may promotes invasion and metastasis of pancreatic cancer cells by MAPK/MEK/ERK signaling pathway. To validate this hypothesis, MEK inhibitor U0126 was used to block the MAPK/MEK/ERK signaling pathway. As expected, the relative level of p-MEK1 (T292), p-MEK1 (T386), p-MEK2 (T394) and p-ERK1/2 were decreased upon the treatment of MEK inhibitor U1026. Furthermore, the migration and invasion ability were significant reduced in PC-1.0 and AsPC-1 cells treated with U0126 (data not shown).

CONCLUSION

The present findings reveal that ITGB4 plays a crucial role in the tumorigenesis of pancreatic cancer. The level of ITGB4 and phosphorylation of ITGB4 at Y1510 were highly expressed in pancreatic cancer tissues, and correlated with local invasion or distant metastasis tissues. Furthermore, the ITGB4 expression was negatively correlated with overall survival of the pancreatic patients. Silencing ITGB4 expression inhibited the migration and invasion abilities
of pancreatic cancer. Mutation analysis demonstrated that phosphorylation of ITGB4 at Y1510 was involved in the MEK1 (T292)-ERK1/2 signaling cascades. Altogether, our study not only revealed the pivotal role of ITGB4 and its Y1510 phosphorylation in the tumorigenesis of pancreatic cancer, but also provided a rationale for the clinical target for the treating pancreatic cancer patients in the future.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.
REFERENCES


FIGURE 1. ITGB4 was highly expressed in pancreatic cancer tissues and associated with poor survival. (A) Immunohistochemical analysis of ITGB4 expression in normal and pancreatic cancer tissues. (B) Quantitative analysis of ITGB4 expression in normal pancreatic tissues compared with pancreatic cancer tissues by quantitative RT-PCR. The data represent the mean ± SD, and differences were considered statistically significant when *P < 0.05. (C) Kaplan–Meier analyses of overall survival rates between 176 pancreatic tumor tissues and 171 normal pancreatic specimens according to ITGB4 expression. P<0.05.
FIGURE 2. ITGB4 promote pancreatic cell migration abilities. (A) After siRNA knockdown of ITGB4 in PC-1.0 and AsPC-1 cells, the relative expression of ITGB4 were determined by Western blotting. (B) Wound-healing cell migration assay of PC-1.0 and AsPC-1 cells upon inhibition of ITGB4 expression. (C) Quantification of the migration ratio of PC-1.0 and AsPC-
1 cells treated with control or ITGB4 siRNA. The error bars represent the standard deviation of three independent experiments. P<0.05 was considered statistically significant.

**FIGURE 3.** ITGB4 promote pancreatic cell invasive abilities. (A) After siRNA knockdown of ITGB4, invasive abilities of PC-1.0 and AsPC-1 cells were determined by transwell. Photography of invasive cells were shown. (B) Quantitative assay of the transwell assay of PC-1.0 and AsPC-1 cells treated with control or ITGB4 siRNA. The cell number passing through the filter in the si-ITGB4 group was significantly lower than that in the si-NC group. The error bars represent the standard deviation of three independent experiments. P<0.05 was considered statistically significant.
FIGURE 4. Phosphorylation of ITGB4 at tyrosine 1510 site was associated with pancreatic cancer, local invasion and distinct metastasis. (A) The expression of p-ITGB4-Y1510 in normal and pancreatic cancer tissues were determined. (B) The expression level of p-ITGB4-Y1510 was examined in normal tissues and local invasion or distinct metastasis pancreatic tissues.
FIGURE 5. Phosphorylation defect ITGB4 mutant reduced ITGB4 expression and eliminated the phosphorylation of ITGB4. (A) PC-1.0 and AsPC-1 cells were transfected with plasmid containing ITGB4 mutation of tyrosine-1510 to alanine, and the expression of ITGB4 and p-ITGB4-Y1510 were determined by Western blotting. (B) Quantitative analysis of ITGB4 and p-ITGB4-Y1510 were shown. The error bars represent the standard deviation of three independent experiments. P<0.05 was considered statistically significant.
FIGURE 6. Phosphorylation of ITGB4 at tyrosine 1510 regulated MEK1-ERK1/2 signaling pathway. (A) PC-1.0 and AsPC-1 cells were transfected with ITGB4-WT or ITGB4-Y1510A plasmids, and the expression of MEK1, MEK2, ERK1/2, p-MEK1 (T292), p-MEK1 (T386), p-MEK2 (T394) and p-ERK1/2 were determined by Western blotting. (B-D) Quantitative analysis of relative expression ERK1/2, p-MEK1 (T292) and p-ERK1/2. The error bars represent the standard deviation of three independent experiments. P<0.05 was considered statistically significant.