Detection of c-K-ras Mutations in Fine Needle Aspirates from Human Pancreatic Adenocarcinomas

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ABSTRACT

Formalin-fixed paraffin-embedded tissue specimens obtained by fine needle aspiration of pancreatic masses from 47 patients were examined retrospectively for cytology and the presence of mutant c-K-ras oncogenes. Point mutations of c-K-ras in codon 12 were detected by RNA-DNA RNase A mismatch cleavage after in vitro DNA amplification of the cellular c-K-ras sequences by the polymerase chain reaction. Of the 36 patients with pancreatic adenocarcinoma, mutant c-K-ras oncogenes were detected in 18 of 25 (72%) with malignant cytologies, 2 of 8 (25%) with atypical cytologies, and 0 of 3 with benign aspiration cytologies. The remaining 11 patients without pancreatic adenocarcinomas did not have mutant c-K-ras genes detectable by the assay. The diagnosis of pancreatic adenocarcinoma was based upon clinical follow-up. The presence of mutant c-K-ras oncogenes did not significantly affect survival in the patients studied. Mutant c-K-ras genes were found at the time of initial clinical presentation in the majority of pancreatic adenocarcinomas, suggesting an important role of the mutation in oncogenesis. In conjunction with cytology, our approach represents an application for cancer diagnosis at the molecular genetic level.

INTRODUCTION

Genetic alterations are frequently associated with neoplasia. These genetic changes (point mutations, translocations, amplification, and deletions) alter cellular proto-oncogenes and/or their "suppressor" genes and contribute to the manifestations of the malignant phenotype (1). The activated proto-oncogenes (oncogenes) most often associated with human neoplasia are the members of the ras gene family (2). These highly conserved genes (c-K-ras, c-H-ras, and N-ras) encode similar membrane-bound proteins possessing guanine nucleotide-binding and hydrolytic activities. It is thought that ras proteins are involved in the process of signal transduction across the cell membrane (3). Single point mutations in ras proto-oncogenes activate their oncogenic potential. Indeed, somatic point mutations at codon 12 in the c-K-ras gene have been recently found in the majority of human pancreatic carcinomas (4-6).

Pancreatic carcinoma accounts for 5% of all cancer deaths in the United States (7). Despite advances in oncology, pancreatic carcinoma is invariably fatal with a median survival of 2.8 months (8) and a 5-year survival of 3-5% (7). FNA of pancreatic masses has been utilized for cytological diagnosis to minimize patient morbidity in this essentially terminal disease. As reported in various studies (9, 10), aspiration cytology can give an accurate diagnosis of carcinoma in 55-100% of pancreatic carcinomas. The cells obtained by FNA can represent the earliest tissue samples of pancreatic tumors and are often the only tissue available for analysis.

Using the RNase A mismatch cleavage method (11, 12), we have successfully analyzed pancreatic aspirates in paraffin-embedded tissue blocks from 47 patients for the presence of single point mutations in the first coding exon of the c-K-ras gene. Since c-K-ras mutations are not found in nonneoplastic tissue (1-5), we reasoned that detection of such mutations could aid in diagnosis independent of cytological criteria. We also wanted to assess the effects of such mutations on clinical outcome.

Since the number of cells obtained by FNA is often small, in vitro DNA amplification by the PCR (13) was utilized to increase the number of c-K-ras sequences present in the 5- to 10-μm sections of formalin-fixed paraffin-embedded aspirates (14) prior to RNA-DNA RNase A mismatch cleavage analysis (4).

MATERIALS AND METHODS

Fine Needle Aspirates. Primary care patients at the Los Angeles County-USC Medical Center with pancreatic masses are routinely studied by fine needle aspiration. Pancreatic masses were aspirated intraoperatively or percutaneously with the guidance of computerized axial tomography or ultrasonography with thin needles (20 or 22 gauge). Cell blocks were made in addition to stained smears. Fifty-four cell blocks of FNAs obtained from 50 patients between the years 1981 and 1987 were available for study. The DNA present in 51 cell blocks from 47 patients could be amplified by the polymerase chain reaction and analyzed for c-K-ras mutations. In three cases no amplifications occurred, despite having cells present. It is unclear why these three specimens were inadequate substrates for the PCR. The aspirates with and without c-K-ras mutations were similar in cellular composition.

The consensus cytological diagnosis of pancreatic adenocarcinoma was made using standard criteria (9, 10) after review by three pathologists (D. S., M. M. C., and S. E. M.). All of the pancreatic tumors were thought to be of ductal cell origin, although exact subclassification (15) was not possible. Clinical follow-up was obtained through review of medical records, death certificates, and the California Tumor Registry. Patients were considered to have pancreatic adenocarcinoma if their clinical outcomes were consistent with terminal carcinoma. Except for one individual, all patients considered to have pancreatic carcinoma died within 1 year after fine needle aspiration. The one patient still alive after 16 months had a pancreatic mass and an aspirate with malignant cells. There were 36 pancreatic adenocarcinomas, 4 tumors in the pancreatic region other than pancreatic adenocarcinoma, and 7 benign lesions among the 47 patients successfully analyzed. Survival was the period between FNA and death. Staging (8) of the patients was not uniform, although disseminated metastases (Stage IV) could be documented by laparotomy or radiological studies. Therefore, extent of disease at the time of aspiration was categorized as Stage IV or less than Stage IV (without disseminated metastases). None of the tumors were considered resectable (Stage I). Treatment of the pancreatic tumors was nonuniform and consisted of chemotherapy, palliative surgery, radiotherapy, or supportive care. The patients with or without mutated c-K-ras genes did not receive treatments which differed significantly (P > 0.05, x²). The log-rank test (16) was used to compare patient outcomes.
Polymerase Chain Reaction. The PCR was performed as previously described (4, 14), although modified for the heat-stable DNA polymerase from Thermus aquaticus (17). One or, in cases where only a small number of cells were present in the aspirate, three 5- to 10-μm paraffin slices from the FNA cell blocks were deparaffinized with one wash of xylene and two washes with ethanol. An additional section was stained to confirm the cytology. The desiccated tissue was then heated at 100°C in 50 μl of water for 7 min. The PCR was performed with an automated thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) for 50 cycles in the presence of two primers (1 μM each) specific for the c-K-ras first coding exon (4). Four units of the heat-stable polymerase (TAQ, Perkin Elmer Cetus) were used for each reaction. Denaturation was at 95°C for 30 sec, annealing at 42°C for 15 sec, and polymerization at 72°C for 1 min. Temperature transitions occurred as fast as possible.

Detection of Mutant c-K-ras Genes. Single point mutations within the first coding exon of the c-K-ras gene were analyzed by the RNAse A mismatch cleavage assay (11), as previously described (4). Uniformly labeled RNA probes corresponding to the first coding exon of the c-K-ras gene were synthesized with SP6 polymerase in vitro transcription system (18) using pAK1NGly (11), as previously described (4), except that 100 μM unlabeled CTP were used. The extent of amplification by PCR of c-K-ras sequences from the aspirates was estimated from non-denaturing polyacrylamide gel electrophoresis and staining with ethidium bromide, as well as hybridization to the c-K-ras probe. Aliquots of the amplified PCR reaction were accordingly adjusted and hybridized to the RNA probe. The conditions for hybridization, RNAse A digestion, and analysis by denaturing polyacrylamide gels and autoradiography were reported previously (4).

RESULTS

Single nucleotide substitutions within the first coding exon of the c-K-ras gene were detected by RNAse A cleavage at single mismatches in RNA-DNA hybrids and polyacrylamide gel electrophoresis of the RNAse A-resistant products (4). Fig. 1 shows the results from two of these experiments. The mismatch-specific RNA bands of 66 and 43 nucleotides (indicated by arrows) are diagnostic of the presence of c-K-ras genes mutant at codon 12 in the SK-CO-1 tumor cell line, used as a positive control (4, 19), as well as in some of the samples (Fig. 1A, lanes 2, 3, 4, 7, 8, and 10; Fig. 1B, lanes 12 through 17 and 19). These bands diagnostic of a mismatch were undetectable in control (4, 19), as well as in some of the samples (Fig. IA, lanes 2, 3, 4, 7, 8, and 10; Fig. IB, lanes 12 through 17 and 19). Although the exact nature of each mutation has not been characterized, based on the mobility of the mismatch bands, it can be deduced that samples 2 and 7 (Fig. 1A) and sample 15 (Fig. 1B) contain a mutation at the first position of codon 12, while the rest of the positive samples are mutant at the second position of codon 12. The RNA-DNA mismatch cleavage assays were interpreted without prior knowledge of the cytological or clinical data. To avoid false positive diagnosis, we have been conservative in the interpretation of the results and only those cases with a clear mismatch pattern have been considered positive. The results of these experiments are summarized in Table 1. Thirty-six of the 47 patients with pancreatic masses in this study had clinical follow-up and outcome consistent with pancreatic carcinoma. c-K-ras mutations were detected in 20 of the 36 (56%) patients. By cytological criteria alone, 25 of these 36 patients had an aspirate consistent with a diagnosis of carcinoma. Among these 25 patients, 18 (72%) had a mutated c-K-ras gene. Eleven of the 36 patients with pancreatic carcinoma had FNA specimens that were not characteristic of pancreatic carcinoma. Eight of these aspirates were judged atypical and three benign. Interestingly, c-K-ras mutations were detected in FNAs from two of the eight patients with atypical cytology. An example of the cytology not diagnostic of carcinoma but containing mutated c-K-ras genes is seen in Fig. 2. Aspirates of four tumors from the pancreatic region which were not pancreatic adenocarcinomas (lymphoma, sarcoma, and gastric and bile duct carcinoma) and seven benign lesions did not contain mutated c-K-ras sequences detectable by our assay.

FNAs of pancreatic masses may score negative in our assay.

Table 1 Aspirates from 47 patients: cytology and c-K-ras mutations

<table>
<thead>
<tr>
<th>Number</th>
<th>ras-mutated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic adenocarcinoma</td>
<td>36</td>
</tr>
<tr>
<td>Cytology malignant</td>
<td>25</td>
</tr>
<tr>
<td>Cytology atypical</td>
<td>8</td>
</tr>
<tr>
<td>Cytology benign</td>
<td>3</td>
</tr>
<tr>
<td>Benign pancreatic disease</td>
<td>7</td>
</tr>
<tr>
<td>Cytology atypical</td>
<td>1</td>
</tr>
<tr>
<td>Cytology benign</td>
<td>6</td>
</tr>
<tr>
<td>Pancreatic region tumors not pancreatic adenocarcinoma*</td>
<td>4</td>
</tr>
</tbody>
</table>

* Lymphoma, sarcoma, and gastric and bile duct carcinoma.

Fig. 1. RNA-DNA mismatch analysis of c-K-ras sequences from paraffin-embedded tissue sections of pancreatic FNAs. A. M, molecular weight marker; P, RNA probe pAKINGly; N, negative control using DNA amplified from normal human fibroblasts; SK-CO-1, positive control using DNA amplified from a colon carcinoma cell line which contains a valine mutation (GTT) at the second position of codon 12. B. Same as A except that the molecular weight marker M was a HaeIII digest of φ X 174 DNA. The arrows at the right indicate the positions of the codon 12-specific mismatch bands. Positive specimens are numbers 2, 3, 4, 7, 8, 10, 12-17, and 19.

1 Unpublished observations.
The factors affecting survival are presented in Fig. 3. Patients with Stage IV disease at the time of aspiration lived significantly shorter ($P < 0.001$), with a median survival of 2.5 months versus 7 months for patients with less than Stage IV disease. This effect of stage on survival has been previously documented (8). The effects of c-K-ras mutations on survival were not statistically significant ($P > 0.05$). The median survival after aspiration was 3.5 months for patients with c-K-ras mutations and 5 months for patients without c-K-ras mutations. Median survival for patients with Stage IV disease was 2.5 months in the presence of c-K-ras mutations and 3.2 months in the absence of c-K-ras mutations. Patients with less than Stage IV disease had median survivals of 7.5 months with c-K-ras mutations and 7 months without c-K-ras mutations.

**DISCUSSION**

The RNAse A mismatch cleavage analysis after gene amplification by the PCR is a powerful tool for detection of point mutations in cellular genes. We have demonstrated that this method is useful for the detection of mutant c-K-ras oncogenes present in routinely obtained clinical specimens of pancreatic adenocarcinoma. Because the c-K-ras mutations were specific for malignant tissue, our approach represents a direct application for cancer diagnosis at the molecular genetic level. The polymerase chain reaction allows in vitro DNA amplification of the scant amounts of DNA present in the small numbers of cells obtained by fine needle aspiration, thereby yielding quantities suitable for analysis. Human papilloma viral sequences have also been detected in fine needle aspirations of metastatic tumors using the PCR (20).

In this study, the majority (72%) of pancreatic adenocarcinomas defined cytologically after FNA had detectable c-K-ras mutations at the time of clinical presentation. In a previous study (4), 95% of pancreatic adenocarcinomas contained mutated K-ras genes. There are several possible explanations for this difference, although the total number of tumors that have been examined in these studies is relatively small and the difference between the two studies is not statistically significant ($P > 0.05, \chi^2$). One possibility is that the distribution of tumor cells containing c-K-ras mutations may not be homogeneous and the smaller number of cells sampled by FNA compared to a typical tissue section may have not contained sufficient cells with the c-K-ras mutation to be detectable in the assay. In addition, while the earlier work was a study of advanced disease (68% of pancreatic tumors were sampled at autopsy), the fine needle aspirations were performed as the initial step for the definitive diagnosis of pancreatic carcinoma and the material we analyzed in this study represented the earliest possible sampling of the tumor cells. Therefore, the differences in the frequency of c-K-ras mutations between these two studies is also consistent with mutations in c-K-ras being acquired during progression of the tumors. Consistent with this hypothesis is the presence of mutant c-K-ras genes in metastatic pancreatic carcinoma (4, 21). In addition, a steady increase in mutated c-K-ras gene incidence has been detected during colorectal early tumor development (22, 23) and progression, including the metastatic stage (23).

The clinical contribution of mutated c-K-ras genes to pancreatic carcinoma is uncertain. The effects of c-K-ras mutations on survival were not statistically significant with the numbers of patients studied. Similar results have also been reported (6). Additional studies are needed to clarify the role of mutated c-K-ras genes in pancreatic carcinoma. The presence of mutated
FIG. 3. Survival curves of patients with pancreatic carcinoma. Only the 25 patients with pancreatic adenocarcinoma and cytology diagnostic of pancreatic adenocarcinoma are presented (see text).

c-K-ras genes in the majority of pancreatic adenocarcinomas at the time of clinical presentation may reflect the aggressive clinical behavior of a disease which is largely resistant to chemotherapy and radiotherapy and seldom resectable. For comparison, using similar highly sensitive DNA probe analysis (19, 22–24), only approximately 40–50% of all colonic carcinomas [5-year survival rate (1), 49–54%] were found to have mutated ras genes. The analysis of pancreatic dysplasia (25, 26) or small resectable pancreatic adenocarcinomas, which were not presently studied and are potentially curable, may yield further insight into the role of ras oncogene mutations in early pancreatic oncogenesis. As multiple genetic alterations may be present in tumors (22), the investigation of multiple oncogenes or anti-oncogenes may yield better understanding of malignancy. The methods used in this study allow selective genetic analysis of human tissues routinely obtained and stored. Further insight into the role of c-K-ras mutations and pancreatic carcinoma may be possible with prospective as well as previously conducted well defined clinical studies if paraffin-embedded tissue samples are available.

The cytological diagnosis of pancreatic carcinoma was more sensitive than diagnosis based on the presence of c-K-ras mutations. The two methods were complementary, however, as more accurate diagnosis was possible if both techniques were utilized. In two cases in which c-K-ras mutations were detected, the cytology was considered atypical but not diagnostic of carcinoma (Fig. 2). The cytological criteria for malignancy must be conservative to avoid a false positive diagnosis, since inflammatory atypia is difficult to distinguish from better differentiated carcinomas (9). Moreover, the cytological diagnosis of malignancy is difficult if only a few atypical cells are present. By using the presence of mutated c-K-ras genes as a criterion for malignancy, two of the eight cytologically atypical aspirates from patients with pancreatic adenocarcinoma could be correctly classified as malignant. Review of the cytology clearly demonstrated cells which had features of malignancy but were too few in number for unequivocal diagnosis. The PCR with subsequent DNA-RNA mismatch assay allows analysis independent of morphology and would be most useful when the cytology is atypical or when the suspicious cells are few in number.

In conclusion, we have detected c-K-ras mutations in cells obtained by fine needle aspiration in the majority of pancreatic adenocarcinomas, suggesting an important and early role of the c-K-ras oncogene in tumorigenesis. However, since there are negative cases, mutations at codon 12 in the c-K-ras gene are not absolutely required for development and/or progression of pancreatic adenocarcinoma. In addition, although none of the pancreatic tumors analyzed other than pancreatic carcinomas has c-K-ras mutations, tumors of a different primary site but metastatic to the pancreas and containing c-K-ras oncogenes could also be detected by our method. Therefore, the detection of c-K-ras oncogenes with mutations at codon 12 in pancreatic aspirations is not completely reliable for diagnosis because neither is their presence diagnostic of pancreatic carcinoma nor is their absence a criterion for benign disease. However, since the mutations appear specific for malignant disease, our oncogene detection approach, in combination with cytomorphological evaluation, allows a more definitive diagnosis of malignancy on aspirates which by cytomorphology alone are not diagnostic of malignancy. Distinguishing between normal and mutant ras-containing tumors and/or between relative oncogene dosage may be useful for classification at the molecular level and may also aid in the evaluation of new therapeutic modalities for pancreatic carcinoma.

REFERENCES

ras MUTATIONS IN PANCREATIC ADENOCARCINOMA


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