Molecular Diagnosis of Pancreas Carcinoma

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Cellular protooncogenes, tumor suppressor genes (antioncogenes), and DNA mismatch repair mutators are generally the key molecular genetic biomarkers undergoing alterations during carcinogenesis, i.e., activation of oncogenes, inactivation of tumor suppressors, and DNA mismatch repair gene defects are essential events in cancer causation. In pancreas cancer, high incidence of oncogene *K-ras* point mutations at the codon 12th is associated with premalignant and malignant transformation. Mutation in *p53* tumor suppressor is also detected in pancreas adenocarcinoma. Concurrent loss of *p53* and *K-ras* function may contribute to the clinical aggressiveness of pancreas cancer. Microsatellite instability and DNA mismatch repair defects may represent new mutator phenotype for pancreas carcinogen-

esis. Mutation of cell cycle regulators, such as inhibitor of CDK4 or *p16* tumor suppressor gene, is a new molecular event in pancreas cancer. Mutation of cyclin-dependent kinases also may be involved in pancreas carcinogenesis. Loss or mutation of a new candidate tumor suppressor, *DPC4* (deleted in pancreas carcinoma locus 4), is reported in pancreas cancer. The protein products of these gene mutations are potential tumor antigens, thus genotype expression can be detected by phenotype. Most of these emerging molecular genetic biomarkers are associated with regulation of cell growth and recognition, as well as gene expression, and may offer new insight into the cellular precursors to and genesis of pancreas cancer. *J. Clin. Lab. Anal.* 11: 225–231, 1997. © 1997 Wiley-Liss, Inc.

Key words: oncogene; tumor suppressor; cell cycle regulator; microsatellite instability; *K-ras*; *p53*; *p16*; *DPC4*

INTRODUCTION: A POOR PROGNOSIS

Cancer of the pancreas is still the most dismal malignant disease today. The mortality and survival rates have not changed during the past decades. Statistically, no one has survived the disease after being diagnosed (Table 1). One major problem is the lack of early detection modality, despite the effort spent in understanding the biology of pancreas carcinoma.

One potential progress is cellular and molecular analysis of pancreas cancer, which includes pancreas or pancreas cancer-associated antigens (POA, PCAA, PaA, PAA, CAMPAS-PX), mucins and glycoconjugates (MUC1, MUC4, lectins, blood group substances), monoclonal antibodies and epitopes (CA19-9, DUPAN-2, SPAN-1, CA50, CA242), cell growth and recognition (oncogenes, tumor suppressor genes, ICAM-1, intermediate filaments, cytokeratins, extracellular matrix molecules, growth factors and receptors EGF, TGF- β , cripto, HGF/met, amphiregulin, angiogenin, cell cycle regulators, candidate tumor suppressor genes), and gene expression (microsatellite instability, chromosome aberrations, DNA mismatch repairing gene defects) (1). The first three groups

may be classified as the traditional topics, whereas the last two as emerging subjects with increasingly important implications.

In a classic report published in 1956, the late Dr. Ernest Witebsky established some fundamental basis for biological analysis of cancer in general and pancreas cancer in particular (2). For example, three important markers, α -fetoprotein, carcinoembryonic antigen, and prostate specific antigen were discovered in 1963, 1965, and 1979, respectively (3–5). In a series of reports published in the 1970s, pancreas oncofetal antigen was noted as the first potential marker of pancreas carcinoma, although its biochemical identity remained totally obscure (6–8). In 1981, the first marker for pancreas cancer

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TABLE 1. Cancer of the Pancreas

New cases:	26,000 (U.S. 1996) and 185,000 (worldwide 1985)
Mortality/incidence:	0.98
5-year survival rate:	1.3%
Median survival:	4.1 months
High risks:	New Zealand Maoris, African Americans, native Hawaiians
Etiology, pathogenesis, and risk factors	
	Cigarette smoking, alcohol consumption
	Industrial carcinogens: chemicals, solvent, gasoline
	Dietary carcinogens: fat, meat
	Medical conditions: diabetes, chronic pancreatitis, gastrectomy
	Genetic alterations
	Inherited predisposition and familial aggregation

in biochemically purified form, pancreas cancer-associated antigen, or PCAA, was reported from this laboratory, along with the first quantitative ELISA primarily designed for serodiagnosis of pancreas cancer (9). Historical perspectives in the development of pancreas tumor-associated antigens and its clinical use had been reviewed previously (10,11). Since 1981, various biochemical and cellular markers have been reported for pancreas cancer, with essentially the same degree of diagnostic accuracy as that of PCAA: ~70%. It is not surprising as PCAA would contain most epitopes of the antigenic moieties of these markers, such as CA19-9, CA50, SPAN-1, DUPAN-2, and sialyl SEA-1 (12–16). It is possible that PCAA also may be related to mucins such as MUC1 (16,17).

With little progress on biochemical and cellular markers of pancreas cancer useful in detecting small or early stage pancreatic tumors, a new approach to the analysis of pancreas carcinoma utilizing molecular genetic techniques has been extensively investigated in recent years.

CELL GROWTH, RECOGNITION, AND GENE EXPRESSION

It is well recognized that carcinogenesis requires several genetic mutations, or “hits,” which are commonly associated with protooncogene inactivation and tumor suppressor gene mutations or deletions, i.e., carcinogenesis is the result of an imbalance of the functions of oncogenes (normal counterpart are protooncogenes) and antioncogenes (18–22). Cells are constantly in the processes of growth, differentiation, and apoptosis. The uncontrolled growth of malignant cells reflects genetic damage to or defect in these fundamental regulatory processes. DNA replication and repair, gene expression, and chromosome recombination are important to the integrity of the cells. Mutations are produced by changes in the base sequence of DNA, which may include the substitution of one base pair for another, the deletion of one or more base pairs, and the insertion of one or more base pairs. If mutations happen to inactivate tumor suppressor genes and activate oncogenes, it may cause cells to grow out of control and thus produce cancer.

Oncogene K-ras

For pancreas carcinoma, the most well known gene inactivation is that involving c-K(Kirsten)-*ras* gene (23–26). *K-ras* gene belongs to the family of p21-*ras* genes (*K-ras*, *H-ras*, and *N-ras*) that code for G-proteins (189 amino acids), which are localized to the inner surface of the plasma membrane and essential for intracellular signaling and thereby cellular proliferation (24,25,27). *Ras* functions as a molecular switch in signal transduction and alternates between an inactive GDP-bound state and an active GTP-bound conformation. *Ras* gene mutations generally “lock” the protein in a constitutively active GTP-bound state and are found disproportionately high in pancreas carcinoma. Point mutations mostly occur at the codon 12th, some at the 13th, and a few at the 61st. The most common mutations are the transition of nucleotides involving guanine to adenine, guanine to cytosine, and guanine to thymine, resulting in wild-type glycine to aspartic acid, arginine, and valine, respectively.

One important issue of *K-ras* mutations and early detection of pancreas carcinoma regards its potential use in cytology and extracellular fluid examination (28–33). Cytologic diagnosis of pancreatic carcinoma is difficult, especially in distinguishing benign atypia from well-differentiated adenocarcinoma. Combination with *K-ras* analysis is a significant development (Table 2). Additionally, *K-ras* mutation is reported in hyperplastic lesions and in situ adenocarcinoma of the pancreas (34). *K-ras* oncogene activation is seen frequently in mucous cell hyperplasia associated with chronic pancreatitis (35). These conditions are commonly viewed as premalignancy of the pancreas. Therefore, *K-ras* mutations can represent a molecular marker for early detection of pancreas carcinoma. Additionally, recombinant mutant *K-ras* proteins can induce T-cell immunity, potentially providing a new avenue for immunotherapy of pancreas cancer (36,37).

Tumor Suppressor Gene p53

p53 is a well-characterized tumor suppressor whose inactivation leads to unconstrained cell proliferation and malig-

TABLE 2. K-ras Mutations in Aspirates of Pancreatic Juice for Detection of Pancreas Carcinoma

Material or result	Reference
Use of aspirates of pancreatic masses	28
Aspirate material from patients with pancreas carcinoma showed mutations whereas those with chronic pancreatitis showed no mutations	29
Use of pancreatic juice and peripheral blood	30
No false positives	31
Increase in diagnostic accuracy of cytology	32
Use of routine cytology preparation (obtaining tumor cells by microdissection)	33

nancy (38,39). Almost 50% of all tumors are found to be associated with a mutated *p53*, i.e., because the core domain of *p53* (amino acids 102–292) exhibits its tumor suppressor function. *p53* binds DNA and turns transcription on and off for many other genes. It regulates entry into the S phase of cell cycle (38,40). It responds when DNA is damaged, either allowing time for the damage to be repaired or helping to put cells to death via apoptosis (39–41).

Although the incidence of mutations of *p53* is similar between pancreas carcinoma and other human tumors, the occurrence of small (1–2 basepairs) microdeletions in pancreas carcinoma is significantly greater than in common types of human tumors (42), suggesting that *p53* inactivation may be more involved in pancreas carcinoma than has been generally perceived. Furthermore, both *K-ras* and *p53* mutations are found in ductal carcinomas of the pancreas and none in nonductal tumors, indicating that mutated *K-ras* and *p53* genes can cooperate in the establishment of ductal pancreatic carcinomas (43,44). Also, *p53* mutations may occur more frequently in metastatic lesions than in primary tumor.

Preneoplastic *p53* mutation has been reported in other cancer. For example, *p53* mutations are detected in nonmalignant gastric mucosa with intestine metaplasia that may represent precursor lesions of gastric cancer (45). Similar reports are unavailable for pancreas cancer.

Inhibitors of Cyclin-dependent Kinases

In the spring of 1994, evidence was reported that the gene for a newly identified inhibitor of the cycle that underlies cell division, a protein with the name p16, may be an important tumor suppressor (46–49). The *p16* gene is located at the segment p21 of human chromosome 9. Earlier work in late 1993 had identified p16 as an inhibitor of one of the key protein kinases, cyclin-dependent kinase (CDK) that drives the cell division cycle, i.e., p16 acts on Rb to form a negative feedback loop that regulates the ability of Rb to prevent cell proliferation (50). Cell cycle progression is controlled by protein complexes composed of cyclins acting as a regulatory molecule and CDK as an enzyme, and cyclin-dependent kinase inhibitors (CKI) (40,41,50,51). For example, G1S phase is controlled by the CDK4, which is activated by cyclin D, which in turn is controlled by the phosphorylation state of Rb protein. p16 inhibits CDK4 by binding in competition with cyclin D. Multiple cyclins CDK and CKI have been isolated and characterized along with their expression during cell cycle progression. There are eight CDK, nine cyclins, and two families of CKI (Table 3). The p16 family binds catalytic CDK subunit and cyclin subunit, whereas the p21 family interacts with CDK-cyclin complexes (52).

The current model of cell cycle control is that the transitions between cell cycle phases are regulated by “checkpoints.” Cell commits at the restriction point to another round of DNA replications, which are positively and negatively controlled

TABLE 3. Cell Cycle Control Components

Key enzymes:	Cyclin-dependent kinases (CDK) CDK 1, 2, 3, 4, 5, 6, 7, 8
Regulatory subunits:	Cyclins Cyclin A, B, C, D, E, F, G, H, I
Cyclin-dependent kinase inhibitors (CKI)	
	Nine mammalian CKI in two families
	p21, p27, p20, p28, p57
	p16(MTS1), p15(MTS2), p18, p19

by internal and external signals as well as the cyclins, CDK and CKI. Many “checkpoints” are deregulated in oncogenesis. The regulatory function of CDK and cyclins is achieved by phosphorylation of fundamental elements in cell cycle transition. Among the CDK inhibitor genes identified so far, *p16*, *p21*, and *p27* have been investigated more extensively than others. Unlike *p21* and *p27*, *p16* is a selective competitive CDK (CDK4 and CDK6) inhibitor (40,41,51,52). Biochemically, *p16* gene encodes a 148 amino acid-protein of Mr 16 kDa (50).

p16 functions as a tumor suppressor and is associated with pancreas carcinoma and other tumors (46–49,53,54). It is frequently inactivated in tumors, typically through deletion, rearrangement, or mutations. As many as 70% of tumor cell lines from various tumor types contain alterations at the *p16* locus. It is altered at a lower, but significant frequency, 10–20% in primary tumors (48,52). In pancreas cancer, altered *p16* is detected in 30–80% of primary pancreas adenocarcinoma (55). Further, the types of mutations in pancreas cancer are different from those of melanoma and lung cancer and may represent the effect of different carcinogens.

p16 is generally accepted to be the same as *MTS1* and also called *CDKN2* or *INKA4* gene (56). It also appears that *p16* is the same as *MLM*, the locus for one type of familial predisposition to melanoma (48,57).

Concurrent inactivations of *p16* gene and *p53* gene have been reported in primary pancreas carcinoma as well as primary and metastatic pancreas tumor cell lines, suggesting that abnormal regulation of *p16* and *p53* and of cyclin-dependent kinases, such as CDK4 and CDK2, may play an important role in the biology of pancreas carcinoma (53). *p16* also is called *DPC3*.

Candidate Tumor Suppressor Genes

One of the most exciting discoveries in pancreas cancer research during the past several years is the most recent report of a new candidate tumor suppressor gene, named *DPC4* (for deleted in pancreas carcinoma, locus 4), which is located at chromosome 18q21.1 and a short distance away from *DCC* (deleted in colorectal carcinoma) (58,59). Other *DPC* also have been reported.

DPC4 is identified by using a relatively new technique of representation difference analysis that was developed in late

1993 (60). The *DPC4* gene carries 11 exons and 552 amino acid coding sequence (58). *DPC4* protein resembles a fruit fly protein Mad that is involved in midgut morphogenesis, related to TGF- β , and may inhibit cell growth. The *DPC4* gene is deleted or mutated in pancreas carcinoma, and possibly others, such as colon, bladder, and biliary tumors (58,59,61). The characteristics and possible function of *DPC4* are summarized in Table 4.

At the present time, there are three loci known to be homozygously deleted in pancreas carcinoma: *DPC4* as just described, *DPC3* (or *p16*) at 9p21 (53), and *DPC1/2* (or *BRCA2*) at 13q12 (62). The frequencies of the deletions are *DPC4* and *DPC3* at 40% each, and *DPC1/2* is rare (59,62). Homozygous deletions at any of the three loci (or essentially *DPC4* and *DPC3*) were ~65%.

When not homozygously deleted, mutations at *DPC4* nucleotide sequence are commonly detected. For example, in 27 pancreas carcinomas that exhibited nonhomozygous deletion, six mutations were identified from sequencing all 11 *DPC4* exons, including a nonsense mutation in exon 8 and exon 9, a splice donor site mutation after exon 10, a nonsense mutation at exon 11, a missense mutation at exon 11, and an 8-bp-frameshift microdeletion (58). Therefore, because of its frequent deletion and mutation, *DPC4* or 18q21.1 indeed is a sensitive hotspot of pancreas carcinoma.

Microsatellite Instability and Mismatch Repair Defect

Mini- or micro-satellite instability, DNA mismatch repair defect, and its association with cancer has received extensive attention lately (63–66), since an instability of microsatellite (MIN) or defect in DNA repair can result in cancer. Instability within the microsatellite was described initially in colorectal cancer. Microsatellites are stretches of DNA found all over the genome, where identical units up to four nucleotides are repeated many times. Functionally, many microsatellites bind to particular transcription factors (63,64). MIN and *K-ras* mutations have been reported with pancreas carcinoma, but also with pancreatitis (67–69). Interesting to note is the detection of MIN and *K-ras* mutations in pancreatic juice DNA. Significantly, all of the cancer patients who had *K-ras* mutations in their pancreatic juice also had evidence of MIN, suggesting that MIN may be associated with the development of *K-ras* mutation. Like other cancers, pan-

creas cancer may involve the DNA mismatch repair defects (65,70,71). One molecular marker of HNPCC, *hMSH2* mutant gene is implicated in pancreas carcinomas, though it may appear rarely (72).

PREDISPOSITION TO PANCREAS CANCER

Some cancers, although perhaps only a small fraction of each, are of hereditary nature (72). Recent evidence of the genetic predisposition to pancreas carcinoma remains to be confirmed from a large number of families and patients (57,73,74). Two interesting recent reports are noted herewith, both relating to *p16* mutations. In a brief note, a kindred with an increased risk of pancreas cancer, melanoma, and possibly additional types of tumor was documented (73). In another, derived from 19 melanoma-prone families, seven cases of pancreas cancer were found in four different families who had *p16* mutations, three of which had two cases each. Overall, four of the 10 kindreds with *p16* mutation had at least one family member each with pancreas cancer, as compared with none of the nine kindreds with *p16* wild-type gene (74).

CONCLUSIONS

The involvement of genetic markers and their molecular alterations in pancreas carcinoma is now well established (75,76). It is noted that these are the factors mostly associated with regulations of gene expression and with cell growth and recognition. As summarized in Table 5, multiple alterations of cancer genes are involved in pancreas carcinoma, which can be the result of several genetic "hits." *K-ras* mutations, mostly point mutation confined to codon 12, are found in 90% of pancreas carcinoma, significantly greater than other carcinomas where mutation frequencies usually are in the range of 50%. *K-ras* mutations are identified in hyperplastic lesions or premalignancy of the pancreas. Detection in aspirates and pancreas juice represents potentially an early diagnosis of pancreas carcinoma. The most commonly mutated tumor suppressor gene *p53* is also detected in pancreas cancer. As an inhibitor of CDK4, *p16* gene is also called *DPC3*, *CDK4I*, *MTS1*, *CDKN2*. The *p16* gene is inactivated in 80% of pancreas carcinomas, most of which are nucleotide sequence changes associated with the loss of wild-type allele, 40% of which are with homozygous deletions of both copies of the gene. Inactivation of *p16* also has been reported in a few families with predisposition of pancreas cancer and melanoma. Inactivations of *DPC4*, the most recently reported *DPC*, are found in 60% of pancreas carcinomas with 40% homozygous deletions of both copies of the gene and 20% nucleotide alterations at *DPC4* locus. The *DPC1/2* or *BRCA2* gene is a

TABLE 4. Candidate Tumor Suppressor in Pancreas Carcinoma: *DPC4* Gene

Deleted in pancreas carcinoma, locus 4, at chromosome 18q21.1, different from <i>DCC</i>
Contains 11 exons and 552 amino acid coding sequence
Deletion or mutations in pancreas carcinoma, and possibly others
<i>DPC4</i> protein resembles a fruit fly protein Mad (involved in midgut morphogenesis), and relates to TGF- β
May inhibit cell growth by TGF- β induced growth inhibition

TABLE 5. Multiple Alterations of Pancreas Cancer Genes

Activation of oncogenes (<i>k-ras</i>)
Inactivation of tumor suppressors (<i>p53</i> , <i>p16</i> , <i>DPC4</i> , <i>DPC1/2</i>)
DNA mismatch pair defect and microsatellite instability

neighbor of *Rb* at 13q12 (77). Although its deletion is rare in one report, but common in another (59,77), LOH is found in ~30% of pancreas carcinomas. LOH of *p21* and *p27*, two other CDK inhibitors, is found in some pancreas carcinomas (78). Finally, the mutation phenotype in DNA mismatch repair system is typified by microsatellite instability. Data from one group revealed six out of nine cases examined showed MIN, whereas from another showed only one out of 200 cases with MIN (67,75).

FUTURE PERSPECTIVE

Although this review primarily deals with molecular diagnostics, the importance of serodiagnosis cannot be overemphasized. For example, recent report has indicated the secretory nature of *BRCA1* gene product (79). This secretory protein will be amiable for serodiagnosis. Polyclonal and monoclonal antibodies are available directed against the first 20 N-terminal amino acids, the last 20 C-terminal amino acids, and other fragments of *BRCA1* protein (79,80). Antibodies are available to almost all oncogene and antioncogene proteins that have been discussed. The protein products of mutations are potential tumor antigens (81). Many products of the pancreas carcinoma genes also can be expected in the circulation and detectable by simple immunochemical means. At the present time, genetic techniques are far more complex than immunologic techniques. Most of the molecular genetic markers can be detected in theory by simpler immunochemical techniques, i.e., genotype can be detected by phenotype (82). New procedures are available to produce the next generation of antibodies of high specificity and affinity, e.g., phase display process (83,84). Therefore, in the not too distant future, genetics and immunology will likely merge to make molecular diagnosis and laboratory analysis of pancreas carcinoma, and other cancers, a simpler and more effective science.

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