The Fanconi anemia/BRCA2 pathway in pancreatic cancer
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CHAPTER 2

GENETIC ALTERATIONS IN PANCREATIC CANCER-ASSOCIATED GENES

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**Introduction**

Pancreatic cancer is fundamentally a genetic disease. This view is supported by the recurrent pattern of genetic changes associated with the transformation of normal pancreatic ductal cells into one of the deadliest forms of cancer (Table 2.1). Each of the key genetic alterations contributes to neoplastic progression by providing the developing neoplastic cells with a selective growth advantage over their neighboring cells. This leads to the serial outgrowth of naturally selected clonal populations of neoplastic cells, and these neoplastic cells can slowly evolve from a noninvasive precursor lesion (pancreatic intraepithelial neoplasia [PanIN]) to an invasive and eventually metastatic carcinoma.

The growth advantage provided by certain genetic changes also implies a pattern of mutually exclusive genetic mutations. That is, a second genetic change in a mutated pathway does not usually result in an additional growth advantage; multiple changes in the same pathway are therefore usually not encountered. The view that pancreatic cancer is fundamentally a genetic disease is also supported by the occurrence of multiple pancreatic cancers in families with a germline genetic alteration in a cancer-associated gene.

The mutational analysis of pancreatic cancer is made difficult by the fact that most often, only a minority of the cells constituting a pancreatic tumor are neoplastic cells. Pancreatic cancers characteristically induce an intense desmoplastic reaction, composed of fibroblasts, inflammatory cells and vessels. The nonneoplastic cells contain vast amounts of normal DNA that can mask the subtle changes in neoplastic cells. To enrich for neoplastic cells, cell culture or the technique of xenografting is used: surgically resected pieces of tumor are transplanted into immunodeficient nude mice, where the population of neoplastic cells expands to form a xenograft - tumors with a very high percentage of malignant cells and few human nonneoplastic cells (Figure 2.1). Using this approach, the mutational changes in pancreatic cancer have been well studied, and pancreatic cancer has emerged as one of the genetically best-understood forms of cancer. Most of the mutational data originate in studies of xenografts and cell lines, subsequently confirmed in primary carcinomas. Metastatic tumors have not been studied molecularly to any significant extent. A routine distinction should be made between conventional ductal adenocarcinoma and a less common, histologically and genetically distinct, variant growing in a medullary pattern. This distinction is clarified in this chapter.
Table 2-1 Summary of genetic alterations in pancreatic cancer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Function</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caretaker genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA2</td>
<td>13q</td>
<td>DNA repair</td>
<td>4-7%</td>
</tr>
<tr>
<td>FANCC, FANCG</td>
<td>9q, 9p</td>
<td>DNA repair</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>Oncogenes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS2</td>
<td>12</td>
<td>Signal transduction</td>
<td>85-95%</td>
</tr>
<tr>
<td>CCNE</td>
<td>19q</td>
<td>G1/S cell cycle transition</td>
<td>NA</td>
</tr>
<tr>
<td>Tumor-suppressor genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16</td>
<td>9p</td>
<td>G1/S cell cycle arrest</td>
<td>98%</td>
</tr>
<tr>
<td>p53</td>
<td>17p</td>
<td>Cell cycle arrest, apoptosis</td>
<td>50-75%</td>
</tr>
<tr>
<td>MADH4</td>
<td>18q</td>
<td>TGFβ/activin pathway</td>
<td>55%</td>
</tr>
<tr>
<td>LKB1/STK11</td>
<td>19p</td>
<td>Serine/Threonine kinase</td>
<td>5%</td>
</tr>
<tr>
<td>TGFβ, activin receptors</td>
<td></td>
<td>TGFβ/activin pathway</td>
<td>5%</td>
</tr>
<tr>
<td>BAX</td>
<td>19q</td>
<td>Apoptosis</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>FBXW7</td>
<td>4q</td>
<td>G1 cell cycle transition</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

Genetic instability and caretaker genes

Pancreatic cancer is caused by a sequence of genetic changes, distributed in time over many years. Evidence is accumulating that genetic instability, leading to a higher frequency of mutations affecting oncogenes and tumor-suppressor genes, is an early event in the development of cancer26-28. Although the mechanisms underlying this instability are still only starting to be understood, important progress has been achieved in recent years. Most pancreatic cancers (>90%), are aneuploid: they contain losses and gains of large portions of chromosomes or whole chromosomes, translocations and rearrangements, leading to a grossly abnormal karyotype, reflecting an underlying chromosomal instability (CIN). A detailed assessment of allelic loss in pancreatic cancer xenografts has been made.
recently by Iacobuzio et al.\textsuperscript{29}, using 386 microsatellite markers (markers for loss of heterozygosity [LOH]) in 93 pancreaticobiliary tumors. The most frequent sites of allelic loss (>60\% of carcinomas) were loci of known tumor-suppressor genes: 9p (\textit{p16/CDKN2A}), 17p (\textit{p53}) and 18q (\textit{DPC4/MADH4}), but moderately frequent losses (40-60\%) were also seen at 1p, 3p, 6q, 8p, 17q, 18p, 21q and 22q, loci not known at present to harbor a tumor-suppressor gene. The average loss was 15\% of all tested markers per tumor. Interestingly, a significant difference in loss of heterozygosity was found between smokers and non-smokers, with carcinomas from smokers displaying more LOH, a difference also noted in lung cancer\textsuperscript{30}. A detailed study of chromosomal arms 17p and 18q in PanINs has indicated that loss of one allele of a tumor-suppressor gene is frequently the first "hit", followed by an intragenic mutation in the second copy of the targeted gene\textsuperscript{31}.

A minority of pancreatic cancers has microsatellite instability (MIN): their chromosome numbers are usually normal, but these cancers have a more subtle defect at the nucleotide level. As is discussed later in greater detail, several specific genetic alterations have been implicated as potential causes of this genetic instability in pancreatic cancer.

\textbf{Telomeres}

Defective telomeres may be the major cause of the chromosomal instability observed in many cancers and in the vast majority of pancreatic cancers. Telomeres are present at the end of chromosomes and consist of specific repeated DNA sequences in association with telomere-binding proteins. Among other functions, telomeres protect chromosomes from self-perpetuating breakage-fusion-bridge cycles: fusion of chromosomal

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Xenografting. \textbf{A} Primary ductal pancreatic adenocarcinoma. \textbf{B} Pancreatic adenocarcinoma xenograft}
\end{figure}
ends, followed by breakage and generation of highly recombinogenic free DNA ends, a phenomenon first described in 1941 by Barbara McClintock\textsuperscript{32}, and later identified in human tumors\textsuperscript{53}. Chromosome fusions preferentially involve the chromosomes with the shortest telomeres, as shown by Hemann \textit{et al.}\textsuperscript{54} by digital karyotyping of splenocytes in genetically defined mice. Telomeres and their chromosome-protective functions can be maintained by the enzyme telomerase; however, most somatic human cells do not express this enzyme. A lack of telomerase leads to the progressive shortening of telomeres, which when proceeding beyond a critical limit will lead to cellular senescence. However, any rogue cell that failed to undergo senescence, perhaps due to the failure of a key regulatory gene for the cell division cycle, would enjoy a tremendous selective advantage to outgrow the otherwise similar, but arrested, cells that surround it. Without telomere caps, unprotected chromosome ends may result in chromosomal fusion, creating fused chromosomes with two centromeres. At the next mitosis, these cells may form \textit{anaphase bridges} in which the two centromeres of this unusual chromosome are pulled to opposite spindle poles, forming an irregular, long chromosome spanning the two centromeres (a bridge). These bridges may subsequently break, leading to daughter cells with highly recombinogenic chromosome ends, and a series of breakage-fusion-bridge cycles, or they may result in cytokinetic failure, with the formation of binucleated cells with supernumary chromosomes. Thus, telomere dysfunction can lead to both structural and numerical instability of chromosomes.

Chromosomal instability provides a tumor with the genetic diversity to overcome certain barriers in carcinogenesis. However, ultimately, chromosomal instability might prove detrimental to tumor growth, which may explain why neoplasms seem to acquire mechanisms to elongate their telomeres at later stages in the development of a malignancy, often through the reactivation of the enzyme telomerase.

An elegant fluorescence \textit{in situ} hybridization assay to detect intact telomeres in archival tissue sections has been developed recently by Meeker \textit{et al.}\textsuperscript{55}. Van Heek \textit{et al.}\textsuperscript{56} used this protocol to show that telomeres are shortened in 79 (96\%) of 82 PanINs (Figure 2.2), perhaps accounting for the early chromosomal instability seen in the development of pancreatic cancer. A reduction in telomere intensity was even seen in 91\% of PanIN-1a, the earliest putative precursor lesion in the pancreatic cancer progression model\textsuperscript{56}. 

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**Figure 2.2** Telomere length in pancreatic intraepithelial neoplasia (PanIN) lesions adjacent to pancreatic adenocarcinoma. Telomeres are labeled red; DNA is counterstained with DAPI (blue). **A.** Weak telomeric signals in the nuclei of low-grade PanIN (PanIN-1A) (double arrows); Intense labeling in adjacent nuclei of normal epithelium (arrows). **B.** Low-grade PanIN-1B. **C.** Sharp transition between high-grade PanIN-3 and normal epithelium. **D.** Weak telomeric signal in cancerized ducts (carcinoma growing into normal ducts, double arrows); interspersed bright signals are lymphocytes (arrow)

**Fanconi anemia/BRCA2 pathway**

Germline mutations in the *BRCA2* gene are present in a significant percentage of patients with familial breast, ovarian, young-onset prostate, and pancreatic cancer\(^37\)\(^-\)\(^40\). Germline mutations in the *BRCA2* gene may also play a significant role in apparently sporadic pancreatic cancers. The cloning of *BRCA2* was greatly aided by the identification of a homozygous deletion in a sporadic pancreatic cancer, and germline mutations in *BRCA2* are found in about 4-7% of sporadic pancreatic cancer\(^38\). The Brca2 protein is thought to play a role in DNA repair through homolo-
gous recombination, a process by which DNA-damage, in particular DNA-interstrand crosslinks, can be repaired by use of the sister chromatid or the homologous chromosome. Other members of the BRCA2 pathway may also play an important role in the development of pancreatic cancer. In 2002, Howlett et al. found that biallelic mutations in the BRCA2 gene are responsible for a subset of Fanconi anemia (FA) cases. FA is a hereditary cancer susceptibility disorder, with the occurrence of hematologic abnormalities or acute myelogenous leukemia at an early age, usually leading to death before the age of 20. Patients who survive into adulthood often develop solid tumors, especially squamous cell carcinomas of the head and neck or the anogenital region. After the discovery of these mutations of the BRCA2 gene in FA, other FA genes were surveyed in pancreatic cancer. In an initial study of 22 pancreatic xenografts and 11 cell lines, two convincing mutations were found: a germline nonsense mutation in the FANCG gene in a cell line and a somatic frameshift mutation in the FANCC gene in a xenograft, both accompanied by loss of the second allele. In addition, several missense mutations were found, of which the functional significance remains undetermined. The FA genes FANCA, FANCC, FANCE, FANCF, FANCG, and FANCL form a nuclear complex that is necessary for the monoubiquitination of Fancd2, the central protein in this pathway. A defect in monoubiquitination, which can be detected by a western blot, indicates that one of the upstream FA genes is defective. A total of 21 pancreatic cancer cell lines (including the already sequenced 11 cell lines) were recently screened using Fancd2 monoubiquitination as a functional assay (Figure 2.3). In this screen, another pancreatic cancer

Table 2.3 Screen for Fanconi anemia gene defects by Fancd2 monoubiquitination assay. The Fanconi proteins Fanca, Fancc, Fance, Fancf, Fancg and Fancl assemble in a nuclear complex that is necessary for the monoubiquitination of Fancd2. The lower band on a Fancd2 immunoblot indicates the nonubiquitinated isoform; the upper band indicates the monoubiquitinated isoform. If any of the Fanconi proteins other than BRCA2 (as in CAPAN1 cells) is defective, only a single band will be visible, as seen in the cell lines Hs766T (Fancg) and PL11 (Fancc).
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cell line was found to be defective in the FA pathway, which led to the discovery of a large homozygous deletion in FANCC in this cell line. Large numbers of carcinomas have not been screened yet, but the frequency of mutations in FANCC and FANCG is expected to be at least 3%. Cells with inactivating mutations in the FA genes (including BRCA2) have an increased in vitro sensitivity to mitomycin C and other DNA-interstrand crosslinking agents. Patients with "sporadic" pancreatic cancers defective in this pathway, are not themselves hypersensitive to these agents. Therefore, the occurrence of FA-defective carcinomas in FA-proficient hosts could provide a very useful therapeutic window for the treatment of these patients with DNA-interstrand crosslinking agents, such as mitomycin C and cisplatin. Why mutations in the FA/BRCA2 pathway are selected for during tumorigenesis is not yet clear. An obvious suggestion would be that defects in this pathway could lead to the initiation of genetic instability. However, genetic instability can be assumed to be present prior to the abrogation of members of the Fanconi anemia/BRCA2 pathway, because the inactivation of the BRCA2, FANCC, or FANCG genes is always due in part to the deletion of one of the alleles. Also, the loss of the second allele in carriers of a BRCA2 germline mutation occurs late in carcinogenesis. Possibly, defects in the FA/BRCA2 pathway lead to a form of genetic instability that is needed to help a neoplasm overcome certain genetic barriers. As noted by Swift in 1971, rare recessive syndromes, such as FA, may be of great value in understanding the origin of clinically common and important neoplasms.

Mitotic checkpoints

In 1998, Cahill et al. showed that all colorectal cancers displaying CIN, but not those displaying MIN, are defective in the mitotic checkpoint normally observed after treatment with mitotic spindle-disrupting agents. In some of these tumors, mutations were found in the BUB1 gene in combination with a wild-type second allele. When these mutants were transfected into MIN tumors, the same type of mitotic checkpoint defect was found, even though the wild type allele was still present, indicating that these mutations have a dominant negative effect. A survey of pancreatic cancer cell lines for these checkpoint abnormalities was conducted by Hempen et al. In contrast to MIN cancer cells, the CIN pancreatic cancer cells did not display a distinct mitotic block after treatment with a mitotic spindle-disrupting agent. In one of these cell lines, two missense mutations were found in a single allele in exon 8 of
the \( BUB1 \) gene, possibly accounting, at least in this cell line, for the observed mitotic checkpoint defect. The occurrence of dominant negative mutations in these checkpoint genes could contribute to aneuploidy and is in accordance with the genetically dominant nature of CIN\(^{54}\).

**Mismatch repair**

Most human tumors, including pancreatic adenocarcinomas, have CIN, producing complex karyotypes with alterations in chromosomal copy numbers (aneuploidy)\(^{55,56}\). However, some carcinomas have an almost diploid (normal) chromosome number. These cancers have a more subtle genetic instability: they have defects in mismatch repair, resulting in elevated rates of sequence mutations and very high rates of mutations in microsatellites: simple repetitive sequences; hence the term microsatellite instability. Defects in mismatch repair in cancer were initially reported in colorectal cancer\(^{57-59}\). Hereditary non-polyposis colorectal cancer syndrome is caused by germline mutations in one of the mismatch repair genes (including \( MLH1 \) and \( MSH2 \)). Proteins encoded by these genes repair single basepair changes and small insertions and deletions. Cancers mutated in these genes accumulate changes of coding and non-coding regions of the genome.

Microsatellite instability is found in approximately 4% of pancreatic cancers\(^{25,24,60}\), and pancreatic cancer has also been reported in some hereditary non-polyposis colorectal cancer kindreds\(^{61,62}\). Pancreatic cancers with microsatellite instability are associated with a medullary histopathology: poor differentiation, a syncytial growth pattern and pushing borders\(^{24,25}\). A medullary histology is always seen in MIN cancers, although half or more medullary tumors do not have MIN. Genetically, they are \( KRAS2 \) wild-type and commonly contain mutations in the \( TGFBRII \), \( ACVR2 \) and \( BRAF \) genes, all in contrast with the usual findings in microsatellite stable pancreatic cancer. Recently, a diagnostic tool has been developed by Montgomery et al., which uses the appearance of anaphase bridges as a distinguishing sign between CIN and MIN tumors. In a study consisting of sarcomas, colorectal and pancreatic tumors, all chromosomally stable tumors (MIN tumors) lacked anaphase bridges, whereas anaphase bridges were found in most CIN sarcomas and carcinomas\(^{65}\).

**Oncogenes**

Oncogenes encode for proteins that when mutationally activated, contribute to neoplastic progression.
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KRAS2
KRAS2 encodes a G (GTP-binding) protein that is involved in the transduction of signals from growth factor receptors and other signaling inputs. The Kras2 protein can be constitutively activated by point mutations in codons 12, 13 or 61. These mutations impair the GTPase activity of the Kras2 protein. The KRAS2 gene is mutated in more than 90% of pancreatic cancers, usually by a mutation in codon 12, the highest rate of mutations for KRAS2 in any type of cancer. Studies in PanINs show KRAS2 to be mutated early in the development of pancreatic cancer, although the prevalence of KRAS2-mutations rises in more advanced lesions. There are a few reports of KRAS2 gene amplification, although whether these serve the same tumorigenic role as classic missense KRAS2 gene mutations remains undetermined.

BRAF
The BRAF gene encodes a serine/threonine kinase located immediately downstream from Kras2 in the Ras signaling pathway, and is a mutational target in several cancers, including melanomas (66%) and colorectal cancer (10%). Mutations in KRAS2 and BRAF seem to be, in large part, mutually exclusive: in a study by Rajagopalan et al., mutations of KRAS2 and BRAF were analyzed in 330 colorectal tumors. The BRAF gene was mutated in 32 (10%) of the carcinomas, and the KRAS2 gene in 169 (51%); none of these tumors contained mutations in both genes. Furthermore, the prevalence of BRAF mutations was much higher in mismatch repair-deficient cancers (31%) than in mismatch repair-proficient cancers (7%). In a study by Calhoun et al., nine KRAS2-wildtype pancreatic cancers were screened for mutations in the BRAF gene; three of these carcinomas (33%) harbored a somatic missense mutation, V599E, previously shown to stimulate the kinase activity of Braf; two of these three carcinomas had a mismatch repair defect. Among 74 KRAS2-mutant cancers, no mutations in the BRAF gene were identified. These observations confirm that neoplasms seem to select for only one mutation in this pathway and that there seems to be nearly a requirement for KRAS2-related signal activation in the development of pancreatic cancer.

Cyclin E
Cyclin E is a known proto-oncogene, overexpressed in several different types of cancer, and the protein product of cyclin E functions as a cell cycle regulator. Cyclin E is targeted for degradation by the ubiquitin lig-
ase Fbxw7 (Ago, Cdc4)\textsuperscript{72, 73}. Using microarrays and immunohistochemical labeling, Calhoun et al. determined that 6\% of pancreatic adenocarcinomas overexpress cyclin E. The authors also reported low-level amplification and ectopic copies of CCNE (coding for Cyclin E) in separate cell lines, as well as a somatic missense mutation in \textit{FBWX7} (H460R) in a xenografted pancreatic cancer\textsuperscript{71}. \textit{FBWX7} functions as a tumor-suppressor gene, with the second allele inactivated by loss of heterozygosity. This biallelic mutation of the \textit{FBWX7} gene was accompanied by strong nuclear immunopositivity for cyclin E, indicating an impaired degradation.

\textbf{Other amplified regions}

Amplification of genomic regions besides CCNE may occur occasionally. Amplified regions include the \textit{AKT2} gene within an amplicon on chromosome 19q and the \textit{MYB} gene on 6q, involving about 10-20\% of cases studied\textsuperscript{74, 76}, although the functional targets of these amplicons are not fully explored.

\textbf{Tumor-suppressor genes}

Tumor-suppressor genes are genes that when inactivated contribute to neoplastic progression. Tumor-suppressor genes usually code for proteins that have a direct or indirect role in governing the cell cycle or apoptosis: roles that restrict the expansion of cell populations. They can be inactivated by a combination of mutations, methylation, or the complete loss of one (LOH) or both (homozygous deletion) alleles.

\textbf{p16/CDKN2A}

Normal cells can only progress through the G1 phase of the cell cycle if they can functionally inactivate the Rb protein by phosphorylation (reviewed in Sherr\textsuperscript{77}). Rb is phosphorylated by a complex of cyclin D and a cyclin-dependent kinase (Cdk4 and Cdk6). p16 inhibits promotion of the cell cycle by competing with cyclin D in binding to Cdk4 and Cdk6, preventing the phosphorylation (inactivation) of Rb. The hyperphosphorylation of Rb releases transcription factors that promote the G1/S transition. Therefore, the p16/Rb pathway can be abrogated by alteration of p16, Rb, Cdk4/Cdk6 or cyclin D.

The \textit{p16/CDKN2A} gene is located on 9p, a site of frequent allelic loss in pancreatic cancer, and is mutated in a variety of cancer types. Caldas et al. demonstrated homozygous deletions of \textit{p16/CDKN2A} in 40\% of pan-

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creatic cancers and inactivating mutations of the gene in another 38% of cancers, by an intragenic mutation coupled with loss of the second allele. In addition, Schutte et al. demonstrated that the p16/CDKN2A gene is inactivated by hypermethylation of its promoter in almost all of the remaining pancreatic cancers. Inactivation of Rb is reported in a small number of pancreatic cancers: Huang et al. found loss of immunohistochemical staining for Rb in 3 of 30 pancreatic cancers. A truncating mutation was found in one of these cancers, and a missense mutation was found on genomic sequencing in another of these three cancers. Thus, the p16/Rb pathway is inactivated in virtually all pancreatic cancers, leading to an inappropriate progression through the G1 phase of the cell cycle.

**TP53**

p53, encoded by the TP53 gene, is a nuclear DNA-binding protein that has an important role in the G1/S cell cycle checkpoint, the maintenance of the G2/M arrest, and the induction of apoptosis. The inactivation of the TP53 gene results in the loss of important restraints on the initiation of replication and the loss of the induction of cell death. The p53 tumor-suppressor gene is located on the short arm of chromosome 17 (17p); this locus shows frequent LOH in pancreatic cancer. The remaining TP53 allele, when sequenced, harbors missense mutations or small frameshift mutations in 50-75% of pancreatic cancers. Thus, the TP53 gene is inactivated in 50-75% of pancreatic cancers.

**TGF-β/activin pathway**

The TGF-β (transforming growth factor type β) pathway is a tumor-suppressive signaling pathway activated by the binding of TGF-β ligands to its cell surface receptors, and the subsequent phosphorylation, complexation and nuclear localization of Smad proteins. This and related pathways can also be activated by the binding of related ligands, such as activin and BMP (bone morphogenic proteins). The long arm of chromosome 18 (18q) is a site of frequent allelic loss in pancreatic cancer: almost 90% of tumors have loss of heterozygosity at this locus. This led Hahn et al. to search for a tumor-suppressor gene on 18q. While screening a panel of pancreatic xenografts, they found a region located at 18q21.1 to be homozygously deleted in a number of tumors; they designated this region DPC4 (deleted in pancreatic cancer). Subsequently, several intragenic mutations were found in a gene
located in this region, in xenografts without a homozygous deletion, but with LOH. This gene, DPC4/MADH4/SMAD4, encodes Madh4, a mediator of the TGF-β pathway, and is inactivated somatically in 55% of pancreatic cancers. In 35% of cancers, MADH4 is inactivated by a homozygous deletion; in 20% by an intragenic mutation in combination with loss of the other allele. The prevalence of MADH4 mutations in pancreatic cancer is much higher than that in other types of cancer. For example, MADH4 is inactivated in 15% or less of breast and colorectal cancers. The immunohistochemical labeling of Madh4 directly mirrors MADH4 gene status. This has been shown for both homozygous deletions and truncating mutations of the MADH4 gene and, more recently, for most inactivating intragenic mutations: these mutant proteins are not stable and/or translated into proteins. Immunohistochemical labeling for the Madh4 protein has been used to investigate the expression of Madh4 in PanINs by Wilentz et al. Madh4 was found to be expressed in all histologically low-grade lesions (PanIN-1 and -2), whereas Madh4 protein expression was lost in 31% of high-grade lesions (PanIN-3). Multiple receptors, including TGF-β receptor type I (Tgfbr1/ALK5), TGF-β receptor type II (Tgfbr2) and the activin receptors type IB (Acvr1b/ALK4) and II (Acvr2) exert their effect through Madh4. In 1998, Goggins et al. studied the genes TGFBR1 and TGFBR2 for inactivating mutations. One of 97 pancreatic and one of 12 biliary adenocarcinomas harbored a homozygous deletion of TGFBR1. In addition, somatic alterations in TGFBR2 were identified in four out of 97 pancreatic adenocarcinomas. Three of these four mutations were homozygous frameshift mutations in a poly(A) tract, in pancreatic cancers with a mismatch repair defect. In a study by Hempen et al., mutations in the ACVR2 gene were found in three mismatch repair defective pancreatic cancers; all of these three mutations were frameshift alterations in a poly(A) tract of the ACVR2-gene, occurring in tumors that also harbored mutations in the TGFBR2-gene. The occurrence of mutations in either gene in MIN-tumors occurred at a higher rate than expected in a mathematical model, based on the prevalence of random alterations in short mononucleotide tracts in these carcinomas. In addition, a CIN pancreatic cancer was found with a frameshift mutation in the ACVR2 gene combined with LOH of the second allele, establishing ACVR2 as a tumor-suppressor gene targeted in pancreatic cancer. In 2001, Su et al. described a homozygous deletion and a 5-bp frameshift deletion in the activin receptor type 1B (ACVR1B/ALK4) gene in two
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pancreatic xenografts. ACVRIB/ALK4 codes for an activin-receptor that signals through Madh4. The two pancreatic cancer xenografts with mutations in the ACVRIB/ALK4 gene both had inactivating mutations in MADH4, creating an apparent contradiction with regard to the dogma that tumor mutations in regulatory pathways are reciprocal: neoplasms are believed to have inactivation of only one member of a linear pathway. However, these findings readily fit a more complex model of a branched signaling pathway in which, at an early stage, MADH4 gene mutations would be detrimental to a developing neoplasm, whereas mutations of TGF-β and activin receptors, with only a partial inactivation of Madh4-mediated signaling, would provide the tumor with a selective advantage. In a later stage, when the cancer cells harbor new defects in cell cycle checkpoints and other regulatory systems, an inactivation of the MADH4 gene would no longer be detrimental, but instead would be advantageous. This theory could explain the stepwise inactivation of members of a branched pathway tumor-suppressive system, like the TGF-β/activin-pathway, and the empiric finding that MADH4 loss in the pancreas appears restricted to late-stage PanINs and cancers.

Other tumor-suppressor genes

Mutations in the LKB1/STK11 are the cause of the autosomal-dominant inherited Peutz-Jeghers syndrome, characterized by mucocutaneous melanin macules and nonneoplastic gastrointestinal hamartomas. In addition, patients with the Peutz-Jeghers syndrome suffer from an elevated risk of cancer: the average age at which cancer is diagnosed ranges from 38-50 years. The risk of death from gastrointestinal cancer is 15- to 50-fold greater than the risk in the general population; Giardiello et al. found a relative risk of 132 for pancreatic cancer in Peutz-Jeghers patients. In a study by Su et al., the LKB1/STK11 gene, encoding a serine/threonine kinase, was found to be homozygously deleted or to harbor a frameshift mutation accompanied by LOH, in 4-6% of 127 sporadic pancreatic and biliary adenocarcinomas. In addition, the wildtype allele in a patient with Peutz-Jeghers syndrome was found to be lost in a pancreatic cancer. The finding of inactivation of LKB1/STK11 in both familial and sporadic cases establishes LKB1/STK11 as a tumor-suppressive gene along the lines of the classical Nicholls/Knudson model. Many other tumor-suppressor genes that are targeted in pancreatic cancer deserve mentioning. Intragenic mutations and homozygous deletions of the MKK4 (Mitogen activated protein kinase kinase 4) gene are
seen in a small percentage of pancreatic cancers\textsuperscript{96}. The \textit{MKK4} gene codes for a component of a stress-activated protein kinase cascade and has roles in apoptosis and growth control. Frameshift mutations in \textit{BAX}, a mediator of apoptosis, have been reported in mismatch repair defective tumors, at a rate higher than predicted based on the occurrence of random frameshifts in microsatellites\textsuperscript{88, 97}. The \textit{EP300} gene codes for p300-a histone acetyltransferase that regulates transcription through chromatin remodeling. A truncating mutation in a pancreatic cancer cell line has been reported by Gayther \textit{et al}.; no larger studies of this gene in pancreatic cancer have been reported\textsuperscript{98}.

**Conclusions**

Through the study of pancreatic cancer precursor lesions, PanINs, a model for the progression of pancreatic ductal epithelium from noninvasive dysplastic intraepithelial lesions to an invasive cancer is emerging (\textit{Figure 2.4})\textsuperscript{99}. The earliest recognizable and prevalent genetic defect is the shortening of telomeres. This defect could plausibly cause CIN, leading to losses and gains of chromosomal arms; loss of heterozygosity of chromosome 9p (the location of \textit{p16}) is seen in 13\% of histologically low-grade duct lesions, and in 90\% of high-grade duct lesions\textsuperscript{23, 79}. All histologically high-grade lesions exhibit LOH at more than one chromosomal locus\textsuperscript{79}. Activating mutations in \textit{KRAS2} can also occur early in this model, although the prevalence rises further down the road towards an invasive malignancy, suggesting that \textit{KRAS2} gene mutations are not
necessarily the first change (gatekeeper) needed for the development of ductal neoplasia. Alterations in the p16 gene occur slightly later than \textit{KRAS2} gene mutations, and the prevalence of loss of this gene also appears to rise with increasing grades of PanIN\textsuperscript{100, 101}. In this genetic progression model of pancreatic cancer, the inactivation of \textit{DPC4}, \textit{p53} and \textit{BRCA2} genes seem to be relatively late events\textsuperscript{99}. The last decade has brought tremendous progress in understanding of the genetic causes of pancreatic cancer. However, it is unlikely that all genetic changes have been found as yet, as was demonstrated by recent discoveries of mutations in \textit{BRAF}, \textit{FBXW7} and the FA genes. Many genes still need to be investigated. The high mortality rate and paucity of therapeutic options for pancreatic cancer highlight the need to apply this extensive genetic knowledge to patient care.