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MicroRNAs encode the diversity between  
pancreatic head and body/tail cancers

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## Abbreviations

5-FU	5-fluorouracile
ADM	acinar-ductal metaplasia
AJCC	american joint commission on cancer
BCA	bicinchoninic acid
BRCA2	breast cancer type 2 susceptibility protein
BSA	bovine serum albumin
CGH	comparative genomic hybridization
CK	cytokeratin
CT	computer tomography
DD	death domain
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ERK	extracellular regulated protein kinases
FCM	flow cytometry
FCS	fetal calf serum
HAS	heat-stable antigen
HCAM	homing cell adhesion molecule
HER-2	human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
HUVEC	human umbilical vein endothelial
IAP	inhibitor of apoptosis protein
IC <sub>50</sub>	half maximal inhibitory concentration
IGF1R	insulin-like growth factor 1 receptor
JAK2	janus kinase 2
LAR	luciferase assay reagent
LPS	lipopolysaccharide
miRNA	microRNA
mRNA	messenger RNA

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PanIN	pancreatic intraepithelial neoplasia
PBS	Phosphate Buffered Saline
PDAC	pancreatic ductal adenocarcinoma
PI3K	phosphoinositide-3 kinase
PET	positron emission tomography
qRT-PCR	quantitative reverse transcription real-time polymerase chain reaction
SEER	surveillance, epidemiology, and end results
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R1	TRAIL-receptor 1
TRAIL-R2	TRAIL-receptor 2
TSG	tumor suppressor gene
UICC	international union against cancer
VEGF	vascular endothelial growth factor

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# 1. Introduction

## 1.1 Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer. It is one of the most lethal human cancers and leads to an estimated number of 227,000 deaths per year worldwide (Raimondi *et al.*, 2009). It has a high metastatic potential and the majority of diseases present themselves at a very late stage. As for other cancers, the 'TNM' system has been used to stage PDAC according to the American Joint Commission on Cancer (AJCC) and International Union against Cancer (UICC). 'TNM' stands for Tumor, Node, and Metastasis. This system describes the size of a primary tumor, whether lymph nodes are involved by cancer cells or not, whether cancer has spread or not. There are 5 stages in the current TNM classification (Table 1) (<http://www.cancerstaging.org/staging/posters>).

**Table 1** AJCC 7<sup>th</sup> Edition TNM Staging System for Pancreatic Cancer

Pancreas				
Tx	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
Tis	Carcinoma in situ			
T1	Limited to pancreas $\leq$ 2 cm			
T2	Limited to pancreas $>$ 2 cm			
T3	Beyond pancreas			
T4	Celiac axis or superior mesenteric artery			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
M0	No distant metastasis			
M1	Distant metastasis			
Stage grouping				
Stage 0	Tis	N0	M0	Localized within pancreas
Stage IA	T1	N0	M0	Localized within pancreas
Stage IB	T2	N0	M0	Localized within pancreas
Stage IIA	T3	N0	M0	Locally invasive, resectable
Stage IIB	T1,2 or 3	N1	M0	Locally invasive, resectable
Stage III	T4	Any N	M0	Locally advanced, unresectable
Stage IV	Any T	Any N	M1	Distant metastasis



The median survival of PDAC is less than 6 months (Xu *et al.*, 2011a). Surgical intervention is possible in about 10% of cases. Despite recent progress in chemotherapy, radiation therapy, and surgical resections, the overall survival rate of pancreatic cancer is still less than 5%. Around 95% of patients diagnosed with PDAC will die of this disease within 5 years, 3/4 within a year (Raimondi *et al.*, 2009).

Pancreatic intraepithelial neoplasia (PanIN) is the presumed precursor lesion to infiltrating PDAC. PanINs are defined as epithelial neoplasms in the smaller pancreatic ducts (usually < 5 mm in diameter) and can be divided into 3 grades based on the degree of architectural and nuclear atypical present (Hruban *et al.*, 2001). The importance of these lesions as precursors to invasive PDAC has been paid great attention to during recent years because it should be possible to detect and treat these non-invasive precursor lesions before an incurable invasive cancer develops. The progression from the precursor lesions (e.g. PanIN) to PDAC suggests the pancreatic duct to be the origin of tumor. A more recent study provided evidence that PDAC might also develop in the centroacinar region, through a process of acinar-ductal metaplasia (ADM) (Aichler *et al.*, 2012). Therefore, study of novel biomarkers with high sensitivity and specificity for early detection of precursors (e.g. PanIN or ADM) is giving hope for improving patient survival.

PanINs are associated with multiple genetic alterations, such as activating point mutations in K-ras oncogene and over-expression of human epidermal growth factor receptor 2 (HER-2)/neu during early stage, and inactivation of the p16 tumor suppressor gene at a later stage followed by the loss of p53, SMAD4, and breast cancer type 2 susceptibility protein (BRCA2) tumor suppressor genes (Harada *et al.*, 2007; Legoffic *et al.*, 2009). However, despite a wealth of molecular studies (Koliopanos *et al.*, 2008), none of the proposed biomarkers are currently recommended for clinical use.

## **1.2 Pancreatic cancer and miRNA**

MicroRNA (miRNA) are non-coding small RNAs, suppressing gene expression or inhibiting translation via binding complementary sequences in the 3'UTR of messenger RNA (mRNA)s. miRNAs regulate biological processes including cell proliferation, differentiation, apoptosis, development, metabolism and neoplastic transformation (Huang *et al.*, 2011). It has been reported that the expression pattern of miRNAs rather than mRNAs are surprisingly informative, highly accurate in reflecting the developmental lineage and differentiation state of tumors (Lu *et al.*, 2005). Comparing with other potential biomarkers, miRNA can be found stabilized in body fluids and tissue samples, which makes it one of the most promising ways for earlier detection of cancer.

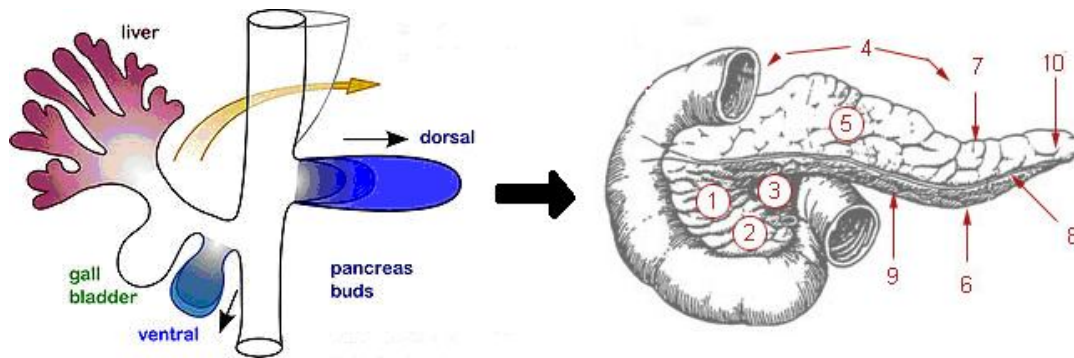
Several studies have focused on the impact of the miRNA expression pattern in PDAC and have shown the potential clinic value in the screening, diagnosis and prognostic prediction of disease using specific miRNAs, such as miR-21, miR-10b, miR-196a, and miR-221 (Basu *et al.*, 2011; Bloomston *et al.*, 2007; Giovannetti *et al.*, 2010; Jamieson *et al.*, 2012; Nakata *et al.*, 2011; Szafranska *et al.*, 2007). Most recently, the miRNA alterations that arise during the development of PanINs has also been identified (Yu *et al.*, 2012). In the future, miRNA expression pattern in PanINs or PDAC may serve as a novel screening tool and potent monitor in PDAC progression. Equally as exciting, it is very promising to research for novel therapies directed against these important disease mediators.

## **1.3 Pancreatic head cancer and body/tail cancer**

### **1.3.1 Difference in anatomy and embryology**

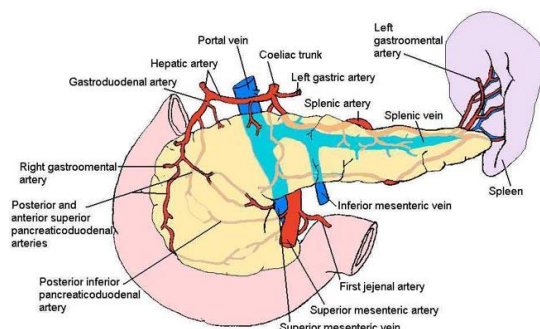
PDAC can be divided into head and body/tail cancers according to the anatomy. Distinctions in anatomical conditions are well known from embryology (In't Veld *et al.*, 2010), histopathology (Atri *et al.*, 1994; Zyromski *et al.*, 2009), and imaging findings (Kawamoto *et al.*, 2009; Schoennagel *et al.*; Wiersema *et al.*, 1995; Yoshikawa *et al.*, 2006).

Pancreatic development begins with the formation of ventral and dorsal buds, which becomes a ventral head (lower head and uncinete process) and dorsal pancreas (upper head, body and tail), respectively (Figure 1).



**Figure 1.** The development of the pancreas from dorsal and ventral buds. 1: Head of pancreas; 2: Uncinate process of pancreas; 3: Pancreatic notch; 4: Body of pancreas; 5: Anterior surface of pancreas; 6: Inferior surface of pancreas; 7: Superior margin of pancreas; 8: Anterior margin of pancreas; 9: Inferior margin of pancreas; (From Wikipedia: [http://en.wikipedia.org/wiki/Main\\_Page](http://en.wikipedia.org/wiki/Main_Page))

This difference in ontogeny leads to significant differences in cell composition, blood supply and innervations between the head and body/tail of pancreas (In't Veld *et al.*, 2010). The superior pancreaticoduodenal artery (a branch of the gastroduodenal artery) and the inferior pancreaticoduodenal artery (a branch of the superior mesenteric artery) run in the groove between pancreas and duodenum and supply the head of pancreas. While the pancreatic branches of splenic artery supply the neck, body and tail of pancreas. The head of pancreas drains into the superior mesenteric and portal veins, while the body and neck drain into splenic vein (Figure 2).



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Figure 2. The blood supply and venous drainage of pancreas. (From EndocrineSugeon: <http://www.endocrinesurgeon.co.uk>)

Pancreas is both an endocrine gland producing important hormones (e.g. insulin, glucagon, somatostatin, and pancreatic polypeptide) and a digestive organ secreting pancreatic juice, which assist the intestinal absorption of nutrients. The endocrine part of the pancreas is made up of approximately one million cell clusters called islets of Langerhans. The endocrine cells have different secretory functions:  $\alpha$  cells secrete glucagon (increase glucose in blood),  $\beta$  cells secrete insulin (decrease glucose in blood),  $\delta$  cells secrete somatostatin (regulates/stops  $\alpha$  and  $\beta$  cells), and pancreatic polypeptide cells secrete pancreatic polypeptide. The endocrine cells ( $\alpha$ -,  $\beta$ -,  $\delta$ - and pancreatic polypeptide cells) are remarkably differentially distributed between head and body/tail of pancreas (In't Veld *et al.*, 2010). The number of endocrine (Langerhans) islets is greater in the body and tail. Insulin-positive endocrine cells are highly refractory to malignant transformation under normal conditions, but a recent study has found that insulin-positive endocrine cells could serve as a cell-of-origin of PDAC under oncogenic mutation inducement in combination with pancreatic injury (Gidekel Friedlander *et al.*, 2009).

In addition, the distinction between pancreas head, body, and tail regions is also reflected by different appearances in ultrasonography (Wiersema *et al.*, 1995), computer tomography (Kawamoto *et al.*, 2009), and magnetic resonance imaging (Yoshikawa *et al.*, 2006). Studies have shown that fatty infiltration is usually most prominent in the anterior aspect of the pancreas head and may stimulate pancreatic neoplasm (Hori *et al.*, 2011; Kawamoto *et al.*, 2009; Zyromski *et al.*, 2009). In this sense, pancreatic head and body/tail cancers may have different malignant potential.

### 1.3.2 Difference in clinical presentation

Although increasing evidence has shown differences on clinical presentation (incidence,

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symptoms, resectability) between pancreatic head and body/tail cancers (Bilimoria *et al.*, 2007; Lau *et al.*, 2010; Matsuno *et al.*, 2004; van Oost *et al.*, 2006), the molecular diversity between the two subtypes of PDAC has not been clarified so far.

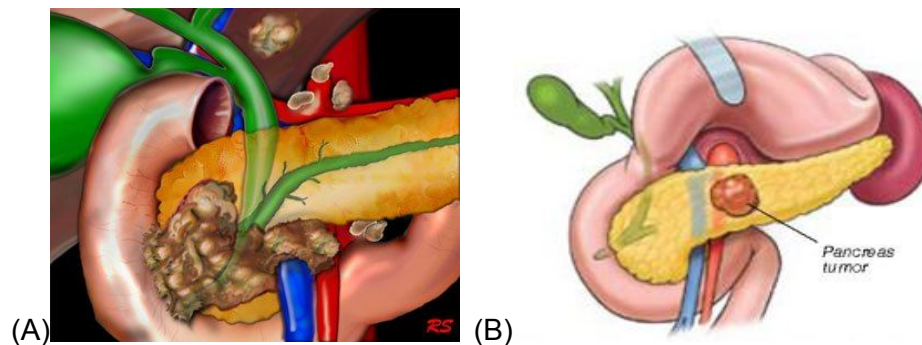
### 1.3.2.1 Incidence

Data from Surveillance, Epidemiology, and End Results (SEER) registries of United States (1973-2002) have shown that about 77.5% (34,072/43,946) of pancreatic cancer originate at the head of the pancreas, on which consequently most discussion on pancreatic cancer has been focused (Lau *et al.*, 2010). The overall annual incidence of pancreatic head cancer is 5.6 per 100,000, compared with 1.6 of pancreatic body/tail cancer (Lau *et al.*, 2010). Data from National Pancreatic Cancer Registry of Japan (1981-2002, n = 9290) (Matsuno *et al.*, 2004) and Eindhoven Cancer Registry of Netherlands (1995-2000, n = 1128) (van Oost *et al.*, 2006) also demonstrated much higher incidences of pancreatic head cancer (62.3% and 56.5%, respectively) than body/tail cancer (17.5% and 12.7%, respectively). For resectable tumors (Stage I: T1N0M0 and T2N0M0), about 70% (6676/9559) located in the head of pancreas as shown by another database in the United States (National Cancer Data Base, 1995-2004) (Bilimoria *et al.*, 2007).

### 1.3.2.2 Symptoms

Symptoms often do not appear until the disease is at an advanced stage, thus making early detection difficult. Notably, a patient's symptoms will vary depending on the location of the cancer within the pancreas. Both pancreatic head and body/tail cancers can cause non-specific symptoms, such as abdominal pain, nausea, loss of appetite and weight loss. However, only tumor blocking the bile ducts, which pass through the head of pancreas, can cause jaundice (Figure 3). A study from China investigated the clinical-pathological characteristics between pancreatic head cancer (n = 541) and body/tail cancer (n = 106) from 1980 to 2003. They found that patients primarily diagnosed with pancreatic body/tail cancer were associated with much less jaundice but more pain, higher serum albumin level, higher carcinoembryonic antigen but lower carbohydrate antigen 19-9 positive rates, and higher

metastasis rate (Wu *et al.*, 2007). Other studies also confirmed that patients with pancreatic body/tail cancer were more likely to be diagnosed at an advanced stage (Eyigor *et al.*, 2010; Sener *et al.*, 1999). SEER registries database reported that patients with pancreatic body/tail cancer had a higher proportion of the distant stage diseases (72.7% vs. 39.2%) compared with patients with pancreatic head cancer (Lau *et al.*, 2010).



**Figure 3.** Pancreatic cancer located in the head (A) and body/tail (B) of pancreas. Bile duct passes through the head of pancreas. Therefore, pancreatic head cancer can easily block the bile ducts and cause jaundice. (A from Doctor Tipster: <http://www.doctortipster.com>; B from CPMC Sutter Health: <http://www.cpmc.org/index.cfm>)

### 1.3.2.3 Diagnostic characterization of genetic markers

Biomarkers are important to the early diagnosis and prompt treatment of diseases. Carbohydrate antigen 19-9 is a routinely used serum biomarker for PDAC (Humphris *et al.*, 2012). A study from China found that pancreatic body/tail cancer was associated with lower serum carbohydrate antigen 19-9 but higher serum carcinoembryonic antigen levels (Wu *et al.*, 2007). However, there is lack of a high sensitive biomarker for PDAC, which make the early diagnosis difficult and lead to a dismal prognosis.

Most recently, as a consequence of technical advances, whole sequencing of the cancer exome has been performed and leads to greater insight into the mutational spectrum of human cancers, including PDAC (Iacobuzio-Donahue, 2012; Jiao *et al.*, 2011; Jones *et al.*,

2004; Sjoblom *et al.*, 2006). In 2008, a comprehensive genetic analysis containing 24 PDACs determined an average of 63 genetic alterations in the sequences of 23,219 transcripts, representing 20,661 protein-coding genes. A core set of 12 cellular signaling pathways and processes explaining the major features of pancreatic tumorigenesis have been defined by these alterations (Jones *et al.*, 2008). Fifteen out of 24 PDAC samples were obtained from the primary tumors. However, the precise locations were not provided, a comparison between pancreatic head and body/tail cancers is impossible in this context.

### 1.3.3 Difference in surgical treatment

A pancreatectomy is the surgical removal of pancreas (part or all). Several types of pancreatectomy exist, such as 'pancreaticoduodenectomy' (Whipple procedure), 'distal pancreatectomy', 'segmental pancreatectomy', and 'total pancreatectomy'. Pancreatic head and body/tail cancers are usually treated with 'pancreaticoduodenectomy' and 'distal pancreatectomy', respectively (Figure 4).

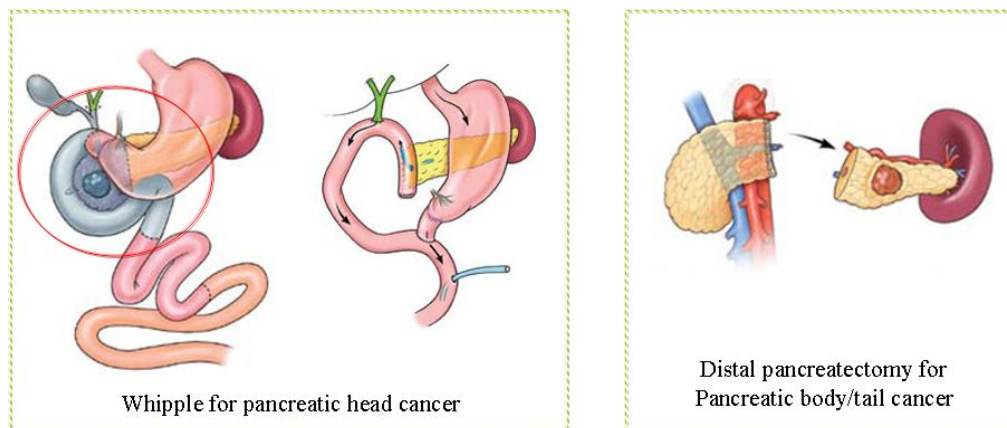


Figure 4. Pancreatic head and body/tail cancers are treated with 'pancreaticoduodenectomy' (Whipple procedure) and 'distal pancreatectomy', respectively. (From CPMC Sutter Health: <http://www.cpmc.org/index.cfm>)

The Whipple procedure consists of removal of the distal half of the stomach (antrectomy), the gall bladder and its cystic duct (cholecystectomy), the common bile duct (choledochectomy),

the head of the pancreas, duodenum, proximal jejunum, and regional lymph nodes. Reconstruction (the original one is called Child's reconstruction) consists of attaching the pancreas to the jejunum (pancreaticojejunostomy), attaching the hepatic duct to the jejunum (hepaticojejunostomy), and attaching the stomach to the jejunum (gastrojejunostomy). So that digestive juices and bile can flow into the gastrointestinal tract and food can also pass through.

A distal pancreatectomy is the resection of pancreatic tissue to the left of the superior mesenteric vascular. It has been considered as the standard technique for management of benign and malignant pancreatic disorders in the body and tail. With the improvement of surgical technique, a surgical procedure is available where the spleen is preserved removing only the pancreas, which is also known as spleen preserving distal pancreatectomy.

#### **1.3.4 Difference in prognosis**

It is not surprising that pancreatic head cancer has a better overall patient survival than those with pancreatic body/tail cancer, because much more patients with pancreatic body/tail cancer are diagnosed at a relatively advanced stage. SEER registries database showed pancreatic head cancer had a 4% lower overall mortality risk compared with pancreatic body/tail cancer (Lau *et al.*, 2010). However, within the same local-stage, pancreatic head cancer had a much lower 3-year survival rate than pancreatic body/tail (Lau *et al.*, 2010). The National Cancer Data Base of United States (1985-1995) including information from 100,313 patients also presented a higher 5-year survival rate for local-stage pancreatic tail cancer (32.4%) compared with local-stage pancreatic head cancer (11.1%) (Sener *et al.*, 1999). Consistent with the results from Western countries, the study from Japan, as mentioned before (Matsuno *et al.*, 2004), showed a significantly higher median survival time (10.2 vs. 9.2 months) and 5-year survival rate (13.8% vs. 10.7%) for pancreatic body/tail cancer (n = 1629) than pancreatic head cancer (n = 5788) in invasive types.



As long as operation is possible, the pancreatic head cancer is surgically treated by pancreaticoduodenectomy, while pancreatic body/tail cancer is treated by a distal pancreatectomy. As mentioned previously, more tumors are diagnosed at early stage and the resectability is higher in pancreatic head cancer. Regarding the complexity of the surgical procedure, pancreatic body/tail cancer shows lower morbidity and mortality than pancreatic head cancer after surgery (Glanemann *et al.*, 2008).

Although surgical resection remains to be the only potential cure for PDAC, only 15-20% of patients newly diagnosed with PDAC are considered for surgical resection (Castellanos *et al.*, 2011). Chemo and/or radiotherapy have emerged as a key factor to improve patient survival both in resectable and non-resectable tumors (Sata *et al.*, 2009). Reports from both Western countries and Japan showed that tumor site (head vs. body/tail) did not relate to progression free-survival or overall survival in patients with advanced or metastatic pancreatic cancer treated with Chemo and/or radiotherapy (Chang *et al.*, 2009; Marechal *et al.*, 2007; Morganti *et al.*, 2003; Tanaka *et al.*, 2008). For resectable tumors, adjuvant chemotherapy was found to be a significant independent predictor of a favorable prognosis in a cohort of 34 Japanese patients with pancreatic body/tail cancer (Murakami *et al.*, 2009), and was thus recommend for patients with pancreatic head cancer (Katz *et al.*, 2010). However, no large study has yet been conducted for comparing the different response to chemo and/or radiotherapy such as toxicity and tumor progression between different tumor sites.

### **1.3.5 Difference in *in vitro* models**

Cancer cell lines recapitulate the genomic events leading to tumor changes seen in clinical tissues and are valuable tools in studies of tumor cell biology. Different PDAC cell lines arising from primary tumors, liver metastasis, ascites, or lymph node metastasis, exhibit a great deal of diversity in structure and function. Among PDAC cell lines originating from the primary tumors, Panc-1 and MIA PaCa-2, which are matched by donor age ( $\pm 10$  years), tumor stage, histological differentiation ([Table 2](#)) and ultrastructural features ([Table 3](#)) (Deer

et al., 2010; Sipos et al., 2003), were selected as the one representing pancreatic head cancer and body/tail cancer, respectively.

**Table 2** Clinical profile and origin of PDAC cell lines originated from primary tumor

Cell line	Age	Gender	Derivation	Stage	Differentiation	Therapy	Survival
BxPC-3	61	Female	Body	II-III	Moderate to poor	Chemotherapy Radiotherapy	6.75 yrs
Capan-2	56	Male	Head	IIA	well	Surgery Chemotherapy	0.5 yrs
MIA PaCa-2	65	Male	Body/tail	II-III	Poor	-	-
Panc-1	56	Female	Head	IIB	Poor	Surgery	-

**Table 3** Grading of ultrastructural features of PDAC cell lines originating from primary tumor

Cell line	Cellular polymorphism	Nuclear polymorphism	Cell membrane structures	Mucin granules	Cell organelles	Lumen formation	Cell polarity	Final score	Grade
Monolayers <sup>a</sup>									
BxPC-3	3	2	1-2	1	1-2	-	-	9	2
Capan-2	1	2-3	2	1-2	1	-	-	8	1
MIA PaCa-2	3	2-3	3	2	3	-	-	13.5	3
Panc-1	3	3	3	3	2-3	-	-	14.5	3
Spheroids <sup>b</sup>									
BxPC-3	3	2	1	1-2	2	1-2	3	14	2
Capan-2	1-2	2	1	1-2	1	1	1-2	9.5	1
MIA PaCa-2	-	-	-	-	-	-	-	20 <sup>c</sup>	3
Panc-1	3	3	2	2	2	2	3	17	3

<sup>a</sup>: Grading of ultrastructural features of monolayer cultures of PDAC cell lines (grade 1 score 5-8, grade 2 score 9-12, grade 3 score 13-15)

<sup>b</sup>: Grading of ultrastructural features of spheroid cultures of PDAC cell lines (grade 1 score 7-11, grade 2 score 12-16, grade 3 score 17-21)

<sup>c</sup>: Estimated score because no spheroid formation.

Compared to MIA PaCa-2, Panc-1 showed a higher binding affinity to collagens (type I and IV), the most abundant protein in the extracellular matrix (ECM), and exhibited higher

adhesion ability to endothelial cells (Deer *et al.*, 2010; ten Kate *et al.*, 2006). In addition, Panc-1 expressed greater levels of a series of cell adhesion molecules (ten Kate *et al.*, 2006). A study using co-culture system with PDAC cells and tumor-derived fibroblasts demonstrated that hepatocyte growth factor produced by the fibroblasts could initiate an apparent invasion-stimulating response in strong c-met expressing Panc-1 cells but not in weak expressing MIA PaCa-2 cells (Qian *et al.*, 2003).

Panc-1 exhibited much higher half-maximal inhibitory concentration (IC<sub>50</sub>) values for 5-fluorouracile (5-FU) and Gemcitabine, and higher irradiation dose than MIA PaCa-2 (Dai *et al.*, 2010; Galloway *et al.*, 2009; Pan *et al.*, 2008). The possible reason was that the expressions of anti-apoptotic proteins were much higher in Panc-1 than those in MIA PaCa-2 (Galloway *et al.*, 2009; Pan *et al.*, 2008).

Both Panc-1 and MIA PaCa-2 presented high intra-pancreatic tumorigenicity, local infiltration and distant metastasis potential in an orthotopic injection of cancer cell model, which could better reflect the clinical microenvironment and provide more convincing data compared to other methods (Hotz *et al.*, 2003; Loukopoulos *et al.*, 2004). However, data directly comparing the tumorigenicity of the two PDAC cell lines were not given in this model. By using immunohistochemical analysis *in vivo*, Panc-1 and MIA PaCa-2 tumors demonstrated quite similar major morphology, mucin accumulation, cytokeratin (CK) profile (CK7/CK19 and CK8/CK18), trans- (Vimentin, CK20, Chr-A and  $\alpha$ 1-chym) and dedifferentiation (pdx-1, shh and ptc) patterns (Neureiter *et al.*, 2005).

## 1.4 Aim of the study

So far, not enough attention has been paid to the molecular diversity between pancreatic head and body/tail cancers and the overall information is limited. The current clinical data support higher incidence and easier detection of pancreatic head cancer compared with pancreatic body/tail cancer. For tumors at the local-stage, pancreatic head cancer has a lower survival than pancreatic body/tail cancer. Although the previous reports describe large cohort of patients, the evidence is still not convincing because there is a lack of strictly case-matched comparison between the two subtypes of PDAC. Further pioneering studies on patient tumor samples are needed.

It is likely that the majority of human genes are regulated by miRNAs. The discovery of miRNAs offers a new opportunity to uncover the aberrantly expressing cellular pathways during the pathognomonic carcinogenetic events for all human cancers.

Our aim was to evaluate the genetic diversity at molecular level between the pancreatic head cancer and pancreatic body/tail cancer. We performed a comparison of miRNA expression profile between pancreatic body/tail cancer and pancreatic head cancer, and found two miRNAs were significantly differentially expressed. We further aim to clarify the role of the differentially expressed miRNA in the phenotype varity between pancreatic body/tail cancer and pancreatic head cancer *in vitro* study.

## **2. Materials and methods**

### **2.1 Tissue samples and cell sources**

Patients with newly diagnosed early stage (I-II) PDAC were considered eligible for this study. Informed written consent was obtained from each patient. Research protocol was approved by the ethical committee of the First Affiliated Hospital, Zhejiang University and strictly followed the guidelines of the Ethical Committee of this hospital and the Declaration of Helsinki. Tumor samples were obtained directly from surgery. The Whipple procedure and distal pancreatectomy was performed for pancreatic head cancer and body/tail cancer, respectively, by the same surgery group, led by Prof. Shusen Zheng. Hematoxylin and Eosin stained tissue sections were reviewed by two independent surgical pathologists. All tumors were classified according to the TNM staging system.

PDAC cell lines (Capan-1, Capan-2, Panc-1, PancII-1, Panc89, BxPc-3 and Colo357) are derived from the Institute for Experimental Cancer Research, UKSH, Campus Kiel, Germany, if not otherwise stated. The detailed information has been given in our previous report (Sipos *et al.*, 2003).

## 2.2 Materials

### 2.2.1 Instruments

Description	Type	Company
Agilent Bioanalyzer	2100	Agilent technologies Inc., USA
Agilent Microarray Scanner	G2565BA	Agilent technologies Inc., USA
Blotting chamber	Mini Trans Blot Cell	Bio-Rad Laboratoeries GmbH, München
Branson Sonifier	PG. 1029	MSE, UK
Cell culture centrifuge	Rotina 48 R	Hettich, Tuttlingen
Cellometer Auto	T4	Nexcelom Bioscience LLC, USA
Centrifuge	Mini Spin Plus	Eppendorf, Hamburg
ECL- chemiluminescence-film	Hyperfilm	GE Healthcare Europe GmbH, München
Film developer	Curix60	Agfa, Gera
Flow cytometry system	BD FACSCalibur™	BD Biosciences, USA
Heidolph duoMax-Rotator shake	1030	Heidolph Instruments GmbH, Schwabach
Heraeus quartz heaters	6000	Heraeus Instruments, Hanau
Hybridization Chamber	G2534A	Agilent technologies Inc., USA
Hybridization oven	G2545A	Agilent technologies Inc., USA
Hybridization oven rotator	G2530-60029	Agilent technologies Inc., USA
Magnetic stirrers	MR300T	Heidolph Instruments GmbH, Schwabach
Microcentrifuge	5417R	Eppendorf, Hamburg
Microscope	Axiovert 135	Carl Zeiss AG, Oberkochen
Minicentrifuge	GalaxiMini	VWR, Darmstadt
Opaque glasses	Diameter 18 mm	Th. Geyer, Renningen
Photometer	Sunrise	Tecan Trading AG, Schweiz

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Real-time PCR system	7700HT	Applied Biosystems, Darmstadt
Roll-mixer	Assistant RM5	Assistant, Sondheim
Standard Power Pack	P25	Biometra GmbH, Goettingen
Thermoblock	Thermomixer compact	Eppendorf, Hamburg
Vortex	Vortex Genie2	Scientific industries Inc., USA
Water bath	WBT12	Reiss-Daimler Medingen
Water Purification System	Milli-Q	Millipore GmbH, Schwalbach
Water-jacketed incubator	3250	Thermo Scientific Forma, UK

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### 2.2.2 Consumables

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Description	Company
Cell culture plates (6-, 24-, 96-well)	Sarstedt, Nümbrecht
Cell culture flasks (25 cm <sup>2</sup> and 75cm <sup>2</sup> )	Sarstedt, Nümbrecht
Cell scraper (16 cm)	Sarstedt, Nümbrecht
Chromatography paper (3 mm)	Whatman GmbH, Dassel
Cryovials	Nalgene, Rochester
Falcons (15ml and 50ml)	Sarstedt, Nümbrecht
Novex® Tris-Glycine gels	Invitrogen, Karlsruhe
Pipette tips	Eppendorf, Hamburg
Pipettes (10 µl, 20 µl, 100 µl, 200µl and 1 ml)	Eppendorf, Hamburg
Powder-free gloves	GE Healthcare Europe GmbH, München
PVDF-transfer membrane	Millipore GmbH, Schwalbach
Serological Pipettes (5 ml, 10 ml, and 25 ml )	Sarstedt, Nümbrecht
ThinCerts™ Cell Culture Inserts (8 µm)	Greiner Bio-One, Frickenhausen
Tubes (1.5ml and 2ml)	Eppendorf, Hamburg

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### 2.2.3 Buffers

Description	Composition
Crystal violet (0.5%)	5 ml crystal violet (5%); 5 ml Methanol (100%); adjust to 50 ml aqua dest
Running buffer 10 ×	30.3 g (0.25 M) Tris Base; 144 g (1.92 M) Glycine; 10 g (1%) SDS; 1000 ml ddH <sub>2</sub> O; Dilute 1:10 with ddH <sub>2</sub> O; PH will be 8.3
Transfer buffer 10 ×	288 g glycine; 60.4 g Tris base; 200 ml Methanol; 1.8 L ddH <sub>2</sub> O
TBST 10 ×	400 g NaCl; 121 g TrisBase; Add aqua dest to a volume of 4 l (about 20 min); Adjust the pH to pH 7.6 (~100ml HCl (25%)); Add 50ml Tween; Add aqua dest to a final volume of 5 l

### 2.2.4 Chemicals and media

Description	Company
Accutase	PAA Laboratories GmbH, Cölbe
Amersham ECL Plus Western blotting Detection Reagents	GE Healthcare Europe GmbH, München
Amersham ECL Western blotting Detection Reagents	GE Healthcare Europe GmbH, München
Aqua for injection	B. Braun Melsungen AG, Melsungen
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH, München
Complete Mini Protease-Inhibitor Cocktail	Roche Diagnostic GmbH, Mannheim
Collagen I (rat tail)	Roche Diagnostics, GmbH, Mannheim
Crystal violet	Sigma-Aldrich Chemie GmbH, München
Curix 60 Developer G153 A and B	Agfa, Gera
Curix 60 Rapid Fixer G354	Agfa, Gera
DharmaFECT 1 siRNA Transfection Reagent	Thermo Fisher Scientific, Bonn



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D-Phosphate Buffered Saline (PBS)	Invitrogen, Karlsruhe
Fetal calf serum (FCS)	Sigma-Aldrich Chemie GmbH, München
Glutamine	Invitrogen, Karlsruhe
Glyceraldehydes	Sigma-Aldrich Chemie GmbH, München
Glycine	Carl Roch GmbH & Co. KG, Karlsruhe
Isopropanol	Otto Fischar GmbH, Saarbrücken
KCl	Merck KGaA, Darmstadt
lipopolysaccharide (LPS)	Sigma-Aldrich Chemie GmbH, München
Methanol	Carl Roch GmbH & Co. KG, Karlsruhe
mirVana™ miR-501-3p inhibitor	Applied Biosystems, Darmstadt
mirVana™ miR-501-3p mimic	Applied Biosystems, Darmstadt
mirVana™ miRNA inhibitor negative control	Applied Biosystems, Darmstadt
mirVana™ miRNA mimic negative control	Applied Biosystems, Darmstadt
NaCl	J.T. Baker, Niederlande
NaOH	Merck KGaA, Darmstadt
Nonfat dried milk power	AppliChem, Darmstadt
Opti MEM culture medium	Invitrogen, Karlsruhe
PhosSTOP Phosphatase Inhibitor Cocktail	Roche Diagnostic GmbH, Mannheim
Power SYBR Green Master Mix	Applied Biosystems, Foster City
Random Primer	New England Biolabs, Frankfurt am Main
RPMI, GlutaMAX™	Invitrogen, Karlsruhe
Spectra Multicolor Broad Range Protein Ladder	Fermentas GmbH; St. Leon-Rot
SYBR Green Master Mix	Qiagen, Hilden
Tris base	Merck KGaA, Darmstadt
Trypsin 10 ×	Sigma-Aldrich Chemie GmbH, München
Tween	Merck KGaA, Darmstadt

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### 2.2.5 Kits

<b>Kits</b>	<b>Company</b>
Dual luciferase® reporter assay kit	Promega, USA
Gene Expression Wash Buffer Kit	Agilent technologies Inc., USA
Human miRNA Microarray Kit version 14.0	Agilent technologies Inc., USA
MicroRNA Spike-In Kit	Agilent technologies Inc., USA
miRNA Complete Labeling and Hyb Kit	Agilent technologies Inc., USA
mirVana™ miRNA Isolation Kit	Ambion, USA
miScript Reverse Transcription Kit	Qiagen, Hilden
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, Bonn
RNA 6000 Nano Kit	Agilent technologies Inc., USA
RNA 6000 Pico Kit	Agilent technologies Inc., USA
RT-for-PCR kit	Clontech Laboratories, Inc., USA

## **2.2.6 Hardware and software**

### **Description**

ABI Prism 7700 SDS software (Applied Biosystems)

Adobe Acrobat Reader 4.0 (Adobe Systems Inc.)

Adobe Photoshop CS3 (Adobe Systems Inc.)

Agilent G4450AA Feature Extraction software 9.5 (Agilent technologies Inc.)

Agilent Gene Spring Software 11.0 (Agilent technologies Inc.)

Agilent GeneSpring software GX10.0 (Agilent technologies Inc.)

Agilent Scan Control software, version A. 7.0 (Agilent technologies Inc.)

Microsoft Internet Explorer 5.5

Microsoft Windows 2000 with SP2 (fully tested on SP4), Windows XP SP2

Pentium IV 1.5 GHz

SBC analysis system (ShanghaiBio Corp.)

SDS 2.1 software (Applied Biosystems)

SPSS windows 13.0 (SPSS Inc.)

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60 GB available disk space

1 GB RAM

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### 2.2.7 Antibodies

Antibody	Source	Dilution	Company	Cata No.
Anti-human Caspase-8	Mouse	1:1000	Cell Signaling, Frankfurt	9746
Anti human E-Cadherin	Mouse	1:2500	R&D systems, Wiesbaden	610181
Anti human VE-Cadherin	Rabbit	1:1000	Cell Signaling, Frankfurt	2158s
Anti human TRAIL R1	Rabbit	1:1000	Milipore GmbH, Schwalbach	16955
Anti human TRAIL R2	Rabbit	1:4000	ProSci Inc., Lörrach	2019
Anti $\beta$ -actin	mouse	1:50,000	Sigma-Aldrich Chemie GmbH, München	A5441
Anti mouse IgG	Goat	1:3000	Cell Signaling, Frankfurt	7076
Anti rabbit IgG	Goat	1:3000	Cell Signaling, Frankfurt	7074
Anti goat IgG	Ape	1:3000	Santa Cruz Biotechnology, Heidelberg	sc-2304

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## 2.3 Methods

### 2.3.1 RNA Isolation

Total RNA was extracted and purified using mirVana™ miRNA Isolation Kit following the manufacturer's instructions. Small pieces of tissues were quickly cut and weighed. Lysis/Binding Buffer (10 volumes of per tissue mass), miRNA Homogenate Additive (1/10 volume) and Acid-Phenol:Chloroform were added into the powdered tissue sample and mixed well. The mixture was centrifuged for 5 min at maximum speed at room temperature. Aqueous (upper) phase was then carefully transferred into a fresh tube, mixed with 1.25 volumes of 100% ethanol, transferred into a Filter Cartridge and centrifuged for 15 sec. miRNA Wash Solution 1 (700 µl) was added into the Filter Cartridge and centrifuged for 5-10 sec. After discarding the flow-through, miRNA wash Solution 2/3 (500 µl) was added and drawn through the Filter Cartridge twice. After discarding the flow-through from the last wash, the Filter Cartridge was transferred into a fresh collection tube. Pre-heated (95°C) nuclease-free water (100 µl) was used to recover the RNA.

### 2.3.2 MicroRNA Microarray

Microarray analysis was performed by SHANGHAI BIOTECHNOLOGY CORPERATION ([www.ebioservice.com](http://www.ebioservice.com)) using the Agilent Human miRNA Microarray Kit (8×15k) version 14.0 (design ID: 31945). miRNA molecular in total RNA was labeled by miRNA Complete Labeling and Hyb Kit. Each slide was hybridized with 100ng Cy3-labeled RNA using miRNA Complete Labeling and Hyb Kit in hybridization Oven at 55°C, 20rpm for 20 hours. After hybridization, slides were washed in staining dishes with Gene Expression Wash Buffer Kit. Slides were scanned by Agilent Microarray Scanner and Feature Extraction software. Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0. The data were analyzed by SBC analysis system (ShanghaiBio Corp.) ([http://www.ebioservice.com/show\\_product.asp?id=398](http://www.ebioservice.com/show_product.asp?id=398)).

### 2.3.3 Reverse Transcription and Real-time PCR

Reverse transcription and Real-time PCR was performed by Zhikun Liu (The First Affiliated Hospital, Zhejiang University, Hangzhou, China) using the Advantage RT-for-PCR kit in the 7700 HT real-time PCR System. All reactions were measured in triplicates in a final volume of 25  $\mu$ l: 12.5  $\mu$ l SYBR Green Mix (2  $\times$ ), 0.2  $\mu$ l cDNA, 1  $\mu$ l primer pair mix (5 pmol/ $\mu$ l per primer), and 11.3  $\mu$ l ddH<sub>2</sub>O. Cycling conditions were chosen according to the manufacturer's protocols. Briefly, real-time PCR started with the incubation of samples at 50°C for 2 min followed by 95°C for 10 min. Following this, quantitative PCR was repeated for 40 cycles with incubation at 95°C for 15 s followed by at 60°C for 1 min. The cycle number at which the real-time PCR reaction reached an arbitrarily determined threshold (CT) was recorded for both the miRNAs/RNAs and U6/GAPDH, and the relative amount of miRNA to U6 was described as  $2^{-\Delta\Delta CT}$  where  $\Delta CT_x = (CT_{miRNA_x} - CT_{U6_x})$  and  $\Delta\Delta CT_x = (\Delta CT_x - \Delta CT_{mean})$ .

### 2.3.4 Cell Lysate Extracts

The 6-well plate was put on ice and washed with ice-cold PBS. Ice-cold modified RIPA buffer (supplemented with Phosphatase inhibitor Cocktail tablet and Protease Inhibitor Cocktail tablet) was used for lysis. After 10 min incubation on ice and 20 min on -80°C, lysed cells were transferred into an eppendorf tube. The cell lysate was then lysed with ultrasound and centrifuged at 14,000  $\times$  g for 15 min. The supernatant was immediately transferred into a fresh centrifuge tube.

### 2.3.5 Pierce Assay

Protein concentration was tested using Pierce BCA Protein Assay Kit according to the standard protocol. Working reagent was prepared by mixing 50 parts of Pierce BCA (bicinchoninic acid) Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). From each protein sample, 20  $\mu$ l was pipetted triply into a 96-well plate. 200  $\mu$ l working reagent was then added into each well and incubated at 37°C for 30 min. The absorbance was measured at near 562 nm. The concentration was calculated using a standard curve.

### **2.3.6 Western Blot**

The extract (10 or 20 µg) was electrophoresed on Novex® Tris-Glycine gels at 125 V for 120 min and electroblotted onto PVDF-transfer membrane at 400 mA for 70 min. The blots were blocked with 5% nonfat milk or BSA in TBST washing buffer for 2 h at room temperature and then incubated at 4 °C overnight with primary antibodies. All antibodies were diluted according to the manufacturer's instructions. After washed at room temperature with washing buffer, the blots were labeled with secondary antibodies for 1 hour at room temperature. Then the blot was washed, incubated with ECL for 1 min, and exposed for 1-5 min in the film developer.

### **2.3.7 Crystal Violet Assay**

After removal of the old medium, cells were incubated with 50 µl of 0.5% crystal violet solution per well at room temperature on a shaker. After 20 min, cells were washed at least 5-6 times with 200 µl water per well and dried at room temperature over night. Then cells were incubated with 200 µl methanol per well for 20 min on the shaker at room temperature and measured at 590 nm.

### **2.3.8 SiRNA Transfection**

In separate tubes, the siRNA (Tube 1: 44 µl of 5 µM siRNA, 66 µl RNase-free water, 110 µl OptiMEM) and DharmaFECT1 transfection reagent (Tube 2: 3.85 µl of DharmaFECT 1, 216.15 µl OptiMEM) were diluted with OptiMEM. The contents of each tube were mixed gently by pipetting carefully up and down, and incubate for 5 min at room temperature. Contents of Tube 1 was then added into Tube 2, mixed and incubated for 20 min at room temperature. For 6-well plate, 400 µl of the transfection mix was added to each well. Cells were incubated at 37°C in 5% CO<sub>2</sub> for 72 hours.

### **2.3.9 Invasion Assay**

PDAC cells were harvested and resuspended in 0.5% FCS to a final concentration of 1 ×

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10<sup>5</sup>/ml. In a Collagen I (50µl at 0.4 mg/ml)-coated transwell chambers, 5 × 10<sup>4</sup> cells were put into upper chamber and 750 µl of 10 % FCS was put into lower chamber. After 24 hours culture at 37°C and 5 % CO<sub>2</sub>, cells that did not migrate through the pores and therefore remained on the upper chamber were gently removed with a cotton swab. Crystal Violet (0.1%) was used to stain the cells and acetic acid (10%) was used to solubilize. Cell numbers were quantified by measuring the intensity of the colored solution using a photometer at 590 nm. The intensity of the untreated transwell was set to 100% as control.

### 2.3.10 Dual Luciferase Activity Assay

Dual Luciferase Activity Assay was performed by Zhikun Liu (The First Affiliated Hospital, Zhejiang University, Hangzhou, China), and technically supported by SHANGHAI GENE PHARMA CORPORATION ([www.genepharma.com](http://www.genepharma.com)). Double-stranded oligonucleotides corresponding to the wild-type (WT-3'UTR) or mutant (MUT-3'UTR) miR-501-3p binding site in the *CDH5* 3'UTR were synthesized and ligated into the psiCHECK<sup>TM</sup>-2 Vector. HUVEC cells were cultured in 6-well plates and transfected with WT-3'UTR or MUT-3'UTR and cotransfected with miR-501-3p mimics/inhibitors or negative controls. Firefly and Renilla luciferase activities were measured by using dual luciferase reporter assay kit 48 h after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity.

### 2.3.11 Statistical analysis

Statistical analyses were performed using windows for SPSS 13.0 and SBC analysis system.  $P < 0.05$  was considered to be statistically significant. The Kolmogorov-Smirnov test was used to check for normality. Quantitative variables were expressed as mean ± SD or median. Categorical variables were presented as values and percentages. Paired-sample *t* test, Wilcoxon-Mann-Whitney test and chi-square test (Fisher's exact test) were used to compare quantitative and categorical variables, respectively. The Kaplan-Meier method and log-rank test were used for survival comparison. Cox regression analysis was used to determine the risk factors of tumor recurrence after curative surgical resection.

### 3. Results

#### 3.1 Differentially expressed miRNAs between pancreatic head and body/tail cancers

##### 3.1.1 Different miRNA profiles between pancreatic head and body/tail cancers

A miRNA microarray platform (Agilent Human miRNA Microarray Kit version 14.0) analysis covering a total of 887 human miRNAs was performed to compare miRNA profiles between 4 pairs strictly matched tumor tissues. They were strictly matched by gender, age ( $\pm 10$  years), CA 19-9 (-:  $< 37$  U/L / +:  $\geq 37$  U/L), TNM stage and histological differentiation. We found the majority of miRNAs, including 66 that have been reported to be associated with pancreatic cancer (Bloomston *et al.*, 2007; Steele *et al.*, 2011; Wang *et al.*, 2011) (Table 4), were not significantly differentially expressed between these two subtypes of pancreatic cancer. This result was not surprising because all the 4 paired tissues were early stage PDACs.

**Table 4** The regulation of pancreatic cancer associated miRNAs in pancreatic body/tail cancer compared with pancreatic head cancer

miRNA	P value	Fold change	Regulation		Expression	
			Body/tail	In PDAC	Head	Body/tail
hsa-miR-126*	0.010409	1.497677	up		5.083503	5.666229
hsa-miR-455-3p	0.015982	2.201055	up		5.655185	6.79338
hsa-miR-501-3p	0.019877	14.17093	down		2.385106	-1.43976
hsa-miR-320d	0.020129	1.211291	down		8.939015	8.66247
hsa-miR-320b	0.043105	1.154092	down	Up	8.651562	8.444804
hsa-miR-375	0.045640	5.612237	up	down	5.595712	8.084288
hsa-miR-146b-5p	0.074065	1.681926	up	up	8.300115	9.050231
hsa-miR-148a	0.076712	1.66357	up	down	8.791904	9.526186
hsa-miR-10b	0.114725	1.345653	up	up	7.734763	8.16307
hsa-miR-146b-3p	0.132901	1.114185	up	up	-3.17074	-3.01476
hsa-miR-190	0.132901	1.114185	up	up	-3.17074	-3.01476
hsa-miR-220a	0.132901	1.114185	up	up	-3.17074	-3.01476
hsa-miR-220b	0.132901	1.114185	up	up	-3.17074	-3.01476
hsa-miR-301b	0.132901	1.114185	up	up	-3.17074	-3.01476
hsa-miR-424	0.148204	1.727851	up	up	7.863314	8.652293

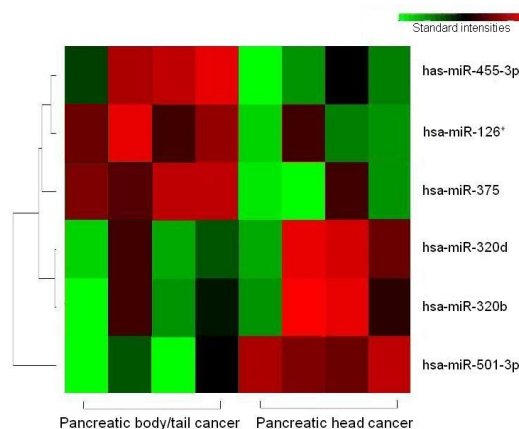


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hsa-miR-21	0.203332	1.126374	up	up	15.59998	15.77166
hsa-miR-142-5p	0.226626	1.823401	up	down	6.074466	6.941097
hsa-miR-155	0.231053	1.420632	up	up	7.461748	7.968281
hsa-miR-139-3p	0.238155	1.480683	up	down	3.542734	4.108997
hsa-miR-148b	0.240651	1.357972	up	down	6.37236	6.813814
hsa-let-7d	0.265628	1.124965	up	up	9.977742	10.14762
hsa-miR-142-3p	0.300062	1.459817	up	down	9.966391	10.51218
hsa-miR-186	0.315269	1.293223	up	up	6.044224	6.415195
hsa-miR-125a-5p	0.336444	1.539021	up	up	7.296905	7.918917
hsa-miR-345	0.336664	6.250811	down	down	-0.37071	-3.01476
hsa-miR-100	0.339273	1.266009	down	up	9.68679	9.346502
hsa-miR-212	0.345962	1.213576	up	up	3.896251	4.175515
hsa-miR-99a	0.346617	1.321411	down	up	9.234546	8.832466
hsa-miR-217	0.353879	4.632326	up	down	-3.17074	-0.95901
hsa-miR-139-5p	0.366812	5.930201	up	down	-1.13349	1.434589
hsa-miR-99b	0.377206	1.456922	up	up	6.921114	7.464037
hsa-miR-95	0.381705	4.517441	up	up	3.342103	5.517609
hsa-miR-92b	0.435211	3.030231	up	up	1.836418	3.435846
hsa-miR-301a	0.436055	1.886369	up	up	3.992251	4.907863
hsa-miR-199a-3p	0.457781	1.272802	up	up	11.92264	12.27065
hsa-miR-181a	0.463916	1.28108	up	up	9.070984	9.428344
hsa-miR-145	0.476132	1.298614	down	up	10.74572	10.36875
hsa-let-7i	0.485352	1.118322	up	up	11.6396	11.80094
hsa-miR-223	0.514289	1.628822	up	up	10.28607	10.9899
hsa-miR-15b	0.517299	1.1142	up	up	9.94014	10.09615
hsa-miR-205	0.529800	1.14686	down	up	-2.81707	-3.01476
hsa-miR-376a	0.531236	1.189124	up	up	6.985436	7.235335
hsa-miR-96	0.551678	1.743802	down	down	6.748685	5.946449
hsa-miR-125b	0.552814	1.101894	down	up	12.1143	11.97432
hsa-miR-10a	0.558727	1.316333	up	up	9.298406	9.69493
hsa-miR-181b	0.561575	1.305739	up	up	6.701516	7.086381
hsa-miR-196a	0.574712	3.273563	up	up	0.89446	2.605321
hsa-miR-92a	0.593379	1.139964	down	up	8.240348	8.051362
hsa-miR-194	0.595127	2.105559	down	up	8.641068	7.566865
hsa-miR-130b	0.642252	1.211653	up	down	6.15882	6.435797
hsa-miR-181d	0.683719	1.22001	down	up	4.655378	4.368486
hsa-miR-27a	0.691632	1.100396	up	up	11.82774	11.96576
hsa-miR-107	0.760746	1.069993	down	up	10.03742	9.939823
hsa-miR-196b	0.787791	2.056219	up	up	1.508813	2.548807
hsa-miR-200b	0.804175	1.330283	down	up	10.12447	9.712735
hsa-miR-199a-5p	0.824088	1.083828	up	up	10.63369	10.74983
hsa-miR-143	0.829075	1.089806	down	up	8.853937	8.729867
hsa-miR-429	0.856847	1.224759	down	up	7.325429	7.032932
hsa-miR-125a-3p	0.863100	1.096062	up	up	6.449531	6.58186

hsa-miR-146a	0.866126	1.108128	down	up	8.158847	8.010722
hsa-miR-23a	0.873887	1.037518	up	up	12.4273	12.48044
hsa-miR-16	0.874665	1.034553	down	up	11.48334	11.43433
hsa-miR-20a	0.887991	1.046063	down	up	9.144461	9.079493
hsa-miR-181c	0.907418	1.059798	up	up	5.025995	5.109785
hsa-miR-222	0.911535	1.06031	down	up	7.131722	7.047236
hsa-miR-24	0.912683	1.036835	up	up	11.71964	11.77182
hsa-miR-23b	0.936822	1.023272	up	up	11.14156	11.17475
hsa-miR-31	0.941169	1.230366	up	up	3.874156	4.173243
hsa-miR-103	0.964565	1.011592	down	up	10.36751	10.35088
hsa-miR-221	0.969799	1.015645	up	up	7.43665	7.459047
hsa-miR-210	0.977395	1.042275	up	up	6.265573	6.325307
hsa-miR-200c	0.994024	1.009893	down	up	9.257704	9.2435

Of note, we found 6 miRNAs that were significantly differentially expressed ( $P < 0.05$ ) (Figure 5). There were higher expressions of miR-126\*, miR-455-3p and miR-375, and lower expressions of miR-501-3p, miR-320d and miR-320b in pancreatic body/tail cancer compared with their matched pancreatic head cancer (Table 4). If we take fold change and Rank Sum difference into consideration, miR-501-3p was the only candidate for further study with  $P < 0.05$ , fold change  $> 2$  and largest Rank Sum difference. But we also take miR-375 into further verification procedures because it was reported to be a potent tumor suppressor in pancreatic cancer (Basu *et al.*, 2011).



**Figure 5.** A hierarchic cluster heat map showing the 6 differentially expressed miRNAs between pancreatic head cancer and pancreatic body/tail cancer samples. Three (miR-126\*, miR-455-3p and miR-375) were higher expressed, and another three (miR-320b, miR-320d and miR-501-3p) were lower expressed in pancreatic body/tail cancer.

### 3.1.2 Lower expression of miR-501-3p and higher expression of miR-375 in pancreatic body/tail cancer

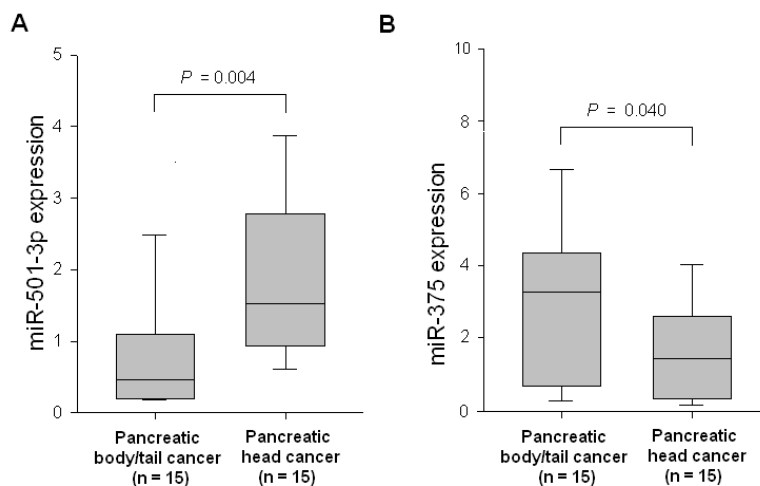
A total of 15 paired tissues were used for the subsequent quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) validations. Clinical and histopathologic variables for the patients included in this study were listed in [Table 5](#).

**Table 5** Tumor samples used for analysis

	Gender	Age (year)	CA 19-9 (ng/ml)	TNM staging	Histological differentiation
Paired 1	Female	68	23	T2N0M0 (I B)	Moderate-poor
	Female	62	38	T2N0M0 (I B)	Moderate-poor
Paired 2	Male	59	12000	T3N1M0 (II B)	Moderate-poor
	Male	59	3885	T2N1M0 (II B)	Moderate-poor
Paired 3	Male	62	2609	T3N1M0 (II B)	Moderate-poor
	Male	59	700	T3N1M0 (II B)	Moderate-poor
Paired 4	Male	61	986	T2N1M0 (II B)	Poor
	Male	66	11525	T2N1M0 (II B)	Poor
Paired 5	Female	61	995	T3N0M0 (II A)	Moderate
	Female	53	531	T3N0M0 (II A)	Moderate
Paired 6	Male	62	150	T3N0M0 (II A)	Moderate
	Male	55	534	T3N0M0 (II A)	Moderate
Paired 7	Male	55	12000	T3N0M0 (II A)	Moderate-poor
	Male	59	700	T3N0M0 (II A)	Moderate-poor
Paired 8	Male	63	894	T3N0M0 (II A)	Poor
	Male	70	961	T3N0M0 (II A)	Poor
Paired 9	Male	70	79	T3N0M0 (II A)	Moderate
	Male	75	271	T3N0M0 (II A)	Moderate
Paired 10	Female	75	572	T3N1M0 (II B)	Moderate
	Female	50	951	T2N1M0 (II B)	Moderate
Paired 11	Male	79	1142	T3N1M0 (II B)	Moderate-poor
	Male	81	240	T3N1M0 (II B)	Moderate-poor
Paired 12	Male	52	471	T3N1M0 (II B)	Moderate-poor
	Male	71	11246	T3N1M0 (II B)	Moderate-poor
Paired 13	Male	61	4	T3N0M0 (II A)	Moderate
	Male	38	17	T3N0M0 (II A)	Moderate
Paired 14	Female	38	54	T3N1M0 (II B)	Moderate
	Female	42	173	T2N1M0 (II B)	Moderate
Paired 15	Male	55	1598	T2N1M0 (II B)	Moderate
	Male	56	71	T3N1M0 (II B)	Moderate

We found the results were consistent with the one from miRNA microarray profiles that miR-501-3p expression was significantly decreased ( $P = 0.004$ , fold change = 2.99) whereas miR-375 expression was increased ( $P = 0.040$ , fold change = 1.88) in pancreatic body/tail cancer than those in pancreatic head cancer (Figure 6).

From the higher expression of miR-375, a tumor suppressor in pancreatic body/tail cancer, we hypothesized that pancreatic body/tail cancer might be 'less' malignant than pancreatic head cancer. However, the role of miR-501-3p in cancer is definitely unknown and should be further evaluated.

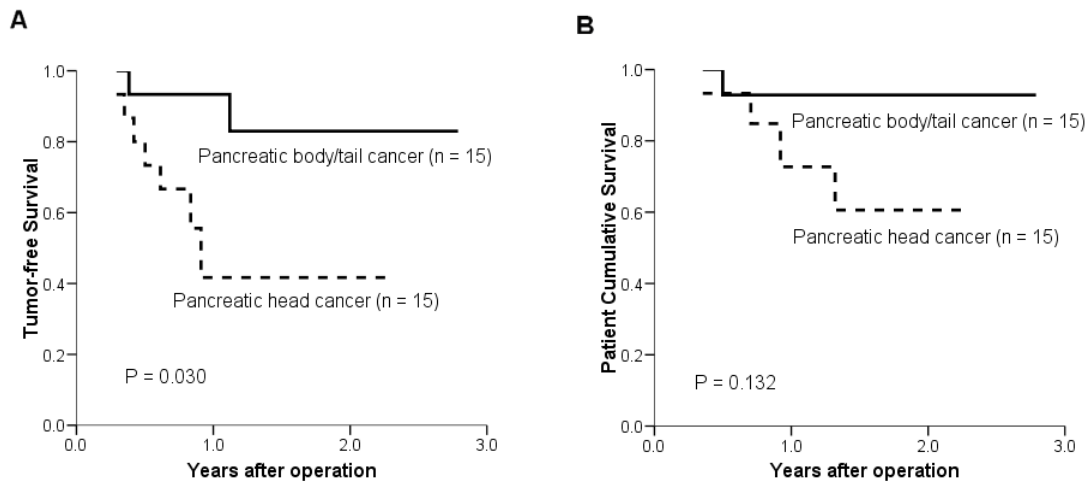


**Figure 6.** Box plot representing the expression of miRNAs. As assessed by real-time quantitative PCR, pancreatic body/tail cancer showed significantly lower expression of miR-501-3p (A) and higher expression of miR-375 (B).

### 3.1.3 Low expression of miR-501-3p contributes to low-risk of tumor recurrence

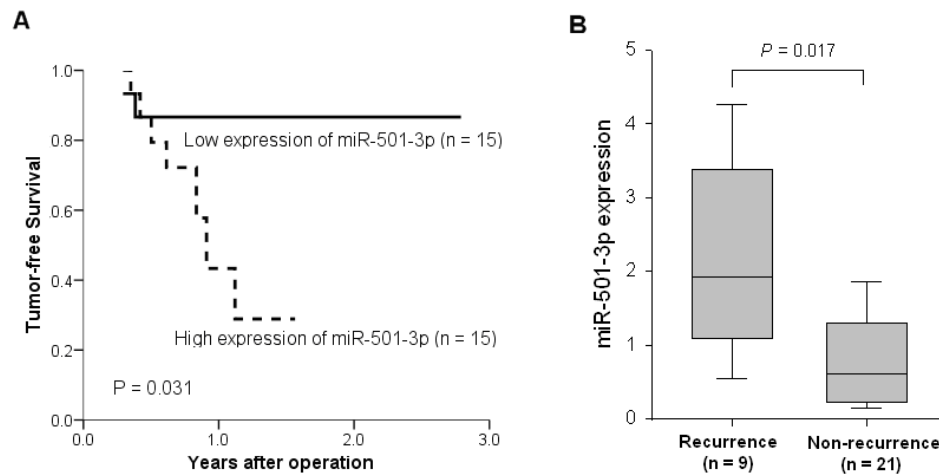
There were 9 out of 30 patients (30.0%) who were found to suffer from tumor recurrence during the follow up ( $2.3 \pm 0.8$  years) and the majority (8/9, 88.9%) occurred within 1 year after operation. The recurrence rate was significantly lower in pancreatic body/tail cancer than that in pancreatic head cancer (13.3% vs. 46.7%,  $P = 0.046$ ). Furthermore, from the Kaplan-Meier survival curves, we could find that both patient cumulative survival and

tumor-free survival were higher in pancreatic body/tail cancer than pancreatic head cancer (Figure 7).



**Figure 7.** Prognosis in patients with pancreatic body/tail cancer and those with pancreatic head cancer. The Kaplan-Meier curves for comparison of tumor-free survival (A) and patient cumulative survival (B) between patients with pancreatic head cancer and those with pancreatic body/tail cancer. Pancreatic body/tail cancer showed significantly higher tumor-free survival than its matched pancreatic head cancer ( $P = 0.030$ ).

To determine the role of miRNAs in tumor recurrence, we then carried out COX regression analysis and found that high expression of miR-501-3p ( $P = 0.034$ ; Risk Ratio = 2.322, 95%CI: 1.068-5.048) but not miR-375 was significantly associated with tumor recurrence after surgery. Patients with high expression of miR-501-3p showed a significantly lower tumor-free survival than those with low expression of miR-501-3p ( $P = 0.031$ , Figure 8A). The cutoff value was selected using receiver operating characteristic curve. In addition, we compared the miR-501-3p expression between patients with and without tumor recurrence and found a significantly higher expression of miR-501-3p in those with tumor recurrence ( $P = 0.017$ , Figure 8B). The data indicated that lower expression of miR-501-3p in pancreatic body/tail cancer might contribute to lower risk of tumor recurrence.



**Figure 8.** Low expression of miR-501-3p contributes to low-risk of tumor recurrence in all 30 patients with PDAC. The Kaplan-Meier curves for comparison between patient with high expression of miR-501-3p and those with low expression of miR-501-3p (A); Box plot represented that patients with recurrence after operation showed significantly higher expression of miR-501-3p (B).

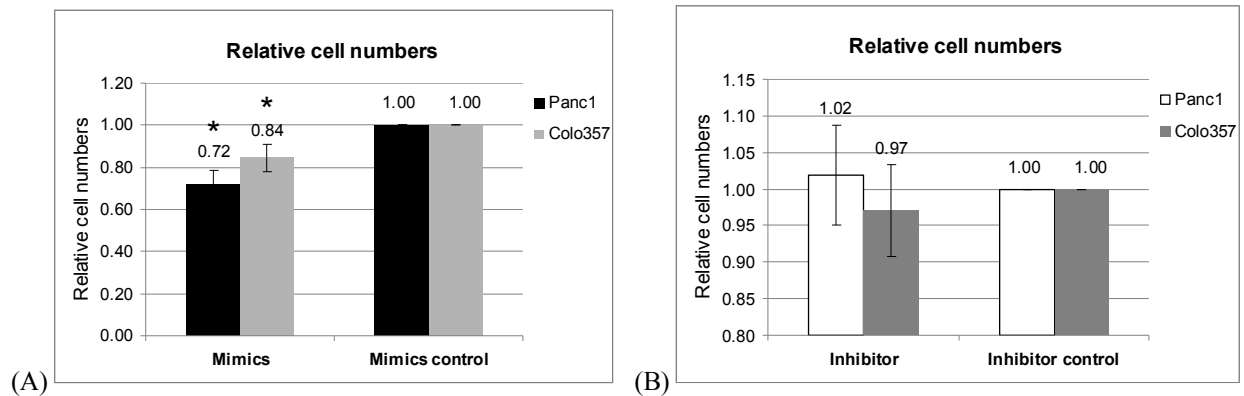
### 3.2 *In vitro* studies: miR-501-3p leads to morphological changes of pancreatic cancer cells

To confirm our hypothesis, we performed an *in vitro* study to evaluate the role of miR-501-3p in PDAC cells. Two frequently used pancreatic cancer cell lines, Panc-1 and Colo357, were transfected with miR-501-3p Mimics and Inhibitors. Since miRNA Mimics and miRNA Inhibitors have different chemical structures, miRNA Mimics are modified double-stranded RNAs while miRNA Inhibitors are modified single-stranded RNA molecules, we will present the results from Mimics and Inhibitors separately. Cell proliferation, viability, invasion, chemo-resistance and TRAIL-sensitivity were compared between Mimic group and its control, and between Inhibitor and its control, respectively. Each experiment was repeated 3 times under the same conditions.

#### 3.2.1 miR-501-3p and proliferation

PDAC cells were transfected with miR-501-3p Mimics, Inhibitors and their controls. After

transfection for 72 hours, cells were trypsinized from 6-well plates and counted. We found significantly fewer cells in the Mimics group than its negative control ( $P = 0.002$  for Panc-1,  $P = 0.013$  for Colo357) but no significant difference was found between Inhibitor group and its negative controls (Figure 9). The result indicates that miR-501-3p may reduce proliferation of PDAC cells.

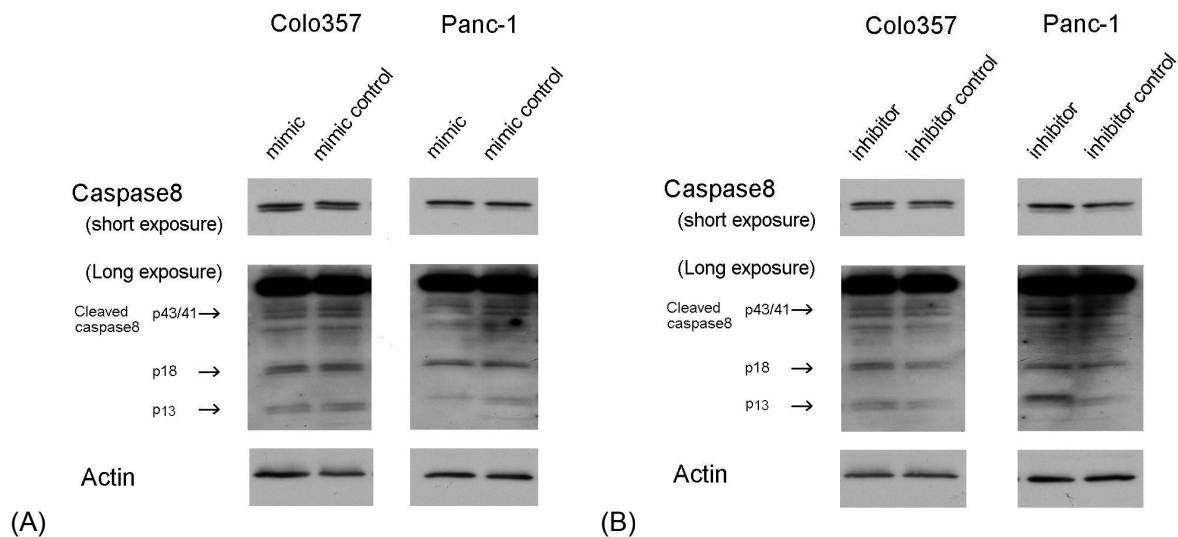


**Figure 9.** Relative cell numbers in the PDAC cells transfected with (A) miR-501-3p Mimics and its negative control, (B) miR-501-3p Inhibitors and its negative control. Cell numbers were normalized to the negative control groups. \*:  $P < 0.05$ . PDAC cells were seeded in the 6-well plates  $1.7 \times 10^5$ /well for Panc-1 and  $2.0 \times 10^5$ /well for Colo357 respectively, and counted 72 hours after transfection. The experiment was repeated 4 times under the same conditions. The figures showed the mean value (bar) and standard deviation (error line).

### 3.2.2 miR-501-3p and cell death

Deregulation of proliferation, together with a reduction of cell death, is necessary for tumor development, progression, and therapy-resistance. From the microscopical observation, we suspected that miR-501-3p may lead to reduced proliferation but also decreased cell death. Therefore, we performed flow cytometry (FCM) to assess the apoptosis and death rates in PDAC cells. Because we did this experiment in Hangzhou, China, using different source of Panc-1 cells compared with the one in Kiel, the results are provided as supplemental data. We found a trend that more apoptotic cells in the Inhibitor group than its negative control (supplemental Figure 1).

In addition, because caspase-8 is an initiator caspase in the extrinsic apoptotic pathway, we tested the caspase-8 protein levels in PDAC cells transfected with Mimics, Inhibitors, or their controls. We found that the cleaved caspase-8 (particularly p18 and p13) was weaker in Mimics group than its negative control, but much stronger in Inhibitors group than its negative control, particularly in Panc-1 cells (Figure 10).



**Figure 10.** The expression of caspase8 protein in Colo357 and Panc-1 cells transfected with (A) miR-501-3p Mimics and its negative control, (B) miR-501-3p Inhibitors and its negative control. Cell lysate was obtained 72 hours after transfection. The arrows indicate the cleaved caspase-8 (p43/41: 43/41 kDa, p18: 18 kDa and p13: 13 kDa).

### 3.2.3 miR-501-3p and invasion

Cell invasion is a crucial step in metastasis and the ability of cancer cells to undergo invasion allow them to enter lymphatic and blood vessels for dissemination (Chambers *et al.*, 2002). Tumor cell motility is an essential step in metastasis and a hallmark of invasion, and ECM adhesion is a key mediator in cell motility and in the process of metastasis (Le Devedec *et al.*, 2010; Ridley *et al.*, 2003). In the first part of our study, we found that miR-501-3p might be associated with metastasis/recurrence of pancreatic cancer. Therefore, evaluating the effect of miR-501-3p on invasion ability *in vitro* could be a strong evidence for proving the clues for

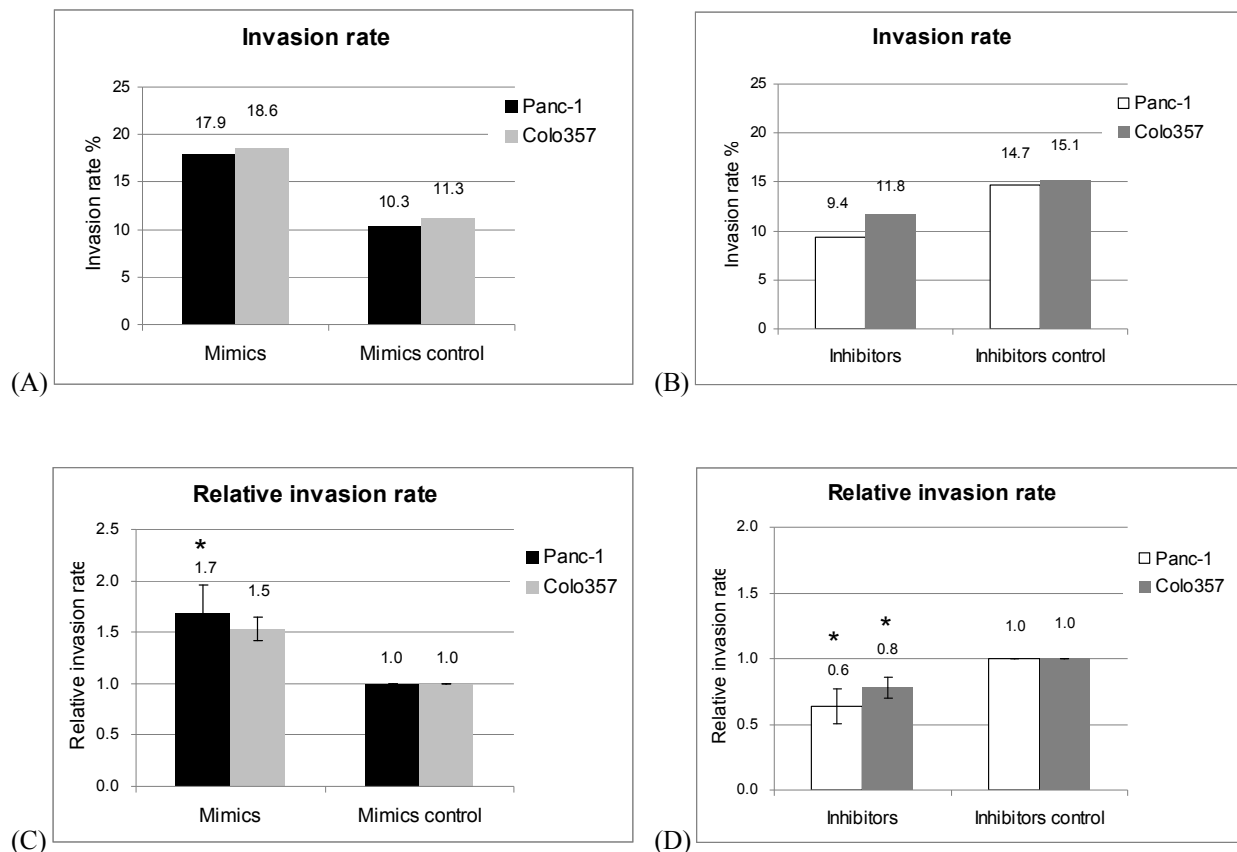


clinical findings.

PDAC is characterized by a pronounced fibrotic reaction composed primarily of type I collagen. PDAC cells have been shown to respond to collagen I by becoming more motile and invasive although collagen I functions as a barrier to invasion (Shields *et al.*, 2011). Here, we used a type I collagen coated transwell chamber for testing the invasion ability of PDAC cells.

After transfection for 72 hours,  $5.0 \times 10^4$  cells/well PDAC cells in reduced serum medium (0.5%) were seeded into the upper chamber. After incubation for 24 hours, the invasion ability was assessed by crystal violet assay. We found significantly higher invasion rate in the Mimics group ( $P = 0.017$  for Panc1,  $P = 0.068$  for Colo357) and less invasion rate in the Inhibitors group ( $P = 0.003$  for Panc1,  $P = 0.001$  for Colo357) compared with their respective negative controls, which indicates that miR-501-3p may promote invasion in PDAC cells (Figure 11).

This result was consistent with the clinical finding, which suggests that pancreatic body/tail cancer is associated with a reduced miR-501-3p expression and consequently a less invasion ability compared with pancreatic head cancer. The possible mechanism might be explained by the decreased E-Cadherin levels in the Mimics group and increased E-Cadherin levels in the Inhibitors group, which is shown in the following part (3.3).

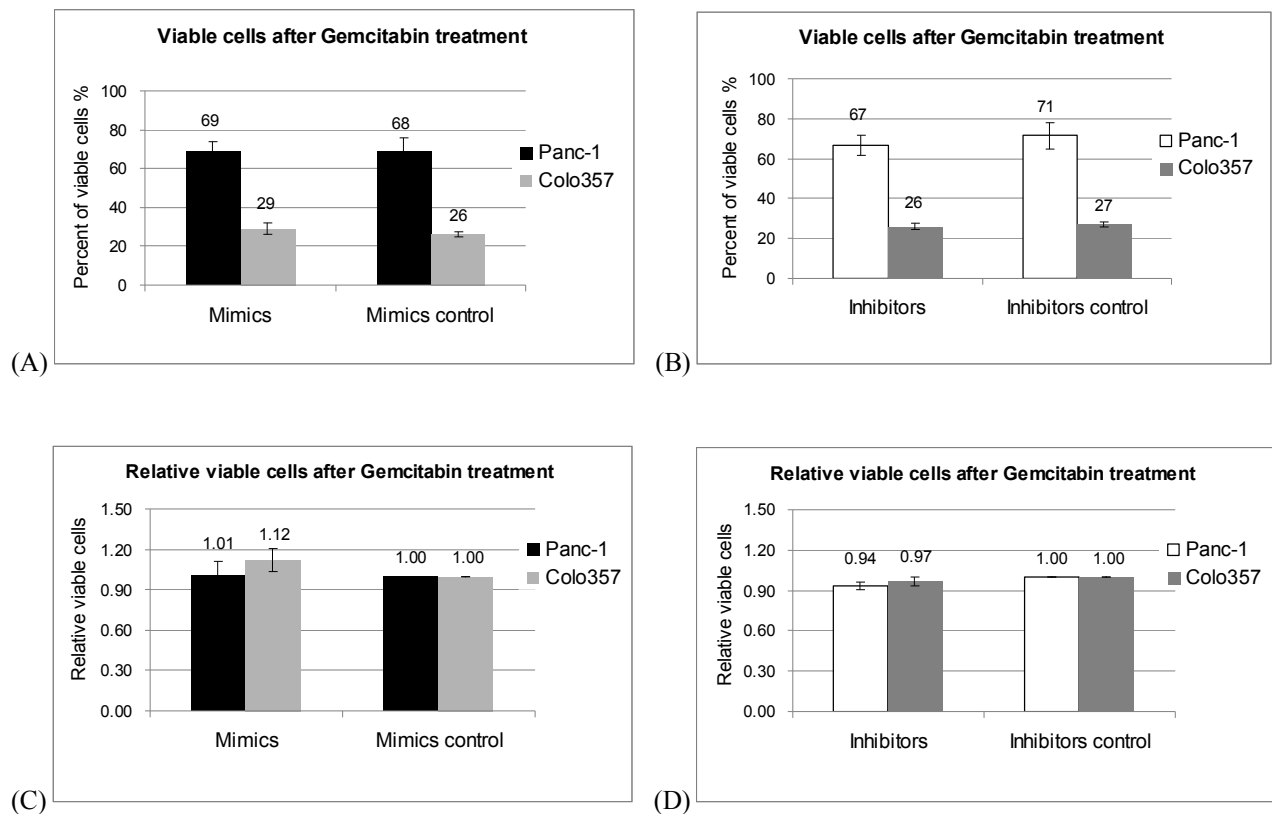


**Figure 11.** Invasion ability in the PDAC cells transfected with miR-501-3p Mimics, Inhibitors and their negative controls. PDAC cells were seeded in the Collagen I-coated transwell chambers as  $5.0 \times 10^4$ /well, and incubated for 24 hours. (A) and (B) showed the median value (bar) of invasion rate (because of non-normal distribution) between Mimics and its negative control, and Inhibitors and its negative control; (C) and (D) showed the mean value (bar) and standard deviation (error line) of relative invasion rate (normalized to the control groups) between Mimics and its negative control, and Inhibitor and its negative control. \*:  $P < 0.05$ . Statistical analysis was performed only in (C) and (D).

### 3.2.4 miR-501-3p and chemo-resistance

Chemo and/or radiotherapy remain the first choice of palliative treatment for PDAC, and promote cancer cell death primarily by the induction of apoptosis. Gemcitabine is now widely used as the first line chemotherapy for the end stage pancreatic cancer. To better evaluate the role of miR-501-3p in the chemo-induced apoptosis, we tested the cell viability in both chemo-resistant cell line (Panc-1) and chemo-sensitive cell line (Colo357).

PDAC cells were transfected with miR-501-3p Mimics, Inhibitors, or their negative controls for 72 hours and then seeded into the 96-well plates. Gemcitabine (1 ug/ml) was given in the treatment group when the cells had settled down. After 48 hours, the viable cells were measured by crystal violet assay (Figure 12). We found more viable cells in Panc-1 than that in Colo357, which proved again that Panc-1 were Gemcitabine-resistant cell line while Colo357 was highly sensitive to Gemcitabine treatment. However, no significant difference was found between Mimics and their control or between Inhibitors and their control in both cell lines. It seems that miR-501-3p is not associated with the chemo-resistance neither in high nor low chemo-sensitive cell lines.

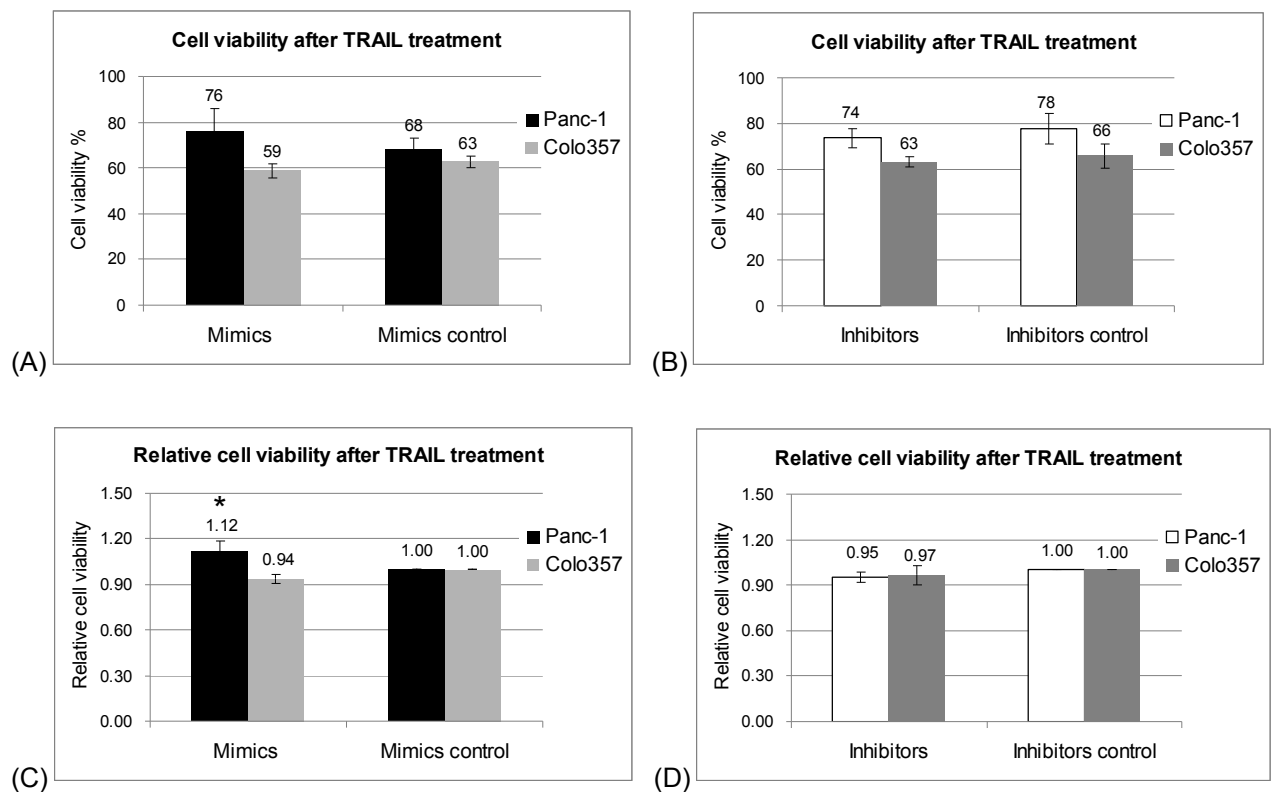


**Figure 12.** Viable cells in Panc-1 and Colo357 transfected with miR-501-3p Mimics, Inhibitors and their negative controls. PDAC cells were seeded in the 96-well plate as  $1.0 \times 10^4$ /well at 72 hours after transfection, and incubated for 48 hours after treatment of Gemcitabine (1 ug/ml). (A) and (B): the percent of viable cells. (C) and (D): relative viable cells. The cell viability was normalized to the control groups. The experiment was repeated 3 times under the same conditions. The figures showed the mean value (bar) and standard deviation (error line).

### 3.2.5 miR-501-3p and TRAIL-induced apoptosis

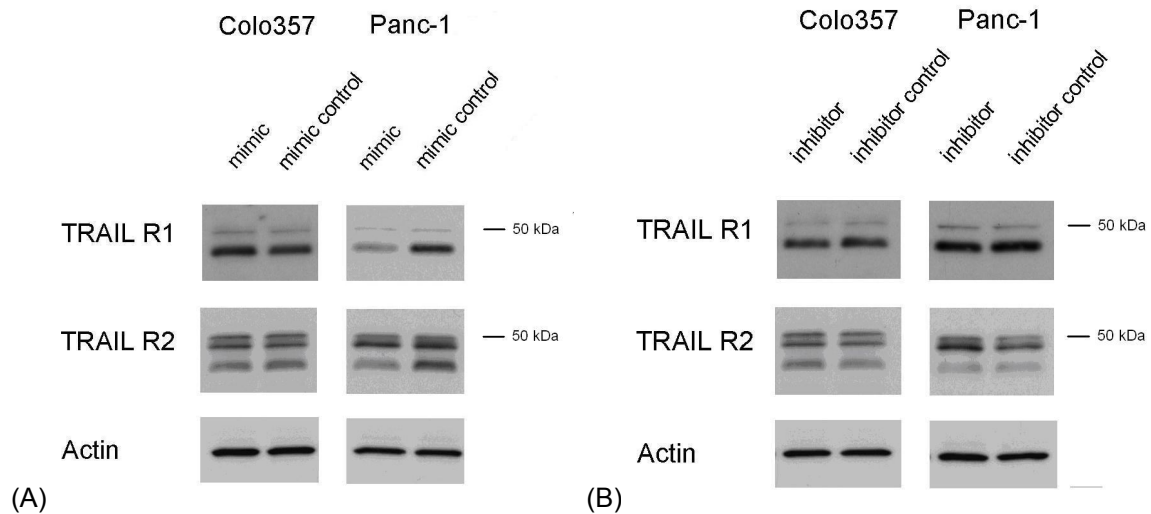
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) superfamily, selectively induces apoptosis in a variety of human cancer cell lines, with no toxicity against normal tissues (Almasan *et al.*, 2003). TRAIL induces programmed death in various cancer cells through its interaction with the death receptors, TRAIL-receptor 1 (TRAIL-R1 or DR4) and/or TRAIL-receptor 2 (TRAIL-R2 or DR5), which contain the intracellular death domain (DD) that is essential for the apoptosis signal. In this study, we evaluate the effect of miR-501-3p on the TRAIL-induced death and test the expression of TRAIL-R1 and TRAIL-R2 after regulation of miR-501-3p levels.

After transfection for 72 hours,  $1.0 \times 10^4$  cells/well PDAC cells were seeded into 96-well plates. TRAIL (100 ng/ml) was added into the treatment group 24 hours after cell seeding. The cell viability was tested using crystal violet assay after additional 24 hours. We found no significant difference between Mimic group and its control, or Inhibitor group and its control in Colo357 cells. However, in Panc-1 cells, Mimic group showed higher viability than its control ( $P = 0.027$ ) (Figure 13).



**Figure 13.** Cell viability in the PDAC cells treated with TRAIL. (A) and (B): cell viability between Mimics and its negative control, and Inhibitor and its negative control. (C) and (D): relative cell viability (normalized to the control groups) between Mimics and its negative control, and Inhibitors and its negative control. \*:  $P < 0.05$ . Statistical analysis was performed only in (C) and (D). PDAC cells were seeded in the 96-well plate as  $1.0 \times 10^4$ /well at 72 hours after transfection, and incubated for 24 hours after treatment of TRAIL (100 ng/ml). The experiment was repeated 3 times under the same conditions. The figures showed the mean value (bar) and standard deviation (error line).

To further study the effect of miR-501-3p on TRAIL signaling pathway, we also tested the TRAIL-R1 and TRAIL-R2 protein levels in different groups, since the decreased expression of death receptors (TRAIL-R1 and TRAIL-R2) in cancer cells is involved in TRAIL resistance (Thorburn *et al.*, 2008). Interestingly, we found decreased expression of TRAIL-R1 and TRAIL-R2 in the Mimics group compared with its negative control in Panc-1 cells but not in Colo357 cells (Figure 14). This result was consistent with the TRAIL treatment experiment and indicated that decreased TRAIL-sensitivity after up-regulation of miR-501-3p may due to the decreased TRAIL-R1 and TRAIL-R2 levels in Panc-1 cells.



**Figure 14.** The expression of TRAIL-R1 and TRAIL-R2 protein in Colo357 and Panc-1 cells transfected with (A) miR-501-3p Mimics and its negative control, (B) miR-501-3p Inhibitors and its negative control. Cell lysates were obtained 72 hours after transfection. The experiment was repeated 3 times and the representative one was shown.

### 3.3 miR-501-3p regulates cell adhesion molecules

#### 3.3.1 miRNA target search

The target genes for miR-501-3p have not been reported so far. Therefore, Open-source software using different algorithms based on sequence complementarity, such as miRBase (<http://www.mirbase.org/>), TargetScan (<http://www.targetscan.org>), PicTar (<http://pictar.mdc-berlin.de/>), DIANA-microT (<http://diana.cslab.ece.ntua.gr/microT/>) and miRanda ([www.microrna.org](http://www.microrna.org)), were used for miRNA target predictions.

MiR-501-3p has not been enrolled in PicTar database. We found 223 conserved targets from TargetScan (TargetScanHuman 6.0), 113 targets from DIANA-microT (Diana microT- v4) with a threshold of 0.5 for high precision, 1676 targets for miRanda with “good” mirSVR score ( $<-0.1$ ) and seed regions. A total of 28 target genes (*AFF4*, *ARID5B*, *CDH5*, *CIT*, *CPEB2*,

*CSDE1*, *DCUN1D5*, *GAN*, *JHDM1D*, *KPNA4*, *NONO*, *NR1D2*, *ODZ2*, *PCDH9*, *PLD3*, *PPP2R5E*, *PPP3CA*, *PPP3CC*, *PTBP2*, *RCC2*, *RNF144A*, *STIM2*, *TARDBP*, *TRERF1*, *UBE2E2*, *ZFHX4*, *ZIC4*, *ZMYM4* and *ZRANB2*) were overlapped among all the 3 databases. Of the 28 genes, *CDH5* (Le Guelte *et al.*, 2011), *JHDM1D* (Osawa *et al.*, 2011), *PCDH9* (Wang *et al.*, 2012), *PPP3CA* (Singh *et al.*, 2008), *PPP3CC* (Hornstein *et al.*, 2008), *PTBP2* (Cheung *et al.*, 2009), *RNF144A* (Marzook *et al.*, 2012) and *STIM2* (Aytes *et al.*, 2012) were reported to be associated with cancer (Table 6).

**Table 6** Potential target gene information \*

Target gene	Product	Function in cancer	Tissue/cell lines	Reports No. **
<i>CDH5</i>	cadherin 5, type 2 (vascular endothelium)	Tumor-associated angiogenesis (oncogene), keep vascular endothelial barrier, reduce permeability (TSG)	Various cell lines	11
<i>JHDM1D</i>	jumonji C domain containing histone demethylase 1 homolog D	suppress angiogenesis (TSG)	Human cervical carcinoma (HeLa), hepatocellular carcinoma (HepG2), epidermoid carcinoma (A431), glioblastoma (T98G)	1
<i>PCDH9</i>	protocadherin 9 protein	Improve prognosis (TSG)	Glioma	3
<i>PPP3CA</i>	phosphatase 3, catalytic subunit, alpha isozyme	Apoptosis (TSG)	Prostate cancer cells	4
<i>PPP3CC</i>	protein phosphatase 3, catalytic subunit, gamma isozyme	reduce recurrence (TSG)	Prostate cancer tissue and cells	2
<i>PTBP2</i>	polypyrimidine tract binding protein 2	Promote proliferation and migration (oncogene)	Human glioma cell lines U251 and LN229	4
<i>RNF144A</i>	ring finger protein 144A	Inhibit migration and invasion (TSG)	Human breast cancer cells (MCF-7), cervical carcinoma cells (HeLa)	1
<i>STIM2</i>	stromal interaction molecule 2	reduce proliferation (TSG)	colon cancer cell lines, breast cancer	11

\*: The genes were listed according to the prediction score.

\*\* : Number of reports was calculated according to the results from PubMed by searching the keywords

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of 'gene name' and 'cancer'.

.TSG: tumor suppressor gene.

### 3.3.2 Differentially expressed E- and VE-Cadherin in various PDAC cell lines

Cadherins are a class of type I transmembrane proteins. They play important roles in cell adhesion, ensuring that cells can bind together in tissues. Because three of the cadherins (cadherin 5, protocadherin 9 and protocadherin 17) have been listed as the potential direct targets of miR-501-3p in TargetScan, DIANA-microT and miRanda databases, we paid great attention and interest in the association between miR-501-3p and cadherins.

E-Cadherin is the most well studied member of the cadherin family. The most important event of EMT is loss of E-Cadherin, which demonstrates a prerequisite for epithelial tumor cell invasion (Guarino *et al.*, 2007). VE-Cadherin (CD144) is traditionally considered a strictly endothelial specific adhesion molecule located at junctions between endothelial cells. Although VE-Cadherin has recently been found in some non-endothelial cells such as breast cancer cells (Labelle *et al.*, 2008), it is still unknown whether VE-Cadherin is expressed in PDAC cells.

We test the expression of E- and VE-Cadherin in several frequently used PDAC cell lines, including Capan-1, Capan-2, Panc-1, PancII-1, Panc89, BxPc-3 and Colo357. Among these seven cell lines, only two (Colo357 and Panc89) expressed VE-Cadherin protein. While the levels of VE-Cadherin were very low in Panc89, only Colo357 showed high levels of VE-Cadherin protein (Figure 15). According to the ultrastructural grading system (Sipos *et al.*, 2003), both Colo357 and Panc89 are grade 2 cell lines. Neither the less malignant grade 1 cell lines (Capan-1 and Capan-2) nor the more malignant grade 3 cell lines (Panc-1, PancTu-I) express VE-Cadherin. The role of VE-Cadherin in the PDAC carcinogenesis and tumor progression should be further studied.

Compared with VE-Cadherin, all studied PDAC cell lines expressed E-Cadherin. Only Panc-1



showed very weak expression but all other cells presented very high E-Cadherin protein levels (Figure 15). E-Cadherin is a marker for EMT and can function as a parameter of 'malignancy'. According to the ultrastructural grading system (Sipos *et al.*, 2003), higher grade of PDAC cells were associated lower expression of E-Cadherin (Figure 15). Taken together, Colo357 (highly expresses VE-Cadherin) and Panc-1 (lowly expresses E-Cadherin) are two special cell lines compared with other PDAC cell lines. Thus, we used these two cell lines as a model for the further study.

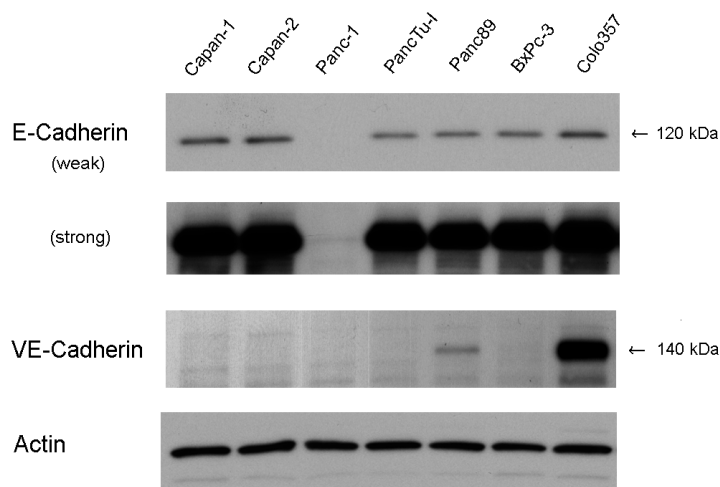
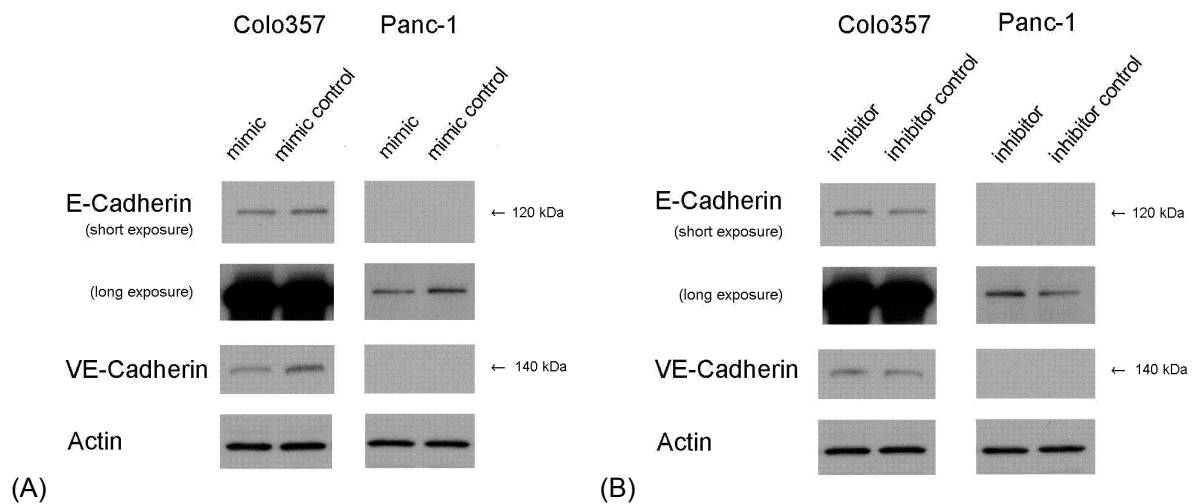


Figure 15. The expression of E- and VE-Cadherin protein in several PDAC cell lines.

### 3.3.3 miR-501-3p regulates E- and VE-Cadherin expression

To verify the possible relation between miR-501-3p and E-Cadherin and VE-Cadherin respectively in pancreatic cancer, we tested the protein levels after regulation of miR-501-3p in Colo357 and Panc-1 cells. We found reduced E-Cadherin protein expression in the Mimics group and increased protein expression in the Inhibitors group compared with their respective negative controls in both cell lines (Figure 16). In Colo357, the only high expressing VE-Cadherin cells, both E- and VE-Cadherin expressions decreased after transfection with miR-501-3p Mimics but increased with miR-501-3p Inhibitors compared to the negative controls (Figure 16). This suggests that miR-501-3p regulates E- and VE-Cadherin

expression. Because there is a direct role for E-Cadherin in the suppression of tumor invasion (Wong *et al.*, 2003), the decreased E-Cadherin in the Mimics group might explain the increased invasion ability in both Colo357 and Panc-1 cells.



**Figure 16.** The expression of E- and VE-Cadherin protein in Colo357 and Panc-1 cells transfected with (A) miR-501-3p Mimics and its negative control, (B) miR-501-3p Inhibitors and its negative control. Cell lysate was obtained 72 hours after transfection.

### 3.3.4 Target VE-Cadherin verification

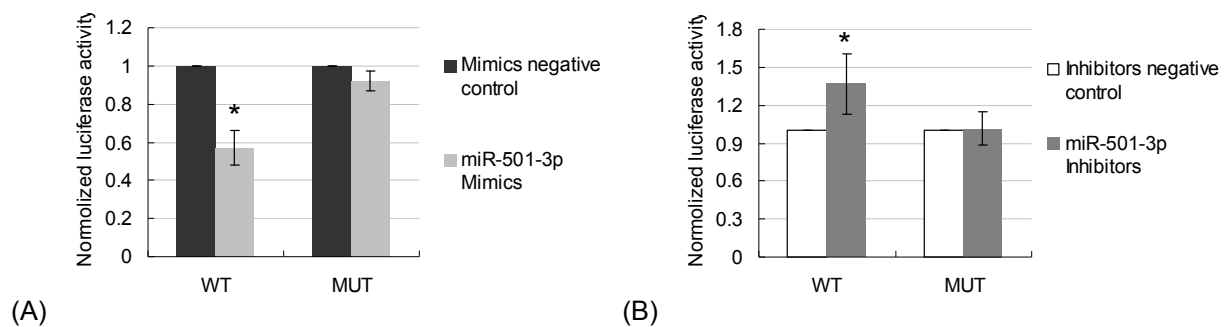
*CDH5* is the leading predicted target genes of miR-501-3p. Its encoded protein VE-Cadherin is well known to be related to tumor invasion and metastasis (Le Guelte *et al.*, 2011) and from the previous Western Blot analysis we have found that miR-501-3p might target VE-Cadherin. Therefore, *CDH5* was chosen for the further verification (Table 7).

We cloned the 3'-UTR region of *CDH5* (WT-3'-UTR) or its mutated (MUT-3'-UTR) downstream of the firefly luciferase reporter gene, and then co-transfected with miR-501-3p Mimics/Inhibitors or its negative control into HUVEC cells (source: The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China). The luciferase activity of the WT construct of *CDH5* 3'-UTR was significantly reduced/increased in the presence/absence of miR-501-3p compared with the negative control ( $P < 0.05$ ). However, such an effect was not

observed in the MUT construct of *CDH5* 3'-UTR (Figure 17). These data suggest a direct and specific interaction of miR-501-3p on *CDH5* 3'UTR.

**Table 7** Targetscanhuman 6.0 for the information of target gene

Gene symbol	Seed match	3'UTR position	Context +score	Conserved branch length	predicted consequential pairing of target region and miRNA
<i>CDH5</i>	7mer-m8	1554-1560	-0.22	2.179	(3' UTR) 5' ...UAU <u>AUUGGCCAAACUGGUGCAUG</u> ... (miRNA) 3' UCUUAGGAACGGG <b>CCACGUAA</b>



**Figure 17.** *CDH5* is a miR-501-3p target gene. (A): The luciferase activity of the WT construct of *CDH5* 3'-UTR was significantly reduced in the miR-501-3p mimics than its negative control. (B): The luciferase activity of the WT construct of *CDH5* 3'-UTR was significantly increased in the miR-501-3p inhibitors compared to its negative control. \*:  $P < 0.05$ . Firefly luciferase activities were normalized to Renilla luciferase activities. Data were normalized to the negative control groups. The experiment was repeated 3 times under the same conditions. The figures showed the mean value (bar) and standard deviation (error line). Cell source: The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

## 4. Discussion

### 4.1 Pancreatic body/tail cancer is associated with better prognosis

The survival rate of pancreatic cancer (i.e. PDAC) is the 'worst' of all major cancers (e.g. lung cancer, liver cancer, gastric cancer, colon cancer, breast cancer, prostate cancer) and mostly depends on the tumor stage. Stage I-II is commonly considered as the stage during which the surgical treatment is most effective. Although surgery has been continuously improved, patients with early stage pancreatic cancer still have a high recurrence rate after curative resection, as was shown by this study that one third of all patients suffered from tumor recurrence during the follow up.

Besides tumor stage, the prognosis of pancreatic cancer is also associated with the tumor site. The theory of different mechanisms in carcinogenesis of tumors at different locations and the importance of subsite division have been supported by several pioneering studies. In colon cancer, a number of studies have demonstrated that right- and left-sided tumors exhibit different genetic, biological and demographical characteristics and risk factors, suggesting that the carcinogenesis and tumor progression of colon cancer may differ with tumor localization (Slattery *et al.*, 2011). In pancreatic serous cystic neoplasm, tumor location in the head of pancreas was independently associated with local invasiveness (Khashab *et al.*, 2011).

For PDAC, pancreatic body/tail cancer has been long-term considered as a 'more' malignant subtype compared with pancreatic head cancer. Clinically, we see patients with pancreatic body/tail cancer more likely to be at advanced stage. The symptoms, such as jaundice and nausea, which could be a signal for early diagnosis, would rarely happen in patients with early stage pancreatic body/tail cancer. Consequently, pancreatic body/tail cancer is commonly associated with a dismal prognosis. The overall survival is much lower in pancreatic body/tail cancer than that in pancreatic head cancer.

However, recently, a large sample study from SEER registries of United States including information from 43,946 patients (Lau *et al.*, 2010) revealed that pancreatic body/tail cancer had a significant higher survival than pancreatic head cancer if both tumors were at the same local-stage. Interestingly, we could find that the old database of United States (1985-1995) also presented a higher survival rate for local-stage pancreatic tail cancer compared with local-stage pancreatic head cancer (Sener *et al.*, 1999). Therefore, we started to hypothesize whether the pancreatic body/tail cancer is 'more' malignant than pancreatic head cancer in nature. However, the data from both clinical and genetical comparison between the pancreatic head cancer and body/tail cancer is rather limited. Given the complex and heterogeneous nature of PDAC, it is reasonable to hypothesize that these two subtypes may be separated from each other and differ not only in phenotype but also in genotype. Furthermore, we suppose that the two subtypes of PDAC may be differing in their malignant characteristics.

This study performed the first comparison between pancreatic body/tail and head cancers using strictly matched early stage cancers. We found that patients with early stage pancreatic body/tail cancers presented higher survival rate than those with paired pancreatic head cancers. This finding was consistent with the previous studies mentioned before. Furthermore, we found patients with early stage pancreatic body/tail cancers had a lower tumor recurrence rate after curative resection.

Tumor recurrence is defined as the return of tumor after radical treatment and after a period of time during which the tumor cannot be detected. There are three types of tumor recurrence: local recurrence (the same tumor occurs where it started), regional recurrence (the same tumor occurs in the lymph nodes near the site of origin), and distant recurrence that is also called metastatic recurrence (the same tumor occurs somewhere else in the body, distant from where it started). The detailed molecular mechanism of tumor recurrence is still unclear. The well-known risk factors are advanced tumor stage, poor histological differentiation and highly malignant tumor subtype. In addition, surgical procedure and surgeon have great impact on the prognosis. For instance, the better observance of tumor-free principle, regional lymph node dissection and appropriate surgical margin are associated with better prognosis. Furthermore, surgery itself by inducing a strong inflammatory stimulation may be a facilitator

of the recurrence/metastatic process (Neeman *et al.*, 2012; Tepel *et al.*, 2006). To some extent, high tumor recurrence rate could be a marker for a high malignant potential, especially under the condition in this study that the same surgeon performed all operations, and tumors were at the same stage and histological differentiation. These results indicate that pancreatic body/tail cancer might be 'less' malignant than pancreatic head cancer.

## **4.2 Lower expression of miR-501-3p in pancreatic body/tail cancer indicates a lower invasive phenotype**

### **4.2.1 miRNA profiles in pancreatic cancer**

In miRBase database (Release 19) (<http://www.mirbase.org>), 2042 mature human miRNAs are reported and more miRNAs are likely to be found. As the development of new technologies, microarray-based approaches has allowed genome-wide, high throughput screening for novel candidates involved in the pathogenesis of a variety of cancers. Aberrant expression of miRNAs has been detected in many human diseases including PDAC. When searching the US National Library of Medicine (PubMed) using keywords 'pancreatic cancer' and 'miRNA', we found that a total of 224 references are listed (<http://www.ncbi.nlm.nih.gov/pubmed>). Among the 224 publications, 204 and 187 were published in the past 5 and 3 years, respectively. It is becoming a very hot and promising field for identifying the PDAC associated miRNAs expression pattern.

Current clinical screening and diagnostic management of patients with PDAC is mainly based on imaging techniques such as contrast-enhanced multi-detector computer tomography (CT) scan, positron emission tomography (PET) scan and diffusion-weighted magnetic resonance imaging. However, due to the relative low sensitivity and specificity, such techniques may only be used for the evaluation of existing of solid tumors (> 20 mm<sup>2</sup>) (Fukukura *et al.*, 2012). Therefore, there is a strong need for identification of novel biomarkers to predict the development of PDAC and early diagnosis.

During the last 5 years, a number of studies have compared the miRNA expression profiles either between PDAC and normal pancreas, or between PDAC and chronic pancreatitis, and even between PDAC and different stages of PanINs, using blood or tissue samples (Bloomston *et al.*, 2007; Jamieson *et al.*, 2012; Szafranska *et al.*, 2007; Wang *et al.*, 2011; Yu *et al.*, 2012). Recently, considering a high volume (~1.5 L) of pancreatic juice production and excretion into the bowel, it has been hypothesized that precancerous or early cancer-related molecular changes may be detectable in feces of pancreatic cancer patients. A specific miRNA pattern has been found in stool as miRNA biomarkers for PDAC screening (Link *et al.*, 2012). Taken together, advanced technologies have helped us to find a series of frequently dysregulated miRNAs in PDAC, such as miR-21, miR-155 and miR-196a (Bhat *et al.*, 2012).

#### **4.2.2 Different miRNA expressions between pancreatic head and body/tail cancers**

Although a number of studies have identified PDAC-associated miRNA expression pattern, there is still a lack of comparison between pancreatic head and body/tail cancers. We could only find indirect evidence from the comparison between two cell lines as typical representatives originating from pancreatic head cancer (Panc-1) and body/tail cancer (MIA PaCa-2), respectively. Studies have shown specifically altered miRNAs between PDAC cell lines and human pancreatic ductal epithelium control cell lines, and between subgroups of PDAC cell lines with different invasion or metastasis properties (Ji *et al.*, 2009; Kent *et al.*, 2009; Mees *et al.*, 2009). From these studies covering a large panel of miRNAs, we could find several differentially expressed miRNAs between Panc-1 and MIA PaCa-2, such as miR-10b, miR-15b, miR-18a, miR-21, miR-22, miR-125, miR-155, miR-181 and miR-196a (Ji *et al.*, 2009; Kent *et al.*, 2009).

For the first time, we performed miRNA microarray screening to provide direct evidence of different miRNA patterns between pancreatic head and body/tail cancers. It is not surprising that the expressions of the majority of miRNAs in this analysis were similar between pancreatic body/tail cancer and head cancer because both subtypes are essentially PDAC. However, as expected, there were also significantly differentially expressed miRNAs between

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the two subtypes. Among 887 detected human miRNAs, 6 were found either remarkably higher or lower expressed in pancreatic body/tail cancer compared with those in pancreatic head cancer. According to the selection criteria, the two most statistically significant miRNAs (miR-501-3p and miR-375) were further verified by qRT-PCR. We hypothesized that miR-501-3p and miR-375 may encode the diversity between pancreatic head and body/tail cancer.

miR-375 was firstly thought to be only expressed in endocrine pancreatic cells (islet  $\beta$ -cells and non- $\beta$ -cells) (Poy *et al.*, 2009), but increasing evidence shows that it could be also expressed in other tissues (Ding *et al.*, 2010; Xu *et al.*, 2011b). miR-375 has great impact on pancreatic islet cell viability and function, and plays important role in the regulation of insulin secretion and glucose metabolism, and acts as a key determinant of blood glucose homeostasis (Lynn, 2009). Furthermore, miR-375 is also closely associated with pancreatic cancer. Studies have demonstrated a down-regulation of miR-375 in pancreatic cancer (Basu *et al.*, 2011; Bloomston *et al.*, 2007; Lee *et al.*, 2007; Szafranska *et al.*, 2007). It can act as a tumor suppressor and targeted several oncogenes such as PDK1 (a master regulator of oncogenic phosphoinositide-3 kinase signaling), Janus kinase 2 (JAK2) and insulin-like growth factor 1 receptor (IGF1R), resulting in decreased cancer cell growth, viability and invasion (Ding *et al.*, 2010; Kong *et al.*, 2012; Xu *et al.*, 2011b). Higher expression of miR-375 in pancreatic body/tail cancer could provide evidence for a 'reduced' malignancy of pancreatic body/tail cancer than pancreatic head cancer.

Unlike miR-375, miR-501-3p has not yet been reported in human cancers. It has not been reported to be highly differentially expressed between PDAC and normal pancreatic tissues. Therefore, the role of miR-501-3p in pancreatic cancer needed further functional analyses.

#### **4.2.3 miR-501-3p is associated with high invasiveness**

Our results demonstrated that miR-501-3p was significantly associated with pancreatic cancer recurrence after surgery. High expression of miR-501-3p was a risk factor of tumor recurrence, which suggests that miR-501-3p may be a potential biomarker for tumor recurrence after surgical therapy. These results indicate that pancreatic body/tail cancer could be



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characterized by a lower expression of miR-501-3p, which contributes to a lower recurrence risk, compared with pancreatic head cancer.

However, as mentioned before, the role of miR-501-3p in PDAC has not been reported yet. We performed *in vitro* study to verify whether miR-501-3p could promote invasion in PDAC cells. In both frequently used cell lines, the invasion rate significantly increased upon the up-regulation of miR-501-3p group and remarkably decreased upon down-regulation of miR-501-3p group compared with their respective negative control groups. Moreover, because miR-501-3p targets cell adhesion molecules as has been reported in the '15th Annual Conference of Rehabilitation in Multiple Sclerosis' ([http://registration.akm.ch/einsicht.php?XNABSTRACT\\_ID=116538&XNSPRACHE\\_ID=2&XNKONGRESS\\_ID=126&XNMASKEN\\_ID=900](http://registration.akm.ch/einsicht.php?XNABSTRACT_ID=116538&XNSPRACHE_ID=2&XNKONGRESS_ID=126&XNMASKEN_ID=900)), we tested the well-known epithelial cell-cell adhesion molecular E-Cadherin among different groups to further understand the mechanism of increased invasiveness via up-regulation of miR-501-3p. The results revealed that up-regulation of miR-501-3p led to a decreased expression of E-Cadherin, while down-regulation of miR-501-3p resulted in an increased expression of E-Cadherin in both cell lines. E-Cadherin is a transmembrane protein localized at the adherens junctions of the epithelial cell basolateral surface and plays a key role in epithelial morphology maintenance. Loss of E-Cadherin expression is a well-recognized marker of EMT and promotes PDAC progression and invasion (Wong *et al.*, 2003). Our results from *in vitro* study provided evidence for the clinical part that down-regulation of miR-501-3p in pancreatic body/tail cancer may lead to a less invasive/metastasis phenotype through up-regulation of E-Cadherin.

In addition, little is known about the molecular targets of miR-501-3p. Although E-Cadherin is regulated by miR-501-3p, no database predicts E-Cadherin can be a direct target of miR-501-3p because of the unmatched sequence. It indicates that miR-501-3p indirectly regulates E-Cadherin expression. Here, we have proven that *CDH5* is a direct target gene of miR-501-3p. VE-Cadherin, also known as Cadherin-5, is a member of cadherin superfamily, expressed by vascular endothelial cells. It appears to be a major adhesive protein involved in the control of endothelial cell-cell contact and is of vital important for maintaining endothelial

barrier integrity and homeostasis (Boda-Heggemann *et al.*, 2009). More importantly, VE-Cadherin-mediated permeability plays a critical role for carcinogenesis including tumor induced angiogenesis and inflammation. It has been demonstrated that tumor cell-secreted vascular endothelial growth factor (VEGF) increased endothelial permeability in association with decreased VE-Cadherin localization at the plasma membrane (Hu *et al.*, 2006). Disruption of VE-Cadherin and subsequently ensuing vascular permeability strongly supports tumor metastasis (Liao *et al.*, 2000; Weis *et al.*, 2004). PDAC cell binding can induce focal disappearance of VE-Cadherin from endothelial cell junctions and facilitate cancer invasion (Nakai *et al.*, 2005). In the present study, we found miR-501-3p directly targeted VE-Cadherin and the down-regulation of miR-501-3p led to an increasing VE-Cadherin expression in the PDAC cell line Colo357. We hypothesize that down-regulation of miR-501-3p in pancreatic body/tail cancer may associate with increased expression of VE-Cadherin, and subsequently a better-preserved 'vascular barrier' to prevent tumor metastasis.

However, the function of VE-Cadherin in PDAC is still unclear. VE-Cadherin is traditionally been considered restrict in endothelial cells. Using a large database including a consensus set of 2984 genes from our previous meta-analysis (Grutzmann *et al.*, 2005), we found that *CDH5*, which encodes VE-Cadherin, could be expressed in both PDAC tissues and cell lines (unpublished data). Here, we provided evidence that PDAC cells (Colo357 and Panc89) could also express the vascular endothelial specific molecular. Only some of the 'moderate' malignant PDAC cells expressed the endothelial-marked molecule. Neither the more nor the less 'malignant' cells expressed VE-Cadherin. The effect of VE-Cadherin on PDAC cells needs further research. For this project, the expression of VE-Cadherin in tumor tissues will be evaluated in the future because of the small amount of the early stage PDAC tissue samples. We are now collecting new samples for immunostaining of VE-Cadherin in tumor tissues.

#### **4.2.4 miR-501-3p reduces proliferation and apoptosis**

Besides invasion, our *in vitro* study also demonstrated that miR-501-3p might reduce proliferation and apoptosis, although the mechanism still needs to be further explored. We

found higher caspase-8 expression in Panc-1 cells upon down-regulation of miR-501-3p compared to the control, and lower cleaved caspase-8 after up-regulation of miR-501-3p. Caspase-8 is engaged in both intrinsic (mitochondria-mediated) pathway and the extrinsic (death receptor-mediated) pathway. In the extrinsic pathway, death receptors (e.g. Fas/Apo-1/CD95, TRAIL-R1, TRAIL-R2) interact with the corresponding ligands, leading to an activation of caspase-8, which could directly activate downstream effector caspases, causing apoptosis (Kruidering *et al.*, 2000). In addition, caspase-8 also could cleave Bid to activate intrinsic apoptotic pathway (Kruidering *et al.*, 2000). Our results indicate that caspase-8 might associate with miR-501-3p-induced decreased apoptosis in PDAC. Moreover, we found that miR-501-3p could reduce the expression of death receptors, TRAIL-R1 and TRAIL-R2, and consequently lead to more resistance to TRAIL-induced apoptosis. This may be another explanation for miR-501-3p-induced decreased apoptosis. To better elucidate the possible mechanism of miR-501-3p reduced apoptosis and proliferation, more apoptosis associated molecules (e.g. caspase-3, -7, -9, and bcl-2 family) and proliferation associated molecules (e.g. Ki-67 and p120) are needed to be studied.

Our previous study has revealed that TRAIL-R1 and TRAIL-R2 show not only apoptotic function but also non-apoptotic functions leading to strong pro-inflammatory responses, which are related to survival, proliferation, migration and invasion (Roder *et al.*, 2011). Pancreatic cancer cells can produce endogenous TRAIL, which binds to TRAIL-R1 or TRAIL-R2 and activate the non-apoptotic pathways to promote tumor progression.

## 5. Summary

So far, not enough attention has been paid to the diversity between pancreatic head and body/tail cancers. Only a few studies have focused on the differences and overall information is limited. The current clinical data support higher incidence and easier detection of pancreatic head cancer compared with pancreatic body/tail cancer. However, for tumors at the local-stage, pancreatic head cancer has a lower survival than pancreatic body/tail cancer. Although the previous reports included large cohorts of patients, the evidence is still not so convincing because there is a lack of strictly matched cohorts between the two subtypes of PDAC. The two groups of patients should be matched not only by tumor stage, but also by other well-known prognostic factors such as age, gender, race, tumor markers and histological differentiation.

This study, for the first time, compares pancreatic head cancer and pancreatic body/tail cancer using strictly matched early stage PDAC tissue samples by using miRNA expression screening. We demonstrate that miR-501-3p and miR-375 are significantly differentially expressed between pancreatic head and body/tail cancers, and may contribute to the diversity. Furthermore, lower expression of miR-501-3p in pancreatic body/tail cancer is associated with a lower tumor recurrence rate after radical surgery therapy compared with pancreatic head cancer. miR-501-3p may be regarded as a potential prognostic marker for tumor recurrence. A limitation of this clinical part is the relative small study cohort. We have collected only 15 pairs of pancreatic head and body/tail cancers during the last 3 years in one of the largest Hepatobiliary Pancreatic Surgery Department of P.R. China. Nevertheless, considering the relative low incidence of pancreatic body/tail cancer and more importantly, the difficulty in strictly matching of age, gender, tumor marker, tumor stage and tumor differentiation between the two groups, the study should have good value. Another limitation is the small amount of tumor tissues. We are collecting more matched samples for further verification and more experiments, such as immunostaining and Western Blot.

By *in vitro* studies, we provide first evidence that miR-501-3p is associated with cancer. It can promote invasion of PDAC cells, possibly via decreasing the expression of E-Cadherin. This

result supports the clinical findings that miR-501-3p is related to tumor recurrence/metastasis. Furthermore, other miR-501-3p-induced biological difference of PDAC cells have been found. miR-501-3p can decrease apoptosis in PDAC cells and reduce TRAIL sensitivity via down-regulation of TRAIL-R1 and -R2. However, the detailed mechanism needs further studies.

Importantly, we demonstrate that E- and VE-Cadherin can be regulated by miR-501-3p, and VE-Cadherin is a direct target of miR-501-3p. Although VE-Cadherin was considered as an endothelial specific molecular, we prove that PDAC cells can express VE-Cadherin. Yet, the specific pathophysiological function of VE-Cadherin in PDAC needs further research.

In conclusion, we suggest that diversity exists between pancreatic head and body/tail cancers, in which the pancreatic body/tail cancer might be 'less' malignant than the pancreatic head cancer. Lower levels of miR-501-3p might contribute to a less invasive phenotype and lower-risk of tumor recurrence/metastasis in pancreatic body/tail cancer than pancreatic head cancer with VE-Cadherin as a putative biomarker. These findings confirm the importance of subsite division and support the development of individual treatment strategies.

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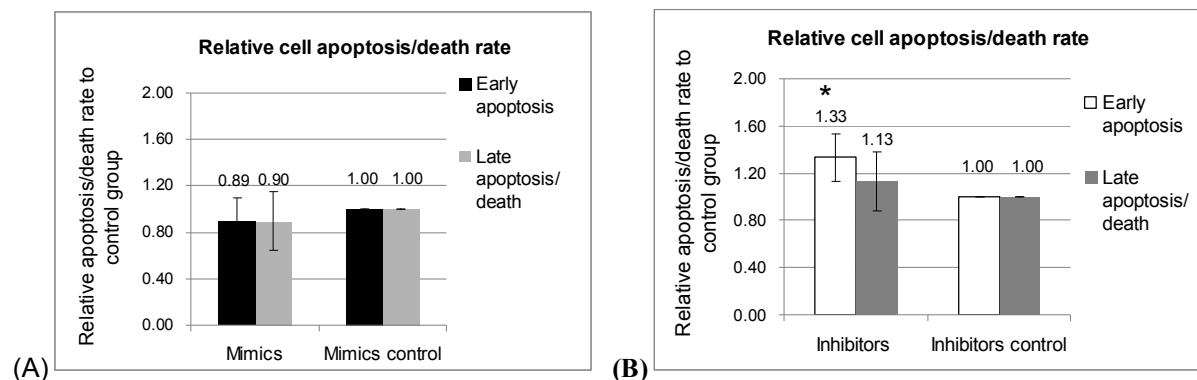
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## 7. Supplemental data

After transfection for 72 hours, Panc-1 cells (source: The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China) were double stained with Annexin V-FITC and PI (Sigma, USA). Viable cells are Annexin V-FITC (-) and PI (-). Cells in early apoptosis (membrane integrity is present) are Annexin V-FITC (+) and PI (-), while cells in late apoptosis (dead) are FITC Annexin V (+) and PI (+). We found that significant more Annexin V-FITC (+) and PI (-) cells in the Inhibitor group than its negative control (11.1% vs. 8.7%,  $P = 0.010$ ), which indicates that miR-501-3p may reduce PDAC cells early apoptosis (Supplemental Figure 1). However, this assay is limited by not distinguishing between cells undergoing apoptotic death versus those dying in a necrotic pathway.



**Supplemental Figure 1.** Cell apoptosis/death rate in the Panc-1 cells. (A): Relative apoptosis/death rate in PDAC cells transfected with miR-501-3p Mimics and its negative control. (B): Relative apoptosis/death rate in PDAC cells transfected with miR-501-3p Inhibitors and its negative control. \*:  $P < 0.05$ . Cell numbers were normalized to the control groups. The experiment was repeated 3 times under the same conditions.



## 8. Acknowledgements

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## 9. Curriculum vitae

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**Education:**

2000–2005

School of Medicine, Zhejiang University      Medical Bachelor

2005–2007

School of Medicine, Zhejiang University      Medical Master

2010–2013

School of Medicine, Zhejiang University      Medical Doctor

**Professional Experience:**

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2007-2010	Department of Surgery First Affiliated Hospital Zhejiang University, School of Medicine Resident
2010-date	Department of Surgery First Affiliated Hospital Zhejiang University, School of Medicine Fellow
2012-2012	Institute for Experimental Cancer Research Comprehensive Cancer Center North University of Kiel Visiting scholar

**Job Experience:**

Over 400 operation performed pre year

Experience in the surgical treatment of following disease:

Hepatocellular carcinoma

Liver cirrhosis

Pancreatic ductal adenocarcinoma

**Research Interests:**

Molecular diagnosis and treatment of pancreatic ductal adenocarcinoma

Prediction of the post-operative complications in liver transplantation

Series management of HBV-related liver diseases:

(Fibrosis-Cirrhosis- Hepatocellular carcinoma)

**Publications in the past five years (as the first two authors):**

1. Ling Q, Xie HY, Lu D, Wei XY, Gao F, Zhou L, Xu X, Zheng SS. Association between donor and recipient TCF7L2 gene polymorphisms and the risk of new-onset diabetes mellitus after liver transplantation in a Han Chinese population. *Journal of Hepatology*, Available online 4 October 2012
2. Ling Q, Xu X, Wei Q, Liu X, Guo H, Zhuang L, Chen J, Xia Q, Xie H, Wu J, Zheng S, Li J. Downgrading MELD improves the outcomes after liver transplantation in patients with acute-on-chronic hepatitis B liver failure. *PLoS One* 2012, 7(1):e30322.
3. Ling Q, Xu X, Wei X, Wang W, Zhou B, Wang B, Zheng S. Oxymatrine induces human pancreatic cancer PANC-1 cells apoptosis via regulating expression of Bcl-2 and IAP families, and releasing of cytochrome c. *Journal of Experimental & Clinical Cancer Research* 2011, 2011 Jun 29;30:66.
4. Ling Q, Xu X, Wei Q, Wei X, Wang Z, Zhou L, Zheng S. Impact of preexisting diabetes mellitus on outcome after liver transplantation in patients with hepatitis B virus-related liver disease. *Dig Dis Sci*. 2011 Mar;56(3):889-93.
5. Ling Q, Xu X, Li J, Wu J, Chen J, Xie H, Zheng S. A new serum cystatin C-based equation for assessing glomerular filtration rate in liver transplantation. *Clin Chem Lab Med*. 2008;46(3):405-10.
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8. Xu X, Ling Q, Wei Q, Wang K, Zhou B, Zhuang L, Zhou L, Zheng S. Korean red ginseng: a new approach for the treatment of graft-versus-host disease after liver transplantation. *Transplant Proc*. 2011 Sep;43(7):2651-5.
9. Xu X, Ling Q, Wei Q, Wu J, Gao F, He ZL, Zhou L, Zheng SS. An effective model for predicting acute kidney injury after liver transplantation. *Hepatobiliary Pancreat Dis Int*. 2010 Jun;9(3):259-63.

10. Xu X, Ling Q, Gao F, He ZL, Xie HY, Zheng SS. Hepatoprotective effects of marine and kuhuang in liver transplant recipients. *Am J Chin Med.* 2009;37(1):27-34.
11. Wu YJ, Ling Q, Zhou XH, Wang Y, Xie HY, Yu JR, Zheng SS. Urinary trypsin inhibitor attenuates hepatic ischemia-reperfusion injury by reducing nuclear factor-kappa B activation. *Hepatobiliary Pancreat Dis Int.* 2009 Feb;8(1):53-8.
12. Xu X, Ling Q, He ZL, Gao F, Zheng SS. Post-transplant diabetes mellitus in liver transplantation: Hangzhou experience. *Hepatobiliary Pancreat Dis Int.* 2008 Oct;7(5):465-70.
13. Xu X, Ling Q, Wu J, Chen J, Gao F, Feng XN, Zheng SS. A novel prognostic model based on serum levels of total bilirubin and creatinine early after liver transplantation. *Liver Int.* 2007 Aug;27(6):816-24.

#### **Awards**

1. 2000, First-class Scholarship and awards for graduate of Zhejiang province
2. 2009, First-class award for development in science and technique of Zhejiang province
3. 2009, First-class award for development in medical science of Zhejiang province
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