TECHNISCHE UNIVERSITÄT MÜNCHEN

Pankreas-Forschungslabor
Chirurgische Klinik und Polikinik
Klinikum rechts der Isar

Association of ALCAM with chemoresistance and tumor cell adhesion in pancreatic cancer

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Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines Doktors der Medizin genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. D. Neumeier

Prüfer der Dissertation:

1. Priv.-Doz. Dr. J. Kleeff

2. Univ.-Prof. Dr. M. Ebert

Die Dissertation wurden am 03. 11. 2009 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 24. 03. 2010 angenommen.

Zusammenfassung

Zielsetzung: Zell-Zell-Kontakte sind wichtig für den Zusammenhalt von Epithelien in ihrem Zellverband. Bei Krebserkrankungen ist die Funktion von Zellkontaktproteinen häufig gestört, was zu erhöhter lokaler Invasivität und zur Fernmetastasierung führen kann.

Methoden: Vorversuche zu unseren Experimenten zeigten, dass die Expression des aktivierten Leukozyten-Adhäsionsmoleküls (ALCAM, CD166), einem Glykoprotein, welches zur Familie der Immunglobuline gehört, im duktalen Adenokarzinom des Pankreas (PDAC) verändert ist. Um diese Daten zu validieren und um die Funktion von ALCAM im PDAC zu untersuchen, wurden quantitative Echtzeit-PCRs, Immunhistochemie-Analysen, RNAi-Experimente, sowie Untersuchungen zu Adhäsion, Migration, Invasion und Chemoresistenz durchgeführt.

Ergebnisse: Wir konnten zeigen, dass ALCAM im Serum von Patienten mit einem PDAC signifikant erhöht war. Im normalen Pankreas wurde ALCAM an den Membranen der Inselzellen exprimiert, während es in den Pankreasgangepithelien nicht nachweisbar war. Im PDAC fand sich ALCAM bei einigen Tumoren an der Zellmembran und im Zytoplasma. Neuroendokrine Pankreastumoren (PNET) zeigten meist ein zytoplasmatisches Färbungsmuster für ALCAM, welches sich vom membranösen Färbungsmuster unterschied, das in normalen Inselzellen beobachtet wurde. Die Ausschaltung der

ALCAM-Expression in vitro mittels RNAi hatte keine Auswirkungen auf Wachstum oder Invasivität von Pankreaskarzinomzellen, bewirkte jedoch eine verminderte Zelladäsion und erhöhte Chemoresistenz. In Zelllinien von neuroendokrinen Pankreastumoren führte die ALCAM-RNAi zu vermindertem Zellwachstum.

Schlussfolgerung: ALCAM ist ein neuer serologischer Biomarker für das Pankreaskarzinom, der mit Zellwachstum, Zelladhäsion und Chemoresistenz in Verbindung steht.

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1. Introduction

1.1. Pancreatic cancer epidemiology

Despite pancreatic cancer (PC) constitutes a group of malignancies, including pancreatic ductal adenocarcinoma (PDAC), serous cystadenocarcinoma, neuroendocrine tumors, sarcoma, acinar cell carcinoma and lymphoma, in the literature the term pancreatic cancer refers almost always to the PDAC. PDAC is a disease with a dismal prognosis and ranks 9th in the incidence of solid cancers and 4th for cancer-related deaths(Kleeff et al., 2007a), with an overall median survival of around 4–6 months. The only cure is surgery(Wagner et al., 2004); however, this option is available only to a maximum of 20% of the patients diagnosed with the disease. Even for these patients, median survival is less than 2 years(Neoptolemos et al., 2004), and only few patients survive significantly longer (Carpelan-Holmstrom et al., 2005). What is so unique about this cancer? Because of the retroperitoneal location of the pancreas, the only early symptom may be obstructive jaundice in the case of cancer formation close to the papilla/common bile duct. Normally, patients report with back pain or a wasting syndrome, generally indicators of advanced/inoperable cancers. Because of the location and composition/texture of the gland, early lesions cannot be visualized as easily as in the hollow organs of the gastrointestinal tract. Further, more PDAC has a particular affinity to grow along nerve sheats—uncommon in most other solid cancers(Bockman et al., 1994). PDAC displays a wide range of genetic and

epigenetic alterations that contribute to its aggressive phenotype(Friess et al., 2003; Kleeff et al., 2006). On the morphological level, PDAC is characterized by a dense cancer stroma, called desmoplasia(Kloppel et al., 2004). This essentially benign cellular component of the cancer raises questions with respect to its contributory role in shielding the neoplastic cells and to various aspects of its pathophysiology.

1.2. Pancreatic cancer pathology

Morphological and molecular studies from the second half of the last century (Cubilla & Fitzgerald, 1976; Kloppel et al., 1980; Kozuka et al., 1979; Moskaluk et al., 1997; Wilentz et al., 1998) led to the identification of the putative precursor lesions of PDAC. Consensus on their nomenclature and classification was reached during the Pancreas Cancer Think Tank, held in Park City, Utah, USA, in September 1999 under the sponsorship of the National Cancer Institute(Kern et al., 2001). According to the original proposal by Klimstra and Longnecker(Klimstra & Longnecker, 1994), these lesions were termed pancreatic intraepithelial neoplasia (PanIN) (Fig. 1). PanINs represent a group of microscopic intraepithelial lesions of small (<5 mm in diameter) pancreatic ducts, and are subclassified into PanIN-1 (A and B), PanIN-2 and PanIN-3, according to the degree of architectural changes (pseudostratification, micropapillary, papillary or cribriform architecture) and cytological abnormalities (transition from cuboidal to tall columnar cells with

abundant supernuclear mucin, nuclear enlargement, crowding and hyperchromatism, increased mitotic rate(Hruban et al., 2001). Molecular analyses have shown that PanINs share many of the genetic alterations that characterize invasive PDAC. These analyses of PanIN lesions demonstrated that the number of genetic alterations increases with the grade of dysplasia of PanINs, further supporting the concept of a multistep pancreatic adenocarcinoma progression model(Hruban et al., 2000a; Maitra et al., 2003). These observational findings have now been substantiated in a number of animal models that recapitulate pancreatic carcinogenesis from PanIN lesions to invasive PDAC(Hruban et al., 2006).

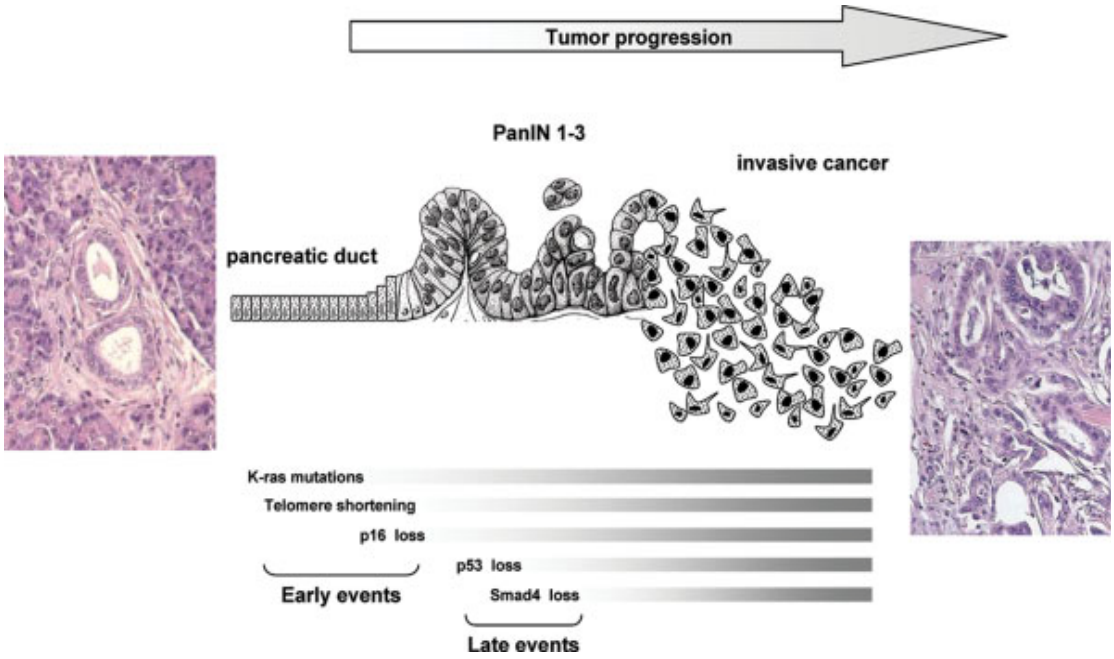

FIGURE 1 Cancer progression model from normal pancreatic ducts via PanIN lesions to invasive pancreatic ductal adenocarcinoma.

1.3. Pancreatic cancer biology

It is well-known that chromosomal abnormalities are involved in the pathophysiology and development of pancreatic cancer. These abnormalities usually present as a loss or gain of alleles in various chromosomes, in a rather random appearance. Established allelic loss has been shown for chromosome arms 1p (50%), 6p (50%), 6q (50%), 8p (56%), 9p (76%), 10p (50%), 10q (50%), 12p (50%), 12q (67%), 17p (95%), 18q (88%), 21q (61%), and 22q (61%). Chromosomal additions involve chromosomes 7 and 20(Lumadue et al., 1995).

Commonly mutated genes in pancreatic cancer include K-ras (in 74–100% of cases), p16INK4a (up to 98%), p53 (43 to 76%), DPC4 (about 50%), HER-2/neu (in about 65%) and FHIT (found in 70% of cases)(Bloomston et al., 2006; Dang et al., 2002; Dergham et al., 1997; Dong et al., 2007; Dugan et al., 1997; Hua et al., 2003; Schutte et al., 1996). Other genes involved are notch1, Akt-2, BRCA2 and COX-2. (Albazaz et al., 2005; Chadha et al., 2006; Goggins et al., 1996; Juuti et al., 2006; Lal et al., 2000; Maitra et al., 2002; Okami et al., 1999; Simon et al., 1998; Wang et al., 2006a; Wang et al., 2006b) K-ras, HER-2/neu, notch1, Akt-2 and likely COX-2 are proto-oncogenes whereas all the other genes are tumor suppressors.

The ductal morphology of PDAC led to postulate that ductal cells were at the origin of transformation. Supporting this hypothesis, PDAC occurs with high frequency in association with dysplastic and hyperplastic ductal lesions(Andea et

al., 2003; Hruban et al., 2001). In this respect, three different ductal preneoplastic lesions have been described: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms and intraductal papillary mucinous neoplasms(Hruban et al., 2000b; Maitra et al., 2005). Common and distinct molecular events have been found among these lesions, suggesting that each precursor lesion may reflect variations leading to malignant transformation(Adsay et al., 2004; Sato et al., 2001). The observation that these lesions display some of the genetic alterations found in PDAC strengthened the hypothesis that PDACs arise from preexisting duct cells. However, this hypothesis has been surprisingly difficult to prove, as direct targeting of oncogenic K-Ras, the most frequent genetic alteration in PDAC, to mature ductal cells using the cytokeratin 19 promoter fails to induce PanINs or PDAC in mice(Brembeck et al., 2003; Schmied et al., 1999).

Other studies using experimental animal models suggest that PDAC may derive from endocrine or acinar cells undergoing metaplasia (or transdifferentiation) processes.

The endocrine hypothesis was based on experiments performed using transformed islet cell cultures that, when transplanted into mouse and hamster(Bardeesy & DePinho, 2002; Schmied et al., 1999; Yoshida & Hanahan, 1994), progressed to PDAC. Furthermore, endocrine lineage markers are frequently expressed in pancreatic cancer cells(Bardeesy & DePinho, 2002). These results have led to speculate that endocrine cells are potential PDAC

precursors(Pour et al., 2002). However, recent data using in vivo lineage tracing in mice suggest that ß-cell transdifferentiation plays no role in regeneration, metaplasia or carcinogenesis.

The frequent observation of acinar-to-ductal metaplasia in humans(Parsa et al., 1985) and in mouse models using acinarspecific promoters suggests an acinar origin of PDAC. In this respect, in Ela-TGF-ß transgenic mice, acinar cells lose zymogen granules and become transitional cells, which subsequently acquire the features of ductal cells. These acinar-derived ductal cells show progressive proliferation and dysplasia – supporting their premalignant nature – and can lead, after long latency, to tumors(Wagner et al., 2001). Similarly, in the Ela-myc model, mice develop pancreatic cancer with 100% penetrance at an early age, half of which are pure acinar carcinomas and the remaining half are ductal adenocarcinomas or mixed ductal and acinar carcinomas(Sandgren et al., 1991).

Mouse models using activated K-Ras have provided further insights into the cell of origin of PDAC. These models have used knock-in strategies for conditional activation of a Kras G12D allele by Cre-loxP recombination. Pancreas-specific activation of K-Ras leads to the development of PanIN lesions (Aguirre et al., 2003; Hingorani et al., 2003). Moreover, when K-Ras activation is combined with mutations in p53(Hingorani et al., 2005) or Ink4a/Arf, a rapid progression to PDAC is observed. Unfortunately, these studies can not address the cell-origin of PDAC,

as they used Pdx1- Cre or p48-Ptf1 alpha- Cre driver strains, which mediate Cre -loxP recombination in all pancreatic cell types.

Tuveson et al.(Tuveson et al., 2006) have recently shown that the K-Ras knock-in in the locus of Mist-1 , a transcription factor required for acinar organization, induces acinar metaplasia and dysplasia, leading to invasive and metastatic pancreatic cancer with mixed acinar, cystic, and ductal features. More recently, Guerra et al. (Guerra et al., 2007) have developed a conditional system for Cre expression under the Elastase promoter. They found that expression of activated K-Ras G12V in nonductal embryonic cells results in PanINs and invasive PDAC. These results suggest that PDAC can originate by transdifferentiation of acinar/centroacinar cells or their precursors into ductal-like cells. Interestingly, centroacinar cells have been also proposed as the origin of ductal lesions in a pancreas-specific knock-out of Pten, a negative modulator of the PI3K/Akt pathway. These mice develop tubular complexes that replace acinar parenchyma and are considered to be the result of the centroacinar compartment expansion (Stanger et al., 2005). Centroacinar cells lie at the interface between acinar cells and adjacent ductal epithelium and could represent a stem- or progenitor-like population in the adult pancreas, particularly in response to pancreatic injury conditions.

Independently of their origin, putative cancer stem cells, defined by their ability to self-renew, to differentiate into the bulk tumor population, and their potential for

tumor formation have been recently identified from human PDAC using cell surface markers (Hermann et al., 2007; Li et al., 2007) .

Stem cell biology has contributed important information regarding epithelial stem cells as potential precursors for human cancer.

During normal pancreatic development, undifferentiated precursor cells are responsible for generating mature acinar, ductal and islet cell types. Although the location and identity of the stem cells in adult pancreas is currently an area of intensive investigation, a precursor cell type could participate in the generation of PDAC(Aguirre et al., 2003; Hingorani et al., 2003). Pointing to this possibility, recent studies have demonstrated that metaplastic and neoplastic ductal epithelium shares features with embryonic pancreas, suggesting that further insights into factors regulating pancreatic development may be useful in identifying initiating events in pancreatic cancer.

Thus, despite enormous progress in our understanding of the pathogenesis of PDAC, we still lack compelling evidence regarding this cancer’s cell of origin and initial development.

1.4. Pancreatic neuroendocrine tumor epidemiology

Pancreatic neuroendocrine tumors (PNETs) are a subgroup of GEP-NETs with unique tumor biology, natural history, and clinical management (Panzuto et al., 2005; Terris et al., 1998; Zikusoka et al., 2005). PNETs are rare pancreatic neoplasms, compared with their more common exocrine counterparts. It is estimated that around 3% of primary pancreatic neoplasms result from neuroendocrine tumors(Ehehalt et al., 2009; Oberg & Eriksson, 2005; Yao et al., 2007). The overall prognosis and long-term survival for PNET patients are far better than for patients with exocrine pancreatic cancer (Bilimoria et al., 2007; Fesinmeyer et al., 2005). The overall 5-year survival rate is in the range of 30% in non-functional (NF) PNETs but up to 97% in insulinomas, a functional (F)-PNET (Mansour & Chen, 2004).

The incidence of PNETs is < 1 per 100,000 in Asian and European population-based studies (Buchanan et al., 1986; Carriaga & Henson, 1995; Eriksson et al., 1989; Lam & Lo, 1997; Lepage et al., 2004; Watson et al., 1989). Recently, Halfdanarson et al. (Halfdanarson et al., 2008) reported an annual incidence of 2.2 in 1,000,000, covering a period of 27 years. These data also showed a male gender preference (males, 2.6; females, 1.8) and a higher incidence of PNETs in recent decades.

1.5. Pancreatic neuroendocrine tumor pathology

By definition, PNETs express neuroendocrine markers—for example, synaptophysin(Wiedenmann et al., 1986), neuron-specific enolase (NSE) (Tapia et al., 1981), and/or chromogranin A (CgA)(Wilson & Lloyd, 1984). The true cell or cells of origin of PNETs, however, are not fully understood. Hormone-producing PNETs can be divided into: (a) common types, that is, insulinoma (17%) and gastrinoma (15%), and (b) rare functional tumors, VIPoma (2%), glucagonoma (1%), carcinoids (serotonin, 1%), somatostatinoma (1%), and exceedingly rare neoplasms like PPoma, adrenocorticotropic hormone (ACTH)oma, growth hormone releasing factor (GRF)oma, calcitonin-producing tumors, parathryroid hormone–related peptide-producing tumors, and others(Kimura et al., 1991) (Chastain, 2001; Garbrecht et al., 2008; Koopmann et al., 2006; Mallinson et al., 1974; O'Toole et al., 2006; Soga, 2005; Soga & Yakuwa, 1998; Srirajaskanthan et al., 2009).

1.6. Pancreatic neuroendocrine tumor biology

The molecular basis of PNET pathogenesis is poorly characterized. The majority of PNETs are sporadic, but PNETs may also be associated with genetic syndromes such as MEN-1, von Hippel-Lindau (VHL) disease, neurofibromatosis 1 (NF-1), and tuberous sclerosis (TSC). The genetic background of these

syndromes may shed light on the molecular mechanisms of PNET pathogenesis. PNETs occur in the majority of MEN-1 patients, typically as numerous pancreatic microadenomas. A minority of these microadenomas acquire the potential to grow and give rise to clinically relevant lesions. When present they are typically in multifocal duodenal and/or pancreatic locations (Akerstrom et al., 2005). PNETs are responsible for premature death in MEN-1 patients (Dean et al., 2000).

The genetic background of sporadic PNETs is complex. Although a number of candidate genes, including MEN-1, RAR-ß, hMLH1, RASSF1, Her2/neu, Cyclin D1, p16INK4a/p14ARF, p18INK4c, p27Kip1, p53, and genes encoding tyrosine kinase receptors, have been implicated in PNET pathogenesis, the genetic and proteomic mechanisms of tumor progression are poorly understood.

Pancreatic endocrine tumors, also known as islet cell carcinomas, are another rare type of neuroendocrine tumor(Oberg & Jelic, 2008). Although localized carcinoids or islet cell tumors are surgically manageable, metastatic disease is present in nearly 50% of patients at the time of diagnosis(Shah et al., 2004). The traditional cytotoxic agents are of limited efficacy in the treatment of neuroendocrine tumors and the treatment of such tumors is a significant challenge in oncology. The use of various chemotherapeutic agents, such as doxorubicin, rapamycin, 5-fluorouracil, cisplatin, carboplatin, etoposide streptozotocin, and temozolomide has led to minimal responses in the treatment of patients with lung carcinoids(Beasley et al., 2000; Jonnakuty & Mezitis, 2007) and pancreatic

endocrine tumors(McCollum et al., 2004; Moertel et al., 1992), mostly of short duration. The low response rates for chemotherapy and the side effects underscore the need for new therapeutic options in these neoplasms.

1.7. Current options of treatment for pancreatic cancer and pancreatic neuroendocrine tumor

Over the past few decades, mortality associated with pancreatic cancer resection has significantly decreased in high-volume centers from around 20% in the 1970s and 1980s to approximately 3% at the current time. Overall, the 5-year survival for all patients with pancreatic cancer is below 5%(Michalski et al., 2007c). The reason for this low survival relates to the aggressive biology of this disease: early development of retroperitoneal and perineural infiltration, angioinvasion, and peritoneal, lymphogenic, and hematogenic dissemination. Surgical resection(Bachmann et al., 2006), the patient's only hope for cure, offers a significantly improved prognosis, with a median survival after resection of 14-20 months and up to 25% 5-year survival rates.

Surgical treatment in specialized referral centers has improved the prognosis of resectable pancreatic cancer considerably despite the generally aggressive behavior of this malignancy(Kleeff et al., 2007b). At the same time, adjuvant therapy for pancreatic cancer has been shown to be effective in providing a

survival benefit. However, some controversy remains over whether to use chemotherapy alone or combined chemoradiation (Chou & Talalay, 1984). Few prospective randomized controlled clinical trials (RCTs) on the use of adjuvant chemotherapy and chemoradiation have demonstrated a distinct survival advantage of systemic chemotherapy (5-FU/FA or gemcitabine) following surgical resection. The most notable published trial is the European Study Group for Pancreatic Cancer (ESPAC)-1 trial. In addition, there are several retrospective analyses and two randomized studies on adjuvant radiation and chemoradiation. Some of these suggested increased survival rates using chemoradiation, which was subsequently widely introduced in clinical routine, especially in the United States. RCTs and a recent meta-analysis of these RCTs confirm, however, the superiority of chemotherapy over chemoradiation, except for a subgroup of patients with positive resection margins. Thus, curative surgery followed by adjuvant systemic chemotherapy should be the standard treatment for patients with resectable, locally confined pancreatic cancer. Further RCTs may clarify potential benefits of chemoradiation in the adjuvant treatment setting. Moreover, the best chemotherapy, or a combination thereof, remains to be determined in large-scale randomized trials.

1.8. Role of Cell Adhesion Molecules

As is the case for all carcinomas, a primary tumor forms in a particular organ. During the formation of a mass lesion, the cancer cells must be attached to each other; therefore, tumors use adhesion molecules to stay together. Tumors can invade adjacent structures directly by growth of the primary tumor, or they can metastasize to distant sites. Metastasis occurs when cells from a primary tumor leave their environment, invade blood vessels or lymphatics, and travel to distant organs/sites(Leber & Efferth, 2009). The process involves attachment of the metastatic cells to the endothelium and invasion into tissue, with proliferation of tumor cells either before or after invasion(Al-Mehdi et al., 2000; Hart & Fidler, 1980). For tumor cells to have a pushing invasive front from the primary tumor, the cells must maintain their adhesion to each other. For tumor cells to metastasize, they must alter their adhesion molecules to detach from the primary tumor mass and then travel to distant sites to establish metastatic lesions.

Adhesion molecules are divided into broad categories, which include immunoglobulins, cadherins, selectins, integrins, and mucins (Ofori-Acquah & King, 2008). Adhesion molecules can be involved in tumor cell–tumor cell adhesion, tumor cell– endothelial cell adhesion, or tumor cell–matrix adhesion; all of these adhesions are essential at different times during primary tumor formation or metastasis. Adhesion molecules can be upregulated or downregulated during the process.

1.9. Structure of ALCAM

Activated leukocyte cell adhesion molecule (ALCAM/CD166), a member of the immunoglobulin superfamily, is a glycoprotein that is involved in both homotypic and heterotypic (to lymphocyte cell-surface receptor CD6) adhesion(Swart, 2002). ALCAM has been cloned in multiple species and has different names, which depend on the species and laboratory that cloned it: chicken neural adhesion molecule BEN/SC-1/DM-GRASP(Burns et al., 1991; Pourquie et al., 1992; Tanaka et al., 1991), rat KG-CAM (Peduzzi et al., 1994), fish neurolin(Paschke et al., 1992), SB10(Bruder et al., 1998), human melanoma metastasis clone D (MEMD)(Degen et al., 1998), mouse/human ALCAM (CD166) (Bowen et al., 1997; Bowen et al., 1995), and HB2(Kurata et al., 1998; Matsumoto et al., 1997). ALCAM has 5 extracellular immunoglobulin domains (2 NH2-terminal, membrane-distal variable-(V)-type and 3 membrane-proximal constant-(C2)-type Ig folds) [D1– D5], a transmembrane region, and a short cytoplasmic tail. The N-terminal domain (D1) regulates affinity, whereas membrane proximal domains D4 and D5 control affinity(Kanki et al., 1994; van Kempen et al., 2001). The cytoplasmic tail contains 32 amino acid residues (Kanki et al., 1994; Laessing et al., 1994). The molecular weight of the native protein is 65 kDa and N-glycosylation at 8 putative sites results in a mature ALCAM species of 110 kDa(Ofori-Acquah et al., 2008; Ofori-Acquah & King, 2008).

1.10. Regulation of ALCAM expression

The gene encoding ALCAM is located on the long arm of human chromosome 3 (3q13.1-q13.2). It is organized into 16 exons that span nearly 150 kb of DNA(Ikeda & Quertermous, 2004). The promoter is TATA-less and enriched with multiple GC-boxes in the proximal region. It contains multiple positive and negative regulatory regions, some with tissue-specific activity, which is consistent with the diametric regulation of the ALCAM gene in different cancers. The promoter contains consensus DNA binding sequences for nuclear factor kappa B and AP-1. DNA-protein binding and reporter gene experiments indicate that the nuclear factor kappa B element is functional, and it is likely involved in increasing expression of ALCAM in tumors because several members of the rel transcription factor family (c-rel, v-rel) induce ALCAM expression in avian lymphoma cell lines(Zhang et al., 1995). That overexpression of Fos-related antigen 2 (Fra-2), a member of the Fos family of AP-1 transcription factors, is associated with decreased expression of ALCAM mRNA and protein highlights a negative regulatory role for AP-1 cis elements in the ALCAM gene.

1.11. Biologic function of ALCAM

The biologic function of ALCAM has been studied using a variety of experimental systems. Virtually all studies that examine ALCAM function have used antibodies

and chimeric, Fc-tagged, soluble ALCAM variants to prevent cell-mediated homotypic and heterotypic ALCAM adhesions. The data from these studies implicate ALCAM in stabilization of the immunologic synapse, T-cell proliferation and activation, monocyte transendothelial migration, and axon fasciculation. The most widely studied is the role of ALCAM in T-cell biology (Aruffo et al., 1997; Bajorath et al., 1995; Bowen et al., 1996; Gimferrer et al., 2004; Hassan et al., 2004; Kato et al., 2006; Patel et al., 1995; Singer et al., 1997; Singer et al., 1996; Starling et al., 1996; Zimmerman et al., 2006). Zimmerman et al reported recently that long-term engagement of dendritic cell ALCAM and CD6 expressed on T-lymphocytes was essential for proliferation of T cells long after the initial contact between the 2 immune cells had been established. This finding is consistent with image analysis of T-cell antigen-presenting cell conjugates, which demonstrates that CD6 and ALCAM colocalize with the T-cell receptor complex at the center of the immunological synapse, and it extends findings by Hassan et al that the ALCAM–CD6 interaction is required for optimal activation of T cells. The costimulatory role of ALCAM in T-cell activation suggests an involvement for ALCAM in the immunologic response to tumor cells. So far, ALCAM has been evaluated in several malignancies, which include melanoma, prostate carcinoma, breast cancer, colorectal carcinoma, bladder cancer, and esophageal squamous cell carcinoma.

2. Aim of this study

Cell-cell adhesion is a prerequisite for the maintenance of epithelial integrity and is thus often altered during carcinogenesis and particularly the metastatic process. Oncogenesis results in the de-regulation of a variety of cell-cell contact proteins to enable a transdifferentiation of the epithelial phenotype into a more mesenchymal cellular structure and function.

ALCAM is a cell adhesion molecule expressed by epithelial cells in several organs. It is expressed at sites of cell– cell contact and is associated intimately with adhesive structures that maintain the structural integrity of the epithelium in various organs. ALCAM expression has been evaluated in melanoma, prostate carcinoma, breast cancer, colorectal carcinoma, bladder cancer, and esophageal squamous cell carcinoma.

Using microarray analysis of normal pancreas and pancreatic tumor tissues, we demonstrated activated leukocyte cell adhesion molecule (ALCAM, CD166), a glycoprotein and a member of the immunoglobulin superfamily, as a de-regulated adhesion protein in pancreatic tumors (unpublished observation). Recently, a mouse to human serum biomarker search demonstrated that ALCAM was significantly increased in sera from pancreatic cancer patients as compared to control groups. However, the major functions of this cell adhesion molecule in cancer remain poorly understood. We hypothesized that – due to its physiological function as a cell-cell contact protein – ALCAM might be involved in oncogenic

transformation in both PNET and PDAC and we propose ALCAM as a novel serum biomarker in human pancreatic cancer which is associated with cell adhesion, growth and chemoresistance.

3. Materials and methods

3.1. Materials

3.1.1. Laboratory equipment

Analytic balance	METTLER
Balance	SCALTEC
Biophotometer	Eppendorf
Centrifuge	Eppendorf
CO_2 incubator	SANYO
Computer Hardware	Fujitsu SIEMENS
Electrophresis/Electroblotting equipment/ power supply	Invitrogen
Freezer -20^{o}C	LIEBHERR
Freezer -80 ^{o}C	Heraeus
Microplate Reader	Thermo Labsystems
Microscope	LEICA
Microwave oven	SIEMENS
PH-meter	BECKMAN
Power supply	BIOMETRA
Refrigerator 4 ^{o}C	COMFORT

Roller mixer	STUART
Scanner	Canon
Sterilgard Hood	THERMO
Thermomixer	Eppendorf
Vortex	IKS
Water bath	MED TECHNIK
Tissue embedding machine	Leica, Germany
Tissue processor	Leica, Germany

3.1.2. Consumables

Biocoat Matrigel Invasion Chamber	BD Biosciences
Cell scraper	BD Falcon
Coverslips	Assistant
Filter (0.2µm)	Neo Lab
Hyperfilm	Amersham
Pure Nitrocellulose membrane (0.45µM)	BIO-RAD
Sterile needles	BD

Tissue culture dishes (60x15mm; 100x20mm)	Cell Star
Tissue culture Flasks (25cm2; 75cm2; 125cm2)	Cell Star
Tissue culture plates (6-well; 24-well; 96-well)	Cell Star
Tubes (15ml; 50ml)	Falcon
Blotting paper	Whatman

3.1.3. Reagents

0.25% trypsin/EDTA	Invitrogen，Germany
2-Mercaptoethanol	Sigma, Taufkirchen
Acetic acid	Merck Biosciences,Schwalbach
Acetic anhydride	Sigma, Taufkirchen
Acrylamide/Bis solution	Bio-Rad, Hercules
Agarose	Invitrogen Germany
Ampicillin	Sigma, Taufkirchen
Annexin-V-Fluos	BD Biosciences
Ammonium per sulfate (APS)	Sigma Taufkirchen
BCA protein assay kit	Thermo Scientific

Biocoat matrigel invasion unit	BD Biosciences, Heidelberg
Bovine serum albumin (BSA)	Roth, Karlsruhe
Bromophenol blue	Sigma, Taufkirchen
Calcium chloride	Merck Biosciences,
Chloroform	Merck Biosciences
Crystal violet	Merck Biosciences
DAKO envision anti-mouse labeled polymer	Dako, Hamburg, Germany
Dextran	Merck Biosciences
Dextran sulfate	Sigma, Taufkirchen
Dimethyl sulfoxide	Sigma, Taufkirchen
Deoxyribonucleotide phosphate	Invitrogen Germany
Dulbecco’s MEM	Invitrogen
RPMI 1640 Medium	Invitrogen Germany
ECL detection reagent	Amersham，Germany
Ethanol	Roth, Karlsruhe, Germany
Ethidium bromide	Carl-Roth, Germany
Fetal calf serum	PAN Biotech
Formamide	Merck Biosciences
Glycerol	Merck Biosciences

Glycine	Roche diagnostics
Haematoxylin	Merck Biosciences
Hydrogen peroxide (30/)	Carl-Roth, Germany
Histowax	Leica,Bensheim, Germany
Humidified chamber	TeleChem
Isopropyl b-D-thiogalactopyranoside	Sigma, Germany
Isopropanol	Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate	Merck Biosciences
Laurylsulfate (SDS)	Sigma,Germany
Liquid nitrogen	Tec-Lab, Germany
Liquid DAB & chromogen substrate	Dako, Hamburg, Germany
Methanol	Merck,Germany
Molecular weight marker	Fermentas, Life Sciences
Dinatrium hydrogenphosphate	Merck,Germany
Normal goat serum	Dako, Hamburg, Germany
Nitrocellulose membranes	Bio-Rad, Hercules, USA
Para-formaldehyde	Fischer, Kehl, Germany
Phosphate buffered saline (PBS) pH 7.4	Invitrogen, Germany

Polyvinylpyrrolidone	Sigma,Germany
Potassium chloride (KCl)	Merck,Germany
Permount	Vector Laboraories
Protease inhibitor cocktail	Roche diagnostics
Proteinase K	Sigma,Germany
QIAquick purification kits	Qiagen, Hilden, Germany
RNAse DNAse-free water	Invitrogen, Germany
Sodium borate	Merck Biosciences
Sodium chloride	Merck Biosciences
Sodium citrate	Merck Biosciences
Sodium phosphate	Merck Biosciences
TEMED	Sigma,Germany
Toluidine blue	Merck Biosciences
Triethanolamine	Sigma, Germany
Tris base	Merck Biosciences
Trypan blue solution	Sigma, Germany
Tween 20	Merck Biosciences
RNAi HiperFect Transfection Reagent	QIAGEN Germany
Hs_ALCAM_6 HP siRNA	QIAGEN Germany

IgG control siRNA	QIAGEN Germany
Human ALCAM ELISA Kit	R&D Systems, Germany
Mouse Anti-human ALCAM/CD166 Monoclonal Antibody	Novocastra Laboratories

3.1.4. Buffers and solutions

Immunohistochemistry

10 x Tris buffered saline (TBS):

Tris base	12.1g
NaCl	85g
H_2O	800ml
pH to 7.4 with	5M HC1
add H_2O to	1000ml

Washing buffer:

10 x TBS	100ml
BSA	1g
add H_2O to	1000ml

Protein extraction and Western Blotting

Cold lyses buffer :

Tris-HCl (pH 7.5)	20 mM
NaCl	150 mM
Na2EDTA	1 mM
EGTA	1 mM
NP-40	1%
Sodium deoxycholate	1%
Sodium pyrophosphate	2.5mM
beta-Glycerophosphate	1mM
NaVO4	1mM
Leupeptin	1ug/ml
PMSF(before use)	1 mM
Protease inhibitor tablet	1
Total:	10ml

Electrophoresis buffer

MOPS	209.2g

Tris base	121.2g
SDS	20g
EDTA free acid	6g
dd H_2O	1L
pH 7.7 with	5M HCl

Transfer buffer

Tris base	29.1g
Glycine	14.7g
Methanol	1000ml
SDS	0.1875g
dd H_2O	5L

500 ml(sigle membrane)

Blocking buffer

5 % dry milk powder in TBS/Tween20

3.2. Methods

3.2.1. Patient data, tissue collection and cell culture

We obtained PDAC (n = 39) and PNET (n=16) tissues from patients in whom pancreatic resections were carried out. Normal human pancreatic tissue samples (n =9) were obtained through an organ donor program from previously healthy individuals. All samples were confirmed histologically. Samples were fixed in paraformaldehyde solution for 24 hr and then paraffin-embedded them for histological analysis. In addition, we preserved a portion in RNAlater (Ambion Europe Ltd., Huntingdon, Ambridgeshire, UK), or snap-froze pieces in liquid nitrogen immediately upon surgical removal and maintained them at -80°C until use. Pancreatic cancer cell lines Aspc-1, Bxpc-3, Capan-1, Colo-357, Miapaca-2, Panc-1, SU86.86, and T3M4 were used. Cells were routinely grown in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin for Aspc-1, Bxpc-3, Colo-357, SU86.86, and T3M4. DMEM with 10% fetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin for Capan-1, Miapaca-2 and Panc-1) at 37°C, saturated with 5% CO_2 in a humid atmosphere.

3.2.2. mRNA and cDNA preparation

All reagents for RNA extraction and cDNA transcription were from Qiagen (Hilden, Germany) and were used as manufacturer's instructions.

3.2.3. Quantitative Real-Time Polymerase Chain Reaction

Quantitative real time PCR was carried out using the LightCyclerTM480 with the SYBR Green 1 Master (Roche). The following primer pairs were used: ALCAM sense: 5'-TAG CAG GAA TGC AAC TGT GG-3; anti-sense: 5'-CGC AGA CATAGT TTC CAG-3'. The expression of ALCAM was normalized to human housekeeping gene beta-actin using the LightCyclerTM480 software release 1.5, version 1.05.0.39 (Roche).

3.2.4. Serum collection and ELISA

Sera from patients and controls were collected, processed and stored in an identical manner to ensure the validity of the results. We collected preoperative blood from 44 pancreatic cancer patients (median age 57 years) undergoing pancreatic resection. The diagnosis was confirmed in all cases by histopathological examination. The 29 control blood samples were collected from healthy volunteers (median age 31 years). We obtained all the sera according to a standardized sampling and coding protocol. Briefly, after sample collection, we incubated the 7.5 ml monovettes (Sarstedt, Nümbrecht, Germany) at 22°C for 30 min and centrifuged at 2,500g for 10 min. Serum was collected, aliquoted into 200µl portions and stored at -80°C until further processing. The study was approved by the ethics committee of the University of Heidelberg and written

informed consent was obtained from all individuals from whom serum samples were collected.

An ALCAM ELISA Kit (R&D systems, Germany) was used following the manufacturer's instructions. Briefly, 96-well Nunc Immuno plates (Nunc, Roskilde, Denmark) were coated overnight at RT with 100 µl (2µg/ml) of ALCAM capture antibodies in PBS (pH 7.0) and washed with PBS 0.05% Tween20. Non-specific binding sites were blocked with 1% bovine serum albumin in PBS for 1h at RT. Either recombinant human ALCAM or serum/supernatant (100 µl per well) were added and incubated for 2h at RT. After washing, biotin-conjugated goat anti-human ALCAM detection antibodies (50ng/ml) were added into each well and incubated for 2h at RT. Horseradish peroxidase-conjugated streptavidin, concentration 1:200 were added to each well and incubated for 20 min at RT. After wash with PBS-Tween20, TMB (BD Biosciences, San Diego, CA) were added for 20 min at RT. Colorimetric reactions were stopped by adding 50 µl of 2N H_2SO_4, and analyzed by microplate reader at 450 nm and 570 nm for correction.

3.2.5. siRNA transfection

For transient mRNA silencing, $15x10^4$ cells per well in 6-well plates were transfected with 5 µM ALCAM RNAi or control RNAi from Qiagen (Hilden, Germany). The ALCAM DNA target sequences were:

5’-.CACCTGCTCGGTGACATATTA-3’. The siRNA sequences were: 5’-CCU GCU CGG UGA CAU AUU ATT-3’ and 5’-UAA UAU GUC ACC GAG CAG GTG-3’. The DNA target sequence for control siRNA is 5’-UUC UCC GAA CGU GUC ACG U-3’. The efficacy of the siRNA transfection was ascertained by Western Blot after 48, 72, 96 and 120 hrs of transfection.

3.2.6. Immunohistochemistry Analysis

Formalin fixed paraffin-embedded tissue sections were dewaxed with Roticlear (Roth, Germany), and gradually rehydrated. Antigen retrieval was achieved by microwaving in 0.01 M citrate buffer for 15 minutes. Peroxidase block was done with 3% water-peroxide in methanol. After block with TBS/3%BSA for 30 min, ALCAM primary antibody (1:500) was applied and incubated overnight at 4°C. On the second day, samples were washed with 1x TBS/0.1%BSA/0.05%Tween20 for 2 times and incubated with apropiated second anti-mouse antibody (DAKO, Glostrup, Denmark) for 1 hour, at RT. Detection was done using DAB Chromogen (DAKO). The slides were counterstained with haematoxylin and mounted. Semi-quantitative evaluation was performed by 2 independent researches. Membranous and cytoplasmic staining intensity of ALCAM were evaluated separately. An immunoreactive score (IRS) was applied. The IRS is the product of staining intensity (graded between 0 and 3) and the percentage of positive cells.

(Michalski et al., 2007a; Michalski et al., 2007b; Michalski et al., 2008a; Michalski et al., 2008b; Michalski et al., 2008c)

3.2.7. Immunoblotting Analysis

3.2.7.1. Protein extraction from cells

Cells were washed twice with ice-cold PBS. After addition of ice-cold modified RIPA buffer/or cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA. 1%NP-40, 1% Sodium deoxycholate, 2.5mM Sodium pyrophosphate, 1mM beta-Glycerophosphate, 1mMNaVO4, 1µg/ml leupeptin, 1 mM PMSF added immediately before use to cells. The cell extracts were homogenized by passing through a syringe G27 needle 10 times. The crude homogenate was then centrifuged at 14,000 g in a precooled centrifuge for 15 minutes. The supernatant was immediately transferred to fresh tubes and aliquoted. The concentration of the extracted protein was determined using BCA Protein Assay kit (Pierce, Thermo Scientific, USA) following manufacturer's intructions. The sample aliquots were stored at -20ºC or used for Western blotting analysis immediately.

3.2.7.2. Western blotting analysis

20µg of total cell lysate was loaded in 10% polyacrylamide gel and eletrophoreticly transferred to nitrocellulose. The membrane was blocked with 20 ml of T-TBS, 5% milk for 1h. Monoclonal mouse anti-ALCAM antibody (Novocastra Laboratories, clone MOG/07,Mouse monoclonal, UK, dilution 1:1000) and GAPDH antibody (Santa Cruz, CA, USA) were incubated overnight at 4°C. After, membrane was washed 3 times with 0.1% Tween20-TBS and incubated with horseradish peroxidase (HRP) conjugated second antibodies (anti-mouse or anti-rabbit) for 1h at RT. Signals were detected using the enhanced chemiluminescence system (ECL, Amersham Life Science Ltd, Bucks, UK).

3.2.8. Cell proliferation Assay

After 24 hours RNAi transfection, SU86.86 cells were seeded into 96-well plates at density of 5000/well. On the second day, the 3-(4, 5-dimethyltriazol-2-yl)-2, 5-dephenyltetrazolium bromide (MTT) test was performed to assess cell viability as T0. For assessment of growth, cells were kept under standard conditions for 48 hours. Then we performed MTT test to assess cell viability as T1. Cell growth was calculated from 3 independent experiments normalized to matching day-0 observations.

3.2.9. Chemotherapy treatment with Gemcitabine and Rapamycin

After 72 hours RNAi transfection, cells were treated as in step 3.2.9 and after 12 hours MTT was performed to assess cell viability as T0. Gemcitabine was added at gradient concentrations: 10nM, 50nM, 100nM, 500nM and 1000nM. 0.01% PBS was used as controls. After 48 hours incubation, MTT test was performed to assess cell viability as T1. The median effective doses of chemotherapeutic drugs on cancer cells grown on 96-well plates were calculated from 3 independent experiments normalized to matching day-0 observations.

For pancreatic neuroendocrine tumor cell line BON, cells were treated as in step 3.2.9 and transferred into 96-well plated after 36 hours of RNAi. 12 hours later, the MTT test was performed to assess cell viability as T0. Then, rapamycin was added at gradient concentrations: 0.1nM, 1nM, 10nM and 100nM. 0.01% DMSO was used as control. After 72 hours incubation, MTT test was performed to assess cell viability as T1. The median effective doses of chemotherapeutic drugs on cancer cells grown on 96-well plates were calculated from 3 independent experiments normalized to matching day-0 observations.

3.2.10. Induction of Apoptosis by Actinomycin D

After 72 hours RNAi transfection, SU86.86 cells were seeded into 96-well plates at density of 5000/well. 12 hours later, MTT test was performed to assess cell

viability as T0. Then we added Actinomycin D at gradient concentrations: 10nM, 100nM, and 1000nM. 0.01% DMSO was used as control. After 24 hours incubation, MTT test was performed to assess cell viability as T1. Cell growth was calculated from 3 independent experiments normalized to matching day-0 observations.

3.2.11. Invasion Assay

BD Biocoat Matrigel invasion chambers, 8µm pore size (BD Biosciences,USA) was used according to the manufacturer's instructions. 22 hours after seeding, chambers were washed and non-invaded cells were removed with a cotton swap. Invaded cells were fixed with cold methanol and stained with hematoxyline. The entire Matrigel was scanned to count invaded cells. The assays were performed 3 times, and results are represented as percent change compared with control (100%).

3.2.12. Adhesion assay

72 h after RNAi transfection cells were, trypsinized collected and seeded into 96-well plates at 1 x 10^4 cells/ well. After 60 min, plates were washed twice with PBS to remove non-adherent cells; this washing step was followed by an MTT assay as described above to determine to number of attached cells.

3.2.13. Statistics

Statistical analysis was performed using the GraphPad Prism 5 software (GraphPad software Inc, San Diego, CA, USA). For survival analysis, a Kaplan-Meier survival curve was plotted followed by group comparisons using a log rank test. Unless otherwise stated, a t-test was used for group-wise comparisons; a p value of < 0.05 was considered significant.

4. Results

4.1. ALCAM is expressed in PDAC and is a prognostic factor in PNET

Microarray analyses revealed upregulation of ALCAM in pancreatic cancer as compared to normal pancreas tissues though this result was not confirmed on a larger set of tissues (normal, CP and PDAC) using QRT-PCR (Fig. 2).

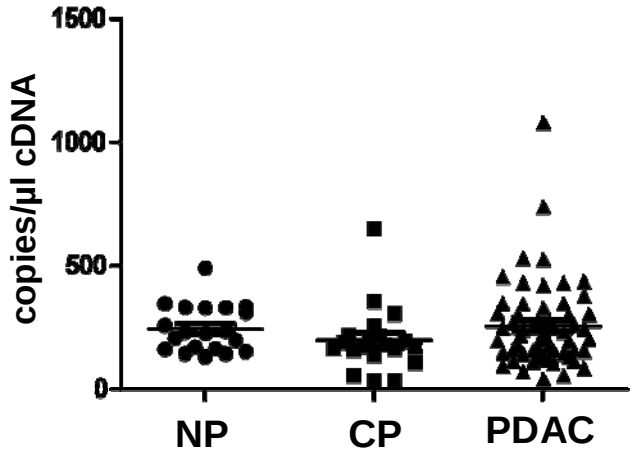

Figure 2: QRT-PCR analysis of ALCAM mRNA levels in the normal pancreas, CP and PDAC tissue samples

We thus performed immunohistochemical analyses revealing weak ALCAM positivity in ductal cells in the normal pancreas while on islet cells; a strong membrane staining was found (Fig. 3A). Some pancreatic intraepithelial neoplasias (in chronic pancreatitis tissues and adjacent to pancreatic cancer; PanIN) were cytoplasm-positive for ALCAM (Fig. 3B). In cancer cells, different staining patterns were observed: while some cancer cells showed various

intensities of ALCAM positivity in the cytoplasm (Fig. 3C), we found cancer cell membrane staining in a minority of slides (6/39 PDAC samples, Fig. 3D. Pancreatic nerves were generally ALCAM-positive (inset, Fig. 3D).

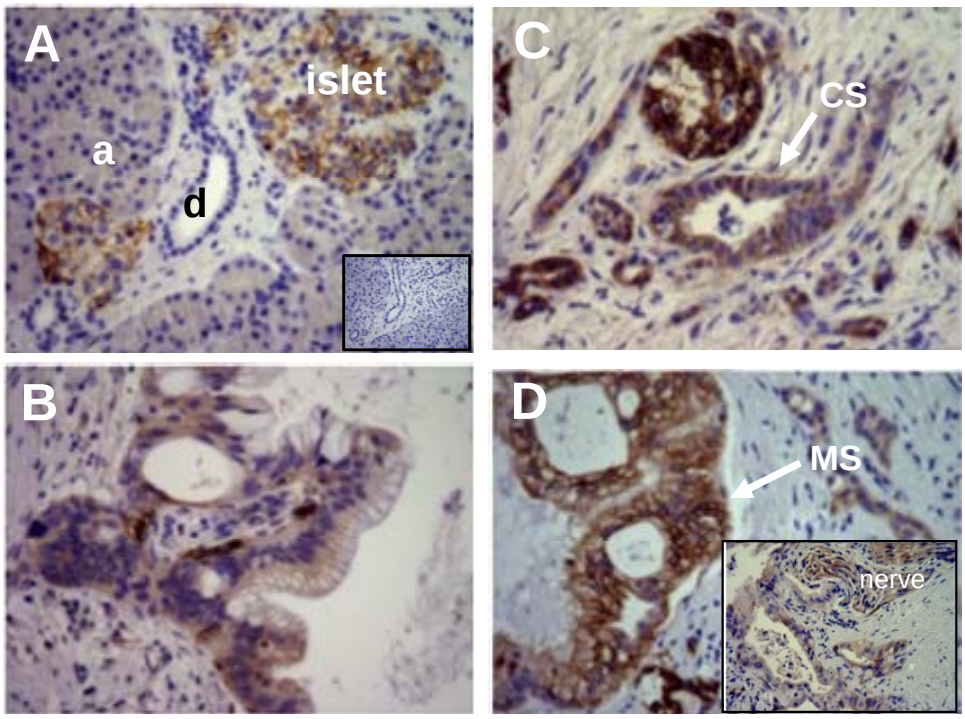

Figure 3: A) ALCAM IHC (1:500) revealed weak positivity in the acinar (a) and ductal (d) cells, however - pronounced membrane staining in islets (islet) in the normal pancreas. Negative controls - insets. B) Cytoplasmic ALCAM staining was observed in PanINs. C), D) Different staining patterns were found in PDAC tissue samples: cytoplasmic staining (C), membrane staining (D), nerves (D, inset).

The PDAC patients were then stratified according to the semi-quantitatively evaluated ALCAM expression levels (according to the tumor cell immunohistochemical stainings: group 1: low levels, group 2: high levels),

respectively; however, no significant influence of ALCAM on survival was observed in Kaplan-Meier survival analysis (median survival low versus high ALCAM levels, 389 days versus 659 days; Fig. 4).

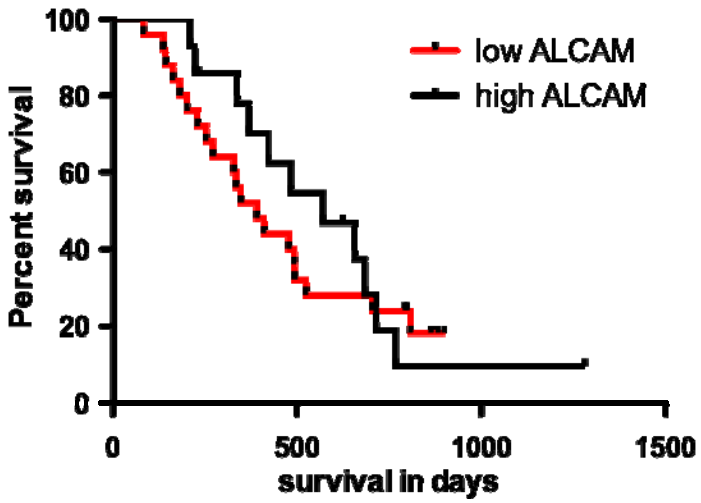

Figure 4: A significant correlation between ALCAM expression and survival could not be observed.

Due to the noticeable membrane staining pattern in islets, we analyzed ALCAM expression in neuroendocrine tumors, as well. In 6 out of 16 samples, the tumor cells were ALCAM-positive with a predominant membrane staining; however, in a few samples, the cytoplasm was immunoreactive for ALCAM (Fig. 5).

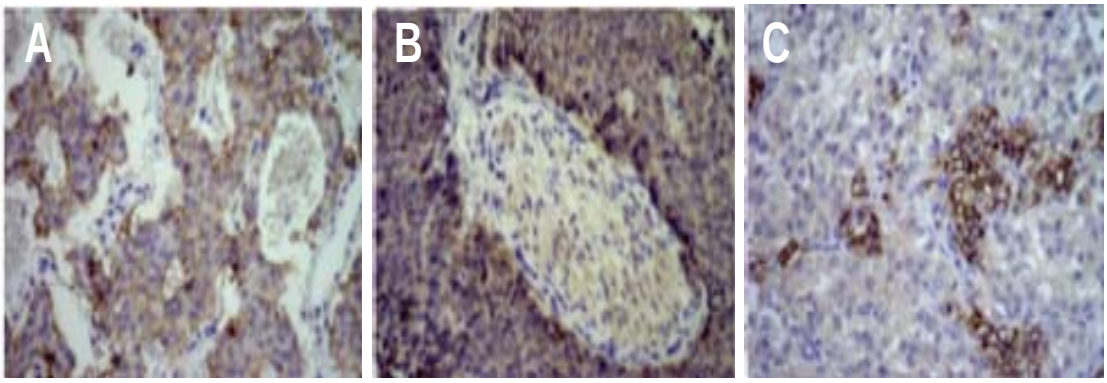

Figure 5: ALCAM expression in PNET: A) membrane staining; B), C) cytoplasmic staining. Original magnification: 200x.

4.2. ALCAM serum levels are increased in PDAC

Since there are reports on secreted ALCAM(Faca et al., 2008), its serum levels were tested in a cohort of 21 healthy donors, 20 CP and 44 PDAC patients revealing significant (two fold) upregulation of soluble ALCAM in PDAC ($p<0.02$, Fig. 6).

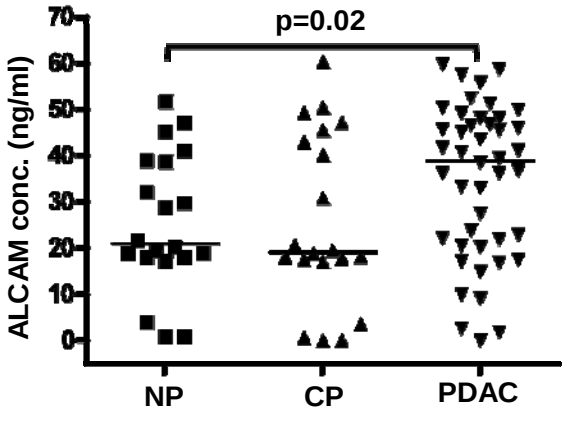

Figure 6: ALCAM serum levels of CP (n=20), PDAC (n=44) patients and healthy controls (n=21) determined by ELISA.

4.3. ALCAM is expressed in PDAC and PNET cell lines and is associated with cell growth, adhesion and chemosensitivity

To test whether our descriptive observations translated into ALCAM function in PDAC and PNET, we first analyzed its expression in cell lines demonstrating a heterogeneous pattern of ALCAM levels in cell lysates (mRNA levels: Fig. 7, protein levels (cell lysates, c/l and supernatants, s/n: Fig. 8) with the highest ALCAM expression levels in BxPC3, Colo357 and Su8686 whereas in MiaPaca2 cells, no ALCAM transcripts were found.

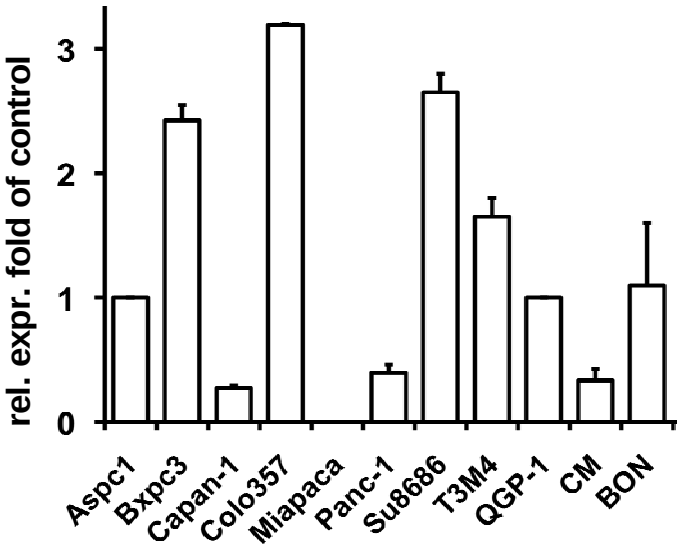

Figure 7: QRT-PCR analysis of ALCAM mRNA levels in PDAC and PNET cells.

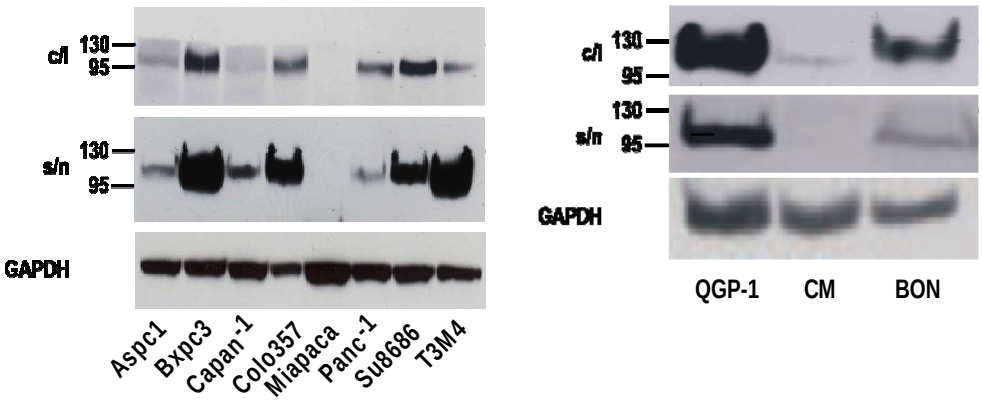

Figure 8: ALCAM protein levels revealed by immunoblot analysis in PDAC and PNET cell lysates (c/l) and supernatants (s/n).

Out of the 3 tested PNET cell lines, QGP-1 and BON transcribed and translated ALCAM while in CM low ALCAM mRNA levels and nearly no protein was found (Fig. 7 & 8).

Using RNAi, ALCAM expression was completely silenced in Su86.86 (PDAC cell line) and BON (PNET cell line) cells at all tested time points (48-120h, Fig. 9).

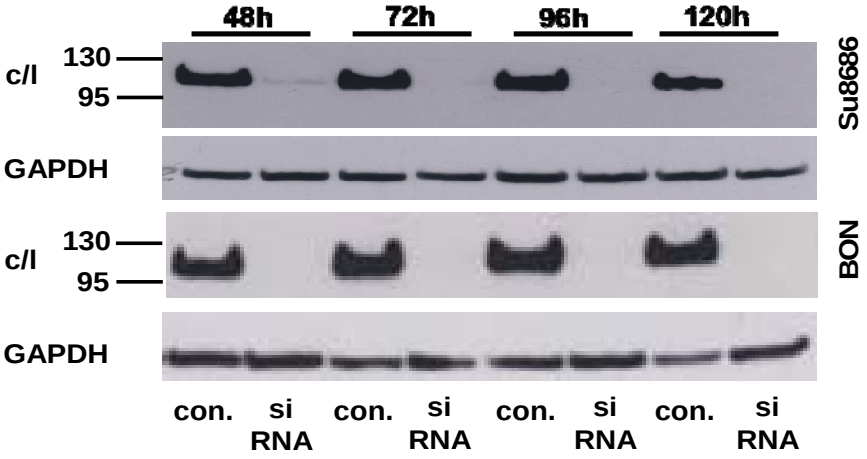

Figure 9: Effect of RNAi on ALCAM protein expression levels in Su 86.86 and BON cells (48 – 120h).

Following ALCAM RNAi, we assessed proliferation of Su86.86 cells showing no significant differences in growth compared to control RNAi-transfected cells (Fig. 10). Since cell-matrix and cell-base membrane contacts are crucial for normal epithelial function and since their disruption is thought to be a prerequisite for tumor cell invasion and potentially also for metastasis, we assessed whether ALCAM silencing effected invasion of Su86.86 cell in a Boyden chamber assay. Using this experimental setup, no differences in the number of invaded cells were observed following ALCAM RNAi (Fig. 10): however, ALCAM silencing significantly reduced Su86.86 cell adhesion (p=0.04, Fig. 10), but the reduced cell adhesion effect was not seen in the BON cell line (data not shown).

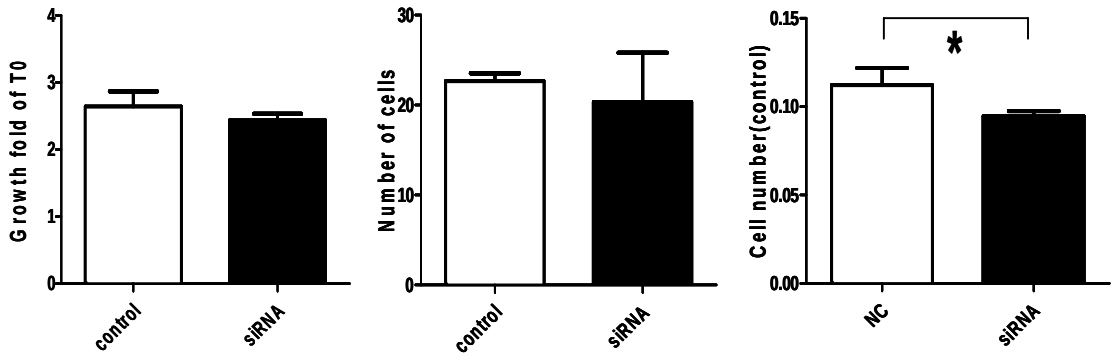

Figure 10: Silencing of ALCAM does not influence pancreatic cancer cell growth (left panel) or invasion (middle panel), but significantly reduces cell adhesion (right panel).

Given the fact that epithelial integrity influences chemosensitivity, chemotherapy with gemcitabine and actinomycin D was performed on Su86.86 cells in which ALCAM had been silenced. These experiments revealed a significant induction of chemoresistance in the ALCAM-silenced cells (gemcitabine: p=0.0429; actinomycin D: p=0.0041; Fig. 11).

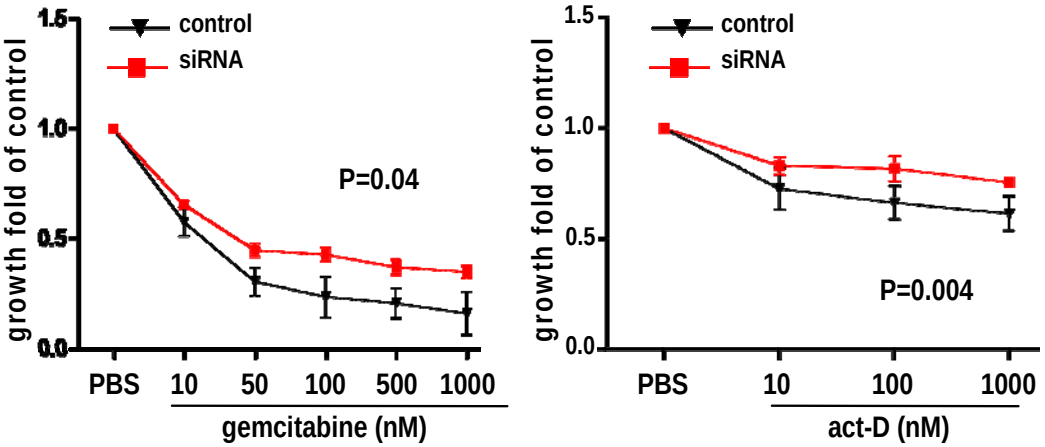

Figure 11: Silencing of ALCAM increases pancreatic cancer cell resistance towards chemotherapeutical agents - gemcitabine and actinomycin D.

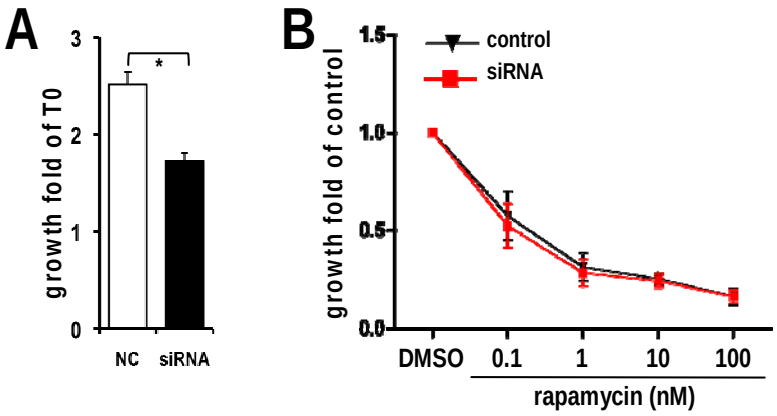

Figure 12: Silencing of ALCAM in PNET BON cells reduces cell growth (A), but has no effect in modulation of BON cell chemosensitivity (B)

In a next step, we assessed the effects of ALCAM RNAi on the growth of theneuroendocrine tumor cell line BON demonstrating that ALCAM silencing significantly reduced the growth of these cells (Fig. 12A). Interestingly and in contrast to the results obtained in the exocrine cancer cell lines, ALCAM RNAi was ineffective in modulating chemosensitivity towards rapamycin in the PNET BON cells (Fig. 12B).

5. Discussion

ALCAM is a cell adhesion molecule expressed by epithelial cells in several organs. It is located at sites of cell– cell contact and is associated intimately with adhesive structures that maintain the structural integrity of the epithelium in various organs. Cis-acting elements and transcription factors that regulate transcription of the ALCAM gene remain virtually unknown; however, emerging data implicate members of the Rel family in ALCAM gene transactivation and of AP-1 related factors in suppressing ALCAM expression. The most widely studied ALCAM function is dendritic cell-mediated T-cell activation and proliferation involving heterotypic interactions with CD6 expressed on T-cells.

ALCAM expression has been evaluated in melanoma, prostate carcinoma, breast cancer, colorectal carcinoma, bladder cancer, and esophageal squamous cell carcinoma. Other malignant cell lines and/or tumors have also been found to express ALCAM: the A-431 epidermoid carcinoma cell line (Northern blot), the HT-1080 fibrosarcoma cell line (Northern blot), 143B PML BK TK osteosarcoma cell line (weak expression by Northern blot), stomach tumors (dot blot analysis using an array), and lung cancer (immunohistochemistry). ALCAM is upregulated in some tumors and downregulated in others. This heterogeneity in different tumors is compounded by variable levels of ALCAM expression at different stages of tumor development in the same type of malignancies. Another source of confusion involves discordant data on the level of ALCAM RNA and protein in

breast cancer tissues; although most studies suggest that reduced ALCAM expression is a bad prognostic indicator in these malignancy. Clearly, additional research is needed to evaluate the role of ALCAM in each tumor type, to determine its interaction with other cellular intermediates, and to determine whether ALCAM is a therapeutic target to treat primary or metastatic tumors.

Immunohistochemical analysis revealed weak ALCAM positivity in ductal cells in the normal pancreas while on islet cells, a strong membrane staining was found. In cancer cells, different staining patterns were observed: while some cancer cells showed various intensities of ALCAM positivity in the cytoplasm, we found cancer cell membrane staining in a minority of slides (6/39 PDAC samples). We also analyzed ALCAM expression in neuroendocrine tumors, in which a minority of samples (6/16) was ALCAM-positive in the tumor cells with a predominant membrane staining.

The following survival analysis of semi-quantitatively evaluated ALCAM immunohistochemistries showed no significant influence of ALCAM on survival in PDAC. These findings are in contrast to other studies where i.e. in human melanocytic lesions, there were associations between high ALCAM expression and melanoma progression (van Kempen et al., 2000). While in low-grade prostate cancer (Gleason grade 1–3) upregulation of ALCAM expression and in high-grade tumors (Gleason grade 4 and 5) ALCAM downregulation was found(Kristiansen et al., 2003). In a colorectal carcinoma study, membranous

ALCAM expression correlated significantly with shortened patient survival. Burkhardt et al(Burkhardt et al., 2006) showed that high cytoplasmic ALCAM expression was associated with shortened patient disease-free survival in breast cancer. In our study, membrane staining of ALCAM was rare in the tested samples, and there was no significant correlation of membrane staining with patient's survival. In breast cancer showed that low levels of ALCAM transcripts in the primary breast tumor correlated with skeletal metastasis and poor prognosis(Davies et al., 2008). These equivocal results suggest that ALCAM function might be dependent on a) the malignant cell type analyzed, b) different tumor-stroma interactions present in various tumor entities and c) potentially also different (unknown) levels of proteases which might cleave ALCAM and could thus be "responsible" for the observed differences in ALCAM functions. In addition, de-regulations of the protein sorting machinery could be the cause for the differences in ALCAM localization between different cancers but also for differences within one particular tumor entitiy (i.e. in this case PDAC).

It is well known that the overexpression or de novo expression of ALCAM at the cell surface can promote cell adhesion and clustering(Uchida et al., 1997), but the observations suggested that delocalization of ALCAM from the cell membrane may be involved in reduced cell adhesiveness and possibly in metastatic behavior. The existence of natural sALCAM is known now, but no data on the biochemical characteristics and the origin of these forms are available. A soluble isoform of

ALCAM, generated by alternative splicing and consisting only of the single amino-terminal immunoglobulin like V1 domain, has been recently described as a promoter of endothelial cell migration and inhibitor of endothelial tube formation. However, the translation into sALCAM protein of this alternatively spliced mRNA by cells naturally expressing this transcript has not been shown. We thus tested its serum levels in a cohort of 20 healthy donors, 20 CP and 44 PDAC patients revealing significant upregulation of soluble ALCAM in PDAC ($p<0.02$). Analysis of the expression of ALCAM in PDAC and PNET cell lines demonstrated a heterogeneous pattern of ALCAM levels. Ombratta et al(Rosso et al., 2007) reported in their ovarian carcinoma study that ALCAM is released from EOC cells by a metalloprotease-dependent mechanism leading to the generation of a natural sALCAM. In our study, we did not test the mechanism of how the sALCAM was generated. Though in vitro ALCAM silencing significantly reduced Su86.86 cell adhesion ($p=0.04$), thus, different levels of proteases which might cleave ALCAM could thus be responsible for the observed differences in ALCAM functions. A reason for the discrepancy between ALCAM IHC and serum levels could be an increased shedding of the protein from the cancer cell membrane; alternatively, disturbed shuttling and anchoring of the protein within the membrane structure might be causative.

Blood constituents, notably plasma proteins, reflect diverse physiologic or pathologic states. The ease with which this compartment can be sampled makes it

a logical choice for screening applications to detect cancer at an early stage. However, the vast dynamic range of protein abundance in plasma and the likely occurrence of tumor-derived proteins in the lower range of protein abundance represent major challenges in the application of proteomic-based strategies for cancer biomarker identification (Hanash, 2003; Hanash et al., 2008). CA19–9 discriminates pancreatic cancer at the time of diagnosis well from healthy controls. CA19–9 levels were elevated in more than 80% of patients compared with healthy controls. However the sensitivity and specificity of CA19–9 in other settings relevant to pancreatic cancer, namely in discriminating between pancreatitis and pancreatic cancer and for detecting cancer at an early stage, are much reduced compared with its power to discriminate newly diagnosed pancreatic cancer and healthy individuals(Koopmann et al., 2006), hence the need for additional markers to constitute a panel with improved sensitivity and specificity for discriminating pancreatic cancer from pancreatitis and for detecting the disease at an early stage prior to onset of symptoms. Elevated levels of ALCAM, IGFBP4, LCN2, and WFDC2 in circulation in pancreatic cancer are novel findings. The panel of candidate markers together with CA19–9 significantly improved sensitivity and specificity in preclinical samples. In our study, we propose ALCAM as a novel serum biomarker in human pancreatic cancer which is associated with cell adhesion, growth and chemoresistance.

Interestingly there were striking differences in tumor cell behaviour comparing PDAC cells to PNET cells following ALCAM RNAi: ALCAM silencing had no effect on pancreatic cancer cell growth while it significantly reduced the growth of PNET cell lines, which is indicative of a minor importance of cell-cell contacts for in vitro pancreatic cancer cell growth; furthermore and since epithelial integrity is disturbed in cell culture, these findings might be a reflection of the model system used in our study.

The administration of cytotoxic agents for the treatment of advanced PDAC has been disappointing in terms of increasing survival and currently, research focuses on the understanding of molecular pathways in order to evaluate the role of targeted therapy, while trials on combinations of newer chemotherapeutic drugs in metastatic disease and adjuvant therapy of pancreatic cancer are ongoing. The goal of systemic chemotherapy is to minimize the patient's disease-related symptoms and to prolong survival. At this time, gemcitabine remains the recommended therapy for patients with advanced pancreatic cancer since its approval in 1996. It is a prodrug which requires cellular uptake and phosphorylation to active metabolites, which inhibit DNA chain elongation and lead to DNA fragmentation and cell death(Noble & Goa, 1997). Its approval was achieved in a phase III trial, in 126 patients who were randomized either to gemcitabine 1000 mg/m^2 weekly x7 followed by 1 week of rest, weekly x 3 every 4 weeks thereafter (63 patients), or weekly bolus 5-FU at a dose of 600 mg/m^2.

The primary end point was the clinical benefit. Clinical response was experienced in 23.8% of gemcitabine-treated patients compared with 4.8% of 5-FU-treated patients (P= .0022). The median overall survival durations were 5.65 and 4.41 months for gemcitabine-treated and 5-FU-treated patients (P = .0025) and the 1-year survival rate was 18% and 2% for the gemcitabine and 5-FU group, respectively(Burris et al., 1997). Since its approval, there has been enough effort to develop gemcitabine combinations for pancreatic cancer patients, which has failed to produce a significant overall survival benefit. In our study, chemotherapy with gemcitabine was performed on Su86.86 cells in which ALCAM had been silenced. These experiments revealed a significant induction of chemoresistance in the ALCAM-silenced cells.

Systemic therapies for carcinoid tumors have had limited antitumor efficacy to date. Since most carcinoid tumors highly express somatostatin receptors, treatment of gastrointestinal carcinoid tumors has largely involved somatostatin analogs or the combination of various chemotherapeutic agents. The antitumor efficacy of rapamycin and its analogs are being actively studied in many tumor types. mTOR has already been validated as a therapeutic target in advanced renal cell carcinoma. Rapamycin analog temsirolimus has been shown to improve overall survival among patients with metastatic renal cell carcinoma, leading to the FDA approval of temsirolimus. Although somatostatin analogs such as octreotide are generally well tolerated and effectively ameliorate many symptoms caused by

neuroendocrine tumors, their antitumor efficacy is limited(Dogliotti et al., 2001; Ricci et al., 2000; Tomassetti et al., 2000; Wymenga et al., 1999). Indeed, rapamycin analogs in combination with octreotide are undergoing clinical trials in neuroendocrine tumors with preliminary evidence of clinical activity. Interestingly, ALCAM RNAi was ineffective in modulating chemosensitivity towards rapamycin in the PNET BON cells in our study.

Actinomycin D (actD) is a potent inducer of apoptosis in a variety of cells in vitro and in vivo. It binds to DNA and inhibits RNA and protein synthesis. High doses (1 mg/ml) of actD block transcription of all RNA species, whereas low doses (100ng/ml) cause preferential inhibition of ribosomal RNA synthesis. A study from Kleeff et al(Kleeff et al., 2000) reported that actD may act via JNK/SAPK and Bax to promote apoptosis in PANC-1 cells and that it may inhibit the growth of other pancreatic cancer cell lines. Our study revealed a significant induction of chemoresistance in the ALCAM-silenced SU86.86 cells.

The observed differences in chemoresistance induction might be attributable to the tested chemotherapeutic substances (i.e. gemcitabine vs. rapamycin) and suggest that ALCAM might have a specific role in relation to gemcitabine. This is of particular importance since gemcitabine is the standard of care both in the palliative and adjuvant treatment of pancreatic cancer. Thus, the molecular mechanisms behind this observation should be further characterized.

In conclusion, we demonstrate in this study that ALCAM expression is de-regulated in pancreatic cancer and in pancreatic neuroendocrine tumors, that it is a serum biomarker in PDAC and that it is associated with neuroendocrine tumor growth and exocrine cancer chemoresistance. These findings lay the basis for in depth analyses of ALCAM protein processing, signaling and of ALCAM-mediated cell-cell and cell-matrix interactions in PDAC and PNET.

6. Summary

PDAC is a disease with a dismal prognosis and ranks 9th in the incidence of solid cancers and 4th for cancer-related deaths, with an overall median survival of around 4–6 months. The only cure is surgery; however, this option is available only to a maximum of 20% of the patients diagnosed with the disease. Even for these patients, median survival is less than 2 years, and only few patients survive significantly longer.

ALCAM - a cell adhesion molecule expressed by epithelial cells in several organs. It is expressed at sites of cell– cell contact and is associated intimately with adhesive structures that maintain the structural integrity of the epithelium in various organs, its expression and function has been evaluated in some desmoplasia including melanoma, prostate carcinoma, breast cancer, colorectal carcinoma, bladder cancer, and esophageal squamous cell carcinoma.

Using our microarray analyses, we demonstrate that the expression of activated leukocyte cell adhesion molecule (ALCAM, CD166) is altered in pancreatic ductal adenocarcinoma (PDAC). Furthermore, ALCAM was found to be increased in the sera of patients suffering from pancreatic cancer suggesting a role in pancreatic carcinogenesis.

We hypothesized that – due to its physiological function as a cell-cell contact protein – ALCAM might be involved in oncogenic transformation in both PNET and

PDAC and we propose ALCAM as a novel serum biomarker in human pancreatic cancer which is associated with cell adhesion, growth and chemoresistance.

Interestingly, the majority of the pancreatic cancer tissue samples tested showed low or no immunoreactivity for ALCAM which is in contrast to the findings in the patient sera. Immunohistochemical analysis revealing weak ALCAM positivity in ductal cells in the normal pancreas while on islet cells, a strong membrane staining was found. In cancer cells, different staining patterns were observed: while some cancer cells showed various intensities of ALCAM positivity in the cytoplasm, we found cancer cell membrane staining in a minority of slides. In the functional experiments, there were striking differences in tumor cell behaviour comparing PDAC cells to PNET cells following ALCAM RNAi. The observed differences in chemoresistance induction might be attributable to the tested chemotherapeutic substances (i.e. gemcitabine vs. rapamycin) and suggest that ALCAM might have a specific role in relation to gemcitabine. This is of particular importance since gemcitabine is the “standard of care” both in the palliative and adjuvant treatment of pancreatic cancer. Interestingly, ALCAM silencing had no effect on pancreatic cancer growth which is indicative of a minor importance of cell-cell contacts for in vitro pancreatic cancer cell growth; since the epithelial integrity is disturbed in cell culture in any way, these findings might be a reflection of the model system used in our study. However, the role of ALCAM in cancer is generally controversially discussed, these equivocal results suggest that ALCAM

function might be dependent on a) the malignant cell type analyzed, b) the different tumor-stroma interactions present in various tumor entities and c) potentially also different (unknown) levels of proteases which might cleave ALCAM and could thus be ‘responsible’ for the observed differences in ALCAM functions. In addition, de-regulations of the protein sorting machinery could be the cause for the differences in ALCAM localization between different cancers but also for differences within one particular tumor entitiy (i.e. in this case PDAC). In our study, we propose ALCAM as a novel serum biomarker in human pancreatic cancer which is associated with cell adhesion, growth and chemoresistance.

In conclusion, we demonstrate in this study that ALCAM expression is de-regulated in pancreatic cancer and in pancreatic neuroendocrine tumors, that it is a serum biomarker in PDAC and that it is associated with neuroendocrine tumor growth and exocrine cancer chemoresistance. These findings lay the basis for in depth analyses of ALCAM protein processing, signaling and of ALCAM-mediated cell-cell and cell-matrix interactions in PDAC and PNET. Clearly, additional research is needed to evaluate the role of ALCAM in each tumor type, to determine its interaction with other cellular intermediates, and to determine whether ALCAM is a therapeutic target to treat primary or metastatic tumors.

7. References

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9. ACKNOWLEDGEMENTS

I am deeply indebted to Professor Helmut Friess who gave me the opportunity to do my research training in the framework of a Medical Doctor degree at the Technische Universität Muenchen.

I am most grateful to Dr. Jörg Kleeff for his friendly supervision and the scientific support, helpful advices and mental support through the years.

Furthermore, I am most grateful to Dr. Christoph W. Michalski. He was encouraging me to develop new projects for better understanding the molecular mechanisms in pancreatic cancer pathogenesis. Moreover, he was deeply concerned with helping me to overcome any obstacles that I may have faced in the laboratory or in my social life.

I am most grateful to Dr. Bo Kong for the scientific and social support as well as his precious advices to complete, correct and conduct this thesis in an easy and understandable way.

I would like to thank all my colleagues, Dr. Zheng Pan, Dr. Danguole Sauliunaite, Dr. Tiago De Oliveira, Dr. Ivane Abiatari, Dr. Weibin Wang, Dr. Tamar Samkharadze, Dr. Simon Rieder, Dr. Kun Wang, Dr. Theresa Klose, Dennis Hedderich, Stephan Rath, Dr. Ekin Demir, Alexandra Boldis, Dr. Sylvia Streit, Dr. Christopher Nicolai, Dr. Charlotte Bruse, Dr. Mert Erkan, Carsten Jäger, Manja Thorwirth and Carmen Marthen for their kind support during my work.

Finally, I would also thank my Chinese colleagues and my family for their encouragement during these years of my oversea’s studies.