The genetic basis of sporadic pancreatic cancer

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1. Introduction

Adenocarcinomas of the pancreatic gland are the fifth leading cause of cancer death in the USA and western Europe with a 5-year survival rate of less than 5% and a median survival of less than 6 months [76]. They bear histological resemblance to the pancreatic ducts and are referred to as pancreatic ductal adeno-carcinoma [5,34,53]. Due to the late occurrence of symptoms and the high metastatic potential, pancreatic adenocarcinomas have a dismal prognosis. Since the pancreaticoduodenectomy, a surgical resection that includes the pancreatic head, the duodenum, common bile duct and the gallbladder was introduced in 1912 by Kausch, surgery still remains the primary curative treatment. However, the 5-year survival for patients undergoing resection, is only about 20% [76] and about 80% of all cancers are unresectable at the time of diagnosis [2]. In order to improve the clinical management of cancer patients, it is mandatory to understand the genetic basis of pancreatic cancers. This review describes the histological progression of pancreatic adenocarcinomas and summarizes the underlying chromosomal and genetic alterations.

2. Histological progression model

The cells of pancreatic adenocarcinomas show morphological similarity to pancreatic ductal cells. Therefore, the latter are believed to be the progenitors of pancreatic adenocarcinomas. They account for approximately 5% of the pancreatic cell population and are cubically shaped with basal nuclei. Usually, pancreatic ductal adenocarcinomas develop from intraductal precancerous lesions, which are characterized by an increase of histoarchitectural disorder and cell or nuclear dysplasia [70]. Intraductal proliferation of epithelial cells was more frequently found in pancreatic tissues with invasive ductal carcinoma than in non-neoplastic tissue, and it was always accompanied by lesions of increased levels of dysplasia [8,43,45,70]. These findings led to the development of a progression-model as well as a standardized nomenclature and classification-system [16,37].

The first histological change, called squamous or transitional metaplasia describes the replacement of the normal ductal epithelium by mature squamous or transitional epithelium without atypia. PanIN-1A (**pan**creatic **i**ntraepithelial **n**eoplasia) denotes the transition to tall columnar cells with basally located non-atypical nuclei (Fig. 1b). PanIN-1B is cytologically indistinguishable from PanIN-1A, but shows a papillary, micropapillary or basally pseudostratified architecture (Fig. 1c). PanIN-2 lesions are flat or papillary and, by definition, these lesions must exhibit nuclear abnormalities, which can include loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyper-chromatism (Fig. 1d). Cytologically, the PanIN-3 lesions resemble carcinoma cells without invasion of the basal membrane, hence they are called "carcinoma *in situ*". In PanIN-3 lesions the epithelium is usually papillary or micro-papillary and rarely flat. True crib-riforming, budding-off of small clusters of epithelial cells into the lumen and luminal necroses can be detected. The cells are characterized by profound signs of nuclear atypia, like loss of nuclear polarity, irregularities in shape, prominent nucleoli and mitoses. Additionally cellular disorders like dystrophic goblet cells with nuclei oriented towards the lumen and mucinous cytoplasm orientated towards the basement membrane can be found [37] (Fig. 1e).

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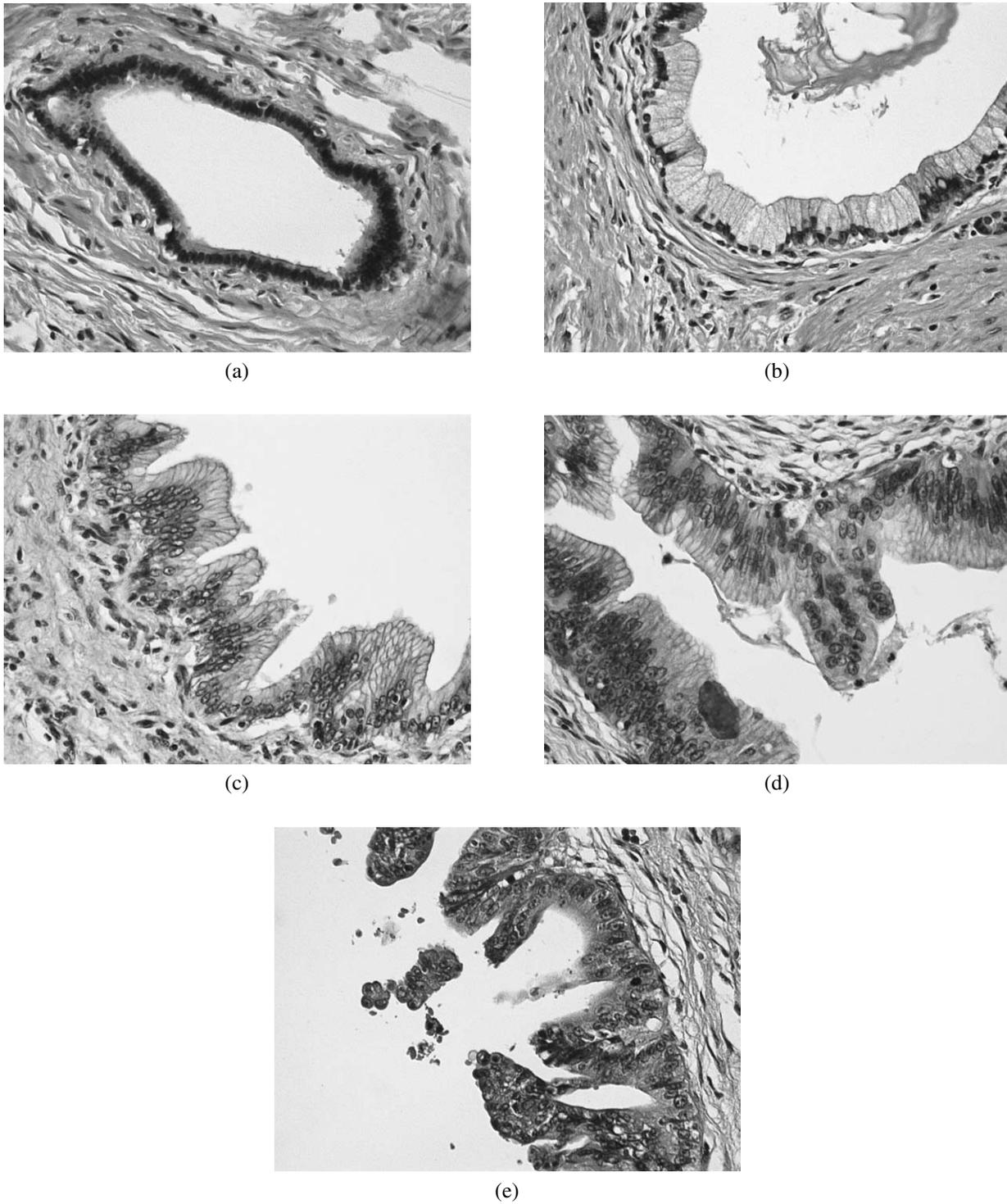
Fig. 1. Representative examples of pancreatic intraepithelial lesions (PanIN), H&E, ×400: (a) normal ductal epithelium, (b) PanIN-1A, (c) PanIN-1B, (d) PanIN-2, (e) PanIN-3.
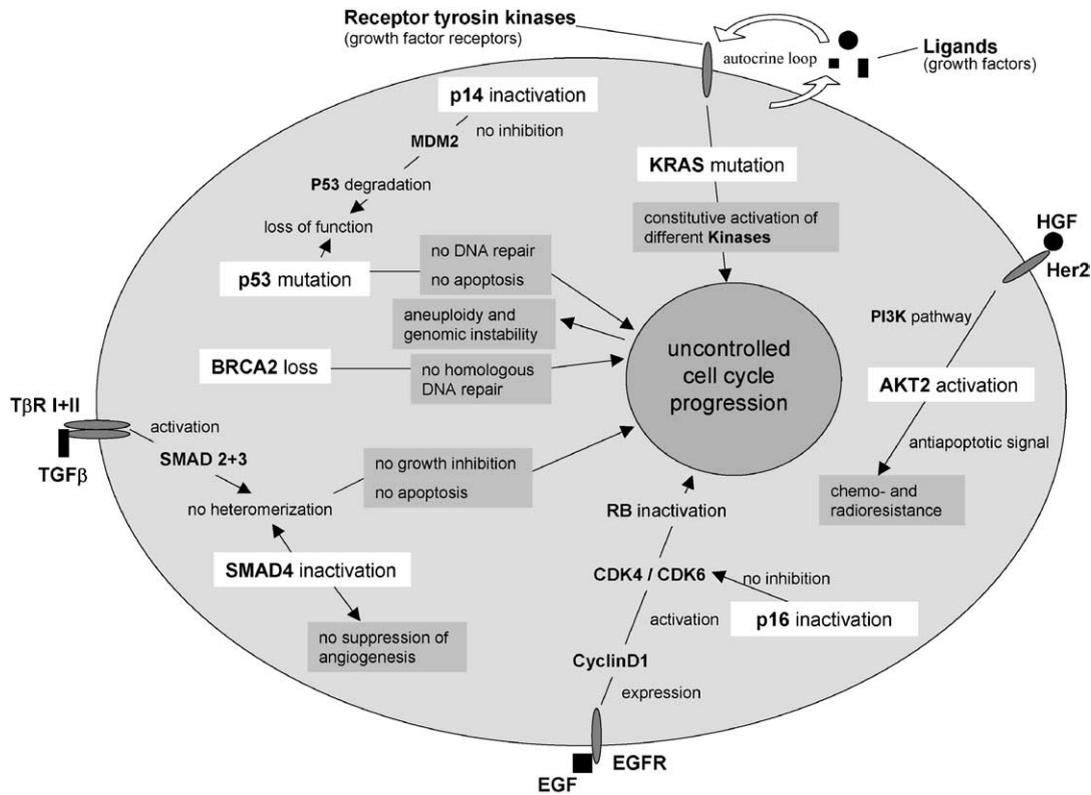
Fig. 2. Schematic diagram of major pathways in the development of pancreatic adenocarcinomas as discussed in the text. Key events (white boxes) and their consequences (grey boxes), which finally lead to the loss of cell cycle control.

3. Genetic alterations

The majority of pancreatic carcinomas show alterations of oncogenes such as *KRAS*, *EGFR* and *HER-2* as well as tumor suppressor genes such as *CDKN2A*, *P53*, *DPC4* and *BRCA2* (often referred to as signature mutations) [5]. According to the suggested progression model, mutations of *KRAS* and activation of *EGFR* and *HER-2* are supposed to be early changes that arise in low-grade PanINs with minimal atypia. In high-level PanINs, losses of *CDKN2A* can be detected, followed by inactivation of *P53, DPC4* and *BRCA2* [5,36].

3.1. KRAS

The oncogene *KRAS* (kirsten rat sarcoma) is located on chromosome 12p12. It encodes a small GTP-binding protein, which is involved in signaling pathways between membrane located receptor tyrosine kinases like TGFR, VGFR, EGFR and HER2 and cytoplasmic kinases. As a consequence of genetic mutations the protein loses its GTPase function and therefore the capability of self-deactivation. This results

in continuous stimulation of different pathways with a number of cellular effects, like induction of DNA synthesis and cell proliferation (Fig. 2). Cells with *KRAS* mutations gain growth advantage through inflammatory stimuli and expression of transforming growth factor alpha (TGFα) and epithelial growth factor (EGF). If additional activation of EGFR (erbb) occurs, it will cause uncontrolled proliferation as a result of autocrine stimulation [5].

Almost all *KRAS* mutations of the pancreatic duct affect codon 12, whereas mutations in codon 13 and 61 are rare [78]. They can be identified in early neoplastic lesions of the pancreatic ducts but also at high frequencies in normal epithelium (19% to 38% [6,48]) and in patients with chronic pancreatitis (18% to 62.5% [49, 56]). The mutation rate increases with disease progression and almost all pancreatic cancers exhibit *KRAS* mutations [5].

3.2. EGFR and HER-2/neu

EGFR (epidermal growth factor receptor) and Her-2/neu (heregulin-, neuregulin- or glial growth factor-

receptor) belong to the *ERBB*-family. All receptors of this gene family are transmembrane proteins and contain two cysteine-rich regions in the extra cellular domain and a tyrosine-kinase domain in the cytoplasmic part.

The *HER-2* oncogene is located on chromosome 17q21.2 and is amplified and over-expressed in many solid tumors [10,67,68]. In normal pancreatic tissue and chronic pancreatitis HER-2 expression remains unaffected, whereas in patients with pancreatic cancer over-expression of HER-2 can be found in early morphologic duct lesions [4,10]. Using immunochemistry, over-expression of HER-2 ranges from 21% to 80%, and only 27% of the immunohistochemical positive cases showed amplification of *HER-2* as detected by FISH [4,60].

The *EGFR* gene is located on chromosome 7p12. Expression of EGFR can be detected in about 50% of pancreatic carcinomas [54,72,80]. EGFR expression seems to play an important role in metastasis (26% in patients without and in 64% of patients with metastasis), especially liver mestastasis and was found more often in male (48%) than in female patients (17%) [72]. Analyses of the pancreatic cancer cell lines BxPC-3 and AsPC-1 exhibited evidence that Cyclin-D1 (*CCND1*) dependent cell cycle progression occurs through the induction of Cyclin-D1 by EGFR. EGF induces *CCND1* mRNA expression, followed by cell cycle progression to G1/S. Expression of EGF, EGFR or Cyclin-D1 could not be detected in normal pancreatic tissue, but at high frequencies in pancreatic carcinomas [54]. These findings indicate that the mitogenic effect of EGF-signaling through EGFR might be jointly responsible for Cyclin-D1 over-expression (Fig. 2). Amplified and unregulated expression of D-cyclins causes phosphorylation and inactivation of the RB protein by the cyclin-dependent-kinases 4 and 6 (CDK4, CDK6). This leads to the unimpeded progression from the G1 to the S phase.

3.3. CDKN2A

CDKN2A (cyclin-dependent kinase inhibitor 2A) is located on chromosome 9p21 and encodes for two tumor suppressor genes via distinct first exons and alternative reading frames in shared downstream exons [66]. The first product is p14^{ARF} and the second is p16^{INK4a}, often referred to as p16. P16 is an inhibitor of CDK4 and CDK6 and counteracts inactivation of RB. P14^{ARF} inhibits MDM2, a protein that is responsible for the proteolytic deactivation of p53. Losses of

p14^{ARF} and p16^{INK4a} lead to the inactivation of the RB and p53 pathways [66] (Fig. 2). Even up to 30% of PanIN-1A and B lesions show inactivating mutations of p16^{INK4a} [32,50,77]. Loss of both gene products of *CDKN2A* by mutation, deletion or promoter hypermethylation can be found in allmost all sporadic pancreatic cancers [57,64]. Germline mutations in the first exon of *p16* are associated with the Familial Atypical Mole-Malignant Melanoma syndrome (FAMMM), a disease associated with a very high penetrance and early onset [5]. This mutation also predisposes to pancreatic cancer but the penetrance is very low and displays latency similar to the sporadic disease [24].

3.4. p53

The tumor suppressor gene *p53* is localized on chromosome 17p13. The "guardian of the genome" maintains genetic stability through its function in cell cycle control [46]. As a response to DNA damage p53 can arrest the cell cycle in order to either initiate DNA repair or, in case of serious damage, through the induction of apoptosis [41]. As a transcription factor, p53 controls the expression of growth inhibiting genes like *p21^{CIP}*, *GADD45* or *IGFBP3* [44]. *P53* mutations can be found in 50% of all human tumors and in up to 81% of pancreatic adenocarcinomas [32, 57]. Loss of p53 function usually results from missense mutations of one and loss of heterozygosity (LOH) of the other allele [50]. As a consequence the adjacent cells accumulate further DNA damages leading to genomic instability and the development of cancer [30] (Fig. 2). The ability of DNA binding is the crucial requirement for the tumor suppressor function of p53, which is found to be lost in all human tumors [42]. Even mutation of one allele can completely disable p53 function since wild-type p53 is deactivated when complexed with mutated p53 [42]. Furthermore, the proteolytic degradation of mutated p53 is delayed, resulting in cellular accumulation. Mutated p53 was found in PanIN-3 with a frequency up to 41% [50], whereas LOH was already found in PanIN-1 lesions with frequencies between 13% and 18% [32,50].

3.5. DPC4/SMAD4

The loss of the long arm of chromosome 18 (18q) is one of the most frequent chromosomal alteration in the development of pancreatic cancer [29]. The gene *DPC4* (deleted in pancreatic cancer, locus 4) is located at chromosome 18q21 and also referred to

as *SMAD4* [29]. As a member of the *SMAD*-family of genes it is involved in transforming growth factor beta (TGFβ)-mediated growth and differentiation signal transduction. TGFβ is an inhibitor of cellular proliferation of most human cells. It induces the transcription of plasminogen-activator-inhibitor-1 (PAI-1) and inhibitors of cyclin dependent kinases such as p15$^{\text{INK4b}}$ and p21$^{\text{CIP}}$. Therefore, disturbance of this signaling pathway results in uncontrolled growth regulation and carcinogenesis [9,33]. If TGFβ binds to the TGFβ type I and II receptors, the SMAD proteins 2 and 3 get phosphorylated, form heteromeric complexes with SMAD4 and translocate to the nucleus, where they function as transcription factors [33]. Additionally, SMAD4 has been shown to suppress angiogenesis [65] (Fig. 2). A loss of SMAD4/DPC4 can be found in up to 60% of pancreatic adenocarcinomas, in nearly 30% through homozygous deletion of both alleles [32, 50]. Nevertheless, complete loss of gene function determined by immunohistochemical expression analyses could not be detected in early PanIN lesions [79]. The *DPC4* locus is more commonly deleted in PanIN-3 lesions than in PanIN-2 lesions, whereas a significant difference could not be established when PanIN-3 lesions and invasive carcinoma were compared [32,50].

3.6. AKT2

Another gene with possible oncogenic function is *AKT2* (**v**-akt murine thymoma viral oncogene homologue 2). It maps to chromosome 19q13 and is a downstream effector of the phosphatidylinositol 3-kinase (PI3K), which is activated by EGF, PDGF and FGF. The latter are all known to be over-expressed in pancreatic cancer [13]. AKT2 is amplified and over-expressed in 20% of pancreatic adenocarcinomas [58]. Activation due to phosphorylation was found in 59% of pancreatic adenocarcinomas and seems to be associated with HER2 over-expression [62]. Constitutive activation of AKT2 provides a potent antiapoptotic signal in cancer cells conferring chemo- and radioresistance [62] (Fig. 2). Additionally it can increase the expression of the insulin-like growth factor I receptor and can promote the invasiveness of human pancreatic cancer cells [71].

3.7. BRCA2

BRCA2 (breast cancer 2) is located on chromosome 13q and encodes for a protein that regulates homologous-recombination-based DNA-repair processes. It therefore maintains genomic stability, whereas BRCA2-deficient cells accumulate chromosomal aberrations [75] (Fig. 2). Patients with germline mutations have a familial predisposition for developing breast and ovarian cancer at younger ages [22]. Additionally, germline *BRCA2*-mutations increase the risk for the development of pancreatic cancers, although the age of onset and the clinical outcome are similar to sporadic pancreatic cancer [22]. BRCA2 is inactivated in about 7% of sporadic pancreatic carcinomas, and loss of both alleles was already found in PanIN-3 lesions [23].

3.8. LKB1/STK11

LKB1/STK11 (serine–threonine kinase 11) is known to play a role in the autosomal dominant inherited Peutz–Jeghers syndrome, which is associated with increased risk of cancer of many organ systems [19]. It is located on chromosome 19p13 and encodes for a cytoplasmic serine–threonine kinase, which may be a member of a yet unidentified signaling pathway. Germline mutations increase risk of developing pancreatic cancers [19]. Additionally STK11 is deactivated in 7% of the sporadic pancreatic cancers and may play a role in tumor growth inhibition [59].

4. Epigenetic changes

Another cause of gene expression changes in the development of pancreatic cancer is DNA-methylation. About 50% of the human genes have 5$'$CpG islands, typically in the 5$'$ regulatory region of the genes. Methylation of these regions usually causes gene silencing of tumor suppressor genes and demethylation can cause transcription of oncogenes. The methylation of the promoter regions of different genes (e.g., *p16*, *RB*, *VHL*, *hMLH1*, *hMSH2*) was also found in pancreatic carcinomas [73]. Beside *p16*, the gene preproenkephalin (*ppENK*), which has growth inhibiting function, was found methylated in 90% of the pancreatic cancers [74]. Methylation of genes with a significant role in carcinogenesis occur early in cancer development. The methylation of *p16* can be observed from 12% in PanIN-1A to 21.4% in PanIN-3 lesions, whereas an increase from 7.7% to 46.2% of *ppENK* methylation can be found in these lesions. The number of methylated loci increases with the size of the tumour and the age of the patients [15,74]. Non-neoplastic epithelium is not methylated [15].

5. Chromosomal alterations

Pancreatic adenocarcinomas are highly aneuploid and genetically heterogeneous. They exhibit a high degree of CIN (chromosomal instability) reflected by ANCA (average number of copy alterations [55]) values of up to 22, the highest known for solid tumors [17, 25,27,39,55]. Patients with complex chromosomal abnormalities have a worse prognosis than those with only a few aberrations [39].

FISH (fluorescence *in situ* hybridization) based techniques like CGH (comparative genomic hybridization) or SKY (spectral karyotyping) established recurrent patterns of chromosomal alterations [17,40,63] (Fig. 3). Genetic losses usually involve chromosome arms and chromosomes 8p, 9p, 17p, 18q, 19p and 21, whereas gains can be mapped to 3q, 5p, 7p, 8q, 12p and 20q [17,51,69]. These chromosomal regions are affected in up to 90% of pancreatic adenocarcinomas and correlate very well with those regions harboring oncogenes and tumor suppressor genes such as *DPC4* at 18q, *p16* at 9p, *p53* at 17p and *Kras* at 12p.

Chromosomal instability occurs early in the development of pancreatic cancers [52]. Caused by inflammation and regenerative processes, the epithelial cells in PanIN lesions show a high degree of proliferation, leading to shortened telomeres [31]. This can lead to chromosomal breakage-fusion-bridge events which lead to chromosomal instability and intratumor heterogeneity [20,21]. Another important point is that chromosomal instability is often accompanied by centrosome abnormalities and amplification [18,61]. A dividing cell with anaphase chromosome bridges and centrosome anomalies could acquire a growth advantage due to partly disrupted chromosomes, if these rearrangements delete one or both alleles of a suppressor gene or would cause an extra copy of an oncogene. This could probably explain the high frequency and recurrence of chromosomal alterations despite intratumor heterogeneity.

6. Summary and future directions

The development of pancreatic adenocarcinomas is associated with inflammation and regeneration of the ductal epithelium, leading to a stepwise accumulation of genetic and histological alterations. But even though many of these mutations can already be found in precursor lesions, it has still not been possible to use these markers to detect pancreatic cancer at early stages or to develop new therapeutical strategies [26]. As discussed above, the onset of chromosomal instability may be the critical point for cancer development. The design of FISH probes to uncover chromosomal abberations often found in pancreatic adenocarcinomas may be useful for early detection. The association between telomere shortening and cancer development has also been shown. But even if this may improve diagnostics, for the therapeutical use one has to look more closer. Since the identification of recurrent patterns of chromosomal gains and losses indicates that numerous loci are involved in pancreatic carcinogenesis, high-throughput technologies like SAGE (serial analysis of gene expression) and gene expression microarrays [7,14,28, 38] will probably improve the classification of tumor types, unveil new prognostic markers and identify new genetic targets [7]. For example the expression profiling of cancer cell lines with different metastatic potential identifies those genes associated with the metastatic or invasive phenotype of pancreatic cancer cells and when done with cells treated or untreated with drugs or, e.g., growth factors, the affected pathways become visible [7]. Additionally, the investigation of the genetic interaction between neoplastic epithelial cells and stromal tissue and the associated desmoplastic reaction could be of relevance for a rational therapeutic approach. In the future, it may be possible to choose the appropriate regimens for the palliative and adjuvant treatment of pancreatic cancer patients based on the individual expression signatures of tissue samples [7]. The main problem of array technology is the generation of a large amount of expression data, which is difficult to handle and to analyze [7]. Many genes have been found to be over expressed in resected cancer specimens like cystatin S, MAT-8, S100A, mesothelin, hsp47, ADAM9 and many more [14,28,38]. However, the issue of selecting the appropriate genes as targets for pharmacological or diagnostic approaches, is still not solved [7]. Further investigations hopefully will contribute to a better understanding of pancreatic tumorigenesis. Especially new animal models that allow the development of premalignant lesions are believed to improve early detection methods [1,35,47]. Mouse models of pancreatic cancer now have reached the level of their counterparts in other organs [47]. The generation of progressive PanIN lesions and low-frequent progression to invasive and metastatic adenocarcinoma following activation of oncogenic KRAS in the mouse pancreas have been made [35,47]. When tissue-specific ink4a/arf deficiency is combined with activation of oncogenic KRAS, accelerated PanIN for-
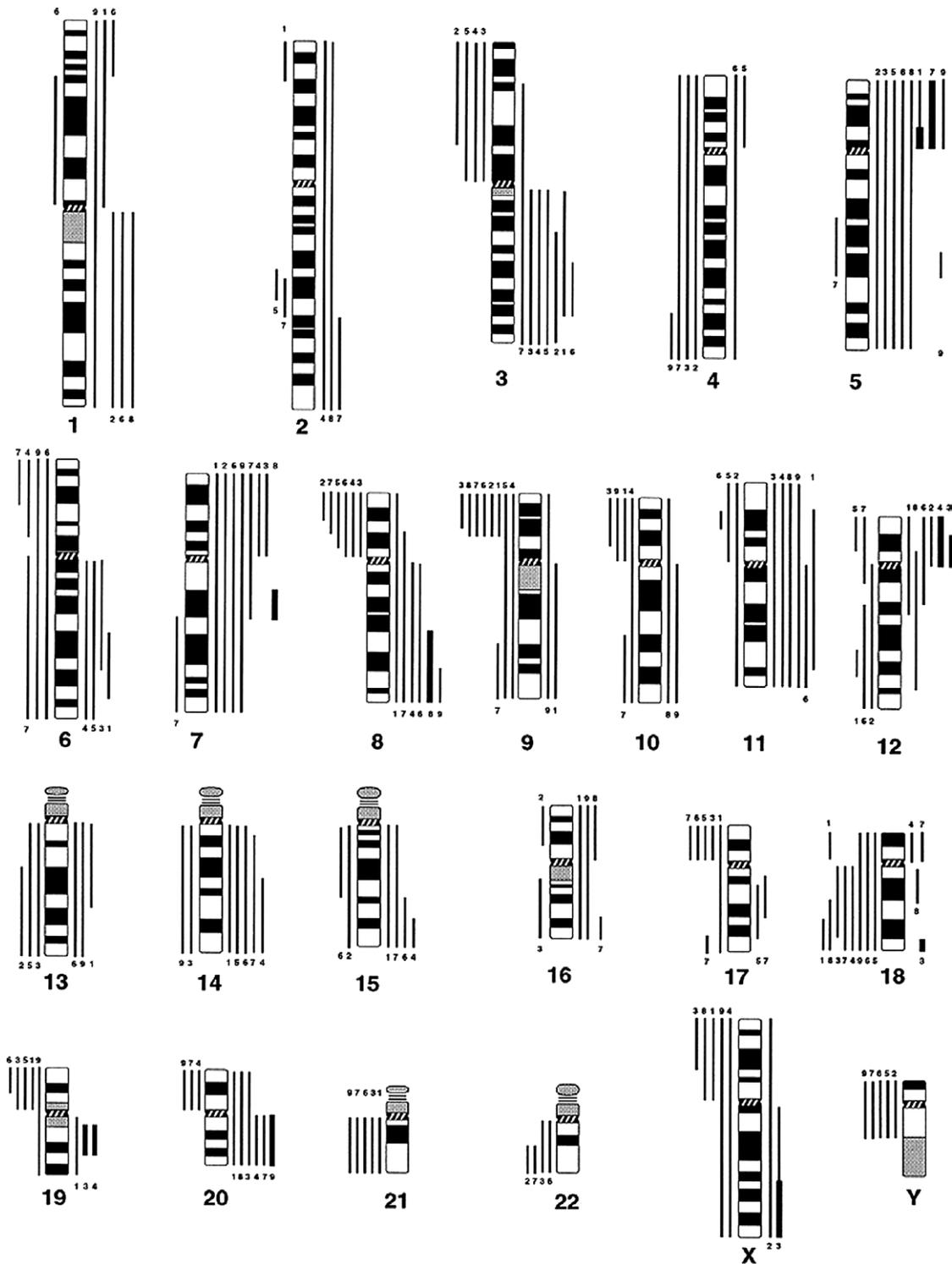
Fig. 3. Karyogram of chromosomal gains and losses in nine pancreatic carcinoma cell lines obtained by CGH. Vertical lines on the left side of each chromosome ideogram represent loss of genetic material, whereas those on the right side correspond to a gain. Amplification sites are represented as solid squares or bars. Each cell line can be identified by the case number on the top of each line: BxPC-3 (1), MIA-PaCa-2 (2), PANC-1 (3), SU.86.86 (4), CFPAC-1 (5), Capan-2 (6), Capan-1 (7), AsPC-1 (8), Hs766T (9) [17].

mation, rapid tumor progression and fatal metastasis occurs [1,47]. Additionally an amplification of the mutated KRAS allele and evolving expression of EGFR and Her2 were observed [1]. These findings in the mouse provide experimental support for the widely accepted model of human pancreatic cancer and may serve as a platform for the identification of early disease markers and for the efficient testing of novel therapeutical strategies [35,47]. For instance, the use of RNA interference (RNAi), mediated by small interfering RNA (siRNA), to silence genes overexpressed in pancreatic cancer has been reported as a new, nontoxic therapy. Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) [11], KRAS [3] and ribonucleotide reductase M2 subunit (RRM2) [12] siRNAs were tested using mouse models and showed complete inhibition of metastasis and suppression of tumor growth [11], inhibition of tumor dissemination in the murine peritoneal cavity [3], attenuated cellular invasiveness and gemcitabine chemo-resistance [12]. These results give hope for the development of rational therapies in the human disease. Even if many discoveries were made in the last few years, still further investigations have to be made to improve our understanding of pancreatic cancer.

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