Table of Contents

Gallbladder and Bile Duct Cancer Research

http://pathology2.jhu.edu/gbbd

The Johns Hopkins Medical Institutions

Creating a Named Fund

Creating a Fellowship Program

Creating an Endowed Chair

Proposed Research Project – The Research Accelerator

Reprints

If you would like to learn more about any of the giving opportunities to support gallbladder and bile duct cancer research, please contact:

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June 27, 2001

Dear Friends:

Gallbladder and bile duct cancers are a significant cause of cancer death among both men and women. Here at Hopkins, we have established a dedicated multiple disciplinary team to fight cancers that arise in the pancreas, bile ducts, and gallbladder. This team includes, surgeons, oncologists, pathologists, gastroenterologists, and many scientists. Remarkably, in a few short years, this team has made most of the major discoveries in the field of gallbladder and biliary tract cancer research. Most of these discoveries have been made in conjunction with ongoing research in pancreatic cancer. The accomplishments of the Hopkins team include the discovery of the pancreas cancer gene (DPC4) and a critical advance which led to the discovery of the second breast cancer gene (BRCA2).

This last year was a particularly exciting year for the Hopkins team. Simply put, the cloning of the human genome has accelerated the pace of research and, importantly, it has provided new avenues for research exploration. We now have many, many more leads than we have resources with which to pursue these leads.

We believe that the success and enthusiasm of the team at Hopkins represents an enormous opportunity for those wishing to have a direct impact on gallbladder and biliary tract cancers. A wide range of giving opportunities is outlined in the enclosed booklet. Importantly, each of these opportunities will not only have a significant impact on our research, they are also wonderful ways to honor loved ones. We hope you find this booklet useful and we encourage you to call if you have any questions at all.

With warm regards,

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Professor, Oncology
Director, Division of Gastrointestinal/Liver Pathology
Director, National Familial Pancreas Tumor Registry
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I

Named Fund for Bile Duct and Gallbladder Cancer Research

Research requires money, and although most of the support for medical research comes from the Federal Government via the National Institutes of Health (NIH), it is private philanthropy that can provide the margin of excellence to a research enterprise. Private funds are flexible and can be deployed quickly to take advantage of new ideas and new people. Private funds also can form a constant base for faculty support upon which grant support can be superimposed. Private funds are particularly needed to support research on bile duct and gallbladder carcinomas. This is because biliary cancers are relatively rare compared to more common cancers like colon cancer and hence biliary research is often given a lower priority by the NIH.

There have been several examples of private giving having a significant impact on a cancer. For example, the Clayton Fund in Basic Colon Cancer Research has laid the foundation for the remarkable success of the colon cancer research team here at Hopkins. Thanks to the Clayton Fund, many of the most significant discoveries in cancer biology in the last decade were made by the colon cancer team at Johns Hopkins (Bert Vogelstein who leads this team is now the most cited scientist in all of science).

Just as the Clayton Fund has had an impact on our understanding of colon cancer, so too would the establishment of a Fund for bile duct and gallbladder cancer research have a fundamental impact on the fight against bile duct and gallbladder cancer.

(Endowments start at $20,000. If they reach the $100,000 level a plaque is placed in the research labs honoring the donor. If they reach the $10 Million level, then the endowment can be used to name a research center).
Fellowship Training Program

New Technologies in Bile Duct and Gallbladder Cancer Research

“The principal mark of genius is not perfection, but originality, the opening of new frontiers.”
Arthur Koestler

Physicians and scientists must make critical decisions when they come to the end of their standard training. They must decide whether or not to pursue an academic career in research. Those who choose a career in research must then choose a sub-specialty area on which to focus their research efforts. These critical career choices are often made for rather trivial reasons. Countless physicians and scientists with enormous potential have chosen not pursue an academic research career because of a lack of a secure fellowship program.

At the same time, young minds are the most creative minds. Human creativity peaks at a rather young age; as our fund of knowledge increases our creativity paradoxically decreases. Indeed, some of the major new ideas in cancer research in the last several years have come from young scientists in their training. For example, Victor Velculescu here at Johns Hopkins created the idea for the revolutionary technology of serial analysis of gene expression (SAGE). Victor did this while he was a post-doctoral student in the Johns Hopkins cancer research laboratories.

We propose to create an endowed fellowship training program in bile duct and gallbladder cancer research at Hopkins. This program will provide secured funding to young scientists and physicians wishing to pursue a career in bile duct and gallbladder cancer research. The research fellowship program will not be a standard fellowship program. Instead it will take advantage of and most importantly encourage the creativity of the trainees. The fellow will not be a mere technician following detailed instructions from a mentor. Instead, the fellows will be given extensive free time and the fellowship will be focused on creating novel new technologies which can be applied to cancer research and on identifying new technologies, developed in other fields, which can be applied to bile duct and gallbladder cancer research.

This approach will bring more minds to the battle against bile duct and gallbladder cancer. Furthermore, the focus on creative spark will mean that our understanding will advance not in safe yet small steps, but rather in daring leaps.

**BUDGET**

<table>
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<tr>
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<tr>
<td>Permanent Endowed Fellowship</td>
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III

Endowed Chair for Bile Duct and Gallbladder Cancer Research

“Everything that is really great and inspiring is created by the individual who can labor in freedom” - A. Einstein

The last five years have brought remarkable advances to our understanding of the genetics of bile duct and gallbladder cancer. This growing understanding of the genetics of bile duct and gallbladder cancer will form the basis of new screening and diagnostic tests for the early detection of bile duct and gallbladder cancer; they will be used to identify patients at risk for familial forms of bile duct and gallbladder cancer; and they can be used to characterize even the most subtle pathologic changes, thereby advancing our understanding of early bile duct and gallbladder neoplasia. In addition, and most importantly, an understanding of the genetic changes associated with the development of bile duct and gallbladder cancer will form the foundation for developing novel, rational, gene-based therapies for bile duct and gallbladder cancer.

The establishment of a named endowed chair for bile duct and gallbladder cancer research would allow us to pursue high-risk research work. We believe this work will advance our understanding of bile duct and gallbladder cancer, not by small steps, but instead by leaps and bounds. All too often, scientists focus their efforts on “evolutionary” work because it is safer, and more of a sure bet. Endowed chairs allow scientists such to pursue revolutionary work. In addition, because endowed chairs are permanent, these named chairs are a wonderful way of permanently honoring the donor.

The cost for a named endowed chair is approximately $2.3 million.
IV

Project Plan:

The Research Accelerator

“Art is I; Science is we” - C. Bernard

Bile duct and gallbladder cancers, because of their rarity, have been grossly understudied. Recent rapid advances in cancer genetics provide a unique opportunity to dramatically advance our fundamental understanding of bile duct and gallbladder cancer. With a significant donation, we could, in one fell swoop, bring bile duct and gallbladder cancer to the forefront of cancer research. Through this program, we propose to set up a shared resource that would facilitate gallbladder and bile duct cancer research nationally. For example, this shared resource would generate, provide, and then share xenografted gallbladder and bile duct cancers. These xenografted gallbladder and bile duct cancers would provide a unique source of pure cancer cells for genetic analysis. Second, this resource would be used to fund fundamental studies of gallbladder and bile duct cancers at the genetic level and at the level of gene expression. These studies would be performed using technologies already established at Hopkins for other cancer types and the results of these analyses would be placed on the Web. Such shared data has proven invaluable in the discovery of new markers and other cancer types and we believe it will greatly facilitate the discovery of new markers in gallbladder and biliary cancers. Finally, this project will establish the infrastructure to maintain a significant long-standing program in gallbladder and biliary cancer research.

In order to push ahead in the battle against bile duct and gallbladder cancer, we have identified five “Impact Areas.” In each area, Johns Hopkins has been the recognized leader. Although bile duct and gallbladder cancer is a significant cause of cancer death, among the media and the general scientific community, bile duct and gallbladder cancer research has not in the past enjoyed the investment that is needed. Therefore, for each Impact Area, we need to increase our commitment and engender greater enthusiasm for fighting this disease.

Impact Areas:

1. The first step in studying the fundamental genetic alterations in a cancer is obtaining pure cancer cells, free from normal cells. We propose to harvest surgically resected biliary and gallbladder cancers and implant a small portion of these cancers into nude mice (a process called "xenografting"). Nude mice lack an immune system and, as a result, the tumor cells that have been implanted grow freely. Although the tumor cells grow freely, normal human cells die out and are replaced with small numbers of normal mouse cells. The resulting tumors are wonderful sources of pure cancer cells. The establishment of a source of pure cancer cells is a critical first step in studying a cancer. We propose to do this with large
numbers of gallbladder and biliary cancers resected at Johns Hopkins. Once established, these xenografted tumors can be harvested for genetic analysis or they can be re-implanted in other nude mice providing a renewable resource for study. As a part of this Research Accelerator, we would share this resource with other investigators throughout the country.

2. Define the **Genetic Mutations** in bile duct and gallbladder cancer. Based upon the initial investment starting in 1990 at Johns Hopkins, the past four years have witnessed a literal explosion in the understanding of the gene mutations that cause bile duct and gallbladder cancer. The vast majority of this work has been generated as an offshoot of our ongoing pancreas cancer research. As a part of our pancreas research, we have discovered several cancer causing genes (DPC4 and BRCA2) and we have identified most of the genes targeted in pancreas and gallbladder/biliary cancers. Just as we have done with pancreatic cancer, we want to discover the fundamental genetic changes that underlie the development of bile duct and gallbladder cancer. Indeed, we have recently shown that Dpc4 is frequently inactivated in bile duct cancers, particularly those of the distal bile duct. In addition, we have shown that p16, a tumor suppressor gene that is inactivated in 100% of pancreas carcinoma, is inactivated in over 70% of gallbladder cancers. Such a fundamental understanding of bile duct and gallbladder cancer is the first step in developing rational treatments.

3. Define the patterns of **Gene Expression** in bile duct and gallbladder cancer. Before a gene can act upon a cell, its code of information must be “expressed”, first as a temporary message (called RNA) and then as a protein. The proteins are what determine the structure and behavior of a cell. There are normally only two copies of each gene, but there are vast differences in the amount of message and protein each gene can produce. For example, a red blood cell produces a single protein, hemoglobin, in levels that exceed all other proteins in the red cell combined. On the other hand, most other cells produce tens of thousands of proteins, in varying amounts, involving a complexity that with conventional technology could not be understood. A revolutionary technique developed by Hopkins researchers has solved this problem in a remarkable way. The technology is called SAGE, for Serial Analysis of Gene Expression. In the past, gene expression was studied one gene at a time, involving months of work due to the inefficiency of these conventional techniques. With SAGE, through the use of sophisticated nucleic acid sequencing and data analysis tools, the expression of tens of thousands of gene messages can be measured simultaneously. This has, for the first time, opened up gene expression patterns for highly efficient study. For example, we have recently published the development of a new marker of pancreas cancer called "PSCA." This was identified as one of the 50,000+ different genes found by SAGE analysis of pancreas cancer, but one that was noted not to be expressed in normal tissues. We have also shown that the protein coded by another gene identified by SAGE, mesothelin, is overexpressed in pancreatic cancers and also gallbladder cancers (this data has been submitted for publication). Without the incredible ability of SAGE to allow us to study thousands of genes simultaneously, such a discovery would probably have taken many decades of work. Just
as we have successfully done for pancreas cancer, we want to define gene expression in bile duct and gallbladder cancer using SAGE, and just as we have done with pancreas cancer, we want to make this data available to all investigators on the Web. The analysis of gene expression by SAGE offers promise of rapid identification of new markers for the early detection of bile duct and gallbladder cancer and the sharing of this data will attract additional investigators into this field.

4. We want to communicate our findings to other scientists and to patients and their families. The Bile Duct and Gallbladder cancer Web Site at Johns Hopkins serves many roles. Patients and families have access to information regarding the disease, lists of publications that are available, and a discussion section (or “chat room”) where personal experiences can be shared and questions posted. A physician monitors this chat room, while a social worker monitors an additional "chat room" on this site. Both are available to advise patients on how to deal with their cancer. For medical researchers in other institutions, basic science information regarding bile duct and gallbladder cancer can be made widely available. This includes detailed descriptions of genetic alterations found by Hopkins investigators in the cancers. Because these cancers can be widely shared, this becomes key information that can be used to spur bile duct and gallbladder cancer research in other laboratories. Data from SAGE analysis can also be made available on the Web. We need support to expand our bile duct and gallbladder cancer Web initiative so that we can share information with physicians, other scientists, patients, and their families.

5. Long-term infrastructure. We need to establish the infrastructure to maintain this effort in the long-term. This is best accomplished through the creation of a named endowed Fellowship training program (see #2) and the creation of a named endowed chair. Fellowships prepare promising young scientists for a future in bile duct and gallbladder cancer. We need to train new investigators and raise their interest in bile duct and gallbladder cancer at an early point in their career. The goals of research training, and the above Impact Areas as well, are well served by the types of research now underway at Hopkins. A number of our key discoveries, such as the association of the breast cancer (BRCA2) gene with pancreas cancers and development of SAGE, have been made by persons in advanced stages of training whose continued participation has only been made possible by special funding awards. These funding opportunities are transient, and a reliable source of support for such advanced research positions would facilitate the translation of basic science to the bedside. As discussed earlier (see #3), named endowed chairs provide the long-term stability needed for established scientists to pursue high impact cutting edge research. Both, because they are named, are wonderful ways to honor a loved one.

The proposal is to assemble the above five impact areas around a joint project that serves as a Research Accelerator. This will take advantage of the strengths of the Johns Hopkins bile duct and gallbladder cancer effort and really could only be done at Hopkins. In brief, we will generate pure populations of cancer cells for research, we will use SAGE technology and a sequencing machine to
define the genetic changes and the genes expressed in these cancers. We will then make this data available to all gallbladder and bile duct cancer researchers over the Internet. Because this huge database will be shared, the Research Accelerator will provide all bile duct and gallbladder cancer researchers a huge “head start” in their research.

The project envisions the participation of a principal investigator who leads a team of technicians and persons in advanced medical training, working in collaboration with informatics specialists having internet-related programming skills. Resources for molecular biology supplies, computer access and Internet access will be provided to the team. Because the technology already exists at Hopkins, startup can be readily accomplished and progress can be proportional to the resources available.

**Budget**

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<tr>
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<td>(to support continued research (supplies) after 5 years)</td>
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Clinical Value of K-ras Codon 12 Analysis and Endobiliary Brush Cytology for the Diagnosis of Malignant Extrahepatic Bile Duct Stenosis


ABSTRACT

Extrahepatic biliary stenosis can be caused by benign and malignant disorders. In most cases, a tissue diagnosis is needed for optimal management of patients, but the sensitivity of biliary cytology for the diagnosis of a malignancy is relatively low. The additional diagnostic value of K-ras mutational analysis of endobiliary brush cytology was assessed. Endobiliary brush cytology specimens obtained during endoscopic retrograde cholangiopancreatography were prospectively collected from 312 consecutive patients with extrahepatic biliary stenosis. The results of conventional light microscopic cytology and K-ras codon 12 mutational analysis were compared and evaluated in view of the final diagnosis made by histological examination of the stenotic lesion and/or patient follow-up. The sensitivities of cytology and mutational analysis to detect malignancy were 36 and 42%, respectively. When both tests were combined, the sensitivity increased to 62%. The specificity of cytology was 98%, and the specificity of the mutational analysis and of both tests combined was 89%. Positive predictive values for cytology, mutational analysis, and both tests combined were 98, 92, and 94%, whereas the corresponding negative predictive values were 34, 34, and 44%, respectively. The sensitivity of K-ras mutational analysis was 63% for pancreatic carcinomas compared to 27% for bile duct, gallbladder, and ampullary carcinomas. K-ras mutational analysis can be considered supplementary to conventional light microscopy of endobiliary brush cytology to diagnose patients with malignant extrahepatic biliary stenosis, particularly in the case of pancreatic cancer. The presence of a K-ras codon 12 mutation in endobiliary brush cytology per se supports a clinical suspicion of malignancy, even when the conventional cytology is negative or equivocal.

INTRODUCTION

Stenosis of the extrahepatic bile ducts is caused by a variety of malignant and benign disorders. To optimally manage such patients, it is often important to determine the etiology of the stenosis. However, it can be difficult to differentiate malignant from benign causes of biliary stenosis, based on clinical presentation and radiological findings alone, and a definitive diagnosis of malignancy can only be established histopathologically. Endobiliary brush cytology can be performed during ERCP to collect material for cytology. Despite the high specificity of brush cytology, the sensitivity is low. An analysis of tumor-specific genetic alterations in these cytology specimens may add to the diagnostic value of brush cytology.

Mutations in the K-ras oncogene are attractive for such analyses for a number of reasons. (a) K-ras mutations are one of the most common genetic alterations in human cancers and are frequent in the two main malignant neoplasms that cause biliary stenosis, pancreatic carcinoma, and bile duct carcinoma (3–9). (b) More than 90% of the K-ras mutations in these neoplasms occur in codon 12, which makes their detection relatively easy. (c) The PCR-based method used for the detection of the K-ras mutations is very sensitive and can identify rare mutant DNA copies among an abundance of wild-type DNA (3). (d) Results from K-ras mutational analyses, as were performed here, can be obtained within 48 h, making the test suitable for routine clinical purposes.

A number of studies have emphasized the diagnostic utility of K-ras mutations in material obtained from the head of the pancreas for the diagnosis of pancreatico-biliary malignancies, but most studies were performed on small groups of selected patients. Furthermore, the specificity of K-ras mutational analysis in the clinical diagnosis of neoplastic disease is unclear, because these mutations are also present in intraductal pancreatic proliferations (called “duct hyperplasia”; Refs. 23–25). This study has prospectively assessed the value of K-ras mutational analysis of endobiliary brush cytology as compared to conventional cytology for the diagnosis of a malignancy.

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1 Supported by The Netherlands Foundation for Scientific Research Grant 950-10-625.
2 To whom requests for reprints should be addressed, at Academic Medical Center, University of Amsterdam, Department of Pathology, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands. Phone: 3120-5665635; Fax: 3120-6960389.

3 The abbreviation used is: ERCP, endoscopic retrograde cholangiopancreatography.
in patients with bile duct stenosis in a large series of consecutive patients with a complete follow-up.

**MATERIALS AND METHODS**

**Patients**

The study population consisted of a series of consecutive patients who underwent ERCP with endobiliary brush cytology for the evaluation of an extrahepatic biliary stenosis at the Academic Medical Center in Amsterdam in the period from January 1, 1993, to February 1, 1996. The Medical Ethical Review Committee of the Academic Medical Center approved the study. If a patient underwent ERCP with brush cytology repeatedly during this period, only the first examination was included. This resulted in 312 patients with a mean age of 63 years; 172 patients were male.

A final diagnosis of the nature of biliary stenosis was based on histological and/or clinical findings (Table 1). In the absence of a tissue diagnosis, a clinical diagnosis was established based on clinical symptomatology, the results of imaging studies prior to the ERCP procedure, and, particularly, the course of the disease. Information concerning the clinical follow-up was obtained from the patient’s physician. All patients were followed for at least 12 months. The 104 patients with a clinical diagnosis of malignant extrahepatic bile duct stenosis had rapidly progressive disease with symptoms such as jaundice, pain, cachexia, and metastases. Importantly, all these patients died of disease within a mean survival of 5.7 months (range, 0–42 months) after the ERCP procedure, which corresponds to survival rates of patients with cancer of the pancreas and extrahepatic biliary tract in general. The mean survival for all of the 220 patients with a malignant etiology of their stenosis was 9 months (range, 0–50 months). Eight of these 220 patients were still alive at the end of follow-up, and their survival ranged from 22 to 50 months: of these, 6 had a surgical resection of the carcinoma and 2 were biopsied only; thus, they were all tissue proven. In contrast, 71 of the 74 patients with benign disease (including the 10 patients with a tissue diagnosis of benign disease) were all alive after a mean follow-up period of 32 months (range, 15–54 months) and had stable disease or regression of their symptoms.

Three patients with benign disease died due to unrelated causes: 2 died from heart disease 10 months and 27 months after ERCP, and 1 died following a hip fracture 18 months after ERCP, all without symptoms of obstructive biliary disease.

In summary, 220 patients (70%) had a malignant etiology for their stenosis, and 74 (24%) had a benign stenosis. In 18 patients (6%), the cause of the stenosis remained unclear because of insufficient information during follow-up, and these patients were excluded from further analysis. The spectrum of the different etiologies of the stenoses in the remaining 294 patients is given in Table 2.

**Materials**

Brushings of the bile duct stenoses were performed with the GRBH-230-3.3.5 (size of brush device) (Wilson-Cook Medical Inc., Winston-Salem, NC). Four cytology smears from each patient were stained with Giemsa and Papanicolaou for routine diagnostic cytology. The remainder of the brush cytology specimen was suspended in 10 ml of DNA buffer and fixed with 10 ml 100% ethanol. The suspensions were stored at 4°C for subsequent K-ras mutational analysis.

Tissue from the area of the bile duct stenosis was available from 71 patients with a malignant cause for their stenosis and from 10 patients with a benign stenosis. These tissues were obtained at resection of the stenotic lesion, from biopsies of the stenotic lesion with malignant findings, and at autopsy (Table 1). In these cases, the available archival tissue blocks were analyzed for K-ras mutations, allowing us to compare directly the mutational status of the patient’s primary pathology with the analysis of the corresponding brush cytology specimens.

**Methods**

**DNA Isolation.** One ml of each brush cytology suspension was used for DNA isolation. In case of the tissue blocks, careful microdissection from 3-μm H&E-stained sections was performed to ascertain a sample of which at least 50% of the
cells comprised the tissue of interest. DNA was extracted as described previously (26).

**K-ras Mutational Analysis.** The protocol of the K-ras codon 12 mutational analysis has been described previously (26). With this assay, DNA is subjected to PCR amplification using primers around codon 12. One of the primers generates a restriction enzyme recognition site with the wild-type codon 12 sequence but not with the mutant codon 12 sequence. Digestion of the PCR products with the restriction enzyme is followed by a second round of amplification, which then yields a PCR product enriched for K-ras codon 12 mutations. The resulting DNA fragments are denatured and dot-blotted onto nylon membranes and subjected to allele-specific oligonucleotide hybridization with radioactive labeled probes, specific for each possible K-ras codon 12 mutation, followed by autoradiography. Cell suspensions with mutant:wild-type ratios of 1:100 and 1:1000 were used as positive controls in every PCR procedure. The suspensions were made of the human colon cancer cell line SW 480 with a homozygous GGT to GTT mutation at codon 12 of K-ras and the human colon cancer cell line HT 29 with wild-type K-ras. Water was used as a control for contamination. placental DNA was used as a control for nonspecific hybridization, and cloned DNA fragments with the six different K-ras codon 12 mutations and the wild-type codon 12 were used as controls for specific hybridization. All PCR products were hybridized with oligonucleotides specific for the wild-type sequence to control for amplification of the patient samples. Both enriched and nonenriched PCR products were dot-blotted next to each other to check the digestion and mutant enrichment. Fig. 1 is an example of an autoradiogram of the K-ras analysis. The above mutational analysis has been validated through comparison with sequence analysis in a previous study (27).

The K-ras mutational analysis results were evaluated without any information regarding the patient. All mutational analyses were performed in duplicate in separate experiments. If there were discrepancies, a third analysis was performed to resolve the discrepancy. A result was called K-ras mutant positive if identical mutations were found in the duplicate analysis and when enrichment for the mutation had occurred.

**Light Microscopy.** All of the cytology smears were independently evaluated by an experienced cytopathologist (L. A. N.). The following diagnostic categories were used: positive for carcinoma, negative for carcinoma, suspect for carcinoma, and material insufficient or not suitable for diagnosis.

**Sensitivity, Specificity, and Positive and Negative Predictive Values.** The following definitions were used for evaluation. Sensitivity was defined as the percentage of patients with disease who had positive test results. Specificity was defined as the percentage of patients without disease who had negative test results. Positive predictive value was defined as the percentage of patients with positive test results who had disease. Negative predictive value was defined as the percentage of patients with negative test results who had no disease.

**RESULTS**

Of the 220 patients with a malignant etiology for their bile duct stenosis, 79 (36%) were diagnosed cytologically, and 92 (42%) had K-ras mutations detected in their cytology specimens (Table 3). Of the 92 patients with mutant K-ras in their cytology specimens, 57 patients were not diagnosed with cytology, and thus the two tests combined were able to identify 136 (79 + 57) patients with malignant disease (62%). Of these 57 patients with mutant K-ras and nondiagnostic cytology, 39 had negative cytology results, 14 had suspect for carcinoma cytology, and 4 had material that was insufficient for diagnosis. Positive predictive values and negative predictive values for the cytology, K-ras mutational analysis, and both tests combined were 98 and 34%, 92 and 34%, and 94 and 44%, respectively.

Eight of the 74 patients with benign disease on follow-up had K-ras mutations identified in their brush cytology (Table 3). All eight patients were alive after a mean follow-up of 30 months (range, 16–50 months), and none had signs of malignant disease at the end of follow-up. Two of these eight patients had a diagnosis of chronic pancreatitis, three had a postsurgical
steno sis, and three patients had primary sclerosing cholangitis. Tissue from the stenotic lesion of one of the patients with primary sclerosing cholangitis was available for K-ras mutational analysis. The patient had undergone a hilar resection because of the suspicion of a cholangiocarcinoma. Histopathological findings were cholecystitis with inflammation and fibrosis of the common hepatic duct. The K-ras mutation found in the brush cytology specimen was not confirmed in the reactive bile duct epithelium in this case.

Two of the eight patients with “false-positive K-ras results,” both with a postsurgical stenosis, also had positive cytology (Fig. 2).

In the 71 patients with a definitive tissue diagnosis of a malignancy, cytology was slightly more sensitive for the diagnosis of carcinomas primary to the bile duct compared to the other causes of malignant biliary stenosis, 33% (6 of 18) versus 23% (12 of 53; Table 4). The sensitivity of K-ras mutational analysis was highest for pancreatic carcinoma, 63% (24 of 38) compared to 27% (9 of 33) for other causes.

Tissue was available for K-ras mutational analysis from 60 of the 71 patients who had a definitive tissue diagnosis of a malignancy (Table 5). Twenty-two of 29 (76%) pancreatic carcinomas had a K-ras mutation compared to 12 of the 31 (39%) nonpancreatic cancers. In 53 of 60 (88%) patients, the K-ras analyses of brush cytology and tissue specimens were concordant: in 27 patients, identical mutations were found, and in 26 patients, both specimens were negative for mutations. In seven patients the results were discrepant. All these patients had wild-type K-ras detected in their cytology specimens, and mutant K-ras was detected in their primary carcinomas; the cytology of these patients was also negative for carcinoma.

No mutations were found in the tissue specimens of the 10 patients with a benign stenosis.

**DISCUSSION**

The clinical value of analyzing endobiliary brush cytology specimens for K-ras codon 12 mutations in establishing the diagnosis of a malignancy in patients with extrahepatic bile duct stenosis was examined. The study materials were prospectively collected from a large series of consecutive patients who underwent ERCP with endobiliary brush cytology to rule out or confirm a neoplastic cause of their bile duct stenosis. Brush cytology accurately diagnosed malignancy in 36% of the patients with a malignant etiology for their biliary stenosis, a sensitivity comparable to two previous studies in which a large consecutive series of patients was analyzed (1, 2). These authors reported similar frequencies of biliary stenosis caused by malignant disease as in our study (57 and 66% versus 70%), and in these previous studies, pancreatic and bile duct carcinoma were also the most frequent carcinomas; the demographics in these two studies are comparable with this series. Thus, the study population in our series can be considered representative for patients undergoing ERCP with brush cytology for the evaluation of a potentially malignant biliary stenosis. Other studies that reported higher sensitivities of biliary cytology dealt with smaller groups of selected patients (28).

The K-ras mutational analysis was especially valuable in the diagnosis of patients with pancreatic carcinoma (63% sensitivity versus 27% in patients with malignancy other than pancreatic carcinoma). One would expect that the K-ras mutational analysis is particularly sensitive for a stenosis caused by pancreatic carcinoma, whereas light microscopic brush cytology is the more sensitive method for carcinomas arising from the bile duct epithelium. Endobiliary brush cytology samples the bile duct epithelium most efficiently, whereas the frequency of K-ras mutations is highest in pancreatic carcinoma (3–7, 9, 26, 28), and the PCR-based technique for detecting K-ras mutations is highly sensitive and thus, in contrast to cytology, less dependent on obtaining a large amount of tumor cells. Because cytology and K-ras mutational analysis have opposite sensitivities for the two most frequent causes of malignant biliary stenosis, a...
from one of these patients with primary sclerosing cholangitis in chronic pancreatitis. Tissue for K-ras mutational analysis in the Methods was available in 71 of the 74 patients (96%) with benign disease. Performing all PCR analyses in duplicate independently minimized the chance of technical errors as a cause for false-positive results. Positive results of the K-ras mutational analysis in the absence of malignancy may be caused by the presence of noninvasive “hyperplastic duct lesions” containing K-ras mutations (23, 24, 29, 30). Hyperplastic duct lesions are frequently found together with cancer in the pancreas, and indeed, there is evidence that these duct hyperplasias can progress to infiltrating carcinoma with K-ras mutation as an early event (30–32). K-ras mutations are also found in hyperplasias in patients with chronic pancreatitis, a condition thought to be a risk factor for developing pancreatic cancer (23, 33). However, it is clear that not all duct hyperplasias progress to invasive carcinoma during the life span of an average patient (24). A longer follow-up would, therefore, be needed to better understand the meaning of the observations in the eight patients in our study without obvious neoplastic disease who harbored K-ras mutations in their brush cytology. A recent study found no cancer in 20 patients with pancreatitis and K-ras mutation in their pancreatic juice after a mean follow-up of 78 months (34). On the other hand, Brat et al. (35) reported three patients with hyperplastic duct lesions who developed pancreatic cancer after 17 months to 10 years, and Berthelemy et al. (13) reported two patients without evidence of cancer at the time of ERCP but with mutated K-ras in their pancreatic juice who developed clinically detectable pancreatic cancers after 18 and 40 months. Nonetheless, it seems best, at present, to consider the eight patients in our study false-positives until it is proven otherwise. Only two of these patients had chronic pancreatitis. Tissue for K-ras analysis was available from one of these patients with primary sclerosing cholangitis in whom the resected biliary stenosis did not harbor a K-ras mutation.

As in our study, the specificity of cytology reported in the literature is often 100% or approaching 100% (1, 2). Interestingly, the two patients with false-positive cytologies also had K-ras mutations detected in their cytology specimens. One patient was a 62-year-old white male. During ERCP, a regular smooth stenosis of the distal common bile duct was seen. He had undergone a cholecystectomy for cholelithiasis in the past; hence, the stenosis was diagnosed as postsurgical. The stenosis was stented, and since then, he has not been jaundiced and had any other complaints. There was no evidence of bile duct obstruction 18 months after brush cytology. The other patient was a 71-year-old white female, also with a postsurgical stenosis. The mid-common bile duct stenosis was treated with a stent. Eighteen months after brush cytology, she had no complaints of extrahepatic bile duct obstruction. These two patients clearly did not meet our criteria for a clinical diagnosis of a malignant bile duct stenosis. Nonetheless, even in retrospect, the cytologies of these two patients were considered positive for carcinoma (Fig. 2, A and B, respectively). One could speculate that these cells came from pancreatic duct lesions with high-grade dysplasia or from an in situ carcinoma, which would also explain the K-ras mutations detected in these patients. Long-term follow-up may then provide a clue to their final diagnosis.

In 60 cases, we were able to directly compare the K-ras mutations identified in brush cytology specimens to those present in the corresponding surgical specimens. We found that the results were identical in 88% (53 of 60) of the cases with malignancy. The main cause for false-negative results was the absence of K-ras mutations in the tumor (26 of 33), mostly cancers other than pancreatic cancer. The discrepant results from the seven patients in which wild-type K-ras was found in the brush cytology but mutant K-ras was detected in the patients’ carcinoma could be due to sampling error because the conventional cytology in these cases was also negative for carcinoma.

More direct sampling of the stenotic lesion could potentially improve the sensitivity of cytology but would diminish specificity of the K-ras mutational analysis. Van Laethem et

<table>
<thead>
<tr>
<th>Final diagnosis</th>
<th>Positive</th>
<th>Negative</th>
<th>Suspect</th>
<th>Insufficient</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
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<td>21</td>
<td>6</td>
<td>3*</td>
<td>38</td>
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<td>4</td>
<td>2</td>
<td>24</td>
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<td>1</td>
<td>14</td>
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<td>4</td>
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<td>5</td>
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</tr>
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<td>1</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
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<td>K-ras negative</td>
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<td>1</td>
<td>5</td>
<td></td>
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</tr>
<tr>
<td>K-ras positive</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-ras negative</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*One specimen could not be amplified and was called K-ras negative.

<table>
<thead>
<tr>
<th>Mutations in carcinomas (a)</th>
<th>Cys</th>
<th>Ser</th>
<th>Arg</th>
<th>Val</th>
<th>Asp</th>
<th>Ala</th>
<th>Gly (b)</th>
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<tr>
<td>Ser</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
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<tr>
<td>Val</td>
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<td>Asp</td>
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<td></td>
<td>1</td>
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<td></td>
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<td>26</td>
</tr>
</tbody>
</table>

Total 1 2 8 15 1 33 60

\(a\) The six possible mutations code for cysteine, serine, arginine, valine, aspartic acid, and alanine.

\(b\) Wild-type codon 12 (GGT) codes for glycine.
al. (22) examined the diagnostic value of K-ras in pancreatic duct brushings and bile duct brushings. Sensitivity of conventional light microscopy of endobiliary brush cytology was similar in their study. They also showed the additional diagnostic value of K-ras mutation analysis in these cytology specimens, especially in the diagnosis of patients with pancreatic cancer, and the high specificity. In contrast, they found that the sensitivity of conventional cytology of pancreatic duct brushings is lower compared to cells derived from carcinomas of the bile duct, the yield of cells from these hyperplastic duct lesions that are frequent in the pancreas with chronic inflammation is lower compared to cells derived from carcinomas because the cells in carcinomas grow less coherently and are easily shed. Following this reasoning, colorectal neoplasms can be diagnosed specifically with the detection of K-ras mutations in the stool despite the frequent occurrence of K-ras mutations present in aberrant crypt foci and hyperplastic polyps, two nonneoplastic lesions that are prevalent in the colorectum without neoplastic disease (36, 37).

In conclusion, PCR-based tests for the detection of K-ras codon 12 mutations can be a valuable diagnostic adjunct to conventional light microscopy of endobiliary brush cytology specimens obtained from patients with a suspicious stenosis of the extrahepatic bile duct, especially in patients with pancreatic carcinoma. The presence of a mutation favors malignancy, even when the cytology reading is negative or equivocal.

REFERENCES


Germline and Somatic Mutations of the \textit{STK11/LKB1} Peutz-Jeghers Gene in Pancreatic and Biliary Cancers


From the Departments of Pathology, Urology, and Surgery and the Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, Maryland; the Department of Pathology, The Academic Medical Center, Amsterdam, The Netherlands; and the Department of Internal Medicine, The Academic Hospital, Erasmus University, Rotterdam, The Netherlands

Peutz-Jeghers syndrome (PJS) is an autosomal-dominant disorder characterized by hamartomatous polyps in the gastrointestinal tract and by pigmented macules of the lips, buccal mucosa, and digits. Less appreciated is the fact that PJS also predisposes patients to an increased risk of gastrointestinal cancer, and pancreatic cancer has been reported in many PJS patients. It was recently shown that germline mutations of the \textit{STK11/LKB1} gene on distal chromosome 19p, which encoded a novel serine/threonine kinase, were responsible for PJS.\textsuperscript{6–9} The increased risk for cancer among PJS patients would suggest that \textit{STK11/LKB1} is a candidate tumor-suppressor gene,\textsuperscript{10} but the role of \textit{STK11/LKB1} gene inactivation in neoplasia has not been conclusively demonstrated.\textsuperscript{11–14}

Pancreatic cancer is an attractive neoplasm to examine for inactivation of \textit{STK11/LKB1}, because it is one of the more common neoplasms to develop in PJS patients. Of the 53 PJS patients reported in four independent studies, six (11%) were diagnosed with pancreatic adenocarcinoma.\textsuperscript{2–5} The demonstration that the \textit{STK11/LKB1} is inactivated in the pancreatic cancer of a PJS patient and in sporadic pancreatic cancers would strongly support a causal link between these mutations and the development of pancreatic cancers and would help establish the tumor-suppressor role of \textit{STK11/LKB1} in neoplasia.

Materials and Methods

PJS Patient and DNA Analysis

Patient PJS1 was an affected family member of a well-followed kindred with PJS.\textsuperscript{15} She had biopsy-proven Peutz-Jeghers polyps of the duodenum (Figure 1A) and was diagnosed with adenocarcinoma at the age of 35 on biopsy of a peripancreatic lymph node, thought originally and on review to be most consistent with a pancreatic origin on the basis of histological features (Figure 1B). DNA was prepared from microdissected histological sections of her surgically biopsied cancer and Peutz-Jeghers polyps. Microdissected samples were incubated overnight at 37°C in 0.04% proteinase K, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, and 1% Tween-20. Proteinase K was inactivated at 95°C for 8 minutes before DNA analysis.

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Peutz-Jeghers syndrome (PJS) is an autosomal-dominant disease characterized by nonneoplastic hamartomas of the gastrointestinal tract and by mucocutaneous melanin macules.\textsuperscript{1} PJS has also been associated with an increased risk of developing cancers.\textsuperscript{2–5} Gastrointestinal, gynecologic, lung, breast, thyroid, basal cell, prostate, and pancreatic cancers have been reported in PJS patients.\textsuperscript{2–5} It was recently shown that germline mutations of the \textit{STK11/LKB1} gene on distal chromosome 19p, which encoded a novel serine/threonine kinase, were responsible for PJS.\textsuperscript{6–9} The increased risk for cancer among PJS patients would suggest that \textit{STK11/LKB1} is a candidate tumor-suppressor gene,\textsuperscript{10} but the role of \textit{STK11/LKB1} gene inactivation in neoplasia has not been conclusively demonstrated.\textsuperscript{11–14}

Pancreatic cancer is an attractive neoplasm to examine for inactivation of \textit{STK11/LKB1}, because it is one of the more common neoplasms to develop in PJS patients. Of the 53 PJS patients reported in four independent studies, six (11%) were diagnosed with pancreatic adenocarcinoma.\textsuperscript{2–5} The demonstration that the \textit{STK11/LKB1} is inactivated in the pancreatic cancer of a PJS patient and in sporadic pancreatic cancers would strongly support a causal link between these mutations and the development of pancreatic cancers and would help establish the tumor-suppressor role of \textit{STK11/LKB1} in neoplasia.
Tissue Samples and Cell Lines

Cancers of the pancreas and distal common bile duct resected at The Johns Hopkins Hospital between 1992 and 1997 were xenografted as described.\(^{16}\) In addition, at the time of the surgery, resected normal duodenal mucosa was frozen and stored at \(-80^\circ\text{C}\). The pancreatic cell lines Su86.86, CFPAC1, AsPC1, Capan1, Capan2, Panc1, MiaPaCa2, BxPc3, and HS766T were purchased from American Type Culture Collection (Manassas, VA) and COLO357 from European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). Pancreatic cell line PL45 was established in our laboratory.\(^{16}\)

Homozygous Deletion Analysis

Genomic DNA samples (40 ng per sample) were screened for homozygous deletions using PCR analysis as previously described.\(^{16,17}\) The primers used to amplify exon 1, 4/5, and 9 of \(STK11/LKB1\) were as reported previously.\(^{8}\) Duplex PCR analyses were performed with pairs of internal control primers and \(STK11/LKB1\)-specific primers. Amplification of \(\text{integrin-}\beta-4\) or \(\text{MKK4}\) was used as a positive internal control. Primers are as listed in Table 1.

Loss of Heterozygosity and Sequence Analyses

Loss of heterozygosity (LOH) was determined using three polymorphic markers, D19S886, D19S565, and D19S216 (Research Genetics, Huntsville, AL). LOH was considered to be conclusive only when analysis of the neoplastic DNA showed the complete loss of one of the two alleles present in the patient’s corresponding normal DNA. When a normal DNA sample was unavailable, LOH status was presumptively shown by the unambiguous presence of only a single allele size at all three polymorphic markers evaluated. All samples which displayed conclusive or presumptive LOH were subject to sequencing. Each exon was amplified by PCR from genomic DNA, treated with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH), and subjected to cycle-sequencing (ThermoSequenase, Amersham, Arlington Heights, IL). The majority of the PCR primers have been reported previously.\(^{8}\) Additional primers are listed in Table 1.

Results

Germline Mutation of \(STK11/LKB1\) and Tumorigenesis

To determine the genetic basis for the increased risk of cancer among PJS patients, we examined the status of the \(STK11/LKB1\) gene in cancer tissues obtained from a patient diagnosed with PJS. In patient PJS1, the known
germline mutation of this family at the splice donor site of intron 3 of STK11/LKB1 was demonstrated in nonneoplastic tissue (Table 3) (Figure 2A). DNA from this patient’s microdissected adenocarcinoma and epithelium of a Peutz-Jeghers intestinal polyp were then sequenced and the second allele of STK11/LKB1 was lost (>80% decrease in allele intensity by densitometry) in the pancreatic cancer, but not in the intestinal polyp (Figure 2B). Due to the limited amount of archival material, only limited sequencing was performed. Because LOH is not the only mechanism of gene inactivation, it is possible that the second allele of STK11/LKB1 in the polyp could be inactivated by methylation, small deletions, or point mutation outside of intron 3. The germline mutation is predicted to affect splicing of the STK11/LKB1 transcript.

**Somatic Inactivation of STK11/LKB1 in Pancreatic and Biliary Cancers**

To further validate STK11/LKB1 as a tumor-suppressor gene, we evaluated the role of somatic mutation in STK11/LKB1 in sporadic pancreatic cancer. Using primers specific for exon 1, 4/5, and 9 of STK11/LKB1, we screened for homozygous deletions among a panel of 100 xenografts of primary pancreatic ductal adenocarcinomas, 16 xenografts of primary distal common bile duct adenocarcinomas, 19 xenografts of other primary carcinomas of the periampullary region (predominantly duodenal and ampullary cancer), and 11 pancreatic cancer cell lines (Table 2). One pancreatic (PX30) and one distal common bile duct (PX115) adenocarcinoma exhibited

### Table 2. Sporadic Neoplasms Analyzed for STK11/LKB1 Mutations

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Total number of samples</th>
<th>Homozygous deletion screening</th>
<th>LOH study</th>
<th>Sequencing</th>
<th>Number of mutated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic adenocarcinoma</td>
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<td>100</td>
<td>92</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Biliary adenocarcinoma</td>
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<td>16</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pancreatic cell lines</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Other carcinomas*</td>
<td>19</td>
<td>19</td>
<td>0</td>
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<td>0</td>
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</tbody>
</table>

*Primary carcinomas of the periampullary region, predominantly duodenal and ampullary cancers.
homozygous deletions of STK11/LKB1 (Figure 3). The entire genomic sequence of STK11/LKB1 was deleted from PX30, whereas only exon 1 of STK11/LKB1 was deleted in PX115. Both homozygous deletions were confirmed by duplex PCR (Figure 3) and verified in parallel xenografts derived from the same primary tumor samples (data not shown). In PX115, adequate DNA was available for Southern blot analysis, which confirmed the absence of STK11/LKB1 sequences (data not shown). The homozygously deleted regions in PX30 and PX115 did not extend to the closest available neighboring markers, D19S886 and D19S565. These markers were originally used to define the distal and proximal boundaries in maps of the PJS gene localization.6,7

LOH frequency at the STK11/LKB1 locus in pancreatic cancer was determined with the highly polymorphic markers D19S886, D19S565, and D19S216 (heterozygosity index = 0.61, 0.81, and 0.76, respectively). Conclusive LOH was found in 22 of the 69 pancreatic cancers for which normal DNA was available (32%) and presumptive LOH was inferred in 8 of the 29 pancreatic cancers (28%). Presumptive LOH of 19p at the STK11/LKB1 locus was seen in 9 of the 11 pancreatic cancer cell lines (82%). In addition, four cancers harbored LOH breakpoints between D19S886 and D19S565. The localization of these breakpoints to the STK11/LKB1 locus further suggests that STK11/LKB1 is the target of the allelic loss observed. All coding sequences and splice junctions of the STK11/LKB1 gene amplified from the genomic DNA of the 39 selected pancreatic xenografts and cancer cell lines exhibiting conclusive or presumptive LOH were sequenced (Table 2). One non-sense and two frameshift mutations were detected (3 of 103 (3%) studied for LOH) (Figure 4, A and B, and Table 3) and confirmed in independent PCR products amplified from the samples. One mutation was in exon 1 and one in exon 5, and both of these were within the catalytic kinase domain of STK11/LKB1 (codons 37–314).8 The third mutation was in exon 8, and it potentially would affect the function of the regulatory domain of STK11/LKB1 that comprises the 119 residues at the carboxyl-terminus.8 Two of the three

![Figure 3. Duplex PCR analysis of homozygous deletions in pancreatic and biliary cancers. Detection of homozygous deletions in the genomic DNAs of pancreatic cancer xenograft PX30 and biliary cancer xenograft PX115 by duplex PCR using pairs of internal control primers (INTB4-B or MKK4-E) and STK11/LKB1-specific primers. The entire coding region of STK11/LKB1 was deleted in PX30. Only exon 1 of STK11/LKB1 was deleted in PX115.](image)

![Figure 4. Somatic mutations in the exonic sequences of STK11/LKB1. A: PX68 (lanes 2) and PX68-1A (lanes 3) are two parallel xenografts of the same pancreatic cancer, and both exhibited the same somatic nucleotide substitution that created a stop codon (a non-sense mutation). The mutation was not detected in the normal tissue of the patient (PN68, lanes 1 and 4). Lanes 5 belong to another pancreatic tumor xenograft, which did not harbor a somatic mutation in STK11/LKB1. The arrow points to the site of the mutation. B: Xenograft tumor PX104 (lanes 3) and its corresponding primary cancer, PC104 (lanes 4), harbored the same nucleotide deletion, which was not detected in the normal tissue of the patient (PN104, lanes 2) or in another pancreatic xenograft (lanes 4). The arrow indicates the site of the mutation.](image)
testicular, and breast cancers. Here, we provide biallelic somatic inactivation of a gene has been cloned, several efforts have failed to show several reports of LOH on 19p in breast, colorectal, and sporadic forms of a cancer. Four independent studies have shown that the risk of death from gastrointestinal cancer among PJS patients is 13- to 30-fold greater than the risk shown that the risk of death from gastrointestinal cancer is diagnosed in patients with PJS ranges from 38–50 years, and there is a reported 20–25 years of latency from the time of PJS diagnosis. There have been several reports of LOH on 19p in breast, colorectal, and pancreatic cancers; however, since the STK11/LKB1 gene has been cloned, several efforts have failed to show biallelic somatic inactivation of STK11/LKB1 in colorectal, testicular, and breast cancers. There have been several reports of LOH on 19p in breast, colorectal, and pancreatic cancers; however, since the STK11/LKB1 gene has been cloned, several efforts have failed to show biallelic somatic inactivation of STK11/LKB1 in colorectal, testicular, and breast cancers. Furthermore, this gene appears to play a role in the development of both sporadic and familial (PJS) pancreatic and biliary cancers. In sporadic cancers, STK11/LKB1 was somatically inactivated in 4% of the pancreatic cancers and in at least 6% of biliary cancers examined. The patient with a familial (PJS) pancreatic cancer inher-

**Table 3. Mutations of the STK11/LKB1 Gene Identified in Pancreatic and Biliary Cancers**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Sample</th>
<th>Allele loss</th>
<th>Position of gene alteration*</th>
<th>Gene alteration†</th>
<th>Predicted product</th>
<th>Origin of gene alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic</td>
<td>PJS1</td>
<td>LOH</td>
<td>Nucleotide +2 Introns 3</td>
<td>CGG gtg to CGG ggtg</td>
<td>Insertion, altered splicing</td>
<td>Germline</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>PX30</td>
<td>LOH</td>
<td>Exons 1 to 9</td>
<td>Homozygous deletion</td>
<td>Absence</td>
<td>Somatic</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>PX68</td>
<td>LOH</td>
<td>Codon 36 Exon 1</td>
<td>TAC CAG to TAA CAG</td>
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<td>LOH</td>
<td>Codon 217 Exon 5</td>
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<tr>
<td>Pancreatic</td>
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<td>LOH</td>
<td>Codon 312 Exon 8</td>
<td>AAA CAT C to AAC ATC</td>
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<td>Somatic</td>
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<td>Biliary</td>
<td>PX115</td>
<td>LOH</td>
<td>Exon 1</td>
<td>Homozygous deletion</td>
<td>Absence</td>
<td>Somatic</td>
</tr>
</tbody>
</table>

*Codons, nucleotides, and exons are numbered according to GDB entries AF032984, AF032985, and AF032986.
†The underlined nucleotides are either deleted or inserted. Exonic sequences are in capital letters and intronic sequences are in lower case. The spaces between trinucleotides denote codon structure.

The xenografted series of pancreatic and biliary cancers, in which we demonstrated the inactivation of STK11/LKB1, have been well characterized genetically, providing additional opportunities to examine the tumor-suppressor role of STK11/LKB1. For example, it would be unusual for two genes in the same pathway to be inactivated in a cancer. We can therefore infer that the STK11/LKB1 suppressive pathway is distinct from the p53, p16, and DPC4 pathways; genetic inactivations of the p53 and p16 genes are known to coexist in tumor PX68, and DPC4 is homozygously deleted from tumors PX30 and PX115. K-ras, which is mutated in 95% of pancreatic cancer cases, is also mutated in tumors PX30, PX68, and PX104.

In summary, we demonstrated the biallelic inactivation of STK11/LKB1 in a pancreatic cancer of a patient with the PJS and in 4–6% of sporadic pancreatic and biliary adenocarcinomas, illustrating the role of this gene in familial and sporadic cancer development.

**Discussion**

PJS predisposes affected family members to the development of cancer. Four independent studies have shown that the risk of death from gastrointestinal cancer among PJS patients is 13- to 30-fold greater than the risk shown that the risk of death from gastrointestinal cancer is diagnosed in patients with PJS ranges from 38–50 years, and there is a reported 20–25 years of latency from the time of PJS diagnosis. There have been several reports of LOH on 19p in breast, colorectal, and pancreatic cancers; however, since the STK11/LKB1 gene has been cloned, several efforts have failed to show biallelic somatic inactivation of STK11/LKB1 in colorectal, testicular, and breast cancers. Furthermore, this gene appears to play a role in the development of both sporadic and familial (PJS) pancreatic and biliary cancers. In sporadic cancers, STK11/LKB1 was somatically inactivated in 4% of the pancreatic cancers and in at least 6% of biliary cancers examined. The patient with a familial (PJS) pancreatic cancer inher-

**Acknowledgments**

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**References**

Advances in Brief

Discovery of New Markers of Cancer through Serial Analysis of Gene Expression: Prostate Stem Cell Antigen Is Overexpressed in Pancreatic Adenocarcinoma


Abstract

Serial analysis of gene expression (SAGE) can be used to quantify gene expression in human tissues. Comparison of gene expression levels in neoplastic tissues with those seen in nonneoplastic tissues can, in turn, identify novel tumor markers. Such markers are urgently needed for highly lethal cancers like pancreatic adenocarcinoma, which typically presents at an incurable, advanced stage. The results of SAGE analyses of a large number of neoplastic and nonneoplastic tissues are now available online, facilitating the rapid identification of novel tumor markers. We searched an online SAGE database to identify genes preferentially expressed in pancreatic cancers as compared with normal tissues. SAGE libraries derived from pancreatic adenocarcinomas were compared with SAGE libraries derived from nonneoplastic tissues. Three promising tags were identified. Two of these tags corresponded to genes (lipocalin and trefoil factor 2) previously shown to be overexpressed in pancreatic carcinoma, whereas the third tag corresponded to prostate stem cell antigen (PSCA), a recently discovered gene thought to be largely restricted to prostate basal cells and prostatic adenocarcinomas. PSCA was expressed in four of the six pancreatic cancer SAGE libraries, but not in the libraries derived from normal pancreatic ductal cells. We confirmed the overexpression of the PSCA mRNA transcript in 14 of 19 pancreatic cancer cell lines by reverse transcription-PCR, and using immunohistochemistry, we demonstrated PSCA protein overexpression in 36 of 60 (60%) primary pancreatic adenocarcinomas. In 59 of 60 cases, the adjacent nonneoplastic pancreas did not label for PSCA. PSCA is a novel tumor marker for pancreatic carcinoma that has potential diagnostic and therapeutic implications. These results establish the validity of analyses of SAGE databases to identify novel tumor markers.

Introduction

SAGE is a recently described technique that allows one to obtain a quantitative and comprehensive profile of cellular gene expression (1, 2). Briefly, in this procedure, cellular mRNA transcripts are converted to cDNA and then cleaved at specific sites by restriction enzymes into small (10–14 bp) fragments, also known as tags. These tags are ligated together into difragments, amplified by PCR, and then concatenated and sequenced as one long fragment of DNA. Each 10–14-bp fragment (tag) should uniquely identify a specific gene transcript because it corresponds to a defined sequence near the transcript’s 3’ terminus, as dictated by the tagging restriction enzyme used (1). The abundance of each tag provides a quantitative measure of the transcript level present within the mRNA sample analyzed, which therefore allows expression levels of specific transcripts to be compared between two samples (2). This ability to quantitate gene expression represents a major advantage of SAGE over other methods of screening cDNA libraries for differentially expressed genes.

In the initial demonstration of the SAGE technique, a gene expression profile of the normal pancreas was constructed and validated by Northern blotting (1). Subsequently, Zhang et al. (2) used SAGE to demonstrate differences in expression patterns between colonic and pancreatic adenocarcinomas and normal colonic epithelium. Such applications of SAGE hold tremendous promise for the identification of diagnostic and/or prognostic markers of malignancy. Indeed, the above-referenced analyses identified several promising serum markers for pancreatic carcinoma, such as tissue inhibitor of metalloprotease 1 (3).

Three recent advances have made analyses of SAGE libraries for differentially expressed genes more feasible. First, rapid progress in the Human Genome Project has facilitated the mapping of specific genes to individual tags specified by SAGE (4). Fewer tags now correspond to ESTs of unknown origin, and more can be assigned to known genes. Second, a large number of normal and neoplastic tissues have now been analyzed by SAGE, creating extremely large databases for study. Third, much of this database is now online and available to the general public (5, 6). As of February 1, 2001, this online database included 88 SAGE libraries, and 3,632,974 tags.

Armed with these tools, we searched an online SAGE database to identify novel markers of pancreatic adenocarcinoma.

Materials and Methods

Based on the identification of differentially expressed genes in our ongoing SAGE investigation of pancreatic cancer, the xProfiler program available online was used to compare gene expression patterns in pancreatic cancer with those in nonneoplastic tissues. In this program, one can select SAGE libraries for analysis and then compare the tags in one group of online SAGE libraries with the tags in another group. We used two queries to determine differentially expressed genes. In the first strategy, we chose a pancreatic adenocarcinoma group composed of the SAGE libraries of four pancreatic cancer cell lines that yielded 96,494 total tags (CAPAN1, 37,926 tags; CAPAN2, 23,222 tags; HS766T, 10,467 tags; and Panc1, 24,879 tags). The nonneoplastic comparison group in this analysis was composed of the SAGE libraries of two short-term cultures of normal pancreatic duct epithelial cells that yielded 64,577 tags (HX, 32,157 tags; and H126, 32,420 tags). In the second query, we expanded both

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3 The abbreviations used are: SAGE, serial analysis of gene expression; PanIN, pancreatic intraepithelial neoplasia; PSCA, prostate stem cell antigen; TFF2, trefoil factor 2; RT-PCR, reverse transcription-PCR; EST, expressed sequence tag.
groups. We expanded the pancreatic cancer group to include the SAGE libraries of two primary pancreatic adenocarcinomas (Panc 91-16113, 33,941 tags; Panc 96-6252, 35,745 tags) in addition to the four above-mentioned pancreatic cancer cell lines. This raised the total number of tags in this group to 166,180. We also expanded the nonneoplastic group to include the SAGE libraries of normal colon epithelium (NC1 and NC2, 50,115 and 49,552 tags, respectively), normal ovarian surface epithelium (HOSE 4 and IOSE29-11, 48,113 and 48,498 tags, respectively), human microvascular endothelial cells (Duke HMEVC and Duke HMEVC + VEGF, 52,532 and 57,928 tags, respectively), normal luminal mammary epithelial cells purified with BER-EP4 antibody conjugated to magnetic beads (mammary epithelium and Br N, 49,137 and 37,558 tags, respectively), and normal prostate (Chen Normal Pr and normal prostate, 66,193 and 13,148 tags, respectively) in addition to the above-mentioned short-term cultures of normal pancreatic ductal epithelium (HX and H126). This raised the total number of tags in this group of 12 nonneoplastic SAGE libraries to 537,681. We set each of the two analyses to display the 100 SAGE tags that were most likely expressed at levels of 10-fold difference between the two groups. The coefficient of variance cutoff settings were kept at the default value of 0%.

The names of genes and ESTs were identified from the tag sequences using an online resource from the National Center for Biotechnology Information.6

Virtual Northern. The online SAGE database also has a feature that allows the user to create “virtual Normars.” This tool allows one to view the expression levels of selected SAGE tags in all of the SAGE libraries. Data are presented as “virtual Normars,” allowing the user to simultaneously visualize the levels of gene expression across multiple samples.

Cell Lines. Human cell lines AsPC1, BxPc3, CAPAN1, CFPAC1, HS766T, MiaPaCa2, and Panc1 were obtained from the American Type Culture Collection (Manassas, VA). The 12 PL cell lines (PL1–6, PL8–11, PL13, and PL14) were low-passage pancreatic carcinoma cell lines generously provided by Dr. Elizabeth Jaffee (7). An immortal human pancreatic ductal adenocarcinoma cell line (HPDE) obtained after transduction of the human papillomavirus 16 E6/E7 genes was kindly provided by Dr. Ming-Sound Taso (Hs.20166, GCCCAGCATT, corresponding to the recently discovered mouse oncogene 24p3) and TFF2 (9 –11). The third tag was predicted to contain such nonneoplastic elements (8). Third, only tags expressing more frequently in the pancreatic cancer groups than in the normal group were considered. Second, tags likely to correspond to normal epithelial cell lines were compared with two short-term cultures of normal pancreatic ductal cells. 67 SAGE tags were identified as more frequently expressed in the cancer group than in the normal group, and 33 were identified as more frequently expressed in the normal than in the cancer group. In the second query, in which the 4 pancreatic cancer cell lines and 2 primary pancreatic cancers were compared with 2 short-term cultures of normal pancreatic ductal cells and 10 other nonneoplastic tissues, 74 SAGE tags were identified as more frequently expressed in the cancer group than in the normal group, and 26 SAGE tags were identified as more frequently expressed in the normal group than in the cancer group.

Four criteria were then used to narrow the candidate tags. First, only tags expressed more frequently in the pancreatic cancer groups were considered. Second, tags likely to correspond to normal entrapped pancreatic parenchyma or stromal elements (such as insulin, pancreatic polypeptide, and collagen type 1 and 3 α) were excluded. These tags were identified only with the second query, which included the libraries derived from primary pancreatic cancers that would be predicted to contain such nonneoplastic elements (8). Third, only tags corresponding to known genes were considered, so that tags corresponding to ESTs or rRNAs were excluded. Fourth, only tags appearing within the top 25 tags of both queries were considered.

When these strategies were applied, three tags emerged as the most promising markers for pancreatic cancer. Two of these corresponded to genes that have been previously shown to be overexpressed in pancreatic carcinomas; these were lipocalin 2 (the human homologue of mouse oncogene 24p3) and TFF2 (9 –11). The third tag was Hs.20166 (GCCCAGCATT), corresponding to the recently discovered PSCA gene (12). This tag was identified 38 times in the 166,180 tags derived from the pancreatic cancers, but was never identified in the 64,577 tags derived from normal pancreatic ductal epithelium. This gene was selected for further analysis.

Using the online SAGE Tag to Gene mapping and Virtual Northern functions, we found that 13 of the 88 SAGE libraries in the database

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contained at least one copy of this tag (Fig. 1). These included four of the six (66%) pancreatic cancer SAGE libraries, along with SAGE libraries derived from normal prostate, primary prostate cancer, and seven other malignancies (listed in Fig. 1).

Among the 13 libraries that contained the tag for PSCA, 10 demonstrated a solitary PSCA tag. Given the known potential error rate inherent in the sequencing procedure used to generate SAGE libraries, one must be leery of accepting tags identified only once in a library because they may have been generated by random error. However, among the three libraries with more than one PSCA tag were two pancreas cancer cell lines, CAPAN1 and CAPAN2, which demonstrated 19 and 17 tags, respectively. On normalization for the number of tags per library, SAGE libraries derived from these cell lines demonstrated over 15 times as many PSCA tags as those derived from any other tissue in the database (Fig. 1).

RT-PCR. Primers corresponding to the PSCA and TFF2 transcripts were designed, and RT-PCR was performed on RNA extracted from 19 pancreatic cell lines. The TFF2 transcript was identified in 16 of the 19 cell lines, and the PSCA transcript was identified in 14 of the 19 cell lines (74%; Fig. 2). Of note, the RT-PCR analyses parallel the results of SAGE analyses. For example, the PSCA transcript was demonstrated in cell line CAPAN1 by RT-PCR, and this cell line demonstrated a high 500 tags/million PSCA expression level with the online SAGE virtual Northern. Also, the PSCA tag was not identified in the online SAGE library corresponding to cell line Panc1, which correlates with the negative RT-PCR result we obtained on RNA extracted from this cell line.

Immunohistochemistry. Using the 1:200 and 1:700 dilutions of the monoclonal anti-PSCA antibody, normal pancreatic tissue did not label, with the exception of a single case in which atrophic pancreatic ducts in an area of chronic pancreatitis labeled weakly. Overall, 36 of 60 tumors (60%) labeled for PSCA (Table 1). In four cases, labeling was focal (1–25% of tumor cells labeled), whereas in four other cases, the labeling was essentially uniform throughout the tumor (75–100% of tumor cells labeled). In the remaining 28 cases, 26–75% of the neoplastic cells were labeled. Similar labeling patterns were seen with both dilutions of the antibody, with the labeling being weaker but still present in all cases at the 1:700 dilution. In general, labeling was intense (3+ or greater) within most (28 of 36) tumors and clearly demarcated them from adjacent normal tissues (Fig. 3). Labeling was most often heterogeneous within malignant glands of the tumors, such that some malignant cells labeled strongly, whereas others were completely negative. Frequently, PSCA labeling often appeared to be accentuated at the luminal border of the neoplastic glands (Fig. 3D), and the luminal contents were frequently labeled. A range of PanIN was identified on the sections studied (13). These consisted of 50 duct profiles containing PanINs derived from 17 cases. These PanINs labeled variably (Table 1). Among the 16 PanIN-1A lesions, 9 labeled, whereas 7 did not label. Among the 20 PanIN-1B lesions, 12 labeled (4 focally), whereas 8 did not label. Among seven PanIN-2

<table>
<thead>
<tr>
<th>Library name</th>
<th>Source</th>
<th>Tags per million</th>
<th>Tag counts</th>
<th>Total tags</th>
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Table 1 PSCA immunolabeling summary

<table>
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<tr>
<th></th>
<th>Normal pancreas (n = 60)</th>
<th>PanIN (n = 50)</th>
<th>Infiltrating pancreatic adenocarcinoma (n = 60)</th>
</tr>
</thead>
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<tr>
<td>Negative</td>
<td>59 (98%)</td>
<td>25 (50%)</td>
<td>24 (40%)</td>
</tr>
<tr>
<td>Focal</td>
<td>0</td>
<td>5 (10%)</td>
<td>4 (7%)</td>
</tr>
<tr>
<td>Positive</td>
<td>1* (2%)</td>
<td>20 (40%)</td>
<td>32 (53%)</td>
</tr>
</tbody>
</table>

* Weak labeling in a case of chronic pancreatitis.

Fig. 1. Online SAGE Tag to Gene Mapping function (Virtual Northern) demonstrating the distribution and frequency of the HS.20166 (GC-CAGCCATT) tag PSCA within the cumulative online SAGE library composed of 3,632,974 tags derived from 88 individual libraries. The tags per million column gives a quantitation of the specific tag’s frequency within a specific library, which reflects the level of the corresponding transcript. Libraries corresponding to the two cell lines derived from pancreatic carcinomas (CAPAN1 and CAPAN2) demonstrate by far the greatest relative number of PSCA tags.

Fig. 2. RT-PCR of 19 pancreatic cancer cell lines, an immortal human pancreatic ductal epithelial cell line (HPDE), and a water control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as a RNA control. PSCA is expressed in 14 of the 19 pancreatic cancer cell lines, and TFF2 is expressed in 16 of the 19 pancreatic cancer cell lines.

Fig. 3. Immunohistochemistry of a case of chronic pancreatitis demonstrating PSCA expression. (A) Low-power view of a chronic pancreatitis lesion with focal (3+) PSCA labeling of atrophic ducts. (B) PanIN-1A demonstrating 1+ focal PSCA labeling. (C) PanIN-1B with 2+ diffuse PSCA labeling. (D) PanIN-2 with 3+ diffuse PSCA labeling. (E) Malignant ductal adenocarcinoma demonstrating diffuse 2+ PSCA labeling.

4322

PSCA OVEREXPRESSION IN PANCREATIC CARCINOMA
lesions, three labeled, whereas four did not label. Among seven PanIN-3 lesions, only one labeled focally, whereas six did not label.

Clinical Correlates. The presence or absence of PSCA immunoreactivity in the pancreatic adenocarcinomas was correlated with a variety of clinicopathological factors. There was a trend for PSCA immunoreactivity to be more frequent in pancreatic carcinomas in men than in women (68% versus 55%; \( P \) nonsignificant). No significant correlations were found between PSCA labeling and tumor size, lymph node status, margin status, race, age, or survival.

Discussion

This year, it has been estimated that 28,000 Americans will be diagnosed with pancreatic cancer, and 28,000 will die from it (14, 15). Tragically, patients are usually asymptomatic until the tumor has reached an advanced stage and is incurable with existing therapy. Current methods of early detection are inadequate. Therefore, there is a great need to develop new markers that will increase our ability to diagnose this deadly cancer.

We analyzed an online SAGE database and identified a new and previously unsuspected marker for pancreatic carcinoma, PSCA. We confirmed the presence of the PSCA mRNA transcript by RT-PCR analysis in pancreatic cancer cell lines and verified protein expression by immunohistochemical analysis of 60 surgically resected pancreatic ductal adenocarcinomas. These studies were concordant because overexpression of PSCA was identified in approximately two-thirds of the pancreatic cancers studied by each technique. In 59 of the 60 pancreata examined immunohistochemically, the adjacent nonneoplastic pancreatic parenchyma did not label. In one case, atrophic pancreatic parenchyma in an area of chronic pancreatitis labeled.

PSCA encodes a 123-amino acid glycoprotein that is anchored to the cell membrane by a glycosylphosphatidylinositol anchor (12). It has been demonstrated to have limited normal tissue distribution by RT-PCR and immunohistochemical studies and is expressed most strongly in the prostate, where it is localized to the putative stem cell component of the prostate, the basal cells. Significantly, PSCA has been demonstrated to be overexpressed by more than 80% of prostatic carcinomas and correlates with the aggressive features of high stage, high Gleason grade, and androgen independence (16). Normal pancreatic tissue does not express PSCA by Northern blotting (12) or by immunohistochemistry (16).

The finding of PSCA overexpression in pancreatic cancer has several immediate applications. The immunohistochemical labeling assay for PSCA could prove useful for diagnostic purposes. Because PSCA is not expressed in normal pancreas, expression of PSCA could support the diagnosis of pancreatic adenocarcinoma, particularly in small biopsy or cytopathology samples. However, our identification of PSCA in some PanINs and in one atrophic pancreas indicates that PSCA labeling in and of itself is not accurate enough to establish the diagnosis of invasive carcinoma in the pancreas.

The immunohistochemical labeling pattern we identified with PSCA, that of frequent accentuation at the luminal borders of the malignant glands, raises the possibility that PSCA may be secreted into pancreatic juice or released into the blood. If so, tests could be devised to detect PSCA in the blood, in duodenal and pancreatic fluids or in stool samples, thereby providing a new marker of pancreatic malignancy. Indeed, PSCA protein has been demonstrated to be secreted in vitro by 293T cells that are transfected with PSCA (12). However, given that approximately one-third of pancreatic cancers do
not overexpress PSCA, this potential marker would not be expected to be 100% sensitive. Indeed, a growing body of evidence now suggests that a panel of markers may be needed to screen for pancreatic cancer (3).

Finally, as a cell surface protein, PSCA has shown promise as a target for immunotherapy of advanced cancers of the prostate (17, 18). Jaffee et al. (19) have recently demonstrated that immunotherapy can be safe and effective in patients with pancreatic cancer, and our findings raise the possibility that PSCA may be a rational immune target in pancreatic cancers that overexpress PSCA.

In summary, we demonstrate that searching an online SAGE database for tags differentially expressed in the libraries derived from neoplastic and nonneoplastic tissues can lead to the discovery of novel neoplastic markers.

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References


Differing Rates of Loss of Dpc4 Expression and of P53 Overexpression among Carcinomas of the Proximal and Distal Bile Ducts

Evidence for a Biologic Distinction

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BACKGROUND. Biliary tract carcinomas are clinically heterogeneous. It is not known if molecular heterogeneity underlies the clinical differences.

METHODS. The authors evaluated 128 bile duct carcinomas, 88 of the distal common bile duct and 40 of more proximal origin (28 perihilar carcinomas, 12 intrahepatic carcinomas), immunohistochemically for abnormalities in the expression of the products of the DPC4 and p53 tumor-suppressor genes. Prognostic factors were evaluated in the series of distal bile duct carcinomas for which follow-up information was available.

RESULTS. The authors found that a significantly higher percentage of distal bile duct carcinomas (55%) demonstrated loss of DPC4 expression than did the proximal bile duct carcinomas (15%; P < 0.001). They also found that a significantly higher percentage of the distal tumors abnormally expressed the p53 gene product (51% vs. 26%; P < 0.001). Among the distal common bile duct carcinomas, the presence of poorly differentiated histology correlated with decreased survival in multivariate analysis, while labeling for p53 or Dpc4, margin status, lymph node status, and tumor dimension did not correlate significantly with survival.

CONCLUSIONS. These results demonstrate that abnormalities in DPC4 and p53 gene expression are frequent in distal common bile duct carcinomas, just as they are in pancreatic ductal adenocarcinoma, suggesting that these two tumor types might share a similar molecular pathogenesis. They also show that proximal and distal bile duct carcinomas have different patterns of inactivation of tumor-suppressor genes, indicating that they often arise through different molecular mechanisms likely reflecting their differing etiologies. Cancer 2001;91:1332–41.

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KEYWORDS: cholangiocarcinoma, tumor-suppressor gene, bile duct, DPC4, p53.

The full length of the human adult biliary tract has a common embryologic origin as a ventral outgrowth from the caudal end of the foregut. Carcinomas of the biliary tract usually share a similar morphology regardless of location within the biliary tract; most are infiltrating adenocarcinomas that incite prominent stromal desmoplasia and demonstrate perineural invasion. However, the clinical presentations and risk factors for biliary carcinomas are markedly different depending on their site of origin within the biliary tract. Relatively little is known about the role played by tumor-suppressor genes in the pathogenesis of biliary carcinoma, and in particular whether genetic differences underlie their clinical heterogeneity.
The p53 gene, one of the most commonly mutated genes in human cancer, has been studied previously in biliary tract carcinomas. Such studies were facilitated by the availability of an immunohistochemical assay for p53 protein expression that is easily applied to many cases and that correlates well (albeit not perfectly) with p53 gene mutation. Native p53 up-regulates expression of the MDM2 gene, which is responsible for p53 degradation, and therefore inactivating mutations of p53 lead to reduced turnover of the mutant p53 protein that then becomes immunohistochemically detectable. However, immunohistochemical investigations of p53 labeling in bile duct carcinomas have produced variable and sometimes conflicting results. A few intriguing studies have suggested that biliary tract carcinomas differ in their frequency of p53 immunoreactivity depending on their site of origin (gallbladder, intrahepatic, proximal common bile duct, and distal common bile duct), perhaps reflecting different pathogenic factors in their respective etiologies. However, many studies have grouped tumors arising from different sites together, making comparisons impossible. In particular, most have grouped perihilar and distal common bile duct tumors together as "extrahepatic bile duct tumors," whereas few have studied these neoplasms separately.

Recent evidence suggests that the DPC4 (MDA9, SMAD4) gene, a tumor-suppressor gene on the long arm of chromosome 18 that is inactivated in 55% of pancreatic adenocarcinomas but less often in other tumors, also is inactivated in bile duct carcinomas. Initially, Hahn et al. demonstrated a homozygous deletion of the DPC4 gene in a single case of biliary tract carcinoma. The rate of involvement then was estimated in a larger series using a partial gene screening technique, single-strand conformational polymorphism analysis, that would miss some DPC4 intragenic mutations and all potential homozygous deletions. Five of 32 (16%) biliary tract carcinomas had point mutations in the DPC4 gene in this analysis. Appropriately, these authors speculated that the frequency of DPC4 genetic alterations in biliary carcinomas actually might be higher than they reported. Of note, these mutations were identified in 4 of 8 common bile duct carcinomas studied, but in only 1 of 24 other biliary tract carcinomas. Recently, Rijken et al. studied 14 distal common bile duct carcinomas for global genetic changes by comparative genomic hybridization and cytogenetics and found a pattern of losses and gains similar to that observed in pancreatic adenocarcinoma. Specifically, the long arm of chromosome 18, which harbors the DPC4 gene, was the most frequent site of loss (8 of 14 cases, or 56%). Taken together, these latter studies suggested that a larger study of biliary tract tumors for DPC4 gene inactivation might be warranted. However, the prominence of the reactive desmoplastic stroma associated with most bile duct carcinomas and their resulting low neoplastic cellularity render genetic analysis particularly challenging.

An immunohistochemical assay for Dpc4 protein expression recently has been developed. Wilentz et al. have demonstrated that immunohistochemical labeling for the Dpc4 protein mirrors the status of the DPC4 gene in pancreatic adenocarcinoma. Specifically, loss of labeling proved to be at least 91% sensitive for DPC4 gene inactivation, whereas intact labeling was at least 94% specific for intact DPC4. Like p53 labeling, this technique has many advantages over direct genetic analysis: it is less labor intensive, avoids the problems posed by contaminating normal DNA, is readily applicable to archival, formalin fixed, paraffin embedded tissue, and allows direct correlation between gene expression and morphology because it is performed in situ.

Therefore, to further clarify the roles of these tumor-suppressor genes, we analyzed 128 biliary tract carcinomas together as "extrahepatic bile duct tumors," whereas few have studied these neoplasms separately.
carcinomas immunohistochemically for Dpc4 protein and p53 protein labeling abnormalities. This series includes a group of 88 distal common bile duct carcinomas for which clinical follow-up information was available, and hence for which the prognostic significance of variables such as immunohistochemical labeling could be determined. For comparison, a group of 40 more proximal bile duct carcinomas also was analyzed immunohistochemically for p53 and Dpc4 labeling.

MATERIALS AND METHODS

Case Selection

Eighty-eight cases of carcinoma of the distal common bile duct were obtained from a database of patients who had undergone pancreaticoduodenectomies (Whipple resections) at The Johns Hopkins Hospital between August 14, 1989 and June 23, 1999 and carried the clinical diagnosis of bile duct carcinoma. For each case, the macroscopic pathology report and all hematoxylin and eosin–stained slides containing carcinoma were reviewed. Carcinomas were accepted as being of distal bile duct origin if they had an in situ component in the biliary tree and/or appeared macroscopically centered on and microscopically emanating from the bile duct. Carcinomas involving the intrapancreatic portion of the bile duct were excluded if they had a prominent pancreatic intraductal carcinoma (high grade PanIN), because these were presumed to be pancreatic in origin.

Clinical and pathologic characteristics were obtained from The Johns Hopkins Hospital Surgical Pathology files and the Johns Hopkins Oncology Center information system database. Characteristics specifically recorded and analyzed were tumor size, presence or absence of lymph node metastases at surgery, de-
gree of tumor differentiation, and presence or absence of carcinoma at a surgical margin. These pathologic factors previously have been shown to independently predict survival in a large series of Whipple resections performed at our institution.22

For comparison to the distal bile duct carcinomas, a set of 28 perihilar and 12 intrahepatic resected cholangiocarcinomas were identified from The Johns Hopkins Hospital Surgical Pathology files. All of the perihilar cholangiocarcinoma patients underwent resection of the extrahepatic biliary tree and gallbladder for carcinomas clinically and pathologically centered on the hepatic duct bifurcation (Klatskin tumors). Those with intrahepatic cholangiocarcinoma did not have a known history of another carcinoma that could microscopically mimic cholangiocarcinoma in a liver metastasis.

**Immunohistochemistry**

For each case, a representative formalin fixed paraffin embedded tissue block containing carcinoma and normal tissue was chosen for labeling. For cases in which an in situ carcinoma component was noted, a block containing this component was specifically chosen for study if available. Unstained 4-micron sections then were cut from the paraffin block selected and deparaffinized by routine techniques. The slides were steamed for 20 minutes in sodium citrate buffer (diluted to 1× from 10× heat-induced epitope retrieval buffer; Ventana-Bio Tek solutions, Tucson, AZ). After cooling for 5 minutes, the slides were labeled with either a 1:100 dilution of a monoclonal antibody to Dpc4 (clone B8; Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:250 dilution of a monoclonal antibody to p53 (clone DO-7; Dako, Carpinteria, CA) by using the Bio Tek 1000 automated stainer (Ventana). Labeling was detected by adding biotinylated secondary antibodies, avidin-biotin complex, and 3, 3′-diaminobenzidine. Sections then were counterstained with hematoxylin. Dpc4 and p53 immunolabeling were evaluated jointly by three authors (P.A., R.E.W., R.H.H.) using a multiobserver microscope. For p53 labeling, a percentage of positive nuclei was determined. Carcinomas were divided into 3 groups: negative (<1% nuclear labeling), intermediate (>1% but <30% nuclear labeling), and positive (>30% nuclear labeling). In statistical analysis, carcinomas showing greater than 30% nuclear labeling were considered positive, given previous studies that have demonstrated that this cutoff point correlates best with the status of the p53 gene.2 For Dpc4 labeling, any area of uniform cytoplasmic labeling and focal nuclear labeling was considered positive. In statistical analysis, any carcinoma showing even focal nuclear and cytoplasmic labeling was considered positive (expresser), whereas carcinomas demonstrating no expression in a background of intact expression by nonneoplastic cells (desmoplastic stroma, normal peribiliary glands, etc., which served as internal controls) were considered negative (nonexpresser). The rationale for considering carcinomas that labeled only weakly as positives came from the previous study of Wilentz et al.,21 who found that pancreatic tumors with this focal staining pattern proved to have an intact DPC4 gene.

**Statistical Analysis**

Cross-tabulations of dichotomous and categoric variables were analyzed with chi-square or Fisher exact tests when appropriate. Means were compared using the Student t test. All of these tests were two-tailed. Estimates of the differences in survival distribution between groups were calculated using the method of Kaplan and Meier23 and were compared using the log rank statistic. The Cox proportional hazards model was used for univariate and
multivariate regression analysis. Tests were performed using STATA software (Intercooled version 6.0; College Station, TX).

RESULTS
Study Group
The pathologic features of the 128 bile duct carcinomas in the study are summarized in Table 1. Most of the 88 distal bile duct carcinomas (84%) were less than 3 cm in dimension, but most (68%) already had metastasized to local lymph nodes at the time of the Whipple resection. Negative surgical margins were obtained in 75% of the resections. Most (67%) carcinomas were moderately differentiated adenocarcinomas, with only a few (3%) well differentiated and the rest (30%) poorly differentiated. One tumor showed focal squamous differentiation and hence qualified as an adenosquamous carcinoma.

Most (64%) of the 28 perihilar carcinomas were moderately differentiated adenocarcinomas, whereas most of the remainder (32%) were poorly differentiated and only 1 (4%) was well differentiated. One of the poorly differentiated tumors was a small cell carcinoma arising in a patient with Clonorchis sinensis infestation. The macroscopic sizes of these ill-defined, fibrosing tumors could only rarely be accurately estimated.

Eleven of the twelve intrahepatic cholangiocarcinomas (92%) were moderately differentiated, with the other being poorly differentiated.

Dpc4 Immunohistochemistry
Cases considered examinable for Dpc4 labeling showed the expected pattern (diffuse cytoplasmic labeling, focal nuclear labeling) in benign ducts, stromal cells, pancreatic acini, islets, and lymphocytes. In general, the intensity of Dpc4 labeling was greater in normal duct epithelial cells than in the other normal tissues. In 16 cases in the study (10 distal tumors, 2 perihilar, 4 intrahepatic), the nonneoplastic cells in the section did not label, and these cases therefore were considered unevaluable.

Among 78 evaluable distal common bile duct carcinomas, 43 (55%) demonstrated loss of Dpc4 labeling. Examples of these lesions are shown in Figures 1 and 2. In these cases, the loss of Dpc4 labeling was clearly distinct from the intact labeling in surrounding normal structures. The 35 (45%) carcinomas that showed intact DPC4 gene expression often labeled less intensely than the surrounding normal tissues, but both nuclear and cytoplasmic labeling were identified at least focally.

In 11 of the 78 distal bile duct carcinomas, an in situ, papillary carcinoma component was present on the section adjacent to the invasive carcinoma. In 9 of these 11 cases, both the in situ and the infiltrating component labeled for Dpc4. In 2 of the 11 cases, the in situ component labeled, but the adjacent invasive component showed loss of DPC4 expression (Fig. 3).

One of the 78 invasive distal common bile duct carcinomas demonstrated heterogeneous labeling. This case contained a moderately differentiated gland-forming component that merged with a second, poorly differentiated component. Although the gland-forming component of the tumor strongly expressed the DPC4 gene, the more poorly differentiated component was completely negative (Fig. 4). We therefore examined the correlation between Dpc4 labeling and differentiation. Dpc4 expression was absent in 68% of the poorly differentiated carcinomas as compared with 49% of the well and moderately differentiated tumors. This difference was not statistically significant, however (P = 0.116).

A lower frequency of loss of DPC4 gene expression (15%) was observed among the 34 evaluable proximal bile duct tumors. Among 26 evaluable perihilar cholangiocarcinomas, 22 (85%) labeled for Dpc4 whereas 4 (15%) showed loss of DPC4 gene expression. In one case with an in situ component, both this in situ component and the invasive carcinoma associated with it expressed the DPC4 gene. Eight of the 12 intrahepatic cholangiocarcinomas were evaluable, and only 1 did not label (12.5%).

The difference between the frequency of labeling in the distal (45%) and proximal (perihilar and intrahepatic grouped together; 85%) cholangiocarcinomas was statistically significant (P < 0.001).

p53 Immunohistochemistry
Among 88 distal common bile duct carcinomas that were studied, 45 (51%) demonstrated greater than 30% nuclear labeling for p53. In most of these cases, greater than 70% of nuclei were labeled in an intense fashion that clearly demarcated tumor from the surrounding normal tissue on low power (Fig. 1C). Twelve cases (14%) demonstrated an intermediate level of labeling (1–30%), whereas in 31 cases p53 labeling was absent. For cases with an in situ component, no significant difference in p53 labeling was noted between the in situ and invasive components in 11 of 12 cases. The one case in which a difference was noted was the adenosquamous carcinoma, in which significant labeling (> 30%) was observed only in the invasive component.

The frequency of greater than 30% p53 immunolabeling was lower among more proximal cholangio-
carcinomas. Among 28 perihilar cholangiocarcinomas, 7 (25%) labeled strongly (>30% nuclei) for p53 (25% of cases) and 18 (64%) labeled intermediately (1–30% nuclei), whereas 3 (11%) did not label. Only 1 (9%) of 11 intrahepatic cholangiocarcinomas labeled strongly (>30% nuclei) for p53, 5 (45.5%) labeled intermediately (1–30% nuclei), and 5 (45.5%) did not label (1 case was unexaminable for p53 due to tissue artifacts in the sections analyzed). The difference in the frequency of greater than 30% labeling between distal and nondistal cholangiocarcinomas was statistically significant ($P < 0.001$).

No significant correlation was identified between p53 labeling and Dpc4 labeling. Dpc4 was absent in 45% of cases with less than 30% p53 labeling, and in 62% of those with greater than 30% p53 labeling. These differences were not statistically significant ($P = 0.130$).

**Survival Analysis on Distal Common Bile Duct Carcinomas**

The results of the survival analysis are summarized in Table 2. Cumulative survival for the patients with distal bile duct carcinoma was 54% (confidence interval [CI], 43–63%) at 12 months and 17% (CI, 10–25%) at 36 months. Simple regression univariate analysis demonstrated four statistically significant variables affecting survival: margin status, degree of differentiation, p53 expression, and lymph node status. Survival was worse for patients with positive margins (41% and 9% at 1 and 3 years, respectively) than those with negative margins (58% and 20% at 1 and 3 years, respectively; $P = 0.021$). Patients with poorly differentiated carcinomas had a lower survival rates (38% and 8% at 1 and 3 years, respectively) than those with moderately and well differentiated tumors taken together (61% and 21% at 1 and 3 years, respectively; $P = 0.010$). Patients

![FIGURE 2.](image-url)
with lymph node metastasis fared worse than those with negative lymph nodes (51% and 12% survival rate at 1 and 3 years vs. 61% and 29%; \( P = 0.05 \)). Surprisingly, patients whose tumors demonstrated greater than 30% nuclear p53 immunoreactivity survived longer (51% and 23% at 1 and 3 years, respectively) than those with less than 30% p53 expression (55% and 8% at 1 and 3 years, respectively; \( P = 0.04 \)). The other variables evaluated (tumor dimension and Dpc4 labeling) did not correlate with outcome. Specifically, among those with positive margins, median survival for patients with tumors with absence of Dpc4 expression (9 months) was not significantly different from those with intact Dpc4 expression (10 months; \( P = 0.51 \)). Among patients with negative margins, median survival for those with intact Dpc4 expression (12 months) did not differ significantly from those with intact Dpc4 expression (15 months; \( P = 0.88 \)).

In multivariable regression analysis, only differentiation remained statistically significant in the final model by using margin status, lymph node status, differentiation, tumor dimension, and Dpc4 or p53 labeling as covariates. Patients with poorly differentiated carcinomas had a significantly worse outcome than those with moderately or well differentiated carcinomas (relative risk, 1.95; CI, 1.15–1.30; \( P = 0.01 \)). Application of other models testing Dpc4 with each of the other covariates and also with different combinations of other covariates did not significantly improve the model or change the estimates. Therefore, we chose a model containing all variables as the final model.

**DISCUSSION**

We have analyzed a large series of biliary tract carcinomas immunohistochemically for loss of \( DPC4 \) gene expression and \( p53 \) overexpression, both of which correlate well with inactivation of these respective tumor-suppressor genes. Compared with their more proximal (intrahepatic and perihilar) counterparts, distal common bile duct carcinomas showed a significantly higher frequency of loss of \( DPC4 \) gene expression and

![FIGURE 3. Loss of DPC4 expression with tumor invasion. (A) Papillary in situ carcinoma component of tumor labels intensely for Dpc4. (B) Associated invasive component of the same tumor does not label for Dpc4. Note surrounding positively labeling stroma.](image-url)
of p53 overexpression. Our results with p53 are similar to those of Diamantis et al. who found a higher percentage of distal extrahepatic bile duct carcinomas (62%) than proximal extrahepatic bile duct carcinomas (30%) labeled for p53, although they used 10% staining as their criterion for positive.13 These results support the concept that biliary tract tumors of different sites arise via different molecular mechanisms. An appreciation of the clinical and epidemiologic heterogeneity of biliary tract carcinomas supports this idea. For clinical and therapeutic purposes, biliary tract carcinomas are typically divided into those arising in four locations: the gallbladder, the liver, the perihilar extrahepatic ducts, and the distal common bile duct.1,24 Many risk factors predispose patients to bile duct carcinomas, including lithiasis (stones), primary sclerosing cholangitis, choledochal cysts, Thorotrast exposure, and infections with agents such as Clonorchis sinensis or Opisthorchis viverrini.1,25 As others have noted, different risk factors affect different sites within the biliary tract preferentially.7,13,26 For example, lithiasis is the major risk factor for carcinoma of gallbladder but does not appear to strongly predispose to carcinomas of the extrahepatic biliary tree. Primary sclerosing cholangitis classically affects the extrahepatic biliary tree and may affect the liver, but only 15% of cases primarily involve the gallbladder.27 Neither of these risk factors classically is centered on the distal portion of the bile duct. Hence, given that the risk factors and their associated carcinogenic influences impact on different locations differently, it is not surprising that the genetic profiles of neoplasms arising from these sites should be different.

The high frequency of loss of DPC4 expression (55%) in the distal common bile duct carcinomas of this study is essentially identical to that observed in infiltrating ductal adenocarcinomas of the pancreas (55%).15,21 Hence, our results establish that distal common bile duct carcinomas are the second tumor type in which inactivation of DPC4, which is uncommon in carcinomas of other organs such as the breast, lung, and colon,16 is frequent. The high frequency (51%) of p53 immunolabeling in these distal common bile duct carcinomas is also comparable to the 50–75% frequency of p53 gene mutations in pancreatic adenocarcinomas.28 Taken together, these results provide more direct evidence that distal common bile duct carcinomas and pancreatic adenocarcinomas share some of the same patterns of genetic alterations. The highly analogous morphology of pancreatic and biliary tract adenocarcinomas previously has been noted.26 Our results suggest that the molecular analogy to pancreatic carcinoma should be limited to distal common bile duct carcinomas. Bile duct carcinomas of this site typically do not arise in the background of risk factors (lithiasis, primary sclerosing cholangitis) that predispose to more proximal biliary tract carcinomas, and it is tempting to speculate that they may share risk factors with pancreatic ductal adenocarcinomas that result in similar genetic changes.

As in pancreatic carcinoma, we suspect that DPC4 alterations occur relatively late in the genetic evolution of distal bile duct carcinomas. Support for this comes from the observation that all of the in situ carcinomas in this study had intact Dpc4 protein, whereas invasive carcinomas associated with two of these in situ carcinomas showed loss of Dpc4 expression. Another invasive carcinoma in this series had intact Dpc4 labeling in its better-differentiated gland forming area, but lost expression in its poorly differentiated, solid component. This latter case is very
similar to one Wilentz et al. reported in their study of pancreatic carcinomas.\textsuperscript{21} Because poorly differentiated carcinomas fared worse than others in our study, we suspected that Dpc4 might serve as a molecular marker of these poor prognosis tumors. Indeed, Dpc4 loss correlated with poor differentiation in this series, although the association was not statistically significant. Although no correlation of Dpc4 status with outcome was observed in this series, larger studies with longer follow-up could be justified to further address this possibility.

REFERENCES


et al., PM-80 pump. Separation was achieved by a BAS microbore column (MF-8949; 1 × 100 mm, with C18 packing of 3-μm particle size, which was attached directly to a Shimadzu UV-9125) and a Waters UV detector (Waters 486 UV detector, outfitted with a Waters microbore cell kit). Adenosine was detected at a wavelength of 258 nm. Chromatographic data were recorded on a chart recorder, and the peak heights of microdialysis samples were compared to the peak heights of adenosine standards (1 pmol/10 μl) for quantification. The detection limit of the assay was 50 fmol (based on a signal-to-noise ratio of 3:1). Repeated assays of standards and pooled samples showed less than 10% variability. Custom-made CMA 10 probes from CMA/Microdialysis had a polyacrylamide membrane with 0.05-μm diameter, a 2-mm microdialysis membrane length, and a 35-μm shaft length. During the experiment, ACSF (composed of 147 mM NaCl, 3 mM KCl, 1.2 mM CaCl2, and 1.0 mM MgCl2, at a pH of 6.8) was pumped through the probe at a flow rate of 1.5 μl/min, the same flow rate used for drug perfusion. Consecutive 10-min dialysis samples were collected on each day to obtain high quality data with low dead space volume (1.2 μl per 10 cm, FEP tubing: CMA/Microdialysis) and correlated with electrophysiologically defined sleep-wakefulness states. Adenosine from a microdialysis probe induced a sharp chromatogram peak with a high signal-to-noise ratio and the same 8-min retention time as the adenosine standard (Fig. 1A).

13. For the analysis of the group data, a sleep cycle was defined as a continuous period that contained all of the behavioral states (W, SWS, and REM sleep), and began and ended with waking periods; the validity of comparisons over time was ensured by rejection of any data where there were suggestions of nonstationarity (adenosine values with >25% change before the first and last waking epochs). Of the samples in this comparison of W and SWS, 66% were 100% in a single state, and the remaining 35% had less than 20% of another state. The mean cycle duration was not different in the basal forebrain and thalamic samples.

14. NBTI actions are discussed in G. Sanderson and C. Schmiedeberg’s Arch. Pharmacol. 337, 561 (1988). These references and our preliminary data confirmed the behavioral states (W, SWS, and REM sleep), and the animal was connected to the polygraph and microdialysis lines. Basal extracellular concentrations of adenosine were determined during the 3-hour baseline period that preceded the drug administration.

15. EEG power spectral analysis was performed during ACSF perfusion, during perfusion with 1 μM NBTI in the basal forebrain and thalamus, and during recovery sleep after NBTI infusion. Partialial EEG screw electrodes were used for EEG acquisition. The data were filtered at 70 Hz (low-pass filter) and 0.3 Hz (high-pass filter) with a Grass electronencephalograph and were continuously sampled at 128 Hz by a Pentium microprocessor computer with a Data-Wave (Data-Wave Technology, Longmont, CO) system. Absolute total power was calculated for the frequency range between 0.3 and 55 Hz. Five different frequency bands were used to calculate the relative power: delta, 0.3 to 4 Hz; theta, 4.1 to 9 Hz; alpha, 9.1 to 18 Hz; beta, 18.1 to 25 Hz; and gamma, 25.1 to 55 Hz. After basal forebrain NBTI perfusion, the relative power was significantly increased in the delta and decreased in the theta, alpha, beta, and gamma bands (<0.05, nonparametric Wilcoxon signed-rank tests, p < 0.05, n = 4), with the power increase between 0.3 and 4 Hz being greater than 20% of another state. The mean cycle duration was not different in the basal forebrain and thalamic samples.

16. In evaluating the physiological relevance of adenosine at various concentrations, it is important to note that in vitro data from our laboratory (3) demonstrated that endogenous adenosine had a consistent inhibitory effect on cholinergic neurons. These data imply that adenosine's physiological effects in vivo are to be expected at baseline that is, without sleep deprivation or NBTI. Rannie et al. (3) did not measure endogenous adenosine concentrations, and thus the precise in vitro effects of doubling adenosine concentrations have not yet been specified, although it is known that this is progresive increases in inhibition of cholinergic neurons (beyond that seen from the endogenous inhibitory effect) with increasing concentrations of exogenously applied adenosine. Furthermore, we believe that the actions of adenosine that we have found in animal studies apply also to humans. First, the increase in EEG sleepiness with increasing duration of wakefulness has been documented in humans (7). Second, the adenosine physiology and pharmacology of experimental animals and of humans appear to be comparable (see reviews in (4–7) and in L. J. Findley, M. Boykin, T. Fallon, L. Belandretti, J. Appl. Physiol. 65, 558 [1988] and H. L. Haas, R. G. Greene, M. G. Yasargil, V. Chan-Palay, Neurosci. Abstr. 13, 156 [1987]). Finally, the adenosine antagonist caffeine increases wakefulness in formal experimental studies (see (7) and H. P. Landolt, D. J. Dijk, S. E. Gaus, A. A. Borbély, Neuropsychopharmacology 12, 229 [1995]) and, as with the adenosine antagonist theophylline, constitutes the sleep-delaying ingredient in coffee and tea.

17. Changes in the entire relative power spectrum with NBTI infusion and in recovery sleep after prolonged wakefulness were, for each band, in the same direction (n = 4 animals).


19. It is also possible that adenosine’s effects in the neocortex may be directly attenuated by cholinergic receptor activation, as has been shown in the hippocampus [P. F. Worley, J. M. Baraban, M. McCasren, S. H. Snyder, B. E. Alger, Proc. Natl. Acad. Sci. U.S.A. 84, 3467 (1987)]. Thus, adenosine’s direct inhibitory effects on cholinergic somata might be enhanced by a consequent disinhibition of adenosine’s effects on neocortical neurons. The specificity of sleep-wakefulness effects of NBTI does not support the idea that adenosine’s effects result from a global action on brain neurons, as suggested by J. H. Benington and H. C. Heller [Prog. Neurobiol. 45, 347 (1996)].

20. We thank R. Shromani, D. Rannie, and D. Stenberg for their advice during this work; L. Carnara and M. Gray for technical assistance; and C. Portas for her preliminary work on this project. Supported by National Institutes of Mental Health, grant R01 MH59, 683 and awards from the Department of Veterans Affairs to R.W.M.

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Gene Expression Profiles in Normal and Cancer Cells

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As a step toward understanding the complex differences between normal and cancer cells in humans, gene expression patterns were examined in gastrointestinal tumors. More than 300,000 transcripts derived from at least 45,000 different genes were analyzed. Although extensive similarity was noted between the expression profiles, more than 500 transcripts that were expressed at significantly different levels in normal and neoplastic cells were identified. These data provide insight into the extent of expression differences underlying malignancy and reveal genes that may prove useful as diagnostic or prognostic markers.

Much of cancer research over the past 50 years has been devoted to analyses of genes that are expressed differently in tumor cells as compared with their normal counterparts. Although hundreds of studies have pointed out differences in the expression of one or a few genes, no comprehensive study of gene expression in cancer cells has been reported. It is therefore not known how many genes are expressed differentially in tumor versus normal cells, whether the bulk of these differences are cell-autonomous rather than dependent on the tumor microenvironment, and whether most differences are cell type-specific or tumor-specific. Technological advances have made it possible to answer such questions through simultaneous analysis of the expression patterns of thousands of genes (1, 2). In this study, using normal and neoplastic gastrointestinal tissue as a prototype, we analyzed global profiles of gene expression in human cancer cells. We used the recently developed method
called serial analysis of gene expression (SAGE) (2) to identify and quantify a total of 303,706 transcripts derived from human colorectal (CR) epithelium, CR cancers, or pancreatic cancers (Table 1) (3). These transcripts represented about 49,000 different genes (4) that ranged in average expression from 1 copy per cell to as many as 5300 copies per cell (5). The number of different transcripts observed in each cell population varied from 14,247 to 20,471. The bulk of the mRNA mass (75%) consisted of transcripts expressed at more than five copies per cell on average (Table 2). In contrast, most transcripts (86%) were expressed at less than five copies per cell, but in aggregate this low-abundance class represented only 25% of the mRNA mass. This distribution was consistently observed among the different samples analyzed and was consistent with previous studies of RNA abundance classes based on RNA-DNA reassociation kinetics (R0 curves) (6). Monte Carlo simulations revealed that our analyses had a 92% probability of detecting a transcript expressed at an average of three copies per cell (7).

Many of the SAGE tags appeared to represent previously undescribed transcripts, as only 54% of the tags matched GenBank entries (Tables 1 and 2). Twenty percent of these matching transcripts corresponded to characterized mRNA sequence entries, whereas 80% matched uncharacterized expressed sequence tag (EST) entries. As expected, the likelihood of a tag being present in the databases was related to abundance; GenBank matches were identified for 98% of the transcripts expressed at >500 copies per cell but for only 51% of the transcripts expressed at ≤5 copies per cell. Because the SAGE data provide a quantitative assay of transcript abundance, unaffected by differences in cloning or polymerase chain reaction efficiency, these data provide an independent and relatively unbiased estimate of the current completeness of publicly available EST databases. Comparison of expression patterns between normal colon epithelium and primary colon cancers revealed that most transcripts were expressed at similar levels (Fig. 1). However, the expression profiles also revealed 289 transcripts that were expressed at significantly different levels [P < 0.01 (8)]; 180 of these 289 were decreased in colon tumors as compared with normal colon tissue (average decrease, 10-fold; examples in Fig. 2A). Conversely, 108 transcripts were expressed at higher levels in the colon cancers than in normal colon tissue (average increase, 13-fold; examples in Fig. 2A). Monte Carlo simulations indicated that the analysis would have detected >95% of transcripts expressed at a sixfold or greater level in normal versus tumor cells or vice versa (9). Because relatively stringent criteria were used for defining differences [P < 0.01 (8)], the number of differences reported above is likely to be an underestimate.

To determine how many of the 289 differences were independent of the cellular microenvironment of cancers in vivo, we compared SAGE data from CR cancer cell lines with that from primary CR cancer tissues (10). Perhaps surprisingly, 130 of 181 transcripts that were expressed at reduced levels in cancer cells in vivo were also expressed at significantly lower levels in the cell lines (Table 3). Likewise, a significant fraction (47 of 108) of the transcripts expressed at increased levels in primary cancers were also expressed at higher levels in the CR cancer cell lines (Table 4). Thus, many of the gene expression differences that distinguish normal from tumor cells in vivo persist during in vitro growth. However, despite these similarities, there were also many differences. For example, only 47 of 228 genes expressed at higher levels in CR cancer cell lines were also expressed at high levels in the primary CR cancers.

Table 1. Overall summary of SAGE analysis.

<table>
<thead>
<tr>
<th></th>
<th>Normal colon</th>
<th>Colon tumors</th>
<th>Colon cell lines</th>
<th>Pancreatic tumors</th>
<th>Pancreatic cell lines</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes‡</td>
<td>62,168</td>
<td>60,878</td>
<td>60,373</td>
<td>61,592</td>
<td>58,695</td>
<td>303,706</td>
</tr>
<tr>
<td>GenBank†</td>
<td>8,753 (59)</td>
<td>10,490 (53)</td>
<td>10,193 (60)</td>
<td>11,547 (56)</td>
<td>8,922 (63)</td>
<td>26,339(54)</td>
</tr>
</tbody>
</table>

†Indicates the number of different genes that match an entry in GenBank. Numbers in parentheses indicate the percentage of the total number of different tags.

Table 2. Summary of SAGE analysis by abundance classes.

<table>
<thead>
<tr>
<th>Copies/cell</th>
<th>Normal colon</th>
<th>Colon tumors</th>
<th>Colon cell lines</th>
<th>Pancreatic tumors</th>
<th>Pancreatic cell lines</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;500</td>
<td>62 (29)</td>
<td>54 (25)</td>
<td>54 (19)</td>
<td>32 (11)</td>
<td>70 (26)</td>
<td>55 (19)</td>
</tr>
<tr>
<td>GenBank‡</td>
<td>59 (95)</td>
<td>52 (96)</td>
<td>53 (98)</td>
<td>32 (100)</td>
<td>70 (100)</td>
<td>54 (98)</td>
</tr>
<tr>
<td>&gt;50 and ≤500</td>
<td>645 (28)</td>
<td>470 (21)</td>
<td>618 (27)</td>
<td>657 (29)</td>
<td>585 (27)</td>
<td>59 (26)</td>
</tr>
<tr>
<td>GenBank†</td>
<td>545 (84)</td>
<td>429 (91)</td>
<td>579 (94)</td>
<td>609 (93)</td>
<td>529 (90)</td>
<td>553 (93)</td>
</tr>
<tr>
<td>&gt;5 and ≤50</td>
<td>4,569 (27)</td>
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<td>2,893 (63)</td>
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<td>14,155 (25)</td>
<td>10,687 (20)</td>
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<td>8,697 (16)</td>
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<td>5,256 (56)</td>
<td>6,805 (48)</td>
<td>5,879 (65)</td>
<td>6,852 (50)</td>
<td>5,155 (59)</td>
<td>21,491 (51)</td>
</tr>
</tbody>
</table>

*For genes, the first number denotes the number of different genes (4) represented in the indicated abundance class. Numbers in parentheses indicate the mass fraction (×100) of total transcripts represented by the indicated abundance class.

†For GenBank entries, the first number indicates the number of different genes that matched an entry in GenBank in the indicated abundance class. Numbers in parentheses indicate the corresponding percentage of genes.

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What are the identities of the differentially expressed genes? Of the 548 differentially expressed transcripts, 337 were tentatively identified through database comparisons. These identifications proved to be legitimate (14), as was expected from previous SAGE analyses (2). Although a large number of differentially expressed genes were identified, some simple patterns did emerge. For example, genes that were expressed at higher levels in normal colon

Table 3. Transcripts decreased in CR cancer. The 20 transcripts displaying the largest decrease in expression in CR cancers (in vivo and in vitro) are listed by fold reduction. The tag sequence represents the 10-base pair SAGE tag, and SAGE UID is the human SAGE tag identifier. Probable GenBank matches are listed and those in boldface were confirmed by Northern blot analysis or by cloning and sequence analysis. Fold changes in expression were calculated as described in Fig. 1. TU, colon tumors; CL, colon cell lines; NC, normal colon. Tables of all 548 differentially expressed genes are available on the Internet at http://welchlink.welch.jhu.edu/~mogen-g/home.htm.

<table>
<thead>
<tr>
<th>Tag sequence</th>
<th>SAGE UID</th>
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<th>CL</th>
<th>NC</th>
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epithelium than in CR tumors were often related to differentiation. These genes included fatty acid–binding protein (15), cytokeratin 20 (16), carbonic anhydrase (17), guanylin (18), and uroguanylin (19), which are known to be important for the normal physiology or architecture of colon epithelium (Tables 3 and 4). On the other hand, genes that were increased in CR cancers were often related to the robust growth characteristics that these cells exhibit. For example, gene products associated with protein synthesis, including 48 ribosomal proteins, five elongation factors, and five genes involved in glycolysis were observed to be elevated in both CR and pancreatic cancers as compared with normal colon cells. Although most of the transcripts could not have been predicted to be differentially expressed in cancers, several have previously been shown to be dysregulated in neoplastic cells. The latter included IGFII (20), B23 nucleolinophin (21), the Pi form of glutathione-S-transferase (22), and several ribosomal proteins (23), all of which were increased in cancer cells, as previously reported. Likewise, Dra (24) and gelosin (25) were decreased in cancer cells, as previously reported. Surprisingly, two widely studied oncopgenes, c-fos and c-erbB3, were expressed at much higher levels in normal colon epithelium than in CR cancers, in contrast to their up-regulation in transformed cells (26).

These data provide basic information necessary for understanding the gene expression differences that underlie cancer phenotypes. They also provide a necessary framework for interpreting the significance of individual differentially expressed genes. Although this study demonstrated that a large number of such differences exist (about 500 at the depth of analysis used), it was equally remarkable that the fraction of transcripts exhibiting significant differences was relatively small, representing 1.5% of the transcripts detected in any given cell type (27). The fact that many, but not all, of the differences were preserved during in vitro culture demonstrates the utility of cultured lines for examination of some aspects of gene expression but also provides a note of caution about relying on such lines to perfectly mimic tumors in their natural environment. Finally, the finding that hundreds of specific genes are expressed at different levels in CR cancers, and that some of these are also expressed differentially in pancreatic cancers, provides a wealth of reagents for future biologic and diagnostic experimentation.

REFERENCE AND NOTES


3. To minimize individual variation, approximately equal numbers of tags (30,000) were derived from two different patients for each tissue. For primary tumors, which were used from pairs with the use of EDTA as described (S. Nakamura, I. Kino, S. Baba, Gut 34, 1240 (1995)), Histopathology confirmed that the isolated cells were >90% epithelial. Isolation of polyadenylate RNA and SAGE was performed as described (2). SAGE data were analyzed with SAGE software and GenBank Release 94 as described (2).

Table 4. Transcripts increased in CR cancer. The 20 transcripts displaying the greatest increase in CR cancers (in vivo and in vitro) are listed by fold induction. Conditions are as described in Table 3.

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<th>Tag sequence</th>
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<th>TU/NC</th>
<th>TU</th>
<th>CL</th>
<th>NC</th>
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<td>TGF-β-induced gene Beta-1 (M77349)</td>
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null hypothesis. We converted this likelihood to an absolute probability value by simulating 40 experiments in which a representative number of transcripts (27,890 transcripts in each experiment) were identified and compared. We derived the distribution of transcripts used for these simulations from the average level of expression observed in the original samples. We then compared the distribution of the p-chance scores obtained in the 40 simulated experiments (false positives) with those obtained experimentally. On the basis of this comparison, a maximum value of 0.0005 was chosen for p-chance. This yielded a false-positive rate that was no higher than 0.01 for the least significant p-chance value below the cutoff.

9. Two hundred simulations, assuming an abundance of 0.0001 in one sample and 0.0006 in a second sample, revealed a significant difference (P < 0.01) 95% of the time.

10. This analysis revealed 208 transcripts that were significantly decreased in CR cancer cell lines as compared with normal colon cells and 228 transcripts that were increased. Venn diagrams and tables illustrating the relation between the in vivo and in vitro differences are available through the Internet at http://welchlink.welch.jhu.edu/~molgen-g/home.htm.

11. It is not possible to obtain pancreatic duct epithelium, from which pancreatic carcinomas arise, in sufficient quantities to perform SAGE. It is therefore not possible to determine whether these transcripts were derived from genes that were highly expressed only in pancreatic cancers or that were also expressed in pancreatic duct cells.

12. Total RNA isolation and Northern blot analysis were performed as described (W. S. el-Deiry et al., Cell 75, 817 (1993)).


14. Northern blot analyses were done on 45 of the 337 differentially expressed transcripts with tentative database matches. In three cases, the pattern of expression was not differentially expressed as predicted by SAGE and, for the purposes of this calculation, they were presumed to represent incorrect database matches.


27. In the case of normal and neoplastic colon cancer tissue, 548 differentially expressed transcripts were identified among the 36,125 different transcripts.

28. We thank K. Poylaik and P. J. Morin for providing colon cancer cell lines; G. M. Nadasy for providing pancreatic primary tumors; and J. Floyd, C. R. Robinson, and Y. Ebleazl-Scalay for technical assistance. Supported by the Clayton Fund and by NIH grants GM07309, CA57345, and CA62924. B.V. is an investigator of the Howard Hughes Medical Institute.

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Brain Regions Responsive to Novelty in the Absence of Awareness

Gregory S. Berns,* Jonathan D. Cohen, Mark A. Mintun

Brain regions responsive to novelty, without awareness, were mapped in humans by positron emission tomography. Participants performed a simple reaction-time task in which all stimuli were equally likely but, unknown to them, followed a complex sequence. Measures of behavioral performance indicated that participants learned the sequences even though they were unaware of the existence of any order. Once the participants were trained, a subtle and unperceived change in the nature of the sequence resulted in increased blood flow in a network comprising the left premotor area, left anterior cingulate, and right ventral striatum. Blood flow decreases were observed in the right dorsolateral prefrontal and parietal areas. The time course of these changes suggests that the ventral striatum is responsive to novel information, and the right prefrontal area is associated with the maintenance of contextual information, and both processes can occur without awareness.

The detection of novelty is a cognitive operation necessary to survival and requires an assessment of both expectedness and context. Events can be familiar in one context but novel in another. More precisely, novelty represents a deviation from the expected likelihood of an event on the basis of both previous information and internal estimates of conditional probabilities (1).

Novelty detection has typically been linked to consciousness because novel events often capture attention. For similar reasons, studies of novelty have often been confounded by awareness (2). Here, we sought to determine whether the response to novelty can occur without awareness and, if so, to identify the associated brain regions in a manner unconfounded by awareness. To do so, we used an implicit learning task.

A large body of research has examined learning mechanisms that operate below the level of awareness. This type of learning is said to occur implicitly because behavioral measures indicate that learning takes place, even though the individuals are unaware of this or are unable to report it explicitly (3). A frequently used paradigm is based on a serial reaction-time task, in which participants observe sequences of visual stimuli and must press buttons corresponding to these. Unknown to the participants, the sequence of stimuli is predetermined by a fixed, repeating order. With practice, reaction times improve (compared with randomly sequenced stimuli), indicating that the participants have learned about the sequential order. However, they are not always conscious of this. When the sequence is sufficiently complex, individuals are unaware of the sequential regularities or that they have learned anything specific about the stimuli, even though their reaction times have improved significantly (4). This indicates that sequential information can be both learned and used in the absence of awareness.

One type of sequence that has been well studied is based on finite-state grammars (5). Such grammars can be used to generate highly complex, context-dependent sequences. With enough practice, individuals show improvements consistent with implicit learning of such grammars. However, because such grammars are typically probabilistic, specific repeating sequences rarely occur, further reducing the likelihood of awareness of the sequential regularities.

Implicit learning of finite-state grammars means that participants have developed expectations for each stimulus, on the basis of the specific stimuli that preceded it in the sequence (that is, its context). Under such conditions, changing the rules of the grammar will cause subsequent stimuli to violate these expectations, by appearing in novel contexts. Thus, a switch in grammars.

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Identification by representational difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the BRCA2 region

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ABSTRACT Homozygous deletions have been central to the discovery of several tumor-suppressor genes, but their finding has often been either serendipitous or the result of a directed search. A recently described technique [Lisitsyn, N., Lisitsyn, N. & Wigler, M. (1993) Science 259, 946–951] held out the potential to efficiently discover such events in an unbiased manner. Here we present the application of the representational difference analysis (RDA) to the study of cancer. We cloned two DNA fragments that identified a homozygous deletion in a human pancreatic adenocarcinoma, mapping to a 1-centimorgan region at chromosome 13q12.3 flanked by the markers D13S171 and D13S260. Interestingly, this lies within the 6-centimorgan region recently identified as the BRCA2 locus of heritable breast cancer susceptibility. This suggests that the same gene may be involved in multiple tumor types and that its function is that of a tumor suppressor rather than that of a dominant oncogene.

Tumor-suppressor genes play a crucial role in the control of cell growth and differentiation. Loss of the function of tumor-suppressor genes is part of the cascade of genetic alterations which drive tumorgenesis (1). The biallelic inactivation of a tumor-suppressor gene typically involves an intragenic change (nucleotide substitution, small insertion, or microdeletion) within one allele, combined with inactivation of the other allele through the loss of a large chromosomal region. Although infrequent, sizable deletions involving both alleles have been observed. Such homozygous deletions have contributed to the discovery of several tumor-suppressor genes (RB1, DCC, and p16) (2–5).

Despite the fact that pancreatic adenocarcinoma is one of the more common human cancers (6), little is known of the genetic alterations in these tumors. One of the reasons is that the tumors generally are diagnosed at a late stage of tumorigenesis. This, together with the aggressive clinical course, severely limits the number of resected specimens available for research. Also, pancreatic adenocarcinomas characteristically exhibit an exuberant host desmoplastic response, resulting in a high admixture of nonneoplastic cells and hampering the molecular genetic analysis of primary tumor samples (7). Finally, familial patterns of pancreatic adenocarcinoma usually do not involve young ages of onset, high penetrance, or extensive pedigrees (8).

We have circumvented some of these problems by the development of a xenograft model of pancreatic adenocarcinoma that generates genetically stable cell expansions, free of infiltrating nonneoplastic human cells (9, 10). Molecular analysis of known oncogenes and tumor-suppressor genes has proven feasible; it is possible to identify both K-ras and p16 alterations in over 80% of pancreatic adenocarcinomas (7, 10, 11) and p53 mutations in at least 70% of the cases (12). However, a conventional search for novel loci of interest presented practical obstacles. Allelotyping had identified frequent loss of heterozygosity (LOH; deletion of only one allele), mainly at sites of known genes, such as 9p (p16), 17p (p53), and 18q (DCC) (7, 10). A limited number of xenografted specimens and the typically large areas involved by LOH precluded a standard search for smaller consensus areas of deletion. An alternative approach for the identification of tumor-suppressor genes preferably would allow high-resolution genome scanning without the need for a statistical analysis of numerous tumor specimens. The newly described technique of representational difference analysis (RDA) (13) suggested a promising approach.

RDA is a means for isolating DNA fragments that are present in only one of two nearly identical complex genomes. It utilizes a subtractive hybridization method but differs from conventional methods (14–16) by using “representations” of the genomes that have a reduction in complexity. Representations are generated by a PCR-based size selection applied to the restriction fragments of both genomes. Moreover, RDA takes advantage of both subtractive hybridization and DNA reassociation kinetics to favor the reiterated PCR amplification of the difference among the two genomes. It has been demonstrated that RDA can enrich difference products over a millionfold after three rounds of selection (13).

Here we apply RDA to the identification of DNA fragments that are deleted in neoplastic tissues. Normal tissue from the patient provides the “tester” sequences, and neoplastic cells provide the “driver” sequences in the hybridization reactions. RDA identifies a simple LOH, when a deletion involves a restriction fragment length polymorphism in such a way that the smaller fragment is deleted in the neoplasm and therefore is present only in the representation of the tester (normal) genome. Due to the PCR-based size exclusion, the larger allele is not present in either of the representations, and the 2:1 allele ratio seen upon comparison of the total genomic DNAs of normal and tumor is converted to a 1:0 ratio in the representations. Thus the existence of the larger allele in the driver will no longer prevent enrichment for the smaller allele in the tester (the “target,” or deleted sequence in the tumor) (Fig. 1). In homozgyously deleted regions, however, both alleles are absent from the driver genome and thus the target alleles do not

Abbreviations: LOH, loss of heterozygosity; RDA, representational difference analysis; cM, centimorgans; STS, sequence-tagged site; YAC, yeast artificial chromosome; FISH, fluorescence in situ hybridization.

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breast carcinoma at age 94, her mother's brother, who died of "stomach" cancer in his 80s, and the patient's brother, who died of colorectal carcinoma at the age of 52. The only siblings in these two generations unaffected by cancer were the patient's sister (alive, age 76) and her mother's sister, who died at the age of 29 from tuberculosis. Both children of the patient are unaffected to date.

**Tissue Samples.** Tissue specimens were obtained from the pancreas upon its resection at The Johns Hopkins Hospital. Histopathological examination revealed a moderately differentiated primary pancreatic ductal adenocarcinoma. The pancreatic cancer was histologically distinct from her previous colorectal carcinoma, slides of which were reviewed. At the time of surgery, normal duodenal mucosa was fresh-frozen at −80°C and xenografts were generated by implantation of 2-mm³ pieces of the primary tumor into athymic nude mice. Xenografts were harvested at a size of 1 cm³, and DNA was prepared as described (10).

RDA. RDA was performed essentially as described by Lisitsyn et al. (13). The restriction endonuclease BamHI and corresponding anchor primers were used for digestion of the DNA samples and subsequent PCR amplifications. For the xenograft-driven RDA, hybridization times were increased to 40 hr. A detailed protocol of the RDA procedure is available from the authors.

The RDA round 2 difference products were cloned by using the pBluescript II plasmid vector (Stratagene). Insert DNAs of individual clones were used as probes for Southern blots containing tester and driver amplicon DNA. These fragments were sequenced by the SequiTherm cycle sequencing method (Epicentre Technologies, Madison, WI) and 20-mer or 24-mer oligonucleotide pairs for sequence-tagged sites (STSs) were designed from these results.

**PCR.** STSs were amplified by using 40 ng of genomic DNA in 67 mM Tris-HCl, pH 8.8/4 mM MgCl₂/16 mM (NH₄)₂SO₄/10 mM 2-mercaptoethanol containing bovine serum albumin at 100 µg/ml, dATP, dCTP, dGTP, and dTTP at 200 µM each, each primer at 1 µM, and 2 units of Taq DNA polymerase (GIBCO/BRL) in a final reaction volume of 15 µl. The enzyme was added after a preheating step of 2 min at 94°C. For 20-mers, 35 cycles of 94°C for 30 sec, 58°C for 1 min, and 72°C for 1 min were followed by a final extension of 5 min at 72°C. For 24-mers, the annealing step was omitted and the extension step was increased to 2 min. Primer sequences for DPC1 were 5'-CAGGTCGCTGAAAACTGATAAGG-3' and 5'-GAGTCAAGTGAGCTACCTC-3', and for DPC2, 5'-CTTCCCAGCTCTTCTATG-3' and 5'-CTTCCCTCACCCTATTTGGAG-3'. Primer sequences for DPC1' were 5'-TCTCTCCACCTCCACTGAAAG-3' and 5'-ATCCACCTTTGGCCAGCAACTAG-3', and for DPC2', 5'-AAGGTCCTCCCAGGCTCAAAGG-3' and 5'-TTCCACAGTACATGGTGGTGTAG-3'. Primer sequences for LC01 were 5'-GCCCTCCGGTAGGCTTATCT-3' and 5'-GAGCGGACACAGGGATGGTT-3'. Dihydropyrimidine dehydrogenase levels were measured by using the chemiluminescent assay (Promega, Madison, WI).

**RESULTS**

RDA. We performed RDA on a human pancreatic adenocarcinoma, essentially as described (13). The strategy is schematically represented in Fig. 2. Tumor DNA was used to drive the substractions, whereas corresponding normal DNA was used as the tester. Tissue from primary tumors, typically infiltrated with nonneoplastic cells, should not effectively drive the subtracts. We therefore used a carcinoma that had been propagated in an athymic nude mouse. Such xenografted tumors are genetically stable and do not contain detectable nonneoplastic human cells (9, 10). As these xenografts contain
up to 50% murine cells, we modified the RDA protocol of Lisitsyn et al. by increasing the time of DNA annealing to 40 hr.

Genomic representations of the xenograft and normal DNA were generated by using the restriction endonuclease BamHI. After two rounds of RDA, a distinct pattern of DNA fragments was visible upon electrophoretic separation of the difference product (Figs. 2A and 3A). The round 2 difference product was cloned by using a plasmid vector (Fig. 2B). True subtraction fragments were detected by using Southern blots of the tester and driver representations (Fig. 2C). This analysis revealed that >80% of 60 randomly selected fragments were subtraction products—i.e., they were absent from the driver and present in the tester.

The sequences of the cloned fragments were used to design primers to amplify STSs (Fig. 2D). Fourteen of 16 STSs derived from unique subtraction fragments were present in normal and xenograft total genomic DNA, consistent with sites of simple LOH in the carcinoma (Fig. 2E). Two STSs, designated DPC1 and DPC2, were present in normal but absent from xenograft DNA, indicating that they were homozgyously deleted in the pancreatic carcinoma. As a control for DNA quality, duplex PCR was performed for both DPC1 (Fig. 3B) and DPC2 with concurrent use of STS primers for an irrelevant locus (LC01), which localized to chromosome 14. To exclude simple insertion/deletion polymorphisms, an adjacent sequence of each cloned fragment was amplified with additional STS primers, designated DPC1' and DPC2'.

As a control, we performed a parallel RDA in which the driver DNA was provided by a cell line derived from the same pancreatic carcinoma. Seven of 8 unique subtraction fragments from this RDA had been identified in the xenograft-driven RDA. These fragments included DPC1.

**Localization and YAC Contig**. The STSs DPC1 and DPC2 both localized to chromosome 13 upon PCR analysis of monochromosomal somatic cell hybrid DNAs of NIGMS mapping panel 2 (Coriell Cell Repositories, Camden, NJ) (18). Both subtraction fragments, DPC1 and DPC2, were used to screen a chromosome 13 phage library (American Type Culture Collection). Two-color FISH, using the whole phage DNAs as probes, localized DPC1 and DPC2 as distinct nonoverlapping nearby sites on a metaphase preparation, below the centromere of chromosome 13 (Fig. 2F).

PCR screening of the Généthon megAYAC library (19) resulted in a YAC contig, encompassing the BRC42 region at 13q12–13. YAC y8808 contained both DPC1 and DPC2 and the markers D13S171, YAC y951a3 contained DPC1 and the markers D13S171 and D13S267, whereas y931f4 contained DPC2, D13S260, and D13S290. Five additional YACs confirmed the contig (Fig. 4). Analysis with the markers D13S289, S290, S260, S171, S267, S219, and S220 did not reveal interstitial deletions within these YACs. YACs suspected to be chimeric, on the basis of Généthon data and our own data, were excluded from the contig.

Dinucleotide markers D13S289, S290, S260, S171, S267, S219, and S220 in this region were all found to be present in the xenograft DNA, exhibiting a pattern of simple LOH upon comparison with normal DNA. Thus the entire homozgyous deletion in the carcinoma mapped between the markers D13S171 and D13S260 at band 13q12.3 (Fig. 4). PCR analysis for the candidate tumor-suppressor genes Brush-1 (21) and RFC3 (22) revealed the expected PCR products in xenograft DNA. None of the eight YACs in the contig contained the Brush-1 sequence. Microsatellite instability was not identified at any loci in the carcinoma.
Pancreatic carcinomas, as well as other carcinomas, can exhibit an average fractional allelic loss at least as high as 20% (7, 27). Overwhelmingly, the detected deletions are LOHs; that is, only one of the two alleles is deleted. Although complete data are not available, the occurrence of deletions involving both alleles is considered to be infrequent. Owing to the total loss of particular genetic information, the cellular effect of most homozygous deletions is assumed to be deleterious. Indeed, the homozygous deletions reported to date are relatively small. The significance of the identification of a homozygous deletion is best illustrated by their contribution to the discovery of several tumor-suppressor genes (RB1, DCC, and p16) (2–5). The potential for identifying homozygous deletions among a high background of heterozygous losses suggests RDA as a powerful approach for the identification of novel tumor-suppressor genes.

The homozygous deletion identified here by RDA maps to chromosome 13q12–13. Allelic loss at 13q is found in pancreatic carcinoma and in a wide variety of other tumor types. The tumor-suppressor gene RB1, located at 13q14, is a candidate target gene within these areas of deletion. However, mutations or other evidence of inactivation of RB1 have been found in only a subset of tumors (28–30). As for pancreatic adenocarcinoma, previous immunohistochemical analyses of Rb protein expression found no evidence of RB1 inactivation (7). The identification of a homozygous deletion at 13q12.3 in a pancreatic adenocarcinoma strengthens the suspicion that, besides RB1, at least one additional tumor-suppressor gene is located on chromosome 13q. Recently, a syndrome of familial breast cancer susceptibility (BRCA2) was linked to a 6-cM region at 13q12–13, between the markers D13S267 and D13S289 (17). Although the BRCA2 candidate region encompasses the deletion we describe here, it is yet not established whether the same genetic target is involved in pancreatic and breast carcinomas. If the target loci were postulated to be identical, the finding of a homozygous deletion would narrow the region for a gene search to the 1-cM region bounded by D13S171 and D13S260. It would also indicate that BRCA2 susceptibility is not due to a dominant oncogene (31) but could be attributed to a tumor-suppressor gene along the model proposed by Knudson, wherein both alleles must be inactivated to achieve the full tumorigenic phenotype (1).

One of our carcinomas under study contains a translocation of 13q, with the breakpoint observed at or near the DPC locus (23). However, our analysis with dinucleotide markers did not reveal LOH at any flanking site of 13q in this particular carcinoma. LOH analysis might underestimate the fraction of cases with genomic alterations in a particular gene. It is also possible that additional cases of pancreatic carcinomas harboring a homozygous deletion would have gone undetected, since the markers flanking the DPC region are located 1 cM apart. We reported a possibly analogous situation with the p16 tumor-suppressor gene, wherein we detected two pancreatic carcinomas as having a homozygous deletion upon the use of two flanking markers, and yet an additional eight homozygous deletions were identified only upon analysis of the p16 gene itself (10). Similarly, the majority of homozygous deletions involving RB1 are intragenic. Conversely, other tumor-suppressor genes, like p53, rarely are inactivated by homozygous deletion. Additional evidence for the involvement of a tumor-suppressor gene of general importance for pancreatic carcinoma includes our finding of LOH that spans 13q12.3 in nearly a quarter of the cases. This frequency of LOH at 13q is comparable with that found for breast carcinoma (32–34) and may be significant even though measurably less than frequencies found at loci of some other tumor-suppressor genes. We postulate that a 1-cM region at 13q12.3, flanked by markers D13S171 and D13S260, contains a tumor-suppressor gene that is involved in pancreatic carcinoma.
The patient in the present study was a member of a familial clustering of adenocarcinomas of various organ sites (see Case Report). Two related points can be elaborated. First, the age of onset in this familial cluster is rather late. Indeed, an onset at older age is the pattern found for most familial pancreatic carcinoma pedigrees (8). Many, if not most, familial clusters of carcinoma in the general population do not reproducibly involve onset at young age. A comprehensive understanding of monogenic and polygenic influences on cancer susceptibility will have to include studies of these less distinctive phenotypic patterns of susceptibility (35). Second, it will be of interest to determine whether the individuals of the presently Thordert familial cluster, k. W. hereditary in the region, which would suggest that the putative tumor-suppressor gene at 13q12.3 might be involved in a variety of malignancies. This would be consistent with the frequent occurrence of allelic loss at 13q in multiple tumor types that is not readily attributable to inactivation of the RB1 tumor-suppressor gene (7, 36–38).

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