

Chapter 2

Molecular Genetics of Pancreatic Cancer

Eric S. Calhoun and Scott E. Kern

1 Introduction

In cancer, genome stability is compromised. Ductal adenocarcinoma of the pancreas is no exception. Considerable progress in the identification and characterization of somatic and/or germline genetic alterations has provided the mechanistic foundations of this genetically complex disease. Numerous alterations, including chromosomal copy-number gains, amplifications and homozygous deletions, loss of heterozygosity (LOH) with and without copy number reduction, and balanced and unbalanced structural re-arrangements, are commonly observed (1–4). Gross chromosomal changes are often complemented by smaller, more subtle alterations affecting the open reading frames of proto-oncogenes, tumor suppressors, and genome caretaker genes (5–8). Continued evaluation of these and additional yet-unidentified genetic alterations should translate into rational diagnostic and treatment strategies.

This chapter describes the spectrum and frequency of genetic alterations observed in ductal adenocarcinomas of the pancreas, as organized by the underlying presence of two, largely mutually exclusive, types of genome instability: chromosomal instability (CIN) and microsatellite instability (MIN). This distinction is justified, as each tumor type exhibits unique histologic and molecular characteristics (9).

2 Chromosomal Instability

The vast majority of pancreatic cancers (97%) have CIN, characterized by numerous chromosomal copy-number gains and losses, amplifications and homozygous deletions, translocations, and inversions. At first glance, these alterations appear random. Careful examination of overlapping genomic alterations has, however, revealed the recurrent targeting of select genes for disruption. The spectrum of altered genes, as well as the types of alterations, makes each pancreatic tumor rather distinctive.

2.1 *Required Genetic Mutations*

Disruptions of at least two genes in pancreatic cancer are nearly universal. The first, K-ras (12p12.1), is an oncogene. A GTP-binding and hydrolyzing enzyme, the K-ras protein functions as a second messenger in growth factor receptor signaling pathways that stimulate the transition through the G1 phase of the cell division cycle. This activity is dependent upon bound GTP, remaining active as long as the nucleotide remains unhydrolyzed. Somatic mutations affecting the GTP-binding pocket can disrupt the ability of K-ras to hydrolyze GTP, rendering the protein constitutively active. Mutations of the codons Gly12, Gly13, or Gln61 are commonly associated with constitutively active K-ras, although recurrent alterations in Ala146 in colorectal cancers suggest additional hotspots may exist (10, 11).

Ninety-five percent of pancreatic cancers harbor activating mutations in K-ras, representing the highest fraction of any tumor type (12, 13). The COSMIC (Catalogue of Somatic Mutations In Cancer) database lists the most common alteration in pancreatic cancer as the Gly12Asp substitution, accounting for ~50% of all pancreatic cancer cases, whereas total alterations in Gly12 account for ~99% of all mutations (14). The paucity of Gly13 mutations is unusual in that significantly higher frequencies are noted in other cancers (e.g., colorectal cancer: Gly13Asp, 15.8%; total Gly13 mutations, 17.3%). Conversely, Gly12Arg substitutions are commonly observed in pancreatic cancer (11.6%), whereas they occur infrequently in colorectal cases (1.0%). The underlying reasons for these tumor-specific mutational preferences are unknown.

The second (virtually required) disruption in pancreatic cancer involves CDKN2A (p16, 9p21.3), one of the most frequently altered genes in cancer. A classical tumor-suppressor, p16 regulates cell cycle progression by inhibiting cyclinD-CDK4/6, the kinase complex responsible for initiating the G1/S phase transition. Inherited alterations in p16 cause FAMMM (familial atypical multiple mole melanoma), a syndrome characterized by numerous dysplastic nevi having atypical shape, color and/or size, a propensity for developing melanoma and a distinct risk of pancreatic cancer (nearly a 20-fold increased risk) (15–18).

The incidence of p16 alterations in sporadic pancreatic cancer is dramatic, with inactivation of this gene occurring in 98% of cases. Unlike K-ras activation, however, gene disruption (i.e., inactivation) occurs through multiple mechanisms including nearly half by homozygous deletion, more than a third by intragenic mutations with LOH, and the remainder by epigenetic promoter silencing (19, 20).

2.2 *Frequently Mutated Genes*

Genes mutated at a frequency >25% suggest they provide a supportive (i.e., selective advantage) role in the development of cancer. The implication that they are not required, however, suggests that other mutations might occasionally substitute for

the more common alterations in these genes. Three examples of genes in this category include SMAD4 (18q21.1), TP53 (p53, 17p13.1), and NCOA3 (AIB1, 20q12), and for each there is limited evidence of substitute gene targets (21, 22).

Inactivation of SMAD4 occurs in approximately 45% (30% by homozygous deletion) of pancreatic cancers, but less frequently in other cancer types (23–26). Functional characterization has shown that the SMAD4 tumor-suppressor protein is TGF β -responsive, translocating to the nucleus (with Smads 2 and 3) after TGF β or activin receptor stimulation to activate expression of genes mediating growth inhibition. Missense mutations in this gene impair protein stability, among other functional defects (27–29).

Another frequently mutated gene in pancreatic cancer, and one of the most frequently altered genes in all cancer types, is TP53 (p53, 17p13.1). The tumor-suppressive properties of p53 lie in its ability to transcriptionally activate target genes in response to cellular stresses such as DNA damage. TP53 blocks G1/S and G2/M transitions by increasing p21WAF1/CIP1 and SFN (14-3-3 sigma) expression, and induces programmed cell death in experimental settings (30–32). Inactivating mutations occur in 70% of pancreatic tumors, with most affecting the ability of the protein to bind to DNA and activate gene transcription (33, 34). Unlike other tumor suppressors, however, homozygous deletions affecting p53 occur very rarely; most mutations are missense mutations. The reason for this remains unknown, but the gene dense region at 17p13.1 may inhibit the formation of homozygous deletions as they would provide a selective disadvantage to cells experiencing the loss of multiple genes.

NCOA3 (AIB1, 20q12) enhances the transcriptional activity of a number of steroid nuclear receptors and is the rate limiting step in estrogen-mediated growth signaling. High-level amplification of this gene has been reported in ~10% of breast cancer tissues and four of nine pancreatic cancer cell lines (35, 36). In archival tissues, copy-number gains were observed in >37% of pancreatic cancers (37). The observance of such high frequencies of AIB1 gains and amplifications in pancreatic cancer was surprising as pancreatic ductal tissues are not normally controlled by endocrine stimulation. Some of these authors suggest that such an amplification “may indicate that estrogen receptor mediated transcriptional activation confers a growth advantage” to these cells. The clinical relevance of these findings remain to be determined, however, as pancreatic cancers have not yet been shown to have hormone dependence.

2.3 *Low-Frequency Mutations*

Despite very little variation in clinical outcome, pancreatic tumors have heterogeneous mutational backgrounds that give each tumor a unique molecular signature. Each genetic mutation, occurring in <25% of tumors, may define a distinct subclass of pancreatic tumors with unique molecular properties. Rational treatment strategies attempt to exploit these tumor-specific properties.

2.3.1 Genes Involved in the G1/S Cell Cycle Checkpoint

Many mutations in pancreatic cancer are found in genes whose functions are linked to the control of the cell cycle. The retinoblastoma protein, RB1 (pRb, 13q14.2), acts as the central control point for regulating cell cycle commitment (38, 39). When cells are not stimulated to divide by growth factors, pRb inhibits E2F transcriptional activity, thereby restricting progression into S-phase. Upon growth factor stimulation, pRb releases E2F, allowing for the expression of S-phase critical genes. Many different tumors commonly inactivate pRb, however, only rare mutations (<6%) are reported in pancreatic cancer (40).

The inactivation of pRb is accomplished by a complex consisting of a regulatory cyclin and a kinase. One member of this complex is CCND1 (Cyclin D1, 11q13.3), working with either cyclin-dependent kinase 4 or 6 to phosphorylate and inactivate pRb. Infrequent Cyclin D1 amplification is reported for several tumor types (41–43), such as 2.5% of head and neck squamous cell carcinomas and 0.5–1.5% of colorectal cancers (>10 gene copies), however, the extent of Cyclin D1 amplification is less clear for pancreatic cancer. Using Southern analysis, Gansauge et al. reported 25% (7/28) of pancreatic cancers to have Cyclin D1 amplification (44). Each case was additionally reported to have increased expression of Cyclin D1 protein.

Such a high frequency of amplification is questioned by Schutte et al., who found no evidence of amplification in 26 pancreatic cancers (at approximate sensitivity six copies or greater) or Huang et al. (greater than fivefold) in 20 pancreatic cancers and 18 cell lines (20, 40). Using SNP arrays, Calhoun et al. found only a single case ($n = 24$) of Cyclin D1 amplification (2). After eliminating overlapping samples analyzed by the studies of Schutte et al. and Calhoun et al., only 2.4% (1/42) had Cyclin D1 amplification. The discrepancies in frequencies between these studies are not readily explained.

Another cyclin, CCNE1 (Cyclin E1, 19q12), combines with Cdk2 to phosphorylate several substrates important for G1/S transition and DNA replicative activities (45–48). Representing a “point of no return,” cells expressing Cyclin E1 become committed to undergoing DNA replication and subsequently mitosis (49).

Cyclin E1 was found to be amplified in various tissues including colon adenocarcinoma (1.2%), metastatic, ductal carcinoma of the breast (2.6%), serous carcinoma of the ovary (8%), and adenocarcinoma of the stomach (13.9%) (50). In total, 23 instances (1.8%) of Cyclin E1 amplification were reported in various types of tumors. Using immunohistochemistry, Cyclin E1 was found to be overexpressed in ~6% of pancreatic cancers, with gene amplification (two separate cell lines) and mutational inactivation of FBXW7 (the E3 ubiquitin ligase responsible for Cyclin E1 degradation) identified as possible mechanisms for the increased expression (51).

2.3.2 Genes Involved in Double-Strand–DNA Break Repair

An additional class of genes mutated in pancreatic cancer is involved in the repair of DNA interstrand crosslinks and double-strand breaks. Collectively, genes involved in

DNA repair are known as genome-maintenance genes or “caretakers” with a primary role in maintaining the sequence integrity of the genome (8, 52). Germline inheritance of an inactive allele often leads to a predisposition to cancer.

BRCA2 (FANCD1, 13q12.3), which participates with Rad51 and BRCA1 to prevent and/or repair DNA double-strand breaks through homologous recombination, is one example of this type of gene (53, 54). Inherited, inactive alleles (such as the common 6174delT truncating mutation) confer an increased risk for developing ovarian and breast cancers as well as a 3.5- to 20-fold increased risk of developing pancreatic cancer (55).

The mutational frequency of BRCA2 was investigated by Goggins et al. BRCA2 was homozygously inactivated in 7% (3/41) of unselected pancreatic cancers (56). All mutations were derived from germline inheritance: Two samples were confirmed with a matched normal tissue, whereas the third, a 6174delT Ashkenazi Jewish founder mutation, was assumed to be inherited. Inactivation of BRCA2 was found to occur late in precursor lesions of the pancreas (57). Only one case of sporadic pancreatic cancer due to the somatic inactivation of both BRCA2 alleles in pancreatic cancer is yet reported (58).

In 2002, Howlett et al. determined that the Fanconi anemia (FA) complementation-group D1 defect was due to biallelic, truncating mutations in BRCA2 (59). This finding linked Brca2 and other FA proteins into a common DNA damage-responsive pathway. Most FA proteins (Fanc A, B, C, E, F, G, L and M) bind to form a heteromeric, E3 ubiquitin ligase complex which monoubiquitinates and activates FANCD2. After translocating to the nucleus, mUb-Fancd2 colocalizes with Brca1, Brca2, and Rad51 to focal areas of DNA damage during S-phase (59, 60).

Due to the known involvement of BRCA2, other FA genes were subsequently examined for somatic alterations in pancreatic cancer. These studies were largely negative, except for occasional mutations found in FANCC (9q22.3) and FANCG (9p13), arising somatically as well as through germline inheritance (61, 62).

2.3.3 Miscellaneous Gene Mutations

An eclectic set of mutations has been identified in pancreatic cancer. However, the functions of most genes cannot be easily cataloged in classical tumor-promoting pathways (i.e., cell cycle G1/S regulation, maintenance of genome integrity, apoptosis). In some instances, a gene's cellular function may not yet be assigned; therefore, the impact of its alteration in cancer is not known. Despite our lack of understanding, the repeated presence of clonal alterations arising during carcinogenesis suggests that these genes may play an important role in the development of individual cancers. Examples are discussed briefly in the following.

Phosphorylated residues on ERBB2 (HER-2/NEU, 17q11.2-12) serve as docking sites for a number of signal transducers that initiate signaling cascades leading to cell proliferation, differentiation, migration, adhesion, resistance to apoptosis, among other effects (63, 64). Overexpression of ERBB2 in pancreatic cancer was first reported by Hall et al. (65), verified by others (66–69), and associated with amplification in varying degrees (67, 70, 71).

AKT2 (19q13.1-13.2) encodes a serine/threonine protein kinase identified as a homolog of the v-akt oncogene and is activated, along with AKT1, in response to the binding of growth factors such as PDGF (72–75). The functional differences between AKT1 and AKT2 activation in cellular signaling pathways important to pancreatic cancer remain to be determined, however. AKT2 is amplified in pancreatic cancer at a frequency of 11% (3/28) to 20% (7/35), suggesting this gene may indeed be important in pancreatic cancer development (76, 77).

The MYB (c-myb, 6q23-24) oncogene has been shown to induce the expression of many genes that regulate proliferation, differentiation, and apoptosis; however, its precise role in cancer remains obscure as normal tissues often express this protein at high levels (78). Examples of MYB amplification have been reported in 29% (5/17) of hereditary BRCA1 breast cancer samples, in 2% (2/100) of sporadic breast cancers as well as in 4–6% of pancreatic cancer cell lines and primary tissues (2, 79, 80).

DCC (deleted in colorectal carcinoma, 18q21.1-21.2) was suggested to play a role as a candidate tumor suppressor gene due to its rare homozygous deletion in colorectal carcinomas and 6% (7/115) of pancreatic carcinomas (25, 81, 82). The common disruption of a neighboring tumor suppressor (SMAD4) and the paucity of intragenic mutations have shed doubt, however, on the role this gene plays in pancreatic cancer.

Inherited mutations in the serine/threonine protein kinase, STK11 (LKB1, 19p13.3), cause Peutz-Jeghers syndrome (83). This rare, autosomal dominant disorder is characterized by hamartomatous polyps of the intestine (predominately in the small intestine), ink-black, spotty pigmentation surrounding the lips, and a dramatically elevated risk of developing many types of cancer. Between the ages of 15 and 64, affected individuals have a 93% cumulative incidence of cancer with a 36% specific incidence of pancreatic cancer (84). The rate of sporadic inactivation of STK11 in pancreatic cancer was reported at 5% (85).

Intragenic mutations and homozygous deletions in MAP2K4 (MKK4, 17p11.2) occur in up to 4% of pancreatic cancers (86–88). This stress-activated protein kinase-cascade pathway member demonstrates both growth control and apoptotic functions. Paradoxically, however, MKK4 has been shown to play a metastasis-suppressing role in an experimental somatic-cell gene knockout model, implying the background mutational environment and other variables may influence the development of a MKK4-null state in pancreatic cancer (89).

The EGFR (7p12) mutation delE746-A750, previously characterized as an activating EGFR mutation in non-small cell lung cancer, was found to occur in 3.6% (2/55) of pancreatic cancers (90). An earlier study failed to find mutations in the kinase domain (exons 18–24) in 92 pancreatic cancer xenografts and nine pancreatic cancer cell lines (91). The involvement of EGFR mutations in pancreatic cancer appears infrequent, but due to the clinical sensitivity of this specific mutation to small molecule inhibitors, further evaluation of this gene's involvement in pancreatic cancer may be warranted (92).

Additional genes, with mutations involving only one or two cases, are reported and listed in Table 2.1. Along with those discussed above, these genes are not frequent players in the development of pancreatic cancer but may be important in the

Table 2.1 Gene mutational frequencies in pancreatic cancer

	Gene	Sporadic	References
CIN	K-ras	95%	12, 13
	CDKN2A	98% ^a	15, 19, 20
	TP53	70%	33
	SMAD4	45%	23, 101
	NCOA3	37–44%	36, 37
	RB1	<6% ^b	40
	ERBB2	<27%	67, 70, 71
	AKT2	11–20%	76, 77
	CCNE1	<6% ^b	2
	DCC	6%	25, 82
	STK11	5%	85
	MYB	4–6%	2, 80
	MAP2K4	2–4%	87, 88, 101
	EGFR	0–4%	90, 91
	CCND1	0–25%	2, 20, 40
	ACVR1B	2%	101, 102
	BRCA2	7% ^c	56, 58
	FANCC	2%	61, 62
	FANCG	1%	61, 62
	GUCY2F	3%	103
	NTRK3	2%	103
	FBXW7	1%	2
	ALK5	1%	21
	TGFBR2	1%	21
MIN	MLH1	100%	104
	TGFBR2	100%	21
	ACVR2A	100%	100
	BRAF	66%	2

^a14% inactivated through promoter methylation. ^bMaximum estimate based upon immunohistochemistry data. ^cNearly all BRCA2 mutations are inherited, even in unselected cases.

development of individual tumors. The ability to characterize the entire background mutational signature of individual tumors will help to characterize the role each of these plays in pancreatic cancer.

3 Microsatellite Instability

MIN tumors (~3% of pancreatic neoplasms) classically have near-diploid genomes exhibiting very few aberrant chromosomes or altered chromosome ploidy. Instead, MIN tumors have replication errors in primary sequences, especially simple repetitive sequences known as microsatellites (93). Mutations or transcriptional silencing in either MSH2 (2p21), MLH1 (3p22.3), or MSH6 (2p16) have been shown to

cause this “replication error-positive” phenotype, with the highest prevalence occurring in colorectal cancers (94–98). Defects in the MSH2 (31%) and MLH1 (33%) are responsible for the majority of HNPCC cases with mismatch repair gene deficiencies (99).

Due to the underlying type of genomic instability, frequent insertions and/or deletions in large mononucleotide tracts (usually 8 or more in length) contained within gene coding sequences are often seen. TGFBR2 (3p24.1) and ACVR2A (2q22.3-23.1) are two such genes that were found to be mutated in all MSI pancreatic tumors examined (3/3) (21, 100).

Providing further evidence that MSI and CIN tumors evolve through distinct mechanisms, MSI pancreatic tumors are K-ras wild-type (51). Activating mutations in BRAF (7q34, a serine/threonine kinase immediately downstream of K-ras signaling) occur in 33% (3/9) of K-ras wild-type tumors and may complement the lack of K-ras mutations. Intriguingly, these mutations (V599E) were not located within a mononucleotide track, even though two of the samples were known to have microsatellite instability. Exactly why BRAF mutations (versus more traditional K-ras alterations) are selected for in this context remains unknown. Examination of K-ras-mutant tumors failed to identify co-existent BRAF mutations, suggesting they may be mutually exclusive alterations in tumors.

4 Negative Observations in Pancreatic Cancer

Negative Observations in Genetic Oncology (www.path.jhu.edu/NOGO) and the Catalogue Of Somatic Mutations In Cancer (www.sanger.ac.uk/genetics/CGP/cosmic) websites detail negative mutational data that can be used by researchers to identify genes that have (or have not) already been investigated for somatic alterations in specific tissues. Such efforts may help maximize the efficiency of mutation detection screens by decreasing the time and cost spent on redundant investigations.

5 Future Perspectives

Cancers of the pancreas, as well as most other types of neoplasms, are very complex genetically. The ability to characterize all of the genetic alterations in an individual tumor remains theoretically possible, but in reality, unattainable due to the high economic and time commitment costs required using current technologies. Significant strides have been made in chip-based technologies that now permit highly detailed maps of gains and losses across an entire genome to be made, including the identification of novel amplifications and homozygous deletions previously missed due to technical resolution. Such studies now permit a more focused and efficient approach for identifying and characterizing the frequencies of mutational targets in larger panels of pancreatic tumors. Without a single-nucleotide-level

resolution, however, arrays will ultimately prove inadequate to describe the mutational background signature of individual tumors. Whole-genome sequencing may one day help catalogue the entire spectrum and characteristics of somatic and germline alterations and enable investigators to fully understand the genetic aspects of cancer.

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