RESEARCH ARTICLE

DLC-1 is a candidate biomarker methylated and down-regulated in pancreatic ductal adenocarcinoma

Yu-Zheng Xue · Tie-Long Wu · Yan-Min Wu ·
Ying-Yue Sheng · Zhe-Qiang Wei · Yu-Feng Lu ·
Li-Hua Yu · Jian-Ping Li · Zhao-Shen Li

Abstract Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive malignancies in humans, and its prognosis is generally poor even after surgery. Many advances have been made to understand the pathogenesis of PDA; however, the molecular mechanisms that lead to pancreatic carcinogenesis are still not clearly understood. The aims of this study were to investigate the relationship between DLC-1 methylation status and clinicopathological characteristics of PDA patients and evaluate the role of DLC-1 methylation status in PDA. The expression of DLC-1 mRNA in PDA tissues was analyzed by real-time PCR. The methylation status of DLC-1 was analyzed by methylation-specific polymerase chain reaction (MSP). Furthermore, we determined the prognostic importance of DLC-1 methylation status in PDA patients. Our results showed that the expression level of DLC-1 mRNA in PDA tissues was lower than that in non-cancerous tissues. The rate of DLC-1 promoter methylation was significantly higher in PDA tissues than in adjacent non-cancerous tissues ($p < 0.001$). Downregulation of DLC-1 was strongly correlated with promoter methylation ($P = 0.003$). The presence of DLC-1 methylation in PDA tissue samples was significantly correlated with clinical stage ($P = 0.005$), histological differentiation ($P = 0.05$), and lymph node metastasis ($P = 0.006$). Kaplan–Meier survival analysis showed that DLC-1 methylation status was inversely correlated with overall survival of the PDA patients. Further, Cox multivariate analysis indicated that DLC-1 methylation status was an independent prognostic factor for the overall survival rate of PDA patients. In conclusion, our data suggest that downregulation of DLC-1 may be explained by DNA methylation; DLC-1 may be a biomarker for PDA.

Keywords DLC-1 · Methylation · MSP · Prognosis

Yu-Zheng Xue, Tie-Long Wu, and Yan-Min Wu contributed equally to this paper.

Y.-Z. Xue (✉) · T.-L. Wu · Y.-M. Wu · Y.-Y. Sheng · Z.-Q. Wei ·
Y.-F. Lu · L.-H. Yu · J.-P. Li (✉)
Department of Gastroenterology,
The Third Hospital Affiliated to Nantong University,
No. 585, Xingyuan North Rd., Wuxi 214041 Jiangsu, China
e-mail: xueyzdoctor@126.com
e-mail: doclijp@yeah.net

Z.-S. Li (✉)
Department of Gastroenterology,
Changhai Hospital of Second Military Medical University,
No. 168, Changhai Rd., Shanghai 200001, China
e-mail: lizhaoshen57@163.com

Introduction

Pancreatic ductal adenocarcinoma (PDA) is a highly lethal disease, which is usually diagnosed in an advanced state for which there is little or no effective therapy [1]. It has the worst prognosis of any major malignancy and is the fourth most common cause of cancer death yearly in multiple countries [2]. Despite advances in surgical and medical therapy, little effect has been made on the mortality rate of this disease [1–4]. Global hypomethylation is often accompanied by dense hypermethylation of the specific promoters in human cancers [5–7]. Promoter hypermethylation results in gene silencing, and such genes have proved to have potent tumor suppressive function and are rather rare [8]. So, there is an urgent need to reveal the underlying mechanisms by which pancreatic cancer cells become invasive and metastatic.

Deleted in liver cancer-1 (DLC-1), isolated from human hepatocellular carcinoma (HCC), is a recently identified tumor suppressor gene [9]. DLC-1 downregulation has been

reported in a variety of human cancers, and its upregulation could inhibit tumor angiogenesis, invasion, and metastasis [10–21]. Recently, the decrease in DLC-1 expression is reported to correlate with hypermethylation of the promoter region [11, 12, 16, 18]. There is increasing evidence showing that gene silencing due to aberrant methylation of DNA is an early event in carcinogenesis and could serve as a potential diagnostic and prognostic biomarker in some cancers [22–24]. In the present study, we investigated the relation between DLC-1 methylation and clinicopathological characteristics of PDA patients and evaluated the role of DLC-1 methylation in PDA.

Materials and methods

Patients and specimens

Specimens of cancer tissues and adjacent non-cancerous tissues were retrieved from 68 primary PDA patients including 40 males and 28 females at the age of 35–77 years who underwent resection of PDA at Changhai Hospital of Second Military Medical University, China, during the period from July 2004 to July 2007. These resected tissue samples were immediately frozen at −80 °C and stored at this temperature until use. All of the samples were histologically verified. Patient survival was defined as the time from the day of surgery to the end of follow-up or day of death due to recurrence or metastasis. The study was approved by the Research Ethics Committee of Changhai Hospital, Second Military Medical University, Shanghai, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to accepted ethical and legal standards.

Real-time RT-PCR

A total of 68 pairs of fresh PDA and adjacent non-cancerous tissues were employed for isolation of total RNA using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was then reverse-transcribed using the M-MLV Reverse Transcriptase Kit (Promega, Madison, WI, USA). The resultant cDNA (20 ng) was mixed with SYBR GreenMasterMix (BioRad, Hercules, CA, USA) and amplified in the CFX96 real-time detection system (Bio-Rad). Each reaction was run in triplicate. The expression of IBSP was normalized against GAPDH by the comparative threshold cycle (ct) method using the following formula: fold difference in expression $= 2^{-(\Delta ct \; of \; target \; gene - \Delta ct \; of \; reference)}$. Primers of DLC-1 used for RT-PCR were designed according to the protocol of Zhang et al. [21].

Methylation-specific PCR

DNA was isolated from tissue samples using the NucleoSpin Tissue kit (Macherey-Nagel, Duren, Germany). Genomic DNA conversion was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). DNA after conversion was used for analyses of the methylation status of the DLC-1 promoter using methylation-specific PCR. Primers of methylated and unmethylated DLC-1 were designed according to the protocol of Peng et al. [9]. Methylation-specific PCR for the DLC-1 promoter was conducted in a total PCR volume of 20 μL. The PCR products were analyzed by 1 % agarose gel electrophoresis.

Statistical analysis

Statistical analyses and graphical representations were performed with GraphPad Prism 5 software (San Diego, CA, USA). Chi-square test was used to analyze the relationship between DLC-1 expression and clinicopathological characteristics. Quantitative values were analyzed using Student's t-test. Survival curves were plotted by Kaplan–Meier method and compared using log-rank test. Survival data were evaluated using Cox proportional hazards model. Independent prognostic factors were determined by a multivariate analysis. All results were considered as significant when P-values were less than 0.05.

Results

The expression of DLC-1 mRNA in PDA tissues and non-cancerous tissues

The expression of PDA mRNA in PDA tissues was analyzed by real-time PCR in 68 pairs of PDA and adjacent non-cancerous tissues. We investigated differences in the expression of DLC-1 mRNA in PDA and adjacent non-cancerous tissues. We found a statistically significant lower expression of DLC-1 in PDA tissues in comparison with adjacent non-cancerous tissues (P=0.002) (Fig. 1a). In addition, we also observed a significantly higher expression of DLC-1 in stage I–II in comparison with III–IV (P=0.016) using the median two-sample test (Fig. 1b).

DLC-1 promoter methylation in PDA tissues and adjacent non-cancerous tissues

We next addressed whether downregulation of DLC-1 in tumor tissues was caused by promoter methylation, an epigenetic alteration that frequently leads to gene silencing. Methylation-specific PCR (MSP) analysis showed that DLC-1 promoter methylation was detected in 35/68

Fig. 1 a Relative expression of DLC-1 mRNA in PDA tissues compared to non-cancerous tissues. b Difference between expression of DLC-1 mRNA in stage I–II and III–IV
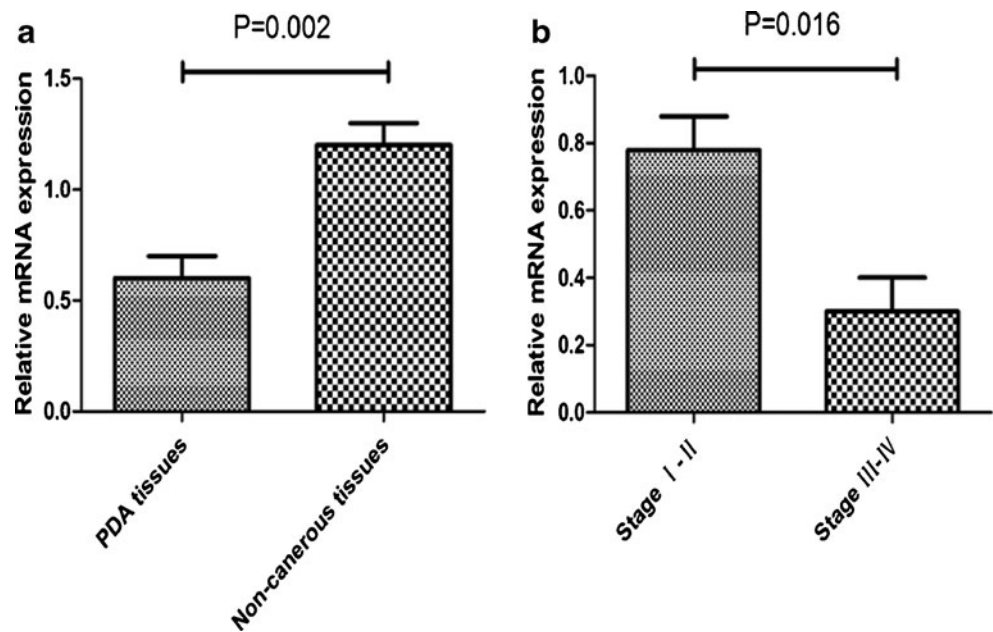
(51.5 %) PDA tissue samples and in 7/68 (10.3 %) adjacent non-cancerous tissue samples (Fig. 1). DLC-1 promoter methylation was significantly higher in PDA tissues than in adjacent non-cancerous tissues ($P<0.001$). Figure 2 demonstrates the methylation status of the DLC-1 promoter of five pairs of PDA tissues and adjacent non-cancerous tissues. We tested the correlation between DLC-1 expression and promoter methylation, and our data indicated that downregulation of DLC-1 was strongly correlated with promoter methylation ($P=0.003$; Table 1).

Correlation between DLC-1 methylation status and the clinicopathological features

The association between DLC-1 methylation status and the clinicopathological features of PDA was further analyzed, as shown in Table 2. We did not find a significant association between DLC-1 methylation status and gender, age, and

tumor location in patients with PDA (all $P>0.05$). Interestingly, we observed that DLC-1 methylation status was closely correlated with clinical stage ($P=0.005$), histological differentiation ($P=0.05$), and lymph node metastasis ($P=0.006$) in patients with PDA.

Correlation of DLC-1 methylation status with overall survival

To investigate the prognostic value of DLC-1 methylation status for PDA, we assessed the association between DLC-1 methylation status and survival duration using Kaplan–Meier analysis with log-rank test. The log-rank test showed that DLC-1 methylation status was inversely correlated with overall survival of the PDA patients (Fig. 3). To determine whether DLC-1 expression is an independent prognostic factor for PDA, we performed a multivariate survival analysis of DLC-1 methylation status and factors including age, gender, tumor location, histological differentiation, lymph node involvement, and TNM stage in patients with PDA. The results showed that DLC-1 methylation status was an independent prognostic factor for PDA (Table 3).
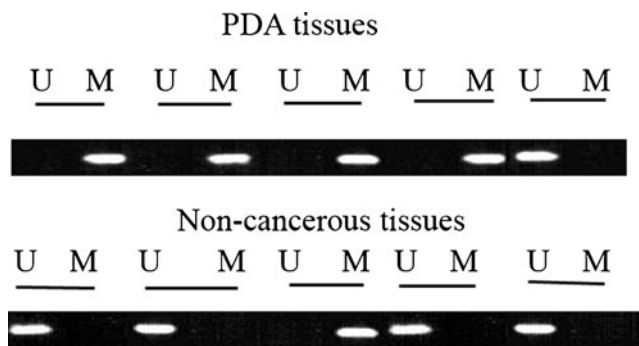

Fig. 2 Methylation status of DLC-1 in five pairs of PDA tissues and adjacent non-cancerous tissues. M methylation, U unmethylation

Table 1 DLC-1 downregulation was associated with promoter methylation

DLC-1 promoter methylation	DLC-1		P value
	No change	Downregulation	
Methylated (n=35)	6	29	0.003
Unmethylated (n=33)	17	16	

Table 2 Relationship between clinicopathological characteristics and DLC-1 methylation in PDA patients

Variable	Number of cases	DLC-1 methylation		P value
		Unmethylated	Methylated	
Age (year)				
≤ 50	21	9	12	0.532
> 50	47	24	23	
Gender				
Male	40	21	19	0.434
Female	28	12	16	
Tumor location				
Head	52	26	26	0.662
Body/tail	16	7	9	
Clinical stage (pTNM)				
I–II	49	29	20	0.005
III–IV	19	4	15	
Histological differentiation				
Well differentiated	23	15	8	0.05
Moderately/poorly differentiated	45	18	27	
Lymph node metastasis				
Absent	37	28	19	0.006
Present	21	5	16	

Discussion

Deleted in liver cancer-1 (DLC-1), isolated from HCC, is a recently identified tumor suppressor gene [9]. DLC-1 downregulation has been reported in a variety of human cancers, and its upregulation could inhibit tumor angiogenesis, invasion, and metastasis [10–21]. Recently, the decrease in DLC-1 expression is reported to correlate with hypermethylation of the promoter region [11, 12, 16, 18]. There is increasing evidence showing that gene silencing

due to aberrant methylation of DNA is an early event in carcinogenesis and could serve as a potential diagnostic and prognostic biomarker in some cancers [22–24]. In the present study, we investigated the relation between DLC-1 methylation and clinicopathological characteristics of PDA patients and evaluated the role of DLC-1 methylation in PDA.

Firstly, the expression of DLC-1 mRNA in PDA tissues was analyzed by real-time PCR. We found a statistically significant lower expression of DLC-1 mRNA in PDA tissues in comparison with adjacent non-cancerous tissues ($P=0.002$). Interestingly, we also observed a significantly higher expression of DLC-1 mRNA in stage I–II in comparison with stage III–IV ($P=0.016$). MSP analysis was used to analyzed DLC-1 methylation status in the same tissue samples that were used in the detection of DLC-1 mRNA. Results showed that DLC-1 methylation status was
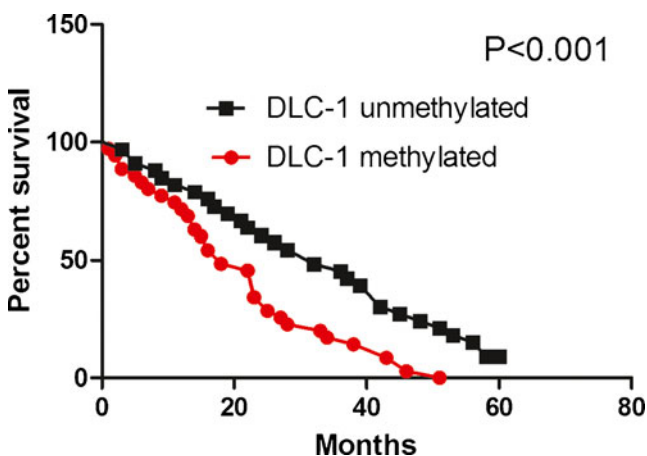

Fig. 3 Patients with methylated DLC-1 had shorter 5-year survival rates than patients with unmethylated DLC-1 when assessed using Kaplan–Meier curves

Table 3 Multivariate Cox survival analysis

Variables	P value
DLC-1 methylation	<0.001
Age	0.732
Gender	0.624
Tumor location	0.562
Clinical stage	0.003
Histological differentiation	0.027
Lymph node metastasis	0.013

significantly higher in PDA tissues than in adjacent non-cancerous tissues. Additionally, our data indicated that downregulation of DLC-1 was strongly correlated with promoter methylation. Furthermore, we examined the correlation between DLC-1 methylation status and the clinicopathological features. Our data indicated that presence of DLC-1 methylation in PDA tissue samples was significantly correlated with clinical stage ($P=0.005$), histological differentiation ($P=0.05$), and lymph node metastasis ($P=0.006$). Kaplan–Meier analysis showed that DLC-1 methylation status was inversely correlated with overall survival of the PDA patients. To determine whether DLC-1 methylation status is an independent prognostic factor for PDA, we performed a multivariate survival analysis of DLC-1 methylation status and factors including age, gender, tumor location, histological differentiation, lymph node involvement, and TNM stage in patients with PDA. The results showed that DLC-1 methylation status, together with histological differentiation, lymph node involvement, and TNM stage, was an independent prognostic factor for PDA.

In summary, the present study indicates that hypermethylation of DLC-1 promoter is a common event in PDA and is correlated with poor prognosis in PDA patients. Although additional work is required to further clarify the mechanism and biological significance of DLC-1 hypermethylation in human carcinogenesis, promoter hypermethylation of the DLC-1 gene is a promising biomarker in early detection and prognosis for PDA patients.

Conflicts of interest None

References

1. Malik NK, May KS, Chandrasekhar R, Wee W, Flaherty L, Iyer R, et al. Treatment of locally advanced unresectable pancreatic cancer: a 10-year experience. J Gastrointest Oncol. 2012;3:326–34. doi:10.3978/j.issn.2078-6891.2012.029.
2. Kaur S, Baine MJ, Jain M, Sasson AR, Batra SK. Early diagnosis of pancreatic cancer: challenges and new developments. Biomark Med. 2012;6:597–612. doi:10.2217/bmm.12.69.
3. McCleary-Wheeler AL, Lomberk GA, Weiss FU, Schneider G, Fabbri M, Poshusta TL, et al. Insights into the epigenetic mechanisms controlling pancreatic carcinogenesis. Cancer Lett. 2013;328:212–21. doi:10.1016/j.canlet.2012.10.005.
4. Sun C, Ansari D, Andersson R, Wu DQ. Does gemcitabine-based combination therapy improve the prognosis of unresectable pancreatic cancer? World J Gastroenterol. 2012;18:4944–58. doi:10.3748/wjg.v18.i35.4944.
5. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;128:683–92.
6. Bird AP. CpG-rich islands and the function of DNA methylation. Nature. 1986;321:209–13.
7. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP. Hypomethylation of DNA from benign and malignant human colon neoplasms. Science. 1985;228:187–90. doi:10.1126/science.2579435.
8. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003;349:2042–54. doi:10.1056/NEJMra023075.
9. Peng D, Ren CP, Yi HM, Zhou L, Yang XY, Li H, et al. Genetic and epigenetic alterations of DLC-1, a candidate tumor suppressor gene, in nasopharyngeal carcinoma. Acta Biochim Biophys Sin (Shanghai). 2006;38:349–55. doi:10.1111/j.1745-7270.2006.00164.x.
10. Ullmannova V, Popescu NC. Expression profile of the tumor suppressor genes DLC-1 and DLC-2 in solid tumors. Int J Oncol. 2006;29:1127–32.
11. Guan M, Zhou X, Soulitzis N, Spandidos DA, Popescu NC. Aberrant methylation and deacetylation of deleted in liver cancer-1 gene in prostate cancer: potential clinical applications. Clin Cancer Res. 2006;12:1412–9. doi:10.1158/1078-0432.CCR-05-1906.
12. Song YF, Xu R, Zhang XH, Chen BB, Chen Q, Chen YM, et al. High-frequency promoter hypermethylation of the deleted in liver cancer-1 gene in multiple myeloma. J Clin Pathol. 2006;59:947–51. doi:10.1136/jcp.2005.031377.
13. Feng X, Li C, Liu W, Chen H, Zhou W, Wang L, et al. DLC-1, a candidate tumor suppressor gene, inhibits the proliferation, migration and tumorigenicity of human nasopharyngeal carcinoma cells. Int J Oncol. 2013;42:1973–84. doi:10.3892/ijo.2013.1885.
14. Liu H, Shi H, Hao Y, Zhao G, Yang X, Wang Y, et al. Effect of FAK, DLC-1 gene expression on OVCAR-3 proliferation. Mol Biol Rep. 2012;39:10665–70. doi:10.1007/s11033-012-1956-6.
15. Chen WT, Yang CH, Wu CC, Huang YC, Chai CY. Aberrant deleted in liver cancer-1 expression is associated with tumor metastasis and poor prognosis in urothelial carcinoma. APMIS. 2013. doi:10.1111/apm.12060.
16. Peng H, Long F, Wu Z, Chu Y, Li J, Kuai R, Zhang J, Kang Z, Zhang X, Guan M. Downregulation of DLC-1 gene by promoter methylation during primary colorectal cancer progression. Biomed Res Int. 2013; 2013: 181384. doi:10.1155/2013/181384.
17. Guan CN, Zhang PW, Lou HQ, Liao XH, Chen BY. DLC-1 expression levels in breast cancer assessed by qRT-PCR are negatively associated with malignancy. Asian Pac J Cancer Prev. 2012;13:1231–3.
18. Liu JB, Zhang YX, Zhou SH, Shi MX, Cai J, Liu Y, et al. CpG island methylator phenotype in plasma is associated with hepatocellular carcinoma prognosis. World J Gastroenterol. 2011;17:4718–24. doi:10.3748/wjg.v17.i42.4718.
19. Feng M, Huang B, Du Z, Xu X, Chen Z. DLC-1 as a modulator of proliferation, apoptosis and migration in Burkitt's lymphoma cells. Mol Biol Rep. 2011;38(3):1915–20. doi:10.1007/s11033-010-0311-z.
20. Zhang T, Zheng J, Jiang N, Wang G, Shi Q, Liu C, et al. Overexpression of DLC-1 induces cell apoptosis and proliferation inhibition in the renal cell carcinoma. Cancer Lett. 2009;283:59–67. doi:10.1016/j.canlet.2009.03.025.
21. Zhang T, Zheng J, Liu C, Lu Y. Expression of DLC-1 in clear cell renal cell carcinoma: prognostic significance for progression and metastasis. Urol Int. 2009;82:380–7. doi:10.1159/000218524.
22. Jin Z, Mori Y, Yang J, Sato F, Ito T, Cheng Y, et al. Hypermethylation of the nel-like 1 gene is a common and early event and is associated with poor prognosis in early-stage esophageal adenocarcinoma. Oncogene. 2007;26:6332–40. doi:10.1038/sj.onc.1210461.
23. Sun D, Zhang Z, Van do N, Huang G, Ernberg I, Hu L. Aberrant methylation of CDH13 gene in nasopharyngeal carcinoma could serve as a potential diagnostic biomarker. Oral Oncol. 2007;43:82–7.
24. Brock MV, Gou M, Akiyama Y, Muller A, Wu TT, Montgomery E, et al. Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. Clin Cancer Res. 2003;9:2912–9.