

Detection of point mutation in K-ras oncogene at codon 12 in pancreatic diseases

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Abstract

AIM: To investigate frequency and clinical significance of K-ras mutations in pancreatic diseases and to identify its diagnostic values in pancreatic carcinoma.

METHODS: 117 ductal lesions were identified in the available sections from pancreatic resection specimens of pancreatic ductal adenocarcinoma, comprising 24 pancreatic ductal adenocarcinoma, 19 peritumoral ductal atypical hyperplasia, 58 peritumoral ductal hyperplasia and 19 normal duct at the tumor free resection margin. 24 ductal lesions were got from 24 chronic pancreatitis. DNA was extracted. Codon 12 K-ras mutations were examined using the two-step polymerase chain reaction (PCR) combined with restriction enzyme digestion, followed by nonradioisotopic single-strand conformation polymorphism (SSCP) analysis and by means of automated DNA sequencing.

RESULTS: K-ras mutation rate of the pancreatic carcinoma was 79%(19/24) which was significantly higher than that in the chronic pancreatitis 33%(8/24) ($P<0.01$). It was also found that K-ras mutation rate was progressively increased from normal duct at the tumor free resection margin, peritumoral ductal hyperplasia, peritumoral ductal atypical hyperplasia to pancreatic ductal adenocarcinoma. The mutation pattern of K-ras 12 codon of chronic pancreatitis was GGT→GAT, GGT and CGT, which is identical to that in pancreatic carcinoma.

CONCLUSION: K-ras mutation may play a role in the malignant transformation of pancreatic ductal cell. K-ras mutation was not specific enough to diagnose pancreatic carcinoma.

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INTRODUCTION

Despite considerable development in sophisticated imaging techniques and cytological examination, an early diagnosis of pancreatic neoplasm is rare. 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^[1-3]. 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. K-ras mutation at codon 12 is one of the most common mutational events in the carcinogenesis of human malignancies^[4]. It occurs mainly in mucin-producing adenocarcinomas^[5]. The highest incidence of K-ras mutation has been found in ductal adenocarcinoma of the pancreas, in which it ranged from 70% to 100%^[6,7]. Because K-ras has been found in very small incidental carcinomas, which were diagnosed only at autopsy^[8], as well as in intraductal portions of ductal carcinomas, it is considered as an initial event in carcinoma development. This may make it a good tool for detecting pancreatic carcinomas at an early stage^[9,10]. However, Yanagisawa *et al.*^[11] showed that K-ras mutations also occur in hyperplastic ductal lesions in pancreas that do not harbor any malignancy. Therefore the K-ras mutation cannot be used as a marker of pancreatic ductal adenocarcinoma. However, this also suggests that ductal lesions, even without dysplasia, may be the forerunners of ductal adenocarcinoma. This assumption also was fostered by the early observation that some of the described ductal lesions frequently are associated with ductal adenocarcinoma. Several studies generally have confirmed the results of Yanagisawa *et al.*, whereas others failed to detect K-ras mutations in nondysplastic epithelium. To address these issues we analyzed 117 ductal lesions for K-ras mutations.

MATERIALS AND METHODS

Tissue selection

The tissues used in this study were derived from patients who underwent partial duodenopancreatectomy for ductal adenocarcinoma of the pancreas and chronic pancreatitis. The basis for selection was the availability of suitable paraffin blocks (blocks age <5 years). 117 ductal lesions were identified in the available sections from pancreatic resection specimens of 24 pancreatic ductal adenocarcinoma, comprising 24 pancreatic ductal adenocarcinoma, 19 peritumoral ductal atypical hyperplasia, 58 peritumoral ductal hyperplasia and 19 normal duct at the tumor free resection margin. 24 ductal lesions were obtained from 24 patients of chronic pancreatitis. 7 surgical specimens of normal pancreas from patients with widely invasive gastric and colonic carcinoma in whom the tumor had not invaded the pancreas and who did not have pancreatic carcinoma or pancreatitis were examined. Multiple 5- and 10- μ m sections were cut from each block and floated onto glass slides. Hematoxylin and eosin staining was performed on 5- μ m sections from each paraffin block. The blade was replaced after each block was cut to prevent carryover of DNA between sections. Pancreatic carcinoma cell line Patu-8988 was obtained from Doctor Elsasser Philips of University of Germany.

DNA extraction

A boiling method of DNA extraction was employed similar to

that previously described by Shibata^[12]. Tissue sections were deparaffinized and digested with a proteinase K digestion buffer (50 mmol/L Tris-HCL, pH 8.5, with 250 µg/mL proteinase K overnight at 55 °C). The tissue was then boiled for 10 min and centrifuged. DNA was quantitated using a spectrophotometer.

Enriched PCR amplification

The first PCR-mediated amplifications were performed as described previously with minor modifications^[13] and produced a 157-base pair fragment that contain K-ras codon 12. Aliquots of the first PCR product were digested with BstN1 (Biolab) at 60 °C for 2 h under the conditions recommended by the supplier. After boiling the mixture for 5 min to inhibit the enzymatic activity, 1/100 volume of the PCR product was subjected to the second PCR under the same conditions but using a different primer set, which flanked internal sequences of 135 bp DNA fragments. The amplified fragments were directly subjected to SSCP. The oligonucleotide primers were purchased from Sangon Co. (Shanghai, China). The sequences of oligonucleotide primers were as follows: 5' ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT 3' (forward primer for first and second PCR); 5' TCA AAG AAT GGT CCT GGA CC 3' (reverse primer for first PCR); 5' TAA TAT GTC GAC TAA AAC AAG ATT TAC CTC 3' (reverse primer for second PCR).

Nonradioisotopic SSCP analysis

Nonradioisotopic SSCP Analyses were performed as described previously, with minor modification^[13]. After denaturation at 100 °C for 5 min, a 10 µL sample was applied and resolved by 120 g/L polyacrylamide gel electrophoresis at 35 V for 21 h at 4 °C. The gels were silver-stained. Each sample was analyzed by SSCP repeatedly to confirm its accuracy.

Direct sequencing of the amplified PCR product

For sequencing the deviant homoduplex bands were cut out of the gel, the DNA eluted by soaking in TE, reamplified, and TA cloned in the pUCm-T vector. Sequencing was performed on an ABI PRISM 377 automated sequencer.

Statistical analysis

A statistical analysis was performed using the chisquare test, the Fisher's exact test and Student *t* test with SPSS.

RESULTS

Detection of K-ras point mutation by nonradioisotopic SSCP analysis and direct sequencing of PCR product

To estimate the sensitivity of this analysis, 1 µg of the DNA extracted from normal peripheral blood lymphocytes and a pancreatic carcinoma cell line were subjected to PCR. Figure 1 shows the electrophoretic profiles of amplified DNA fragments by SSCP. The positive percentage of K-ras mutation of the pancreatic carcinoma was 79% (19/24) which was significantly higher than that in the chronic pancreatitis 33% (8/24) ($P < 0.01$). It was also found that K-ras mutation rate was progressively increased from normal duct at the tumor free resection margin, peritumoral ductal hyperplasia, peritumoral ductal atypical hyperplasia to pancreatic ductal adenocarcinoma (Table 1).

The nucleotide sequences of K-ras mutation showed the following transitions: GGT to GAT, GGT to GTT, and GGT to CGT. (Table 2).

Relationship between K-ras gene mutation and location, histological grade and clinical stage of tumors

The diagnostic accuracy of K-ras mutations in pancreatic

carcinoma relative to the location, histological grade and clinical stage of the tumor is summarized in Table 3. There are no apparent correlation between the location, histological grade, clinical stage of the tumor and the presence of K-ras gene mutations.

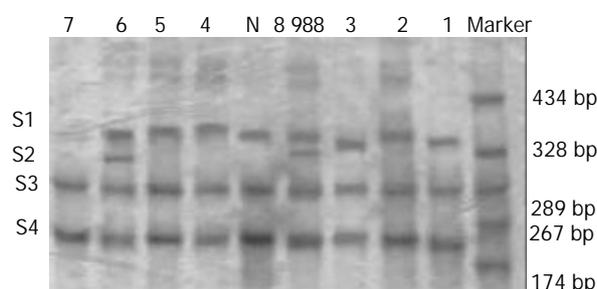


Figure 1 Detection of point mutations of the K-ras oncogene in pancreatic diseases. Lane N, healthy control subject DNA from normal peripheral blood lymphocytes. Lane 8988, pancreatic carcinoma cell line Patu-8988 DNA with confirmed mutant K-ras. Lane 6, patients with pancreatic carcinoma with mutant K-ras. Lane 7, normal pancreatic tissue. Lane 1, 3, 4, patients with chronic pancreatitis with mutant K-ras. Lane 2, 5, patients with chronic pancreatitis with wild type K-ras.

Table 1 Relative frequency of K-ras mutation in ductal lesions with pancreatic diseases

Ductal lesion	n	K-ras (-)	K-ras (+)	Positive percentage (%)
Pancreatic ductal adenocarcinoma	24	5	19	79.2
Peritumoral ductal atypical hyperplasia	19	15	14	73.6
Peritumoral ductal hyperplasia	58	38	20	34.5
Normal duct at the tumor free resection margin	16	16	0	0
Chronic pancreatitis	24	16	8	33.3
Normal pancreas	7	7	0	0

Table 2 K-ras mutation pattern of ductal lesions and the corresponding primary pancreatic carcinoma

Ductal lesion	Nucleotide sequence of K-ras12 codon		
	GAT	GTT	CGT
Pancreatic ductal adenocarcinoma	8	4	7
Peritumoral ductal atypical hyperplasia	7	2	3
Peritumoral ductal hyperplasia	10	3	7
Chronic pancreatitis	4	2	2

Table 3 Relationship between K-ras gene mutation and location, histological grade and clinical stage of pancreatic carcinomas

Pathologic factor	n	Positive rate (%)
Tumor location		
Head	18	55.6
Tail/Corpus	6	66.7
Histological grade		
G1	6	66.7
G2	10	50
G3	8	62.5
Clinical stage		
I	1	100
II	3	66.7
III	9	66.7
IV	11	45.5

Clinical and morphologic data on 24 patients with chronic pancreatitis

The occurrence of mutations was unrelated to clinical and morphologic indexes. The difference was not statistically significant (Student *t* test) (Table 4).

Table 4 Clinical and morphologic data on 24 patients with chronic pancreatitis

	K-ras positive cases (n=8)	K-ras negative cases (n=16)
Mean age of patients (yrs)	39.9±15.5(19-68)	51±14.9(23-67)
Gender ratio (M:F)	6:2	10:6
Mean duration of CP (yrs)	6.7±3.2(2-27)	5.7±4.5(3-25)
Mass-CP of pancreatic head	5 cases	10 cases
Weight loss (>10 kg)	3 cases	7 cases
Diabetes (insulin dependent)	4 cases	7 cases
Nicotine(>20 cigarettes/day, >5 yrs)	5 cases	8 cases

CP: chronic pancreatitis.

DISCUSSION

Pancreatic ductal carcinoma is known to have the highest K-ras mutation rate among all tumors. The codon 12 of this gene is affected in 80-100% of the cases. Mutated K-ras also has been found in ductal lesions such as mucinous cell hypertrophy and ductal papillary hyperplasia; therefore these lesions have been regarded as precursor lesions of carcinoma^[11]. However, this assumption is based on rather contradictory results, with K-ras mutation rates in ductal lesions ranging from 0-94%^[14]. The discrepancies between the results of the various studies are based on the wide range of investigated cases, the selection of lesion types, and PCR employed.

Matsubayashi *et al.*^[15] analyzed 317 carcinoma-associated ductal lesions, 39% were positive for K-ras mutations. Moskaluk *et al.*^[14] focused on atypical papillary hyperplasia and found K-ras mutations in 75% of their samples. Both study groups used a detection method (nested PCR-RLFP) with a sensitivity of 5%. But SSCP analysis is one of the simplest and most sensitive methods for detection of mutations based on PCR technology^[16-18]. Since its first report, the SSCP analysis has been widely used to detect mutations in genes responsible for various hereditary diseases and somatic mutations of oncogenes. In an earlier study, Lemoine *et al.*^[19] found no mutations in cases of ductal hyperplasia, but they did find mutation in all severely dysplastic lesions that they regarded as intraductal extensions of the associated ductal adenocarcinoma. It appears that their method, which was based on slot-blot analysis, was not sensitive enough to detect K-ras mutations in nondysplastic ductal lesions.

Chronic pancreatitis, regardless of its etiology, is considered a risk factor for the development of pancreatic ductal adenocarcinoma^[20]. The risk seems to increase with the duration of CP. Some histological studies have searched for possible carcinoma precursor lesions in this disease. Cylwik *et al.*^[21] reported severe dysplasia in 8.6% of 70 resection specimens from patients with CP; advanced fibrosis was associated with dysplasia in 65%. Because of these results, they concluded that surgical removal of the pancreas should be recommended. We cannot confirm these data. We were unable to identify any severe dysplasia carcinoma in situ changes in 24 resection specimens from patients with CP and varying duration of the disease.

K-ras mutation is considered an early event in the tumorigenesis of pancreatic carcinomas. In our study the positive percentage of K-ras mutation of the pancreatic carcinoma was 79%(19/24) which was significantly higher than

that in the chronic pancreatitis 33%(8/24) ($P<0.01$). It was also found that K-ras mutation rate was progressively increased from normal duct at the tumor free resection margin, peritumoral ductal hyperplasia, peritumoral ductal atypical hyperplasia to pancreatic ductal adenocarcinoma. It appeared that the locations, histological grade, clinical stage of pancreatic carcinomas were all not related to the presence of K-ras mutations. The occurrence of K-ras mutations was not associated with the duration of CP and also not with mass-CP of pancreatic head. Rivera *et al.*^[22] reported K-ras mutations in 2 of 11 cases and Yanagisawa *et al.*^[11] even 62.5% mutations in 10 of 16 lesions from 10 cases. K-ras mutations were not detected by Kubrusly *et al.*^[23]. Their negative results probably were due to the comparatively insensitive PCR method and the tissues they selected for investigation. To summarize, when sensitive enough methods are applied, K-ras mutation frequently can be detected in nondysplastic ductal epithelium of patients with CP. Berger *et al.*^[24] reported a clear correlation between K-ras mutations and the nicotine history of their patients. In our study, the relationship was unclear. Therefore, nicotine seems to be a major factor in the induction of the mutation but does not inevitably induce it.

The relation between CP and pancreatic adenocarcinoma remains difficult to explain. Continuous epithelial regeneration and proliferation are thought to promote carcinogenesis in many organs. In our study, the incidence of the ductal lesions and K-ras mutations did not increase with the duration of CP. It therefore seems that hyperplastic ductal changes and K-ras mutations will not inevitably lead to the development of ductal adenocarcinoma.

Future prospective studies should be performed to get a well documented long term follow-up of K-ras positive cases to elucidate the role of K-ras mutations in pancreatic carcinogenesis in CP patients. In addition, other mutations, such as those of the p16, DPC4, and BRCA2 genes or promoter methylation of tumor suppressor, which evidently play role in a linear tumor progression model of pancreatic carcinoma, also should be looked for in lesions that might be responsible for the presumed pancreatic carcinoma sequence in chronic pancreatitis^[25-30].

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