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Genome-wide Characterization of Genetic Aberrations in Pancreatic Cancer

■

ACADEMIC DISSERTATION

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To Eero and Mona

Genomin laajuinen geneettisten muutosten karakterisointi haimasyövässä

Haimasyöpä on haiman ulkoeritteisten eli eksokriinisten rauhasten pahanlaatuinen kasvain, joka saa alkunsa rauhastiehyeiden pintakerroksesta. Haimasyöpään sairastuu Suomessa vuosittain noin 700 ihmistä. Taudin ennuste on erittäin huono, sillä lähes kaikki sairastuneet kuolevat viiden vuoden kuluessa diagnoosista. Huono ennuste johtuu pääasiallisesti siitä, että haimasyöpä aiheuttaa yleensä oireita vasta taudin varsin myöhäisessä vaiheessa. Tällöin syöpä on tavallisesti jo ehtinyt levitä paikallisesti ja lähettää etäpesäkkeitä muualle elimistöön. Tässä väitöskirjatyössä selvitettiin erilaisin solu- ja molekyyligeneettisin menetelmin haimasyövän syntyyn ja kehittymiseen liittyviä geneettisiä muutoksia. Käytetyt menetelmät kattavat koko genomin erilaisella herkkyydellä ja selventävät haimasyövän syntyyn liittyviä tapahtumia lähtien kromosomitason muutoksista aina yksittäisten geenien osallisuuden tutkimiseen.

Kromosomaalisella vertailevalla genomisella hybridisaatiolla analysoitiin 31 haimasyöpäsolulinjaa ja 13 haimasyöpänäytettä. Tutkimuksessa löydettiin useita kromosomialueita, kuten kromosomikäsivarret 8q, 11q, 12p, 17q ja 20q, joissa esiintyi yleisesti perimäaineksen lisääntymistä eli monistumaa haimasyövässä. Samalla tavoin tunnistettiin useita kromosomialueita, joissa esiintyy yleisesti perimäaineksen häviämistä. Näihin kuuluivat mm. kromosomialueet 18q, 9p, 4q, 3p ja 8p.

Tutkimuksessa tunnistetuilla monistuvilla kromosomialueilla sijaitsee useita geenejä, joiden on jo aiemmin osoitettu olevan osallisena muiden kiinteän kudoksen kasvainten, kuten rintasyövän, synnyssä. Tutkimuksessa selvitettiinkin

14 tällaisen geenin mahdollista osuutta haimasyövän synnyssä käyttäen fluoresenssi in situ hybridisaatio -menetelmää. Tunnetuista syöpägeeneistä *MYC* todettiin monistuneeksi 54 %:ssa solulinjoista ja *CCND1* 28%:ssa. Sen lisäksi 17q-kromosomikäsivarressa sijaitsevat *ERBB2*-, *TBX2*- ja *BIRC5*-geenit olivat monistuneita 20 %:ssa, 50 %:ssa ja 58 %:ssa tapauksista. Kromosomi 20q -alueelta löytyi useita geenejä, jotka olivat erittäin yleisesti monistuneita haimasyöpäsolulinjoissa. Näistä *CTSZ*-geeni oli kaikkein useimmin monistunut 83 %:ssa solulinjoista. Tutkimuksessa etsittiin lisäksi tarkemmin 12p-kromosomialueen monistuman mahdollisia kohdegeenejä. Saadut tulokset osoittivat, että monistuma-alue on kooltaan 3,5 megaemäsparia. Tällä alueella sijaitsevien geenien ilmenemistasoja tutkittiin käyttäen mikrosirumenetelmää ja nämä tutkimukset osoittivat, että *KRAS2*-, *DEC2*- ja *PPFIBP1*-geenit olivat yliekspressoituneita monistuneissa tapauksissa ja siten edustavat mahdollisia monistuman kohdegeenejä.

Viimeisessä osatyössä tutkittiin genomin laajuisella mikrosirutekniikalla 12232 geenin monistuma- ja ekspressiotasot 13 haimasyöpäsolulinjassa. Tutkimuksessa paikannettiin 24 erillistä monistuma-aluetta, joiden sijainti voitiin määrittää erittäin tarkasti. Statistisen testin avulla tunnistettiin 105 geeniä, jotka olivat sekä monistuneita että yliekspressoituneita haimasyövässä. Näistä osa oli aiemmin syövässä monistuneiksi todettuja geenejä, kuten *AURKA*(*STK15*) ja *MLN51*, tai tunnettuja onkogeenejä, kuten *RAB4A* ja *RELA*. Lisäksi oli joukko geenejä, joiden ei ole aiemmin tiedetty monistuvan syövässä. Näihin kuuluu mm. *PAK4*-geeni, joka toimii mm. solujen migraatiossa ja adheesiossa. Suuri osa tutkimuksessa tunnistetuista 105 geenistä (78 %) on osallisena sellaisissa solun sisäisissä prosesseissa, kuten DNA:n jakautumisessa, transkriptiossa ja solun signaloinnissa, joilla voidaan olettaa olevan merkitystä syövän synnyssä.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, which are referred to by their Roman numerals.

I. Mahlamäki EH, Höglund M, Gorunova L, Karhu R, Dawiskiba S, Andrén-Sandberg A, Kallioniemi OP, Johansson B: Comparative genomic hybridization reveals frequent gains of 20q, 8q, 11q, 12p, and 17q, and losses of 18q, 9p, and 15q in pancreatic cancer. *Genes Chromosomes Cancer 20: 383-391, 1997*.

II. Mahlamäki EH, Bärlund M, Tanner M, Gorunova L, Höglund M, Karhu R, Kallioniemi A. Frequent amplification of 8q24, 11q, 17q, and 20q-specific genes in pancreatic cancer. *Genes Chromosomes Cancer 35: 353-358, 2002*.

III. Heidenblad M, Jonson T, Mahlamäki EH, Gorunova L, Karhu R, Johansson B, Höglund M. Detailed genomic mapping and expression analyses of 12p amplifications in pancreatic carcinomas reveal a 3.5-Mb target region for amplification. *Genes Chromosomes Cancer 34: 211-223, 2002.*

IV. Mahlamäki EH, Kauraniemi P, Monni O, Wolf M, Hautaniemi S, Kallioniemi A. High-resolution genomic and expression profiling reveals 105 putative amplification target genes in pancreatic cancer. (Submitted)

ABBREVIATIONS

ARF	alternate open reading frame gene
AURKA	aurora kinase A
BAC	bacterial artificial chromosome
BIRC5	baculoviral IAP repeat-containing 5 (survivin)
BMI	body mass index
cDNA	complementary DNA
CCND1	cyclin D1
CDKN2A	cyclin-dependent kinase inhibitor 2A
CGH	comparative genomic hybridization
CI	confidence interval
CTSZ	cathepsin Z
DAPI	4', 6'-diamidino-2-phenylindole
DEC2	basic helix-loop-helix domain containing, class B, 3
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiotreitol
dNTP	deoxynucleotidetriphosphate
EGFR	epidermal growth factor receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
EST	expressed sequence tag
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
INK4A	alternate symbol for CDKN2
KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog
MADH4	MAD, mothers against decapentaplegic homolog 4
Mb	megabase

mRNA	messenger ribonucleic acid
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
P1	P1 artificial chromosome
PAC	P1 derived artificial chromosome
PAK4	p21 (CDKN1A)-activated kinase 4
PanIN	pancreatic intraepithelial neoplasia
PCR	polymerase chain reaction
PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)
Rad51	RAD51 homolog (S. cerevisiae)
RB1	retinoblastoma 1
RNA	ribonucleic acid
RR	relative risk
RT-PCR	reverse-transcriptase polymerase chain reaction
SSC	standard saline citrate
SDS	sodium dodecyl sulfate
STS	sequence tagged site
TBX2	T-box 2
TP53	tumor protein p53
YAC	yeast artificial chromosome

ABSTRACT

Pancreatic cancer was the fourth most common cause for cancer deaths in males, and the third most common in females in 2001 (Finnish Cancer Registry, 2003). The prognosis of this cancer is poor and almost all patients die within five years of diagnosis. Although several genes have been implicated in the pathogenesis of pancreatic cancer, the entire spectrum of genetic aberrations leading to the development of this disease is poorly characterized. The main aim of this study was to identify genetic aberrations and specific genes having a crucial role in the pathogenesis of pancreatic cancer.

Copy number aberrations were analyzed in 31 pancreatic cancer cell lines and 13 tumor biopsies by chromosomal CGH. Several common regions of gain and amplification were detected, including those at 3q, 7p, 8q, 11q, 17q, 19q, and 20q. Similarly, frequent losses were observed at 4q, 8p, 9p, 18q, and 21q. The chromosomal regions involved in frequent copy number gains contain several genes, such as the *MYC*, *CCND1*, and *ERBB2* oncogenes, with an established role in cancer pathogenesis. The potential involvement of these oncogenes as well as that of several genes from 17q and 20q -regions, was explored in 30 pancreatic cancer cell lines. Amplification of the *MYC* oncogene was observed in 54% of the cell lines and *CCND1* in 28%. At the 17q region, *ERBB2*, *TBX2* (17q23), and *BIRC5* (17q25) were amplified in 20%, 50%, and 58% of the cell lines respectively. At 20q, the *CTSZ* gene (20q13) was most commonly amplified in 83%, *NCOA6* (20q11) in 71%, and *PTPN1* in 70% of cases.

Detailed characterization of the commonly observed 12p amplicon was performed in 15 pancreatic cancer cell lines to identify possible amplification target genes. FISH analysis using YAC clones allowed delineation of the region

of interest to an approximately 5 Mb segment at 12p11-p12. Semiquantitative PCR was then used to further narrow down the amplification to a 3.5 Mb segment, between markers D12S1617 and sts-N38796. A chromosome segment-specific cDNA microarray containing 29 expressed sequences from the D12S1617 and sts-N38796 interval was constructed to explore expression levels of genes from this region in eight pancreatic cancer cell lines. This expression survey revealed overexpression of four ESTs, including the *DEC2* and *PPFIBP1* genes. In addition, increased expression of the *KRAS2* gene, located in the distal part of the amplicon, was observed in all cell lines with amplification.

A genome-wide 12 232 clone cDNA microarray was used for high-resolution mapping of copy number increases and for the identification of putative amplification target genes in 13 pancreatic cancer cell lines. The CGH microarray analysis implicated 24 independent amplified regions. These included several chromosomal segments, such as 3q, 5p, 7q, 8q24, 11q13, 15q, 17q, 19q, and 20q, previously shown to be gained or amplified by chromosomal CGH or by FISH, whose exact boundaries were now delineated on a base-pair scale. A statistical analysis revealed 105 genes that were systematically overexpressed when amplified. These included previously described amplified genes, such as *STK15* and *MLN51*, as well as novel targets for copy number alterations, such as *p21-activated kinase 4* (*PAK4*) involved in cell migration, cell adhesion, and anchorage-independent growth. Functional characterization indicated that 78% of the 105 genes are associated with cellular processes, such as signal transduction, transcription, and DNA replication, that could be directly associated with cancer pathogenesis. The 105 genes identified in this study to be activated by increased copy number are therefore likely to be part of the tumorigenesis of pancreatic cancer.

INTRODUCTION

In the past 20 years the basic elements of cancer have been subjected to intensive research and many specific genetic alterations involved in cancer pathogenesis have been detected. Cancer development is known to be a multi-step process where an accumulation of numerous genetic changes gradually leads to the transformation of a normal cell into a tumor cell (Kinzler and Vogelstein, 2001). Genetic alterations in cancer, such as mutations, translocations and changes in gene copy number i.e. deletions and amplifications, typically lead to inactivation of tumor suppressor genes and activation of oncogenes. Such gene abnormalities may be acquired in somatic cells, for example through exposure to radiation or carcinogens, or they may be inherited. Inherited gene defects typically lead to increased cancer susceptibility and cause so-called cancer syndromes, for instance the Li-Fraumeni syndrome that is caused by mutations e.g. in the *TP53* gene (Malkin et al., 1990). Besides tumor suppressor genes and oncogenes, gene abnormalities may also target DNA repair genes, whose malfunction leads to accelerated mutation rate and genetic instability (Fearon, 2001). Chromosomal instability leads to cancer cell aneuploidy, which is also very typical for pancreatic cancer (Griffin et al., 1994; Griffin et al., 1995; Gorunova et al., 1998).

Various experimental model systems have been used to investigate the early events in cancer initiation. These studies have, for example, aimed to identify the minimum number of gene defects required to transform a normal cell into a tumorigenic cell (Lundberg et al., 2000; Hahn and Meyerson, 2001). They have suggested that at least four signaling pathways must be disrupted to create tumorigenic human cells from normal mesenchymal or epithelial cells (Hahn et al., 1999). These pathways are regulated by large-T antigen, oncogenic *ras* and telomerase. Large-T antigen perturbs at least two distinct cellular control

pathways through its ability to bind and functionally inactivate the *RB1* and *TP53* tumor-suppressor proteins. Oncogenic *ras* activates the mitogen-response pathway, and telomerase has a central role in the maintenance of functional telomeres (Hahn et al., 1999). However, research by Seger et al. (2002) showed that telomerase activation is not necessary for transformation, but combined expression of adenovirus *E1A*, oncogenic *ras*, and *MDM2* is sufficient to convert a normal human cell into a cancer cell (Seger et al., 2002). In this combination, *RB1*, p300 and p400 pathways are disrupted by E1A, and *MDM2* is responsible for the disruption of the *TP53* pathway. Given these findings, the role of telomere maintenance in the transformation of human cells remains controversial.

The model proposed by Fearon and Vogelstein has been for more than ten years the paradigm for the development of colorectal carcinoma (Fearon and Vogelstein, 1990). According to this model colorectal tumors progress through a series of clinical and histopathological stages. These comprise phases from normal epithelium through early, intermediate, and late adenomas and finally culminating into invasive and metastasizing carcinomas. Each one of these histopathological stages are accompanied by specific genetic alterations. For example, the transition from normal to dysplastic epithelium is characterized by loss of the *APC* tumor suppressor gene and similarly, transition from late adenoma to carcinoma is associated with mutations of the *TP53* gene. Similar sequential acquisition of genetic aberrations associated with a distinct histomorphological phenotype has also been observed e.g. in melanoma (reviewed by Bastian, 2003) and in the early phases of pancreatic cancer (Wilentz et al., 1998; Wilentz et al., 2000; Luttges and Kloppel, 2001; Swartz et al., 2002). The main focus of this study was to use genome wide methods, including CGH and large scale cDNA microarrays, to identify genetic changes involved in pancreatic cancer pathogenesis.

REVIEW OF THE LITERATURE

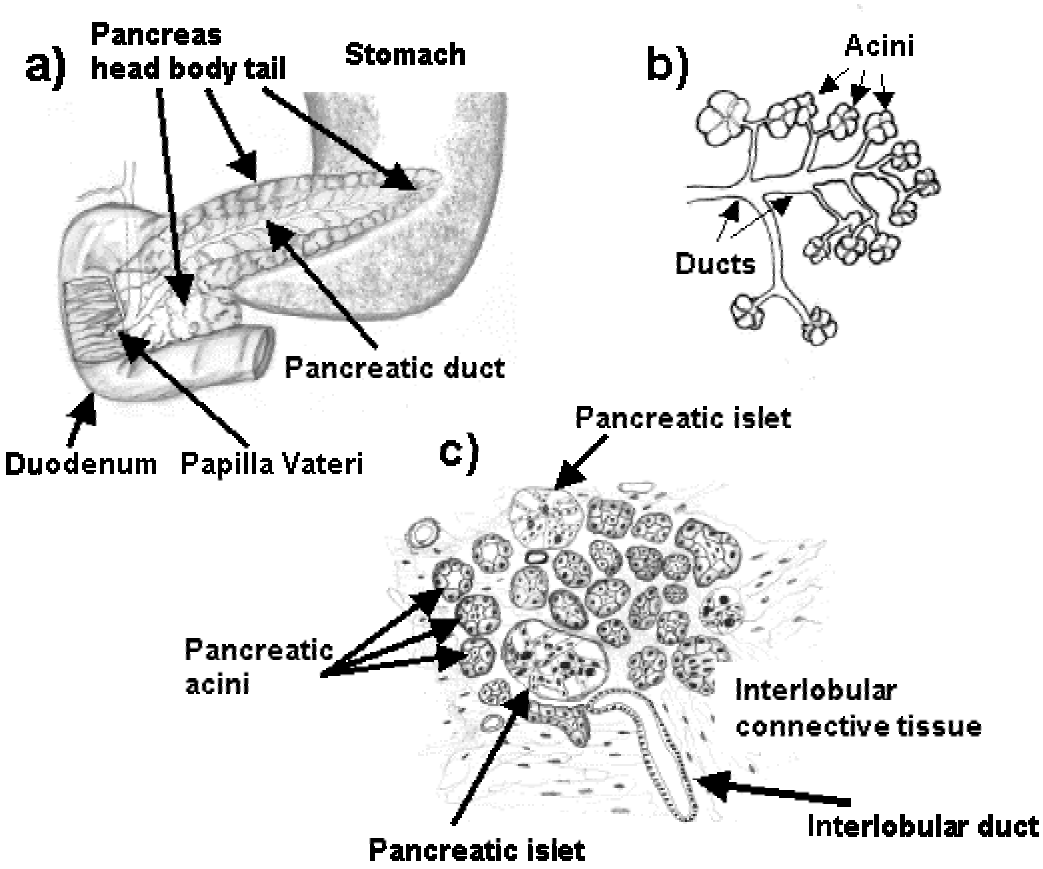
1. Pathology of pancreatic cancer

The pancreas is involved in two different and very important physiological processes, the regulation of digestion (exocrine pancreas) and glucose metabolism (endocrine pancreas). The exocrine pancreas consists of acinar and duct cells that make up the majority of the pancreatic tissue (Figure 1). The acinar cells produce digestive enzymes and the duct cells add mucous and bicarbonate to the enzyme mixture. The duct size increases from the acini to the main and accessory pancreatic ducts that empty into the duodenum. The endocrine pancreas consists of four specific cell types that are organized as islets and secreting hormones into the bloodstream (Beckingham, 2001). Cancers of the pancreas can occur both in the exocrine pancreas (classic pancreatic adenocarcinomas) and in the endocrine pancreas. This thesis is concerned with exocrine pancreatic adenocarcinoma.

The most common exocrine pancreatic cancer is ductal pancreatic adenocarcinoma and its variants (Brat et al., 1998), accounting for about 85-90% of cases. Rare subtypes of exocrine pancreatic cancer include acinar cell carcinoma, intraductal papillary-mucinous carcinoma, mucinous cystadenocarcinoma, serous cystadenocarcinoma, pancreatoblastoma, and solid-pseudopapillary carcinoma. Macroscopically ductal pancreatic adenocarcinomas are firm and poorly defined masses. According to the World Health Organization (WHO) histological classification of tumors of the exocrine pancreas (edited by Hamilton and Aaltonen, 2000) most ductal adenocarcinomas are well to moderately differentiated and are characterized by well-developed glandular structures embedded in desmoplastic stroma consisting of excessively proliferating fibroblasts and components of extra cellular matrix. The

desmoplastic reaction is characteristic for pancreatic cancer and is apparently caused by inappropriate expression of the connective tissue growth factor (Wenger et al., 1999). Intratumoral heterogeneity regarding the differentiation status is also frequent in pancreatic adenocarcinoma. At the advancing edge of the carcinoma the tumor is scattered in the pancreatic stroma as small clusters of neoplastic cells (Hamilton and Aaltonen, 2000). The most common way for the spread of pancreatic adenocarcinoma is through the perineural sheaths into the retroperitoneal fatty tissue but lymphatic spread is also frequently observed (Hamilton and Aaltonen, 2000).

Figure 1. *Schematic illustration of structure and topography of pancreas a) Pancreas, duodenum and stomach b) Pancreatic acini c) Microscopic scheme from pancreatic tissue*



The majority (60-70 %) of pancreatic adenocarcinomas occur in the head of the organ, a minority of cases is found in the body or tail of the pancreas (see Figure 1). The size of carcinomas of the head of the pancreas ranges from 1.5 to 5 cm, whereas carcinomas of the body and tail are usually larger at the time of diagnosis (Hamilton and Aaltonen, 2000). Due to the fact that there is a lot of space for the tumor to grow and spread, the first symptoms of pancreatic cancer come typically rather late in the disease progression. Symptoms are usually caused by the growing tumor obstructing the common bile duct and pancreatic ducts or perineural invasion to the celiac plexus. Complete obstruction of the common bile duct causes jaundice whereas obstruction of main pancreatic duct leads to duct dilatation and haustration and to fibrous atrophy of the pancreatic parenchyma. In carcinomas of the body and tail, local extensions are more common because of the late diagnosis.

Pancreatic cancer is thought to develop through a series of duct lesions. Hyperplastic and metaplastic pancreatic duct lesions are recommended to be designated as pancreatic intraepithelial neoplasias (PanIN) (Hamilton and Aaltonen, 2000). PanIN-1 lesions have a flat or papillary mucinous epithelium without cellular atypia, whereas PanIN-2 lesions show increasing signs of cellular atypia and a prevalence of papillary architecture. PanIN-3 lesions correspond to carcinoma in situ lesions (Luttges et al., 2001).

2. Epidemiology of pancreatic cancer

Pancreatic cancer is the tenth most common cancer in men and the ninth most common in women, and the disease is the fourth leading cause of cancer death in the United States (Greenlee et al., 2001). The incidence rates are higher for men than for women (Lowenfels and Maisonneuve, 1999) and increase with age, so that 80% of cases manifest between the ages of 60 and 80 years (Gold and Goldin, 1998). In Finland, 689 new cases of pancreatic cancer were diagnosed in

2001, 320 of these occurring in men and 369 in women (Finnish Cancer Registry, 2003). During the year 1999, 308 men (mortality rate 7.8/100 000) and 323 women (mortality rate 4.8/100 000) died from pancreatic cancer in Finland. The overall 5-year survival rate ranges between 1% and 17%, depending on the stage of the disease, with a median survival between 8.5 to 10.1 months (Greenlee et al., 2001; Pernick et al., 2003). The poor prognosis of pancreatic cancer is largely due to the late symptoms leading to a situation where most tumors have already metastasized and are therefore inoperable at the time of diagnosis (Schnall and Macdonald, 1996; Lowenfels et al., 1999).

Pancreatic cancer is thought to develop through exposure to various environmental risk factors. There are several environmental factors influencing our cells, including carcinogens, alcohol, and radiation. Smoking is a well-documented risk factor for the development of pancreatic cancer and has been shown to be associated with a two-fold increase in the risk (Falk et al., 1990; Zatonski et al., 1993; Ahlgren, 1996; Gold and Goldin, 1998; Shapiro et al., 2000; Villeneuve et al., 2000; Schuller, 2002). The roles of other factors, such as alcohol consumption and the associated development of pancreatitis, are controversial (Lowenfels et al., 1993; Karlson et al., 1997; Gold and Goldin, 1998; Silverman, 2001; Schuller, 2002; Ye et al., 2002), although a recent study showed that patients with chronic pancreatitis have a markedly increased risk of pancreatic cancer with a standardized incidence ratio of 19.0 (95% CI 5.2-48.8) (Malka et al., 2002). An association between diabetes and pancreatic cancer has also been observed (Everhart and Wright, 1995), but in these cases the diabetes is most likely caused by the cancer (Gullo et al., 1994; Gullo, 1999). Helicobacter pylori carriage (Stolzenberg-Solomon et al., 2001) and previous cancer history are estimated to lead to an approximately 2-fold increase in pancreatic cancer risk (Travis et al., 1997; Poole et al., 1999). Occupations associated with exposures to metal and textile dusts or certain chemicals, such as pesticides, and working in a biological research laboratory may also slightly increase the risk of pancreatic cancer (Pietri et al., 1990; Ji et al., 1999; Alguacil et al., 2000; Rachet et al., 2000).

The possible involvement of dietary factors in the development of pancreatic cancer has been studied extensively. A study involving 900 000 individuals showed that high body mass index (BMI) increases the relative risk (RR) of pancreatic cancer with persons with a BMI of 35-39.9 having an RR of 1.49 (95% CI 0.99-2.22) (Calle et al., 2003). Another study involving 163 689 individuals also implicated obesity as a risk factor as persons with a BMI of at least 30 kg/m^2 had a RR of 1.72 (95% CI 1.19-2.48) of pancreatic cancer (Michaud et al., 2001). Diets with high intake of saturated fat and red meat, especially grilled red meat, have been associated with increased risk of pancreatic cancer (Anderson et al., 2002; Stolzenberg-Solomon et al., 2002). On the contrary, other dietary factors, such as high carbohydrate intake (Stolzenberg-Solomon et al., 2002) and consumption of fruits, vegetables, and green tea (Ji et al., 1997; Gold and Goldin, 1998) have been associated with decreased risk for pancreatic cancer.

Genetic predisposition is also thought to play a role in the development of pancreatic cancer. A significant association has been observed between family history of pancreatic cancer and pancreatic cancer (RR ranging between 3.0 and 18.0) (Fernandez et al., 1994; Schenk et al., 2001; Tersmette et al., 2001) and hereditary factors may account for approximately 5% of the total pancreatic cancer burden (Lynch et al., 2002). Familial clustering has been connected to an autosomal dominant inheritance pattern in approximately 10% of all cases (Banke et al., 2000). Among 44 788 pairs of twins, monozygote twin men had an RR of 14.0 (95% CI 3.2-60.9), dizygote men an RR of 12.7 (95% CI 3.0-54.1), and monozygote women an RR of 9.6 (95% CI 1.3-73.0) for developing pancreatic cancer (Lichtenstein et al., 2000). Interestingly, dizygote women did not have an increased risk of pancreatic cancer compared to normal population (Lichtenstein et al., 2000). In a genomewide screening of 373 microsatellite markers, significant linkage was found on chromosome 4q32-34, providing evidence for a major locus for dominantly inherited pancreatic cancer (Eberle et al., 2002). Pancreatic cancer is also part of the disease spectrum in several hereditary cancer syndromes, including hereditary breast cancer (*BRCA2*) (Goggins et al., 1996), familial atypical mole-malignant melanoma syndrome

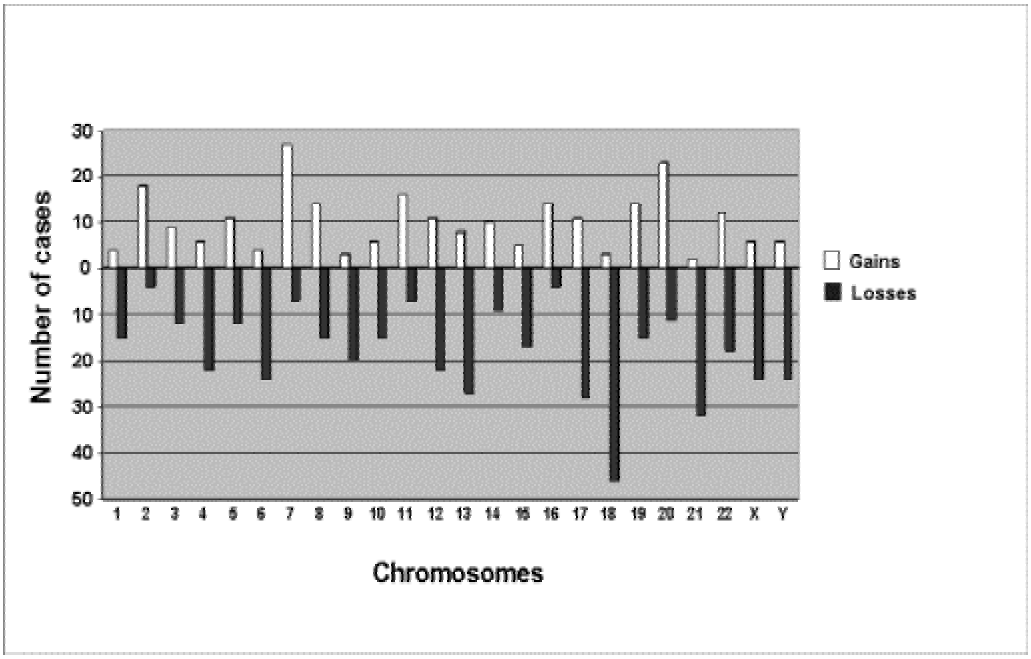
(Vasen et al., 2000), Peutz-Jeghers syndrome (Giardiello et al., 2000), hereditary nonpolyposis colorectal cancer (Lynch et al., 1996), and hereditary pancreatitis (Lowenfels et al., 1997; Lowenfels et al., 2000). Patients with hereditary pancreatitis caused by mutations in the cationic trypsinogen gene *PRSS1* have a 53-fold risk of pancreatic cancer (Whitcomb et al., 1996).

3. Genetic and epigenetic changes in pancreatic cancer

3.1. Cytogenetic findings in pancreatic cancer

Genome-wide analyses of genetic changes in pancreatic carcinomas have been performed using traditional cytogenetic analyses as well as comparative genomic hybridization. To date, cytogenetic analyses have been done on low-passage cell lines derived from a total of 220 primary pancreatic tumors or metastases (Johansson et al., 1992; Bardi et al., 1993; Griffin et al., 1994; Johansson et al., 1994; Griffin et al., 1995; Gorunova et al., 1998; Höglund et al., 1998a; Höglund et al., 1998b). These analyses showed abnormal karyotypes in 52-72% of cases and complex karyotypes with more than three abnormalities per tumor were observed frequently. The most common abnormalities in cytogenetic analyses were the loss of complete copies of chromosomes 1 (in 11-29% of cases), 6 (19-33%), 12 (4-23%), 13 (8-25%), 17 (11-29%), 18 (23-35%), 21(19-34%), and Y (7-17%), as well as gains of chromosome 1 (7-38%), 7 (8-35%), 8 (4-42%), 11 (19-26%), 12 (8-26%), and 20 (12-33%). Overall, chromosome losses were observed more frequently than gains. Figure 2 represents a summary of losses and gains occurring in 190 pancreatic cancer cases according to the Mitelman Database of Chromosome Aberrations in Cancer (http://cgap.nci.nih.gov/Chromosomes/RecurrentAberrations).

Figure 2. *Summary of numerical chromosomal aberrations in 190 pancreatic cancers by traditional cytogenetic analysis (reviewed by Mitelman, 2003). The number of cases with gain or loss are shown for each chromosome.*



In addition to losses and gains of whole chromosomes, structural chromosomal aberrations were also frequently detected in the cytogenetic analyses of pancreatic cancer. According to the Mitelman Database, 65 recurrent (i.e. occurring in more than two cases) unbalanced chromosomal abnormalities have been reported in 190 pancreatic cancer cases. Surprisingly, no balanced aberrations were observed in this large set of samples. The recurrent unbalanced chromosomal aberrations are listed in Table 1 and include deletions, additions of unknown material, and isochromosomes. However, marker chromosomes, that are rearranged chromosomes that could not be identified by G-banding, were also frequently observed, emphasizing the complexity of the chromosomal aberrations in these tumors. Moreover, intratumoral heterogeneity has been shown to be very common in pancreatic cancer. An extreme example was reported by Gorunova et al. (1995) who identified more than 50 clones with unrelated numerical and structural chromosome changes in a single tumor where the number of karyotypic anomalies per clone varied from one to eight

(Gorunova et al., 1995). Finally, complex karyotypes have been shown to correlate with poor differentiation of the tumor and short patient survival in pancreatic cancer (Johansson et al., 1994).

Table 1. *Chromosome abnormalities in pancreatic cancer reviewed by Mitelman.*

CHROMOSOME	ABNORMALITY, NUMBER OF CASES IN BOLD
1	del(1)(p13) **2**, del(1)(p21) **2**, del(1)(p32) **2**, add(1)(p36) **4**, i(1)(q10) **9**, del(1)(q11) **3**, del(1)(q12) **5**, del(1)(q21) **3**
3	add(3)(p11) **2**, del(3)(p11) **5**, del(3)(p12) **2**, del(3)(p21) **2**, i(3)(q10) **4**
4	del(4)(q21) **3**, del(4)(q25) **2**
5	i(5)(p10) **5**
6	i(6)(p10) **2**, del(6)(q15) **4**
7	add(7)(p22) **3**, del(7)(q11) **2**, del(7)(q32) **2**
8	del(8)(p12) **2**, del(8)(p21) **3**, i(8)(q10) **4**, add(8)(q24) **2**
9	add(9)(p11) **2**, del(9)(p13) **4**
10	del(10)(p11) **2**, i(10)(q10) **2**, add(10)(q26) **4**
11	add(11)(p11) **3**, del(11)(p13) **2**, add(11)(p15) **2**, dup(11)(q13q23) **2**, del(11)(q14) **2**, add(11)(q21) **3**, del(11)(q23) **2**, dup(11)(q13q23) **2**
12	add(12)(p11) **2**
13	add(13)(p11) **2**, der(13;13)(q10;q10) **2**, der(13;13)(q10;q10) **2**, der(13;15)(q10;q10) **3**, i(13)(q10) **2**
14	add(14)(p11) **5**, der(14;15)(q10;q10) **3**, i(14)(q10) **2**
15	add(15)(p11) **4**, der(13;15)(q10;q10) **3**, der(14;15)(q10;q10) **2**, i(15)(q10) **2**
16	add(16)(p13) **3**, del(16)(q22) **2**
17	add(17)(p11) **6**, i(17)(q10) **4**
18	add(18)(q12) **3**, del(18)(q12) **2**
19	add(19)(p13) **2**, i(19)(q10) **2**, add(19)(q13) **8**
20	add(20)(q13) **3**
21	add(21)(p11) **3**, i(21)(q10) **2**
22	add(22)(p11) **3**
X	add(X)(q22) **2**
i=isochromosome, add=additional unknown material in the arm, del=lost material in the arm, der=derivative chromosome,	

3.2 Comparative genomic hybridization studies of pancreatic cancer

Due to the extreme complexity of the genetic aberrations occurring in pancreatic cancer, it is not possible to completely solve their genetic composition by traditional cytogenetic analysis. Different methods are therefore needed to reveal the genetic changes in this disease. Comparative genomic hybridization (CGH) is a useful technique that provides information on DNA copy number alterations, i.e. gains and losses, across the whole genome (Kallioniemi et al., 1992). CGH analysis does not require the preparation of metaphase chromosomes from the tumor but instead maps the genetic aberrations on normal human chromosomes. Therefore this technique is especially helpful in the analysis of complex chromosomal changes, such as those occurring in pancreatic cancer. One of the disadvantages of CGH, as of all other techniques based on isolated DNA, is that the sample should contain at least 50% tumor cells (Kallioniemi et al., 1994). The desmoplastic reaction that is so characteristic of pancreatic cancer may make it difficult to obtain such samples. However, despite such problems, CGH studies have revealed chromosomal abnormalities in almost 100% of pancreatic cancer cell lines and in 67-100% of primary tumors (Solinas-Toldo et al., 1996; Fukushige et al., 1997; Curtis et al., 1998; Ghadimi et al., 1999; Schleger et al., 2000; Shiraishi et al., 2001; Harada et al., 2002). The frequency of aberrations with CGH in pancreatic cancer ranges from 5-25 per primary tumor and 14-27 per cell line. Almost all CGH studies have indicated common losses affecting chromosome arms 6q (in 30-50% of cases), 9p (30-89%), and 18q (42-89%), as well as gains at 7q (56-67%), 8q (24-67%), 7p (4-78%), and 20q (15-83%) in pancreatic adenocarcinomas (Figure 3). High-level amplifications have been detected in 15-60% of uncultured tumors (Solinas-Toldo et al., 1996; Harada et al., 2002). Surprisingly, the number of chromosomal aberrations observed with CGH was shown not to correlate with tumor grade and stage in pancreatic cancer (Schleger et al., 2000).

The same chromosomal regions have been shown by CGH to be involved both in primary pancreatic tumors and cell lines (Solinas-Toldo et al., 1996; Fukushige

et al., 1997; Curtis et al., 1998; Ghadimi et al., 1999; Schleger et al., 2000; Shiraishi et al., 2001; Harada et al., 2002) indicating that the cell lines can serve as a valuable model in the study of pancreatic cancer. In a recent study by Harada et al., (2002), three to four separate samples were microdissected from 20 pancreatic tumors and analyzed by CGH. The CGH results showed a wide variety of different genetic changes between adjacent neoplastic glands within a single tumor, confirming the previous knowledge of the wide intratumoral heterogeneity in pancreatic cancer.

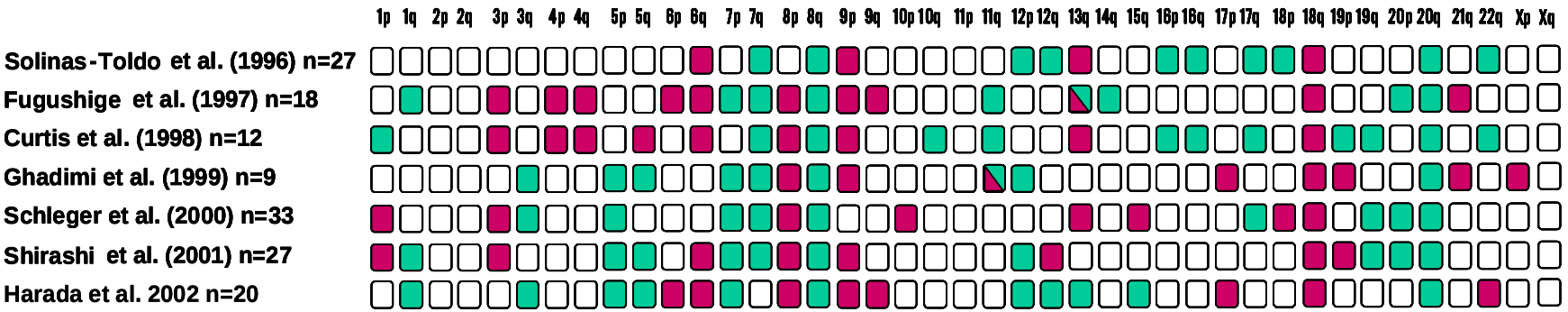


Figure 3. *Summary on the most common losses and gains in primary pancreatic cancers and pancreatic cancer cell lines reported in seven CGH studies. The number of samples analyzed in each study is indicated after the reference. Approximately eight most common aberrations which appear in at least 10% of the samples are shown. Gains are indicated in green and losses in red.*

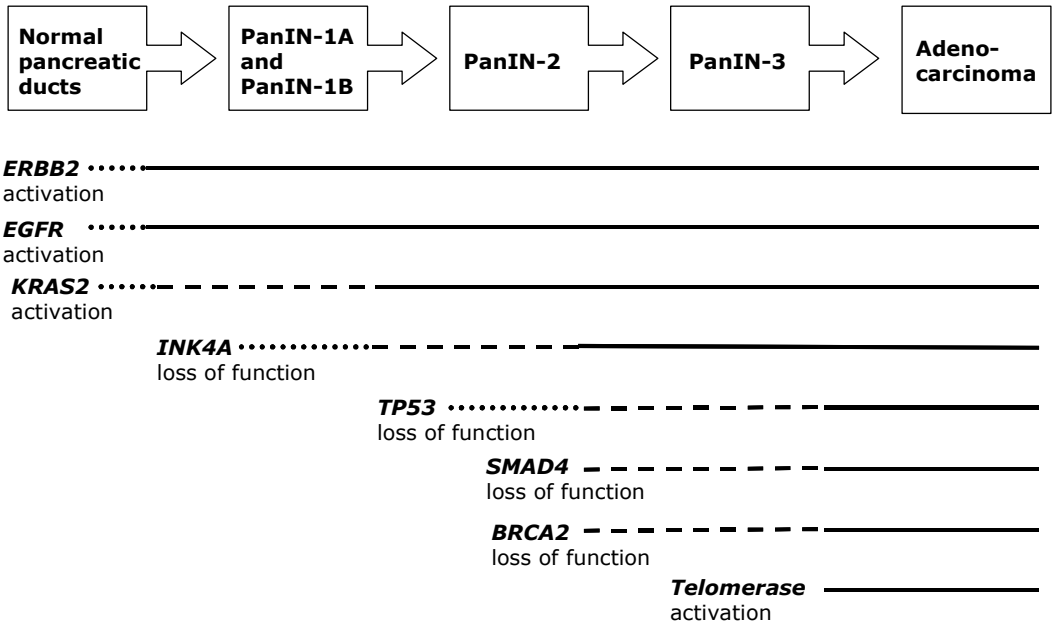
3.3 Gene alterations in pancreatic cancer

The role of known oncogenes and tumor suppressor genes in the development of pancreatic cancer has been fairly well established (reviewed in Bardeesy and DePinho, 2002). Activation of the *KRAS2* oncogene by point mutation is the most common genetic change in pancreatic cancer occurring in nearly all primary pancreatic cancers (Almoguera et al., 1988; Rozenblum et al., 1997). The activation of *KRAS2* leads to a number of cellular changes including induction of proliferation, invasion, and survival (reviewed in Shields et al., 2000). *KRAS2* mutations have been shown to occur exclusively in three hotspots (codons 12, 13, and 61), of which codon 12 is most commonly affected in pancreatic cancer (Grunewald et al., 1989; Minamoto et al., 2000). *KRAS2* mutations have been found in normal pancreas as well as in noninvasive neoplastic precursor lesions (Figure 4), indicating that they represent an early event in the pathogenesis of pancreatic cancer (Moskaluk et al., 1997; Luttges et al., 1999b). In addition, multiple different *KRAS2* mutations have been found more frequently in pancreatic cancers with previous pancreatic intraepithelial neoplasia (PanIN) than without, suggesting that clonally distinct precursor lesions may contribute to tumor development in pancreatic cancer (Laghi et al., 2002).

Pancreatic cancers frequently overexpress multiple growth factors and growth factor receptors. These include the epidermal growth factor receptor (*EGFR*) and related receptors, multiple ligands that bind to EGFR, certain fibroblast growth factor receptors and ligands, as well as insulin-like growth factor and its receptor (reviewed by Korc, 1998). For example, EGFR has been shown to be overexpressed in 30-50% of pancreatic cancers (Yamanaka et al., 1993; Tobita et al., 2003). Specific drugs targeting the EGFR, i.e. monoclonal antibodies and tyrosine kinase inhibitors, are currently available for the treatment of tumors with activation of the *EGFR* pathway and have also produced promising results in pancreatic cancer (Xiong and Abbruzzese 2002). *ERBB2* amplification and overexpression is a relatively common event in pancreatic cancer (reviewed by Sakorafas et al., 2000). *ERBB2* was shown to be amplified in 27% of pancreatic

adenocarcinomas and overexpressed in about 20% of the tumors (Hall et al., 1990; Safran et al., 2001). Both *EGFR* and *ERBB2* activation ase considered as early events pancreatic cancer development as they already occur in pancreatic cancer precursors.

Figure 4. *Model of the accumulation of genetic aberrations in pancreatic intraepithelial neoplasia (PanIN) and pancreatic cancer. The type of the line indicates the frequency of the lesion. (Modified from to Bardeesy and DePinho, 2002.)*



Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) at 9p21 encodes for two tumor suppressors, *INK4a* (p16) and *ARF* (p14) (Sherr, 2001). Both of these proteins act as cell cycle regulators, INK4a through the retinoblastoma tumor suppressor pathway and ARF by stabilizing the p53 tumor suppressor protein (Quelle et al., 1995; Stott et al., 1998). Germ line mutations of *CDKN2A* are found in melanoma-prone families and are also known to cause the familial atypical mole-malignant melanoma syndrome, both of these are characterized by increased risk of pancreatic cancer (Goldstein et al., 1995; Whelan et al., 1995). In sporadic pancreatic carcinomas, homozygous deletions of *INK4a* have been detected in 41% of tumors and sequence changes in 38% (Caldas et al., 1994). Rozenblum

and coworkers showed that *CDKN2A* was inactivated either by mutation or deletion in 76% of primary pancreatic cancers (Rozenblum et al., 1997). The *INK4a* seemed to be the primary target in pancreatic cancer because mutations affecting *INK4a* but sparing *ARF* have been identified (Rozenblum et al., 1997; Bardeesy and DePinho, 2002). Moreover, *INK4a* inactivation has been shown to occur already in early-stage PanIN-1 lesions (Figure 4), indicating that it is an early event in the development of pancreatic carcinoma (Bardeesy and DePinho, 2002).

The tumor suppressor protein *TP53* (p53), a nuclear DNA-binding protein, plays an essential role in the regulation of the cell cycle (reviewed in Bullock and Fersht, 2001; Vousden and Lu, 2002). Inactivation of the *TP53* gene located at chromosome 17p13.1, occurs in about 50% of human tumors (Carson and Lois, 1995). Germline mutations of *TP53* cause the Li-Fraumeni syndrome that is characterized by diverse mesenchymal and epithelial neoplasms at multiple sites (Srivastava et al., 1990). In pancreatic cancer, *TP53* has been shown to be either deleted or mutated in 50-75% of cases (Ruggeri et al., 1992; Scarpa et al., 1993; Rozenblum et al., 1997; Coppola et al., 1998). In almost all cases, loss of one allele has been shown to be coupled with an intragenic mutation in the other allele, leading to the inactivation of *TP53*. Allelic loss of *TP53* has been shown to be present in the PanIN-2 lesions (Figure 4) and is particularly common in those lesions with moderate-grade dysplasia, suggesting that this genetic change occurs fairly early in the development of pancreatic cancer (Luttges et al., 2001).

MADH4 (mothers against decapentaplegic homolog 4, also abbreviated *SMAD4*, *DPC4*) is located at 18q21.1, encodes a key intracellular messenger in the transforming growth factor beta (*TGFB*) signaling cascade. *TGFB* is a potent inhibitor of growth and differentiation of epithelial cells and it has been assumed that loss of *MADH4* function relieves this inhibition (reviewed by Massague, 1998). Recent studies have also indicated that *MADH4* is involved in the suppression of angiogenesis (Schwarte-Waldhoff et al., 2000). About 90% of human pancreatic carcinomas show allelic loss at chromosome 18q and 30% of tumors have been found to contain a homozygous deletion at the *MADH4/DPC4*

locus (Hahn et al., 1996). *MADH4* was also found to be inactivated by mutation in 22% pancreatic carcinomas without homozygous deletions (Hahn et al., 1996). Rozenblum et al. (1997) confirmed the involvement of *MADH4* in pancreatic cancer and showed that it is either deleted or mutated in 53% of tumors. Patients with *MADH4* protein positive tumors have shown longer survival than *MADH4* negative patients (Tascilar et al., 2001). *MADH4* mutations have been observed in PanIN-3 lesions (Figure 4) and therefore seem to occur later than *INK4A* and *TP53* mutations in the development of pancreatic carcinomas (Bardeesy and DePinho, 2002).

3.4 Epigenetic changes in pancreatic cancer

In addition to genetic aberrations, epigenetic changes including DNA hypomethylation or hypermethylation and histone acetylation or deacetylation have been shown to have an essential role in cancer progression. In tumor tissues, many genes have hypermethylated promoter regions, which is associated with inappropriate transcriptional silencing of genes (Jones and Baylin 2002). In pancreatic cancer, hypermethylation of the *ras* association domain family 1A (*RASSF1A*) and p16 (*INK4A*) genes has been detected in 64% and 43% of primary adenocarcinomas respectively (Dammann et al. 2003). Silencing of the *TSLC1* tumor suppressor gene by methylation has been detected in about one third of pancreatic adenocarcinomas and high-grade PanIN-3 lesions, but not in low-grade PanIN lesions or in normal pancreatic tissue, suggesting that it is a late event in tumor progression (Jansen et al. 2002). Several other genes, including C*CND2*, *3-OST-2*, *SPARC*, *RARB*, and *TIMP3* have been reported to be methylated in pancreatic cancer (Ueki et al. 2000; Matsubayashi et al. 2003; Miyamoto et al. 2003; Sato et al. 2003a) and the list of genes is likely to grow in the future.

Hypomethylation has also been observed in tumor cells in comparison to normal cells. The hypomethylation of structural elements, such as centromeric DNAs, might cause enhanced genomic instability (Jones and Baylin 2002). Sato et al. (2003b) analyzed a set of 32 genes to investigate the relationship between

hypomethylation and gene expression in pancreatic cancer. They identified seven genes, among them *CLDN4*, *LCN2*, *TFF2*, *S100A4*, and *PSCA* that were hypomethylated and overexpressed in pancreatic carcinoma cell lines and primary tumors but not in normal pancreatic ducts. These results indicate that hypomethylation is also a common event in pancreatic cancer and leads to increased expression of affected genes.

The functional significance of histone deacetylation has been studied using a deacetylase inhibitor trichostatin A (TSA) in pancreatic cell lines (Donadelli et al. 2003). The cellular growth of nine pancreatic cancer cell lines with mutated p53 seemed to be greatly inhibited by TSA, suggesting that histone deacetylation inhibitors may offer new possibilities in the treatment of pancreatic cancer. This study, together with ongoing DNA methylation studies, attempts to understand the epigenetic changes in pancreatic cancer cells.

4. DNA microarrays

4.1 DNA microarray technology and its applications in cancer research

Microarrays permit the analysis of gene expression, DNA sequence variation, protein levels, tissues, cells and other biological and chemical molecules in a massively parallel format. DNA microarrays were first developed for high throughput analysis of differential gene expression patterns (Schena et al., 1995; DeRisi et al., 1996; Lockhart et al., 1996) and the currently available arrays theoretically allow the analysis of all genes in the human genome in a single experiment. There are basically three kinds of DNA arrays: cDNA, oligonucleotide, and genomic arrays. The main applications of the cDNA and oligonucleotide arrays are expression analyses, whereas genomic arrays are used for copy number analysis.

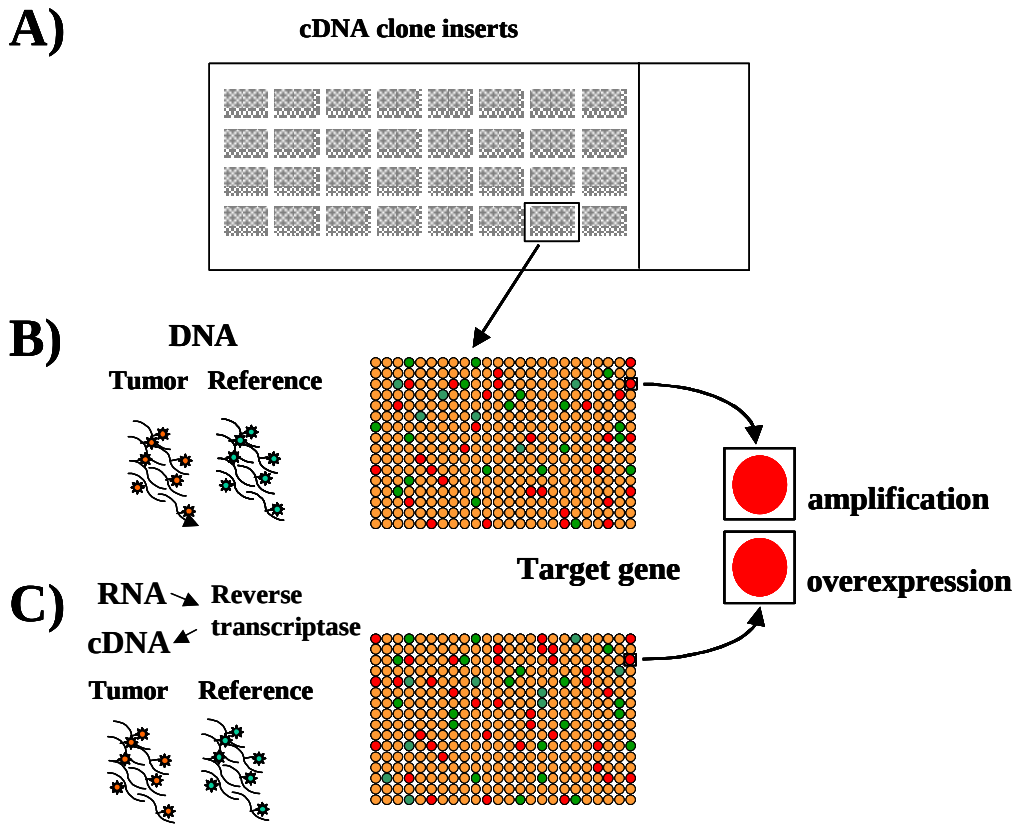
The cDNA microarrays contain spotted PCR amplified inserts from cDNA clones. The expression levels in two samples can be directly compared with each other when differently fluorescent-labeled sample and reference cDNA are hybridized on the cDNA array. The ratio of the fluorescence intensities reflects the down- or up-regulation of the genes examined (Schena et al., 1995; DeRisi et al., 1996). The oligonucleotide microarrays contain oligonucleotides that are either synthesized in situ by photolithography or ink-jet technology (Lockhart et al., 1996; Hughes et al., 2001) or spotted on the array (Barczak et al., 2003). Several oligonucleotides representing each individual gene and its possible splice variants can be placed on an array. In the case of arrays made by photolitography, comparisons of different samples are done on separate hybridizations instead of comparing two samples on the same array using different colors (Wodicka et al., 1997). In addition to expression analyses, the oligonucleotide arrays can also be used for detection of DNA polymorphisms and mutations (Lipshutz et al., 1999). Genomic microarrays are used for copy number analyses and are typically constructed by spotting DNA or PCR products from large insert size genomic clones, such as P1, PAC, and BAC clones, on glass slides (Solinas-Toldo et al., 1997; Pinkel et al., 1998).

The applications of DNA microarray technologies in cancer research are numerous. First of all, large-scale microarray based expression studies have illustrated that different tumor types can be distinguished based on their expression profiles (Alizadeh et al., 2001). In addition, histologically similar tumors can be subclassified into specific categories. Such subclassification of tumors has been successfully performed in many different tumor types, including lymphomas, melanomas, breast cancer, and pediatric tumors (Alizadeh et al., 2000; Bittner et al., 2000; Perou et al., 2000; Khan et al., 2001). Expression profiling can also classify tumors according to clinical characteristics. For example, in diffuse large B-cell lymphoma molecular profiling enabled the identification of patient groups with different clinical outcomes and the expression patterns predicted patient outcome better than previous clinical and histopathological criteria (Shipp et al., 2002). Similar results have been obtained e.g. in breast cancer (van 't Veer et al., 2002) and in gliomas (Nutt et al., 2003).

Recently, microarray based expression profiling has also been used to predict the ability of primary tumors to metastasize (Ramaswamy et al., 2003).

In addition to expression profiling, DNA microarrays have been adapted for the analysis of copy number changes in cancer by CGH. The use of DNA microarrays in copy number analysis enables both high throughput data collection and increased mapping resolution and therefore facilitates the subsequent identification of genes involved in copy number changes. The first high-resolution CGH studies used arrayed large-insert size genomic clones, such as cosmid, P1, PAC, and BAC clones, as hybridization targets (Solinas-Toldo et al., 1997; Pinkel et al., 1998). The CGH microarray technique was shown to reliably detect not only high-level copy number differences, such as amplifications, but also gains and both homozygous and heterozygous deletions (Solinas-Toldo et al., 1997; Pinkel et al., 1998; Snijders et al., 2001). Copy number analysis using cDNA microarrays was pioneered by Pollack and coworkers (1999) and has been shown to be applicable in the detection of both increased and decreased copy numbers. The main advantage of the use of cDNA clones as hybridization targets is that an identical array can be applied in parallel expression analysis, providing a means of rapid correlation between gene copy number alterations and gene expression changes (Kauraniemi et al., 2001; Monni et al., 2001; Hyman et al., 2002; Pollack et al., 2002) (Figure 5). The resolution of CGH microarray technologies is dependent on several factors, including the number of clones on the array, the local clone density, the accuracy of the localization of the clones along the genome, and, in the case of genomic clones, the clone insert size. Arrays containing approximately 3000 clones would provide an average resolution of 1 Mb, assuming that the clones were evenly distributed across the human genome. A single gene resolution can be theoretically achieved by using cDNA clones as hybridization targets.

Figure 5. *Schematic illustration of gene copy number and expression analysis using cDNA microarray technique. A) PCR-amplified cDNA clone inserts are printed robotically on glass microscope slides in defined array format. B) In copy number analysis, differentially labeled tumor and reference DNAs are hybridized on the cDNA microarray. C) In expression analysis, differentially labeled tumor and reference cDNAs are hybridized on the cDNA microarray. The ratio between the tumor and reference intensities is quantified for each cDNA clone and reflects gene copy number (e.g. amplification) or gene expression (e.g. overexpression) changes in the tumor sample.*



4.2 DNA microarray studies in pancreatic cancer

To date, seven studies utilizing DNA microarray technology for large-scale expression surveys in pancreatic cancer have been published (Crnogorac-Jurcevic et al., 2001; Crnogorac-Jurcevic et al., 2002; Han et al., 2002;

Iacobuzio-Donahue et al., 2002; Tseng et al., 2002; Iacobuzio-Donahue et al., 2003; Logsdon et al., 2003). Crnogorac-Jurcevic et al. (2001) applied an array containing 588 cancer-related genes for the search of new candidates that could be used as markers of pancreatic cancer. Analysis of bulk tumor tissue revealed differentially expressed genes belonging mostly to the stromal component of the tumor, reflecting the presence of the typical desmoplastic reaction in pancreatic cancer. In contrast, the analysis of fine needle aspiration samples revealed several differentially expressed genes previously implicated in pancreatic cancer. In addition, dysregulated expression of genes not previously associated with pancreatic cancer was also discovered. These included genes such as *Rac1*, *GLG1*, *NEDD5*, *RPL-13a*, and *RPS9* as well as members of the *Wnt5A* gene family (Crnogorac-Jurcevic et al., 2001). Based on these data, fine needle aspiration provides a practical source of material that also offers an efficient (more than 95%) enrichment of tumor cells for microarray based expression analyses in pancreatic cancer (Crnogorac-Jurcevic et al., 2001). Another way to enrich for tumor cells in mixed cell populations is microdissection. The laser capture microdissection technique was applied to obtain samples from both normal and malignant pancreatic epithelium (Crnogorac-Jurcevic et al., 2002). Expression analysis by cDNA microarray revealed 15 differentially expressed genes in the microdissected pancreatic samples. Eleven genes, including *ABL2*, *Notch4*, and *SOD1*, were upregulated and four genes, such as *XRCC1*, were dowregulated in the malignant epithelial cells as compared to the normal pancreatic ducts (Crnogorac-Jurcevic et al., 2002).

Han and coworkers (2002) used cDNA microarray technology to identify new diagnostic markers and therapeutic targets for pancreatic cancer. They compared the expression profiles obtained from nine pancreatic cancer cell lines using a 5760 clone cDNA chip to those obtained from normal pancreas. This analysis revealed 30 genes whose expression levels were significantly upregulated (an expression ratio greater than 2 SD from the mean in at least three of the nine cell lines studied) in pancreatic cancer (Han et al., 2002). These genes belong to several functional categories including transcription or translation-related genes (e.g. *c-MYC*), cell adhesion and migration-related genes (e.g. *uPAR* and

S100A11), and DNA replication and mitosis-related genes (e.g. *AURKA (STK15)* and *Rad51*). The overexpression of 25 of the upregulated genes was also confirmed with RT-PCR and Northern blotting. In addition, the overexpression of *MYC* and *Rad51* was validated in patient samples with RT-PCR and by immunostaining (Han et al., 2002).

Iacobuzio-Donahue and collegues used both oligonucleotide-based microarrays (Iacobuzio-Donahue et al., 2002) and cDNA microarrays (Iacobuzio-Donahue et al., 2003) to identify differentially expressed genes in pancreatic cancer. Analysis of normal pancreas, pancreatic cancer, and pancreatic cancer cell lines using an Affymetrix GeneChip containing 60 000 gene fragments revealed 97 genes that were upregulated in pancreatic cancer compared to normal tissue (more than fivefold increase in expression level). Of these, 28 genes had previously been implicated in pancreatic cancer, whereas 69 genes represented potential novel tumor markers or therapeutic targets for pancreatic cancer (Iacobuzio-Donahue et al., 2002). Similar analysis of normal pancreas, pancreatic cancer, and pancreatic cancer cell lines using a 45 000 gene cDNA microarray revealed a set of more than 400 genes that were differentially expressed in the pancreatic cancer tissues and cell lines compared to normal pancreas (Iacobuzio-Donahue et al., 2003). These genes were linked to multiple cellular processes, such as cell-cell and cell-matrix interactions, cytoskeletal remodeling, proteolytic activity, and Ca^{++} homeostasis. A set of 149 genes was more highly expressed in pancreatic cancers compared with normal pancreas and contained 103 genes not been previously reported to be associated with pancreatic cancer. Therefore these 103 genes represent putative new tumor markers for pancreatic cancer (Iacobuzio-Donahue et al., 2003).

As outlined above, the most recent microarray based expression studies in pancreatic cancer have mostly focused on the identification of novel differentially expressed genes that could be used as diagnostic markers or as targets for development of new therapies against pancreatic cancer. Although a considerable number of such differentially expressed genes has been identified in these studies, the real clinical value of these findings remains to be seen.

AIMS OF THE PRESENT STUDY

The aims of the study were:

1. To characterize chromosomal aberrations in pancreatic cancer by CGH

2. To study the amplification status of genes commonly amplified in human solid tumors in pancreatic cancer

3. To identify new amplified and overexpressed genes in the12p region in pancreatic cancer

4. To perform a genome-wide gene copy number and expression survey in pancreatic cancer using cDNA microarrays

MATERIALS AND METHODS

1. Cell lines and primary tumors

Thirteen established pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA; 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, SW 1990) and six from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany; DAN-G, HUP-T3, HUP-T4, PA-TU 8902, PA-TU 8988S, PA-TU 8988T). The cell lines were grown under recommended culture conditions.

Twenty-five tumor biopsies were obtained from twenty-four patients with exocrine pancreatic cancer who were treated at the Department of Surgery, Lund University Hospital, Sweden. Thirteen biopsies (1672-88p, 2561-88m, 513-89p, 2087-91p, 3324-92p, 1653-93m, 1707-93m, 1727-93p, 1820-93p, 1840-93m, 1853-93m, 1864-93p, and 1950-93p) were used directly for CGH analyses, whereas twelve biopsies (LPC1p, LPC2p, LPC3p, LPC4p, LPC5m, LPC6p, LPC7m, LPC8p, LPC10m, LPC11p, LPC11m, and LPC12m) were subcultured 4-8 times for expansion of malignant cells as described (Gorunova et al., 1998; Jonson et al., 1999).

2. Comparative genomic hybridization (CGH)

Genomic DNA was extracted from primary biopsies and cell lines according to standard procedures (Sambrook et al., 1989) and analyzed by CGH as previously described (Kallioniemi et al., 1994). Test samples were labeled with FITC-12-dUTP (DuPont, Boston, MA) and sex-matched normal reference DNA with

Texas Red-6-dUTP (DuPont). The labeled DNAs, 600 ng test DNA and 400ng reference DNA, were hybridized with 10μg unlabelled Cot-1 DNA (Gibco BRL, Gaithersburg, MD) to normal lymphocyte metaphase chromosomes. The hybridization was carried out for 2 days in a humid chamber at 37°C. After washing, the chromosomes were counterstained with 0.5μM 4',6-diamidino-2-phenylindole (DAPI) in an antifade solution.

Three consecutive images matching the DAPI, FITC, and Texas Red fluorescence were acquired from 4-8 metaphases using an Olympus BX 50 epifluorescence microscope (Olympus, Tokyo, Japan) and a CCD camera (Xillix Inc., Vancouver, B.C). The test to reference fluorescence ratios were quantified using either the Scil-Image software (National Research Institute, Delft, The Netherlands) or the Quips digital image analysis system (Vysis, Inc., Downers Grove, IL). The Y chromosome and other regions containing repetitive DNA sequences were excluded from the analysis. Chromosomal regions with copy number ratios under 0.85 were considered to be lost, above 1.15 to be gained, and above 1.5 to be amplified.

3. Fluoresence in situ hybridization (FISH)

A total of 27 DNA probes was used in the FISH analyses (Table 2). Locus- and gene-specific probes were obtained by screening of large-insert size P1, BAC, and PAC libraries (Genome Systems Inc., St. Louis, MO; Research Genetics Inc., Huntsville, AL; Roswell Park Institute, Buffalo, NY). A probe for *CTSZ* (20q13) was kindly provided by Dr. Inigo Santamaria (University of Oviedo, Spain), and the probes for the *MYC* (8q24.1), *CCND1* (11q13) and *ERBB2* (17q12) oncogenes as well as centromere-specific probes for chromosomes 8, 11, and 17 were obtained from Vysis. A pericentromere-specific probe for chromosome 20 (RMC20L116) was acquired from the Resource for Molecular Cytogenetics (UCSF, San Francisco, CA). The YAC clones were selected based on the information available at the Whitehead Institute for Biomedical Research database (http://www-genome.wi.mit.edu/) and were obtained from the

Foundation Jean Dausset-CEPH (http://www.cephb.fr). Inter-ALU PCR was used for YAC probe preparations.

Table 2. *Probes used in FISH analyses.*

Probe	Type	Gene	Location
MYC		MYC	8q24
CCND1		CCND1	11q13
ERBB2		ERBB2	17q12
210A19	PAC	TBX2	17q23
203D6	BAC	BIRC5	17q25
RMC20P0	P1	BCL2L1	20q11
3917	P1	NCOA6	20q11
103A1	P1	NCOA3	20q12
208K10	BAC	MYBL2	20q12
64H10	P1	PTPN1	20q12
97	BAC	ZNF217	20q13
cK20.10e9	cosmid	-	20q13
189F4	PAC	AURKA	20q13
60016	PAC	CTSZ	20q13
CEP 8		-	8p11.1-q11.1
CEP 11		-	11cen
CEP 17		-	17cen
RMC20L1	plasmid	-	20cen
942e1	YAC	-	12p11-12
899f8	YAC	-	12p11-12
870g11	YAC	-	12p11-12
894g1	YAC	-	12p11-12
753f12	YAC	-	12p11-12
754c1	YAC	-	12p11-12
965g6	YAC	-	12p11-12
891f1	YAC	-	12p11-12
WCP 12	-	-	Chr 12

FISH analyses were performed as previously described (Tanner et al., 1994) (Höglund et al., 1995). Briefly, two differentially labeled probes were hybridized to interphase nuclei or metaphase chromosomes and 50-100 cells were scored to determine the mean copy number for each of the probes. A 1.5-fold increase in the ratio of test probe copy number relative to the reference centromere copy number was considered to represent gain and a greater than 3-fold increase to represent high-level amplification.

4. KRAS2 mutation analysis

The *KRAS2* oncogene mutation was studied from the low passage cell lines. The PCR-amplified cDNAs were sequenced using the BigDye sequencing kit (Applied biosystems, Warrington, UK) and the reactions were analyzed on an ABI PRISM 310 (Perkin Elmer, Foster City, CA).

5. Semiquantitative PCR analyses

Primer sequences and mapping data for semiquantitative PCR analyses were obtained from National Bioscience, Plymouth, MN (*RBBP2*, *CCND2*, *KRAS2*, EST 9, EST 15, EST 21, EST 25), Whitehead Institute for Biomedical Research (*WI-6757*, *WI-7330*, *WI-6700*, *WI-7785*, *WI-9915*, *WI-10249*), and the National Center for Biotechnology Information, Bethesda, MD (*D12S1591*, *D12S1617*, *stSG31947*, *D12S1640*, *SHGC-81184*, *sts-N38796*, *SHGC-150640*). The multiplex PCR-reactions were performed using 1.25 mM MgCl, 200 µM of each dNTP, 0.5 µM of each primer, 1 x PCR buffer, and 2.5 U Platinum *Taq* DNA polymerase (Life Technology, Täby, Sweden). As template, 25 µg DNA was used in a 50 µl reaction volume. The PCR conditions were 24-25 cycles of 96ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 1.5 min, followed by a final 10 min extension at 72ºC. Primers for the internal standard *L1Hs* were added to the reaction after 12-13 cycles. The PCR reactions were performed in duplicate and were quantified by phosphorimaging (FLA-3000, Fujifilm, Japan). Copy number

profiles were generated by comparing the mean intensity ratio between *L1Hs* and each STS/EST in the tumor DNA to that obtained from normal DNA. Expression analyses were performed using cDNA derived from 15 ng of total RNA as a template and *ACTB* and *GAPDH* as internal standards in the PCR reactions. Cell lines LPC3p and LPC10m with no 12p gains or amplifications were used as references and the expression levels for each gene were presented as a mean of four assays.

6. cDNA MICROARRAY

6.1 Chromosome segment specific cDNA microarray

A total of 29 ESTs (stSG16473, sts-N27112, stSG8911, WI-7371, stSG42378, sts-N22720, sts-N36106, sts-H14650, sts-R68240, SHGC-24297, stSG41517, stSG13184, WI-6757, stSG4534, stSG48132, WI-14142, WI-6700, sts-U46837, WI-11450, stSG51826, sts-H00695, stSG53121, stSG46483, sts-AA033590, sts-N26544, WI-20340, WI-7785, stSG49224, and sts-N38796) between markers D12S1617 and sts-N38796 were selected for the microarray analysis and cDNA clones corresponding to the 3´ends were obtained from the IMAGE consortium. The clone inserts were amplified using vector specific primers. Around 50 ng of each PCR product were spotted onto Gene Screen nylon filters (NEN Life Science Products, Boston, MA) using a floating pin replicator (V&P Scientific, San Diego, CA).

The probes were prepared as previously described (Jonson et al., 2000). Prior to hybridization, the nylon filters were prehybridized for 2 hr at 42°C in 5 ml standard hybridization solution with 5 µg Cot-1 DNA (Life Technologies), and 5µg poly-dA (Research Genetics). The hybridizations were carried out for 16-18 hours at 42°C. The filters were then washed. Hybridizations were quantified by electronic autoradiography using an FLA-3000 (Fujifilm). The spot intensity ratios of tumor cell lines without 12p aberrations were used as references. Two

hybridizations were performed for each sample and the spots were analyzed and quantified using Array Gauge software (Research Genetics).

6.2 Genome-wide cDNA microarray

The genome-wide cDNA microarray contained a total of 12 232 cDNA clones. Preparation and printing of the cDNA clones on glass slides was done as previously described (DeRisi et al., 1996; Mousses et al., 2000). Copy number and expression analyses on cDNA microarray were performed as described (Pollack et al., 1999; Monni et al., 2001). For copy number analysis, genomic DNA was extracted from cell lines using standard protocols and sex-matched DNA from normal lymphocytes was used as a reference. Genomic DNA was digested for 14–18 h with *AluI* and *RsaI* (Life Technologies, Inc.) and purified by phenol/chloroform extraction. Six µg of digested cell-line DNA was labeled with Cy3-dUTP and 6 µg of normal DNA with Cy5-dUTP (Amersham Pharmacia, Piscataway, NJ) using Bioprime Labeling Kit (Life Technologies, Inc.). One hundred fifty µg Cot-1 DNA (Life Technologies, Inc., Rockville, MD), 300 µg yeast tRNA (Gibco/BRL), and 60 µg each poly dA and poly dT were added to the labeled probes. The hybridization mixture was denatured at 100ºC for 1.5 min, incubated for 30 min at 37ºC, and hybridized on the microarray slide for 16-24 hours at 65ºC in a sealed, humidified chamber. The slides were washed in 0.1% SDS, 0.5xSSC/0.01% SDS, and 0.06xSSC for 2 min each.

For expression analysis, mRNA was extracted from cell lines using FastTrack® 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA). A pool of mRNA derived from all 13 cell lines was used as a standard reference. Labeled cDNA was synthesized from four µg mRNA in an oligo(dT)-primed polymerization with SuperScript II reverse transcriptase (Life Technologies, Inc.) in the presence of either Cy5 (test) or Cy3 (reference) labeled dUTP. The Cy5-labeled test cDNA and Cy3-labeled reference cDNA were combined with 12 µg poly (dA)

(Pharmacia, Bridgewater, NJ), six µg tRNA, and 10µg Cot-1 DNA (Life Technologies, Inc., Rockville, MD) in 0.25 % SDS, 2xSSC. The probe mix was incubated at 98°C for 2 minutes and at 4 °C for 10 sec, and hybridized on the cDNA microarray. The hybridization was carried out at 65°C for 16 hours, and the slides were then washed as described above.

6.2.1 Image acquisition and data analysis

The fluorescence intensities were detected by using a laser confocal scanner (Agilent Technologies, Palo Alto, CA). Intensity data were integrated over 225-μm^2 pixels and recorded at 16 bits. The two fluorescent images were formed by randomizing tumor intensity values into the red channel and control intensity to the green channel. The image analysis was performed using the DeARRAY software (Chen et al., 1997; Monni et al., 2001). After background subtraction, average intensity at each clone in the test hybridization was divided by the average intensity of the corresponding clone in the control hybridization. Within-slide normalization for each cDNA and CGH microarray was performed using Local Weighted Scatter Plot Smoother (LOWESS) method (Cleveland, 1979; Yang et al., 2001) for each print-tip group. After within-slide normalization, low quality measurements (i.e. copy number data with mean reference intensity less than 50 fluorescent units, and expression data with both test and reference intensity less than 100 fluorescent units and/or with spot size less than 50 units) were excluded from the analysis and were treated as missing values.

The genomic locations of the cDNA clones on the microarray were determined on the basis of using information from the human genomic sequence. The chromosome and base pair positions for each cDNA clone were obtained from the November 2002 freeze of the University of California Santa Cruz's GoldenPath database (www.genome.ucsc.edu) as described (Hyman et al., 2002). The CGH copy number data was then arranged according to the position of the clones along chromosomes. Genes with copy number ratio >1.4 (representing the upper 5% of the CGH ratios across all experiments) were

considered to be amplified. Amplicons were defined using the following criteria: (1) 6 or more adjacent clones with a copy number ratio >1.4 or (2) at least 3 adjacent clones with a copy number ratio >1.4 and no less than one clone with a ratio >2.0. To ensure as accurate amplicon mapping as possible, the amplicon start and end positions were extended to include neighboring non-amplified clones (ratio <1.4). Thus, the amplicon size determination was partially dependent on local clone density.

The influence of gene copy number on gene expression level was evaluated as described (Hautaniemi et al., 2003). Briefly, within-slide normalized CGH and cDNA ratios in each cell line were log-transformed and median centered. In addition, cDNA data were median centered using values across all 13 cell lines. For each gene, the CGH data were represented by a vector that was labeled 1 for amplification ratio >1.4 and 0 for no amplification. Amplification was correlated with gene expression using the signal-to-noise statistics (Hautaniemi et al., 2003). A weight, w_g, was calculated for each gene:

$$w_g = \frac{m_{g1} - m_{g0}}{\sigma_{g1} + \sigma_{g0}}, \quad (1)$$

where m_{g1}, σ_{g1} and m_{g0}, σ_{g0} denote the means and standard deviations for the expression levels for amplified and non-amplified cell lines respectively. To assess the statistical significance of each weight, 10,000 random permutations of the label vector were performed. The probability that a gene had a larger or equal weight by random permutation than the original weight was denoted by α. A low α (<0.05) was taken to indicate a strong association between gene expression and amplification.

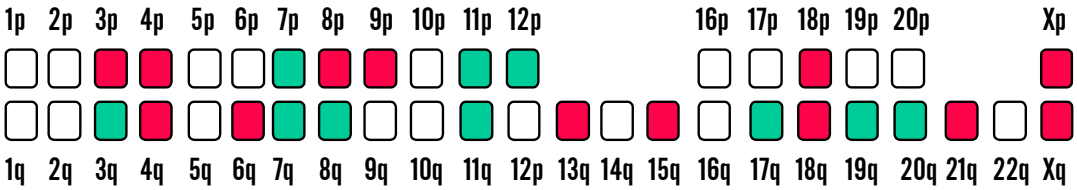
RESULTS

1. Analysis of copy number changes in pancreatic cancer by CGH (I, II)

A genome-wide survey of copy number aberrations was performed in 31 pancreatic cancer cell lines and 13 pancreatic adenocarcinoma tumor biopsies by CGH. All 31 cell lines showed DNA copy number alterations by CGH and, on average, 10 losses (range 4-17) and 9 gains (range 1-16) were observed per cell line. The losses were most commonly seen at 18q (in 97% of cases), 9p (77%), 4q (65%), 3p (58%), 8p (55%), and 21q (55%). The 18q loss was observed in all but one cell line (Mia PaCa-2). Gains at 20q, 11q, 3q, 8q, 7p, and 17q were seen in more than half of the cell lines. A total of 27 high level amplifications was seen in cell lines, most commonly affecting 20q (in six cases), 8q and 12p (five cases each), and 7q (four cases). Among the tumor biopsies, six samples (46%) showed no chromosomal changes by CGH, and overall the aberrations were less frequent than in cell lines. On average, two losses and three gains were seen per biopsy sample, but no high-level amplifications were observed. The most common losses and gains are described in Figure 6. The most common losses were detected at chromosome 18 (in 31% of cases), as well as at 6q, 9p, and 17p (in 23% each). Gains were most frequently observed at 7p (in 31% of cases), 8q (31%), chromosome 5 (23%), chromosome 11 (23%), 12p (23%), and 18q (23%). Comparison of CGH data between cell lines and biopsies indicated a few differences. For example, total loss of chromosome 18 was a common event in biopsies, whereas loss of only the q-arm was preferentially seen in cell lines. In addition, gains affecting chromosomes 5 and 18q were more frequent in biopsies than in cell lines.

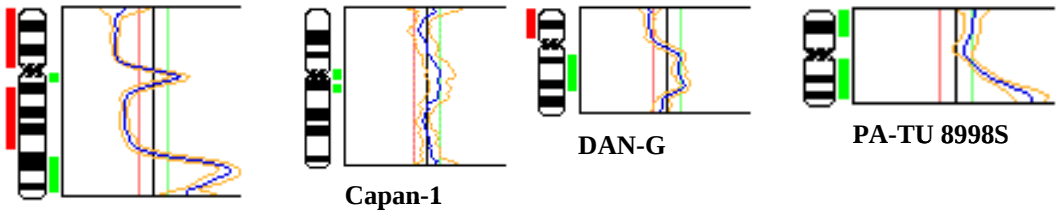
Figure 6. ***A****. Summary of the most common losses and gains in 31 pancreatic cancer cell lines. Aberrations occurring in more than 40% of the samples are indicated. Gains are shown in green and losses in red.* ***B.*** *Examples of CGH copy number ratio profiles from pancreatic cancer cell lines.* ***C****. Examples of FISH analyses of gene amplification in pancreatic cancer cell lines. The cell lines and probes used are indicated in each image.*

A.



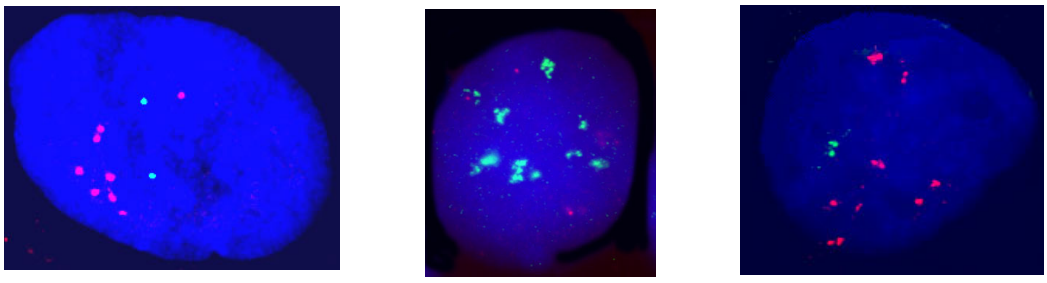
B.

Chromosome 8 Chromosome 11 Chromosome 17 Chromosome 20



C.

BIRC5 / cen 17 **ZNF217 / cen 20** **CTSZ / cen 20**



Hs 766T **PA-TU 8988S** **Capan 2**

2. Targeted copy number analysis of 8q24, 11q13, 17q, and 20q by FISH (II)

Based on the CGH results, chromosomal regions 8q24, 11q13, 17q, and 20q were frequently involved in copy number increases in pancreatic cancer and therefore candidate genes from these regions were further evaluated by interphase FISH. A total of 30 pancreatic cancer low-passage or established cell lines was studied at 14 different loci and all cell lines showed amplification with at least one of the probes tested. Amplification of the *MYC* oncogene at 8q24.1 was seen in 13/24 cases (54%) with five tumors (21%) showing high-level amplification (> 3-fold increase in the copy number relative to chromosome 8 centromere). The *CCND1* gene (at 11q13) was amplified in 28% of the cases (8/29) with high-level amplification in a single cell line. At 17q, copy numbers of three genes, *ERBB2* at 17q12, *TBX2* at 17q23, and *BIRC5* at 17q25, were evaluated and the amplification frequency increased towards the telomere of chromosome 17. *ERBB2* was amplified in 5/20 cell lines (20%), *TBX2* in 10/20 (50%), and *BIRC5* in 11/19 (58%). High-level amplification of *ERBB2* and *TBX2* was observed in the same two cell lines, whereas *BIRC5* was highly amplified in four cell lines.

At chromosome 20, a detailed copy number analysis of nine different genes/loci covering the entire q-arm was carried out. The amplification frequencies at 20q varied from 32% to 83%. The *CTSZ* gene was most frequently amplified in 19/23 cell lines (83%), whereas *MYBL2* was amplified in only 9 cell lines (32%). High-level amplification of 20q sequences were observed relatively infrequently, except for *CTSZ* and *ZNF217*, which were highly amplified in 13% and 10% of the cases respectively. The amplification of *BIRC5* in cell line HS766T, *ZNF217* in PA-TU 8988S and *CTSZ* in Capan2 is demonstrated in Figure 4 C.

3. Detailed characterization of the 12p amplicon in pancreatic cancer (III)

Gain or amplification of 12p and especially the 12p11-p12 region was observed in 13/31 (42%) pancreatic cancer cell lines in CGH. The initial characterization of the 12p amplicon was performed in 13 cell lines by FISH using a total of eight YAC clones that mapped to this region. High-level amplification was observed in four cell lines (LPC4p, LPC5m, LPC11p, and LPC11m) and involved a common amplified segment from YAC 753f12 to YAC 891f1 at 12p11-p12. The size of the amplified region was approximately 5 Mb.

The common region of amplification identified by FISH was further defined using semiquantitative DNA analysis. Copy numbers of fourteen STS/EST markers selected from this region were assayed in six pancreatic cancer cell lines LPC4p, LPC5m, LPC11p, LPC11m, DANG and SU.86.86 that had shown copy number increases at 12p11-p12 by CGH and/or FISH. The semiquantitative PCR analysis revealed a 3.5 Mb amplified region between markers *D12S1617* and *sts-N38796* in all six cell lines. The LPC11p and LPC11m cell lines showed a 5-fold amplification peak at the distal end including the *KRAS2* gene. SU.86.86 showed two amplification peaks, the distal including *KRAS2* and the proximal located between markers *WI-7785* and *sts-N38796*. The LPC5m and DANG cell lines showed large amplicons covering nearly the entire 3.5 Mb region and LPC4 showed low-level amplification with all markers tested.

Next, the mutation and expression status of *KRAS2* was evaluated because it was included in the distal part of the 12p amplicon. Codons 12, 13, and 61 were selected for the mutation analysis as these had been previously shown to be frequently affected in pancreatic cancer and PanIN lesions (Almoguera et al., 1988; Moskaluk et al., 1997; Luttges et al., 1999a; Laghi et al., 2002). No mutations were found in codons 13 and 61, whereas codon 12 was mutated in 10 out of 14 low passage cell lines, including all 5 tested cell lines with local

amplification, as well as in SU.86.86 cells. Increased *KRAS2* expression was detected in all six cell lines with amplification between *D12S1617* and *sts-N38796*.

A chromosome segment specific cDNA array was used to analyze expression levels of 29 expressed sequences from chromosome region 12p in six cell lines with local amplification (LCP4p, LPC5m, LPC11p, LPC11m, DANG, AND SU.86.86) and in two with gains at 12p (LPC1p and LPC12m). Four of the transcribed sequences, *DEC2* (EST 9), *Hs.173074* (EST 15), *PPFIBP1* (EST 21), and *Hs.284270* (EST 25), showed increased expression levels in the test cell lines compared to the average from two reference cell lines without 12p amplification. The expression levels of these four ESTs were further evaluated by RT-PCR. *DEC2* showed increased expression in SU.86.86 cells. Two- to fourfold overexpression of *Hs.173074* was detected in LPC5m, DANG, and SU.86.86 whereas Hs.284270 showed similar overexpression in LCP4p and LCP5m. *PPFIBP1* showed at least twofold increased expression in all six cell lines with local 12p amplification with three cell lines, LPC5m, DANG, and SU.86.86, showing 12, 15, and 5-fold increased expression levels respectively.

4. Genome wide expression and amplification survey in pancreatic cancer by cDNA microarray (IV)

In Study IV, a genome-wide copy number and expression survey was performed to obtain high-resolution information on copy number changes in pancreatic cancer, and also to identify novel genes activated by amplification. Thirteen pancreatic cancer cell lines were screened using a cDNA microarray containing 12 232 clones. Chromosomal and base-pair locations were obtained for 10 389 clones providing an average resolution of 308 kb throughout the human genome. A total of 24 separate amplicons was identified, ranging in size from 130 kb to 11 Mb with an average of 2 Mb. The extent of the amplicons reflects both the actual size of the amplicon but also the local density of the clones on the array.

The amplicons were located on 12 different chromosomes with multiple separate amplicons observed on chromosomes 15q, 17q, and 19.

Comparison between data obtained in Studies I, II, and IV indicated that regions most commonly gained by chromosomal CGH also showed increased copy number in CGH on cDNA microarray. Most of the amplicons defined in Study IV were located at chromosomal regions frequently showing gain by chromosomal CGH. For example, two separate amplicons were defined at 3q region that showed gain in a large fraction of cell lines by chromosomal CGH. However, a few discrepancies were noted. No amplicons were observed at 7p, 11p, 12p, and 20p by CGH microarray although these chromosomal regions were commonly affected by chromosomal CGH. Such differences might be explained by local clone densities or low sensitivity of array CGH in detecting low level copy number increases.

The expression profiles of the 13 cell lines were then analyzed using an identical 12 232 clone microarray. The expression information was correlated with the CGH array data to allow direct identification of genes whose expression levels were elevated due to increased copy number in the pancreatic cancer cell lines. A statistical analysis with random permutation tests revealed 105 genes whose expression levels were heavily dependent on gene copy number, i.e. these genes were activated by increased copy number (see Study IV Table II). The set included genes previously shown to be amplified in pancreatic cancer (e.g. *AURKA (STK15)*, serine/threonine kinase 15) as well as known oncogenes (e.g. *RAB4A*, member of the *RAS* oncogene family). To obtain further information on the possible role of these 105 genes in pancreatic cancer pathogenesis, the cellular functions of these genes were explored using the SOURCE database (http://source.stanford.edu). Functional information was retrieved for 84 (80%) genes, whereas 17 genes represented hypothetical proteins and 21 were known genes or ESTs with no functional annotation. A majority (78%) of the remaining 67 genes was involved in key cellular processes including signal transduction (17 genes), protein processing (11), metabolism (8), RNA processing (7), transcription (5), and DNA replication (4).

DISCUSSION

1. Identification of recurrent chromosomal copy number changes in pancreatic cancer by CGH (I, II)

In order to achieve an overview of the genetic aberrations present in pancreatic cancer, a total of 31 cell lines and 13 tumor biopsies derived from pancreatic cancer primary tumors and metastases were studied by CGH. In the cell lines, CGH analyses revealed numerous copy number changes affecting every single chromosome. An average of 19 aberrations was detected per specimen, with gains and losses being about equally prevalent. These results confirmed previous observations in cytogenetic studies where complex karyotypes have been commonly reported in pancreatic cancer (Johansson et al., 1992; Bardi et al., 1993; Griffin et al., 1994; Johansson et al., 1994; Griffin et al., 1995; Gorunova et al., 1998; Höglund et al., 1998a; Höglund et al., 1998b). Conversely, CGH analyses in tumor biopsies revealed a considerably smaller number of aberrations. Only an average of two losses and three gains were detected per sample. This result likely reflects the presence of excessive amounts of connective tissue in the primary tumor samples, the so-called desmoplastic reaction that is a common characteristic of pancreatic cancer.

This study identified several chromosomal regions with recurrent copy number changes in the pancreatic cancer cell lines. The CGH analyses showed common losses at chromosomal arms 18q, 9p, 4q, 3p, 8p, and 21q, whereas gains were commonly found at 20q, 11q, 3q, 8q, 7p, and 17q. In addition, a total of 27 separate high level amplification sites was observed in the cell lines, most commonly affecting 20q, 8q, 12p, and 7q. Similarly to cell lines, losses of 18q and 9p as well as gains of 7p, 8q, and 11q were common in the primary tumors. However, losses at 6q and 17p as well as gains at chromosomes 5, 12p, and 18q

were also frequently observed in the primary tumors. No high-level amplifications were observed in the primary tumors, again possibly due to the presence of connective tissue contamination. Overall, the results obtained with chromosomal CGH in this study concur with other published CGH studies of pancreatic cancer (Solinas-Toldo et al., 1996; Fukushige et al., 1997; Curtis et al., 1998; Ghadimi et al., 1999; Schleger et al., 2000; Shiraishi et al., 2001; Harada et al., 2002). The CGH data generated in this study highlight the complexity of chromosomal copy number changes in pancreatic cancer and pinpoint several chromosomal regions that are commonly lost or gained.

2. Evaluation of the involvement of known amplification target genes in pancreatic cancer (II)

The CGH results revealed common gains and amplifications on specific chromosomal regions, such as 8q, 11q, 17q, and 20q, that have also been found to be frequently involved in copy number increases in other solid tumors (El-Rifai et al., 2000; Forozan et al., 2000; Guan et al., 2000; Koo et al., 2001; Reutzel et al., 2001). In addition, these chromosomal regions contain several genes that are known to be amplified in various epithelial tumors. Some of these genes, such as the *ERBB2* (at 17q12), *MYC* (at 8q24), and *CCND1* (at 11q13) oncogenes, have well-established roles in cancer and their amplification has been shown to have clinical significance e.g. in breast cancer (Ross and Fletcher, 1999; Liao and Dickson, 2000; Ormandy et al., 2003). Furthermore, several genes along the q-arms of chromosomes 17 and 20 have been postulated as putative amplification target genes (Tanner et al., 1996; Ambrosini et al., 1997; Anzick et al., 1997; Monni et al., 2001). In the light of this information, we decided to explore the possible involvement of these candidate genes in copy number increases in pancreatic cancer. FISH analyses with a panel of fourteen gene- and locus specific probes were performed in 30 pancreatic cancer cell lines. The FISH results showed amplification with all probes tested with amplification frequencies ranging from 20% to 83%. The amplification

frequencies observed for the *ERBB2* (20% of cases) and *MYC* (54%) oncogenes were in good concordance with previous results from primary pancreatic tumors and pancreatic cancer xenografts (Armengol et al., 2000; Safran et al., 2001). Interestingly, on chromosome 17 the amplification frequencies increased towards the q-telomere with the *BIRC5* gene (at 17q25) being amplified most frequently in 58% of the cases. In general, the most commonly amplified genes were located on chromosome 20q, where *CTSZ* was amplified in 83%, *NCOA6* in 71%, and *PTPN1* in 70% of the cell lines. *BIRC5* has been previously found to be overexpressed in pancreatic cancer (Satoh et al., 2001; Sarela et al., 2002), whereas no alterations involving *CTSZ*, *NCOA6* and *PTPN1* have been reported. In conclusion, the FISH analyses performed in this study reveal frequent amplification of several known amplification target genes in pancreatic cancer and especially pinpoint the frequent involvement of several chromosome 20q-specific genes.

3. Targeted analysis of the 12p amplicon in pancreatic cancer (III)

The CGH analyses also revealed frequent gains and amplifications affecting the 12p region in the pancreatic cancer cell lines. 12p gains have also been commonly observed in other solid tumors, such as testicular germ cell tumors (TGCTs) and less frequently in bladder cancers, colon cancers, ovarian cancers and liposarcomas (Knuutila et al., 1998; Harding et al., 2002; Rieker et al., 2002; van Echten et al., 2002; He et al., 2003). A combination of two different techniques, FISH analysis with YAC clones and semiquantitative PCR with STS and EST markers, was used in this study to narrow down the common region of amplification in pancreatic cancer to a 3.5 Mb segment at 12p11-p12 spanning from marker D12S1617 to sts-N38796. Recent studies in testicular germ cell tumors have also narrowed down the involvement of amplification to a region that overlaps with the one defined here (Roelofs et al., 2000, Rodriguez et al., 2003).

The amplified region at 12p11-p12 identified in this study consisted of two separate amplification peaks. The distal peak included the KRAS2 gene that is known to be commonly activated by mutations in pancreatic cancer (Almoguera et al., 1988; Rozenblum et al., 1997). Codon 12 mutations were detected in 10 of the 14 pancreatic cancer cell lines tested and increased expression was seen in all cell lines with amplification. Recently, Hoa et al. showed amplification and overexpression of KRAS2 in head and neck squamous cell carcinomas (Hoa et al., 2002). These results implicate KRAS2 as an obvious putative target gene for the distal amplification peak. A more detailed characterization of the consequences of the 12p11-p12 amplification in pancreatic cancer was performed by evaluating the expression levels of 29 ESTs from the 3.5 Mb amplified region using cDNA microarray analysis. This expression survey identified four potential targets, DEC2, PPFIBP1, and two anonymous ESTs. Of these, only PPFIBP1, located at the proximal end of the amplified region, showed consistent overexpression (two- to 15-fold) in all cell lines with 12p11-p12 amplification, making it a promising target gene for the proximal amplification peak. The PPFIBP1 gene (PTPRF interacting protein, binding protein 1) belongs to the family of liprins, leukocyte common antigen-related (LAR) transmembrane tyrosine phosphatase-interacting proteins that have been implicated in cell-matrix interaction (Serra-Pages et al., 1998). PPFIBP1 was recently shown to interact with S100A4, a calcium-binding protein related to tumor invasiveness and metastastasis (Kriajevska et al., 2002). Taken together, these results implicate the KRAS2 and PPFIBP1 genes as putative targets for 12p11-p12 amplification in pancreatic cancer.

4. High throughput genome-wide screening of amplified and overexpressed genes in pancreatic cancer (IV)

In Study IV, 12 232 arrayed cDNA clones were applied for the analysis of gene expression and gene copy number changes in 13 pancreatic cancer cell lines. The high-resolution copy number analysis by CGH microarray revealed 24 independent regions of copy number increase, ranging in size from 130 kb to 11

Mb. These regions included several chromosomal segments, such as 3q, 5p, 7q, 8q24, 11q13, 15q, 17q, 19q, and 20q, that have previously been shown to be commonly gained or amplified by chromosomal CGH or by fluorescence in situ hybridization in pancreatic cancer (Solinas-Toldo et al., 1996; Fukushige et al., 1997; Curtis et al., 1998; Ghadimi et al., 1999; Schleger et al., 2000; Shiraishi et al., 2001; Harada et al., 2002). Besides confirming data from these previous studies, the CGH microarray analysis allowed determination of the exact base-pair boundaries for each aberration and therefore permitted mapping of the copy number increases much more accurately than has previously been possible. For example, copy number increases affecting chromosomal regions 19p13.3 and 19q13.3 were delineated to segments spanning 130 kb and 390 kb respectively, representing a mapping resolution far beyond the capabilities of chromosomal CGH. The high resolution copy number data obtained in this study considerably advances the current knowledge on the genetic changes occurring in pancreatic cancer and provides an excellent starting point for the identification of specific genes involved in these chromosomal aberrations.

Several large-scale expression surveys implicating hundreds of overexpressed genes in pancreatic cancer have been published (Crnogorac-Jurcevic et al., 2001; Crnogorac-Jurcevic et al., 2002; Han et al., 2002; Iacobuzio-Donahue et al., 2002; Iacobuzio-Donahue et al., 2003; Logsdon et al., 2003). Although such information is extremely interesting, it is very difficult to assess whether these hundreds of differentially expressed genes highlight primary changes that have a central role in cancer pathogenesis or whether they reflect secondary events. In study IV, the genome-wide copy number and expression surveys were combined in order to identify genes whose expression levels were altered through increase in copy number in pancreatic cancer because such genes might represent primary mediators of cancer development. As mentioned earlier in this discussion, the use of cDNA microarrays for parallel gene copy number and expression analysis provides a direct correlation of copy number and expression data on a gene-by-gene basis throughout the genome (Pollack et al., 1999; Monni et al., 2001). Results from the genome-wide copy number and expression survey in Study IV illustrate the considerable influence of gene copy number on gene expression

patterns. First of all, several genes located within regions of increased copy number, such as the *FBL*, *PD2*, *SUPT5H*, and *SARS2* genes located at the 19q13.1 amplicon, were highly expressed in cell lines with increased copy number. More importantly, a statistical approach (Hautaniemi et al., 2003), applied to systematically evaluate the input of gene copy number to gene expression level, revealed a set of 105 genes whose expression levels were linked to gene copy number increase across all 13 cell lines. This set included genes previously shown to be amplified in human tumors, such as *AURKA (STK15)* and *MLN51*, as well as known oncogenes, such as *RAB4A* and *RELA* (Sen et al., 2002; Varis et al., 2002; Li et al., 2003; Liptay et al., 2003). In order to explore the possible roles of these 105 genes in pancreatic cancer pathogenesis, information on their functional characteristics was retrieved from the SOURCE database (http://source.stanford.edu), a scientific resource that brings together publicly available data on gene functions. According to this analysis, 78% of these genes were associated with essential cellular processes, including signal transduction, transcription, and DNA replication, indicating that they may have a role in cancer development. Overall, these results imply that the set of 105 genes identified in the genome-wide survey is activated by increased copy number in pancreatic cancer and therefore likely to be actively involved in the pathogenesis of this disease.

5. Putative amplification target genes in pancreatic cancer

A relatively small number of genes has previously been shown to be activated by amplification in pancreatic cancer. These include the *ZNF146* (Blottiere et al., 1999) and *AKT2* (Miwa et al., 1996) genes, both located at 19q13.1. *CCND1* at 11q13 (Gansauge et al., 1997), *MYB* at 6q24 (Wallrapp et al., 1997), *MYC* at 8q24.1 (Schleger et al., 2002) and *AURKA* (*STK15*) at 20q13 (Li et al., 2003) are also found to be amplified and overexpressed in pancreatic cancer. Our genome-wide cDNA microarray survey and the subsequent statistical approach revealed a list of 105 putative amplification target genes. Most of these genes have not

previously been linked to pancreatic cancer, although some of them, such as *PAK4* (p21-activated kinase 4, located at 19q13.1) have been implicated in tumor development. The p21-activated serine/threonine kinases (*PAK*s) play an important role in a variety of cellular functions including cell morphogenesis, motility, survival, angiogenesis, and mitosis (reviewed by Kumar and Vadlamudi, 2002). *PAK4* has been shown to regulate cell migration, cell adhesion, and anchorage-independent growth both in human cancer cell lines and in fibroblasts suggesting a central role in oncogenic transformation and tumorigenesis (Chang et al., 1999; Callow et al., 2002). In addition to *PAK4*, twelve other genes from the 19q13.1 amplicon, such as *SUPT5H* [suppressor of Ty 5 homolog (S. cerevisiae)], *SARS2* (seryl-tRNA synthetase 2), *RPS16* (ribosomal protein S16), *RBT1* (RPA-binding trans-activator), and *FBL* (fibrillarin), were included in the list of putative amplification target genes, suggesting that amplification in this regions leads to the simultaneous activation of multiple genes. In conclusion, this study identified a large set of genes previously not known to be amplified and overexpressed in pancreatic cancer.

6. Future prospects

In this study, several chromosomal regions as well as specific genes that are likely to be involved in the development and progression of pancreatic cancer were identified using established cell lines and short-term cultures of primary pancreatic carcinomas. A similar pattern of genetic aberrations has been observed in CGH studies performed in primary pancreatic carcinomas, indicating that the cell lines and short-term cultures can be used as a representative model system. However, it is always possible that the specific gene copy number and expression changes observed in the cDNA microarray approach in this study reflect genetic aberrations related to in vitro cell culture conditions rather than to cancer pathogenesis. Therefore, it is extremely important to validate the results obtained here using uncultured primary tumors. The possible clinical significance of the genes identified, e.g. as prognostic factors, needs to be explored using a large collections of primary pancreatic tumors. If such clinical

validation is able to confirm the results obtained in cell lines, the next logical step will be the analysis of their function both in normal and in cancer cells. These could include e.g. transfection assays to overexpress these genes or the use of the newly developed siRNA technology to downregulate the putative amplification target genes. Such analyses are also likely to reveal further information on the possible clinical applicability of these genes, e.g. as therapeutic targets. However, it is clear that it is not possible to perform detailed clinical and functional validation for all of the 105 genes identified in this study. Therefore, prioritization of the genes of interest according to several factors, such as their frequency of involvement, cellular localization, and functional characteristics, will be essential for the success of this approach.

Summary and conclusions

The main aim of this study was to identify chromosomal regions and specific genes involved in the development and progression of pancreatic cancer. The major findings were:

Genome-wide analysis of copy number changes was performed in 31 pancreatic cancer cell lines and 13 tumor biopsies using chromosomal CGH and several chromosomal regions were found to be frequently altered in pancreatic cancer. Gains and amplifications were most commonly observed at 20q, 11q, 3q, 8q, 7p, 17q, and 19q, whereas losses were most frequently seen at 18q, 9p, 4q, 8p, and 21q.

Chromosomal regions 8q, 11q, 12p, 17q, and 20q were selected for further studies where FISH analyses were applied to study copy numbers of selected genes from these regions in 30 pancreatic cancer cell lines. These analyses indicated frequent amplification of the *MYC* (in 54% of the cell lines) and *CCND1* oncogenes (28%) as well as the *BIRC5* (at 17q25, in 58% of the cases) and *CTSZ* genes (20q13, 83%), suggesting that they might have a role in the pathogenesis of pancreatic cancer.

Detailed evaluation of the 12p amplicon revealed a 3.5 Mb amplified region. A chromosome segment-specific cDNA microarray analysis implicated overexpression of four ESTs, including the *DEC2* and *PPFIBP1* genes. In addition, the *KRAS2* gene located in the amplified region was shown to be consistently overexpressed in the amplified cell lines. These results highlight a distinct set of genes that are likely to be activated by 12p amplification in pancreatic cancer.

A genome-wide high-resolution mapping of copy number increases and expression changes was performed using a 12 232 clone cDNA microarray. The CGH microarray analysis identified 24 independent amplified regions whose exact boundaries were precisely defined on base-pair scale. A statistical approach revealed a set of 105 genes whose expression levels were closely linked to gene copy number. These included genes such as *p21-activated kinase 4* (*PAK4*), that have not previously been implicated as amplification target genes. The set of 105 genes activated by amplification is likely to have a central role in the tumorigenesis of pancreatic cancer.

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