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Original Article

KRAS mutational analysis in ductal adenocarcinoma of the pancreas and its clinical significance

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ABSTRACT

Mutations of *KRAS* are detectable in 70–90% of pancreatic duct adenocarcinomas (PDAC), using direct sequencing. We used a highly sensitive molecular method in order to investigate: (a) the frequency and prognostic significance of different *KRAS* mutations and, (b) whether the presence of *KRAS* mutations in histologically-negative resection margins of PDAC could explain local tumor recurrence after surgery.

Twenty-seven patients with histologic diagnosis of PDAC, radical pancreaticoduodenectomy and histologically-negative margins were evaluated. *KRAS* mutations were searched for mutant-enriched PCR in tumor and negative resection margins.

KRAS mutations were detected in 85.2% of the cases; the most frequent mutation was G12D (48.1%). Shorter OS was found in patients with G12D (25 months; 95% CI, 20.5–29.5), *vs* patients with other mutations (31.5 months; 95% CI, 25.6–37.1) (N.S.). *KRAS* mutation in histologically-negative margins was detected in one patient who died of locoregional recurrence; six patients had tumor recurrence but no mutations in surgical margins.

The high frequency of *KRAS* mutations suggests a search for *KRAS* status to improve the diagnosis in suspected cases; the G12D mutation could be related to poor prognosis, but without statistical significance. No correlation was found between the frequency of cancer recurrence and *KRAS* mutations in surgical margins.

Introduction

Pancreatic duct adenocarcinoma (PDAC) is one of the most aggressive epithelial tumors in both males and females worldwide [1]. The short overall five-year survival rate (less than 5%) is mainly due to the late tumor stage at the time of diagnosis [6], but, even for the small group of patients with resectable tumors, the prognosis remains poor [7]. This could reflect the inadequate removal of the tumor or the presence of precancerous cells in surgical margins that, despite normal morphology, may harbor molecular abnormalities responsible for local cancer recurrence. Molecular genetic analyses indicated that activating mutations of *KRAS* oncogene are early events in pancreatic carcinogenesis, and occur in 70–90% of PDAC [3,10]. Point mutations involve most of all the codon 12 [5], in contrast to colorectal cancer (CRC) in which mutations in codon 13 are not rare, occurring in about 20% of the cases [23]. The role of *KRAS* mutation in predicting response to target therapies in CRC is widely accepted [2], whereas its prognostic significance is doubtful. A recent methanalysis conducted in 1261 CRCs seems to indicate a poor prognosis for patients harboring G12V mutation *vs* patients with *KRAS* wild-type or harboring other *KRAS* mutations, suggesting the importance of an accurate molecular characterization of KRAS gene in order to establish its real prognostic significance [11].

Frequency and type of *KRAS* mutations in PDAC have been analyzed in few studies, all using low-sensitive direct sequencing, obtaining conflicting results concerning statistical correlation with mutation and survival or local recurrence [12,13,16,18,20].

In this study, the prognostic significance of *KRAS* mutation status was analyzed in 27 patients with PDAC by using a highly sensitive mutant-enriched PCR that is able to recognize one copy of mutated

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Table 1
Sequences of primers and PCR reaction protocol.

Gene	Primer name	Sequence	Cycle	Length
KRAS codon 12 (Outer)	3F 10B	5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′ 5′-ACTCATGAAAATGGTCAGAGAAACCTTTAT-3′	95 °C × 10 min; (95 °C × 30 s, 50 °C × 1 min, 72 °C × 1 min) × 20 cycles; 72 °C × 3 min	143
KRAS codon 13 (Outer)	9F 10B	5′-ACTGAATATAAACTTGTGGTAGTTGGCCCTGGT-3′ 5′-ACTCATGAAAATGGTCAGAGAAACCTTTAT-3′	95 °C × 10 min; (95 °C × 30 s, 54 °C × 1 min, 72 °C × 1 min) × 20 cycles; 72 °C × 3 min	113
KRAS codon 12 (Inner)	3F 14B	5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′ 5′-TCAAAGAATGGTCCTGGACC-3′	95 °C × 10 min; (95 °C × 30 s, 54 °C × 1 min, 72 °C × 1 min) × 45 cycles; 72 °C × 3 min	143
KRAS codon 13 (Inner)	9F 4B	5′-ACTGAATATAAACTTGTGGTAGTTGGCCCTGGT-3′ 5′-TCAAAGAATGGTCCTGCACC-3′		113

DNA among 1000 copies of wild-type DNA. *KRAS* status was also searched for in histologically-negative surgical margins in order to evaluate whether *KRAS* mutations may correlate with local tumor recurrence independently to the histologic evidence of cancer or dysplasia.

Materials and methods

Patients and tissue specimens

Twenty-seven patients who underwent radical pancreaticoduodenectomy for pancreatic adenocarcinoma from 1996 to 2010 were investigated.

The patients were selected on the basis of the following criteria: radical surgical procedure with complete tumor ablation, clinical follow-up of at least 12 months and resection margins histologically negative. All operations were performed by the same surgeon (M.G.), and all patients underwent radical pancreaticoduodenectomy following the Whipple – Child procedure, without pylorus preservation, and with extended adenectomy.

Histological classification and staging were performed according to TNM classification, and tumor grade was assessed as well (G1), moderate (G2) and poorly differentiated (G3) [9].

Hematoxylin-Eosin slides were collected from the archives of the Division of Pathology and were revised by one of us (R.B.) in order to select the area with a percentage of tumor cells more than 70% and area of resection margin with normal cells.

DNA extraction

Manual macrodissection of the tumor and normal margin area from formalin-fixed, paraffin-embedded tissue was done while five 5-μm thick sections were prepared and then collected in a 1.5 mL tube. A microtome blade was cleaned with xylene between each block in order to avoid sample cross contamination.

DNA was extracted using EDTA-SDS/proteinase K follow by phenol–chloroform and resuspended with 30 μL of DEPC-treated and RNAse free water.

DNA concentration was evaluated using a spectrophotometry (Eppendorf, Hamburg, Germany) in order to diluted samples to a final concentration of 25 ng/μL.

KRAS mutational analysis

KRAS status was analyzed by means of mutant-enriched PCR (ME-PCR) to detect two hotspots in codons 12 and 13 of exon 2 that include more than 95% of mutations in this gene. Outer PCR reaction was performed using Eppendorf Mastercycler gradient PCR system (Eppendorf, Hamburg, Germany). Fifty ng of DNA was added to the reaction mix in a final volume of 50 μL, containing: DEPC-treated and RNAse free water (Promega, Madison, USA), 0.1 μM of each primer, 1.5 mM $MgCl_2$, 0.08 mM dNTPs and 0.2 U/μL of Taq Polymerase in the presence of 1X PCR buffer (Promega, Madison, USA). Codon 12 was amplified using primers 3F and 10B, but codon 13 with primers 9F and 10B (Table 1). Fifteen μL of amplification products underwent enzymatic digestion using BstnI enzyme (NewEngland BioLabs, Ipswich, USA), specific for codon 12, and BglI (Fermentas, Canada) specific for codon 13. Reaction was done in a final volume of 20 μL containing DEPC-treated and RNAse free water (Promega, Madison, USA), 1X enzyme buffer and 5 U/μL of each enzyme. Overnight incubation was performed following the manufacturer's instruction.

Inner PCR was carried out using primers 3F and 14B to amplify codon 12, and primers 9F and 4B to amplify codon 13. Two μL of enzymatic digestion was added to reaction mix prepared as follows: DEPC-treated and RNAse free water (Promega, Madison, USA), 0.5 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 U/μL of Taq Polymerase in the presence of 1X PCR buffer (Promega, Madison, USA). PCR protocols are shown in Table 1.

Fifteen μL of PCR product underwent enzymatic digestion as described above.

Inner PCR product and second enzymatic digestion of each sample were analyzed by means of 3% agarose gel electrophoresis, and visualized using ethidium bromide staining. DNA purification was performed using NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany). Sequencing analysis of positive samples was performed as previously described [17].

Statistical analysis

Fisher's exact test was performed in order to evaluate correlation between survival and clinico-pathological findings. Overall Survival (OS) and Disease Free Survival (DFS) curves were carried out using Kaplan–Meier methods and compared with Log-Rank test.

To compare the average and the median time of OS between the groups analyzed, we performed the non-parametric *t*-student test and the Mann Whitney test.

Significant values were considered when $P < 0.05$, with an interval of confidence of 95%.

Results

Clinical material

Fourteen patients were males (52%) and 13 were females (48%), with a mean age of 67 years at surgery (range 51–80; median 68). No perioperative mortality occurred. The postoperative morbidity rate was 11.11% (one case of peritoneal bleeding needing re-operation, one case of postoperative gastrointestinal bleeding and one case of pancreatic fistula, treated conservatively).

In Table 2 the clinico-pathological findings of the series are summarized. No statistical correlation was found, comparing survival data with age, gender, staging, grading and perineural invasion.

Twenty of 27 (74.1%) patients died with a mean of 23 months after surgery (median 20, range 12–51 months), and 7/27 (25.9%)