EEVA LAURILA

Novel Regulators of Pancreatic Cancer Cell Growth and Mobility

ACADEMIC DISSERTATION
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Basically, I'm not interested in doing research and I never have been.
I'm interested in understanding, which is quite a different thing.

– *David Blackwell*

YHTEENVETO

Haimasyöpä on harvinainen mutta erittäin aggressiivinen syöpätyyppi, johon sairastuu Suomessa vuosittain noin tuhat henkilöä. Vaikka haimasyövän osuus uusista syöpätapauksista on vain hieman yli 3%, se on kolmanneksi yleisin syöpäkuolemien syy niin miehillä kuin naisillakin. Haimasyövän ennuste on siis huono. Suurin syy tälle on se, että keinoja haimasyövän diagnosointiin ja hoitoon on todella vähän, ja hoidot ovat usein tehottomia. Tämän työn tarkoituksena oli tutkia haimasyövässä toistuvasti monistuvia kromosomialueita sekä kartoittaa ns. mikroRNA:iden ilmenemismuutoksia ja siten löytää uusia mahdollisia kohdegeenejä niin diagnostisiin kuin hoidollisiinkin tarkoituksiin.

Kromosomimuutokset ovat tyypillisiä suurimmalle osalle kiinteitä syöpäkasvaimia, kuten haimasyövälle. Toistuvasti monistuvilla kromosomialueilla sijaitsee suurella todennäköisyydellä geenejä, joilla on vaikutusta sairauden syntyyn, ja jotka siten voisivat toimia myös kohdegeeneinä syövän diagnoosissa tai hoidossa. Tässä työssä tutkittiin 7q21-q22 kromosomialueen monistumaa haimasyövässä ja pyrittiin siten löytämään uusia kohdegeenejä. Kyseistä monistumaa todettiin esiintyvän n. 25% sekä haimasyöpäsolulinjoista että haiman primäärikasvaimista, ja se johti useiden monistuman ydinalueella sijaitsevien geenien yli-ilmentymiseen. Jatkotutkimukset kohdistuivat kolmeen monistuneeseen geeniin, *ARPC1A*, *ARPC1B* ja *KPNA7*, ja osoittivat, että näiden toiminta vaikuttaa haimasyövän ominaisuuksiin.

ARPC1A ja *ARPC1B* koodaavat ARP2/3 proteiinikompleksin ARPC1-alayksikköä. ARP2/3 proteiinikompleksi toimii soluissa aktiinin polymerisaatiossa ja siten osaltaan säätelee solujen liikkumista. Näiden kahden geenin hiljentäminen haimasyöpäsoluissa vähensi huomattavasti solujen migraatiota ja invaasiota, todennäköisesti ARP2/3 proteiinikompleksin toiminnan häiriintymisestä johtuen. KPNA7 puolestaan kuuluu alfa-karyoferiinien proteiiniperheeseen ja toimii tumakuljetusreseptorina. *KPNA7*:n ilmentymisen hiljentäminen vähensi dramaattisesti haimasyöpäsolujen kasvua. Kasvun hidastuminen johtui p21-proteiinin lisääntymisestä ja siitä seuranneesta solusyklin osittaisesta pysähtymisestä

G1-vaiheeseen. Nämä tulokset viittaavat siihen, että 7q21-q22 monistuma-alueella ei olisi yhtä ainoaa kohdegeeniä, vaan ennemmin useamman geenin joukko, jotka kaikki ovat monistuneita ja yli-ilmentyneitä ja yhdessä vaikuttavat syöpäsolujen eri ominaisuuksiin. Sekä ARPC1 että KPNA7 toimivat tärkeissä solun toimintaan vaikuttavissa tehtävissä, ja ovat siten potentiaalisia uusien syöpähoitojen kohteita.

MikroRNA:t ovat lyhyitä yksijuosteisia RNA-molekyylejä, joilla on tärkeä tehtävä geenien ilmentymisen säätelyssä. Myös niiden ilmentyminen on usein häiriintynyt syövässä. Mikro-RNA:iden ilmentymistä tutkittiin haimasyöpä-solulinjoissa sekä normaalista haimasta peräisin olevissa näytteissä, ja tarkoituksena oli tunnistaa sellaiset mikro-RNA:t, joiden ilmentyminen oli muuttunut syövässä. Työssä löydettiin 72 mikro-RNA:ta, joiden ilmentymisen perusteella voitiin erottaa syöpänäytteet normaaleista näytteistä. Näiden joukosta valittiin miR-31 tarkempiin toiminnallisiin kokeisiin sen erityisen mielenkiintoisen ilmentymistavan vuoksi. Yllättäen sekä miR-31:n ilmentymisen estäminen että lisääminen johtivat haimasyöpäsolujen migraation vähenemiseen, mistä voidaan päätellä, että miR-31:n toiminnan kannalta ratkaisevaa on sen määrä solussa. Useissa tutkimuksissa miR-31:n on todettu olevan tärkeä solujen liikkumisen säätelijä monissa eri syövissä. Tämä tekee siitä erityisen houkuttelevan kohteen syövän etäpesäkkeisiin kohdistuville hoitomuodoille.

Yhteenvetona voidaan todeta, että tässä tutkimuksessa osoitettiin kolmella 7q21-q22 monistuman kohdegeenillä olevan onkogeenisiä ominaisuuksia haimasyövässä. Kaikki nämä kolme geeniä, *ARPC1A*, *ARPC1B* ja *KPNA7*, olivat yli-ilmentyneitä haimasyöpäsoluissa ja niiden toiminta osaltaan vahvisti näiden solujen pahanlaatuisia ominaispiirteitä. Lisäksi miR-31:n todettiin yli-ilmentyvän osassa haimasyövistä, ja sen osoitettiin olevan tärkeä syöpäsolujen liikkumisen säätelijä. Tämä tutkimus tarjoaa uutta tietoa haimasyövän patogeneesin geneettisestä taustasta.

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following communications, which are referred to in the text by their Roman numerals.

I **Laurila E**, Savinainen K, Kuuselo R, Karhu R, Kallioniemi A. Characterization of the 7q21-q22 Amplicon Identifies ARPC1A, a Subunit of the Arp2/3 Complex, as a Regulator of Cell Migration and Invasion in Pancreatic Cancer (2009). Genes Chromosomes Cancer 48:330-339.

II **Laurila E**, Savinainen K, Kallioniemi A. KPNA7, a nuclear transport receptor, promotes malignant properties of pancreatic cancer cells in vitro. Submitted.

III **Laurila E**, Sandström S, Rantanen LM, Autio R, Kallioniemi A. Both inhibition and enhanced expression of miR-31 lead to reduced migration and invasion of pancreatic cancer cells (2012). Genes Chromosomes Cancer 51:557-568.

ABBREVIATIONS

ABP	actin binding protein
APBB2	amyloid beta precursor protein-binding, family B, member 2
AR	androgen receptor
ARID1A	AT rich interactive domain 1A (SWI-like)
ARM repeat	armadillo repeat
ARP2/3 complex	actin related protein 2/3 complex
ARPC1-5	actin related protein complex, subunit 1-5
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
BSA	bovine serum albumin
BUD31	BUD31 homolog
CAS	cellular apoptosis susceptibility protein
CCND1	cyclin D1
CDK2	cyclin-dependent kinase 2
CDK4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
CDKN1A	cyclin-dependent kinase inhibitor 1A, p21
CDKN1B	cyclin-dependent kinase inhibitor 1B, p27
CDKN2A	cyclin-dependent kinase inhibitor 2A, p16
cDNA	complementary DNA
CGH	compratative genomic hybridization
DNA	deoxyribonucleic acid
dUTP	2'-deoxyuridine 5'-triphosphate
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
FAMMM	familial atypical multiple mole melanoma
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
GUSB	glucuronidase beta
HNPCC	hereditary nonpolyposis colorectal cancer
HPRT	hypoxanthine phosphoribosyltransferase 1
IBB domain	importin beta binding domain
KPNA1-7	karyopherin alpha 1-7
KPNB	karyopherin beta
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LATS2	large tumor suppressor, homolog 2

LUC	luciferase
MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5
MDM2	p53 E3 ubiquitin protein ligase homolog
miRNA	microRNA
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2
mRNA	messenger RNA
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1
MYC	v-myc myelocytomatosis viral oncogene homolog
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
NLS	nuclear localization signal
NPC	nuclear pore complex
NPF	nucleation promoting factor
NPTX2	neuronal pentraxin II
PAC	P1 derived artificial chromosome
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
PALB2	partner and localizer of BRCA2
PanIN	pancreatic intraepithelial neoplasia
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PDAP1	platelet-derived growth factor alpha associated protein 1
RHOA	ras homolog family member A
PPP2R2A	protein phosphatase 2, regulatory subunit B, alpha
PTCD1	pentatricopeptide repeat domain 1
PVDF	polyvinylidene difluoride
RAN	RAN, member RAS oncogene family
RanGTP	GTP-bound RAN
RDX	radixin
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RSBN1	round spermatid basic protein 1
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SMAD4	Sma- and Mad-related protein family member 4
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
SNP	single nucleotide polymorphism
TACC1	transforming, acidic coiled-coil containing protein 1
TACC2	transforming, acidic coiled-coil containing protein 2
TBP	TATA-box binding protein
TGF-β	transforming growth factor β
TMEM130	transmembrane protein 130
TP53	tumor protein 53
TRRAP	transformation/transcription domain-associated protein
WASP	Wiskott-Aldrich syndrome protein
WAVE3	Wiskott-Aldrich syndrome protein family, member 3
WCA	Wiskott-Aldrich homology domain 2, central region, acidic region

ABSTRACT

Pancreatic cancer is a rare but very aggressive malignancy affecting yearly approximately 1,000 individuals in Finland. Although it accounts only for a little over 3% of all new cancer cases, it is the third leading cause of cancer deaths for both genders. The main cause for the poor prognosis is the fact that the diagnostic and therapeutic tools for pancreatic cancer are truly limited and inefficient. This study aimed to characterize both recurrently amplified chromosomal regions as well as microRNA expression patterns in pancreatic cancer and thus to identify novel putative targets for diagnostic and therapeutic purposes.

Large chromosomal aberrations are typical for most solid tumors, including pancreatic cancer. Recurrently amplified regions are likely to contain genes which contribute to the development of the disease and might thus serve as targets for early detection or treatment of the disease. Here, a detailed characterization of the 7q21-q22 amplicon in pancreatic cancer was performed in order to identify novel target genes. The amplification was found to exist in 25% of both pancreatic cancer cell lines and primary tumors and to result in overexpression of several genes within the amplicon core. Further functional studies on three of the amplified genes, *ARPC1A*, *ARPC1B*, and *KPNA7* confirmed that these genes do contribute to the pathogenesis of pancreatic cancer.

ARPC1A and *ARPC1B* both encode for the ARPC1 subunit of the ARP2/3 protein complex which participates in actin polymerization and thus regulates cell mobility. Silencing of these two genes in pancreatic cancer cells resulted in a significant reduction of cell migration and invasion, presumably due to defective function of the ARP2/3 complex. KPNA7 belongs to the karyopherin alpha protein family of nuclear import receptors. Silencing of *KPNA7* expression dramatically decreased the growth of pancreatic cancer cell lines via a p21 induced G1 arrest. These data suggest that rather than a single target gene, the 7q21-q22 amplicon contains a set of genes which are all amplified and overexpressed and together

contribute to different features of the cancer cells. Both ARPC1 and KPNA7 have important cellular functions and might serve as potential novel therapeutic targets.

MicroRNAs are short single-stranded RNA molecules which have a crucial role in regulating gene expression, and are also widely misregulated in cancer. The expression levels of miRNAs in a panel of pancreatic cancer cell lines and normal samples were screened to identify miRNAs that are aberrantly expressed in pancreatic cancer. A set of 72 differentially expressed miRNAs was found to provide a molecular signature discriminating the cancer and normal samples. Of these, miR-31 was further functionally studied based on its unique on-off expression pattern. Interestingly, both inhibiting and inducing miR-31 expression decreased the migration of pancreatic cancer cells, indicating that not only the presence but also the amount of miR-31 is important for its function. The role of miR-31 as a regulator of cancer cell mobility has also been established in various other cancers, making it a tempting target for anti-metastasis therapy.

To conclude, three target genes of the 7q21-q22 amplicon, *ARPC1A*, *ARPC1B*, and *KPNA7* were shown to have oncogenic properties in pancreatic cancer, as they were all overexpressed and promoted the malignant properties of the disease. Furthermore, miR-31 was shown to be overexpressed in a subset of pancreatic cancers and to regulate cancer cell mobility. Overall, this study provides novel information on the genetic background of pancreatic cancer pathogenesis.

INTRODUCTION

Cancer is a large heterogeneous class of diseases of uncontrollable cell overgrowth. Common to all these diseases is that they all arise from accumulation of genetic alterations in the cells (Vogelstein and Kinzler 2004, Chin and Gray 2008, Stratton et al. 2009). A certain number of genetic changes need to occur for a normal cell to become a cancer cell, and a number of additional changes are needed for that single cancer cell to be able to grow to a large tumor, and later on to invade into surrounding tissues. This process, which leads to the transformation of a normal cell to a malignant tumor, starts with a series of random mutations. Most of these mutations are either irrelevant or lethal, but some alterations may be beneficial for the cell. Accumulation of advantageous mutations in a single cell may lead to clonal expansion and eventually development of cancer. The genes involved in tumorigenesis have been traditionally classified in three groups: oncogenes, tumor suppressor genes and stability genes (Vogelstein and Kinzler 2004). Oncogenes and tumor suppressors are genes which in normal cells regulate cell growth - oncogenes can be described as the gas pedal of the tumor and tumor suppressors as the brakes. The third class is so-called stability genes, which do not directly contribute to the tumor growth but are needed to maintain genomic integrity, and thus mutations in them leads to increased mutation rate in other genes. Moreover, additional genes have been identified, which do not fit into any of these classes but do play a role in tumorigenesis by for example enabling the formation of new blood vessels. As the result of these genetic mutations, initially normal cells acquire advantageous characteristics, eventually transforming them to malignant cancer cells (Vogelstein and Kinzler 2004, Chin and Gray 2008, Stratton et al. 2009).

In two famous reviews, Hanahan and Weinberg specified the hallmark properties of cancer cells which distinguish them from normal cells (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). Probably the most characteristic feature of cancer cells in contrast to normal cells is their ability to divide and grow unlimitedly. In normal tissue, cell growth is strictly controlled but cancer cells have

become independent of external growth factors and also are able to ignore anti-proliferative signals. Mutations in members of the growth control pathways, such as KRAS, may lead to continuous downstream signaling, even with no stimulus from outside the cell. Similarly, the pathways mediating the antigrowth signals, such as the Rb pathway, are usually defective. Furthermore, the lifespan of a normal cell is restricted and they can undergo only a limited number of cell divisions. In cancer cells the telomers used as the "counting mechanism" are faulty and the cells can continue dividing endlessly. Cancer cells have also acquired the ability to avoid apoptosis, and so the normal balance between cell division and cell death is lost. In order to the single cancer cell to evolve to a metastasizing tumor, it has to gain additional features to be able to invade into surrounding tissues and eventually also to metastasize to other organs (Hanahan and Weinberg 2000). The ability to activate the invasion- metastasis cascade is the most distinctive feature of malignant cells, and in fact, the major difference between malignant and benign tumor cells (Lazebnik 2010, Hanahan and Weinberg 2011). Furthermore, to continue growing the malignant tumors also have to acquire mechanisms for example to avoid immune destruction (Hanahan and Weinberg 2011).

Pancreatic cancer is an extreme aggressive malignancy, which typically is diagnosed at late stages when the disease has already gained all the hallmarks described above, and is not curable anymore (Bilimoria et al. 2007, Hidalgo 2010, Bond-Smith et al. 2012). Despite enormous efforts, no effective therapeutic targets or tools for early diagnosis have been found. This study was carried out to increase the understanding of pancreatic cancer genetics. Characterization of a specific chromosomal amplification and microRNA expression patterns were performed to identify novel target genes which might be eventually used as diagnostic, prognostic and therapeutic tools for pancreatic cancer.

REVIEW OF THE LITERATURE

1. Pancreatic cancer

1.1. Epidemiology and clinical factors

Pancreatic cancer is one of the deadliest malignancies in Finland as well as the rest of the western world. In 2010, there were a little over 1,000 new pancreatic cancer patients in Finland (Finnish Cancer Registry, http://www.canceregistry.fi). The age-adjusted incidence rates (new cases per 100,000 individuals) of pancreatic cancer in Finland were 6.7 for females and 9.6 for males, making it the ninth and tenth most common cancer in females and males, respectively. The corresponding mortality rates were 6.2 for females and 9.4 for males, which adds up to approximately 1,000 deaths per year, and results in pancreatic cancer being the third most common cause of cancer deaths for both genders (Finnish Cancer Registry, http://www.canceregistry.fi). These rates are very similar to those in other western countries (Bilimoria et al. 2007, Siegel et al. 2012).

The 5-year survival of pancreatic cancer is only a little over 5% and the median survival less than 6 months, and there has been very little improvement in the survival rates for the last decades (Bilimoria et al. 2007, Siegel et al., 2012). The extremely poor prognosis is mostly due to rapid and aggressive progression of the disease and lack of methods for early detection, which result in pancreatic cancer typically not being detected until late stages of the disease where no curative treatment is available. More than 50% of the pancreatic cancers are at stage IV (metastasized cancer) at the time of diagnosis (Bilimoria et al. 2007). Moreover, although the patients with locally advanced disease have a slightly better prognosis when compared to those with metastasized cancer, the survival rates are still rather poor (median survival 10 vs. 2.5 months, respectively) (Bilimoria et al. 2007).

Early diagnosis of pancreatic cancer remains a challenge since the first symptoms of the disease typically are non-specific, including weight loss, abdominal pain, and

nausea (Hidalgo 2010, Bond-Smith et al. 2012). Even the more severe symptoms, such as abnormalities in the liver function, appear usually not until the later stages, and are dependent on the location of the tumor (Hidalgo 2010). The majority of the tumors are located at the head of the pancreas (Bilimoria et al. 2007) and often cause jaundice because the bile duct is blocked (Hidalgo et al. 2010).

Treatment methods of pancreatic cancer are rather limited and also widely dependent of the disease stage. Tumors either localized within the pancreas (stage I) or locally invasive tumors (stage II) are usually resectable whereas locally advanced (stage III) and metastasized (stage IV) tumors cannot be operated. Since most of the patients are diagnosed with advanced disease, only 15% of patients are eligible for surgery (Bilimoria et al. 2007, Hidalgo 2010, Bond-Smith et al. 2012). Operative treatment options include several types of pancreatectomy (surgical removal of the entire or part of the pancreas), the most common of those being pancreatoduodenectomy or "the Whipple procedure" where the head of the pancreas is removed along with parts of the stomach, duodenum, bile duct and the gall bladder (Bond-Smith et al. 2012). Both chemo- and radiotherapy are commonly used with or without surgery, but unfortunately pancreatic cancer is widely resistant to these (Hidalgo 2010, Bond-Smith et al. 2012). For advanced pancreatic cancer, the treatment is mostly palliative (Hidalgo 2010) and the need for novel therapeutic options is evident. Targeted molecular therapy by delivery of drugs targeting specific proteins or pathways is used, and several novel drugs are in clinical trials (Herreros-Villanueva et al. 2012). Also immune therapy by boosting the patient's immune system is already partially in use, and pancreatic cancer vaccines are currently studied in clinical trials (Koido et al. 2011).

1.2. Risk factors and genetic predisposition

Since early detection of pancreatic cancer is challenging, efforts have been made to identify those individuals with higher risk for the disease. Screening of the high-risk individuals might result in the diagnosis being made at earlier stages when the disease is still curable (Sakorafas et al. 2012). Several risk factors, both environmental and genetic, have been identified, but still the challenge remains

mainly unsolved. Medical history plays a major role since some diseases have been proven to be associated with increased risk for pancreatic cancer. Patients with chronic pancreatitis have up to 13 times increased risk of pancreatic cancer (Lowenfels et al. 1993, Lowenfels and Maisonneuve 2004, Hassan et al. 2007, Raimondi et al. 2009) and diabetes has been proven to at least double the risk of pancreatic cancer (Chari et al. 2005, Hassan et al. 2007, Maisonneuve et al. 2010). Environmental factors have been widely studied but only few have been shown to actually cause increased risk for pancreatic cancer. Cigarette smoking is known to be a major risk factor for pancreatic cancer, as well as many other malignancies (Hassan et al. 2007, Bond-Smith et al. 2012, Pandol et al. 2012). Heavy alcohol consumption can increase the risk for pancreatic cancer and alcohol drinking is also associated with chronic pancreatitis (Lowenfels and Maisonneuve 2004, Hassan et al. 2007). Dietary factors have been studied but no association has been confirmed (Lowenfels and Maisonneuve 2004). However, obesity does increase the risk for pancreatic cancer (Bond-Smith et al. 2012). Finally, the risk for pancreatic cancer strongly increases with age and the majority of the patients are elderly (Bardeesy and DePinho 2002, Finnish Cancer Registry 2012).

Approximately 10% of all pancreatic cancers have an underlying hereditary component, either a germline mutation or another disorder which results in an increased risk of developing cancer (Bardeesy and DePinho 2002, Lowenfels and Maisonneuve 2004, Raimondi et al. 2009, Shi et al. 2009). Some of these factors are associated with a hereditary genetic syndrome, which predisposes the affected person to different tumor types. Hereditary syndromes associated with pancreatic cancer include Peutz-Jeghers syndrome (Giardiello et al. 2000), hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (Kastrino et al. 2009), hereditary pancreatitis (Lowenfels et al. 1997), hereditary breast and ovarian cancer (The Breast Cancer Linkage Consortium 1999), familial atypical multiple mole melanoma (FAMMM) (Vasen et al. 2000, Bartsch et al. 2002, Goldstein et al. 2004), familial adenomatous polyposis (FAP) (Giardiello et al. 1993), Li-Fraumeni syndrome (Kleihues et al. 1997) and cystic fibrosis (Maisonneuve et al. 2007). The relative risk of pancreatic cancer in these syndromes vary from only a slightly increased risk of hereditary breast and ovarian cancer syndrome to more than 100-fold risk in Peutz-Jeghers syndrome (Hruban et al. 2010, Klein 2012). The genes affected in these syndromes are typically tumor suppressor genes, such as *CDKN2A*

in FAMMM, *BRCA1*, *BRCA2*, and *PALB2* in hereditary breast and ovarian cancer and DNA mismatch repair genes *MLH1* and *MSH2* in HNPCC (Erkko et al. 2007, Raimondi et al. 2009, Klein 2012). However, the majority of the pancreatic cancers, which appear to be of familial background, are due to factors that are still unknown (Raimondi et al. 2009, Shi et al. 2009, Klein 2012).

1.3. Pathology of pancreatic cancer

The pancreas is a gland located in the upper abdomen, between the stomach and the small intestine. The pancreas can be divided into two separate compartments: the endocrine and the exocrine pancreas, which have completely separate functions (Bardeesy and DePinho 2002, Hezel et al. 2006, Balic et al 2010). Exocrine pancreas accounts for the vast majority (80%) of the organ mass and appears as a branched network of acinar and duct cells. Acinar cells which produce multiple digestive enzymes, are organized into clusters at the end of ducts and cover the majority of the exocrine pancreas. Ductal cells form the pancreatic duct network, add mucous and bicarbonate into the enzyme mix and finally merge into the main pancreatic duct releasing the enzymes into the duodenum (Bardeesy and DePinho 2002, Balic et al. 2012). Endocrine pancreas exists as cell clusters called the Islets of Langerhans, which are embedded in the exocrine pancreas. The islets consist mainly of α- and β-cells which secrete insulin and glucagon, hormones responsible for regulating glucose metaboly (Bardeesy and DePinho 2002, Balic et al. 2010).

Pancreatic cancer is a group of different tumor types, originating from both endocrine and exocrine parts of the pancreas. However, usually the term refers to pancreatic ductal adenocarcinoma (PDAC), which is the most common malignancy of the pancreas accounting for 85% of all pancreatic cancers and more than 90% of malignancies of the exocrine pancreas (Winter 2006, Bond-Smith et al. 2012, Samuel and Hudson 2012). Other, more uncommon types of pancreatic cancers of exocrine origin are for example acinar cell carcinomas and intraductal papillary mucinous neoplasms (Bond-Smith et al. 2012). Cancers arising from the endocrine pancreas, such as insulinomas and gastrinomas are far more rare and represent completely different tumor types (Bond-Smith et al. 2012). In this study, the term pancreatic cancer is used to refer to pancreatic ductal adenocarcinoma.

2. Genetic changes in pancreatic cancer

2.1. Progression model of pancreatic cancer

Development of pancreatic cancer is a stepwise process. A progression model for pancreatic cancer development was first suggested a little over ten years ago (Hruban et al. 2000) and has been later on shown to be rather accurate. The model describes how the normal pancreatic epithelial cells gradually accumulate genetic changes in crucial genes and eventually progress to invasive carcinoma through a series of intermediate stages (Hruban et al. 2000) (Figure 1). These stages, pancreatic intraepithelial neoplasias (PanINs) are microscopic, histologically separable, and are graded 1-3, according to increasing histological abnormalities (Hruban et al. 2000, Hezel et al. 2006, Koostra et al. 2008). Numerous genetic changes in key cancer genes have been associated with specific stages of the pancreatic cancer progression model. The most common genetic alterations have been shown to take place already in the earliest PanIN lesions (Kanda et al. 2012).

The *KRAS* oncogene is the most commonly altered gene in pancreatic cancer, being mutated in 95% of the cases, most often in codon 12 (Almoguera et al. 1988, Hruban et al. 1993, Maitra et al. 2006, Jones et al. 2008). *KRAS* has been found to be mutated already in over 90% of the PanIN lesions, indicating that *KRAS* mutation is one of the earliest genetic events in pancreatic carcinogenesis (Feldmann et al. 2007, Kanda et al. 2012). These mutations in *KRAS* make it permanently active, leading to the activation of multiple molecular pathways, such as the RAF/ERK pathway which has a major role in transcriptional regulation, and the PI3K pathway which is involved for instance in cell cycle progression (Malumbres and Barbacid 2003, Hezel 2006, Mihaljevic et al. 2010). Moreover, pathways downstream of KRAS are often mutated when KRAS itself is not affected (Jones et al. 2008, Hong et al. 2011). In addition, overexpression of the epidermal growth factor receptor *ERBB2*, often due to gene amplification, is a frequent early event in pancreatic cancer (Hruban et al. 2000).

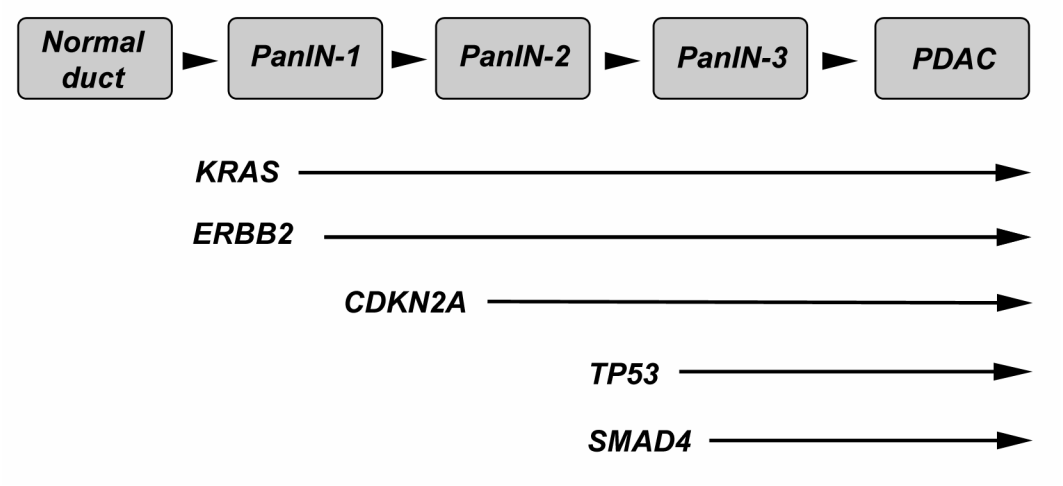

Figure 1. The stepwise progression model of pancreatic cancer. The genetic changes gradually accumulate during the progression from normal duct via PanIN lesions to pancreatic ductal adenocarcinomas (PDAC).

Besides the *KRAS* mutation and *ERBB2* activation, three tumor suppressor genes are commonly inactivated at the early stages of pancreatic cancer development, resulting in the loss of cell cycle control (Ottenhof et al. 2011). Of these, *p16/CDKN2A* (*INK4A*) is altered, either deleted, mutated or hypermethylated, in more than 95% of pancreatic cancers and often already in PanIN-2 lesions (Caldas et al. 1994, Schutte et al. 1997, Ueki et al. 2000, Ottenhof et al. 2011). The p16 protein plays an important role in the negative regulation of the cell cycle as it binds to the cyclin dependent kinases CDK4 and CDK6 and thus inhibits progression of the cell cycle at the G1/S checkpoint. In cells where p16 is lost, the G1/S transition of the cell cycle is not properly regulated which may lead to uncontrolled cell growth (Mihaljevic et al. 2010, Ottenhof et al. 2011). Another very commonly inactivated gene is *TP53* which is altered in at least 50% of pancreatic cancers, typically by mutations in the DNA binding domain (Rozenblum et al. 1997). The p53 protein is often described as “the guardian of the genome”, highlighting its crucial role in the cell. It prevents the cell from continuing the cell cycle with damaged DNA, and loss of p53 might be one of the reasons for the vast genomic instability commonly observed in pancreatic cancer (Vogelstein and Kinzler 2004, Mihaljevic et al. 2010). Third tumor suppressor commonly inactivated in pancreatic cancer is *SMAD4/DPC4*, which is non-functional in 55% of the cases, typically in

later stages of carcinogenesis. SMAD4 acts in the TGF-β pathway which regulates normal cell growth and loss of SMAD4 may thus lead to uncontrolled growth (Hahn et al. 1996, Wilentz et al. 2000, Iacobuzio-Donahue et al. 2004). Mutations of *TP53* and *SMAD4* are typically found in the PanIN-3 lesions which are already in transition to invasive growth (Ottenhof et al. 2011).

In the last decade, several mouse models of pancreatic cancer have been produced by using this knowledge of the genetic changes behind the disease (Herreros-Villanueva et al. 2012). Since KRAS is mutated in almost all pancreatic cancers, it is usually the basis of the transgenic models, accompanied by mutations or deletions of different genes, including *SMAD4* and *TP53*. These mice show a variety of pancreatic cancer phenotypes, many of those progressing via PanIN lesions to invasive and metastatic cancer (Herreros-Villanueva et al. 2012).

2.2. Chromosomal aberrations

In addition to mutations affecting single genes, large chromosomal aberrations, such as amplifications and deletions, are also very common in pancreatic cancer. Pancreatic cancer karyotypes are usually extremely complex, with enormous amount of gains, losses and translocations (Bardeesy and DePinho 2002, Karhu et al. 2006, Samuel and Hudson 2012). For example, Kowalski et al. (2007) reported several cases where the tumor cells had a total of over 70 chromosomes, with some chromosomes being presented in four or five copies, some completely absent and many having abnormal structures. Telomere shortening as well as mutations in the key regulators of the cell cycle are likely to be the main contributors to the formation of these chromosomal rearrangements (Hezel et al. 2006, Campbell et al. 2010).

Most of the chromosomal changes are considered to be totally random and solely reflect the genetic instability of the disease (Bardeesy and DePinho 2002). However, some chromosomal changes have been shown to be recurrent, suggesting that genes in these regions might play a crucial role in the disease pathogenesis (Hezel et al. 2006, Samuel and Hudson 2012). To identify such genes, different array based methods have been used to search for recurrent genomic gains and losses in pancreatic cancer. In addition to the chromosome and array CGH (comparative

genomic hybridization) studies, also SNP (single nucleotide polymorphism) arrays and sequencing technologies have been utilized (Karhu et al. 2006, Samuel and Hudson 2012). Based on these analyses, the most common chromosomal aberrations in pancreatic cancer are the losses at the chromosomes 6q, 9p, 13q, 17p, and 18q, and gains at the chromosomes 7q, 8q, 11q, 17q, and 20q (Karhu et al. 2006, Campbell et al. 2010, Gutiérrez et al. 2011, Samuel and Hudson 2012). Frequencies of these aberrations vary largely from one study to another (~15-90%), depending on the platform and the sample set. Overall, losses appear to be more frequent than gains, and gains typically cover smaller regions (Karhu et al. 2006, Gutiérrez et al. 2011). However, all the aberrations mentioned above have been recurrently observed both in pancreatic cancer cell lines and primary pancreatic tumors.

Chromosome 7q is one of the regions recurrently amplified in pancreatic cancer. High-level amplifications of the 7q21-q22 locus have been observed in several array-based CGH studies (Aguirre et al. 2004, Heidenblad et al. 2004, Holzmann et al. 2004, Mahlamäki et al. 2004, Bashyam et al. 2005, Gysin et al. 2005, Loukopoulos et al. 2007, Suzuki et al. 2008). However, whereas the 8q amplifications for example often target the *MYC* oncogene located at 8q24, the exact targets of the 7q21-q22 amplification were not revealed by the array studies. During the course of this study, several putative target genes were identified and further studied, these including *ARPC1A* and *ARPC1B* which are two genes encoding a subunit of a protein complex involved in actin polymerization, and a novel nuclear import receptor *KPNA7*.

Selective increase or decrease of the copy number of a given gene is an efficient way of altering its activity (Schwab 1999, Albertson et al. 2006, Myllykangas and Knuutila 2006). However, cells do not have a proficient way of actually selecting the gene to be amplified or deleted, making the event random. Most of these gains or losses just disappear, but some can give the cell a growth advantage and are thereby preserved (Albertson et al. 2003). For example, deletions of the tumor suppressors *CDKN2A* and *SMAD4* and amplifications of the oncogene *MYC* are beneficial for the cell and thus selectively maintained, making them common findings in pancreatic cancer (Jones et al. 2008, Samuel and Hudson 2012).

Since amplicons often cover large chromosomal regions, they usually contain many genes which are co-amplified and subsequently overexpressed. However, all of them do not necessarily contribute to the formation of the tumor and are therefore

considered as so-called passenger genes. Identification of the driver genes, or amplification target genes, which actually promote the tumor development, is challenging, since one amplicon may indeed contain dozens of genes which are all amplified and overexpressed (Copeland and Jenkins 2009, Stratton et al. 2009, Bell 2010, Santarius et al. 2010, Eifert and Powers 2012). It is also known that the size of the amplified region commonly varies from one tumor to another. Therefore it is crucial to identify the minimal region of amplification, also known as the amplicon core, which is amplified in most of the samples and thus most likely to hold the critical gene (Samuel and Hudson 2012). In an excellent review, Santarius et al. (2010) listed 77 amplicon target genes that are likely to have a causal role in cancer. Genes were divided in three separate classes based on the amount of evidence on their contribution to cancer. All genes summarized in the article are located at the minimal region of amplification and also overexpressed. However, hundreds of such genes have been identified in genome-wide screens, and thus the 77 putative target genes listed in the article were required to have further evidence for causative role in cancer. Additional criteria for classification were clinical correlation (expression associated with with clinical outcome), knowledge of the gene or the pathway (for example, other genes in the same pathway also amplified or mutated), biological evidence (overexpression or knockdown causes biological effect), and supporting results from animal studies. Three genes, *ERBB2*, *EGFR* and *AR*, were categorized as Class I genes, which have the strongest evidence for their involvement in tumorigenesis. Most of the 77 genes had evidence of causal role only in one malignancy and very few genes, such as *ERBB2* and *CCND1*, were listed in more than one cancer type. For pancreatic cancer, three genes, *ARPC1A*, *SMURF1*, and *MED29*, were on the list (Santarius et al. 2010). One has to keep in mind that as the knowledge of cancer cell characteristics increases (Hanahan and Weinberg 2011), also further criteria for defining amplicon target genes could become important to consider.

Despite the above mentioned criteria, functional characterization of the amplified genes is absolutely essential to establish which genes actually do have a role in tumor development. Knockdown of amplified genes in overexpressing cells and on the other hand, forced expression of the same genes in non-expressing cells, followed by studies on the functional consequences of the abnormal expression, is an efficient way to separate driver genes from passengers (Santarius et al. 2010,

Eifert and Powers 2012). For example, cell proliferation, anchorage independent growth and cell migration and invasion are typical features which are altered after manipulation of the expression of an amplification target gene.

2.3. High-throughput sequencing data

Modern whole-exome sequencing studies provide valuable information on the genetic changes in pancreatic cancer and are able to reveal both mutations and larger chromosomal changes such as amplifications and deletions. Large-scale analyses of several tumors give insight into the frequency of a given mutation and enable the evaluation of its significance (The International Cancer Genome Consortium 2010, Iacobuzio-Donahue et al. 2012). The first large-scale sequencing study concerning pancreatic cancer was done by Jones et al. (2008) and reported the sequencing of all exons of protein-coding genes in 24 advanced pancreatic adenocarcinomas. An average of 63 genetic alterations was found in these cancers, most of which were point mutations. Alterations in the key genes, such as *TP53*, *CDKN2A*, *SMAD4* and *KRAS* were commonly found, but the study also revealed a number of less common mutations, the relevance of which remains to be solved. However, rather than single genes, the mutations were found to accumulate in twelve key pathways or cellular processes, such as apoptosis, regulation of the cell cycle or KRAS signaling, that have been implicated in cancer (Table 1). Six of these pathways were affected in all 24 samples and the rest in at least 67% of the cases.

Later on, similar studies with different sample sets and study designs have been performed (Jones et al. 2009, Wu et al. 2011, Roberts et al. 2012). In a recent study both tumor and germline DNA of one individual (patient with familial pancreatic cancer from the study by Jones et al. 2008) were sequenced, and the *PALB2* gene (partner and localizer of BRCA2, functions as a tumor suppressor gene) was found to be recurrently mutated already in the germline, resulting in higher risk for pancreatic cancer, as well as other malignancies (Jones et al. 2009). Furthermore, germline *ATM* deletions (ataxia telangiectasia mutated, a cell cycle checkpoint kinase) were found from 2 of 16 pancreatic cancer families (Roberts et al. 2012). Also pancreatic cysts and pre-neoplastic lesions have been screened and an average of ten mutations per sample was found to exist, giving valuable knowledge of the

early events of pancreatic cancer development. In fact, some of the genetic alterations typical for late stage pancreatic cancer, such as KRAS mutations, were found already in these early lesions, supporting the stepwise progression model of the disease (Wu et al. 2011). In the future, tumor genome sequencing might become a standard for pancreatic cancer patients, and the treatment of the disease may depend on the genetic alterations of the tumor.

Table 1. *Core signaling pathways or cellular processes involved in pancreatic cancer. Adapted from Jones et al. 2008.*

Pathway or process	Examples of altered genes
Apoptosis [1]	*CASP10, VCP, CAD, HIP1*
Control of the G1/S phase transition [1]	*CDKN2A, FBXW7, CHD1, APC2*
DNA damage control	*ERCC4, ERCC6, EP300, RANBP2, TP53*
Hedgehog signaling [1]	*TBX5, SOX3, LRP2, GLI1, GLI3, BOC, BMPR2, CREBBP*
Homophilic cell adhesion	*CDH1, CDH10, CDH2, CDH7, FAT, CDH15, PCDH17, PCDH18, PCDH9, PCDHB16, PCDHB2, PCDHGA1, CDHGA11, PCDHGC4*
Invasion	*ADAM11, ADAM12, ADAM19, ADAM5220, ADAMTS15, DPP6, MEP1A, PCSK6, APG4A, PRSS23*
Integrin signaling	*ITGA4, ITGA9, ITGA11, LAMA1, LAMA4, LAMA5, FN1, ILK*
JNK signaling	*MAP4K3, TNF, ATF2, NFATC3*
KRAS signaling [1]	*KRAS, MAP2K4, RASGRP3*
Small GTPase signaling (non-KRAS)	*AGHGEF7, ARHGEF9, CDC42BPA, DEPDC2, PLCB3, PLCB4, RP1, PLXNB1, PRKCG*
TGF-β signaling [1]	*TGFBR2, BMPR2, SMAD4, SMAD3*
Wnt/Notch signaling [1]	*MYC, PPP2R3A, WNT9A, MAP2, TSC2, GATA6, TCF4*

[1] Pathway was altered in all 24 samples

2.4. MicroRNAs

MicroRNAs (miRNAs) are short (~22 nucleotides) non-coding RNA molecules which have a crucial role in post-transcriptional regulation of gene expression (He and Hannon 2004, Bartel 2009, Krol et al. 2010, Pritchard et al. 2012). To date, 1600 miRNA precursors and more than 2000 mature miRNAs have been identified and the number has been constantly increasing (miRBase release 19,

http://www.mirbase.org/). Each miRNA can regulate dozens or even hundreds of genes, affecting the activity of entire pathways and networks, and one gene can have up to 50 miRNA binding sites (Gunaratne et al. 2010, Pritchard et al. 2012). Identification of miRNA target genes remains a challenge due to the small size of miRNAs and the fact that only partial complementarity is needed between the miRNA and its target (Bartel 2009, Krol et al. 2010). A number of softwares have been developed to predict putative miRNA targets but all of those produce both false negative and false positive results, and thus experimental verification of the predicted targets is always needed (Bartel 2009, Iorio and Croce 2012).

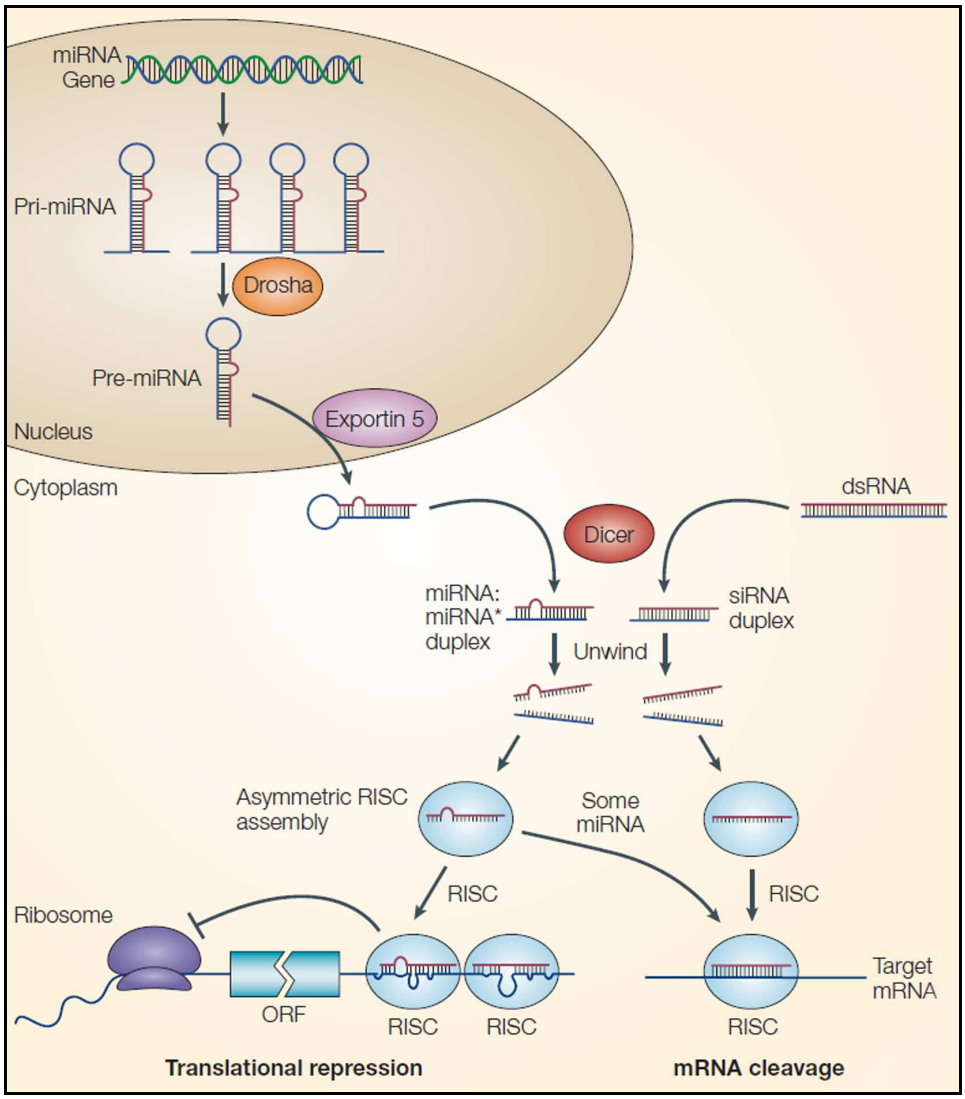

Figure 2. MicroRNA biosynthesis. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, He and Hannon, 2004.

MicroRNA biogenesis begins in the nucleus where the miRNA is transcribed by RNA polymerase (usually RNA polymerase II) into a long (up to several kilobases) hairpin-structured primary molecule called pri-miRNA which may hold several miRNA sequences (He and Hannon 2004, Krol et al. 2010, Iorio and Croce 2012). Still in the nucleus, an enzyme called Drosha processes the pri-miRNA molecule into a ~70 nucleotide long precursor hairpin called pre-miRNA, which is then transported into the cytoplasm. In the cytoplasm, the pre-miRNA molecule is further processed by another enzyme, Dicer. The miRNA molecule is now a short double-stranded RNA molecule. One strand is the "passenger" strand (also known as complementary miRNA or *miRNA) and is usually quickly degraded, whereas the other represents the mature miRNA molecule. The mature miRNA is directed to the RNA-induced silencing complex (RISC) by specific Argonaute proteins. The miRNA molecule, now attached to the RISC complex, recognizes its target mRNA by the sequence (He and Hannon 2004, Krol et al. 2010, Iorio and Croce 2012). The miRNA binding sites are typically located in the 3' untranslated region (3' UTR) of the target mRNA. The sequences of the miRNA and the target mRNA may be only partially or fully complementary, leading to translational inhibition or degradation of the target mRNA, respectively (Krol et al. 2010, Iorio and Croce 2012). The biosynthesis of the microRNAs is illustrated in Figure 2.

Similar to the traditional protein coding genes, also miRNAs are widely deregulated in cancer. A great number of miRNAs have been demonstrated to be up- or downregulated in various malignancies, including pancreatic cancer (Lu et al. 2005, Iorio and Croce 2012). Some miRNAs, such as miR-21 and the let-7 miRNA family, show altered expression across many different cancer types, but also more cancer-type specific miRNA expression patterns have been found to exist (Lu et al. 2005). These miRNA expression profiles have been shown to be even more accurate in classifying cancer specimens than the traditional mRNA expression profiling (Rosenfeld et al. 2008). Furthermore, miRNAs can be categorized as onco-miRs or tumor suppressors, depending on the genes they regulate (Hammond 2006, Iorio and Croce 2012). For example, commonly overexpressed miRNAs at the miR-17-92 cluster typically suppress the expression of several tumor suppressor genes and thereby act themselves in an oncogenic fashion (Oliver et al. 2010). On the other hand, the members of the let-7 miRNA family normally inhibit the expression of oncogenes, and their downregulation in cancer results in increased expression of the

target genes (Torrisani et al. 2007, Wang et al. 2012). Generally, downregulation of miRNAs seems to be more common in cancer than upregulation (Lu et al. 2005, Hammond 2006).

There is a rapidly increasing number of reports on altered miRNA expression patterns in pancreatic cancer (Bloomston et al. 2007, Lee et al. 2007, Szafranska et al. 2007, Kent et al. 2009, Olson et al. 2009, Park et al. 2009, Zhang et al. 2009, Ali et al. 2010, Bhatti et al. 2011, Mees et al. 2011, Zhang et al. 2011, Donahue et al. 2012, Hamada et al. 2012, Jiao et al. 2012, Jung et al. 2012, Munding et al. 2012, Panarelli et al. 2012, Papaconstantinou et al. 2012, Piepoli et al. 2012, Schultz et al. 2012). These studies have been made utilizing both clinical tumor samples and commercially available cell lines. Many miRNAs commonly misregulated in other cancers are aberrantly expressed also in pancreatic cancer. Interestingly, there have been shown to be significantly more upregulated miRNAs than downregulated (Wang and Sen 2011), a pattern opposite to that observed in most of the other tumor types (Lu et al. 2005, Hammond et al. 2006). Nevertheless, all studies have identified both up- and downregulated miRNAs in pancreatic cancer but the number of miRNAs with altered expression vary by the criteria used to define differential expression.

2.4.1. miR-31

Several reports have revealed aberrant expression of miR-31 in various types of cancers, suggesting that it might possess a universal role in carcinogenesis. Interestingly, it has been reported to be both up- and downregulated in a wide range of different malignancies (Table 2). In pancreatic cancer, miR-31 has been shown to be frequently upregulated, already in the early PanIN lesions, and was also recently associated with poor prognosis (Szafranska et al. 2007, Jamieson et al. 2012, Yu et al. 2012).

The functional role of miR-31 has been widely studied (studies summarized in Table 2). It was first demonstrated to play a crucial role in regulating migration, invasion and metastasizing of breast cancer cells, as inhibition of miR-31 induced breast cancer metastasis formation (Valastyan et al. 2009). Later on, its role in

regulating cell mobility has been supported by several studies in various different malignancies, highlighting the significance of miR-31 in cancer (Table 2). For example, in mesothelioma, the miR-31 locus is frequently lost and the loss is associated with an aggressive tumor type. Re-expression of miR-31 in mesothelioma cells suppresses migration, invasion, cell proliferation and clonogenity (Ivanov et al. 2010). On the other hand, in lung cancer miR-31 is upregulated and inhibition of its expression reduces cell growth (via G1 arrest) and tumorigenity both *in vitro* and *in vivo* (Liu et al. 2010). Several target genes acting in cell adhesion or mobility have been suggested for miR-31, and include RDX (radixin, cytoskeletal actin-associated protein), WAVE3 (Wiskott-Aldrich syndrome protein family, member 3, acts in complex which links actin with receptor kinases), and several integrin family members (Valastyan et al. 2009, Augoff et al. 2011, Sossey-Alaoui et al. 2011). Furthermore, miR-31 also seems to play a role in the resistance to chemo- and radiotherapy, which are characteristics of pancreatic cancer (Bhatnagar et al. 2010, Wang et al. 2010, Lynam-Lennon et al. 2012).

Table 2. *Studies on miR-31 function in various cancers*

Cancer	Expr.	Function	Putative target genes	Study
adult T Cell leukemia	down	miR-31 locus frequently lost or epigenetically silenced, which triggers oncogenic signaling. miR-31 downregulation leads to NF-κB activation and apoptosis resistance.	MAP3K14 (NIK)	Yamagishi et al. 2012
bladder carcinoma	down	Low expression in invasive bladder carcinoma compared to superficial tumors. Overexpression decreases bladder cell invasion.		Wszolek et al. 2009
breast cancer	down	miR-31 expression inversely correlates with metastasis in breast cancer patients. Overexpression of miR-31 suppresses metastases and inhibition induces metastases. No effect on viability or cell proliferation.	ITGA5 RDX RHOA	Valastyan et al. 2009
breast cancer	down	miR-31 suppresses expression of several integrins and alters cell mobility.	ITGA2 ITGA5 ITGAV ITGB3	Augoff et al. 2011
breast cancer	down	Overexpression of miR-31 suppresses WAVE3 expression, leading to inhibition of invasion. miR-31 expression gradually decreases during breast cancer progression.	WAVE3	Sossey-Alaoui et al. 2011

cancer-associated fibroblasts	down	Overexpression of miR-31 inhibits migration and invasion	SATB2	Aprelikova et al. 2010
colon cancer	up	miR-31 expression induced by TNF-α and β. Overexpression in colon cancer cell lines enhances motility and invasiveness.	TIAM	Cottonham et al. 2010
colorectal cancer	up	Inhibition of miR-31 expression sensitizes cells to 5-FU and reduces cell migration and invasion but does not affect cell cycle or colony formation.		Wang et al. 2010
esophageal carcinoma	down	miR-31 downregulated in radioresistant cells. Re-expression sensitizes cells to radiation. Regulates a set of 13 genes involved in DNA repair.	PARP1 SMUG1 MLH1 MMS19	Lynam-Lennon et al. 2012
glioma	down	Re-expression inhibits migration and invasion.	RDX	Hua et al. 2012
head and neck squamous cell carcinoma	up	Inhibition reduces cell viability and migration. Overexpression increases proliferation, migration, anchorage-independent growth and growth in nude mice.	FIH	Liu CJ et al. 2010
Kaposi sarcoma	up	miR-31 stimulates endothelial cell migration	FAT4	Wu et al. 2011
lung cancer	up	Inhibition reduces cell growth (G1 arrest) and tumorigenity both in vitro and in vivo.	LATS2, PPP2R2A	Liu X et al. 2010
lung cancer	up	Cigarette smoke increases miR-31 expression, resulting in increased cell proliferation and tumorigenicity.	DKK-1 DACT-3	Xi et al. 2010
melanoma	down	Overexpression inhibits cell proliferation and tube formation.		Greenberg et al. 2011
mesothelioma	down	miR-31 locus is frequently lost and loss is associated with an aggressive tumor type. miR-31 overexpression suppresses migration, invasion, cell proliferation and clonogenity.		Ivanov et al. 2010
ovarian cancer	down	miR-31 overexpression in cells with mutant p53 pathway leads to growth inhibition.	STK40 CEBPA E2F2	Creighton et al. 2010
prostate cancer	down	Expression sensitizes prostate cancer cells to chemotherapy-induced apoptosis.	E2F6	Bhatnagar et al. 2010
prostate cancer	down	Overexpression inhibits cell proliferation, invasion and migration		Fuse et al. 2012

3. ARP2/3 complex

3.1. Actin cytoskeleton

The actin network determines the shape of the cell. However, the cell is not static or fixed but the shape is constantly changing as the cell is growing or moving, meaning that the actin network also needs to be very dynamic (Pollard and Borisy 2003, Revenu et al. 2004, Goley and Welch 2006). The actin cytoskeleton is not only a structural element of the cell but it also acts in a variety of cellular events from cell migration and vesicle trafficking to endocytosis and cell division (Pollard and Porisy 2003, Gourlay and Ayscough 2005, Goley and Welch 2006). Dynamic regulation of actin filaments by a large group of actin binding proteins (ABPs) is required for the proper execution of these cellular processes and changes in the structure of the cell (Revenu et al. 2004, Goley and Welch 2006).

The actin cytoskeleton consists of polymeric actin filaments which are futher arranged to form large networks. Monomeric globular actin (G-actin) is an ATP-binding protein which easily is self-arranged into polymeric filaments (F-actin) forming helical structures (Pollard and Porisy 2003, Nurnberg et al. 2011). Spontaneous actin polymerization is a cellular event which occurs very rapidly *in vitro* and must be strictly regulated *in vivo* by a great variety of proteins (Nürnberg et al. 2011). However, the initiation of new actin filaments, called nucleation, needs to be triggered by specific proteins and is the rate-limiting step of actin polymerization (Goley and Welch 2006). Different classes of actin nucleation regulators have been identified. For example, formins promote nucleation of new unbranched filaments whereas the ARP2/3 protein complex acts in nucleating new actin branches from existing filaments (Kovar 2006, Goley and Welch 2006, Chhabra and Higgs 2007, Campellone and Welch 2010).

Polymeric actin filaments are polarized, the fast-growing end known as the plus or the barbed end, and the other as the minus or the pointed end (Pollard and Porisy 2003, Revenu et al. 2004). To prevent further elongation of the filament as well as dissociation of the actin monomers, capping proteins bind to the filament ends and thus regulate the length of the actin polymers. Tropomodulins bind to the minus end and CapZ to the plus end to stabilize the filaments (le Clainche and Carlier 2008, Nürnberg 2011). However, often actin filaments are constantly recycled so that the

elongation of the barbed end and the depolymerization of the pointed end are in balance, keeping the length of the filament rather stable. This phenomenon, sometimes called actin treadmilling, enables dynamic and rapid modifications of the actin cytoskeleton (le Clainche and Carlier 2008).

Polymeric filaments are further organized to bundles, branched networks and gels to increase their strength and stability and to serve in various cellular functions. For example, filamins cross-link actin fibers to loose networks and α-actinin binds the filaments into parallel bundles (le Clainche and Carlier 2008, Nürnberg 2011). Also the cutting, debranching and depolymerization of actin filaments are regulated by proteins like cofilin and gelsolin (Revenu et al. 2004, Nürnberg et al. 2011). Furthermore, the regulators of actin are also themselves strictly controlled, and dependent for example on pH and Ca2+ concentration, providing additional levels of control for actin dynamics (Revenu et al. 2004).

In addition to being the structural element of the cell, actin also has a central role in cell migration and adhesion (Goley and Welch 2006, le Clainche and Carlier 2008). The barbed ends of actin branches are usually at the leading edge of the cell, towards the movement or growth (Pollard and Borisy 2003, Arjonen et al. 2011). Rapid reassembly of actin at the leading edge enables the formation of protrusions and promotes cell migration (le Clainche and Carlier 2008, Nürnberg 2011). In addition to cell shape and mobility, actin network has a central role in endocytosis and vesicle trafficking, together with the motor protein myosin (Gourlay and Ayscough 2005, Cingolani and Goda 2008, Arjonen et al. 2011). Moreover, during apoptotic cell death, caspases target actin filaments and cleave off small actin fragments which further accelerate apoptosis (Gourlay and Ayscough 2005). Abnormalities in all roles described for actin interfere with key cellular functions and may thus promote tumorigenesis (Arjonen et al. 2011, Nürnberg et al. 2011).

3.2. Structure and function of the ARP2/3 complex

One of the regulators of actin polymerization is the ARP2/3 protein complex which was first described in 1994 and has been later established to control branching of the actin filaments (Machesky et al. 1994, Pollard and Beltzner 2002, Padrick et al. 2011). The human ARP2/3 protein complex consists of seven subunits and is the

key regulator of actin branching (Figure 3) (Mullins et al. 1997, Goley and Welch 2006, Chhabra and Higgs 2007, Campellone and Welch 2010). The subunits ARP2 and ARP3 (actin related protein 2 and 3) are structurally similar to actin and form the actin binding core of the protein complex. The other parts of the complex are more diverse and are named ARPC1-5, referring to actin related protein complex 1-5 (Pollard and Beltzner 2002, Goley and Welch 2006, Nurnberg et al. 2011).

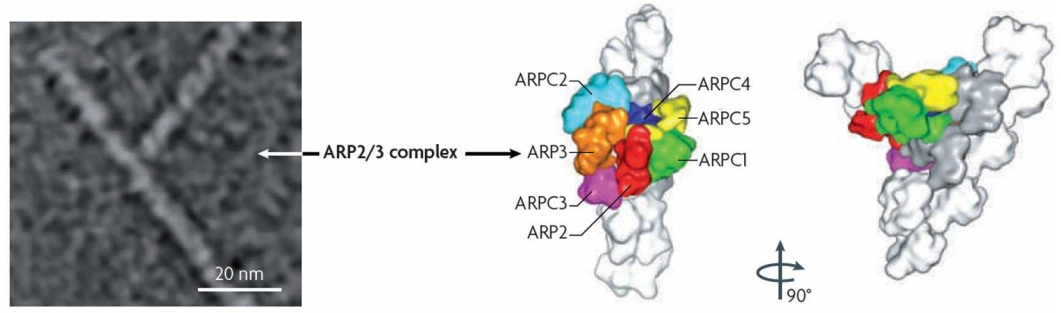

Figure 3. Structure and function of the ARP2/3 complex in nucleating new actin branches from existing filaments. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, Campellone and Welch, 2010.

The ARP2/3 complex acts in nucleating new branches from existing actin filaments as illustrated in Figure 3 (Goley and Welch 2006, Campellone and Welch 2010). It binds to the side of the existing mother filament and initiates the growth of a lateral daughter filament (le Clainche and Carlier 2008). The structural core of the ARP2/3 complex is formed by ARPC2 and ARPC4 subunits, which are critical for the integrity of the complex and which attach the ARP2/3 complex to the mother filament (Gournier et al. 2001, Rouiller et al. 2008). The ARP2 and ARP3 subunits of the complex are believed to mimic an actin dimer, providing a template for the new branch (Mullins et al. 1998, Rouiller et al. 2008). The three other subunits, ARPC1, ARPC3, and ARPC5, participate in the activation of the nucleation function of the complex (Gournier et al. 2001, Rouiller et al. 2008). Activity of the ARP2/3 complex is ATP-dependent and regulated by several nucleation-promoting factors (NPFs), including the WASP and WAVE protein family members (Goley and Welch 2006, Campellone and Welch 2010, Padrick et al. 2011). The NPF

proteins bind to the ARP2/3 complex via a specific WCA domain, causing a conformational change which enables the nucleation of a new actin filament. The NPF proteins also assist in recruiting actin monomers to the nucleation site (Goley et al. 2004, Campellone and Welch 2010).

3.3. ARPC1A and ARPC1B

ARPC1A and ARPC1B are structurally highly similar proteins which both act as the ARPC1 subunit of the ARP2/3 protein complex (Goley and Welch 2006). ARPC1 is 41 kDa in size (and was thus previously named p41) and consists of seven WD repeats which form a seven-bladed β-propeller protein (Welch et al. 1997, Goley and Welch 2006). The exact function of ARPC1 in the complex is uncertain but it has been suggested to be needed for the structural organization of the nucleation site (Winter et al. 1999). Also a role in the regulation of the activity of ARP2/3 has been proposed, possibly through binding of nucleation promoting factors (Winter et al. 1999, Gournier et al. 2001, Kelly et al. 2006). ARPC1 binds directly to WASP which is a regulator of ARP2/3 activity, suggesting that ARPC1 might have a regulatory role. Moreover, it has been shown to interact with PAK1 (p21 activated kinase 1, functions in the regulation of cell mobility and morphology; Vadlamudi et al. 2004) and bind and activate Aurora A which is a kinase needed for cell cycle progression (Molli et al. 2010). Gene disruption studies in yeast showed that the ARPC1 subunit is essential for cell viability (Winter et al. 1999). Based on functional studies, ARPC1A and ARPC1B have been suggested to have slightly different functions (Molli et al. 2010) but the exact roles of these two proteins remain still to be found.

4. Nuclear transport

4.1. Mechanisms of nuclear transport

In eukaryotic cells, the nuclear membrane divides the cell into two separate compartments, the nucleus and the cytoplasm. Transporting RNA, proteins and other molecules to their correct locations, both in and out of the nucleus, is crucial for normal cell function (Kau et al. 2004, Mosammaparast and Pemberton 2004, Pemberton and Paschal 2005, Faustino et al. 2007). For instance, RNA is transcribed in the nucleus and needs to be transported into the cytoplasm to be translated. At the same time, nuclear proteins, such as histones and transcription factors, are produced in the cytoplasm and are then transported into the nucleus (Kau et al. 2004). Bidirectional trafficking between these two cellular compartments is needed for various cellular events, from regulation of gene expression to control of the cell cycle. Thus, it is evident that this transport machinery is a key player in the maintenance of cellular homeostasis. Malfunction of nuclear import or export results in incorrect localization of RNA and proteins, which might subsequently lead to a variety of diseases, including cancer (Faustino et al. 2007).

Both import and export of molecules occurs via nuclear pore complexes (NPC) which are cylindrical structures on the nuclear membrane, connecting the nuclear and cytoplasmic compartments (Strambio-De-Castillia et al. 2010). Transport of proteins through the nuclear membrane can be roughly divided into two categories, passive diffusion and active transportation (Faustino et al. 2007). However, recently also a third mechanism was suggested for nuclear export, as ribonucleoprotein particles were shown to be transported out of the nucleus via nuclear envelope budding (Speese et al. 2012).

Passive diffusion through the nuclear pore complexes is possible only for small, maximum of 40 kDa proteins (Faustino et al. 2007, Stewart 2007). Larger molecules need to be actively transported through the membrane. The proteins which are aimed to be transported to the nucleus, contain a nuclear localization signal (NLS) in their amino acid structure (Pemberton and Paschal 2005, Faustino et al. 2007). There are different types of NLS sequences, which can be recognized by diverse import proteins (Stewart 2007). Although different import pathways exist, the classic and most common import pathway is the karyopherin-mediated import via

nuclear pore complexes as illustrated in Figure 4 (Kau et al. 2004, Mosammaparast and Pemberton 2004, Pemberton and Paschal 2005, Stewart 2007). The import cycle begins by the recognition of the cargo protein NLS by karyopherin-α (importin-α / KPNA), which binds to the cargo protein. Alpha karyopherin acts as an adaptor protein and is the link between the cargo and the karyopherin-β (importin-β / KPNB), which is the actual transporter. Once formed, the cargo:KPNA:KPNB complex is docked to the nuclear pore complex and can now enter the nucleus via the NPC. In the nucleus, RanGTP binds to the karyopherin-β, resulting in dissociation of the trimeric protein complex and subsequent cargo release. The RanGTP-bound karyopherin-β is recycled back to the cytoplasm, and karyopherin-α binds to RanGTP-bound CAS (cellular apoptosis susceptibility protein) which is its export receptor (Kau et al. 2004, Mosammaparast and Pemberton 2004, Pemberton and Paschal 2005, Stewart 2007).

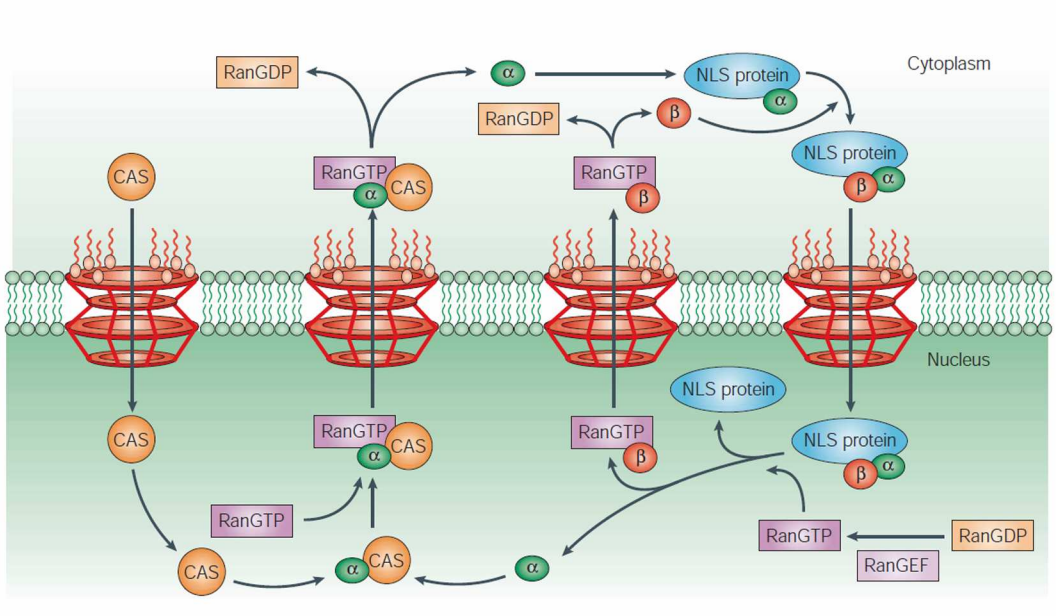

Figure 4. Nuclear import of nuclear localization signal (NLS) containing proteins and recycling of the karyopherin alpha and beta import receptors. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Kau, 2004.

4.2. Karyopherin alpha protein family

The human karyopherin-α protein family consists of seven highly conserved members (Goldfarb et al. 2004, Kelley et al. 2010). The basic molecular structure of all karyopherin-α proteins is similar, and consists of ten Armadillo (ARM) repeats which form the body of the curved shape protein. The N terminus of the karyopherin-α acts as the importin-β binding (IBB) domain and in the inner curve are located the two NLS binding sites. The major site is located at the ARM repeats 1-4 and the minor site at repeats 6-8 (Conti et al. 1998, Conti and Kuriyan 2000, Fontes et al. 2000, Stewart 2007). The IBB domain also has an important role in regulating cargo binding, as its amino acid sequence mimics NLS sequence and it can also bind the NLS binding site, competing with the NLS containing cargo (Stewart 2007). When karyopherin-α is not bound to karyopherin-β, the IBB domain is bound to the NLS binding site, allowing only proteins with stronger affinities to bind to it. As the karyopherin-β binds to the IBB domain, the NLS binding sites are revealed and open for cargo proteins also with lower affinity to bind. The IBB domain thus also exhibits an autoinhibitory role and regulates cargo binding in the cytoplasm as well as cargo release in the nucleus (Kobe 1999, Matsuura and Stewart 2004).

The cargo proteins are recognized by the transporters by their NLS sequence which can be either monopartite (based on one amino acid cluster) or bipartite (two clusters separated usually by a ~10-12 amino acid spacer) (Stewart 2007). Monopartite NLS sequences usually bind to the major site of karyopherin-α, whereas bipartite NLS sequences occupy both binding sites (Stewart 2007). Many cargo proteins also bind directly to karyopherin-β, and are transported without karyopherin-α. However, karyopherin-α is an adaptor protein and cannot act alone but always needs to bind karyopherin-β to enter the nucleus (Pemberton and Paschal 2005, Stewart 2007).

Although the basic mechanism of cargo binding is known, there is very little knowledge of the roles of different karyopherin-α proteins. The human genome encodes seven karyopherin-α genes, whereas the yeast S. cerevisiae genome only has one single karyopherin-α gene (Goldfarb et al. 2004). Although all seven karyopherin-α proteins are structurally similar, they are likely to have different roles in terms of time and context as well as cargo specificity. There is evidence of

different karyopherin-α proteins acting at different tissue and cell types at different times during development, and some karyopherin-α proteins have been shown to have specific roles in embryogenesis (Tsuji et al. 1997, Kamei et al. 1999, Köhler et al. 2002). In adult tissues, different expression patterns of KPNAs have also been observed (Kamei et al. 1999) but information on the possible cargo specificity is truly limited. KPNAs have also been shown to bind cargoes with different affinities, suggesting another level of diversity in nuclear import (Kelley et al. 2010).

4.3. KPNA7

Karyopherin alpha 7 (KPNA7) is the newest member of the karyopherin-α family, and was first identified in 2010 (Kelley et al. 2010). It is structurally most closely related to KPNA2 but shows significant sequence similarity also to the other members. As the other karyopherin-α proteins, KPNA7 also consists of ten ARM repeats with the NLS binding pocket, and N terminal IBB domain (Stewart 2007, Kelley et al. 2010).

Expression of KPNA7 orthologs has been studied in various animals, including bovine, porcine and mouse tissue (Tejomurtula et al. 2009, Hu et al. 2010, Park et al. 2012). In all these studies, KPNA7 expression has been linked to embryogenesis and fertility. Both in mouse and cattle, when screening diverse adult tissues, KPNA7 expression was found predominantly in the ovary (Tejomurtula et al. 2009, Hu et al. 2010). Furthermore, in all three animals, KPNA7 expression was found in oocytes or early stage embryos, and in mouse cells the protein was localized in the nucleus (Hu et al. 2010). In human HeLa cells, KPNA7 was shown to be predominantly expressed in the nucleus (Kelley et al. 2010) but the function of the human KPNA7 still remains completely unknown. Whether KPNA7 has a specific role in human fertility, remains to be solved.

AIMS OF THE STUDY

The aim of this study was to characterize both a recurrently amplified chromosomal region as well as microRNA expression patterns in pancreatic cancer in order to identify novel putative targets for diagnostic and therapeutic purposes. The specific aims were the following:

1. To delineate the 7q21-q22 amplicon in pancreatic cancer and identify the putative amplification target genes.
2. To functionally characterize the amplification target genes and evaluate their significance in pancreatic cancer.
3. To screen microRNA expression patterns in pancreatic cancer, and to functionally characterize differentially expressed microRNAs.

MATERIALS AND METHODS

1. Cell lines (I, II, III)

Sixteen human pancreatic cancer cell lines were used in the study (Table 3). Thirteen of those, AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, Hs 700T, Hs 766T, MIA PaCa-2, Panc-1, SU.86.86, and SW1990, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). DAN-G, HUP-T3, and HUP-T4 were obtained from the German Collection of Microorganisms and Cell Cultures (Brunswick, Germany). The normal pancreatic ductal cell line hTERT-HPNE was obtained from the ATCC. All cell lines were authenticated to avoid misidentification and were grown under recommended culture conditions.

Table 3. *Properties of the pancreatic cancer cell lines used in the study.*

Cell line	Distributor	Origin	Age of donor	Gender
AsPC-1	ATCC [1]	Ascites	62	F
BxPC-3	ATCC	n.a.	61	F
Capan-1	ATCC	Liver	40	M
Capan-2	ATCC	n.a.	56	M
CFPAC-1	ATCC	Liver	26	M
Dan-G	GCMCC [2]	n.a.	n.a.	n.a.
HPAC	ATCC	n.a.	64	F
HPAF-II	ATCC	n.a.	44	M
Hs700T	ATCC	Pelvis	61	M
Hs766T	ATCC	Lymph node	46	M
Hup-T3	GCMCC	n.a.	n.a.	n.a.
Hup-T4	GCMCC	n.a.	n.a.	n.a.
MIA PaCa-2	ATCC	n.a.	65	M
Panc-1	ATCC	n.a.	56	M
SU.86.86	ATCC	Liver	57	F
SW1990	ATCC	Spleen	56	M

[1] ATCC: American Type Culture Collection
[2] GCMCC: German Collection of Microorganisms and Cell Cultures

2. RNA samples (I, II, III)

Normal pancreatic RNA samples were obtained from commercial sources (Ambion, Austin, TX; Biochain, Hayward, CA; and Clontech, Mountain View, CA). The panel of normal tissue RNA samples was purchased from Ambion.

Table 4. *Clinicopathological data of the pancreatic cancer speciments on the tissue microarray slide used in Study I.*

No	Sex	Age	Histology	Grade	TNM Stage
1	M	51	Adenocarcinoma	G1	-
2	M	70	Adenocarcinoma	G1	-
3	M	39	Adenocarcinoma	G1	-
4	M	58	Adenocarcinoma	G3	T3N0M0(IIA)
5	F	60	Adenocarcinoma	G3	T3NxM0(IIA)
6	F	42	Adenocarcinoma	G1	-
7	M	63	Adenocarcinoma	G2	T3N1M0(IIB)
8	M	60	Adenocarcinoma	G2	-
9	F	58	Adenocarcinoma	G3	-
10	M	66	Adenocarcinoma	G3	T3N0Mx(IIA)
11	F	54	Adenocarcinoma	G2	-
12	F	72	Adenocarcinoma	G2	T3N1M0(IIB)
13	M	41	Adenocarcinoma	G2	T3N1M1(IV)
14	M	53	Adenocarcinoma	-	T2N1M1(IV)
15	M	64	Mucinous adenocarcinoma	-	T3N0M0(IIA)
16	M	60	Adenocarcinoma	G2	T2N0M1(IV)
17	F	75	Adenocarcinoma	G2	T2N0M0(IB)
18	F	64	Adenocarcinoma	G2	T3N1M1(IV)
19	M	64	Adenocarcinoma	-	T3N0M1(IV)
20	F	62	Mucinous adenocarcinoma	-	T3N0M0(IIA)
21	M	62	Adenocarcinoma	G2	T3N1M0(IIB)
22	M	62	Adenocarcinoma	G2	T3NxM1(IV)
23	M	58	Adenocarcinoma	G2	-
24	M	72	Intraductal papillary mucinous carcinoma	-	-
25	M	41	Neuroendocrine carcinoma	-	-
26	M	62	Adenocarcinoma	G2	T3N1M0(IIB)
27	F	47	Adenocarcinoma	G2	-
28	M	81	Adenocarcinoma	G2~3	-
29	F	64	Carcinoma	-	-
30	F	53	Anaplastic carcinoma, papillary adenocarcinoma	G4	T3N1M1(IV)
31	M	44	Adenocarcinoma	G2~3	T3N1M1(IV)
32	M	55	Adenocarcinoma	G3	T3N1M1(IV)
33	M	49	Adenocarcinoma	G3	-
34	M	51	Non-neoplastic	-	-
35	M	70	Non-neoplastic	-	-
36	M	60	Non-neoplastic	-	-
37	F	58	Non-neoplastic	-	-
38	M	62	Non-neoplastic	-	-
39	F	47	Non-neoplastic	-	-
40	M	81	Non-neoplastic	-	-
41	M	49	Non-neoplastic	-	-

3. Tissue microarray (I)

Commercially available AccuMax A207 (III) tissue microarrays were purchased from Petagen Incorporation (Seoul, Korea). The tissue microarrays contained 33 pancreatic cancer specimens in duplicate and eight non-neoplastic pancreatic tissue samples. The clinicopathological features of the samples are detailed in Table 4.

4. Genomic clones (I)

Fourteen bacterial artificial chromosome (BAC) or P1 derived artificial chromosome (PAC) clones were selected to cover the 3 Mb amplicon area at 7q21-q22 (Table 5). The BAC and PAC clones were selected using the public genome databases, NCBI (National Center for Biotechnology Information) Map Viewer (http://www.ncbi.nlm.nih.gov/mapview) and UCSC (University of California Santa Cruz) Genome Browser (http://genome.ucsc.edu). Clones were obtained from CHORI (Children's Hospital Oakland Research Institute, Oakland, CA). Chromosome 7 centromeric probe (p7alphaTET) was used as a reference.

Table 5. *BAC and PAC clones used in fluorescence in situ hybridization*

Clone	Type	Start (bp)	End (bp)	Size (bp)
RP11-94N7	BAC	96 033 106	96 170 067	136 960
RP11-525A11	BAC	96 327 722	96 426 137	98 415
RP11-172J11	BAC	96 424 137	96 510 872	86 735
RP11-356B17	BAC	96 607 609	96 714 001	106 392
CTB-94H21	BAC	96 794 459	96 919 295	124 836
RP5-1090P18	PAC	97 047 406	97 169 536	122 130
RP5-1111F22	PAC	97 216 826	97 354 933	138 107
RP11-177C9	BAC	97 449 432	97 565 939	116 507
RP11-725M1	BAC	97 740 402	97 906 781	166 379
RP5-1186C1 [a]	PAC	98 088 214	98 201 066	112 846
RP11-62N3 [a]	BAC	98 187 801	98 361 362	173 562
RP11-405I21 [a]	BAC	98 334 645	98 473 080	138 435
RP4-550A13	PAC	98 512 376	98 591 892	79 516
RP11-136B3	BAC	98 676 638	98 760 751	84 113

[a] Clone was included in the contig probe

5. Fluorescence *in situ* hybridization (I)

Fluorescence *in situ* hybridization (FISH) was performed on interphase nuclei of the pancreatic cancer cell lines to carry out copy number analysis of the 7q21-q22 locus. BAC and PAC clone DNA was extracted using the standard alkaline lysis method and the probes were labeled with Spectrum Orange dUTP (Vysis, Downers Grove, IL) by the random priming method. Chromosome 7 centromeric reference probe was labeled with fluorescein-12-dUTP (Perkin-Elmer, Boston, MA). The labeled probes were then purified using the BioSpin P6 columns (Bio-Rad, Hercules, CA).

Dual-color FISH on interphase nuclei of the sixteen cell lines was performed as previously described (Bärlund et al. 2000) and the signals were analyzed using the Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan). For all probes, control experiments on normal lymphocytes were performed to verify correct localization of hybridization signals. Fifty intact nuclei were analyzed for each probe and cell line, and the relative copy number was counted as the ratio of locus specific probe versus control probe. A relative value above 1.5 was considered increased copy number.

FISH on tissue microarray was done as described (Alarmo et al. 2006). A contig of three overlapping BAC/PAC clones covering the 7q21-q22 amplicon core (RP11-1186C1, RP11-62N3, and RP11-405I21) was used as a hybridization probe. Control experiment on normal lymphocyte nuclei was performed to ensure that the probe contig gave a single hybridization signal. Hybridization signals from at least twenty nuclei were counted and the absolute mean copy numbers were determined.

6. Quantitative RT-PCR

6.1. mRNA expression (I, II, III)

Real-time quantitative RT-PCR was used to quantify the mRNA expression levels of the ten genes located in the 7q21-22 amplicon (I), and the nine putative miR-31 target genes (III). All gene expression analyses were performed using the LightCycler instrument (Roche, Mannheim, Germany). Total RNA was extracted from the cells using the Trizol reagent (Invitrogen, Carlsbad, CA) and first-strand

cDNA synthesis was performed using the SuperScript III First Strand Synthesis kit (Invitrogen).

Table 6. *Sequences of all primers used in the qRT-PCR experiments.*

Gene	Primers (5'->3')	Study
APBB2	CCTGGTGATCCATGTCAGAA TCGGAGGTTAAGGGTGTTTG	III
ARID1A	GTCAGTATGGCCCACAAGGT GGCACCCATGGGGTTTAT	III
ARPC1A	CAGAGTGTTTTCTGCCTACATT ACTTAGGAGCGGCAGGA	I
ARPC1B	GTTATTTCGAGCAGGAGAATGAC GTAGGCTGAAAAGATCCGACA	I
BUD31	AAAAGATGACCGTGACCTTGAAC TGTGGGTCAGGTTGTACGCT	I
KPNA1	GACTTGTGGAACTGCTGATGC TCCCCTGTGACAATGTTTCC	II
KPNA2	GTTATCCTGGATGCCATTTCA AGCCTCCACATTCTTCAATCA	II
KPNA3	TGAGCCATCAGGAAGTCAAA CGGTGCCAGTCACTATGTTG	II
KPNA4	GGCAGAAACCATAGGCAATCT TCATTTTCATGATTTTGAAGTTGTTC	II
KPNA5	TGGCTAAAAAGGGTAGCTTCA CATGATATTTTTCCTCTTGGCATA	II
KPNA6	GAGGAACCCCTGAGCAGAT AGCAAGTCACATAGGGGTTTG	II
KPNA7	CGGTGATGGCCCAGAGT GCGGAGAGAAGGAGTCAAGAC	I
KPNA7	CCAGTCAATATGCCGACCTT AGACTGACCGCCATCCTCT	II
LATS2	AACTGGTGAACGCAGGATG CCCATCTTGCTGATGTACTCC	III
MAP4K5	CAGACCATGGCGATGTAAAA TCGTTTTGCAATGGTAGCTG	III
NPTX2	CAGACCCTCAAGGACCG AGGCAGCGTCTTCTTGAT	I
PDAP1	GACCCCAAAAAGGAGAAGAAAT TCTCTTCTCGTTCTCTCCTCGAA	I
PPP2R2A	GGTGGTAGAGTTGTCATCTTTCAA TCTCCTCTGCTATGAGACTGGA	III
PTCD1	GCAACTACACGGTGCTGATTG ACTCGGCACAGACGTTGAAC	I
RHOA	GGGAGCTAGCCAAGATGAAG GTACCCAAAAGCGCCAATC	III
RSBN1	GGGGTTTGACTGGCAGAGT GGTTATGCGAGGTTGGTCAC	III
SMURF1	AAGAAATCTTTGAGGAGTCTTACC CATTTCATGGCACAGCAAGTA	I
TACC1	AAGACGGGTCCACTGTGC CTCCACAGGACACCGACAC	III
TACC2	CCCCACTATTCGCTCAGAAA AGGGCTTCTATCCGCATGAT	III
TBP	CATGACTCCCATGACCC TGGTTCGTGGCTCTCTTA	I
TMEM130	AAAAGATGACCGTGACCTTGAAC TGTGGGTCAGGTTGTACGCT	I
TRRAP	TGCTGCGTCTGCTGAAC GGGGTTGTCATGCTCGAT	I

For the 7q21-22 amplicon genes, the PCR primers and probes were purchased from TIB MolBiol (Berlin, Germany). For the miR-31 target genes, probes from the Universal Probe Library (Roche) were used along with primers from Sigma (St. Louis, MO). All primers used in the qRT-PCR experiments are listed in Table 6. All gene expression levels were normalized against a house-keeping gene, either *TBP* (TATA-box binding protein, Study I), *HPRT* (hypoxanthine phosphoribosyl-transferase, Study III), or *GUSB* (glucuronidase beta, Study II). *TBP* primers and probes were obtained from TIB MolBiol and *GUSB* and *HPRT* reference gene assays were purchased from Roche.

6.2. MicroRNA expression (III)

TaqMan microRNA assays (Applied Biosystems, Carlsbad, CA) were used to quantify miRNA expression levels for the microarray data validation. Twenty-five nanograms of total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit and quantitative RT-PCR was performed as instructed using the LightCycler instrument (Roche). All miRNA expression levels were normalized against RNU48.

7. Transfections

7.1. Transfection of siRNAs (I, II)

Gene-specific siRNAs were used to study the functional roles of *ARPC1A*, *ARPC1B*, and *KPNA7* in pancreatic cancer. All gene-specific siRNAs were designed using the siRNA Selection Program of the Whitehead Institute, Cambridge, MA (Yuan et al. 2004). For *ARPC1A* and *ARPC1B*, the siRNA molecules were purchased from Proligo (Paris, France) and for *KPNA7*, four gene-specific siRNAs were purchased from Dharmacon (Lafayette, CO), and a pool with equal concentrations of each of the four was prepared. For all cell lines, 30 000 or 150 000 cells per well were plated on 24- or 6-well plates, respectively. Twenty-four hours after seeding the cells were transfected with 10 nM siRNA or siRNA pool, using the

Interferin reagent (Polyplus-Transfection, San Marcos, CA) according to the manufacturer's instructions. A siRNA targeting the firefly luciferase (*LUC*) gene (Sigma, St. Louis, MO) was used as a control in all experiments. Efficient gene silencing was verified each time by qRT-PCR and with either *TBP* or *GUSB* as a reference gene.

7.2. Transfection of miRNA inhibitors and precursors (III)

Functional consequences of abnormal miR-31 expression were studied by transient transfection of anti-miR-31 inhibitors or pre-miR-31 precursors and their corresponding controls (Ambion) into AsPC-1, HPAF-II, and MIA PaCa-2 cells. Cells were plated either on 24- or 6-well plates using following cell numbers: AsPC-1 and HPAF-II 30 000 or 150 000 cells per well, and MIA PaCa-2 10 000 or 80 000 cells per well, for 24- or 6-well plates, respectively. Twenty-four hours after seeding the cells were transfected with a final concentration of 30 nM miRNA precursor or inhibitor using the Interferin reagent (Polyplus-Transfections). The efficacy of miR-31 silencing or expression was verified each time using qRT-PCR and with the housekeeping gene RNU48 as a reference.

8. Functional assays

8.1. Cell proliferation (I, II, III)

Cell proliferation assays were performed on 24-well plates and the cells were counted 24-96 hours after siRNA or miRNA transfection using the Coulter Counter instrument (Beckman Coulter, Fullerton, CA). All assays were done in six replicates and repeated at least twice.

8.2. Cell cycle analyses and apoptosis assays (I, II, III)

In the cell cycle analyses and apoptosis assays, cells were grown on 6-well plates, collected at the designed time point after siRNA or miRNA transfection, and suspended in 500 µl of hypotonic propidium iodide staining buffer (0.1 mg/mL sodium citrate tribasic dehydrate, 0.03% Triton X-100, 50 µg/µL propidium iodide, 2 µg/mL RNase A). For the apoptosis assay the Annexin V FITC Apoptosis Detection Kit was used (Calbiochem, Nottingham, UK). The cell cycle distributions and the number of apoptotic cells were analyzed using the Accuri C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI) and the ModFit LT software (Verity Software House Inc, Topsham, ME). All experiments were performed in six replicates and repeated at least twice.

8.3. Migration and invasion assays (I, II, III)

Cell migration and invasion studies were performed using 8.0 µm BD Falcon migration chambers or BD BioCoat Matrigel invasion chambers (BD Biosciences, CA) according to the manufacturer's instructions. Cells were placed in the chamber 48 hours after transfection, and a 1% vs. 10% FBS gradient was used as a chemoattractant. After 22 hours, the migrated and invaded cells were fixed with methanol and stained with toluidine blue. Stained cells were photographed with Aperio ScanScope XT microscope (Aperio Technologies, Vista, CA) and the total area of cells from four images per insert was analyzed using the ImageJ software (Abramoff et al. 2004).

8.4. Colony formation (II)

Potential for anchorage independent growth was assayed by growing cells on 0.35% agarose on six-well plates. After 14 days, twelve images per well were captured with the Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan) using the Capture Pro 6.0 program. The number, size and total area of colonies were quantified using the ImageJ software (Abramoff et al. 2004).

9. MicroRNA array (III)

9.1. Sample preparation and array hybridization

The miRNA array hybridizations were performed according to manufacturer's instructions using Agilent's miRNA labeling and hybridization kit (Agilent, Santa Clara, CA). Briefly, 100 ng of total RNA was dephosphorylated, denatured, and labeled with pCp-Cy3 dye. Labeled RNA was purified using the Micro BioSpin 6 columns (Bio-Rad, Hercules, CA). Samples were denatured and hybridization was allowed to occur at 55°C for 20 hours. Each microarray slide contains eight identical subarrays. The normal pancreas samples were pooled and hybridized to each slide to allow comparison of the data between the slides. Post-hybridization washes were performed as recommended. Arrays were scanned by using Agilent DNA microarray scanner (Agilent).

9.2. Data analysis

For the data analysis, the miRNA array images were transformed to spot intensity data with Agilent Feature Extraction Software (version 9.5.1.1). The Limma package of Bioconductor (Gentleman et al. 2004, Smyth et al. 2005) was used for both preprocessing of the data and the actual data analysis. In preprocessing, 64 viral RNAs on the array were excluded. Also control spots and spots that were flagged as saturated, nonuniformity outliers, or population outliers were omitted. The background of the data was first corrected using the normexp method with offset 50 (Ritchie et al. 2007) and the data were normalized with quantile normalization (Pradervand et al. 2009). The mean value of the replicate probes in log2 scale was used for each miRNA in each sample, resulting in altogether 470 miRNAs in the actual analysis. In the differential expression analysis, the group of 16 cancer cell lines was compared with the group of four normal samples and the pooled normal samples. Differentially expressed miRNAs were identified utilizing empirical Bayes linear model and the Benjamini–Hochberg adjustment for the P-values with the Limma package (Smyth et al. 2005). The miRNAs with adjusted P-value below 0.05 and fold change over 1.5 between the groups were considered as

differentially expressed. Further, the relationships between the samples were revealed using the hierarchical clustering method with correlation distance and average linkage within each sample.

9.3. miRNA target gene analysis

Predicted target genes for selected miRNAs were identified using GOmir application (version 9/2009), which combines data from four different miRNA target prediction databases, TargetScan, PicTar, miRanda, and RNAhybrid, and allows the comparison of the results (Roubelakis et al. 2009).

10. Western blot (II, III)

Total protein from the cell lines was collected by first washing the cell monolayer twice with PBS and then lysing the cells into RIPA buffer (1% PBS, 1% non-idet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing Complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany).

Nuclear and cytoplasmic protein fractions were collected as described earlier, with minor modifications (Abmayr et al. 2006). Shortly, cells were collected and resuspended in hypotonic buffer containing protease inhibitors and incubated 15 min on ice. Cytoplasmic protein fraction was collected after centrifugation (3300g 15 min 4°C). Next, the pellet was resuspended in low-salt buffer and high-salt buffer was carefully added. Solution was incubated on ice for 30 min and nuclear fraction was collected after intense centrifugation (25 000g 30 min 4°C). For all protein extractions, the protein content was measured using the Bradford reagent (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

40 or 50µg of protein extract was used for western blot analyses. Gel-electrophoresis and blotting were done as described previously (Alarmo et al. 2009). Shortly, proteins were separated by SDS-PAGE on 12% polyacryleamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics GmbH) using a Trans-blot SD Semidry transfer apparatus (Bio-Rad Laboratories). After blotting, the membrane was blocked overnight followed 1 hour incubation with primary and secondary antibodies. All antibodies used in western blot analyses

are summarized in Table 7. Finally, proteins were visualized by using the BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Roche Diagnostics GmbH) according to manufacturer's instructions.

11. Immunofluorescence (III)

Immunofluorescence was performed as earlier described (Kallio et al. 2011). Briefly, cells were first fixed with 4% paraformaldehyde for 30 min and rinsed with PBS. Cells were pretreated with BSA-PBS solution (1% BSA, 0.05% saponin in PBS) for 30 min. Incubations with primary antibody (diluted in BSA-PBS, dilutions are summarized in Table 7) and Alexa-Fluor 488 or 568 secondary antibody (diluted 1:200 in BSA-PBS, Molecular Probes, Eugene, OR) were performed for 1 hour, both followed by 3 x 5 min washes in BSA-PBS. Phalloidin was purchased from Invitrogen (Carlsbad, CA). Immunostained cell were photographed with the Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan) using the Capture Pro 6.0 program. All antibodies used in immunofluorescence are summarized in Table 7.

Table 7. *Antibodies and dilutions used in western blot and immunofluorescence.*

	Dilution used in		
Antibody	**Western blot**	**Immunofluorescence**	**Manufacturer**
APBB2	1:1000	-	Abcam, Cambridge, UK
β-actin	1:10 000	-	Molecular Probes, Eugene, OR
Caspase-3	-	1:500	Cell Signaling, Danvers, MA
CDK2	1:200	-	Santa Cruz Biotechnology, Santa Cruz, CA
CDK6	1:200	-	Santa Cruz Biotechnology
Cyclin A	1:200	-	Santa Cruz Biotechnology
Cyclin E	1:200	-	Santa Cruz Biotechnology
E-cadherin	1:1000	1:500	Abcam
Histone H3	1:200	-	Santa Cruz Biotechnology
KPNA7	1:500	1:1000	GenWay Biotech, San Diego, CA
KPNA7	1:500	1:1000	LifeSpan Biosciences, Seattle, WA
KPNA7	1:500	1:1000	Sigma, St. Louis, MO
p21	1:100	-	Santa Cruz Biotechnology
p27	1:500	-	Santa Cruz Biotechnology
RSBN1	1:1000	-	Abcam
Tubulin	1:20 000	-	Sigma
Vimentin	1:1000	1:500	Sigma

12. Statistical analyses (I, II, III)

The Mann-Whitney test was used to compare the medians of the test and control groups in all functional studies as well as in amplicon gene expression analyses in Study I. All p values are two-sided.

RESULTS AND DISCUSSION

1. Detailed characterization of the 7q21-q22 amplicon in pancreatic cancer (I)

Gene amplification is a common mechanism for oncogene activation in solid tumors (Albertson et al. 2003, Vogelstein and Kinzler 2004, Albertson 2006). In pancreatic cancer, a great number of recurrently amplified regions have been identified but only very few target genes have been functionally verified and characterized (Santarius et al. 2010, Samuel and Hudson 2012). Functional analysis of the genes within the amplicons is needed to evaluate their significance for the disease and possibly reveals novel diagnostic or therapeutic targets. Earlier studies have highlighted several genes which are frequently amplified in cancer and the amplification is associated with for example poor survival rates or drug resistance. For example, *MYCN* amplification in neuroblastomas has been associated with more aggressive disease and poor survival, and amplifications of *MYC*, *ERBB2*, *CCND1*, *EGFR*, and *MDM2* in breast cancer are associated with high tumor grade (Al-Kuraya et al. 2004, Vogelstein and Kinzler 2004, Albertson 2006). This study aimed to perform a detailed characterization of the 7q21-q22 amplicon, which one of the chromosomal regions recurrently amplified in pancreatic cancer.

1.1. Fluorescence *in situ* hybridization delineates a 0.77 Mb amplicon core region (I)

Previous array CGH studies by us and others have revealed pancreatic cancer cell lines and primary tumors to have a ~3 Mb commonly amplified chromosomal region at 7q21-q22 (Aguirre et al. 2004, Heidenblad et al. 2004, Holzmann et al. 2004, Mahlamäki et al. 2004, Bashyam et al. 2005, Gysin et al. 2005, Loukopoulos et al. 2007, Suzuki et al. 2008). However, at the time this study was started, the CGH

studies only provided a rough overview of the amplicon with rather poor resolution. More detailed studies are always needed to delineate the amplicon core region and to identify the putative target genes.

Fluorescence *in situ* hybridization using thirteen evenly distributed BAC/PAC probes was performed on 16 established pancreatic cancer cell lines to determine the more exact structure and boundaries of the 7q21-q22 amplicon. Increased copy number (relative copy number >1.5-fold) was found in four cell lines out of the sixteen. The AsPC-1 cell line harbored high-level amplification with the relative copy numbers reaching up to 8.7-fold, whereas in the other three cell lines, Capan-1, Hs700T, and HPAF-II, lower level gains were detected (relative copy numbers up to 1.7-, 2.9-, and 2.3-fold, respectively). The copy number profiles of the three cell lines with lower level gains were rather uniform across the entire 7q21-q22 amplicon. However, the AsPC-1 copy number profile had a clear peak of high level amplification at the distal end of the amplicon, which was used to define the amplicon core region. This minimal region of amplification was 0.77 Mb of size, stretching from the end of the BAC clone RP11-725M1 to the start of the clone RP11-136B3 (Figure 5). All clones between these two demonstrated a high level amplification (relative copy number over 8-fold) in the AsPC-1 cell line.

The existence of the 7q21-q22 amplicon was verified also in 32 primary pancreatic tumors in order to evaluate its clinical significance. A contig of three overlapping BAC probes (RP5-1186C1, RP11-62N3, and RP11-405I21; Figure 5) representing the amplicon core region were used as the probe. Increased copy numbers were detected in 7 out of 29 tumors (24%), where the hybridization was successful. Unfortunately we were unable to link the amplification to any of the clinicopathological characteristics of the samples, but this is likely to be at least partially because of the rather small sample size.

To summarize, the 7q21-q22 amplification was found to exist in ~25% of both pancreatic cancer cell lines as well as in primary pancreatic tumors, indicating that it does have relevance to the disease rather than being just a cell culture artifact. In addition to pancreatic cancer, the 7q21-q22 amplification has been also found in other malignancies, including gastric, esophageal and hepatocellular carcinomas, and melanoma (Balazs et al. 2001, Riegman et al. 2001, Morohara et al. 2005, Sy et al. 2005), further indicating that the amplicon actually has significance beyond

pancreatic cancer. However, no target genes for the 7q21-q22 amplification had been suggested at the time of this study.

1.2. Amplification of the 7q21-q22 locus leads to overexpression of a specific set of genes (I)

One of the main criteria for the definition of an amplification target gene is that the increased copy number leads to overexpression of the corresponding gene. To address this issue, two public genome databases, NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview) and UCSC Genome Browser (http://genome.ucsc.edu) were used to identify the genes within the 0.77 Mb amplicon core region, and altogether ten transcripts were identified. Genomic locations of the genes within the amplicon core are shown in Figure 5, and a summary of the ten amplified transcripts and their functions are shown in Table 8. Recently, one microRNA, miR-3609, has also been localized in the amplicon core. However, since this data was not available at the time of the study, the research was focused on the gene transcripts.

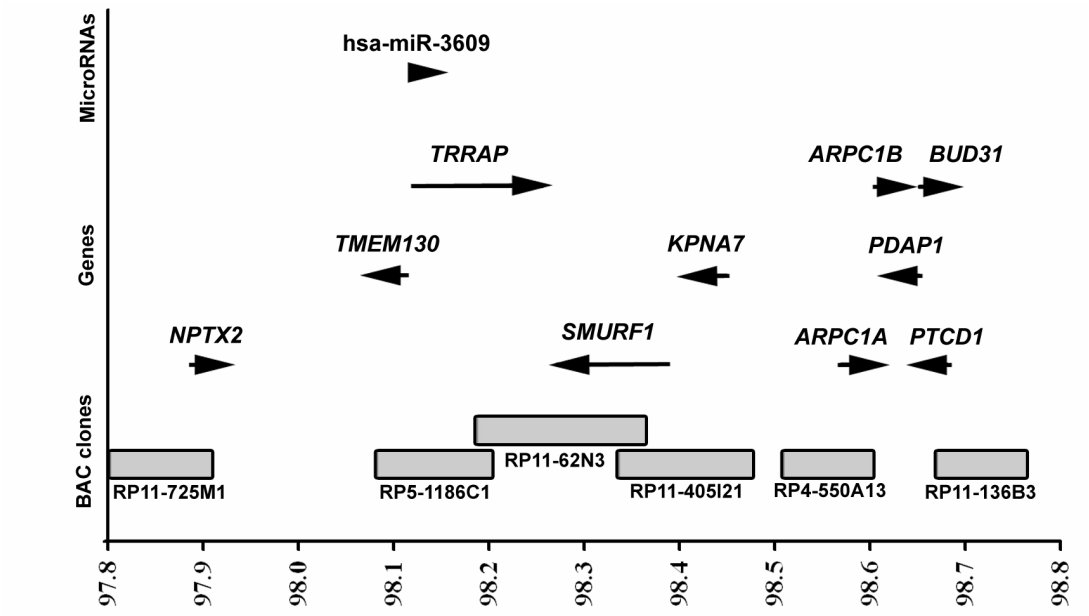

Figure 5. Genomic locations of the BAC/PAC clones, gene transcripts and microRNAs located at the 7q21-q22 amplicon core. Arrowheads indicate the transcription direction of the genes and microRNAs.

First, the expression of these ten transcripts was studied using regular RT-PCR in the AsPC-1 cell line. Two genes, *NPTX2* and *TMEM130*, were excluded from further studies since they had very weak expression or were not expressed at all in this cell line with the most intense amplification and were thus not likely to be the target genes of the amplification. Interestingly, *NPTX2* has been reported to be frequently hypermethylated at the promoter region, possibly explaining its low expression in the AsPC-1 cells (Park et al. 2007, Zhang et al. 2011, Zhang et al. 2012). The expression of the remaining eight genes was comprehensively studied using quantitative RT-PCR in the same panel of sixteen pancreatic cancer cell lines used in the copy number analyses. Four commercially available samples of normal pancreatic RNA were also included in the panel.

Table 8. *Gene transcripts located at the 7q21-q22 amplicon core region.*

Gene symbol	Gene name	Function
NPTX2	Neuronal pentraxin II	Participates in synapse formation and synaptic remodeling (Bjartmar et al. 2006, Koch and Ullian 2010)
TMEM130	Transmembrane protein 130	No known function.
TRRAP	Transformation/transcription domain-associated protein	Large protein which acts in transcription and DNA replication and repair by recruiting histone acetyltransferase (HAT) complexes to chromatin (Murr et al. 2007)
SMURF1	SMAD specific E3 ubiquitin protein ligase 1	E3 ubiquitin ligase which induces the translocation of TGFβ pathway inhibitor SMAD7 into the cytoplasm, and promotes destruction of SMAD4 (Ebisawa et al. 2001, Morén et al. 2005)
KPNA7	Karyopherin alpha 7	Nuclear transport receptor which acts in the import of proteins into the nucleus (Kelley et al. 2010)
ARPC1A	actin related protein 2/3 complex, subunit 1A	One of the seven subunits of the ARP2/3 protein complex, which acts in actin polymerization. Alternative to ARPC1B (Goley and Welch 2006).
ARPC1B	actin related protein 2/3 complex, subunit 1B	One of the seven subunits of the ARP2/3 protein complex, which acts in actin polymerization. Alternative to ARPC1A (Goley and Welch 2006).
PDAP1	PDGFA associated protein 1	Phosphoprotein which associates with PDGFA which may be involved in regulating fibroblast growth (Fischer and Schubert 1996)
BUD31	BUD31 homolog (*S. cerevisiae*)	Yeast Bud31 acts in spliceosome assembly and promotes mRNA splicing (Masciadri et al. 2004, Saha et al. 2012)
PTCD1	Pentatricopeptide repeat domain 1	Mitochondrial matrix protein, participates in the 3' end processing of tRNAs (Rackham et al. 2009, Sanchez et al. 2011)

For each gene, the expression levels in the four amplified cell lines were compared to those in the non-amplified cell lines and the normal pancreas. Three genes located at the most distal end of the amplicon, *PDAP1*, *BUD31*, and *PTCD1*, did not show any significant association between amplification and overexpression, with the most important finding being that they were not overexpressed in the most intensely amplified AsPC-1 cells. In fact, *PDAP1* and *PTCD1* even had lower expression in the AsPC-1 cells than in the normal pancreas. The remaining five genes, *TRRAP*, *SMURF1*, *KPNA7*, *ARPC1A*, and *ARPC1B*, all had a clear association between amplification and overexpression and thus represent the putative 7q21-q22 amplification target genes. The median expression levels of the groups of the amplified and non-amplified cell lines showed a statistically significant difference ($p<0.05$) and they all were also extremely highly overexpressed in the AsPC-1 cells.

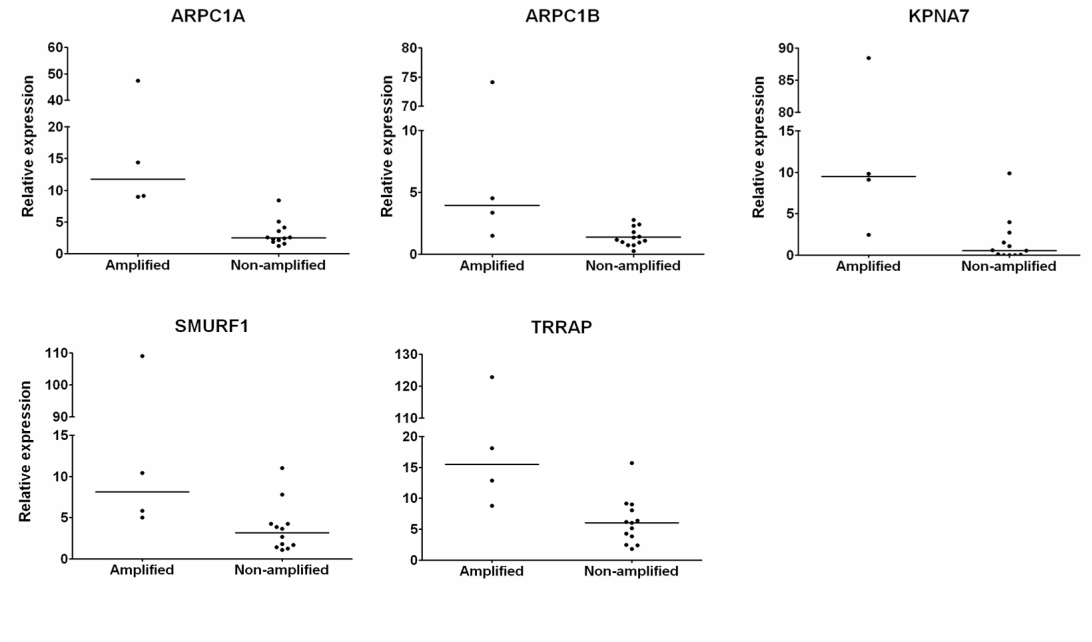

Figure 6. Expression of five putative 7q21-q22 amplicon target genes, *ARPC1A*, *ARPC1B*, *KPNA7*, *SMURF1*, and *TRRAP* in the groups of amplified and non-amplified cell lines.

The focus of this study was on functional evaluation of the roles of *ARPC1A*, *ARPC1B*, and *KPNA7* in the pancreatic carcinogenesis. All five genes are located at

the centre of the amplicon core region and have a strong association between gene amplification and overexpression (Figure 6). Of these, *ARPC1A* had the highest p value for the association between amplification and overexpression, and *ARPC1B* was selected based on its functional similarity. Moreover, *KPNA7* had an extremely interesting expression pattern with almost absent expression in normal pancreas, and was therefore included in the functional studies. However, *SMURF1* and *TRRAP* have also been suggested to be the targets of the 7q21-q22 amplicon in pancreatic cancer (Suzuki et al. 2008, Kwei et al. 2011). Inhibition of *SMURF1* and *TRRAP* expression reduced cell proliferation and induced *SMURF1* expression significantly increased colony formation (Suzuki et al. 2008). Furthermore, *SMURF1* amplification and subsequent overexpression has also been shown to increase the invasiveness of pancreatic cancer cells (Kwei et al. 2011). Several studies have shown that rather than one single amplification target gene, amplicons often have a set of genes that are concurrently amplified, overexpressed, and together cause the malignant phenotype (Yang et al. 2006, Pärssinen et al. 2007, Brown et al. 2008, Carvalho et al. 2009, Wu et al. 2012). Interestingly, the famous review by Santarius et al. (2010) listed only three amplification target genes with good evidence on their role in pancreatic cancer, and both *ARPC1A* and *SMURF1* were on the list. It appears evident that the 7q21-q22 amplicon is also likely to contain several target genes.

2. Functional evaluation of the putative 7q21-q22 amplicon target genes (I, II)

2.1. *ARPC1A* and *ARPC1B* regulate cell migration and invasion in pancreatic cancer (I)

The *ARPC1A* and *ARPC1B* genes are successively located at the 7q21-q22 amplicon, highly similar to each others, and both encode for the p41 subunit of the human Arp2/3 protein complex (Welch et al. 1997). They were selected for further studies based on their function and gene expression data. Functional consequences of abnormal *ARPC1A* and *ARPC1B* expression were studied by silencing the genes

both individually and simultaneously, using RNA interference (RNAi). Two gene specific siRNAs were designed for both genes and efficient (at least 80%) downregulation of mRNA levels was verified in all studies. Also the gene specificity of the siRNAs was verified by confirming that *ARPC1A* siRNA did not silence *ARPC1B*, and vice versa.

The effects of *ARPC1A* and *ARPC1B* silencing on cellular functions were studied by multiple different assays in AsPC-1 cells harboring high-level amplification and overexpression of both genes. Non-amplified Panc-1 cells were used as a control in each experiment. Silencing of *ARPC1B* did not alter the growth of the AsPC-1 cells but silencing of *ARPC1A* resulted in small yet statistically significant decrease in AsPC-1 cell proliferation at 96 hours after transfection (12% decrease as compared to the *LUC* control, $p<0.005$), and the same phenomenon was observed with both *ARPC1A* siRNAs. As expected, silencing of *ARPC1A* or *ARPC1B* did not alter Panc-1 cell growth or any other features studied further on. Next, the consequences of silencing on cell mobility were assessed. Interestingly, silencing of *ARPC1A* and *ARPC1B* resulted in a dramatic reduction of cell migration (Table 9). Simultaneous silencing of both genes also led to significantly reduced migration but did not produce any additive effect. Moreover, silencing of *ARPC1A*, either individually or together with *ARPC1B*, significantly decreased the invasion ability of AsPC-1 cells, while silencing of *ARPC1B* alone did not have an effect.

Table 9. *Effect of ARPC1A and ARPC1B silencing on AsPC-1 cell migration and invasion.*

siRNA name	siRNA identifier	Migration[a]	Invasion[a]
siLUC	-	100 %	100 %
siARPC1A	489	25 % **	55 % **
	196	48 % **	55 % *
siARPC1B	446	55 % **	106 % n.s.
	272	59 % *	85 % n.s.
siARPC1A + siARPC1B	489 + 446	31 % **	54 % **

[a] Percentage (%) of siLUC control
* $p<0.05$, ** $p<0.005$

ARPC1A and *ARPC1B* genes both encode for the p41 subunit (ARPC1) of the ARP2/3 protein complex which regulates actin polymerization and thus cell mobility (Goley and Welch 2006). Although the exact function of the ARPC1 is not fully understood, it has been shown to be essential for cell viability, indicating an central role in the protein complex (Winter et al. 1999, Gournier et al. 2001). ARPC1 has been suggested to act as the regulatory subunit of the ARP2/3 protein complex, as it is phosphorylated by the p21 activated kinase 1 (PAK1) and the phosphorylation is required for cell mobility (Vadlamudi et al. 2004). This study shows that silencing of both *ARPC1A* and *ARPC1B* impairs the migration ability of pancreatic cancer cells, most likely via incorrect function of the ARP2/3 protein complex. Overexpression of the entire ARP2/3 protein complex or some of its subunits has been found in several malignancies, including breast, colorectal, and gastric cancers (Otsubo et al. 2004, Wang et al. 2004, Semba et al. 2006, Zheng et al. 2008). Moreover, coexpression of the ARP2 subunit of the complex and one of its activators, WAVE2, has been linked to poor prognosis in breast, colorectal, and lung cancer because of increased risk of metastases (Semba et al. 2006, Iwaya et al. 2007a, Iwaya et al. 2007b).

Interestingly, the pancreatic cancer cells overexpressing both of these genes appear to be more dependent on *ARPC1A*, since *ARPC1A* seems to be capable of compensating the lack of *ARPC1B*, but *ARPC1B* cannot fully cover the loss of *ARPC1A* expression. This suggests that the proteins encoded by these two genes either have slightly different roles in the Arp2/3 complex, or the cells may under certain conditions become more dependent on one protein than the other. In breast cancer, silencing of *ARPC1B* but not *ARPC1A* reduced the proportion of cells entering the G2/M phase of the cell cycle, and ARPC1B was shown to be involved in the Aurora A kinase activation, a cellular process needed in proper progression through mitosis (Molli et al. 2010). However, in our study silencing of *ARPC1B* did not alter pancreatic cancer cell growth, but the phenotypes in tissues of diverse genetic background are known to vary a lot, which may explain these differences (Moore et al. 2001, Deer et al. 2010). Further studies are needed to reveal the possible preferences in the expression of ARPC1A and ARPC1B in different tissues, cell types and cellular conditions, and to find out the exact functions of these two proteins. Also studies on the function of the six other subunits of the ARP2/3 protein complex are needed to truly understand its role in both pancreatic and other

cancers. Recently, miR-133a mediated silencing of ARPC5 was demonstrated to inhibit cell migration and invasion in head and neck squamous cell carcinoma (HNSCC) (Kinoshita et al. 2012). The suggested role for the ARPC1 subunit as the regulator of the ARP2/3 complex makes it an exceptionally interesting drug target in cancer.

2.2. Overexpression of *KPNA7* promotes the malignant phenotypes of pancreatic cancer (II)

The *KPNA7* gene is located in the middle of the 7q21-q22 amplicon core and was highly overexpressed in several pancreatic cancer cell lines as the result of gene amplification, whereas in normal pancreas *KPNA7* expression is nearly absent. Due to this interesting expression pattern, together with the literature lacking almost any information about *KPNA7* expression, a qRT-PCR screen in a panel of 20 additional normal human tissues was performed. Also in these, *KPNA7* expression levels were very low, with only marginal expression detected in ovary and trachea. The *KPNA7* expression levels in primary pancreatic tumors were queried from the several microarray databases but almost no data was available. Only a single cervical cancer dataset demonstrating at least 2.5-fold increase in *KPNA7* expression in approximately 25% of the samples was found (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE20167). This scarcity of information can be mainly explained by the fact that until very recently, KPNA7 was only a hypothetical protein predicted by sequence similarity. The *KPNA7* gene was originally isolated from LNCaP prostate cancer cells and was shown to be expressed in HeLa cervical cancer cells (Kelley et al. 2010) as well as in BT-474 breast cancer cells (unpublished data), suggesting that it is indeed expressed in a subset of cancer samples. Unfortunately all of the commercial KPNA7 antibodies as well as the custom-made antibody failed to recognize the KPNA7 protein, allowing the evaluation of its expression only at the mRNA level.

Functional consequences of aberrant *KPNA7* expression were studied by silencing the gene in the AsPC-1 and Hs700T pancreatic cancer cell lines which

both harbor a high-level amplification and subsequent overexpression of *KPNA7*. Panc-1 cells with no amplification and only very marginal expression were used as a control. Using a pool of four different siRNAs (*siKPNA7*), efficient (at least 80% as compared to *LUC* siRNA control) silencing of *KPNA7* mRNA levels was observed already at 24 hours after transfection and persisted for at least 96 hours. The mRNA levels of other alpha karyopherins were also screened to ensure that the siRNAs did not alter their expression.

Silencing of *KPNA7* led to a dramatic reduction of cell growth in both AsPC-1 and Hs700T cell lines. In both cell lines, a trend for slower cell proliferation could be seen already at 72 hours after transfection, and at 96 hours a striking and statistically significant growth reduction was evident (Table 10). As expected, *KPNA7* silencing did not alter the growth of Panc-1 cells with low endogenous *KPNA7* expression. Next, apoptosis and cell cycle analyses were performed to determine whether the reduction in cell growth was caused by increased rate of cell death or decreased rate of cell proliferation. No differences in the number of apoptotic cells were detected but instead, a marked G1 arrest could be seen in both AsPC-1 and Hs700T cells after *KPNA7* silencing. At 72 hours after *KPNA7* siRNA transfection, the fraction of cells in the G1 phase was dramatically increased in both cell lines (Table 10). To explore the cellular mechanisms of the G1 arrest in more detail, the expression of six well-known cell cycle regulator proteins (CDK2, CDK6, Cyclin A, Cyclin E, p21, and p27) was assessed in AsPC-1 and Hs700T cells after *KPNA7* silencing. Since the KPNA7 protein acts in nuclear transport, the nuclear and cytoplasmic protein fractions were studied separately, to reveal not only changes in the expression levels but possibly also in the subcellular localization of the proteins. Interestingly, a clear induction of the p21 protein levels was observed in both cell lines and in both nuclear and cytoplasmic protein fractions. For the remaining five proteins no significant alterations were detected.

Table 10. *Summary of the functional consequences of KPNA7 silencing in AsPC-1 and Hs700T cells.*

	AsPC-1[a]	**Hs700T**[a]
Proliferation (at 96 hours)	37 % **	54 % **
Cell cycle	G1 arrest G1 fraction 66% vs. 46% **	G1 arrest G1 fraction 64% vs. 48% **
Colony formation	29 % *	79 % ***
Migration	45 % **	Not altered
Invasion	29 % n.s.	Not altered
Cell morphology	Not altered	Change from raft-like to fibroblast-like

[a]Reduction (%) as compared to the LUC control.
* $p<0.05$, ** $p<0.005$, *** $p<0.0005$

Potential for anchorage independent growth was studied by growing *siKPNA7* transfected AsPC-1 and Hs700T cells in soft agar for 14 days. For AsPC-1 cells, the total colony area was decreased 29% in *siKPNA7* transfected cells as a result of decreased of colony size. For Hs700T cells, a dramatic decrease in both colony size and number of colonies was observed, adding up to a 79% decreased total colony area. Silencing of *KPNA7* also reduced migration and invasion ability of AsPC-1 cells (Table 10) whereas the mobility of Hs700T cells was not altered. However, in Hs700T cells silencing of *KPNA7* dramatically altered the cell morphology, causing the cells normally growing as raft-like structures to acquire a fibroblast-like shape. However, despite various experiments assessing apoptosis and EMT (epithelial-mesenchymal transition) related factors, the underlying reasons for this phenomenon could not be discovered.

The nuclear transport machinery is responsible for carrying various proteins and RNA in and out of the nucleus, making it a key player in maintaining cellular homeostasis (Pemberton and Paschal 2005). Abnormalities and malfunctions in this complex protein network can lead to incorrect localization of proteins and therefore cause various diseases, including cancer (Faustino et al. 2007). For example, in addition to mutations, the tumor suppressor protein p53 has been shown to be inactivated by incorrect localization in the cytoplasm, impairing its proper function in the nucleus (Moll et al. 1992). In some cases, the mislocalization was proven to

be due to a truncated form of the import receptor alpha karyopherin (Kim et al. 2000).

KPNA7 is the newest member of the karyopherin-α protein family and operates in nuclear import. This study shows that *KPNA7* expression is absent in almost all human adult tissues, but overexpression of the gene is a frequent event in pancreatic cancer. Studies in several animals have shown that KPNA7 is expressed during embryogenesis, suggesting that the gene might normally act during the embryo development and is then silenced in adult differentiated cells (Tejomurtula et al. 2009, Hu et al. 2010, Wang et al. 2012). Molecular pathways normally needed during the embryogenesis are known to be frequently activated in cancer, and aggressive tumors, such as pancreatic cancer, often overexpress genes that are enriched in embryonic stem cells (Miller et al. 2005, Kelleher et al. 2006, Ben-Porath et al. 2008). These data suggest that KPNA7 mainly functions during embryonic development, is normally silenced in adults, but abnormally activated in cancer cells.

Silencing of *KPNA7* resulted in a remarkable decrease of cell proliferation as well as a great reduction in the anchorage independent growth, both of which are key features of cancer cells (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011). Furthermore, the growth decrease was shown to be caused by a p21 induced G1 arrest of the cell cycle. The p21 protein is a cyclin-dependent kinase inhibitor which inhibits the G1/S cyclin-dependent kinases, mainly the activity of CDK2–Cyclin-E complexes (Malumbres and Barbacid 2001, Abbas and Dutta 2009). Silencing of p21 as well as other abnormalities in the regulation of the cell cycle are very common in all human cancers (Malumbres and Barbacid 2001, Abbas and Dutta 2009, Malumbres and Barbacid 2009). In addition to the universal changes in cell growth, *KPNA7* silencing also caused decreased migration ability in AsPC-1 cells and changed the morphology of the Hs700T cells. These cell line specific phenotypes are likely to be explained by the different genetic and phenotypic characteristics of the cells (Moore et al. 2001, Deer et al. 2010).

Overexpression of other alpha karyopherins, especially KPNA2, has been frequently reported in several malignancies, such as bladder, breast, esophageal, lung, ovarian, and prostate cancers (Dahl et al. 2006, Gluz et al. 2008, Sakai et al. 2010, Zheng et al. 2010, Jensen et al. 2011, Mortezavi et al. 2011, Wang et al. 2011). In breast cancer cells, *KPNA2* overexpression induced colony formation and

increased cell migration whereas silencing led to opposite phenotypic effects (Noetzel et al. 2012). Moreover, in lung and prostate cancers *KPNA2* silencing led to reduced cell migration, cell viability, and cell proliferation (Mortezavi et al. 2011, Wang et al. 2011). Although this study clearly demonstrates that overexpression of *KPNA7* promotes the malignant properties of pancreatic cancer, further studies are still needed to determine the exact function of KPNA7 in embryonic, adult and cancerous cells. Moreover, identification of the actual cargoes of KPNA7 is crucial to uncover the consequences of its abnormal expression in cancer. However, the fact that *KPNA7* expression is extremely low in practically all normal human adult tissues makes it an especially attractive therapeutic target for both pancreatic cancer and other malignancies.

3. miRNA expression patterns in pancreatic cancer (III)

3.1. 72 differentially expressed miRNAs provide a molecular signature for pancreatic cancer (III)

Agilent miRNA microarrays were utilized to screen the miRNA expression patterns in a panel of sixteen established pancreatic cancer cell lines and four normal pancreatic RNA samples. The internal control sample (pool of the normal samples) hybridized on all microarray slides in the study showed highly similar expression profiles, thus indicating good consistency between data derived from different microarray slides and allowing slide-to-slide comparison. Hierarchical clustering was able to separate the samples into two discrete groups, one containing the four normal pancreatic samples and the other all pancreatic cancer cell lines. Although the cancer cell lines did fall into a few subgroups, no link between those groups and specific cell line characteristics, such as such as site of origin (primary tumor vs. metastasis), differentiation or mutation status, could be found (Moore et al. 2001, Deer et al. 2010).

Next, differentially expressed miRNAs between the two sample groups were identified using the Bayes method and the Benjamini-Hochberg adjustment. Altogether 72 miRNAs with differential expression (at least 1.5-fold expression

change, adjusted p-value <0.05) between these two groups were identified, approximately half of these being up- and half downregulated in pancreatic cancer cells as compared with normal samples. As expected, many of the differentially expressed miRNAs, such as the let-7 family and miR-21, have been already shown to be frequently altered in multiple malignancies, and the results obtained are in line with the literature of miRNA expression in pancreatic cancer cell lines and primary tumors (Bloomston et al. 2007, Lee et al. 2007, Szafranska et al. 2007, Kent et al. 2009, Olson et al. 2009, Park et al. 2009, Zhang et al. 2009, Ali et al. 2010, Bhatti et al. 2011, Mees et al. 2011, Zhang et al. 2011, Donahue et al. 2012, Hamada et al. 2012, Jiao et al. 2012, Jung et al. 2012, Munding et al. 2012, Panarelli et al. 2012, Papaconstantinou et al. 2012, Piepoli et al. 2012, Schultz et al. 2012). However, the study also identified novel cancer related miRNAs, such as miR-801 that has been later on suggested to be a marker for early detection of breast cancer (Cuk et al. 2012).

To validate the microarray data, a set of twelve differentially expressed miRNAs was selected for qRT-PCR analysis. The selected miRNAs represented different expression ranges, varying from very low to very high expression. A median correlation of 0.66 (Spearman's rank correlation coefficient, range from 0.398 to 0.926) was observed between the two methods, thus confirming the reliability of the microarray data.

One miRNA is capable of regulating the expression of dozens or even hundreds of genes (Gunaratne et al. 2010, Pritchard et al. 2012). The GOmir application was used to predict the targets for the 20 miRNAs with most significant differential expression in this study. GOmir utilizes four different target prediction programs, TargetScan, miRanda, RNAhybrid, and PicTar, and thus gives more reliable predictions than the use of only a single program. When used individually, the four different programs were able to identify tens or even hundreds of possible target genes for each miRNA but the number of common target genes was significantly lower (range of 0-73 common targets per miRNA, median value 12).

MiRNA expression signature has been shown to correctly classify cancer and normal samples, and to be even more accurate than mRNA profiling (Rosenfeld et al. 2008). In this study, the expression profile of 72 miRNAs was sufficient to separate the normal and cancer samples. However, for this miRNA signature to have actual diagnostic value, several issues must be solved. First of all, the cancer

samples used in this study were merely cell lines. It is possible, that some of the expression changes observed in this study represent either artefacts related to cell culturing or are cell line specific events. Yet, many of the miRNAs with most significant differential expression have been shown to be altered also in primary pancreatic cancer samples (Bloomston et al. 2007, Lee et al. 2007, Szafranska et al. 2007, Olson et al. 2009, Zhang et al. 2009, Bhatti et al. 2011, Zhang et al. 2011, Donahue et al. 2012, Hamada et al. 2012, Jiao et al. 2012, Munding et al. 2012, Panarelli et al. 2012, Papaconstantinou et al. 2012, Piepoli et al. 2012, Schultz et al. 2012). Second obstacle in using the signature as a diagnostic tool is the number of miRNAs. Screening of 72 miRNAs is time-consuming and expensive, and to actually have potential in the pancreatic cancer diagnosis, the miRNA signature should contain a far smaller number of miRNAs and still to be accurate. Thus, this study mainly provides novel information for understanding the pathogenesis of pancreatic cancer, but also demonstrates that the commercially available pancreatic cancer cell lines indeed provide proper models for studying miRNAs in pancreatic cancer.

3.2. miR-31 regulates migration and invasion in pancreatic cancer cells (III)

Of the 72 differentially expressed miRNAs, miR-31 demonstrated a specially interesting expression pattern. In normal pancreas and in six of the cell lines it was expressed at very low levels, but in ten cell lines it had strikingly high expression. Based on this on-off type expression profile, miR-31 was selected for further functional studies where the molecular consequences of both inhibition and overexpression of miR-31 were evaluated. miR-31 expression was inhibited in two cell lines with high endogenous miR-31 levels (AsPC-1 and HPAF-II) and induced in a cell line with almost absent miR-31 expression (MIA PaCa-2). Quantitative RT-PCR was used to verify efficient miR-31 silencing (over 80% decrease in relative expression) and expression (up to 200-fold increased expression). Control experiments were performed by silencing miR-31 in non-expressing cells and inducing expression in cells with high endogenous expression.

Inhibition of miR-31 expression resulted in statistically significant cell growth reduction at 96 hours after transfection, both in AsPC-1 and HPAF-II cells (12%

and 24% reduction compared to control cells, respectively). Interestingly, induced expression of miR-31 in MIA PaCa-2 cells also led to reduced cell growth (24% reduction at 96 hours after transfection). Despite the similar changes in cell growth rates, alterations in the cell cycle were seen only in the MIA PaCa-2 cells in which an apparent G1 arrest was detected at 72 hours after transfection. Silencing of miR-31 also significantly altered migration ability of AsPC-1 and HPAF-II cells and also influenced cell invasion although no statistical significance was achieved (Table 11). Furthermore, induced miR-31 expression in MIA PaCa-2 cells also reduced both migration and invasion ability of the cells (Table 11). In all other control experiments no differences in either cell growth or migration and invasion ability were detected, but inducing miR-31 expression in AsPC-1 cells, already endogenously overexpressing miR-31, surprisingly resulted in dramatic decrease in both migration and invasion (Table 11). Although previous studies have shown the cellular consequences of both up- and downregulation of miR-31 but none of those has reported the phenomenon seen in the AsPC-1 cells in this study. Only in one report studying HNSCC, miR-31 expression has been both induced and inhibited in the exactly same cell line, but the consequences were also opposite (Liu et al. 2010).

Table 11. *Effects of manipulating miR-31 expression on migration and invasion of three pancreatic cancer cell lines.*

Treatment	**Function**	**Cell line[a]**		
		AsPC-1	**HPAF-II**	**MIA PaCa-2**
Anti-miR-31	Migration	33 % **	64 % **	Not altered
	Invasion	27 % n.s.	20 %	n.d.
Pre-miR-31	Migration	61 % **	n.d.	58 % **
	Invasion	74 % **	n.d.	35 % *

[a]All values are shown as reduction (%) compared to the control cells.
n.d., not determined
* $p<0.05$, ** $p<0.005$

To explore the function of miR-31 and also validate the results from the miRNA target gene prediction programs, the putative target genes of miR-31 were studied in more detail. The GOmir application was able to identify a total of seven common

targets for miR-31, *APBB2*, *ARID1A*, *MAP4K5*, *PPP2R2A*, *RSBN1*, *TACC1*, and *TACC2*. In addition to these genes, two additional previously confirmed miR-31 targets, *LATS2* and *RHOA*, were selected (Valastyan et al. 2009, Liu et al. 2010). One of the genes, *TACC2*, was not expressed in any of the samples analyzed and was thus excluded from further analyses. No differences in the basal expression of the putative target genes were detected when comparing the cell line groups with low and high miR-31 levels. However, in the cell lines with manipulated miR-31 expression, at least 50% change in the expression of four genes (*APBB2*, *LATS2*, *PPP2R2A*, and *RSBN1*) was observed, both when inhibiting and inducing miR-31 expression. Two most likely miR-31 targets, *LATS2* and *RSBN1*, were further studied by western blot. Unfortunately no changes in the protein expression levels were seen although both of them were confirmed to harbor at least two miR-31 binding sites.

Multiple studies have implicated miR-31 as an important regulator of the metastasis process in many tumor types (studies summarized in Table 2) but this was the first time when its role in pancreatic cancer was studied. Interestingly, both up- and downregulation of miR-31 have been reported in different cancers. However, although the expression patterns vary largely between different malignancies, the effect of abnormal miR-31 expression seems to be mainly affecting cell invasion and development of metastases. Both upregulation (Valastyan et al. 2009, Wszolek et al. 2009, Aprelikova et al. 2010, Ivanov et al. 2010, Sossey-Alaoui et al. 2011, Fuse et al. 2012, Hua et al. 2012) and downregulation (Cottonham et al. 2010, Liu et al. 2010, Wang et al. 2010, Wu et al. 2011) of miR-31 have been demonstrated to inhibit cancer cell mobility. Based on this study, the phenotypes induced by aberrant miR-31 expression appear to be dependent not only on tissue type but also the cell line and more studies utilizing several different cell lines are needed to solve this question. However, since a single miRNA may regulate even hundreds of target genes (Friedman et al. 2009), the consequences of altering such a master regulator of gene expression are indeed expected to vary greatly, depending on the genetic and epigenetic background of the cells.

4. Future perspectives

This study provides novel in vitro data on both the expression patterns of microRNAs and the function of one miR-31 and three amplification target genes, *ARPC1A*, *ARPC1B*, and *KPNA7*, in pancreatic cancer.

The functional studies of the amplicon target genes were performed using the RNAi technology. Although several siRNAs were used for each gene, the possibility of off-target effects must still be taken into consideration. Thus, it would be important to show the functional consequences of overexpression of the same genes in cells with absent or low endogenous expression. Furthermore, this study was performed mainly on mRNA level, and the obvious next steps would be first to verify the results on protein level and then to explore the cellular roles of these proteins in more detail. Since ARPC1A and ARPC1B are components of a large protein complex, it would be interesting to also study the other subunits of the complex, and to evaluate the roles of the individual subunits as well as the entire protein complex. KPNA7 acts as a nuclear import receptor, and thus exploring its cargoes would be a logical step to further study its role in pancreatic cancer.

This study revealed a specific microRNA profile for pancreatic cancer, which is able to separate cancer and normal samples. To gain clinical value, the profile should be tested also with primary tumor samples, and the number of miRNAs used in profiling should be decreased. Furthermore, since the putative miR-31 target genes tested in this study could not be confirmed, the role of other predicted targets should be assessed in the future. This would help in determining the molecular pathways that are altered by abnormal miR-31 expression and thus in understanding the complex phenotype observed in this study.

The study was carried out by using mainly cell lines, an approach that has both advantages and disadvantages. Cell lines provide a fast and an inexpensive way of studying cancer genes but when interpreting the results it must kept in mind that they may lack many of the properties of the actual tumors. When studying cellular properties, the lack of the tumor microenvironment, such as the surrounding tissues and the immune response is a major point to consider. Thus, although the results from cell line studies provide a good basis, they need to be validated in primary tumor samples or in in vivo models to increase their reliability. For this study, proceeding to animal models would be a logical step, and xenograft models with

both silencing and overexpressing the target genes could provide additional evidence on the role these genes in pancreatic cancer pathogenesis.

CONCLUSIONS

This study was aimed to identify and functionally characterize both genes and microRNAs aberrantly expressed in pancreatic cancer. The major findings of this thesis were the following:

1. As a result of a detailed study on the 7q21-q22 amplification, a 0.77 Mb amplicon core region was identified and found to exist in 25% of both pancreatic cancer cell lines and primary tumors. The amplification was found to lead to overexpression of several genes, some of which were proven to have an important role in pancreatic carcinogenesis.

2. Functional characterization of three putative 7q21-q22 amplicon target genes, *ARPC1A*, *ARPC1B*, and *KPNA7* confirmed their functional role in pancreatic cancer. *ARPC1A* and *ARPC1B* were shown to regulate mobility of pancreatic cancer cells whereas *KPNA7* had a more diverse function. *KPNA7* silencing resulted in a p21 induced G1 arrest of the cell cycle, but also led to changes in cell migration and morphology. All three genes have potential to serve as novel targets for anti-cancer therapy.

3. Pancreatic cancer was shown to possess a distinct expression pattern of microRNAs. A set of 72 miRNAs was identified, which were differentially expressed between pancreatic cancer and normal pancreatic samples, and thus provide a molecular signature for the disease.

4. miR-31 was found to be strongly overexpressed in a subset of pancreatic cancer cell lines. Functional characterization of miR-31 proved that it plays an important role in regulating migration and invasion of pancreatic cancer cells.

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