RESEARCH ARTICLE

Expression and Prognostic Significance of lncRNA MALAT1 in Pancreatic Cancer Tissues

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Abstract

Background: Long non-coding RNAs (lncRNAs) have been recently observed in various human cancers. However, the role of lncRNAs in pancreatic duct adenocarcinoma (PDAC) remains unclarified. The aim of this study was to detect the expression of lncRNA MALAT1 in PDAC formalin-fixed, paraffin embedded (FFPE) tissues and to investigate the clinical significance of the MALAT1 level. Methods: The expression of MALAT1 was examined in 45 PDAC and 25 adjacent non-cancerous FFPE tissues, as well as in five PDAC cell lines and a normal pancreatic epithelium cell line HPDE6c-7, using qRT-PCR. The relationship between MALAT1 level and clinicopathological parameters of PDAC was analyzed with the Kaplan-Meier method and Cox proportional hazards model. Results: The relative level of MALAT1 was significantly higher in PDAC compared to the adjacent normal pancreatic tissues (p=0.009). When comparing the MALAT1 level in the cultured cell lines, remarkably higher expression of MALAT1 was found in aspc-1 PDAC cells compared with the immortal pancreatic duct epithelial cell line HPDE6c-7 (q=7.573, p<0.05). Furthermore, MALAT1 expression level showed significant correlation with tumor size (r=0.35, p=0.018), tumor stage (r=0.439, p=0.003) and depth of invasion (r=0.334, p=0.025). Kaplan-Meier analysis revealed that patients with higher MALAT1 expression had a poorer disease free survival (p=0.043). Additionally, multivariate analysis indicated that overexpression of MALAT1, as well as the tumor location and nerve invasion, was an independent predictor of disease-specific survival of PDAC. Conclusion: MALAT1 might be considered as a potential prognostic indicator and may be a target for diagnosis and gene therapy for PDAC.

Keywords: Pancreatic cancer - long non-coding RNA - MALAT1 - survival - prognosis

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Introduction

Pancreatic cancer (PC) is a highly malignant tumor with increasing incidence and mortality in the world (Canyilmaz et al., 2013; Siegel et al., 2013; Zahir et al., 2013), which leads to disproportionately high percentage (6.58%) of cancer-related deaths (Siegel et al., 2011). In USA, it is the fourth leading cause of cancer -related deaths, with deleted estimated 43, 920 new cases and 37, 390 deaths in 2012 (Siegel et al., 2012). In China, it is the sixth main cause of cancer death with 3.66% incidence and 2.55% mortality of all cancer incident cases in 2013 (Chen et al., 2013c). Pancreatic duct adenocarcinoma (PDAC) accounts for approximately 90% of primary PC (Haugk, 2010), with an overall 5-year survival rate of 5% and a median survival time of 6 months (Schneider et al., 2005). This poor prognosis is due to the late diagnosis and lack of effective treatments (Shrikhande et al., 2011; Tajiri et al., 2012). Despite the recent advances in clinical and experimental oncology, the prognosis of PC still remains poor (Mardin et al., 2013). Thus a thorough understanding of the mechanism underlying the development and progression of PC is essential to improve the diagnosis, prevention and treatment (Prassas et al., 2012). Recently, there has been growing evidence to indicate that non-coding RNAs (ncRNAs) can influence cancer onset, progression and outcome, which provides new insights into the biology of PC (Gutschner et al., 2013; Kim et al., 2013). In the human genome, the ratio of non-coding DNA to total genomic DNA is nearly 98.5%. Recent studies have shown that transcription is not limited to protein-coding regions, but is available in the whole genome (>90%), including non-coding regions. This yields large numbers of ncRNAs (Consortium, 2007). Based on transcript size, regulatory ncRNAs can be further divided into two subclasses: small ncRNA (20-200nt) and long ncRNAs (lncRNAs, >200nt) (Tano and Akimitsu, 2012; Tzadok et al., 2013;Liu et al., 2014). Some lncRNAs can execute a wide range of vital functions, for instance, gene regulation (Yang et al., 2011), splicing control (Zong et al., 2011) or X chromosome dosage compensation (Tian et al., 2010) in the cell. The lncRNAs are also associated with human diseases, especially cancer, since lncRNAs can be deregulated and actively contribute to tumorigenesis (Gutschner and Diederichs, 2012; Spizzo et al., 2012). Metastasis Associated Lung adenocarcinoma

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Transcript1 (MALAT1), also known as nuclear-enriched abundant transcript 2 (NEAT2), has an evolutionarily highly conserved, long noncoding 8.7-kb transcript, which locates on chromosome 11q13 (Ji et al., 2003). MALAT1 presents no protein expression *in vitro* translation because there are no open reading frames. MALAT1 co-localizes with SC35 splicing domains, which is known as nuclear speckles in mouse and human cells, suggesting a role in RNA (Hutchinson et al., 2007). MALAT1 regulates gene expression and post-transcriptionally modifies primary transcripts (Schorderet and Duboule, 2011), which is highly conserved among mammals and widely expressed in normal mouse and human tissues, such as lung and pancreas, as well as in multiple cancer types, including lung, breast, colon, prostate and liver cancers (Lin et al., 2007). However, the relationship between the expression of MALAT1 and PDAC development and progression remains unclear. The aim of the current study is to detect the expression of MALAT1 in PDAC tissues and to explore the relationship between MALAT1 level and clinicopathological features and patient survival.

Materials and Methods

Patients and tissue samples

Forty-five formalin-fixed, paraffin embedded (FFPE) PADC tissues were obtained from the patients who underwent primary surgical resection of PADC between January, 2010 and November, 2011 at the First Affiliated Hospital of Guangxi Medical University in China. Twenty-five cases contained adjacent non-tumor (ANT) pancreatic tissues. The diagnosis of PADC was confirmed by two experienced pathologists. Clinicopathological features were collected, including age, gender, clinical stage, grade, venous invasion, nervous invasion, status of lymphatic metastasis, distant metastasis, tumor node metastasis (TNM) stage (Qureshi et al., 2011), carbohydrate antigen 19-9 (CA19-9) and carcinomacmbryonic antigen (CEA). Post-surgery follow-up was performed every 6 months till November 30, 2013. All patients had completed follow-up information. The disease specific survival (DSS) was defined as the length of time between the surgery and death. The study was approved by the Research Ethics Committee of the First Affiliated Hospital of Guangxi Medical University, China. Informed written consents were obtained from all patients who participated in this study.

Cell lines

The human pancreatic cancer cell lines including Panc-1, Bxpc-3, Aspc-1, Capan-1, Miapaca-2 and the human immortal pancreatic duct epithelial cell line HPDE6C-7 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Wuhan, China). Panc-1, Capan-1 and Miapaca-2 cell lines were maintained routinely in Dulbecco's modified Eagle's medium (wisent, China), while Bxpc-3, Aspc-1 and HPDE6C-7 were cultured in supplemented 1640 (wisent, China) with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100mg/ml streptomycin at 37°C in a 10% CO_2 atmosphere.

RNA preparation, reverse transcription and quantitative real-time PCR

Total RNAs were extracted from FFPE cancer and ANT tissues by using RNeasy FFPE Kit (QIAGEN, Germany), abiding by the manufacturer protocol. RNA was isolated from the cultured cells by using an RNA isolation kit (TRI Reagent, Invitrogen, USA) according to the manufacture instructed protocol. Reverse transcription (RT) and qPCR kits were applied to evaluate expression of MALAT1 from tissue and cell samples. The 20μl RT reactions were performed using a Maxima First Strand cDNA Synthesis Kit (Fermentas, K1641, Canada) and were incubated for 30min at 37°C, 5s at 85°C, and then stabilized at 4°C. For relative qPCR, 2μl diluted RT products were mixed with 12.5μl of 2×SYBRPremix Ex TaqII (Roche, Switzerland), 1μl forward and reverse primers and 8.5μl nuclease-free water in a final volume of 25μl according to manufacturer instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an internal control. The PCR primers were as follows: MALAT1 sense, 5'AGTACAGCACAGTGCAGCTT3', reverse, 5'CCCACCAATCCCAACCGTAA3'; GAPDH sense 5'GTAAGACCCCTGGACCACCA3'; reverse, 5'CAAGGGGTCTACATGGCAACT3'. All reactions were operated on the Eppendorf Master cycler EP Gradient S (Eppendorf, Germany) with following conditions: 95°C for 30s , followed by 40 cycles at 95°C for 15s and 60°C for 1min. Real-time PCR was performed in triplicate, including no template controls. Amplification of the appropriate product was confirmed by melting curve analysis and gel electrophoresis. Relative mRNA expression of MALAT1 was calculated with the comparative threshold cycle (CT) ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001;Chen et al., 2013a; 2013b).

Statistical analysis

The Student test, one ANOVA test, Mann-Whitney test or χ^2 test were performed to study the significance of differences between groups using SPSS 19.0 software (Chicago, USA). Spearman correlation was applied to study the relationship between MALAT1 expression and clinicopathological parameters. Receiver operator characteristic curve (ROC) was employed to identify the diagnostic value. DSS rates were calculated by Kaplan-Meier method with the log-rank test. Variables with a value of $p<0.05$ in univariate analysis were used in subsequent multivariate analysis on the basis of Cox proportional hazards mode. p values less than 0.05 were considered statistically significant.

Results

Expression of MALAT1 in PADC tissues

The expression of MALAT1 was significantly higher in PADC compared with ANT tissues ($p=0.009$; Figure 1A). Furthermore, ROC curve was performed to identify the diagnostic value of MALAT1 level in PDAC. The area under curve (AUC) of MALAT1 was 0.69 (95%CI 0.561~0.829, $p=0.009$). The cut-off value for MALAT1 was 0.1035. The sensitivity and specificity were 77.8% and 60%, respectively (Figure 1B).

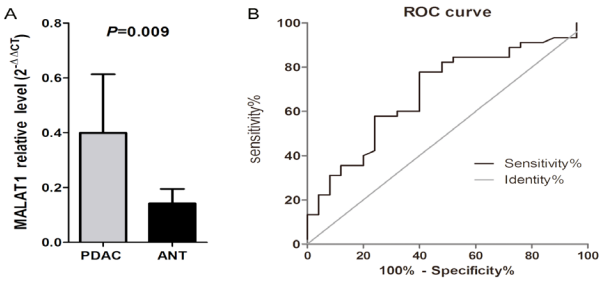

Figure 1. MALAT1 Expression and its Diagnostic value in PDAC. Quantitative real-time RT-PCR was performed to detect the expression of MALAT1 in pancreatic duct adenocarcinoma tissue (PDAC) and adjacent non-tumor (ANT) tissue (A). ROC curve of MALAT1 level in PDAC (B) . The area under curve (AUC) of MALAT1 was 0.69 (95%CI 0.561~0.829, p=0.009).

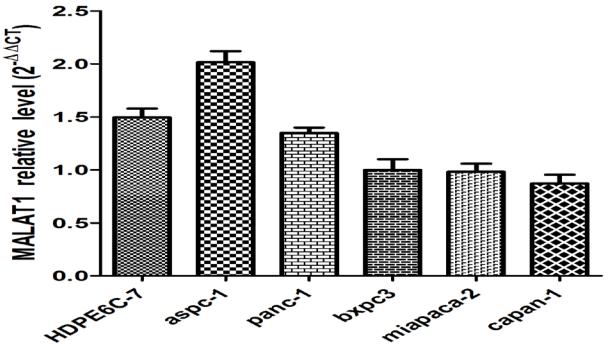

Figure 2. Expression of MALAT1 in Five Human Pancreatic Cancer Cell Lines and an Immortal Pancreatic Duct Epithelial Cell Line HPDE6C-7. A significant higher expression of MALAT1 was found in aspc-1 than in HPDE6C-7 (q=7.573, p<0.05).

Expression of MALAT1 in PC cell lines and HPDE6C-7cells

MALAT1 expression in PC cancer cell lines and HPDE6C-7cell line were also quantified. A significant higher expression of MALAT1 was found in aspc-1 than in HPDE6C-7 (q=7.573, p<0.05). However, lower expression of MALAT1 was detected in Bxpc-3, miapaca-2 and capan-1 same as above (q=7.573, q=7.446, q=9.45 respectively, p<0.05). There was no significant difference of MALAT1 expression between panc-1 and HPDE6C-7 cells (q=2.130, p>0.05, Figure 2).

Relationship between MALAT1 expression and clinicopathological factors in PDAC

To assess the correlation of MALAT1 expression with clinicopathological data, expression of MALAT1 in tumor tissues were categorized as low or high according to the mean value. The higher expression of MALAT1

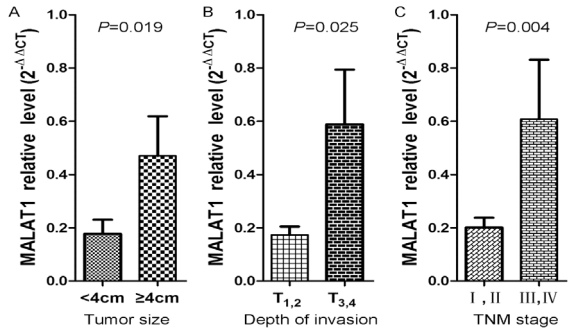

Figure 3. Relationship between MALAT1 Expression and Clinicopathological Factors in PDAC. MALAT1 and Tumor size (A), Depth of invasion (B) and TNM stage (C)

Table 1. Relationship between MALAT1 Expression and Clinicopathological Features of PDAC

Characteristics		Number of case	MALAT1 expression High (n=26)	%	Low (n=19)	%	p value
Age (years)	<58	21	14	53.85%	7	36.84%	0.259
	≥58	24	12	46.15%	12	63.16%	
Gender	Male	26	15	57.69%	11	57.89%	0.989
	Female	19	11	42.31%	8	42.11%	
Tumor size	<4 cm	16	5	19.23%	10	52.63%	0.019
	≥4 cm	29	21	80.77%	9	47.37%	
Location	Pancreatic head	31	17	65.38%	15	79.95%	0.321
	Pancreatic tail	14	9	34.62%	4	21.05%	
Histological grade	Well	11	5	19.23%	6	31.58%	0.334
	Moderately	25	14	53.85%	11	57.89%	
	Poorly/others	9	7	26.92%	2	10.53%	
Depthof invasion	T1,T2	22	9	34.62%	13	64.42%	0.025
	T3,T4	23	17	65.38%	6	31.58%	
Lymphatic metastasis	Absent	22	11	42.31%	11	57.89%	0.369
	Present	33	15	57.69%	8	42.11%	
Venousinvasion	Absent	30	18	69.23%	13	68.42%	0.954
	Present	15	8	30.77%	6	31.58%	
Nervous invasion	Absent	31	17	63.58%	14	73.68%	0.553
	Present	14	9	34.62%	5	26.32%	
Distant metastasis	Absent	38	20	76.92%	18	94.74%	0.103
	Present	7	6	23.08%	1	5.26%	
Tumor stage	Iv and II	24	9	34.62%	15	78.94%	0.004
	III and IV	21	17	65.38%	4	21.05%	
CA199	<37U/ml	11	7	26.92%	4	21.05%	0.651
	≥37U/ml	34	19	73.10%	15	78.94%	
CEA	<5ng/ml	28	16	61.54%	12	63.15%	0.912
	≥5ng/ml	27	10	38.46%	7	36.84%	

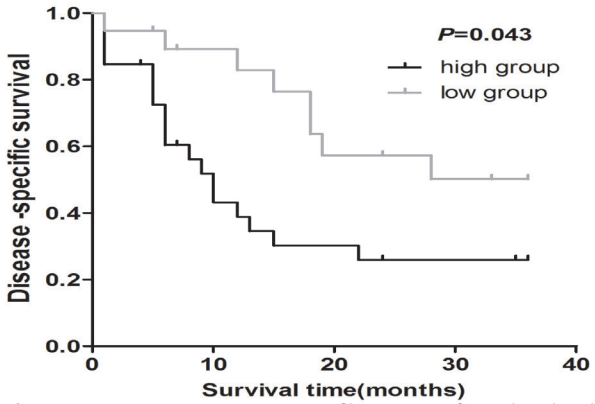

Figure 4. Kaplan-Meyer Curves of MALAT1 expression in PDAC. Patients with high MALAT1 expression had a significantly poorer prognosis than those with low expression (p=0.043)

Table 2. Univariate Analysis of Clinicopathological Factors for Disease-specific Survival in PDAC

Variable	PC(N)	HR (Hazard ratio)	95%CI	p value
Age (years)				
<58	21	1	0.328-1.488	0.35
≥58	24	0.696		
Gender				
Male	26	1	0.333-1.822	0.38
Female	19	0.711		
Tumor size				
<4 cm	16	1	0.32-1.644	0.444
≥4 cm	29	0.721		
Location				
Pancreatic head	31	1	0.98-4.650	0.013
Pancreatic tail	14	2.103		
Histological grade				
Well	11	1	0.777-2.614	0.262
Moderately	25	1.419		
Poorly/others	9			
Depth of invasion				
T1,T2	22	1	0.999-4.965	0.021
T3,T4	23	2.136		
Lymphatic metastasis				
Absent	23	1	0.658-3.093	0.368
Present	22	1.427		
Venous invasion				
Absent	30	1	0.446-2.359	0.939
Present	15	1.034		
Nervous invasion				
Absent	31	1	0.893-4.847	0.01
Present	14	2.08		
Distant metastasis				
Absent	38	1	0.211-1.800	0.423
Present	7	0.641		
Tumor stage				
I and II	24	1	0.296-1.456	0.298
III and IV	21	0.654		
CA199				
<37U/ml	11	1	0.471-2.859	0.738
≥37U/ml	34	1.167		
CEA				
<5ng/ml	28	1	0.307-1.636	0.402
≥5ng/ml	27	0.709		
MALAT1				
Low	19	1	1.027-2.205	0.036
High	26	1.505		

Table 3. Multivariate Analysis of Clinicopathological Factors for Disease-specific Survival in PDAC

Variable	HR	95% CI	p value
PDAC Location	3.482	1.414-8.547	0.007
Depth of invasion	1.731	6.90-9.312	0.241
Nervous invasion	4.631	1.86-11.553	0.001
MALAT1	1.798	1.177-7.747	0.007

was found in the groups of larger tumor size, later tumor stage and deeper invasion than in the corresponding groups (all p<0.05, Table 1, Figure 3). Moreover, analyzed with Spearman coefficient of correlation, MALAT1 expression level showed closed correlations with tumor size (r=0.35, p=0.018), tumor stage (r=0.439, p=0.003) and depth of invasion (r=0.334, p=0.025). However, no significant relationship between MALAT1 expression and other clinicopathological features was found, such as age, gender, tumor location, histological grade, lymphatic metastasis, venous invasion, nervous invasion, CA19-9 and CEA (all p>0.05, Table1).

Correlation between MALAT1 expression and prognosis of PDAC patients

As is shown in Figure 4, patients with high MALAT1 expression had a significantly poorer prognosis than those with low expression (p=0.043, Figure 4). Univariate analysis of DSS revealed that the relative level of MALAT1 expression (p=0.036), tumor depth (p=0.021), nervous invasion (p=0.010) and tumor location (p=0.013) were prognostic indicators. Other clinicopathological features, such as age, gender, tumor size, histological grade lymphatic metastasis, venous invasion, CA199 and ECA were not statistically significant prognosis factors (all p>0.05, Table 2). Variables with a value of p<0.05 were selected for multivariate COX analysis (Table 2). Multivariate analysis indicated that MALAT1 expression level, nervous invasion and tumor location were independent prognostic indicators for DSS in patients with PDAC (p<0.05, Table3).

Discussion

With the advance of high resolution microarray and genome wide sequencing technology, lncRNAs have recently caught increasing attention (Ma et al., 2012; Tang et al., 2013; Liu et al., 2014). Some studies suggest that dysexpression of lncRNAs is associated with numerous diseases including cancer (Wapinski and Chang, 2011; Tang et al., 2013). Furthermore, lncRNAs have been identified to play a major role in the development and progression of different cancers. Some well-defined lncRNAs, including HOTAIR (Gupta et al., 2010; Kim et al., 2013; Nakagawa et al., 2013), MEG3 (Lu et al., 2013) and LOC285194 (Qi et al., 2013) have been reported to be strongly associated to survival of cancer patients, thus they have been determined as prognostic indicators for a delete certain types of cancers.

MALAT1 was first discovered to be three folds higher expressed in metastasizing human non-small-cell lung carcinomas (NSCLCs) compared to the non-metastasizing tumor using RT-PCR by Ji P, et al in 2003 (Ji et al.,

2003). Other groups also studied the expression level and functions of MALAT1 in different types of malignancies. However, no report of MALAT1 in PDAC was found. MALAT1 has been found to be up-regulated in several solid tumors, such as NSCLSs (Schmidt et al., 2011) endometrial stromal sarcoma (Yamada et al., 2006), HCC (Lin et al., 2007), bladder urothelial carcinoma (Han et al., 2013) and prostate cancer (Ren et al., 2013). Thus, we speculated that MALAT1 also had similar impact on the tumorigenesis, development and progression of PDAC, which might be linked to prognosis of PDAC patients. To confirm these hypotheses, we firstly detected the MALAT1 level in 45 pairs of PDAC and 25 adjacent normal tissues by RT-qPCR. The relative expression level of MALAT1 was significantly higher in tumor compared with adjacent normal tissues. This result was confirmed in the *in vitro* sample. The MALAT1 expression in Aspc-1 cell lines was also remarkably higher compared with immortal pancreatic duct epithelial cell line HDPE6C-7. The overexpression of MALAT1 in PDAC tissues and PDAC Aspc-1 cells suggested that MALAT1 could act as an oncogene in PDAC similar to other tumors. We also performed the ROC curve to identify the diagnostic significance of MALAT1 expression in PDAC and the AUC area was 0.69, suggesting a possible diagnostic value of MALAT1. However, this was based on a limited numbers of patients. More evidence should be provided with a larger cohort in the future. Most recently, based on small numbers, MALAT1 was shown to be detectable in the cellular fraction of peripheral human blood, showing different expression levels between NSCLC patients and cancer-free controls. For the discrimination of NSCLC patients from cancer-free controls a sensitivity of 56% was calculated conditional on a high specificity of 96%. The results of this study by Weber, et al (Weber et al., 2013) indicated that MALAT1 complied with key characteristics of diagnostic biomarkers, i.e., minimal invasiveness, high specificity, and robustness. It remains interesting to investigate the plasma MALAT1 level as a biomarker in PDAC to improve the entire diagnostic performance.

Then we went further to investigate the relationship between MALAT1 expression and different clinicopathological parameters. The significantly higher MALAT1 expression was found in the groups of larger tumor size, deeper invasion and advanced stage, delete compared to their corresponding groups. Spearman correlation also showed the positive relationships between MALAT1 level and tumor size, depth of invasion and tumor stage. Since the clinicopathological parameters of tumor size, depth of invasion and tumor stage represent partially the deterioration and progress of the tumor, MALAT1 might be a factor related to the tumor progression. Similarly to our finding, MALAT1 expression levels were reported to be greater in invasive bladder urothelial carcinoma than in noninvasive carcinoma (Han et al., 2013). In the same study, MALAT1 expression levels were greater in high-grade bladder urothelial carcinomas than in low-grade carcinoma (Han et al., 2013), which was inconsistent with our current study, indicating that the function and contribution of MALAT1 could be tumor dependent. No correlations were found between MALAT1 expression and other clinicopathological features in the current study, including age, gender, tumor location, lymphatic metastasis, venous invasion, nervous invasion, distant metastasis, CA199 and CEA levels. Further large-scale studies are needed to confirm our findings.

Next, we studied the influence of MALAT1 expression on patient survival. Kaplan-Meier analysis indicated that patients with high MALAT1 expression had a poorer disease free survival than those of low MALAT1 expression in PDAC. Moreover, Univariate analysis of DSS revealed that the relative level of MALAT1 expression, tumor location, depth of invasion and nervous invasion could be prognostic indicators. Multivariate analysis further showed that expression of MALAT1, together with tumor location and nervous invasion, was an independent predictor of disease specific survival for PDAC. This was in agreement with the prognostic role of MALAT1 in colorectal cancer (Ji et al., 2013), HCC (Lai et al., 2012) and NSCLC (Schmidt et al., 2011). Thus, high MALAT1 expression in several tumors was associated with a poor prognosis and MALAT1 could act as an independent prognostic factor for predicting tumor prognosis, including PDAC.

Over-expression of MALAT1 may influence the development, progression and prognosis of PDAC. However, the molecular mechanism involved is unclear. MALAT1 was associated with epithelial mesenchymal transition (EMT) that allowed cancer cells to obtain invasive capacity. Ying et al. (2012) demonstrated that down- regulation of MALAT1 caused a decrease of ZEB1, ZEB2 and Slug levels, and an increase of E-cadherin levels in EMT of bladder cancer cell. Qing et al. (Ji et al., 2013) reported that in colorectal cancer, MALAT1 might indirectly interact with ß-catenin to affect its signal cascade. Target genes of MALAT1 may differ between specific tissues and cell types. The specific target genes and signal-pathway controlled by MALAT1 in PDAC require detailed investigation in the future.

Some studies had proved that down-regulation of MALAT1 level can regulate apoptosis genes expression such ascaspase-3, caspase-8, Bax, Bcl-2, and Bclxl, which leads to inhibit cervical cancer on cell growth, cell cycle progression and invasion (Guo et al., 2010). In A549 NSCLCs, RNAi-mediated suppression of MALAT1 RNA suppressed migration and clonogenic growth (Schmidt et al., 2011). Similarly, cell proliferation inhibition, increased apoptosis, and decreased motility were observed in MALAT1 small interfering RNA-transfected bladder urothelial carcinoma T24 and 5637 cells (Han et al., 2013). These studies indicated that MALAT1 could be a potential target for molecular therapy for cancers. However, it remains to be investigated how MALAT1 could contribute in the treatment of PDAC.

In conclusion, above all, our results showed that MALAT1 mRNA level was significantly higher in PADC tissues and some PC cell lines. A high expression of MALAT1 was detected in tumors of larger size, advanced tumor stage and deeper invasion. In addition, the overexpression of MALAT1 was associated with poor prognosis. These findings suggested that MALAT1 might be a potential prognostic indicator in PDAC and may be

a possible target for diagnosis and gene therapy, although more samples are needed to confirm these results. Further *in vitro* and in vivo experiments are now being carried out by our group to investigate the effect of MALAT1 on the malignant phenotypes of pancreatic carcinoma.

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