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MOLECULAR PATHOBIOLOGY OF PANCREATIC ADENOCARCINOMA

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1. ABSTRACT

Pancreatic adenocarcinoma is a major cause of cancer death in the United States. Most cases are sporadic and are discovered at late stage when they are not curable by surgery. Information about the molecular biology of pancreatic adenocarcinoma has increased significantly in the last five years with the identification of alterations in the K-ras proto-oncogene and the $p16^{INK4a}$, p53, FHIT, and DPC4 tumor suppressor genes in a high percentage of tumors. Pancreatic adenocarcinoma is not homogeneous genetically, however, and other genes are clearly involved in some sporadic and heritable tumors. This review summarizes recent data relating to the molecular biology of pancreatic adenocarcinoma with emphasis on features which may be of clinical significance for diagnosis and/or therapy. Molecular genetic alterations that disturb cell cycle regulation in tumor cells can affect their response to chemotherapeutic agents and radiation and many of these genes are targeted in pancreatic adenocarcinoma. Knowledge of these genetic alterations in individual tumors may allow selection of optimal therapeutic strategies for individual patients. Furthermore, molecular detection of oncogene and tumor suppressor gene mutations may find application as screening tests for pancreatic adenocarcinoma at least in high risk populations. Biological therapy aimed at specific oncogenes and tumor suppressor gene replacement therapy protocols for pancreatic adenocarcinoma are beginning and may offer promise in the future.

2. INTRODUCTION

Pancreatic cancer is the fifth leading cause of cancer death in the United States. Based on population data and cancer incidence rates collected by the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program, it is estimated that 29,000 new cases of cancer of the pancreas will be diagnosed in the United States in 1998, and 28,900 cancer deaths will result from pancreatic cancer (1). In 50 percent of cases, pancreatic cancer will be locally advanced at diagnosis precluding cure by surgical means. The overall five year survival for pancreatic cancer is less than 5 percent. Until

Table 1. Heritable predisposition to pancreatic adenocarcinoma; AD = autosomal dominant; AR = autosomal recessive

Syndrome	Pattern of Inheritance	Relative Risk	Gene (Locus)
Familial Clustering	AD (subset)	variable	Unknown
BRCA2 (males)	AD	1.7	BRCA2 (13q12)
Hereditary Pancreatitis	AD	unknown	Unknown (7q35)
Ataxia Telangiectasia	AR	unknown	ATM (11q22-23)
HNPCC	AD	unknown	hMSH2 (2p15-16) hMLH1 (3p21-23)
FAMMM (subset)	AD	13.0	$p16^{INK4a}$ (9p21)
Peutz-Jegher's syndrome	AD	100.0	STK11 (19p13.3)

recently, therapeutic approaches including chemotherapy and radiation have been unrewarding. The recent application of novel chemoradiation protocols has shown encouraging activity in locally advanced pancreatic adenocarcinoma with acceptable toxicity (2). These hybrid protocols may permit useful therapy for patients with this deadly disease in the near future.

Identified predisposing factors for the development of pancreatic adenocarcinoma are advanced age and cigarette smoking. The majority of cases occur in the seventh and eight decades and are infrequent before age forty. Rare cases have been described in children, however. Cigarette smoking in college has been estimated to increase the risk of developing pancreatic adenocarcinoma later in life by 2.6 fold. The association with smoking is dependent on the duration and number of cigarettes smoked but the possible role of passive smoking has not been addressed (3).

The vast majority of cases of pancreatic cancer are adenocarcinomas arising from the pancreatic ducts. The typical histomorphology of ductal adenocarcinoma is one of small neoplastic glands surrounded by an intense non-neoplastic stromal reaction together with inflammatory cells. Rare tumors arise from pancreatic acinar tissue or from neuroendocrine cells in the islets of Langerhans. These tumors tend to have a much different biologic behavior than usual ductal pancreatic adenocarcinoma and are not considered further here. Cystic tumors of the pancreas (both mucinous and serous) also occur and in their pure form have a substantially better prognosis than ductal carcinomas. These cystic lesions are also excluded from consideration here.

Accompanying some ductal adenocarcinomas are precursor lesions ranging from flat mucinous lesions to atypical papillary intraductal lesions. Many of these precursor lesions are absent or have been overgrown by the invasive tumor. In some cases, these precursor lesions have been shown to harbor genetic mutations (K-ras gene mutations are most common) suggesting a multi-step progression to malignancy analogous to adenomatous polyps of the colon (4-6).

Although a full understanding of the biology of pancreatic adenocarcinoma is currently out of reach, its genetic composition is being unraveled at a steady pace. The study of cases that appear to have a hereditary basis has permitted the identification of genetic loci through linkage studies that may influence tumorigenesis and tumor progression. By analogy to other tumors, some of these oncogenes and tumor suppressor genes are likely involved in development of sporadic cases of pancreatic adenocarcinoma. Other previously characterized oncogenes and tumor suppressor genes appear to play an important role in sporadic tumors as well. It is increasingly clear that molecular genetic alterations in tumors are a significant determining factor for response to specific therapeutic protocols. Identification of specific gene defects in individual tumors may permit the choice of the most beneficial treatment modality for each patient. In this article we review the literature on the genetics of pancreatic adenocarcinoma and discuss the possible implications for genetic screening [which might permit diagnosis at an earlier stage] as well as the impact of gene alterations on novel treatment modalities.

3. HERITABLE PANCREATIC CANCER

Heritable pancreatic adenocarcinomas account for 3-10% of all pancreatic adenocarcinomas 3-10%. Heritable pancreatic adenocarcinoma can be divided into three subgroups (1) defined genetic syndromes associated with pancreatic adenocarcinoma; (2) clustering of pancreatic adenocarcinoma in a family not associated with a specific genetic syndrome; and (3) pancreatic adenocarcinoma in primary relatives of patients with non-pancreatic malignancies (3). While these groups can be defined operationally, there is potential overlap in that some family clusters may represent undiagnosed genetic syndromes and the association of some pancreatic adenocarcinomas with breast and ovarian cancer appears to be through the BRCA2 gene. Heritable cases are potentially helpful in identifying specific genes involved in pancreatic carcinogenesis. These patients may also be a useful study group for assessing new screening methods which might then be applied to the wider population.

3.1.Genetic syndromes predisposing to pancreatic adenocarcinoma

A number of well characterized genetic syndromes are associated with a significantly increased risk

of developing pancreatic adenocarcinoma. Some of these, such as hereditary pancreatitis, are organ specific. Other syndromes, such as ataxia telangiectasia and Peutz-Jegher's Syndrome, are associated with the development of tumors at many sites in addition to the pancreas. In still other syndromes, such as hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome II) and familial atypical mole-malignant melanoma syndrome (FAMMM), pancreatic tumors are much less frequent than the characteristic tumors which define these syndromes (colon cancer and melanoma respectively). Although pancreatic adenocarcinoma is a relatively rare in some of these genetic diseases, it's lethality makes it a clinically important complication of these syndromes. The precise risk of developing pancreatic adenocarcinoma is difficult to define in most of these syndromes but available relative risk data is summarized in table 1.

3.1.1. Hereditary pancreatitis

Hereditary pancreatitis is an autosomal dominant disorder characterized by recurrent, severe episodes of pancreatitis which is linked to a gene at 7q35 (7). Family studies of this condition are consistent with an autosomal dominant disorder with 80% penetrance. Predisposition to pancreatic adenocarcinoma may be due to chronic injury and regeneration by analogy to patients with nonhereditary chronic pancreatitis. Tumors usually develop later in life with an age distribution similar to that of sporadic tumors. Some caution must be used in making this analogy, however, since chronic pancreatitis is associated with some of the same environmental risk factors (ethanol and tobacco) as is pancreatic adenocarcinoma.

3.1.2. Ataxia telangiectasia

Ataxia telangiectasia is an autosomal recessive disorder. The gene responsible for the disorder resides on chromosome 11q22-23 and encodes for a protein similar to phosphatidylinositol 3' kinase which is involved in signal transduction, meiotic recombination and cell cycle control. Loss of one normal allele of this gene results in cells that are deficient in DNA repair and are prone to accumulate gene mutations. Patients are thus predisposed to many different types of cancers including leukemias, breast cancer, gastric cancers and pancreatic adenocarcinomas.

3.1.3. FAMMM syndrome

The FAMMM syndrome (familial atypical mole-malignant melanoma syndrome) is inherited in an autosomal dominant fashion and predisposes to the development of multiple malignant melanomas. This syndrome appears to be genetically heterogeneous with some but not all kindreds having germline mutations in the $p16^{INK4a}$ gene. $p16^{INK4a}$ is a negative regulator of cell cycle progression at the G1S restriction point (see below) and is frequently altered in sporadic pancreatic adenocarcinomas. Goldstein *et al.* found that the risk of developing pancreatic adenocarcinoma in FAMMM kindreds was increased by a factor of 13 in the kindreds with $p16^{INK4a}$ mutations (8). No excess cases of pancreatic adenocarcinoma occurred in the FAMMM kindreds with wild-type $p16^{INK4a}$, however. Interestingly, the risk of developing invasive malignant melanoma did not differ significantly between the two groups with a 75x relative risk of developing melanoma in kindreds with the germline $p16^{INK4a}$ mutations versus 38x in the kindreds without $p16^{INK4a}$ mutations. These data provide compelling evidence of a direct role for $p16^{INK4a}$ gene alterations in the genesis of hereditary pancreatic adenocarcinoma.

3.1.4. Hereditary nonpolyposis colorectal cancer

HNPCC (hereditary nonpolyposis colorectal cancer) is inherited as an autosomal dominant trait predisposing affected individuals to colon cancer. Two genes which can produce this syndrome map to chromosome 2p and 3p respectively (9). In addition, affected individuals are at increased risk of cancers of breast, endometrium, ovary and pancreas, documented by pedigree studies (10). HNPCC is associated with replication errors and microsatellite instability resulting from an abnormality in DNA mismatch repair (3,11,12) .

3.1.5. Peutz-Jegher's Syndrome

Peutz-Jegher's syndrome is an autosomal dominant disorder characterized by multiple hamartomatous polyps of the gastrointestinal tract and mucocutaneous melanin deposits. The gene resulting in this disease, STK11, has been recently mapped but has not been fully characterized (13,14). This syndrome is associated with a significantly increased risk for developing tumors of the gastrointestinal tract at multiple sites. In a cohort of 31 patients followed by Giardiello *et al.* for 13 years, 15 developed some type of gastrointestinal cancer (48%). Four of these were pancreatic adenocarcinomas corresponding to a 100x relative risk compared to the general population estimated from this small group of patients (15).

3.2. Familial Pancreatic Cancer

Family clusters of pancreatic adenocarcinoma have been extensively reviewed by Lynch *et al* (16). Families in which three generations have been afflicted with pancreatic adenocarcinoma have been described, some in association with non-insulin dependent diabetes mellitus. Families with up to four affected siblings with pancreatic adenocarcinoma have also described and an autosomal dominant mode of transmission suggested. Since different authors have used different definitions of familial pancreatic adenocarcinoma to identify index cases, it is difficult to compare reported data (16). Family studies have shown an age presentation comparable to that of sporadic pancreatic adenocarcinoma with tumors developing relatively late in life. Since pancreatic adenocarcinoma is uncommon, but not rare, some case

Table 2. Oncogenes and Tumor Suppressor Genes Commonly Altered in Pancreatic Adenocarcinoma

Gene	Chromosomal Locus	Alteration	Frequency of Alteration	Reference
K-ras	12p13	Point mutation codon 12,13, or 67	74-100%	20, 21
HER-2/neu	17q11	Overexpression	66-69%	64, 65
$p16^{INK4a}$	9p21	Homozygous deletion point mutation hypermethylation	27-98%	21, 22, 41
$p19^{ARF}$	9p21	Homozygous deletion point mutation	27-82%*	21, 22, 41
$p15^{INK4b}$	9p21	Homozygous deletion	27-48%**	21, 22, 41
p53	17p 13	Point mutation LOH	43-76%	20, 21
DPC4	18q21	Homozygous deletion point mutation LOH	50%	21
FHIT	3p14	Intragenic deletion abnormal splicing homozygous deletion	66-70%	23
Rb1	13q14	Point mutation	0-10%	41, 66, 67

* Calculated from reference 21 assuming that reported $p16^{INK4a}$ gene deletions and point mutations but not promotor hypermethylation result in loss of $p19^{ARF}$. ** Calculated from reference 21 assuming that reported $p16^{INK4a}$ gene deletions but not point mutations and promotor hypermethylation result in loss of $p15^{INK4b}$.

clustering in families may be coincidental. Conversely, since environmental carcinogens clearly predispose to pancreatic adenocarcinoma, some familial clustering may result from shared environmental risk factors rather than from genetic predisposition. Nevertheless, it seems clear that some families have a genetically determined increased risk of pancreatic adenocarcinoma. Such cases have been estimated to account for 3-10% of all cases of pancreatic adenocarcinoma. Some of these families may fit into previously defined genetic syndromes (e.g. HNPCC) (3,16). More uniform definition of familial clusters may aid in better defining the population at risk and ultimately in identifying the genes and/or environmental risk factors involved.

3.3. Pancreatic adenocarcinoma risk in families with other sporadic cancers

The final group of patients with potentially heritable pancreatic adenocarcinoma are those occurring in association with non-pancreatic cancer in the same the family. Of particular interest is the association with ovarian and breast cancers. Tulinius *et al* in their analysis of cancer risk of family members of randomly selected female breast cancer patients found that first degree male relatives had a 1.66 relative risk of developing pancreatic adenocarcinoma (17). This level of risk is orders of magnitude less than in most of the previously discussed genetic syndromes. An increased risk of ovarian cancer was also associated with a family history of pancreatic adenocarcinoma. Kerber and Slattery estimated that up to 4.8% of ovarian cancers were associated with a family history of pancreatic adenocarcinoma (18). Clearly there may be some overlap between these patients and those with defined genetic syndromes (see above). Nevertheless, further analysis of these kindreds may identify as yet undescribed genetic mutations involved in the pathogenesis of pancreatic adenocarcinoma.

4. CHROMOSOMAL ABNORMALITIES IN PANCREATIC ADENOCARCINOMA

Cytogenetic analysis of pancreatic tumors has been useful in identifying specific chromosomal abnormalities although most tumors show complex karyotypes with numerous anomalies. G-banded metaphase spreads have suggested that additions to chromosomes 7 and 20, and loss of chromosome arms 1p and 6q are important in the pathogenesis of pancreatic adenocarcinoma (10). Molecular probes specific for each chromosomal arm have been used to refine genetic mapping and allelic loss studies have been helpful in identifying tumor suppressor gene candidates in pancreatic adenocarcinomas. Comprehensive evaluation of 17 pancreatic adenocarcinomas showed a high frequency of non random allelic loss for chromosome arms 1p (50%), 6p (50%), 6q (50%), 8p (56%), 9p (76%), 10p (50%), 10q (50%), 12p (50%), 12q (67%), 17p (95%), 18q (88%), 21q (61%), and 22q (61%) (10). These allelic loss data correlate with known locations of several tumor suppressor genes. Chromosome 17p is the normal location of the p53 tumor suppressor gene, chromosome 18q is the site of the DPC4 gene (and the DCC gene implicated in colorectal carcinoma) and chromosome 9p is the site of the $p16^{INK4a}$(MTS1) gene. These individual genes and their potential mechanisms of action in the genesis of pancreatic adenocarcinoma are discussed further below.

DNA Ploidy has been investigated by some workers in an effort to select candidates for surgical resection (19). Aneuploid tumors have been reported to be associated with a poorer prognosis than diploid tumors. These data must be interpreted with caution, however, since almost all ductal pancreatic tumors are known to contain multiple genetic abnormalities and are expected to be aneuploid. Ploidy analysis may identify less common, lower grade tumors (e.g. cystadenomas and low grade

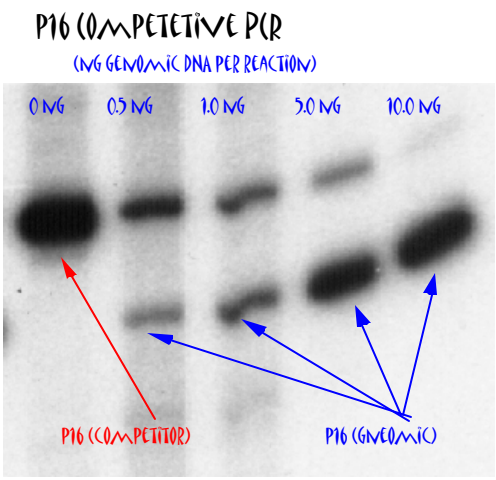

Figure 1A. Autoradiograph of gel electrophoresis of $p16^{INK4a}$ competitive PCR assay after restriction digestion. A constant amount of competitor was added to each reaction with the indicated amount of control genomic DNA added. Note equivalence of band intensities with 1.0 nanogram of control DNA.

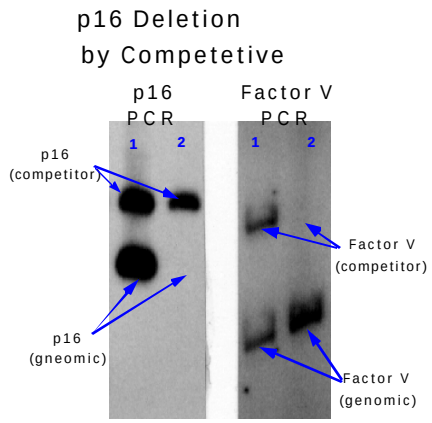

Figure 1B. Autoradiograph of gel electrophoresis of $p16^{INK4a}$ and control (Factor V) competitive PCR assays after restriction digestion. Two tumor samples, 1 & 2, are shown in each PCR assay. Tumor 1 shows no evidence of $p16^{INK4a}$ deletion with equal band intensity ratios for the $p16^{INK4a}$ and control (Factor V) PCR assays. Tumor 2 shows evidence of homozygous $p16^{INK4a}$ gene deletions with complete loss of the signal for genomic $p16^{INK4a}$ with preservation of the control (Factor V) genomic band.

cystadenocarcinomas) which would be expected to have a significantly better prognosis.

5. GENETIC ALTERATIONS IN PANCREATIC ADENOCARCINOMA

The most commonly altered genes in pancreatic adenocarcinoma are: K-ras (74-100%), $p16^{INK4a}$ (up to 98%), p53 (43 to 76%) and DPC4 (53%) (20-24). As shown in table 2 K-ras is a proto-oncogene whereas all the others are tumor suppressor genes. Additional genes have been found to be altered at lower frequency and some as yet uncharacterized genetic lesions may be important in tumorigenesis. Understanding the technical aspects of genetic analysis in these tumors is important in interpreting the significance of reported data and in designing rational diagnostic and therapeutic strategies. Assessment of proto-oncogene mutations such as K-ras is straight forward and reliable. Since only a limited number of genetic changes are expected, these can usually be assessed with good sensitivity. The same is not true for most tumor suppressor gene alterations which may correspond to homozygous deletions or novel point mutations. Methodologies for screening for p53 gene mutations are well established and have good sensitivity both for identifying novel mutations and for detecting mutations in a relatively small percentage of cells in a tissue sample. Unfortunately, detection of homozygous deletion (a common alteration for $p16^{INK4a}$) or promoter mutations (which occur in Rb1) is much more problematic. In particular, deletion analysis is hampered by the presence of normal tissue in biopsy specimens (endothelial cells, fibroblasts) which contribute to background and can lead to false negative results.

For deletion analysis of tumor tissue, it is necessary to quantitatively assess the amount of DNA present in the sample to determine whether homozygous deletion has occurred. Since tumor necrosis and formalin fixation damage DNA to a variable extent in different tumor biopsies, it is necessary to monitor for DNA quality as well. This can be accomplished by employing a synthetic competitive substrate lacking a Kpn I restriction enzyme site present in the native $p16^{INK4a}$ sequence. A constant amount of this competitor is added to PCR reactions containing known amounts of tumor DNA. PCR products made from the two templates can then be distinguished by digestion with Kpn I followed by gel electrophoresis as shown in figure 1A. The ratio of the intensity of the two bands reflects the number of intact copies of the gene present in the sample. This ratio is insensitive to PCR conditions and allows a true estimate of the number of amplifiable gene copies present in a sample. To control for the quality of DNA in different samples, a second competitive PCR assay is performed using identical amounts of sample DNA with primers for a control gene which produce a PCR product of nearly identical size. A decrease in normalized $p16^{INK4a}$ gene copy number of more that 50% is then indicative of homozygous deletion in the tumor sample as shown in figure 1B.

The use of tissue culture cell lines or xenografts from tumors can overcome the difficulties posed by contaminating normal tissue by producing a pure tumor cell population. Unfortunately, these techniques have been shown to select for *in vitro* deletion of the gene of interest in some tumors including pancreatic

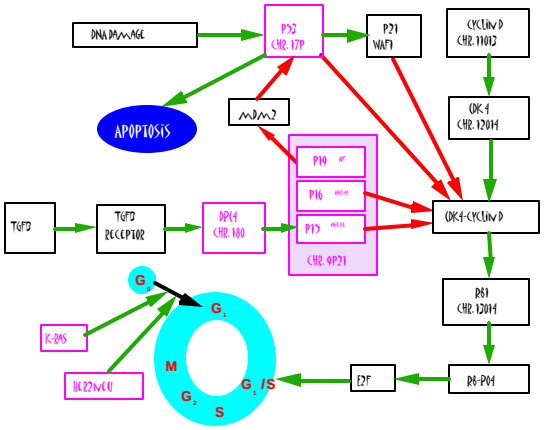

Figure 2. Cells cycle control pathways altered in pancreatic adenocarcinoma. Schematic diagram of gene products controlling entry into the cells cycle (G_0 --> G1) and G1S progression. Genes frequently altered in pancreatic adenocarcinoma are in magenta with their chromosomal location noted. Normal positive and negative regulatory interactions are indicated in green and red respectively. Gene mutation typically results in the loss of a negative regulatory signal (e.g. $p16^{INK4a}$) or the enhancement of a positive signal (e.g. K-ras).

adenocarcinoma and can thereby overestimate the frequency of gene deletions (25). These alternate methodologies clearly have the potential to significantly under or over estimate the frequency of homozygous deletion and these technical limitations must be considered when evaluating the literature.

5.1. K-ras proto-oncogene

The K-ras gene which is located on chromosome 12p13 has been found to be mutated in many precursor lesions associated with pancreatic adenocarcinoma as well as in the vast majority of pancreatic adenocarcinomas. Mutations have been identified in up to 28-81% of dysplasias and noninvasive intraductal lesions (4,5). The differences in frequency of K-ras mutations may be explained by the inclusion of low and high grade dysplasia and adenomas in different studies. These findings are consistent with a model in which K-ras gene mutations are an early and essential step in the development of most pancreatic adenocarcinomas.

The K-ras gene encodes a guanine nucleotide binding protein involved in signal transduction. Abnormal activation of the K-ras oncogene can occur as a result of point mutations at codons 12,13 or 61. Specific amino acid substitutions at these sites then alter it's GDP/GTP binding site and result in retention of GTP (by blocking it hydrolysis to GDP) producing a constituitively active K-ras protein. One of the steps in the normal attachment of K-ras to the cell membrane involves farnesylation of the ras protein by farnesyl transferase. Without this step, the ras protein cannot associate with the cell membrane and so cannot mediate signal transduction. Blockage of farnesylation can then potentially inhibit both normal signal transduction as well as cell transformation by mutant ras proteins. This pathway may have implications for treatment as discussed below.

Hruban *et al.* observed point mutations in 68 of 82 (83%) of surgically resected or biopsied adenocarcinomas of the pancreas using primer mediated, mutant-enriched, polymerase chain reaction-restriction fragment length polymorphism analysis and allele-specific oligonucleotide hybridization (26). 49% of these mutations were guanine to adenine transitions, 39% guanine to thymine transversions, and 12% guanine to cytosine transversions. Of note, the overall prevalence of K-ras mutations in patients with a history of smoking at some point in their lives was 88% versus 68% in patients who never smoked.

5.2. $p16^{INK4a}$ /$p15^{INK4b}$/$p19^{ARF}$ tumor suppressor genes

The INK4 family of cyclin dependent kinase (CDK) inhibitors is comprised of multiple gene products which repress cell cycle progression at G_1S (27-29) (figure 2). Loss of these gene products then favors cell cycle progression at G_1S. $p16^{INK4a}$ (MTS-1, INK4A) was the first member of the INK4 family identified and it is altered in a significant percentage of human tumors (30). The $p16^{INK4a}$ gene product inhibits the interaction of cyclin D with CDK4. This cyclin D-CDK4 complex phosphorylates the retinoblastoma gene product (Rb1) preventing the formation of the E2F-Rb1 complex (figure 2). This phosphorylation step frees E2F to act as a transcription factor with the resultant progression of the cell cycle to S phase. Loss of $p16^{INK4a}$ activity results in the elimination of these inhibitory effects at the level of cyclin D-CDK4 interaction, thereby promoting cell cycle progression (31). $p16^{INK4a}$ deletion is associated with progression in follicular center cell lymphoma to high grade lymphoma and with decreased survival in nonsmall cell lung cancer (32,33).

Loss or decreased activity of $p16^{INK4a}$ can occur through a variety of mechanisms. Many tumors with $p16^{INK4a}$ alteration show evidence of homozygous deletion at this locus. In some tumors point mutations in the $p16^{INK4a}$ coding sequence are more frequent than deletions while in other tumor types, inactivation of the $p16^{INK4a}$ promotor by methylation has been reported without evidence of mutation or deletion of the structural gene (34).

The $p16^{INK4a}$ gene located on chromosome 9p21 is composed of three exons: a 5I region of 126 bp (coding exon 1), a middle region of 307 bp (coding exon 2) a 3 region of 11 bp (coding exon 3). A second INK4 family member is closely linked to $p16^{INK4a}$. The

$p15^{INK4b}$ gene is located 30 kb downstream from the $p16^{INK4a}$ gene and is deleted in most tumors with $p16^{INK4a}$ deletions (35). $p15^{INK4b}$ is upregulated by TGF beta (Fig 2) and inhibits the formation of activated CDK4 in the same manner as $p16^{INK4a}$ (36). Another putative tumor suppressor gene is imbedded in the $p16^{INK4a}$ locus. $p19^{ARF}$ derives in part from an alternate reading frame in the $p16^{INK4a}$ gene. It utilizes the same second exon as $p16^{INK4a}$ with a separate first exon and promotor. $p19^{ARF}$ is therefore inactivated in all tumors with $p16^{INK4a}$ gene deletions and in many but not all tumors with $p16^{INK4a}$ point mutations (37). The biologic significance of the unusual genetic organization of the $p16^{INK4a}$ locus is unclear. Recently, $p19^{ARF}$ has been shown to bind to the mdm2 protein and thereby inhibit its interaction with p53 (38,39). Normally, mdm2 regulates p53 by binding to it and accelerating its degradation and blocking its effects on gene transcription. Overexpression and /or gene amplification of mdm2 in some types of tumors (but not in pancreatic adenocarcinoma) results in functional loss of normal p53 function and a transformed phenotype. Loss of $p19^{ARF}$ then mimics the effects of mdm2 overexpression by permitting mdm2 to freely interact with p53 and down regulating it. Consequently, most homozygous deletions at this locus result in the coordinate loss of three gene products involved in regulating the G_1S checkpoint with potentially synergistic facilitation of cell cycle progression. No other single genetic target has the potential to result in this number of genetic alterations targeting and single cellular pathway.

$p16^{INK4a}$ gene deletions and/or mutations have been found in a significant number of pancreatic cancers but their prevalence varies substantially in different reports (27-82%) (40). Schutte *et al.* suggested that $p16^{INK4a}$ gene alterations are present in virtually all pancreatic carcinomas when promotor inactivation by methylation is considered as well as gene mutation and deletion (41). These investigators found alterations in $p16^{INK4a}$ in 49 of 50 pancreatic carcinoma xenografts and cell lines.

This viewpoint is controversial, however, since other investigators have found substantially lower frequencies of $p16^{INK4a}$ gene alterations (27% and 38%) in primary pancreatic adenocarcinomas (22,42). Huang *et al.* carried out deletion and mutational analyses of $p16^{INK4a}$ in 30 microdissected primary human ductal pancreatic carcinomas from patients who were not subjected to radiotherapy or chemotherapy prior to surgical resection of the tumors, and 18 human pancreatic carcinoma cell lines (22). Single-strand conformation polymorphism and DNA sequence analyses, and PCR-based deletion analyses were performed for mutations and homozygous deletions of the $p16^{INK4a}$ gene respectively. The overall frequency of deletions and mutations was 66% for the cell lines compared to 27% for primary tumors. It is clear from the study of other types of human tumors that the frequency of detectable $p16^{INK4a}$ gene alterations is substantially increased in tumor cell lines as an artifact of *in vitro* culture (43). Observed differences in the frequency of $p16^{INK4a}$ deletion by different investigators is likely methodologic (see above) and that the true frequency of $p16^{INK4a}$ gene deletion in primary pancreatic cancers is uncertain (25).

In addition to gene inactivation by intragenic mutation and gene deletion, transcriptional inactivation of the $p16^{INK4a}$ gene secondary to hypermethylation of its 5'-CpG island was investigated by methylation-specific PCR. In one study using xenografts and cell lines, seven of nine pancreatic adenocarcinomas without demonstrable $p16^{INK4a}$ mutations showed evidence of hypermethylation. Conversely, nine tumors with identified $p16^{INK4a}$ mutations did not show evidence of hypermethylation (41).

5.3. p53 tumor suppressor gene

The human p53 gene, located on chromosome 17p13, is a tumor suppressor gene that plays a central role in genetic stability and cell survival. p53 gene mutations are common in pancreatic adenocarcinoma (43-76%). Germline p53 gene mutations (Li-Fraumeni syndrome) result in early onset tumors of various types and although the risk of pancreatic adenocarcinoma is low compared to the incidence of other malignancies, it is still substantially greater than in the general population. Li *et al* identified 4 cases of pancreatic cancer in 151 patients with Li-Fraumeni syndrome (2.6%) (44). This cohort of patients had a total of 169 primary tumors. Interestingly, three of the four pancreatic tumors occurred in individual over 45 years of age, similar to the demographics of sporadic pancreatic adenocarcinoma. The shortened life expectancy of these patients may have contributed to the relatively low incidence of pancreatic adenocarcinoma in these patients.

The p53 tumor suppressor gene serves a critical role at the G_1S transition by blocking cell entry into S phase in response DNA damage. The p53 gene has been proposed to exert an inhibitory effect on CDK4 directly, and indirectly through p21/WAF1 as shown in figure 2. Wild-type p53 is also necessary for the efficient activation of apoptosis in sensitive cells in response to DNA damage (45). Apoptosis is the major mechanism by which ionizing radiation and many chemotherapeutic agents cause tumor cell death. There is now substantial *in vitro* and *in vivo* evidence that p53 mutations are associated with decreased response to many types of chemotherapy and may be a predictor of decreased survival in some tumors (46-49). This has been supported by studies in pancreatic carcinoma cell lines whereby reintroduction of wild-type p53 activates apoptosis (50). Treatment protocols which aim to restore normal p53 function in tumor cells by gene therapy are now underway in some centers. p53 mutations may also result in phenotypic changes in tumor cells by

virtue of its role as a transcription factor which may affect the aggressiveness of pancreatic carcinoma (50).

Numerous different p53 point mutations can occur and their precise phenotype cannot always be predicted from their DNA sequence. Mutations in the p53 gene typically result in an abnormal protein that is longer-lived than the wild-type protein. Because p53 normally functions as a dimer, mutant p53 protein can sequester wild-type protein in nonfunctional heterodimers resulting in a dominant negative phenotype for many p53 point mutations (51). Many tumors with p53 mutations develop loss of heterozygosity (LOH), discarding the wild-type p53 allele. The long half-life of mutant p53 has allowed its detection by antibody staining of tissue sections (52). It has become clear, however, that overexpression of wild-type p53 can occur in some tumors so that immunochemical detection of p53 is not necessarily indicative of p53 gene mutation (53).

5.4. DPC4 (Smad4) tumor suppressor gene

DPC4 is located on chromosome 18q21 and shows homology to the mad family of proteins which play a critical role in signal transduction through the TGF beta superfamily of cell surface receptors. They are thought to act through $p15^{INK4b}$ to inhibit CDK4 interaction with cyclin D. Therefore inactivation of this gene promotes progression from the G_1S restriction point as described above (figure 2) . Recent evidence suggests that TGF beta expression may be a positive prognostic factor in pancreatic adenocarcinoma (54).

This gene shows biallelic inactivation in approximately 50% of cases of pancreatic adenocarcinoma. In most cases, DPC4 is inactivated by homozygous deletion with point mutation followed by loss of heterozygosity in the remaining cases. Rozenblum *et al* noted a high concordance for DPC4 and $p16^{INK4a}$ inactivation. Interestingly, all of these cases with DPC4 inactivation also had $p16^{INK4a}$ inactivation but not vise versa (21). These findings are consistent with a model in which genetic inactivation of $p16^{INK4a}$ increases the selective advantage of developing a DPC4 mutation. Since DPC4 appears to act primarily through $p15^{INK4b}$, it would be surprising if DPC4 deletion would be effective if $p15^{INK4b}$ was deleted with $p16^{INK4a}$ (figure 2) .

5.5. FHIT, fragile histidine triad, candidate tumor suppressor gene

The FHIT gene encodes an Ap_3A hydrolase, a member of the histidine triad gene family. Diadenosine oligophosphates appear to act as cytokines and their concentration is normally elevated in cells at G_1S (55). The FHIT gene product can inactivate the signaling function of diadenosine oligophosphates by hydrolyzing them. Inactivation of FHIT may then promote cell cycle progression at G_1S by virtue of higher diadenosine oligophosphate levels. FHIT has been localized to the 3p14.2FRA3B site which is targeted for homozygous deletion in colon and gastric carcinoma and pancreatic adenocarcinoma (56). Ohta *et al* identified aberrant FHIT transcripts in these tumors, suggesting a role for this gene in carcinogenesis (57). Various investigators have implicated FHIT as a putative tumor suppressor gene in malignancies such as breast, renal and lung carcinoma and a similar role in pancreatic adenocarcinoma has been suggested. Simon *et al* found that full-length FHIT transcripts were absent in 10 of 14 pancreatic carcinoma cell lines (71%). Most of these cases had intragenic homozygous deletions of FHIT exons 3, 4 and 5 (23).

Some have argued that FHIT alterations are not functionally important in tumorigenesis but simply result from its coincident location at an active fragile site (58). This innocent bystander hypothesis is plausible since most of the reported data derives from human tumor cells lines. Aberrant FHIT transcripts observed in such cell lines may be an *in vitro* artifact in some cases. Indeed, aberrant FHIT transcripts have been observed in non-tumor tissues and are more common in aging cells. Some of these abnormalities may be due to reduced RNA-splicing fidelity in aging cells.

Conversely, the frequent loss of heterozygosity at 3p14 in premalignant conditions argues for a role for FHIT in tumorigenesis (59). Experiments transfecting wild-type FHIT into tumor cell lines with FHIT abnormalities have produced conflicting results. Otterson *et al* found that stable overexpression of wild-type FHIT did not alter cell morphology, inhibit colony formation, or inhibit cell proliferation *in vitro* (60). In addition overexpression of wild-type FHIT did not lead to altered cell cycle kinetics in dividing cells. In contrast, Siprashvili *et al* found that transfection of wild-type FHIT into several tumor cell lines resulted in a reduction of the frequency and size of tumors developing after transfer into nude mice (61). These divergent results can be reconciled if the effects of FHIT were only exposed *in vivo*, in nude mice, and were not evident in tissue culture. Clearly more information is needed to decide this question and whether there could be a role for FHIT in gene therapy. The effects of FHIT replacement in pancreatic adenocarcinoma have not been reported.

5.6. HER-2/neu (erbB2) proto-oncogene

HER-2/neu is a proto-oncogene that encodes a transmembrane glycoprotein with tyrosine kinase activity, which is closely related to the epidermal growth factor (EGF) receptor. Up regulation or amplification of this gene has been associated with a poor prognosis of cancers of the breast and ovary. Increased expression of HER-2/neu has been observed in noninvasive intraductal lesions of the pancreas as well as pancreatic adenocarcinoma.

Yamanaka *et al* showed that both HER-2/neu protein and mRNA are detectably expressed in the acinar

cells and ductal cells of normal human pancreas. HER-2/neu immunostaining was demonstrated in normal pancreas and in 34 of 76 (45%) pancreatic carcinomas. They also found that HER-2/neu mRNA was overexpressed in pancreatic adenocarcinoma in the absence of HER-2/neu gene amplification assessed by Southern hybridization (62).

Other investigators did not find consistent immunohistochemical evidence of HER-2/neu expression in normal pancreatic tissue but did find expression in some tumors and intraductal lesions. HER-2/neu expression was demonstrated in 82% of ducts with flat mucinous hyperplasia, 86% of ducts with papillary mucinous hyperplasia without atypia, 92% of ducts with atypical papillary mucinous hyperplasia and all specimens with carcinoma in situ (63). HER-2/neu expression was absent in all but one case of normal pancreatic ducts and ductules. HER-2/neu expression was observed in 69% of moderately differentiated infiltrating carcinomas but none of the poorly differentiated carcinomas.

Dugan *et al* also showed increased expression of HER-2/neu in pancreatic adenocarcinoma by immunohistochemical methods (64). In a series of 79 primary pancreatic adenocarcinomas, they found significantly lower expression of HER-2/neu in poorly differentiated areas of tumor compared to well and moderately differentiated areas. Most well and moderately differentiated invasive tumors (62%) showed moderate or strong expression of HER-2/neu while a minority of poorly differentiated tumors (19%) showed similar expression (63).

The observed differences in staining of normal pancreatic parenchyma almost certainly result form differences in the sensitivity of the immunohistochemical stains employed. Antibodies for HER-2/neu have been problematic in the evaluation of breast carcinoma because of the effects of tissue fixation on antigen retrieval and the lack of internal controls for staining. It will be of interest to see if the reported lack of HER-2/neu gene amplification in pancreatic adenocarcinoma is confirmed by FISH. Available evidence suggests that HER-2/neu overexpression is a relatively early event in the development of pancreatic adenocarcinoma which is lost in advanced, poorly differentiated tumors. If this model is validated in subsequent studies, biologic therapy with Herceptin^{TM} (human anti-HER-2/neu antibody) may not be of much advantage in patients with poorly differentiated pancreatic adenocarcinoma.

5.7. BRCA2, hereditary breast Cancer

BRCA2 has been implicated as a potential tumor suppressor in familial aggregates of pancreatic carcinoma with a history of breast carcinoma. Goggins *et al.* have also shown germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas(65). In an unselected panel of 41 pancreatic carcinomas (30 xenografts and 11 pancreatic cell lines) screened for alterations of BRCA2, 15 (27%) showed loss of heterozygosity. Four (10%) had abnormalities in the second allele when the entire BRCA2 gene was screened using a protein truncation assay. Three of these four mutations were identified as germline in origin. One of the patients with a germline mutation had a single relative with breast cancer while another had a single relative with prostate cancer. None had a family history of pancreatic adenocarcinoma. Based on these limited data, the incidence of germline mutations of BRCA2 in pancreatic adenocarcinoma may be as high as in breast and ovarian cancer. The significance of loss of heterozygosity for BRCA2 in some cases of pancreatic adenocarcinoma is less clear since loss of heterozygosity has not been found in breast cancers from BRCA2 kindreds.

5.8. Rb1, retinoblastoma tumor suppressor gene

Rb1 has been shown to be altered in less than 10% of pancreatic carcinomas investigated (66,67). Due to the large size of the gene and the nature of alterations in it in different tumors, it may be difficult to reliably identify mutations and this is probably a minimum estimate for the number of Rb alterations in pancreatic adenocarcinoma. Tumors with $p16^{INK4a}$ alterations do not usually have coexistent Rb mutations since these would tend to mitigate the effects of $p16^{INK4a}$ loss. Since $p16^{INK4a}$ alterations are common in pancreatic adenocarcinoma, Rb mutations are expected to be uncommon (41)

6. DIAGNOSTIC IMPLICATIONS

The ideal screening test for pancreatic carcinoma would be a specific marker detectable in peripheral blood or feces which would enable noninvasive screening and detection of early tumors prior to the onset of symptoms. No such test is currently available and the prospects for developing such a marker are not promising. K-ras mutations have been assessed in stool samples and this marker has shown good sensitivity but limited specificity for pancreatic adenocarcinoma (68). Positivity can also result from non-neoplastic conditions of the pancreas such as pancreatitis, as well as from adenomatous colon polyps.

Recently Nomoto *et al* advocated the clinical application of mutations of the K-ras oncogene for the detection of micrometastases (69). Using a hemi-nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis to detect mutations of K-ras at codon 12, tumor cells could be detected in liver and lymph node tissue that were not apparent on histological examination. The authors suggest that this technique can detect subclinical micrometastases. This assay detected tumor derived K-ras mutations in the peripheral blood of all pancreatic cancer patients tested at the time of surgery emphasizing the degree of systemic involvement in this disease. A related PCR strategy has been successfully employed for the detection of tyrosinase

mRNA in the blood or lymph nodes of patients with malignant melanoma and carcinoembryonic antigen mRNA in lymph nodes from patients with colon cancer(70). Early results in these systems suggest that molecular detection of tumor cells can be predictive of adverse outcome.

p53 mutations can be detected in duodenal brushing specimens and their presence has been shown to correlate with the presence of pancreatic adenocarcinoma (71). Iwao *et al* also suggested that detection of a specific p53 mutation at codon 273 (GGT to CAT in exon 8) was predictive of massive metastases and may be an indicator of unusually poor prognosis. This finding is provocative but must clearly be reproduced by other investigators. Caution must be used in basing a diagnosis of pancreatic adenocarcinoma on the presence of p53 mutations alone, since p53 mutations have been identified in dysplastic and precursor lesions in other systems.

Rozenblum *et al* pointed out that the coexistence of K-ras and $p16^{INK4a}$ alterations is quite uncommon in tumors other than pancreatic adenocarcinoma in which they found 83% concordance (21). They further suggested that this concordance could be used as a diagnostic criterion for pancreatic adenocarcinoma. This observation is interesting scientifically but has no obvious application to clinical diagnosis or management of patients with pancreatic adenocarcinoma.

Recently, anti-p53-autoantibodies (a-p53-aab) have been investigated as a potential tumor marker for pancreatic adenocarcinoma. a-p53-aab can develop as a humoral response to increased intracellular p53 protein in tumor tissue. Since p53 is frequently mutated and overexpressed in pancreatic adenocarcinoma, Gansauge *et al*. evaluated 145 patients with pancreatic adenocarcinoma for the presence of a-p53-aab using ELISA and Western-blotting (72). 16% of the pancreatic adenocarcinoma patients had demonstrable a-p53-aab in their serum. The presence of a-p53-aab was more frequent in higher stage patients with lymph node metastases Using similar methodology, Raedle *et al.* found a similar frequency of positivity for a-p53-aab in patients with pancreatic cancer (73). Unfortunately, a similar percentage of patients with chronic pancreatitis were also positive (12%) as well a lesser percentage of patients with acute pancreatitis (5%). a-p53-aab would not expected to be specific for pancreatic adenocarcinoma since p53 is overexpressed in a wide variety of tumors.

At the present stage of our knowledge of pancreatic carcinoma, none of the available screening methodologies are appropriate for the general population based on their cost and lack of specificity. Patients at high risk for the development of pancreatic adenocarcinoma based on genetic syndromes or strong familial clustering might benefit from some of these strategies, however. Evaluation of peripheral blood and/or liver and lymph node tissue from patients with pancreatic adenocarcinoma may be useful in identifying patients at high risk for local progression. On the other hand, overall prognosis for pancreatic adenocarcinoma is currently so poor that such information may not be of great clinical utility. With the development of more effective therapeutic strategies for pancreatic adenocarcinoma (see below), this type of prognostic information may be of considerable value in the future.

7. THERAPEUTIC IMPLICATIONS

Molecular genetic alterations that disturb cell cycle regulation in tumor cells can affect their response to chemotherapeutic agents and radiation. Many of the genes which regulate the critical cell cycle checkpoint at G1/S are altered pancreatic adenocarcinoma. Knowledge of these genetic alterations in individual tumors may allow selection of optimal therapeutic strategy for individual patients. Phase II studies are currently ongoing to assess the potential role of p53 and $p16^{INK4a}$ alterations in determining response to Taxol chemoradiation (2). Furthermore, the availability of biologic therapies which target specific oncogenes such as farnesyl transferase inhibitors of K-ras and Herceptin^{TM} for HER2/neu require knowledge of a tumor's genetic composition to permit rational therapeutic decisions.

Gene therapy protocols for pancreatic adenocarcinoma are beginning and offer promise for the future. Strategies include tumor suppressor gene replacement and the use of antisense RNA to prevent the expression of oncogenes (74). Wild type p53 can potentially be introduced into tumors to promote apoptosis and chemosensitivity as has been accomplished in nonsmall cell lung cancer. Similar strategies are planned for $p16^{INK4a}$ replacement. Liposome mediated antisense K-ras constructs have been successfully employed to inhibit dissemination of pancreatic adenocarcinoma in the murine peritoneal cavity. The development of appropriate and effective vectors and transfection strategies which can target tumor cells is currently a major limiting factor in developing clinically useful gene therapy protocols.

Adoptive immunotherapy is another form of gene therapy that is being explored as a possible treatment for pancreatic adenocarcinoma. McCarty *et al.* recently reported the use of high affinity human p53-specific cytotoxic T-lymphocytes (CTLs) from transgenic mice to suppress the growth of p53-over expressing human tumors in severe combined immunodeficient mice (75). The p53-specific CTLs lysed p53-overexpressing pancreatic carcinoma cell lines and inhibited the growth of established human tumor xenografts. Undoubtedly, additional novel strategies will be developed as well.

8. PERSPECTIVE

Knowledge of the molecular biology of pancreatic adenocarcinoma has grown rapidly and has potential implications for the surgical, radiation, and chemotherapeutic management of this deadly disease. Understanding the genetics of pancreatic adenocarcinoma will become even more important as more effective therapeutic strategies are developed and tested. While all pancreatic adenocarcinomas are not genetically identical, they share many common features with a very high

frequency of alterations in K-ras, p53, and $p16^{INK4a}$ genes. The $p16^{INK4a}$ gene and the closely linked p15 and p19 genes appear to be of special significance in that they are more frequently targeted in pancreatic adenocarcinoma than in any other neoplasm. In addition, the genetic predisposition for developing pancreatic adenocarcinoma in the FAMMM syndrome with $p16^{INK4a}$ gene mutations suggests that $p16^{INK4a}$ can be involved in the genesis of these tumors. Other oncogenes and tumor suppressor genes are clearly involved in producing most pancreatic adenocarcinomas so that the genotype of these tumors is quite complex.

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