

ORIGINAL ARTICLE

Association of chloride intracellular channel 4 and Indian hedgehog proteins with survival of patients with pancreatic ductal adenocarcinoma

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SUMMARY

Pancreatic cancer is the fourth most common cause of cancer-related mortality. Novel molecular biomarkers need to be identified for personalized medicine and to improve survival. The aim of this study was to examine chloride intracellular channel 4 (CLIC4) and Indian Hedgehog (Ihh) expression in benign and malignant lesions of the pancreas and to examine the eventual association between CLIC4 and Ihh expression, with clinicopathological features and prognosis of pancreatic cancer. A retrospective study of specimens collected from January 2000 to December 2011 at the Department of Pathology of the Second and Third Xiangya Hospitals, Central South University was undertaken to explore this question. Immunohistochemistry of CLIC4 and Ihh was performed with EnVision™ in 106 pancreatic ductal adenocarcinoma specimens, 35 paracancer samples (2 cm away from the tumour, when possible or available), 55 benign lesions and 13 normal tissue samples. CLIC4 and Ihh expression in pancreatic ductal adenocarcinoma were significantly higher than in paracancer tissue and benign lesions (CLIC4: $P = 0.009$ and Ihh: $P < 0.0001$; CLIC4: $P = 0.0004$ and Ihh: $P = 0.0001$ respectively). CLIC4 and Ihh expression was negative in normal pancreatic tissues. The expression of CLIC4 and Ihh was associated significantly with tumour grade, lymph node metastasis, tumour invasion and poor overall survival. Thus CLIC4 and Ihh could serve as biological markers for the progression, metastasis and/or invasiveness of pancreatic ductal adenocarcinoma.

Keywords

chloride intracellular channel 4, chronic pancreatitis, Indian Hedgehog, pancreatic ductal adenocarcinoma, pancreatic intra-epithelial neoplasia

Introduction

Pancreatic cancer represents only 3% of all newly diagnosed cancers, but it is the fourth most common cause of cancer death (Siegel *et al.* 2013). About 40% of the patients have locally advanced or metastatic disease at diagnosis, and only about 15% of the tumours are operable (Stathis & Moore 2010). The prognosis remains unsatisfactory despite recent therapeutic advances. The median survival of pancreatic ductal adenocarcinoma (PDA) is 13.4 months after resection combined with gemcitabine (Zhou *et al.* 2014). The currently available markers cannot accurately predict survival

and identifying novel markers is required for personalized medicine which could lead to better outcomes.

High cell proliferation rate, migration and invasiveness are specific properties of tumorigenesis, and these features involve chloride channels. The chloride intracellular channel (CLIC) is a novel protein family consisting of seven members (Berryman & Bretscher 2000). Among these, CLIC4 (also called mtCLIC) is expressed in the mitochondria and regulates intracellular pH and cell volume (Jentsch *et al.* 2002). CLIC4 is present in the mitochondrial membrane, cytoplasm, and nucleus as a soluble protein (Harrop *et al.* 2001). Under normal physiological conditions, CLIC4 localizes in the nucleus

through the nuclear localization signal (NLS) (Suh *et al.* 2004). Littler *et al.* 2005 showed that the functional activity of CLIC4 was determined by its redox state: oxidant conditions enhance its membrane binding capacity and channel activity. CLIC4 is also involved in multiple cellular processes including apoptosis (Shiio *et al.* 2006), inflammation (Ogawa *et al.* 2005; Sukiennicki & Fowell 2006) and endothelial tubulogenesis (Bohman *et al.* 2005; Ulmasov *et al.* 2009). Recent studies revealed that CLIC4 is highly expressed in cancer tissues and tumour-related stromal fibroblasts, but it is almost absent in normal stromal tissues, suggesting that CLIC4 could be a potential target against some cancers (Shukla *et al.* 2014; Peretti *et al.* 2015).

Hammerschmidt *et al.* 1997 discovered that in *Drosophila* the Hedgehog signalling pathway plays a critical role during embryonic development. It is also involved in tissue regeneration and carcinogenesis in various adult tissues, as well as in the maintenance of both cancer stem cells and adult stem cells (Azoulay *et al.* 2008). The three mammalian homologs of the Hedgehog ligand (*Hh*) gene are Sonic Hedgehog (*Shh*), Desert Hedgehog (*Dhh*), and Indian Hedgehog (*Ihh*) (Echelard *et al.* 1993; Marigo *et al.* 1995). The Hh signal transduction is mediated by two receptors: patched (PTCH) and GPCR-like protein smoothened (Smo) (Ingham & McMahon 2001; Lum & Beachy 2004). The activation of Hedgehog signalling is involved in the growth of various tumours such as basal cell carcinoma of the skin (Daya-Grosjean & Couve-Privat 2005), medulloblastoma (Dahmane *et al.* 2001; Berman *et al.* 2002), lung cancer (Watkins *et al.* 2003), gastrointestinal cancer (Berman *et al.* 2003) and prostate cancer (Thayer *et al.* 2003; Karhadkar *et al.* 2004). *Ihh* expression in some malignant tumours is higher than in benign lesions, and *Ihh* expression is related to the incidence, development, biological behaviours, and prognosis of breast (Xuan & Lin 2009), gastric (Xu *et al.* 2012), pancreatic (Xu *et al.* 2013), colorectal (Fu *et al.* 2010), ovarian (Ray *et al.* 2011), cervical (Chaudary *et al.* 2012) and kidney (Jager *et al.* 2014) cancers.

Nevertheless, the relationship between CLIC4 and *Ihh* in pancreatic cancer is poorly understood. Therefore, the aim of this study was to use immunohistochemistry to examine CLIC4 and *Ihh* expressions in benign and malignant lesions of the pancreas and to examine the eventual associations between CLIC4 and *Ihh* expression with clinicopathological features and prognosis of pancreatic cancer.

Materials and methods

Study design and specimens

This was a retrospective study of specimens collected from January 2000 to December 2011 at the Department of Pathology of the Second and Third Xiangya Hospitals, Central South University.

Exclusion criteria were as follows: (i) pre-operative radiotherapy or chemotherapy; (ii) presence of any tumour not originating from the pancreatic duct (e.g. pancreatic acinar

cell carcinoma or neuroendocrine carcinoma); or 3) any treatment complication that could affect survival (e.g. pancreatic fistula). This study included 106 PDA specimens, 35 paracancer samples (2 cm away from the tumour, when possible or available, from the 106 tumour specimens), 55 benign pancreatic lesions (from 55 patients) and 13 normal pancreatic tissue samples (from patients that underwent pancreaticoduodenectomy due to duodenal papilla tumour). All specimens were consecutively and prospectively collected. All diagnoses were reviewed for the present study.

Immunohistochemistry

All specimens were routinely fixed in 4% formaldehyde, embedded in paraffin and sectioned at 4 μ m, which were mounted on poly-L-lysine-coated slides. The rabbit-anti-human CLIC4 (catalog #AP7564a) and the rabbit-anti-human *Ihh* (catalog #AP2704a) polyclonal antibodies were purchased from Abgent (San Diego, California, USA). Immunohistochemistry of CLIC4 and *Ihh* was carried out using the EnVision™ system (ChemMate™ EnVison+/HRP/DAB, rabbit/mouse two-step staining method), according to the manufacturer's protocol (DAKO laboratories, California, USA). Briefly, slides were dried overnight at 65°C, deparaffinized in xylene and dehydrated using a series of graded alcohols. Heat-induced epitope retrieval was conducted with sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) at 96°C for 30 min. Endogenous peroxidase activity was inhibited by incubating the sections in 3% hydrogen peroxide for 15 min at room temperature. Non-specific binding sites were blocked with 10% normal goat serum for 10 min. The slides were incubated with 50 μ l of rabbit-anti-human CLIC4 or *Ihh* polyclonal antibodies (1:50 dilution) at 37°C for 1 h. Slides were washed in PBS three times for 5 min. The sections were incubated with 50 μ l of solution A (ChemMate™ EnVison+/HRP) at 37°C for 30 min and washed in PBS three times for 5 min. DAB (DAKO Real DAB+Chromogen, K5007) was applied for about 2 min and removed by rinsing with distilled water. Slides were counterstained with haematoxylin. The sections were dehydrated, soaked in xylene and mounted with neutral resin. The positive control for CLIC4 was human breast carcinoma tissue sections provided by Abgent. The positive control for *Ihh* was human lung carcinoma tissue sections provided by Abgent. The 0.01 mol/L PBS liquid (pH 7.4) was used instead of CLIC4 and *Ihh* antibodies as negative control.

Staining positivity was determined by two senior pathologists blind to clinical information. Disagreements were solved by discussion. Ten high-power and non-overlapping fields were randomly selected to examine 400 cancer cells. Cases with $\geq 25\%$ positive cells were determined as positive and those with $< 25\%$ were negative (Leppilampi *et al.* 2003; Span *et al.* 2003; Madan *et al.* 2006; Liao *et al.* 2010).

Follow-up

Because of the poor prognosis of pancreatic cancer (Siegel *et al.* 2013), most patients do not survive over 2 years.

Three patients survived >2 years and their survival data were censored at 2 years.

Statistical analysis

Data were tested for normality using the Kolmogorov–Smirnov test. Continuous data were presented as mean \pm standard deviation. Categorical data were presented as n (%). Data were analysed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The association of CLIC4 and Ihh expressions with clinicopathological features was analysed using the chi-square or the Fisher's exact test, as appropriate. The Kaplan–Meier survival analysis and log-rank tests were used for univariate survival analysis. The Cox proportional hazards model (LR method; $P < 0.05$ for inclusion) was used for multivariate survival analysis and to determine the 95% confidence interval (95% CI) of odds ratios (ORs). $P < 0.05$ indicated statistical significance.

Ethical approval statement

This study was approved by the ethics committee for human studies of Central South University, China. It complied with the Code of Ethics of the World Medical Association (Helsinki Declaration of 1964, as revised in 2002).

Results

Characteristics of the subjects

Among the included patients, 61 were male (57.5%) and 45 were female (42.5%); mean age was 54.5 ± 11.5 years.

There were 38 (35.8%) well-differentiated PDA, 35 (33.0%) moderately differentiated PDA and 33 (31.1%) poorly differentiated PDA; 29 patients (27.3%) had positive regional lymph nodes; and 64 patients (60.4%) had surrounding organ invasion. When considering the TNM classification, 11 patients (10.4%) were of stage I, 42 (39.6%) were of stage II, 37 (34.9%) were of stage III and 16 (15.1%) were of stage IV. Paracancer tissue was available for 35 patients: tissue was normal in 12 patients, ten showed mild atypical hyperplasia, eight showed moderate atypical hyperplasia, and five showed severe atypical hyperplasia.

Regarding the 55 specimens of pancreatic benign lesions, they were from 29 males (52.7%) and 26 females (47.3%). Among them, there were 20 cases of chronic pancreatitis (36.4%), 20 of adenoma (36.4%) and 15 of intra-epithelial neoplasia (27.3%). Among the patients with chronic pancreatitis, there were 10 cases of mild pancreatitis, six of moderate pancreatitis and four of severe pancreatitis. Among these patients with chronic pancreatitis, the glandular epithelium of three of them showed mild atypical hyperplasia, two had moderate atypical hyperplasia, and one had severe atypical hyperplasia. Among the patients with adenoma, 15 were serous adenomas, while five were mucous adenomas. Among these cases, the glandular epithelium of four patients showed mild atypical hyperplasia, three had moderate atypical hyperplasia and two had severe atypical hyperplasia. For the patients with pancreatic intra-epithelial neoplasia (PIN), six were of grade I, five were of grade II and four were of grade III. Normal pancreatic tissues of 13 patients were also collected as controls.

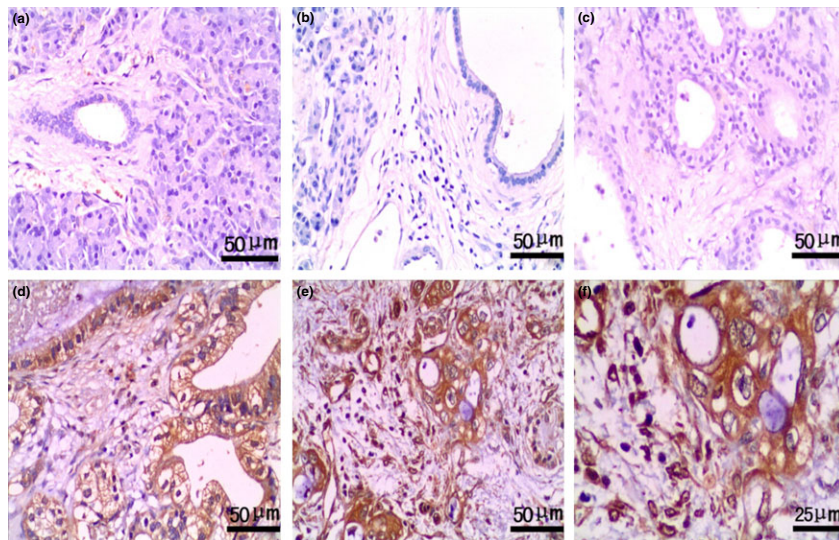


Figure 1 Expression of CLIC4 in pancreatic with benign and malignant lesions. EnVision™ immunohistochemistry: (a) negative CLIC4 expression in pancreatic normal tissues ($\times 200$); (b) negative CLIC4 expression in chronic pancreatitis tissues ($\times 200$); (c) negative CLIC4 expression in pancreatic serous adenoma tissues ($\times 200$); (d) positive CLIC4 expression in well-differentiated PDA ($\times 200$), tumour size was 2.5 cm and TNM stage II; (e) positive CLIC4 expression in poorly differentiated PDA ($\times 200$), tumour size was 4 cm and TNM stage IV; (f) magnified image from e, CLIC4 staining was localized in the cytoplasm of epithelial cells and in the nucleus of fibroblasts in poorly differentiated PDA ($\times 400$).

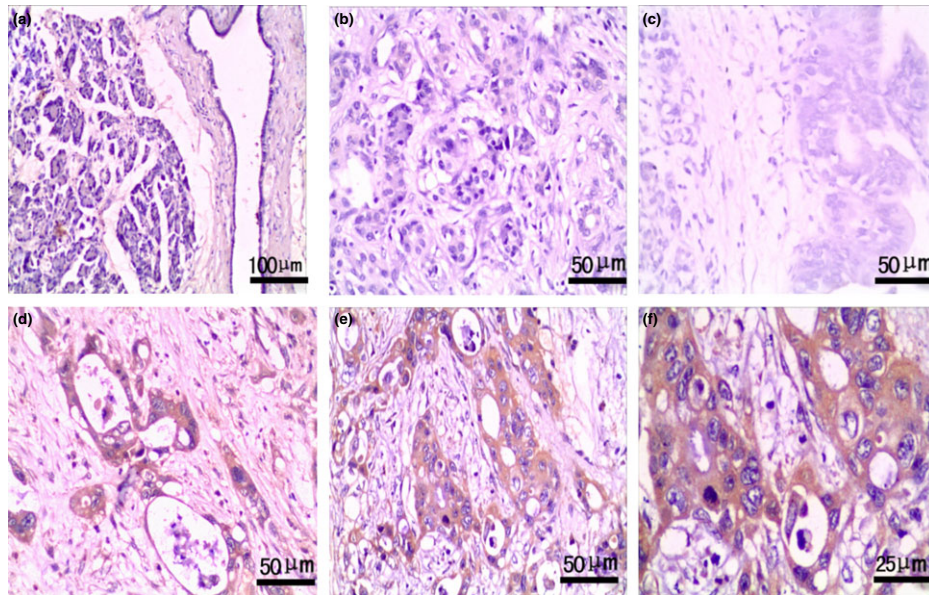


Figure 2 Expression of Ihh in pancreas with benign and malignant lesions. EnVision™ immunohistochemistry: (a) negative Ihh expression in pancreatic normal tissues (×100); (b) negative Ihh expression in chronic pancreatitis tissues (×200); (c) negative Ihh expression in PIN II tissues (×200); (d) positive Ihh expression in well-differentiated PDA (×200), tumour size of 3 cm and TNM stage I; (e) positive Ihh expression in poorly differentiated PDA (×200), tumour size of 4 cm and TNM stage IV; (f) magnified image from e, Ihh staining was localized in the cytoplasm of epithelial cells in poorly differentiated PDA (×400).

Table 1 CLIC4 and Ihh expression in benign and malignant lesions of pancreas

Tissue types	N	CLIC4 positive, n (%)	Ihh positive, n (%)
PDA	106	54 (50.9)	63 (59.4)
Paracancer	35	9 (25.7)	10 (28.6)
Benign lesions	55	12 (21.8)	10 (18.2)
Normal tissue	13	0	0

For CLIC4: PDA vs. paracancer: $P = 0.0092$; PDA vs. benign lesions: $P = 0.0004$; PDA vs. benign lesions: $P = 0.0004$; PDA vs. normal: $P = 0.0005$.

For Ihh: PDA vs. paracancer: $P < 0.0001$; PDA vs. benign lesions: $P = 0.0001$; PDA vs. normal tissues: $P = 0.0015$.

CLIC4 and Ihh protein expression

Immunohistochemistry revealed that CLIC4 was localized in the cytoplasm of epithelial and tumour stromal cells (including fibroblasts), while Ihh was localized in the cytoplasm of the epithelial cells only (Figures 1 and 2). As shown in Table 1, among the 106 patients with PDA, CLIC4 and Ihh were positive in 54 (50.9%) and 63 (59.4%) patients respectively. Among the 35 patients with available paracancer tissue, CLIC4 and Ihh were positive in nine (25.7%) and 10 (28.6%) patients respectively. Among the 55 patients with benign lesions of the pancreas, 12 (21.8%) were CLIC4 positive and 10 (18.2%) were Ihh positive. Among the 13 normal pancreatic tissues, CLIC4 and Ihh were negative in all samples. The rate of CLIC4 and Ihh positivity in PDA was significantly

Table 2 Association of CLIC4 and Ihh expression with the clinicopathological characteristics of PDA

Characteristics	N	CLIC4		Ihh	
		Positive, n (%)	P	Positive, n (%)	P
Age (years)					
≤45	22	15 (68.2)	0.069	16 (72.7)	0.154
>45	84	39 (46.4)		47 (56.0)	
Gender					
Male	61	31 (50.8)	0.976	34 (55.7)	0.367
Female	45	23 (51.1)		29 (64.4)	
Differentiation					
Well	38	14 (36.8)	0.008	14 (36.8)	<0.0001
Moderate	35	33 (48.5)		21 (60.0)	
Poor	33	24 (72.7)		28 (84.8)	
Tumour size (cm)					
≤3 cm	13	4 (30.8)	0.075	5 (38.5)	0.069
3–5 cm	68	33 (48.5)		39 (57.4)	
>5 m	25	17 (68.0)		19 (76.0)	
Lymph node metastasis					
No	77	30 (39.0)	<0.0001	38 (49.4)	0.001
Yes	29	24 (82.8)		25 (86.2)	
Invasion					
No	42	11 (26.2)	0.032	14 (33.3)	<0.0001
Yes	64	43 (67.2)		49 (76.6)	
T stages					
I	11	4 (36.4)	0.018	4 (36.3)	<0.0001
II	42	16 (38.1)		17 (40.5)	
III	37	21 (56.8)		28 (75.7)	
IV	16	13 (81.3)		14 (87.5)	

Table 3 Relationship between CLIC4 and Ihh expressions, clinicopathological characteristics and survival of patients with PDA

Characteristics	N	Median survival [range] (months)	P value
Age (years)			
≤45	22	8.2 (3–19)	0.143
>45	84	9.7 (2–24)	
Gender			
Male	61	10.0 (2–24)	0.198
Female	45	8.6 (2–21)	
Differentiation			
Well	38	11.3 (3–24)	<0.0001
Moderate	35	9.7 (3–21)	
Poor	33	6.9 (2–14)	
Tumour size (cm)			
≤3 cm	13	13.5 (5–21)	0.023
3–5 cm	68	9.3 (2–22)	
>5 m	25	7.4 (3–24)	
Lymph node metastasis			
No	77	10.6 (2–24)	<0.0001
Yes	29	6.4 (2–12)	
Invasion			
No	42	13.3 (5–24)	<0.0001
Yes	64	6.8 (2–17)	
T stages			
I	11	16.5 (11–24)	<0.0001
II	42	11.4 (3–22)	
III	37	7.1 (2–17)	
IV	16	4.6 (2–8)	
CLIC4			
–	52	12.3 (3–24)	<0.0001
+	54	6.6 (2–16)	
Ihh			
–	43	12.5 (5–24)	<0.0001
+	63	7.3 (2–22)	

higher than in the paracancer samples ($P = 0.009$ and $P < 0.0001$), benign lesions ($P = 0.0004$ and $P = 0.0001$) and normal pancreatic tissue ($P = 0.0005$ and $P = 0.002$). Interestingly, the ductal epithelium of paracancer and benign samples with positive CLIC4 and Ihh expressions

displayed mild to severe atypical hyperplasia or intra-epithelial neoplasia of grades II–III.

Among the benign lesions, the rates of CLIC4 positivity in chronic pancreatitis, adenoma and intra-epithelial neoplasia were 15.0% (3/20), 25.0% (5/20) and 26.7% (4/15), respectively, while those of Ihh were 15.0% (3/20), 20.0% (4/20) and 20.0% (3/15) respectively (all $P > 0.05$).

Associations between CLIC4 and Ihh expressions and clinical pathological features of PDA

CLIC4 and Ihh positivity was associated with poorly differentiated PDA ($P = 0.008$ and $P < 0.0001$), lymph node metastasis ($P < 0.0001$ and $P = 0.001$), surrounding organ invasion ($P = 0.03$ and $P < 0.0001$) and advanced TNM stage ($P = 0.02$ and $P < 0.0001$) (Table 2). CLIC4 and Ihh were not associated with age, gender and tumour size.

Association between CLIC4 and Ihh in PDA

Among the 54 patients with CLIC4 expression, 38 showed Ihh expression. Of the 52 patients without CLIC4 expression, 27 exhibited no Ihh expression. There was an association between the expressions of CLIC4 and Ihh ($P = 0.023$).

Associations between survival, clinicopathological features and expression of CLIC4 and Ihh in patients with PDA

Follow-up was carried out in 106 patients with PDA through mail or outpatient visits every 3 months for up to 2 years. Patients surviving ($n = 3$) over this period were censored. Twenty-nine patients survived >1 year and 77 patients survived <1 year. Mean survival was 9.4 ± 0.7 months. According to the Kaplan–Meier survival analysis, poor survival of patients PDA was associated with poor differentiation ($P < 0.0001$), tumour size ($P = 0.02$), advanced TNM stage ($P < 0.0001$), lymph node metastasis ($P < 0.0001$) and surrounding organ invasion ($P < 0.0001$)

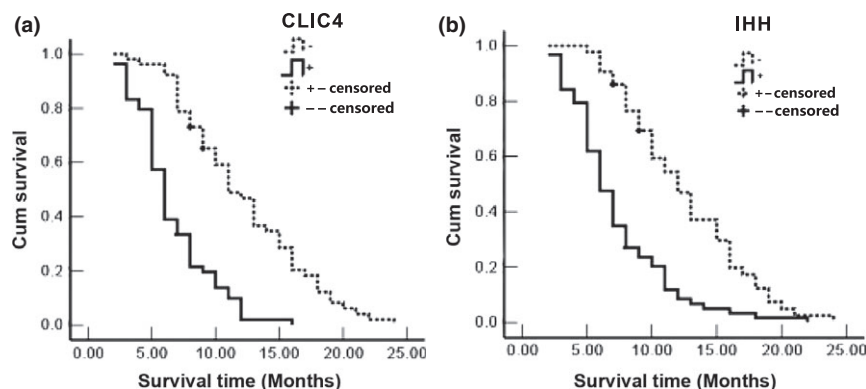


Figure 3 Association between survival and expression of CLIC4 and Ihh in patients with PDA. (a) Survival curves for CLIC4 expression in PDA. Median survival was 12.3 months for negative cases and 6.6 months for positive ones ($P < 0.001$). (b) Survival curves for Ihh expression in PDA. Median survival was 12.5 months for negative cases and 7.3 months for positive ones ($P < 0.001$).

Table 4 Multivariate Cox regression analysis of overall survival in patients with PDA

Groups	Factors	B	SE	Wald	P	RR	95% confidence interval	
							Lower	Upper
Differentiation	Well/moderate/poor	0.689	0.244	7.970	0.005	1.992	1.234	3.213
Tumour mass size	<3 cm/3–5 cm/>5 cm	1.098	0.397	7.651	0.006	2.997	1.377	6.524
Lymph node metastasis	No/yes	0.974	0.338	8.285	0.004	2.648	1.364	5.140
Invasion	No/yes	0.821	0.345	5.680	0.017	2.274	1.157	4.468
TNM stages	I/II/III/IV	0.777	0.245	10.085	0.001	2.175	1.346	3.513
CLIC4	–/+	0.891	0.265	11.320	0.001	2.437	1.450	4.094
Ihh	–/+	0.697	0.247	7.948	0.005	2.007	1.237	3.259

B, regression coefficients; SE, standard error; RR, Relative risk.

(Table 3). The overall survival of patients with positive CLIC4 and Ihh expressions was obviously shorter than that of the CLIC4- and Ihh-negative cases (CLIC4: negative: 12.3 vs. positive: 6.6 months, $P < 0.0001$; Ihh: negative: 12.5 vs. 7.3 months, $P < 0.0001$) (Figure 3).

Multivariate analysis

The Cox multivariate analysis showed that overall survival was independently and negatively associated with poor differentiation, tumour size >3 cm, TNM stage III or IV, lymph node metastasis, surrounding organ invasion, CLIC4 expression and Ihh expression (Table 4).

Discussion

Pancreatic cancer is the fourth most common cause of cancer-related mortality. There is a need for novel molecular biomarkers to improve personalized medicine and survival. The aim of this study was to examine CLIC4 and Ihh expression in benign and malignant lesions of the pancreas and to attempt to determine associations between CLIC4 and Ihh expressions and clinicopathological features and prognosis of pancreatic cancer. Results showed that both CLIC4 and Ihh expression in PDA was significantly higher than in paracancer tissue and benign lesions. In normal pancreatic tissue, CLIC4 and Ihh expression was negative. The expression of both CLIC4 and Ihh was significantly associated with tumour grade, lymph node metastasis, tumour invasion and poor overall survival.

Several studies have investigated the expression and regulation of CLIC4 in tumours. Significant alterations in CLIC4 expression have been documented in bladder cancer (Dyrskjot *et al.* 2004), uterine leiomyoma (Bae *et al.* 2004), glioma (Zhong *et al.* 2012) and melanoma (Alonso *et al.* 2007). In colon cancer, CLIC4 was positive in 67.2% of 421 patients, while only 2.1% of the matching mucosa was positive. The expression of CLIC4 in colorectal cancer was considered to be related to the distribution of cancer stem-like cells (Deng *et al.* 2014). This was considered as to be an innate factor contributing to the aggressiveness of the metastatic cancer stem-like cells of colorectal cancer. CLIC4 expression was

reported to be a marker of colon cancer stem cells and to be associated with poor prognosis (Deng *et al.* 2014). It also enhanced matrix metalloproteinase 9 expressions and invasion in cancer cell lines escaping photodynamic therapy (PDT). Overexpression of CLIC4 in A375 and MDA-MB-231 cancer cells constrained the PDT-induced suppression of invasiveness (Chiang *et al.* 2013). CLIC4 is present in the exosomes released from human ovarian cancer cells (Liang *et al.* 2013) and high levels of circulating CLIC4 were identified as a marker of prognosis in these patients (Tang *et al.* 2013). Furthermore, CLIC4 could be a potential biomarker to monitor tumour progression and recurrence in human cancers, but this is controversial. However, another study revealed loss of CLIC4 in multiple human epithelial tumours including renal, ovarian and breast cancers and reported that CLIC4 expression is increased in stromal cells with the progression of malignancy (Suh *et al.* 2007). Suh *et al.* (2005) suggested that decreasing CLIC4 expression by using siRNA inhibited the growth of human osteosarcoma cells, increased apoptosis and decreased cell proliferation. Ronnov-Jesson *et al.* (2002) proposed that CLIC4 has an important function in the myofibroblast stroma of breast cancer patients wherein the transcript was upregulated after TGF- β treatment. Thus, the exact mechanisms by which CLIC4 functions as a tumour suppressor or stromal activator are still open questions.

In 1980, Nusslein-Vollhard and Wieschaus showed that the Hedgehog signalling pathway was related to growth, organ development and congenital malformations (Nusslein-Vollhard & Wieschaus 1980). Later, other studies showed that the Hedgehog signalling pathway was closely related to the growth and differentiation of cells, playing a pivotal role in embryonic development, homeostasis of mature tissue and tumorigenesis (Dahmane *et al.* 2001). It has been found in recent years that among common malignant tumours (basal cell carcinoma, small cell lung cancer, upper gastrointestinal cancer, prostate cancer and endometrial cancer), tumour development was closely related to the abnormal activation of the Hedgehog signalling pathway (Pasca di Magliano & Hebrok 2003; Feng *et al.* 2007). In breast cancer, increased expression of Ihh was associated with increased proliferating index of Ki-67. Ihh expression was also associated with lymph

node metastasis and clinical stage of breast cancer. Hedgehog signalling molecules are essential to the progression of invasive ductal carcinoma of the breast (Thayer *et al.* 2003). The discovery of cyclopamine made the Hedgehog signalling pathway more clinically significant. These steroid alkaloids mainly block the function of the SMO receptor to inhibit the activation of the Hedgehog signalling pathway, therefore inhibiting the growth of cancer cells (Xuan & Lin 2009). Kayed *et al.* (Kayed *et al.* 2004) proposed that *Ihh* and its receptors *PTCH* and *Smo* were expressed in pancreatic cancer and that blocking the Hedgehog signalling pathway could result in the inhibition of pancreatic cancer cell growth, thereby suggesting that aberrant activation of the *Ihh* pathway contributes to pancreatic tumour development. Recent studies showed that *Ihh* expression level is closely associated with the incidence, development, biological behaviours and prognosis of some malignant tumours (breast, gastric, pancreatic, colorectal, ovarian, cervical and kidney cancers) (Berman *et al.* 2003; Thayer *et al.* 2003; Karhadkar *et al.* 2004; Xuan & Lin 2009; Fu *et al.* 2010; Xu *et al.* 2012, 2013).

Although the expression of *CLIC4* and *Ihh* has been previously reported in some cancers, to our knowledge, this is the first report showing *CLIC4* and *Ihh* expression in benign and malignant lesions of the pancreas. In this study, an extensive collection of PDA and benign pancreatic lesions were used to demonstrate the clinical and pathological significance of *CLIC4* and *Ihh* expressions in PDA. In accordance with results observed in other types of cancers, the present study showed that *CLIC4* and *Ihh* expressions was associated with PDA. Among patients with PDA, *CLIC4* and *Ihh* expression was associated with poor prognosis and poor survival. Therefore, *CLIC4* and *Ihh* could play a critical role in carcinogenesis and progression of PDA.

The present study is not without limitations. The sample size was relatively small and all cases were from the same institution. In addition, the retrospective nature of the study prevented examining factors that were not identified in the charts. It would be interesting to observe the expression of other molecular markers of prognosis in relation to *CLIC4* and *Ihh*. Additional studies are still necessary to grasp a better understanding of these markers in PDA.

In conclusion, *CLIC4* and *Ihh* expression was associated with poor survival of patients with PDA. *CLIC4* and *Ihh* could serve as potential biological markers indicating progression, metastasis and/or invasiveness of pancreatic ductal adenocarcinoma.

Conflict of interests

The authors declare that they have no conflict of interests.

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