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ORIGINAL ARTICLE

Expression and significance of TLR4 and HIF-1α in pancreatic ductal adenocarcinoma

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Abstract

AIM: To investigate the expression of toll-like receptor (TLR) 4, nuclear factor-κB (NF-κB) p65 and hypoxia-inducible transcription factor 1α (HIF-1α) in pancreatic ductal adenocarcinoma and their clinical significance.

METHODS: The mRNA of TLR4 and HIF-1α were investigated by real-time polymerase chain reaction in 30 cases of pancreatic ductal adenocarcinoma and its adjacent tissues, and expression of TLR4, NF-κB p65 and HIF-1α protein were detected by immunohistochemistry in 65 cases of pancreatic ductal adenocarcinoma tissues and 38 cases of corresponding adjacent tissues. The relationship between TLR4 or HIF-1α and pathologic features, as well as the association between TLR4 and HIF-1α, were also analyzed. Kaplan-Meier method was used to assess the impact of expression of TLR4 and HIF-1α on survival of patients with pancreatic cancer.

RESULTS: The relative quantification of TLR4 and HIF-1α mRNA in tumor tissues was 0.81 ± 0.10 and 0.87 ± 0.11, respectively, significantly higher than that in adjacent tissues (0.81 ± 0.10 *vs* 0.70 ± 0.16, $P = 0.002$; 0.87 ± 0.11 *vs* 0.68 ± 0.13, $P = 0.000$). The protein expression of TLR4, NF-κB p65 and HIF-1α in tumor tissues was 69.20%, 66.15% and 70.80%, respectively, being significantly higher than that in adjacent normal tissues (69.20% *vs* 39.50%, $P = 0.003$; 66.15% *vs* 31.58%, $P = 0.001$; 70.80% *vs* 36.80%, $P = 0.001$). There was no significant correlation between TLR4 or HIF-1α expression and the age, gender, tumor location, the degree of tumor differentiation in the patients ($P > 0.05$). However, there was significant correlation between the expression of TLR4 or HIF-1α and tumor size, lymph node metastasis, venous invasion and clinical staging ($P < 0.05$). The expression of TLR4 and HIF-1α had a significant impact on survival of patients with pancreatic adenocarcinoma.

CONCLUSION: TLR4, NF-κB p65 and HIF-1α are overexpressed in pancreatic adenocarcinoma, TLR4 may be partly involved in up-regulating HIF-1α, and both synergestically promote development of pancreatic adenocarcinoma.

Key words: Pancreatic ductal adenocarcinoma; Toll-like receptor 4; Nuclear factor-κB p65; Hypoxia-inducible factor 1

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INTRODUCTION

Because of pancreas insidious nature and anatomic location, pancreatic ductal adenocarcinoma (PDAC) is difficult to be detected early, often becomes advanced by the time of diagnosis. The 5-year survival rate is less than 5% and a median survival time is 5-6 mo[1]. Although 10%-15% of patients underwent potentially surgical resection, the median survival was only 10-18 mo with a 5-year survival of 17%-24%[2]. Most failures occur within 1-2 years after surgery because of local recurrence, hepatic metastases, or both[3]. Therefore, understanding the molecular biology of pancreatic cancer is important for diagnosis, prevention and treatment of PDAC.

Toll-like receptors (TLRs) are a family of pattern recognition receptors that are best-known for their role in host defense against infection. In human, 13 types of TLRs have been identified, mainly expressed by immune cells and epithelial cells. Recently, TLRs have also been detected in many tumor cell lines or tumors, especially epithelium-derived cancers[4]. TLRs may promote the proliferation and inhibit the apoptosis, and lead to migration, invasion and angiogenesis of tumor[5]. However, it remains unknown whether TLR4 is expressed and what role it plays in PDAC.

Hypoxia-inducible transcription factor 1 (HIF-1) is a heterodimer that consists of hypoxic response factor HIF-1α and the constitutively expressed HIF-1β[6]. HIF-1α is unstable in well-oxygenated tissues due to ubiquitin-mediated degradation, but rapidly becomes stable in hypoxic conditions[7]. In human cancers, HIF-1α is overexpressed as a result of intratumoral hypoxia and genetic alterations[8], HIF-1 targets the transcription of over 60 genes involved in many aspects of cancer biology including cell survival, glucose metabolism, cell invasion and angiogenesis[9]. However, the regulatory mechanisms of HIF-1α in tumor microenviroment are complex and still not fully understood. Recently, some studies have demonstrated that bacteria or Lipopolysaccharide (LPS)-induced HIF-1α stabilization even under normoxic conditions[10-12], and LPS-induced HIF-1α accumulation was TLR4 dependent[13] in immune cells, such as macrophage[10,12,13], monocytes[11]. Thus, we wonder whether there is some association between HIF-1α and TLR4 in tumor cells similar to immune cells.

In this study, TLR4 and HIF-1α mRNA and protein were detected in PDAC and its adjacent tissues by real-time polymerase chain reaction (PCR) and immunohistochemical methods. The association between TLR4 and HIF-1α, and impact of their expression on patients' survival were analyzed. The nuclear factor-κB (NF-κB) p65, which is an essential component in both TLR4 signal pathway and HIF-1 signal pathway, was also detected by immunohistochemistry.

MATERIALS AND METHODS

Patients and samples

The study group consisted of 65 patients with primary PDAC, 40 males and 25 females, ranged in age from 36 to 82 years, with a median age of 52 years. They underwent pancreaticoduodenectomy or distal pancreatectomy with lymph node dissection at Pancreatic Center of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, between January 2007 and December 2008. Sixty-five cases of pancreatic cancerous tissues and 38 cases of corresponding adjacent normal tissues were collected, the adjacent normal tissues were taken from the edge of the tumor and confirmed by pathology. All samples were divided into two parts immediately after removed from patients, one was put into liquid nitrogen for real-time PCR, the other was fixed in 4% buffered formaldehyde and embedded in paraffin wax for immunohistochemistry. None of the patients received preoperative chemotherapy or radiation therapy. Among 65 patients, 25 (38.5%) cases were moderately differentiated, 21 (32.3%) well differentiated, and 19 (29.2%) poorly differentiated. Forty cases (61.5%) had lymph node metastases. Nineteen cases (29.2%) had venous invasion. According to the sixth edition of the TNM classification of the International Union Against Cancer (UICC), 24 patients were at pathological stage Ⅰ or Ⅱ, and 41 patients were at stage Ⅲ or Ⅳ. All patients received chemotherapy routinely after operation, and were followed up regularly for 5-35 mo (mean, 14 mo). The procedure had been approved by the Ethics Committee of Union Hospital, Tongji Medical College.

RNA isolation, reverse transcription and real-time PCR

The total RNA of PDAC and adjacent tissues were extracted using Trizol (invitrogen, USA) according to the manufacturer's protocol. The concentration was analyzed by ultraviolet spectrophotometer. Two micrograms of RNA was used for the reverse transcription. TLR4, HIF-1α and β-actin genes were amplified with SYBR Green in a fluorescence reader FTC-2000 (Feng Ling Bio-engineering Co., Ltd., Shanghai, China). The primer sequences for TLR4 gene were as follows: 5'-ACCTGTCCCTGAACCCTATGAA-3' (forward) and 5'-CTTCTAAACCAGCCAGACCTTG-3' (reverse); the primer sequences for HIF-1α gene were: 5'-TGAAGTGTACCCTAACTAGCCG-3' (forward) and 5'-AATCAGCACCAAGCAGGTCATAG-3' (reverse); and the primer sequences for β-actin gene were: 5'-GAAACTACCTTCAACTCCATC-3' (forward) and 5'-CGAGGCCAGGATGGAGCCGCC-3' (reverse). Cycling conditions were as follows: initial denaturation at 94℃ for 5 min, followed by 30 cycles at 94℃ for 30 s, at 58℃ for 30 s and at 72℃ for 30 s. The negative control was performed using normal pancreatic tissue taken far away from the cancerous tissues. The results were analyzed by the fluorescence reader program [calculation formula: folds = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct$ = [(Ct target gene - Ct β-actin) experiment sample - (Ct target gene - Ct β-actin) control sample][14]. Each experiment was carried out in triplicate

using β-actin as an internal standard; the results were expressed as mean ± SD of representative triplicates.

Immunohistochemistry

The tissue specimens were fixed in 10% formalin buffered at pH 7.0 for 24 h and paraffin-embedded. Histologic slides (5 μm) were deparaffined in xylene and rehydrated through a series of graded ethanol. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was performed by microwaving the slides in citrate buffer (pH 6.0) for 20 min at 120℃. Then the sections were washed and blocked with normal goat serum (Streptavidin/Peroxidase immunohistochemical kit, Zhongshan Goldenbridge Biological Technology Co., LTD, Beijing, China) for 30 min at room temperature, subsequently exposed to the primary antibody (rabbit polyclonal anti-TLR4, 1:50, Santa Cruz, CA, USA; mouse monoclonal anti-HIF-1α, 1:50, Santa Cruz, CA, USA; NF-κB p65 rabbit monoclonal antibody, 1:50, Cell Signaling Technology Inc, MA, USA). overnight at 4℃. After washing in phosphate buffered saline (PBS), the slides were incubated with a biotinylated goat anti-mouse or anti-rabbit secondary antibody (1:200, Streptavidin/Peroxidase immunohistochemical kit) for 30 min at room temperature. After reactions with freshly prepared diaminobenzidine-tetrahydrochloride (DAB kit, Zhongshan Goldenbridge Biological Technology Co., LTD, Beijing, China), the slides were counterstained in haematoxylin and coverslipped in a systemic mounting medium. Negative controls were prepared without the primary antibodies.

The assessment of TLR4, HIF-1α and NF-κB p65 was based on previously described guidelines[15], which consist of the percentage of stained tumor cells and the staining intensity. At least 3 different high-power (× 400) fields of tumor infiltration were examined. The percentage of positive tumor cells was rated as follows: 1 point, ≤ 10% positive tumor cells; 2 points, 11%-50% positive tumor cells; 3 points, 51%-80% positive tumor cells; and 4 points, ≥ 81% positive tumor cells. The staining intensity was rated as follows: 1 point, weak intensity; 2 points, moderate intensity; and 3 points, strong intensity. Points for expression and percentage of positive cells were added, and specimens were attributed to four groups according to their overall score: negative, ≤ 10% of cells stained positive, regardless of intensity; weak expression, 3 points; moderate expression, 4-5 points; and strong expression, 6-7 points. Weak expression was rated as negative, and moderate and strong expressions were rated as positive for analysis. Two independent investigators (Zhang JJ and Tian Y) blinded to clinical data performed the analysis. Specimens scored differently by the two investigators were reinvestigated together using a multiheaded microscope.

Statistical analysis

For statistical analysis, SPSS 15.0 was used. The independent *t* test was used to compare the means of TLR4 or HIF-1α mRNA between PDAC and its adjacent tissues. The χ^2 test was used to analyze the difference in expression of TLR4, NF-κB p65 and HIF-1α protein between PDAC and its adjacent tissues, as well as correlation between TLR4, HIF-1α and clinicopathologic features. One-way analysis of variance was used for three groups. Survival curves were calculated using the Kaplan-Meier method and analyzed using the log-rank test. Differences at $P < 0.05$ were considered to be statistically significant.

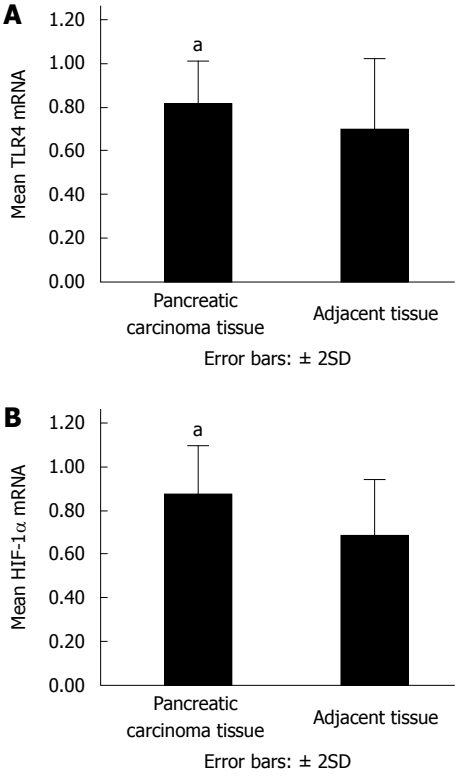

Figure 1 Toll-like receptor (TLR) 4 and hypoxia-inducible transcription factor-1α (HIF-1α) mRNA in pancreatic carcinoma and adjacent tissues. [a]*P* < 0.05 *vs* adjacent tissues. A: TLR4 mRNA in 30 cases of pancreatic carcinoma and adjacent tissues was determined by real-time polymerase chain reaction (PCR); B: HIF-1α mRNA in 30 cases of pancreatic carcinoma and adjacent tissues was determined by real-time PCR.

RESULTS

TLR4 is overexpressed in PDAC

To investigate whether TLR4 is expressed in PDAC, we first detected TLR4 mRNA by real-time PCR. The results showed that the mean level of TLR4 mRNA was 0.81 ± 0.10 in tumor tissues, and it was 0.70 ± 0.16 in adjacent normal tissues, with significant difference between them ($P = 0.002$, Figure 1A). We then detected TLR4 protein by immunohistochemistry, and found that TLR4 protein was localized in cytoplasm and membrane of tumor cells (Figure 2A and B), and the expression of TLR4 was 69.2% (45/65) in cancerous tissues, which was significantly higher than that in adjacent normal tissues (69.2% *vs* 39.5%, $P = 0.003$, Table 1). When analyzing the relationship between TLR4 and clinicopathological features, we found that the expression of TLR4 mRNA or protein was not correlated with age, gender, location and differentiation of the tumor ($P > 0.05$), but

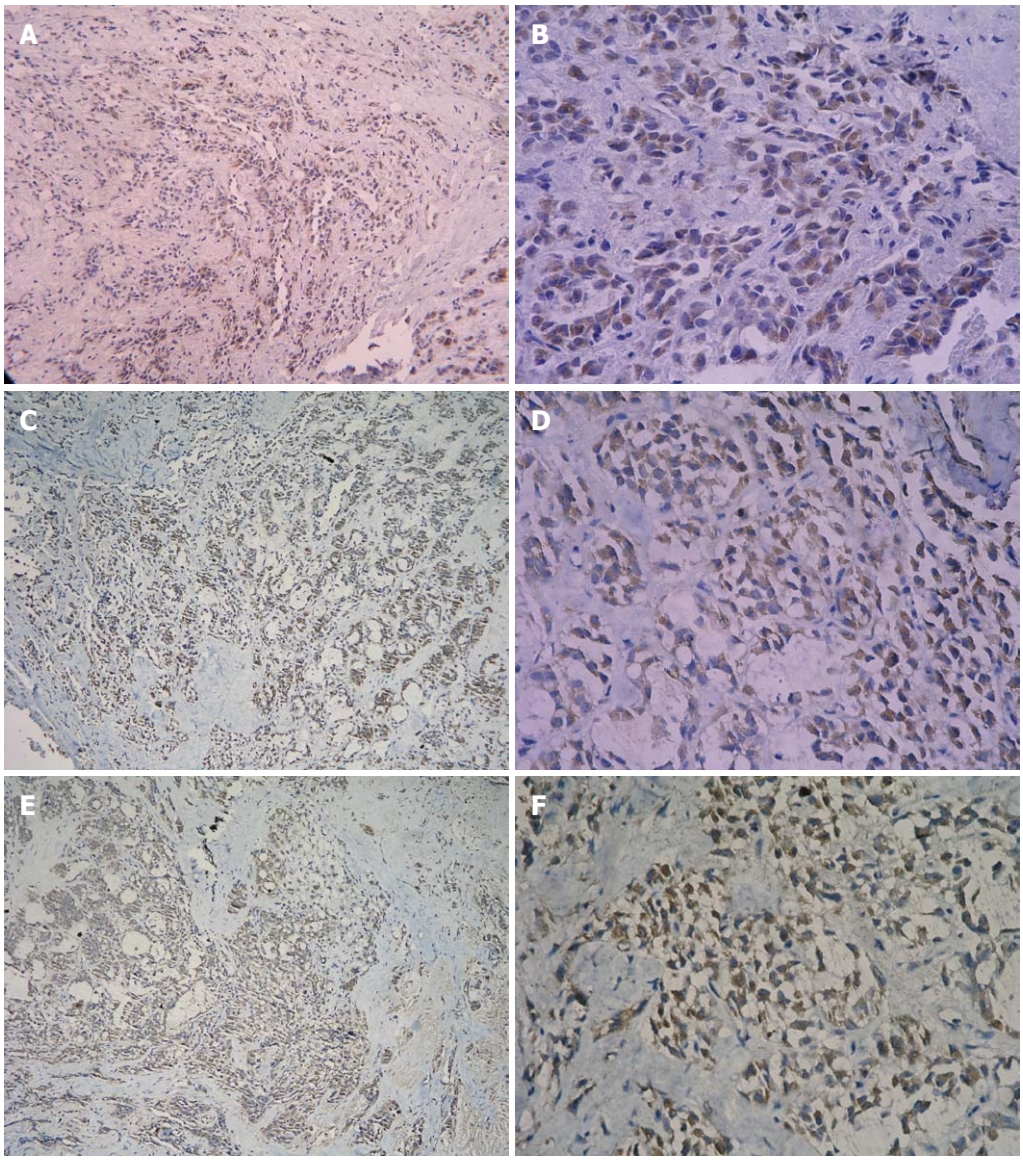

Figure 2 Expression of TLR4 and HIF-1α protein in pancreatic carcinoma tissue. Brown color displays the positive expression. A: Expression of TLR4 protein in carcinoma tissue (SP, × 100); B: Expression of TLR4 protein in carcinoma tissue (SP, × 400); C: Expression of HIF-1α protein in carcinoma tissue (SP, × 100); D: Expression of HIF-1α protein in carcinoma tissue (SP, × 400); E: Expression of NF-κB p65 protein in carcinoma tissue (SP, × 100); F: Expression of NF-κB p65 protein in carcinoma tissue (SP, × 400).

Table 1 Expression of TLR4 and HIF-1α protein in pancreatic carcinoma and adjacent tissues *n* (%)

	Cases	TLR4 protein		HIF-1α protein	
		Positive	*P* value	Positive	*P* value
Tumor tissues	65	45 (69.20)	0.003	46 (70.80)	0.001
Adjacent tissues	38	15 (39.50)		14 (36.80)	

TLR4: Toll-like receptor 4; HIF-1α: Hypoxia-inducible transcription factor-1α.

significantly correlated with tumor size, lymph node involvement, venous invasion and pathological stage ($P < 0.05$, Table 2).

HIF-1α is overexpressed in PDAC

We continued to detect HIF-1α in PDAC, and found that the mean level of HIF-1α mRNA in tumor tissues was 0.87 ± 0.11, being significantly higher than that in adjacent tissues, which was 0.68 ± 0.13 ($P = 0.000$, Figure 1B). Immunohistochemical results showed that HIF-1α was expressed in the nuclei and/or the cytoplasm of tumor cells (Figure 2C and D). The expression of HIF-1α was found in 46 of 65 (70.8%) cases of tumor tissues, and 14 of 38 (36.8%) cases of adjacent tissues, with significant difference between them (Table 1, $P < 0.05$). Furthermore, we also found that the expression of HIF-1α was correlated with tumor size, lymph node involvement, venous invasion and pathological stage ($P < 0.05$, Table 2).

Correlation of TLR4 and HIF-1α expressions in PDAC

In macrophages, LPS can raise the level of HIF-1α in a TLR4-dependent fashion[13], but it remains unknown in tumor cells. To study whether TLR4 is associated with HIF-1α in PDAC, we performed the correlative analysis.

Table 2 Relationship between TLR4, HIF-1α and clinical pathologic features

	TLR4 mRNA		TLR4 protein			HIF-1α mRNA		HIF-1α protein		
	mean ± SD	*P*	+	-	*P*	mean ± SD	*P*	+	-	*P*
Age (yr)		0.258			0.381		0.150			0.229
≤ 59	0.83 ± 0.10		24	13		0.90 ± 0.13		24	13	
≥ 60	0.79 ± 0.09		21	7		0.84 ± 0.08		22	6	
Gender		0.133			0.137		0.744			0.464
Male	0.79 ± 0.09		25	15		0.88 ± 0.11		27	13	
Female	0.84 ± 0.10		20	5		0.86 ± 0.12		19	6	
Location of tumor		0.374			0.280		0.197			0.680
Pancreas head	0.80 ± 0.11		31	11		0.89 ± 0.13		29	13	
Pancreas body-tail	0.83 ± 0.09		14	9		0.84 ± 0.08		17	6	
Histology		0.654			0.585		0.279			0.224
Well	0.84 ± 0.11		13	8		0.88 ± 0.11		12	9	
Moderate	0.80 ± 0.11		19	6		0.89 ± 0.12		20	5	
Poor	0.80 ± 0.07		13	6		0.82 ± 0.08		14	5	
Tumor size (cm)		0.021			0.014		0.030			0.039
≤ 2	0.77 ± 0.07		9	10		0.82 ± 0.08		10	9	
> 2	0.85 ± 0.10		36	10		0.91 ± 0.12		36	10	
pN		0.012			0.017		0.049			0.038
Positive	0.85 ± 0.09		32	8		0.90 ± 0.12		32	8	
Negative	0.77 ± 0.08		13	12		0.82 ± 0.08		14	11	
Venous invasion		0.021			0.023		0.018			0.033
Positive	0.85 ± 0.09		17	2		0.91 ± 0.12		17	2	
Negative	0.77 ± 0.09		28	18		0.82 ± 0.07		29	17	
pStage		0.031			0.010		0.016			0.024
Ⅰ, Ⅱ	0.77 ± 0.09		12	12		0.81 ± 0.07		13	11	
Ⅲ, Ⅳ	0.84 ± 0.09		33	8		0.91 ± 0.12		33	8	

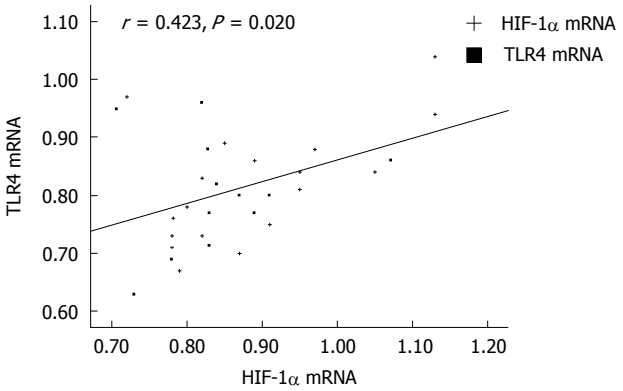

Figure 3 Correlation between TLR4 mRNA and HIF-1α mRNA.

The results showed that there existed a significant correlation between TLR4 mRNA and HIF-1α mRNA (r = 0.423, P = 0.020, Figure 3), similar result was also found between TLR4 and HIF-1α protein (r_p = 0.451, P < 0.05, Table 3), suggesting that TLR4 has a positive correlation with HIF-1α in PDAC.

NF-κB p65 is overexpressed in PDAC

NF-κB is an essential downstream component of TLR signal pathway[16], and HIF-1α is a target gene of NF-κB[17]. To further explore the association between TLR4 and HIF-1α, we investigated NF-κB p65 expression in PDAC by immunochemistry. The results showed that NF-κB p65 was expressed in the nuclei and/or the cytoplasm of tumor cells (Figure 2E and F), and the positive expression of NF-κB p65 was found in 43 (66.15%) of 65 cases of tumor tissues, being significantly higher than

Table 3 Correlation between TLR4 and HIF-1α protein

	TLR4 expression		r_p	*P* value
	Positive (n = 45)	Negative (n = 20)		
HIF-1α expression			0.451	0.000
Positive (n = 46)	38	8		
Negative (n = 19)	7	12		

in 12 (31.58%) of 38 cases of adjacent tissues (P = 0.001). Considering NF-κB is an essential component in TLR4 signal pathway and an important modulator of HIF-1α, we could speculate that NF-κB p65 may be a link in the association between TLR4 and HIF-1α.

Overexpression of TLR4 and HIF-1α in PDAC has a negative impact on patient survival

In order to understand whether over-expression of TLR4 and HIF-1α in PDAC has an impact on patient survival, the relationship between the expression of TLR4 or HIF-1α and life span of patients was analyzed. The mean survival of patients without over-expression of TLR4 in tumor tissues was 19.7 mo, and it was 12.4 mo in those with over-expression of TLR4, log-rank analysis showed there was a significant difference (P = 0.011, Table 4 and Figure 4A). Similarly, the mean survival of patients without over-expression of HIF-1α in tumor tissues was 20.1 mo, being significantly longer than those with over-expression of HIF-1α, which was 12.8 mo (P = 0.005, Table 4 and Figure 4B). In addition, the survival time of group with neither TLR4 nor HIF-1α over-expression

Table 4 Impact of TLR4 and HIF-1α on survival time

Protein	Expression	Estimated survival	95% CI		*P*
			Lower bound	Upper bound	
TLR4	Positive	12.4	10.8	14.0	0.011
	Negative	19.7	15.4	23.9	
HIF-1α	Positive	12.8	11.1	14.4	0.005
	Negative	20.2	15.2	25.1	
TLR4 positive	HIF-1α positive	11.8	10.1	13.4	0.132
	HIF-1α negative	15.0	10.9	19.2	
TLR4 negative	HIF-1α positive	16.5	12.5	20.6	0.089
	HIF-1α negative	21.6	15.1	28.2	0.014[1]

[1]*P* value compared between patients with both negative TLR4 and HIF-1α expression and those with both positive TLR4 and HIF-1α expression.

was 21.6 mo, significantly longer than those with both over-expressions, which was 11.8 mo ($P = 0.014$, Table 4 and Figure 4C).

DISCUSSION

TLR4 was studied first in TLRs, which is mainly expressed in immune cells and epithelial cells. The activation of TLR4 facilitates the activation of two pathways: the MyD88 (myeloid differentiation primary-response protein 88)-dependent and MyD88-independent pathways. The MyD88-dependent pathway involves the early phase of NF-κB activation, which leads to the production of inflammatory cytokines. The MyD88-independent pathway activates interferon (IFN)-regulatory factor (IRF3) and involves the late phase of NF-κB activation, both of which lead to the production of IFN-β and the expression of IFN-inducible genes. In 1998, TLR4 was identified as the signal transducer for LPS, a major cell-wall component of Gram-negative bacteria[18], in addition to microbial ligands, an increasing number of endogenous ligands are being reported as candidate stimulators of TLR4, including heat shock proteins (HSP60, HSP70, endoplasmin, HSPB8 and α-crystallin A chain), high mobility group box 1 (HMGB1), and various products of the extracellular matrix such as fibronectin, heparan sulphate, biglycan, fibrinogen, oligosaccharides of hyaluronan and hyaluronan breakdown fragments[5].

There are increasing evidences suggesting that TLR4 is also expressed in many tumor cell lines or tumors, especially epithelium-derived cancers[4]. In human head and neck squamous cell carcinoma[19], lung cancer[20], gastric carcinoma[21], ovarian tumors[22] and melanoma cells[23], TLR4 was all over-expressed, but not or lowly expressed in corresponding adjacent tissues or normal tissues. However, whether human pancreatic cancer cells express TLR4 remains to be understood.

In the present study, we found that not only TLR4 mRNA, but also TLR4 protein was over-expressed in PDAC compared with adjacent tissues. In addition, we found that the over-expression of TLR4 was related to tumor size, lymph node involvement, venous invasion and pathological stage. Kaplan-Meier analysis showed that the mean survival of patients without TLR4 expression in tumor tissues was significantly longer than those with TLR4 expression. These data indicated that TLR4 expressed in PDAC cells may contribute to its progression. Up to date, there have been increasing studies about effect and corresponding mechanism of TLR4 signal pathway on tumor progression. Pidgeon *et al*[24] found that endotoxin, a TLR4 agonist, introduced during surgery is associated with the enhanced growth of metastases following surgical trauma. In ovarian tumors, functional activity for TLR4 was demonstrated by stimulation of cell lines with specific ligands and subsequent activation and translocation of NF-κB and release of the proinflammatory cytokines interleukin-6 and CCL-2[22]. In human lung cancer cells[25], TLR4 ligation promoted production of immunosuppressive cytokines TGF-β, vascular endothelial growth factor (VEGF), proangiogenic chemokine IL-8, NF-κB was activated and contributed to apoptosis resistance of human lung cancer cells induced by LPS. Similar to other solid tumors, in pancreatic tumor microenviroment, there may also exist endogenous TLR4 ligands, which might be released during matrix degradation associated with tumor progression including HMGB1, heat shock proteins, hyaluronic acid and fibronectin fragment[22]. Many of these endogenous ligands may activate TLR4 signal pathway, induce inflammatory response, and promote tumor cells proliferation, apoptosis resistance, infiltration and metastasis. Thus, TLR4 might be a new marker of PDAC progression.

In solid tumors, cancer cells proliferation may outpace the rate of angiogenesis, result in tissue hypoxia, and cellular adaptation to hypoxia is a key step in tumor progression. This adaptation is regulated mainly by HIF-1 that is known to play an essential role in oxygen homeostasis[26]. In this study, we found the expression of both HIF-1α mRNA and protein was significantly higher in PDAC tissues than those in adjacent tissues, and the increased level of intracellular HIF-1α was associated with tumor size, lymph node involvement, venous invasion and pathological stage. These results were consistent with a previous study[27]. Wei *et al*[27] found that expression of HIF-1α was significantly higher in pancreatic cancer tissues, and there was significant difference in the expression of HIF-1α mRNA between Japanese Pancreatic Society stages Ⅰ-Ⅱ and stages Ⅲ-Ⅳ. Kaplan-Meier analysis also showed there was no significant

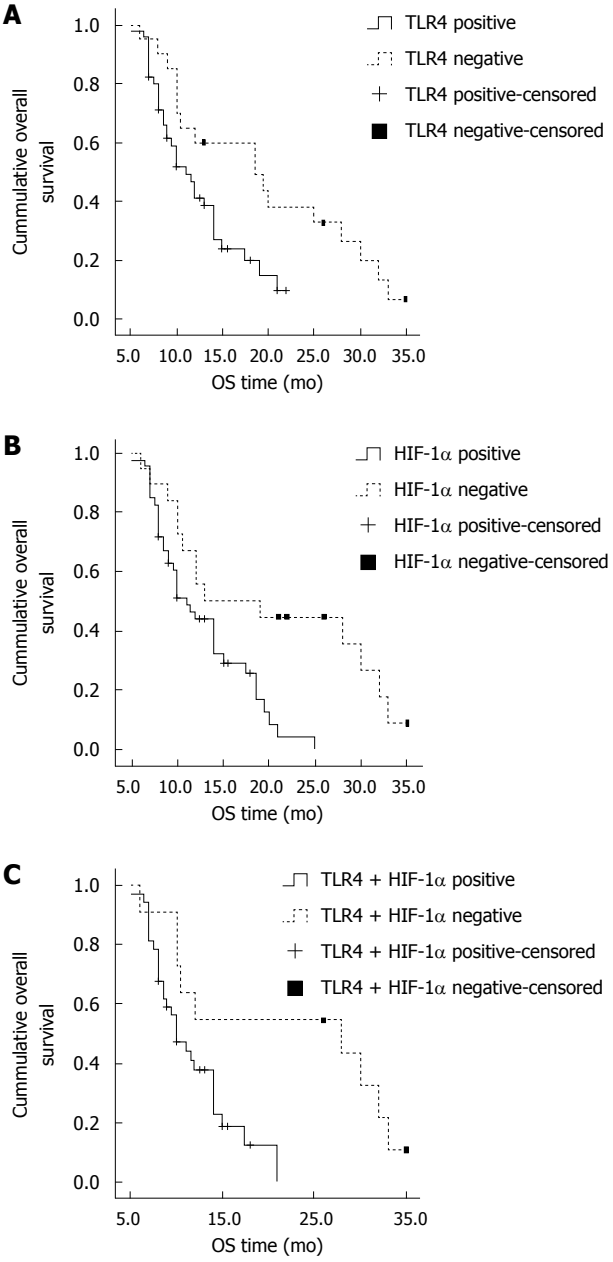

Figure 4 Kaplan-Meier analysis overall survival depending on TLR4 and HIF-1α expression in pancreatic ductal adenocarcinoma. A: The mean survival of patients with negative TLR4 expression in tumor tissues was significantly longer than those with TLR4 positive expression (P = 0.011); B: The mean survival of patients with negative HIF-1α expression in tumor tissues was significantly longer than those with HIF-1α positive expression (P = 0.005); C: Survival of patients with both negative TLR4 and HIF-1α expression was significantly longer than those with both positive TLR4 and HIF-1α expression (P = 0.014).

difference in the early stage of postoperation between negative and positive HIF-1α expression groups, but as time passed, the mean survival of patients without over-expression of HIF-1α in tumor tissues was significantly longer than those with over-expression of HIF-1α, which further demonstrated that patients with positive HIF-1α expression have worse prognosis. The main cause may be that upregulated and active HIF-1α forms complex with HIF-1β, translates into nuclear and activates the transcription of many genes that code for proteins involved in angiogenesis, glucose metabolism, cell proliferation, survival and invasion, metastasis[28]. Thus, the mortality of patients with over-expression of HIF-1α in the late stage increases because of recurrence, metastasis, chemotherapy and radiotherapy resistance. Thus, HIF-1α may serve as a useful molecular marker for prognosis of PDAC.

In the present study, we found that there was positive correlation between TLR4 and HIF-1α, indicating that there may exist a crosstalk between TLR4 signal pathway and HIF-1 signal pathway, which may act synergistically to promote the progression of PDAC. Recently, HIF-1α has also been shown to be up-regulated under normoxia in response to LPS[29]. In several reports, LPS, a ligand of TLR4, was found to induce accumulation and DNA-binding activity of HIF-1α protein in murine microglial cells[30], macrophage-differentiated cells[12], macrophages[31], and human myeloid monocytic leukaemia cells[32], which suggested that TLR4 signal pathway might regulate HIF-1α expression *via* some mechanism. van Uden *et al*[28] demonstrated that NF-κB was a direct modulator for HIF-1α expression. In our study, we also found that NF-κB p65 was over-expressed in PDAC. Considering NF-κB was an essential downstream component of TLR4 signal pathway[16], we speculated that the activation of TLR4 signal pathway in the microenviroment of PDCA might be partly involved in up-regulating HIF-1α *via* NF-κB pathway, then promoted progression of pancreatic adenocarcinoma.

In conclusion, the present study revealed that TLR4 and HIF-1α were over-expressed in pancreatic cancer, and both were related to the survival of patients with PDAC. On the other hand, there was positive association between TLR4 and HIF-1α. TLR4 signal pathway and HIF-1α signal pathway may synergistically promote development of the pancreatic cancer. However, the exact mechanism of crosstalk between HIF-1α and TLR4 in PDAC needs to be further studied.

COMMENTS

Background

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant digestive tumor with a very poor prognosis. Hypoxia-inducible transcription factor-1α (HIF-1α) is involved in malignant progression in many solid tumors, including PDAC, up-regulation of HIF-1α accelerates PDAC progression, but the exact regulatory mechanisms of HIF-1α in PDAC has not been unequivocally addressed. Recently, an increasing number of studies reported that toll-like receptors (TLRs) were upregulated in epithelial malignancies and involved in tumor progression, but whether TLRs, such as TLR4, is expressed on PDAC cells remains unknown. In immune- related cells, TLR signal pathway may induce expression of HIF-1α, but it is also still unclear whether there exists some association between TLR4 and HIF-1α in tumor microenviroment, such as PDAC.

Research frontiers

Activating TLRs pathway in tumor cells could promote the proliferation and inhibit the apoptosis, leading to migration, invasion and angiogenesis of tumor, but it remains unknown whether TLR4 is expressed and what role it plays in PDAC. In human cancers, HIF-1α is overexpressed as a result of intratumoral hypoxia. Recently, HIF-1α has also been shown to be up-regulated under normoxia in response to LPS, a potent agonist of TLR4, in immune-related cells, however, whether HIF-1α is regulated by TLR4 signal pathway in tumor is unknown. In this study, the authors demonstrate that TLR4 and HIF-1α are over-expressed in PDAC, and TLR4 expression is associated with HIF-1α. Nuclear factor-κB (NF-κB) p65, an important component in both TLR pathway and HIF-1 pathway, is also over-expressed in PDAC. The expression of TLR4 and HIF-1α has a

significant impact on survival of patients with PDAC. These results might indicate that TLR4 could be a potential factor for mediating HIF-1α in PDAC.

Innovations and breakthroughs

This is the first study to report that TLR4 is over-expressed in PDAC, and it is also the first report to show that TLR4 is associated with HIF-1α in PDAC.

Applications

The study demonstrated that TLR4, NF-κB p65 and HIF-1α were over-expressed in PDAC. The expression of TLR4 and HIF-1α was associated with tumor size, lymph node metastasis, venous invasion and pathological stage. Furthermore, the expression of TLR4 was positively correlated with expression of HIF-1α. Thus, TLR4 may be a novel marker for the progression and prognosis of PDAC, and may provide a new strategy for therapeutic intervention in the treatment of patients with PDAC in the future.

Peer review

This is an interesting study with valuable information regarding the expression and clinical correlation of TLR4 and HIF-1α in pancreatic ductal adenocarcinoma.

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