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Detection of K-*ras* Point Mutation at Codon 12 in Pancreatic Diseases: A Study in a Brazilian Casuistic

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ABSTRACT

Objective To clarify the sensitivity and the validity of K-*ras* point mutational analysis at codon 12 in Brazilian patients with pancreatic diseases, and the possible correlation between the presence of the mutation and the histopathological findings.

Patients Ninety-seven Brazilian patients with pancreatic ductal adenocarcinoma, pancreatic neuroendocrine tumors and chronic pancreatitis were enrolled in this study. Forty-five patients (46%) were female and 52 patients (54%) were male, having an average age of 60.2±9.2 years for adenocarcinoma (n=52), 45.1±19.4 years for pancreatic neuroendocrine tumors (n=20), and 46.4±11.2 years for chronic pancreatitis (n=25). DNA extracted from 11 normal human peripheric lymphocytes was utilized as a control.

Results The sensitivity of K-*ras* mutational analysis was 83.3% (25/30) in paraffin-embedded samples and 72.7% (16/22) in surgically resected specimens of the malignancy. On the other hand, no mutations were found in pancreatic neuroendocrine tumors or in chronic pancreatitis. Regarding the histopathological grading, the higher positivity rate was found in poorly-differentiated adenocarcinoma (100%), and progressively decreased in moderately-differentiated adenocarcinoma (72.2%), and well-differentiated adenocarcinoma (66.6%). The positivity rate in non-classified adenocarcinoma was 81.8%.

Conclusion K-*ras* point mutation, in our study, is notably prevalent in malignancies and is absent in chronic pancreatitis and pancreatic neuroendocrine tumors. These results encourage us to consider the possibility of treatment strategies for this oncogene in the future.

INTRODUCTION

Despite considerable development in sophisticated imaging techniques and cytological examination, an early diagnosis of pancreatic neoplasm is rare. Hence, the rate of cure of this disease is less than 10%. Furthermore, surgical therapy for pancreatic cancer is frequently not curative, most often as a consequence of this tumor's propensity to metastasize. Only in a minority of cases is the diagnosis made at a very early stage, when curative surgery might significantly ameliorate the 5-year survival rate [1, 2, 3]. Therefore, a better understanding of the molecular basis of transformation into malignant tumor may contribute to the establishment of new criteria for diagnosis, prognosis and treatment of human neoplasms. Genetic mutations are associated with many types of tumors. In general, these changes involve genes, called protooncogenes and tumor suppressor genes, engaged in the control of cellular growth and differentiation.

Protooncogenes, such as *ras* genes (N-*ras*, H-*ras* and K-*ras*), are able to code proteins commonly referred to as $p21^{ras}$ which act as molecular switches in the intracellular signal transduction process, binding GTP and GDP with intrinsic GTPase activity [4, 5, 6, 7, 8, 9]. This gene is converted to an active oncogene by point mutations and plays an important role in tumorigenesis by maintaining the active GTP-bound form, thus favoring the constitutive transmission of a positive signal for cell growth. In pancreatic cancer, this type of mutation usually arises at codon 12, the hot spot of the gene itself, although mutations at codons 13 and 61 may also occur with substitution of the correspondent aminoacid in the *ras* protein [5, 7, 8]. Altered protein products are an expression of this mutation which is capable of transforming cells into a malignant phenotype [8, 9].

Mutational activation of the K-*ras* at codon 12 has been demonstrated in 71 to 100% of the cases of pancreatic adenocarcinoma [5, 7, 8, 9]. This very high prevalence of mutation has never been identified in other types of human tumors. Apparently, the substitution of a nucleotide at the first or second base of codon 12, for example, from GGT (glycine) to GTT (valine) or to AGT (serine) or to GAT (aspartic acid) or to CGT (arginine), may precede the development of malignancy [9].

The high prevalence of mutation in pancreatic tumors suggests that point mutations in the K-*ras* gene might be used in future screening protocols for pancreatic cancer. *ras* is associated with more aggressive and recurrent tumors. Moreover, the K-*ras* oncogene has been utilized in the process of metastasis and aggressiveness of tumoral cells [10]. Therefore, it seems to be an important target for novel anti-cancer therapies.

Because of the great importance of K-*ras,* as stated above, the aim of the present study was to clarify the sensitivity and the validity of K-*ras* point mutation at codon 12 in Brazilian patients with pancreatic diseases, and the possible correlation between the presence of the mutation and the histopathological findings.

MATERIAL AND METHODS

Patients

A total of 97 Brazilian patients from Hospital das Clínicas-FMUSP and Hospital Sírio-Libanês with histologically proven pancreatic ductal adenocarcinoma (52 patients), pancreatic neuroendocrine tumors (20 patients), and chronic pancreatitis (25 patients) were enrolled in this study.

Forty-five patients (46.4%) were female and 52 patients (53.6%) were male, having an average age of 60.2±9.2 years (range: 36-82) for adenocarcinoma, 45.1±19.4 years (range: 16-81) for pancreatic neuroendocrine tumors, and 46.4±11.2 years (range: 34-71) for chronic pancreatitis (Table 1). DNA extracted from blood samples taken from 11 healthy subjects (4 females and 7 males) was used as a negative control.

Concerning the 52 patients with pancreatic ductal adenocarcinoma, 30 (57.7%) were smokers and 35 (67.3%) were not alcoholics. They underwent pancreaticoduodenectomy in resectable cases and palliative surgery in unresectable cases. When unresectable tumors are found in the exploratory laparotomy, our group does not perform a biopsy of the primary pancreatic tumor due to the high morbidity rate from this procedure. Therefore, extra pancreatic biopsies were performed in adjacent or invaded organs, such as lymph nodes, the liver, and the stomach. This fact may explain the necessity of formulating a

Table 1. Characteristics of the 97 patients enrolled in the study.

	Sex (M/F)	**Age: mean±SD (Range)**
Pancreatic adenocarcinoma (52 patients)	30/22	60.2±9.2 (36-82)
Neuroendocrine tumors (20 patients)	4/16	45.1±19.4 (16-81)
Chronic pancreatitis (25 patients)	18/7	46.4±11.2 (34-71)
Total (97 patients)	52/45	53.5±14.2 (16-82)

fourth category, namely non-classified adenocarcinoma.

All the 25 patients with chronic pancreatitis were alcoholics and 23 patients (92.0%) were heavy smokers. Nineteen patients (76.0%) had pancreatic calcifications on plain X-ray, confirmed by endoscopic retrograde cholangiopancreatography. Ten patients (40.0%) had pseudocysts. All patients had untractable pain not responsive to narcotic drugs at the time of surgery. Thus, these patients underwent surgery as a result of pseudocysts and clinical persistent untractable pain. A pancreatic resection or a lateral pancreaticojejunostomy was performed.

Eighteen patients with pancreatic neuroendocrine tumors (90.0%) did not smoke and none of them had alcoholic antecedents. All tumors were non-metastatic and enucleation was performed.

All surgical specimens were analyzed histopathologically. Regarding the histological grading of adenocarcinoma, exocrine pancreatic tumors were classified according to the differentiation grade as well-differentiated adenocarcinoma (6 patients), moderately-differentiated adenocarcinoma (18 patients), poorly-differentiated adenocarcinoma (6 patients), and non-classified adenocarcinoma (22 patients) [11]. Concerning the pathological features of the neuroendocrine tumors, they were classified as functioning neuroendocrine tumors (12 insulinomas and 4 apudomas) and 4 non-functioning neuroendocrine tumors. Chronic pancreatitis was classified according to the Marseille diagnostic criteria [12].

The study was divided into two phases. In the first phase (from January 1994 to December 1998), 52 formalin-fixed and paraffin-embedded samples from archival tissue were studied retrospectively, to clarify whether the experimental design targeting the detection of K-*ras* point mutation had some validity. Twenty-six non-tumoral tissue samples taken from surgical margins, lymph nodes, the gallbladder, the stomach, and the common bile duct were used as negative controls. In the second phase (from January 1999 to December 2001), 45 surgically resected specimens, frozen in liquid nitrogen, were used to analyze the frequency of K-*ras* mutations. Normal human peripheric lymphocytes were used as negative controls.

Detection of K-*ras* Point Mutation

DNA Extraction from Paraffin-Embedded Pancreatic Tissue

DNA was extracted from three consecutive 10-µm sections of the paraffin block that best represented each tumor, previously selected from hematoxylin-eosin stained slides. To prevent cross contamination from tissues with flakes of paraffin, disposable microtome blades were used and the instruments were cleaned with xylene after each section was cut. Three baths in 500 µL of xylene at 95 ºC followed by three baths in 500 µL of 100% ethanol were used to deparaffinize the tissue. After that, the tissue samples were centrifuged for 5 minutes at 13,000 rpm. The resulting pellets were incubated overnight at 37 ºC in 500 µL of digestion buffer (10 mM Tris pH 7.4, 100 mM NaCl, 25mM disodium EDTA, 0.5% sodium dodecyl sulfate) containing 10 µL of proteinase K (200 µg/mL final concentration). The enzyme was inactivated by heating the specimens for 10 minutes at 95 ºC; protein was removed by adding an equal volume of phenol:chlorophorm:isoamyl alcohol (25:24:1), and DNA was precipitated by adding 1/4 volume 8 M ammonium acetate and 1 mL of 100% ethanol. The DNA samples were then incubated at –20 ºC for 1 hour and centrifuged for 10 minutes at 13,000 rpm at 4 ºC. The resulting pellets were washed in 70% ethanol to remove salt, air dried, and resuspended in 50 µL of Tris 10mM, EDTA 1 mM buffer [13, 14].

DNA Extraction from Surgically Resected Specimens

The specimens were extracted with a genomic DNA isolation reagent (guanidine-detergent lysing solution, DNAzol®, Gibco BRL, Life Technologies, Gran Island, NY, USA) according to the manufacturer's protocol.

Polymerase Chain Reaction/Restriction Fragment Length Polymorphism (PCR/RFLP)

Mutations in codon 12 of the K-*ras* gene were detected by PCR/RFLP analysis with *Mva*I (Amersham Life Science, Buckinghamshire, England) as the restriction enzyme. The PCR reactions were carried out in a final reaction volume of 25 µL containing 1X reaction buffer, 0.2 mM deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 1.5 mM magnesium chloride, 1 µM of each primer, 1.5 units of *Taq* polymerase (Gibco BRL, Life Technologies, Gran Island, NY, USA) and 2.5 µL of extracted DNA (50-100 ng). All necessary procedures to prevent contamination were followed. Synthesized primers (Bio-Synthesis Inc., Lewisville, TX, USA) (sequence 5'- 3') were used. The sequence is as follows: A (sense) ACTGAATATAAACTTGTGGTAGTTGGA CCT, B (antisense) TCAAAGAATGGTCCTGGACC, and C (antisense) TAATATGTCGACAAAACAAGATTTACC TC. Underlined bases represent mismatches from the K-*ras* DNA sequence [7, 15].

Two PCR amplification rounds were performed. After the first-round amplification, using primers A and B, a 157 base pair fragment was amplified, which would be digested by *Mva*I (CC**A**/**T**GG). When codon 12 is normal (glycine), it contains two restriction sites or just one site if the codon 12 contains a mutation in either of its first two bases. Therefore, wild-type fragments cleave to yield products with sizes of 114, 29, and 14 base pairs, whereas mutant fragments cleave to yield products of 143 and 14 base pairs. When a second-round amplification is performed with primers A and C, just fragments of 143 base pairs are amplified. This second amplification gives rise to a product of 135 base pairs, which is cleaved with *Mva*I at one site if the codon 12 is normal, but fails to cleave if a mutation exists in the first two bases of codon 12 [7, 15].

The PCR reactions were performed in a thermocycle (Gene Amp PCR System 2400, Perkin Elmer, Foster City, CA, USA); each cycle was performed at 96 ^{o}C for 1 min for denaturation, 55 ^{o}C for 1 min for annealing and 73 ^{o}C for 30 s for extension. The first PCR was comprised of 12 cycles, followed by digestion of PCR products (10 µL aliquots) with *Mva*I 4U (90 min at 37 ºC) and, after that, a second amplification of 30 thermal cycles, using primers A and C, was performed. Except for the number of PCR cycles, all other conditions were identical to those reported in the first PCR. The product of this second PCR was again digested with the enzyme *Mva*I. After this second digestion, adenocarcinoma samples showed a non-cutting 135 base pair product, while negative-control samples showed a 106 base pair fragment.

Electrophoresis was performed using 2% agarose gel within a Horizon 58 apparatus (Gibco BRL, Life Technologies, Gran Island, NY, USA), stained with ethidium bromide, and then photographed using an ultraviolet transilluminator and analyzed by computerized imaging system (EDAS 120, Kodak, Rochester, NY, USA).

ETHICS

This was an observational study. All patients were managed in accordance with normal clinical practice. The protocol was approved by the Ethics Committee of the University of São Paulo School of Medicine.

STATISTICAL ANALYSIS

Frequencies, mean values, standard deviations (SD) and ranges were used as descriptive statistics. The relationship between the positivity of the K-*ras* mutation and the histopathological grading was tested by means of the Mantel-Haenszel chi-square test for linear association. The statistical analysis was performed by running the SPSS/PC+ statistical package (SPSS Inc., Chicago, IL, USA) on a personal computer. A two-tailed P of 0.05 was chosen as the cut-off for detecting statistically significant values.

RESULTS

The overall sensitivity of K-*ras* mutational analysis was 78.8% (41/52); in particular, the positivity of the K-*ras* mutation was 83.3% (25/30) in paraffin-embedded samples and 72.7% (16/22) in surgically resected specimens of pancreatic ductal adenocarcinomas (Table 2). On the other hand, no mutations were found in the 20 pancreatic neuroendocrine tumors nor in the 25 chronic pancreatitis (Figure 1).
Regarding the histopathological grading, the higher positivity rate was found in poorly-differentiated adenocarcinoma (100%), and progressively decreased in moderately-differentiated adenocarcinoma (72.2%), and well-differentiated adenocarcinoma (66.6%) (Table 2). The relationship between the positivity of the K-*ras* mutation and the histopathological grading did not reach statistical significance (P=0.180). The positivity of the K-*ras* mutation was 81.8% in non-classified adenocarcinoma.

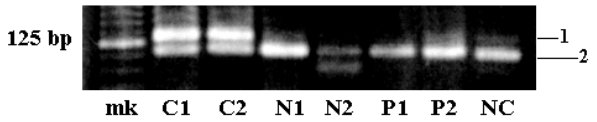

Figure 1. Detection of the codon 12 mutation in the K-*ras* oncogene in pancreatic diseases by PCR/RFLP analysis. Ethidium bromide-stained agarose gel of PCR-RFLP analysis showing specimens of different pancreatic diseases.

1: upstream, mutant band
2: downstream, wild band
mk: marker
C1, C2: pancreatic adenocarcinoma from two patients
N1, N2: neuroendocrine pancreatic tumors from two patients
P1, P2: chronic pancreatitis from two patients
NC: healthy subject blood sample (negative control)
See text for more details

DISCUSSION

The survival rate over 5 years in pancreatic cancer patients in the United States, has not been higher than 3-5% over the last decade. The poor prognosis of this disease could be ameliorated if curative surgery was performed in its early stage [1, 2, 3, 16].
The main problem in diagnosing pancreatic cancer, especially at the early stage, is characterizing and identifying which group of the population has a higher risk of tumor. Subjects with diseases such as chronic pancreatitis (mainly hereditary chronic pancreatitis), mucinous ductal dilatation (intraductal tumor) and long-standing diabetes, have been considered as population groups having an increased risk of developing pancreatic cancer [17, 18].
Using the advantages of the genomic amplification technique *in vitro*, we have

Table 2. Frequency of K-*ras* gene mutation positivity in patients with adenocarcinoma according to the histopathologic grading study.

	Paraffin embedded samples	Surgically resected specimens	Total
Well-differentiated adenocarcinoma	2/2 (100.0%)	2/4 (50.0%)	4/6* (66.7%)
Moderately-differentiated adenocarcinoma	8/11 (72.7%)	5/7 (71.4%)	13/18* (72.2%)
Poorly-differentiated adenocarcinoma	6/6 (100.0%)	0/0	6/6* (100.0%)
Non-classified adenocarcinoma (metastatic sites)	9/11 (81.8%)	9/11 (81.8%)	18/22 (81.8%)
Total	**25/30 (83.3%)**	**16/22 (72.7%)**	**41/52 (78.8%)**

* P=0.180 by Mantel-Haenszel chi-square test for linear association. Non-classified adenocarcinoma patients were excluded from analysis.

shown that mutations in the K-*ras* oncogene can be readily detected in fresh tumoral tissues or paraffin embedded samples. The facility in using paraffin embedded samples, stored at room temperature for many years enormously magnified the potential for applications of these methods. Therefore, a retrospective analysis of the frequency of mutated oncogenes in some types of tumors became possible. In the present study, the sensitivity was 83.3% according to previous reports. However, no statistical significance between this positivity rate and the histological grading was found. Concerning the sensitivity of 72.7% found in surgically resected specimens, it also corresponds to literature findings (71-100%) [5, 7, 8, 9, 19].

Point mutations in K-*ras* are particularly helpful in diagnosis for several reasons. Firstly, mutations in this oncogene are usually limited to just one codon. Moreover, the incidence of K-*ras* point mutation at codon 12 is definitely higher when compared with mutations at codon 13 or 61 in pancreatic cancer [5, 9]. Secondly, point mutations are sufficiently frequent in pancreatic adenocarcinoma suggesting that this gene may be a sensitive marker for the diagnosis of pancreatic carcinoma and finally, mutations in the K-*ras* oncogene are present in pancreatic carcinoma *in situ* which makes the early detection of a tumor possible, thereby increasing the possibility of cure.

Pavelic *et al.* [20] detected K-*ras* mutations only in metastatic malignant insulinoma, while we did not find any K-*ras* mutations in pancreatic neuroendocrine tumors. Their results support the idea that the progression of pancreatic cells to a more malignant phenotype requires further genetic damage. On the other hand, Yashiro *et al.* did not find any mutations in endocrine tumors which corresponds to our results [21].

Recently, Löhr *et al.* [22], in an extensive review study, found the K-*ras* mutation in chronic pancreatitis ranging from 0 to 100%. The reason for this discrepancy is not clear. In our study, no single K-*ras* mutation was detected in chronic pancreatitis. The findings of Löhr *et al.* may be related to the advanced age (average: 71 years) of the chronic pancreatitis patients in which K-*ras* mutations were observed. In contrast, in our study the mean age of patients with chronic pancreatitis was 46 years. In fact, if patients are evaluated over longer periods, they are more likely to harbor such mutations [23, 24, 25]. Moreover, another possible variation is the etiology of pancreatitis, because the outcome of this disease regarding cancer development is different in alcoholic and hereditary pancreatitis [26]. In this study, all the patients with chronic pancreatitis have alcohol abuse as the etiology of the disease. Therefore, considering that hereditary chronic pancreatitis is possibly a risk factor for the preneoplasic process, further studies using patients with this etiology will be necessary to understand pancreatic tumoral behavior. It is also possible that in the future, the finding of K-*ras* mutations in pancreatic juice in those patients with hereditary pancreatitis might indicate the necessity for a resection procedure before the development of pancreatic cancer.

The K-*ras* point mutation occurs in the early stage of pancreatic carcinogenesis process, however it has not been clarified whether the frequency of this oncogene could be correlated with the grade of cellular atypism [27, 28, 29, 30]. On the contrary, colorectal cancers have been used to study the timing of activation of *ras* genes since this type of cancer has a well-defined premalignant stage in the form of adenomas or polyps [31].

The high prevalence of the K-*ras* mutation in pancreatic cancer, as verified in this study and previous reports suggests the possibility of detecting the mutation in pancreatic juice collected endoscopically or after pancreatic duct brushing or cholangiopancreatography, in duodenal juice and even in stools [32, 33, 34, 35, 36, 37, 38, 39, 40, 41].

In conclusion, the K-*ras* point mutation in our study is considerably prevalent in malignancies and is absent in chronic pancreatitis and pancreatic neuroendocrine tumors. These results encourage us to consider the possibility of treatment strategies of this oncogene in the future.

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Key Words: Genes, *ras*; Human; Neuroendocrine Tumors; Pancreatic Neoplasms; Pancreatitis; Polymerase Chain Reaction; Polymorphism, Restriction Fragment Length

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