Detection of ras Gene Mutations in Pancreatic Juice and Peripheral Blood of Patients with Pancreatic Adenocarcinoma

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Abstract

Pancreatic adenocarcinomas are known to have a high incidence of K-ras gene mutations. Differential diagnosis of pancreatic cancer and chronic pancreatitis sometimes presents a clinical dilemma. We recently developed a highly sensitive and specific polymerase chain reaction capable of detecting 3–30 copies of mutant K-ras genes harboring codon 12 single base changes in the presence of 300,000 normal copies. Mutant ras genes were detected in DNA purified from pancreatic juice from all 6 cases of pancreatic adenocarcinoma and 1 case of intraductal papillary neoplasm of the pancreas. In 2 of 6 other cases with pancreatic adenocarcinoma, circulating metastatic cells were detected in DNA purified from peripheral blood. Activated ras genes were not found in pancreatic juice of three control cases (chronic pancreatitis and cholelithiasis) or in the peripheral blood of two patients with insulinomas. Notable conclusions of this study are that there can be significant levels of shed tumor cells in peripheral blood and an even higher number in pancreatic juice. In addition, two different K-ras mutations were found in some patients.

Introduction

Over 90% of pancreatic adenocarcinomas contain mutated ras genes and the site of mutation is restricted to codon 12 of K-ras gene (1–3). Since mutant K-ras is not found in nonmalignant disease such as chronic pancreatitis, detection of mutant K-ras in pancreatic disease provides a definitive diagnosis of pancreatic adenocarcinoma (3, 4). Solid organ tumors are known to shed cells. Only an extremely small fraction of these cells have been shown to establish metastatic disease. Several laboratories have reported using the PCR to detect metastatic cells in peripheral blood. These studies made use of the amplification of tissue-specific mRNA of tyrosine hydroxylase, tyrosinase, and prostate-specific antigen for neuroblastoma (5), melanoma (6), and prostate cancer (7), respectively, and relied on multiple copies of mRNA in each cell. We chose to target DNA rather than RNA for our studies to circumvent the restrictions on sample collection and preservation of cellular RNA. Sidransky et al. (8) recently described a method using PCR and phage cloning coupled to a radioactive probe-based detection strategy to identify mutant ras genes from shed colorectal tumor cells in feces. Unfortunately the described procedure is technically challenging since it requires sophisticated molecular biological methods.

The multiple PCR procedures that have been developed for the detection of point-mutated genes in an excess of unaltered genes have been reviewed (9). These procedures have compromised specificity or use incorrect statistical considerations for providing estimates of discrimination. We recently developed a highly sensitive PCR to specifically amplify target mutant oncogenes in the presence of a vast excess of normal genes. This method was used to analyze the DNA samples extracted from the pancreatic juice of patients with pancreatic adenocarcinoma. On the basis of the dramatic increase in sensitivity, we also analyzed DNA samples from peripheral blood to determine if circulating metastatic cells containing a K-ras mutation could be detected.

Materials and Methods

Pancreatic juice was collected from ten Japanese patients at our hospitals during endoscopic retrograde cholangiopancreatography with injection of secretin (10). Two to 8 ml of pancreatic juice were obtained and examined by cytology. In two cases, pancreatic juice was divided in half and DNA was extracted separately. For extraction of DNA, 0.5 to 1 ml of pancreatic juices was centrifuged to collect cells; then the pellets were washed with PBS and resuspended in 100 μl of 10 μm Tris (pH 8.0), 0.9% polyoxyethylene 10 lauryl ether (Sigma), and 0.05 mg/ml proteinase K. The mixtures were incubated at 56°C for 1 h, heated at 95°C for 10 min, and centrifuged briefly, and then the DNAs were collected using a Centricon 100 (Amicon). DNAs were resuspended in 40 μl of 10 μm Tris (pH 8.0)-0.1 μm EDTA. Ten μl of each sample, containing 5 to 100 ng of DNA as determined by the method of Walsh et al. (11), were used for PCR.

Peripheral blood (5 ml) from eight different patients was collected in the presence of EDTA. The samples were centrifuged, the supernatants were discarded, and the pellets were resuspended in 4 ml of PBS at 4°C before 8 ml of cold distilled water were added to lyse RBC. After centrifugation and washing with PBS, the pellets were digested with proteinase K followed by phenol-chloroform extraction (12). The DNAs were then quantitated by UV absorption. About 100 μg of DNA were extracted and 1 μg of DNA in 10 μl of 10 μm Tris (pH 8.0)-0.1 μm EDTA was used for each PCR. The highly sensitive and specific PCR method used is described separately. Since only three kinds of mutations at codon 12 of K-ras gene (CGT, GTT, or GAT) were reported in cases of Japanese patients with pancreatic adenocarcinoma (3), three different sense primers were used, the 3′-nucleotide of which was complementary to one of the mutant codons (e.g., CGT, GTT, or GAT). The sequences of the primers were: R1 = GGTTAGTGGACGCTC-R2 = GTAGTTGGAGCTGAA; and the antisense primer, R4 = CTATTGGTGATCATAGTCGG. The primer directly amplification of an 88- or 89-base pair product from exon 1. Three amplification reactions were performed for each sample; primer combinations used were R1–R4, R2–R4, and R3–R4. Briefly, up to 1 μg of sample DNAs was used in 50 μl amplification reactions containing 20 μm Tris (pH 8.4), 50 μm KCl, 2.5 μm MgCl₂, 50 μm concentrations of each dATP, dCTP, dGTP, and TTP, 0.25 μm concentrations of each primer, and 5 units of AmpliTaq DNA polymerase, Stoffel Fragment (Perkin-Elmer) incorporating AmpliWax (Perkin-Elmer) for greater specificity (13). For the primer combinations R1–R4 and R2–R4, PCR was performed for 45 cycles (95°C for 10 s, 56°C for 10 s) and for R3–R4, 35 cycles were used (95°C for 10 s, 56°C for 5 s) using a GeneAmp PCR system 9600 (Perkin Elmer). The thermal cycler was precooled (at least 5 cycles) to ensure accurate temperature control for the initial annealing steps. Following amplification, 10 μl of the reaction mixtures were loaded on 8% acrylamide gels and stained with ethidium bromide.

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2 The abbreviations used are: PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

Results

Following a systematic and comprehensive survey of reaction-conditions, thermal cycling parameters, alternative DNA polymerases, and the sequestering of reaction components prior to the initial denaturation step, we identified a highly sensitive and specific PCR protocol for the identification of rare cancer cells harboring a single nucleotide alteration. The optimized PCR assay provides for the detection of 0.01 ng of mutant DNA (equivalent of 3 copies of activated K-ras genes) for either CGT or GTT codons in 1 μg of DNA (equivalent of 150,000 cells or 300,000 copies of K-ras genes) that did not contain an activated oncogene. Detection of the GAT codon requires 10-fold more copies in 1 μg of DNA.

Pancreatic juices were initially analyzed cytologically. Although there were no cases that proved malignant by cytology, mutations of K-ras codon 12 were detected in all 6 cases with pancreatic adenocarcinoma and 1 case with papillary neoplasm of the pancreas, whereas 3 control cases (2 cases with chronic pancreatitis, 1 case with choledocholithiasis) were not found to contain mutations (Fig. 1: Table 1). Of the 7 cases, 2 cases contained a GAT mutation. 2 cases showed a GTT mutation, 2 cases showed both GAT and GTT mutations, and 1 case showed both GAT and CGT mutations (Table 1). Two DNA samples (from patients 4 and 5) were divided in two and the separated fractions were extracted independently; the results were the same (data not shown).

The mutations detected in pancreatic juice correlated with those shown by directly analyzing tumor samples (3). Tumor samples from four of seven patients (Table 1) had been analyzed previously by amplification of exon 1 of K-ras gene followed by direct sequencing (3). In 3 cases, a point mutation shown by direct sequencing matched the results from the single nucleotide discriminatory PCR method. In another patient, only a GAT mutation was demonstrated by direct sequencing; both GAT and GTT mutations were detected by the optimized PCR.

Discussion

The clinical diagnosis of pancreatic adenocarcinoma has improved but there are still cases in which a definitive diagnosis of pancreatic cancer from chronic pancreatitis cannot be made. These two diseases may share similar clinical and radiological characteristics (14). In such cases, pathological diagnosis is required for specific diagnosis. Fine needle aspiration cytology or biopsy have been used for specific diagnosis of pancreatic adenocarcinoma (10). Because of the location of the pancreas, biopsy is not as straightforward as gastrointestinal tract tumors of the stomach and colon. In addition, a risk that tumor cells may spread following biopsy has been reported (15). Cytological examination of cells in samples of pancreatic juice is another option (10); however, the earlier reports that showed high sensitivity for diagnosis have not been repeated (16). Although the PCR method, as used in this study, is not quantitative, comparison to a panel of copy number controls and extrapolation permitted estimation of the approximate number of activated oncogenes detected. As much as 1–10% of

Table 1 Detection of ras gene mutations in pancreatic juices

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Tumor size (cm)</th>
<th>Cytology</th>
<th>Mutant in pancreatic juice</th>
<th>Tumor mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>M</td>
<td>Pancreatic adenocarcinoma</td>
<td>5 x 8</td>
<td>Class 1</td>
<td>GGT</td>
<td>GTT</td>
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<tr>
<td>2</td>
<td>61</td>
<td>M</td>
<td>Pancreatic adenocarcinoma</td>
<td>4 x 4</td>
<td>Class 2</td>
<td>GAT</td>
<td>GAT</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>M</td>
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<td>4 x 4</td>
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<td>GAT</td>
<td>GAT</td>
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<tr>
<td>4</td>
<td>76</td>
<td>M</td>
<td>Intraductal papillary neoplasm</td>
<td>4 x 5</td>
<td>Class 2</td>
<td>GAT/GTT</td>
<td>N/A^b</td>
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<tr>
<td>5</td>
<td>57</td>
<td>M</td>
<td>Pancreatic adenocarcinoma</td>
<td>3 x 4</td>
<td>Class 2</td>
<td>GAT/GTT</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>71</td>
<td>M</td>
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<td>3 x 4</td>
<td>Class 2</td>
<td>GAT/GTT</td>
<td>N/A</td>
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<tr>
<td>7</td>
<td>75</td>
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<td>3 x 4</td>
<td>Class 2</td>
<td>GAT/GTT</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>73</td>
<td>M</td>
<td>Chronic pancreatitis</td>
<td>N/A</td>
<td>Class 2</td>
<td>GAT/GTT</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>F</td>
<td>Chronic pancreatitis</td>
<td>N/A</td>
<td>Class 2</td>
<td>GAT/GTT</td>
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<tr>
<td>10</td>
<td>53</td>
<td>M</td>
<td>Choledocholithiasis</td>
<td>N/A</td>
<td>Class 2</td>
<td>GAT/GTT</td>
<td>N/A</td>
</tr>
</tbody>
</table>

^a The sequence of wild type is GGT. These sequences were determined by PCR followed by direct sequencing as described elsewhere (3).

^b N/A, not available; N/D, not detected.
the cells in the pancreatic juices from patients examined harbored point mutations. The basis for the discrepancy of the results of molecular biological and cytological methods is unclear but may be due to degenerative changes of tumor cells in pancreatic juice (16). Confirmation of these results with larger numbers of samples is required to resolve this question. This molecular approach provides a more sensitive and specific as well as more convenient diagnosis of pancreatic adenocarcinoma.

Three of the 7 cases were shown to have two different mutations at codon 12 of K-ras (Table 1). Previous analysis of tumor material from one of these cases (patient 5) using entire exon amplification and direct sequencing of PCR products detected only one of these mutations. The presence of two different mutations of ras genes from the same sample, although previously reported, are rare (17). Preliminary quantitation indicated that the GAT mutation was in approximately a 4–10-fold excess of other mutations. There are multiple possible explanations for the presence of multiple ras mutations. The ras mutations may not be the primary genetic insult of the oncogenic process in pancreatic cancer but instead may be a result of the genetic liability initiated by an earlier genetic alteration. Alternatively, the genetic instability introduced by the first ras mutation may increase the likelihood of a mutation in the other copy of the ras gene in the same cell with advanced tumors. It would be interesting to determine if both mutations are found in the same cell. The dramatic increase in sensitivity of this PCR method for activated oncogenes presumably is the reason for the surprising large proportion of samples with two different mutations. Additional studies with this highly sensitive procedure will be necessary to determine the prevalence and significance of this observation.

The optimized single nucleotide discriminatory PCR assay was capable of detecting mutant DNA in peripheral blood in a fraction of the pancreatic adenocarcinomas tested. When samples of peripheral blood were studied, a GAT mutation was not detected although this was the most common type of mutation in cases of Japanese pancreatic adenocarcinoma (3). Whether this represents a statistical anomaly or the fact that the GAT mutation is detected with 10-fold less sensitivity is unclear. By extrapolation, these data indicate that there may be as many as 2 million circulating metastatic cells in peripheral blood alone. This result suggested that large numbers of tumor cells were circulating in the blood stream, in some cases, which may reflect one reason for the poor prognosis of pancreatic adenocarcinoma. Unlike the PCR procedure that targets tissue-specific mRNA to detect metastatic cells in peripheral blood (5–7), the PCR method described here does not identify the affected tissue or organ. On the other hand, the detection of activated ras genes can be used for all cancers in which this oncogene participates in the carcinogenic process thereby reducing the number of different assays required. Increasing the sensitivity to detect GAT mutations and studying larger numbers of cases including early stage pancreatic adenocarcinoma should be carried out to confirm these results and, by comparison to clinical features, to determine utility beyond differential diagnosis.

Acknowledgments

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References


Table 2 Detection of ras gene mutations in peripheral blood

<table>
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<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Tumor size (cm)</th>
<th>Metastases to remote organ</th>
<th>Mutant in peripheral blood</th>
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<tbody>
<tr>
<td>11</td>
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<td>Pancreatic adenocarcinoma</td>
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<td>+</td>
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<td>12</td>
<td>42</td>
<td>F</td>
<td>Insulinoma</td>
<td>3.5 x 3.5</td>
<td>-</td>
<td>Not detected</td>
</tr>
<tr>
<td>13</td>
<td>60</td>
<td>M</td>
<td>Insulinoma</td>
<td>3 x 4</td>
<td>+</td>
<td>Not detected</td>
</tr>
<tr>
<td>14</td>
<td>50</td>
<td>M</td>
<td>Pancreatic adenocarcinoma</td>
<td>4 x 4</td>
<td>+</td>
<td>Not detected</td>
</tr>
<tr>
<td>15</td>
<td>70</td>
<td>M</td>
<td>Pancreatic adenocarcinoma</td>
<td>2 x 4</td>
<td>-</td>
<td>GTT</td>
</tr>
<tr>
<td>16</td>
<td>56</td>
<td>M</td>
<td>Pancreatic adenocarcinoma</td>
<td>4 x 3</td>
<td>+</td>
<td>GTT</td>
</tr>
<tr>
<td>17</td>
<td>63</td>
<td>M</td>
<td>Pancreatic adenocarcinoma</td>
<td>4 x 3</td>
<td>+</td>
<td>Not detected</td>
</tr>
<tr>
<td>18</td>
<td>60</td>
<td>F</td>
<td>Pancreatic adenocarcinoma</td>
<td>6 x 9</td>
<td>-</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

* Liver, pleura, peritoneum, and distant lymph nodes.
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