Pathol. Oncol. Res. (2015) 21:267–272
DOI 10.1007/s12253-014-9817-3

RESEARCH

High Phosphorylation Status of AKT/mTOR Signal in DESI2-Reduced Pancreatic Ductal Adenocarcinoma

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Received: 19 September 2013 / Accepted: 25 June 2014 / Published online: 31 July 2014
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Abstract Desumoylating isopeptidase 2 (DESI2) is a recently identified protein with unclear functions. In this study, a total of 132 tissue samples of pancreatic ductal adenocarcinoma and 73 samples of pancreatic normal tissues were explored to assess DESI2 expression and its implications to AKT/mTOR signal. Immunohistochemistry showed DESI2 expression is significantly decreased in cancer tissues versus normal tissues, presenting lowest level in poorly differentiated cancer. Unlike DESI2, the key factors in AKT/mTOR pathway including p-AKT, mTOR, p-mTOR and p-P70S6K present high expression in pancreatic cancer. It is notable that p-mTOR is significantly increased in DESI2-lower cancer compared with DESI2-higher cancer, although mTOR presents no difference in the two groups. The relative p-mTOR/mTOR ratio is also significantly elevated in DESI2-lower cancer. Moreover, the samples whose p-AKT and p-mTOR scores both exceed the median are obviously increased in DESI2-lower cancer compared with DESI2-higher cancer. As a downstream molecule of AKT/mTOR pathway, p-P70S6K was found to display higher level in DESI2-lower pancreatic cancer. High phosphorylation status of those proteins in DESI2-reduced pancreatic cancer indicates that there is high activity of AKT/mTOR signal in condition of DESI2 reduction, which could provide clues to reveal the implications of DESI2 in carcinogenesis.

Keywords DESI2 · AKT · mTOR · Pancreatic ductal adenocarcinoma

Introduction

Desumoylating isopeptidase 2 (DESI2), also known as PPPDE peptidase domain-containing protein 1 (PPPDE1), is a recently identified protein that may be involved in the reverse process of proteins modification by small ubiquitin-like modifier (SUMO) [1–3]. Analysis of amino acid sequence shows DESI2 belongs to the DESI family that contains a highly conserved PPPDE peptidase domain [4, 5]. As another member of DESI family, DESI1 is demonstrated to have a role of deSUMOylase, but not the deubiquitination [6]. Previous studies indicated that DESI2 could be involved in embryonic development and apoptosis [7–9]. However, the definite functions of DESI2 remain to be unknown.

By means of immunofluorescence and tissue screening, we found DESI2 is located at Golgi apparatus in cell and presents ubiquitous distribution in most tissues. However, low expression of DESI2 was disclosed in pancreatic cancer, suggesting certain implications of DESI2 in this cancer [10]. Pancreatic cancer is a common encountered carcinoma with high malignance in clinic [11, 12]. Previous studies revealed that AKT activation is increased in nearly half of pancreatic cancer and related to prognosis [13, 14]. As a downstream key factor of AKT,mTOR were also reported to play essential functions in progression of pancreatic cancer, which leads to development of inhibitors against mTOR [15, 16]. However, several drugs targeting mTOR were not up to expectations in clinical trials, which prompted us to review the regulation of AKT/mTOR signal in pancreatic cancer [17, 18]. These confusions present a hypothesis that there may be other undiscovered factors to impact the decision of AKT/mTOR signal in pancreatic cancer.

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In the present study, we investigated DESI2 expression in pancreatic ductal adenocarcinoma and normal tissues by means of a tissue library containing 205 samples. We also assessed the expression of key factors in AKT/mTOR pathway in pancreatic ductal adenocarcinoma with various DESI2 levels, and further analyzed the activation patterns of AKT/mTOR signal in pancreatic cancer. Based on above exploration, we hope to find clues about DESI2 to influence on AKT/mTOR signal in pancreatic cancer.

Materials and Methods

Antibodies

A rabbit polyclonal antibody against DESI2 is from Proteintech (20517-1-AP). Antibodies against p-AKT1 (Thr 308) (sc-135650), mTOR (sc-8319), p-mTOR (Ser 2448) (sc-101738), p-P70S6K (sc-101768) andβ-actin (sc-10731) were from Santa Cruz. A secondary antibody conjugated with horseradish peroxidase against rabbit IgG (sc-2030) was also from Santa Cruz.

Immunohistochemistry

The clinical specimens including pancreatic ductal adenocarcinoma and normal tissues were collected from the West China Hospital of Sichuan University. The sections were performed using an avidine-biotine-peroxidase complex (ABC) method and visualized with diaminobenzidine (DAB) according to the manufacturer's instructions. For statistical analysis, total staining was scored as the product of the staining intensity (negative=0, mild=1, moderate=2, and strong=3)×the percentage of positive cells (recorded on an ordered categorical scale: 0–5 %=0, 6–30 %=1, 31–70 %=2, and 71–100 %=3), resulting in a scale of 0–9 [19].

Western Blot

To extract proteins, the tissue samples were pulverized in liquid nitrogen, lysed in RIPA buffer for 40 min on ice, and subjected to centrifugation at 13,000 rpm for 40 min at 4 °C. The proteins in supernatant was assayed with a Protein Assay kit (Bio-Rad), separated by 12 % SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked in TBST containing 6 % skimmed milk for 1 h at 37 °C, and then incubated with primary antibodies for 1 h at 37 °C. After washing three times with TBST, the membranes were probed with secondary antibodies for 40 min at 37 °C. Finally, the membranes were washed three times and developed with Immobilon Western Chemiluminescent reagent (Millipore).

Statistical Analysis

Quantitative data were recorded as means±SD. One-way analysis of variance was used to analyze differences among multiple groups. The criterion for significance was set at $P<0.05$ for all analyses. Computations were performed by the statistical software SPSS 11.5.

Results

Expression Analysis

A total of 132 tissue samples of pancreatic ductal carcinoma and 73 samples of pancreatic normal tissues were assessed for expression levels of DESI2, p-AKT, mTOR, p-mTOR and p-P70S6K by means of immunohistochemistry. The results showed DESI2 presents strong staining in normal tissues, but weak staining in cancer tissues (Fig. 1). For p-AKT, mTOR, p-mTOR and p-P70S6K, their higher level were detected in cancer tissues compared with normal tissues (Fig. 1). To confirm the expression of DESI2, p-AKT, mTOR, p-mTOR and p-P70S6K in tissues, Western blot was performed to verify the representative tissues and showed the similar levels to immunohistochemistry (Fig. 2). The clear bands of Western blot also reflected the specificity of the antibodies in this work, indicating reliable staining of tissue immunohistochemistry.

Clinicopathological Features

Clinicopathological association of DESI2 levels with various pancreatic parameters was evaluated, which included gender, age, differentiation and clinical stage (Table 1). DESI2 levels showed significant differences between cancer and normal tissues ($P<0.001$), with reduced expression in cancer. It is noteworthy that immunohistochemistry showed nominal staining in poorly differentiated cancer tissues compared with well and moderately differentiated cancer tissues (Fig. 3). Consequently, analysis showed significant differences of DESI2 levels were caught among the cancer tissues with various differentiations ($P<0.05$), showing extremely low scores in poorly differentiated cancer (Table 1). Moreover, gender, age, and clinical stage failed to exhibit significant difference for DESI2 expression between cancer and normal tissues (Table 1).

Activation of AKT/mTOR Signal and DESI2 Expression

To explore the implications of DESI2 in pancreatic ductal adenocarcinoma, we further assess the status of AKT/mTOR activation in cancer with different DESI2 levels through immunohistochemistry. In this regard, the samples of pancreatic caner were classified into two groups: DESI2-lower cancer and

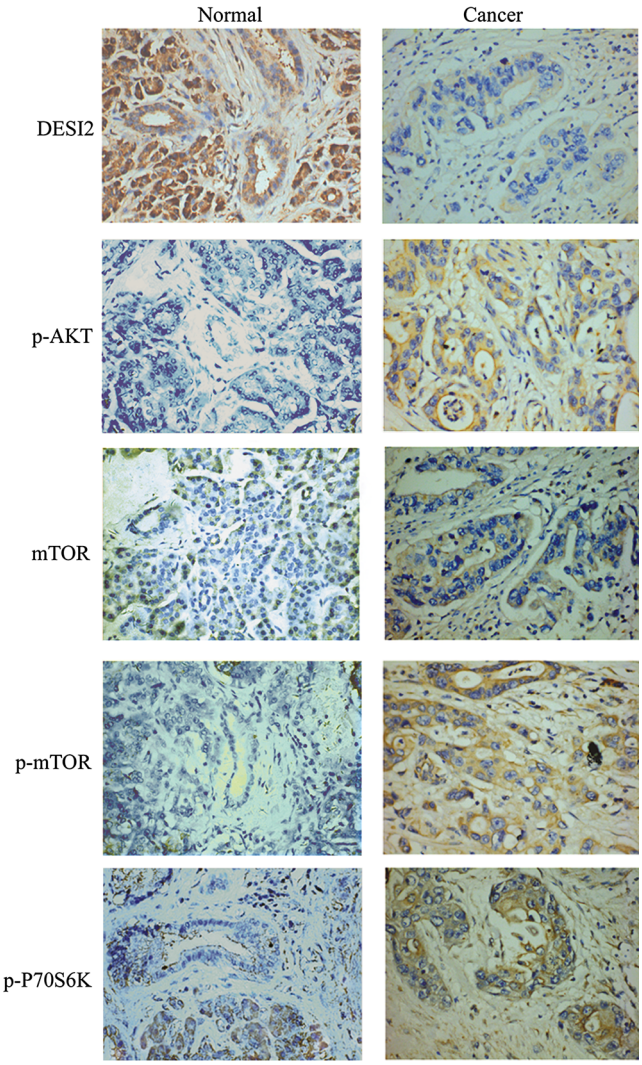

Fig. 1 Expression analysis in pancreatic ductal adenocarcinoma and pancreatic normal tissues. The staining intensities of DESI2 are obviously lower in cancer tissues compared with normal tissues. However, p-AKT, mTOR, p-mTOR and p-P70S6K were found to present higher staining in cancer tissues compared with normal tissues

DESI2-higher cancer. We analyzed levels of p-AKT and found there is no difference in the two groups of pancreatic caner (Table 2). It is notably that p-mTOR is significantly increased in DESI2-lower cancer compared with DESI2-higher cancer, although mTOR present no difference in the two groups of pancreatic caner (Table 2).

Further analysis revealed the interesting results. The relative p-mTOR/mTOR ratio is significantly elevated in DESI2-lower cancer (Fig. 4a). Moreover, the samples whose p-AKT and p-mTOR score both exceed the median are obviously increased in DESI2-lower cancer compared with DESI2-higher cancer, with 43/70 versus 11/62 (Fig. 4b). As a downstream molecule of AKT/mTOR signal, p-P70S6K was found to display higher level in DESI2-lower pancreatic cancer compared with DESI2-higher cancer (Table 2), indicating increased phosphorylation role of AKT/mTOR signal for

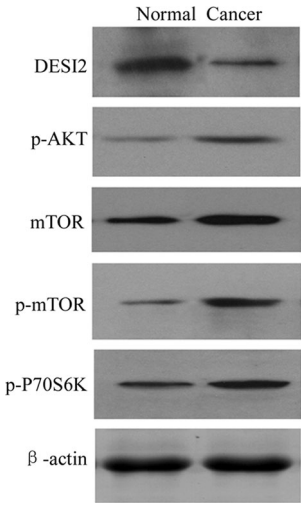

Fig. 2 Western blot shows expression of DESI2, p-AKT, mTOR and p-mTOR in representative cancer and normal tissues. Equal loading of protein was determined by β-actin

P70S6K in condition of DESI2 reduction. These results indicate that active status of AKT/mTOR signal in pancreatic ductal adenocarcinoma with reduced DESI2 expression.

Disscusion

To date, the nature of DESI2 is still unknown. In previous study, we found DESI2 is down-regulated in pancreatic ductal

Table 1 Clinicopathlogical association of DESI2 with pancreatic ductal adenocarcinoma

Factors	Number	Average score	*P*-value
Group			
Cancer	132	3.25±1.73	< 0.001
Normal	73	6.71±2.59	
Gender			
Male	76	2.96±2.26	0.273
Female	56	3.64±2.29	
Age			
31–59	87	2.98±2.17	0.231
≥60	45	3.77±2.31	
Differentiation			
Well	21	4.38±2.81	0.032
Moderate	58	3.66±2.45	
Poor	53	2.35±1.52	
Clinical stage			
IA + IB	49	3.96±2.41	0.059
IIA + IIB	76	2.85±2.09	
III	3	2.53±0.92	
IV	4	2.61±0.74	

Clinical stage is according to American Joint Committee on Cancer: AJCC Cancer Staging Manual 6th edition

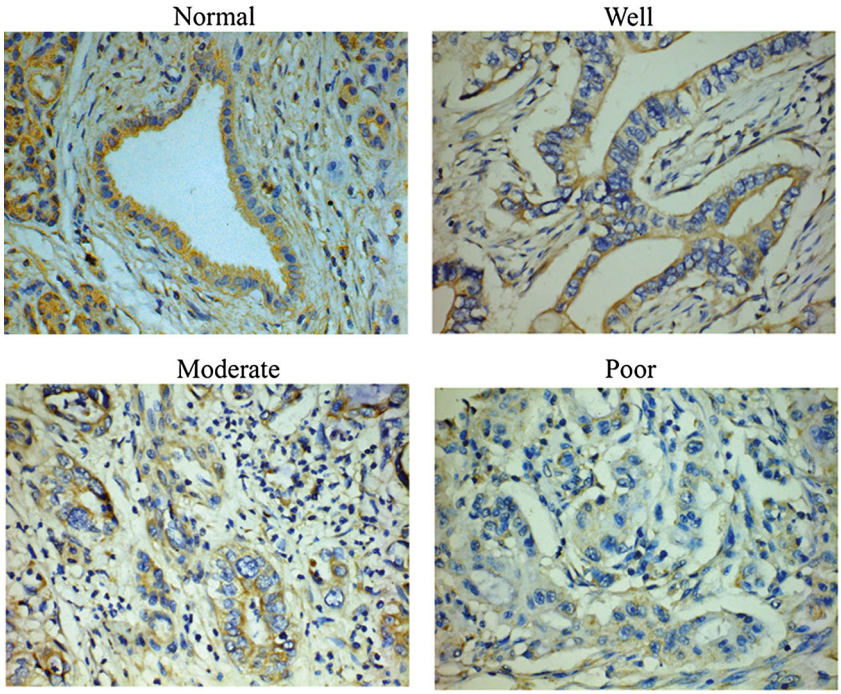

Fig. 3 Features of DESI2 expression in clinical tissues of pancreatic ductal adenocarcinoma. DESI2 intensities decrease as tissue differentiation declines in cancer tissues, showing slightest staining in poorly differentiated caner

adenocarcinoma and skin cancer. However, DESI2 expression is relatively stable in most other cancer, indicating the specific implications of DESI2 in those cancers. In this study, we continued to expand the library of tissue samples to assess the feature of DESI2 expression and key factors in AKT/mTOR pathway in pancreatic ductal adenocarcinoma, and the obtained data confirm our and other's previous findings. However, further analysis of our data reveals some interesting implications of DESI2 in pancreatic ductal adenocarcinoma.

As a member of DESI family, bioinformatics analysis show DESI2 has very high homology to DESI1 that has been demonstrated to be a deSUMOylase [4–6]. Our previous study identified that DESI2 is located at Golgi apparatus, suggesting it could be involved in post-translational modification of proteins [10]. It is known that deSUMOylation is a reverse process for SUMO, mainly involving in protein stability and gene transcription [20–22]. Although the substrate of DESI1 has been identified to be BZEL, a new transcriptional repressor, the target of DESI2 is still unclear [6]. We previously found DESI2 overexpression could cause to obvious degradation of vimentin, but it is necessary to identify the precise mechanism responsible to the stability of vimentin [9].

Table 2 p-AKT, p-mTOR, mTOR and p-P70S6K expression in pancreatic ductal adenocarcinoma with various DESI2 levels

Factors	Lower DESI2 level	Higher DESI2 level	*P*-value
Score	≤4	>4	
Number	70	62	
p-AKT	7.62±2.37	6.53±2.19	0.26
p-mTOR	7.36±3.56	4.27±2.68	0.021
mTOR	5.93±2.85	7.13±2.94	0.12
p-P70S6K	8.13±3.65	4.25±2.31	< 0.001

In view of post-translational modification of proteins is a foundation action in cell and play wide roles to affect downstream processes such as phosphorylation pathway [23]. Therefore, we focused on AKT/mTOR, an essential signal corresponding to pancreatic cancer, and investigated its potential connections to DESI2 expression. By analysis of clinical tissues, we found p-mTOR is significantly increased in DESI2-lower cancer, suggesting there are some links between the higher malignancy and DESI2 reduction, which is also consistent with our finding that poor differentiation with DESI2 reduction. It is interesting that the ratio of p-mTOR/mTOR is significantly elevated in DESI2-lower cancer, indicating the factors participating in process of p-mTOR phosphorylation are activated in condition of DESI2 reduction. The factors that impact AKT/mTOR signal are very complicated, covering PTEN, Erk, AMPK, et al., which can influence PI3K, TSC2 and Raptor in AKT/mTOR pathway [24, 25]. The different activation status of AKT/mTOR signal with various DESI2 expressions suggests that DESI2 is not only a biomarker, but also play certain roles in progression of pancreatic carcinoma through affecting AKT/mTOR signal.

As a classic downstream molecule, higher p-P70S6K expression also suggests more active AKT/mTOR signal in DESI2-reduced cancer, which further provides the evidence of DESI2 about its influence on phosphorylation status of AKT/mTOR signal [26]. Apart from the methods such as DESI2 interference in vitro, clinical tissues were applied in this study to assess activation status of AKT/mTOR signal in

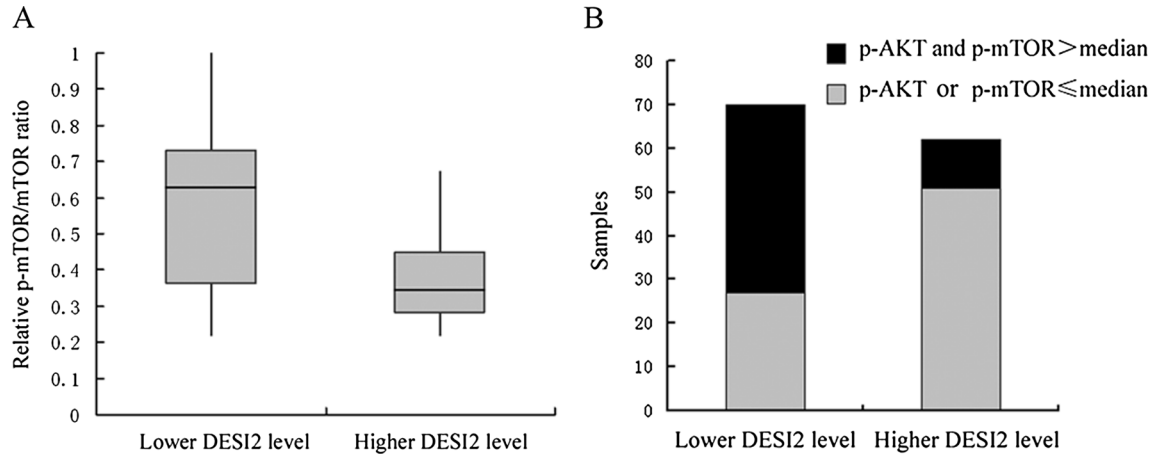

Fig. 4 Assessment of AKT/mTOR status in pancreatic ductal adenocarcinoma with various DESI2 levels. **a** Relative p-mTOR/mTOR ratio is exhibited in chart. For each set of values, *the box* represents the middle half of the data distributing from 25 to 5 %. The *line across the box* represents the median. The *lines above and below the box* represent the relative maximum and minimum values, respectively. **b** Number and scale of samples whose p-AKT and p-mTOR both exceed the median are exhibited in chart

DESI2-reduced pancreatic cancer. It is accepted that occurrence and development of cancer are extremely complex [11]. Many events such as tumor microenvironment, metabolism and immunity are believed to contribute to carcinogenesis [11]. Therefore, it is difficult to elucidate the progression of pancreatic cancer just by means of cell lines in vitro. Depending on clinical tissues, we investigated the potential connections of DESI2 and AKT/mTOR signal in the real pathologic state; however, these clues in clinical tissues could be ignored in vitro conditions.

In summary, our data suggest potential value of DESI2 as a reduced biomarker for pancreatic ductal adenocarcinoma. Significant activation of AKT/mTOR signal in DESI2-reduced state indicates the functional implications of DESI2 in cancer progression. Further investigations on interaction network of DESI2 will reveal its precise roles, which should contribute to the treatment of pancreatic cancer.

Acknowledgments This study was supported by the National Natural Science Foundation of China (81101530).

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