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World J Gastroentero, 2000; 6(6):917-919
World Journal of Gastroenterology
 ISSN 1007-9327

Detection of k-ras gene point mutation in fine needle aspiration and pancreatic juice by sequence special primer method and its clinical significance

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Subject headings pancreatic neoplasms/ diagnosis; polymer*as*e chain reaction; biopsy, needle; genes, *ras*; pancreatic diseases; pancreatic juice; gene amplification; cytodiagnosis

Liu XL, Dai CC, Miao Y, Du JH, Zhang ZS, Chen SZ. Detection of -*ras* gene point mutation in fine needle aspiration and pancreatic juice by sequence special primer method and its clinical significance. ***World J Gastroentero***, 2000;6(6):917-919

INTRODUCTION

The point mutation rate of k-*ras* gene at codon 12 in pancreatic adenocarcinoma is reported to be as high as 90%[1-30], and with no mutations in normal pancreas tissues or other pancreatic disorders. We have detected the presence of k-*ras* gene mutation and its mutant styles since 1994 by PCR-SSP in the FNA or pancreatic juice obtained from pancreatic adenocarcinoma tissues.

MATERIALS AND METHODS

Sources of samples

Eighty-eight copies of samples were collected by fine needle aspiration preoperatively under ultrasound guidance or with direct viewing intraoperatively from January 1994 to December 1996, among which there were 35 pancreatic adenocarcinoma, 20 chronic pancreatitis, 8 ampullary carcinoma, 7 bile duct carcinoma, 6 insulinoma and 12 normal pancreas tissues. All the aspirates were routinely smeared, then mixed with 50μL lysis solution and stored in the Eppendorf tubes. Another 47 pancreatic juice samples were obtained by ERCP or puncturing from pancreatic duct intraoperatively or from external drainage postoperatively, including 17 pancreatic adenocarcinoma. The juice volume was more than 1.5mL. All the samples were immediately frozen in liquid nitrogen and strored at -70℃.

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Supported by Natural Science Foundation of Jiangsu Scientific Committee
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Received 2000-06-25 **Accepted** 2000-07-25

Preparation of samples

FNA samples The aspirates were quickly made into 2 - 5 pieces of smears for light microscopic observation. The other aspirates mixed with 50μL lysis solution were added in proteinase K, making the final concentration of 500mg/L. The mixture was then incubated at 55℃ for 2 hours and put into water bath at 95℃ for 10 minutes to inactivate proteinase K, then 15μL supernatant was collected after centrifugation for PCR detection.

Pancreatic juice samples Pancreatic juice was put into 1.5mL Eppendorf tube and underwent high speed centrifugation. Some parts of the sediments were used for smears (2-5 pieces), the residual parts were completely washed with PBS, centrifugated and added 50μL lysis buffer solution. The subsequent procedures are the same as used in FNA specimens.

PCR detection

Our primers were synthesized and supplied by Shanghai Bio-Engineering Research Center. The sequences of primers were: R1=5'GGTAGTTG-GAGCTC3', R2=5'GTAGTTGGAGCTGT3', R3=5' GTAGTTGGAGCTGA3', R4=5'CTATTGTTG-GATCAT ATTCG3'. The primers combination were R1-R4 to amplify 89bp fragment of CGT mutation and R2-R4, R3-R4 to produce 88bp fragments of GTT and GAT mutation respectively. The PCR kits were purchased from Shanghai Huamei Biological Products Corporation (PCR KitA system) and the DNA amplifier is the Perkin-Elmer 2400 model. The reaction volume was 50μL containing 50mmol/L KCl, 10mmol/L Tris-HCl pH = 8.5, 1.7mmol/L $MgCl_2$, 0.01% gelatin, 0.08% Triton-X-100, 1.0μmol/L of each primer, 200μmol/L of each dNTP and 1.5 units of Taq DNA polymerase. Three amplification reactions were performed for each sample. There were 35 circles including denaturation at 94℃ for 45 seconds, annealing at 55℃ for 45 seconds and extension at 72℃ for 45 seconds. Each reaction was set with positive and negative control, the primers and templates of positive control were included in the PCR kitA system. The template was prepared from human genome DNA with its amplification fragment of 110bp. Fifteen ìL amplifying products were loaded on 8% acrylamide gel electrophoresis under 120 volts for 50 minutes, stained with ethidium bromide

and then observed, and photographed under UV transillumination.

RESULTS

PCR findings

There were 32(91.4%) positive cases and 3(8.6%) negative ones in FNA samples of pancreatic adenocarcinoma. The mutant styles included 15 GTT, 11 GAT and 6 CGT. Among the 17 pancreatic juice specimens, 16 (94.1%) were positive and 1 (5. 9%) negative with 9 cases of GTT, 4 GAT and 3 CGT. No mutations occurred in aspirates or pancreatic juice specimens of insulinoma, chronic pancreatitis, ampullary carcinoma, bile duct carcinoma, duodenal papilla carcinoma and normal pancreas tissues.

Cytological results (Table 1)

Table 1 Cytological findings of FNA and pancreatic juice in PA, AC and BDC

	FNA(No. %)				Pancreatic juice (No. %)			
	No	P	SP	N	No	P	SP	N
PA	35	20(57.1)	9(25.7)	6(17.2)	17	2(11.8)	2(11.8)	13(76.4)
AC	8	4(50.0)	2(25.0)	2(25.0)	3	0(0)	0(0)	3(100.0)
BDC	7	4(57.1)	2(28.6)	1(14.3)	3	0(0)	0(0)	3(100.0)

FNA=fine needle aspirates, PA = pancreatic adenocarcinoma, AC = ampullary carcinoma, BDC = bile duct carcinoma, P = positive, SP=suspicious positive, N = negative

DISCUSSION

Comparison of different methods for detecting k-ras gene point mutation in pancreatic adenocarcinoma

It has been reported that the k-*ras* gene at codon 12 had a high incidence of mutation in pancreatic adenocarcinoma, usually presented with CGT, GTT, and GAT styles, occasionally showed TGT, AGT ways. At present, the available methods for detecting its mutation include PCR-RMCA[1,2], PCR-ASO[3-11,24-26], PCR-DSM[12-17], PCR-SSP[18,31], PCR-RFLP[17,19-27,33] and PCR-SSCP[28-30,32], among which PCR-SSP is the simplest and quickest one. No enzyme cut, hybridization, radioactive and non-radioactive imaging technique were needed. It only took a few hours to complete the entire procedure. Tada *et al* collected 9 samples of pancreatic juice for detection by PCR-SSP in 1993[18]. The results showed that all the 6 cases of pancreatic adenocarcinoma had positive findings and one cholelithiasis, two chronic pancreatitis had no mutation. The number of samples, however, was too small to have any statistical significance. We have used the PCR-SSP method on FNA and pancreatic juice samples of pancreatic adenocarcinoma since 1994. The point mutation rate of k-*ras* gene was 91.4% and 94.1% respectively without false positive.

Comparison of FNA and pancreatic juice cytological results with PCR-SSP findings

Currently, the accuracy rate for diagnosis of pancreatic neoplasms by FNA technique is about 58%-83%, and the positive rate of pancreatic juice cytology is less than 10%. Our research in FNA and pancreatic juice cytology also supports these results. It indicates that PCR-SSP has advantages in the differential diagnosis of benign and malignant pancreatic diseases compared with cytological examination, but it has little diagnostic value in ampullary carcinoma and bile duct carcinoma.

Significance of detecting k-ras gene point mutation by PCR-SSP

Pancreatic adenocarcinoma is one of the commonly encountered tumors and the incidence is increasing with age. By now there has been no satisfactory method for early diagnosis. It is still very difficult to define the character of the pancreatic mass and to differentiate between tumor-like pancreatitis and pancreatic adenocarcinoma or chronic pancreatitis and whole-pancreas adenocarcinoma. The commonly used imaging examinations (such as type B ult*ras*ound, CT) have no qualitative diagnostic value. Determination of serum tumor markers (CA19.9, CA50, CA242, etc.) has only 60%-70% sensitivity or specificity and the false positive rate may be as high as 30%-40%. The positive rate of pancreatic juice cytology is too low (<10%) and FNA method could yield indefinite results because of the insufficient samples or atypical cellular manifestation. It is, therefore, helpful for us to use PCR-SSP technique to detect the point mutation of k-*ras* gene at codon 12 when we are not sure about the diagnosis of pancreas disorders. It may serve as a practical method for distinguishing pancreatic benign masses from malignant ones, and making a definitive diagnosis of pancreatic adenocarcinoma.

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Edited by Ma JY