

ANALYSIS OF K-RAS GENE CODON 12 MUTATION IN PANCREATIC TISSUE OF PATIENTS WITH PANCREATIC CANCER

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Abstract — The aim of this study was to analyze K-ras codon 12 mutation in the pancreatic tissue of Serbian patients with pancreatic cancer and assess whether the given mutation can be used as a molecular marker for this disease. The study was performed on pancreatic tissue samples obtained from 40 patients with clinical diagnosis of pancreatic cancer. The presence of K-ras codon 12 mutation was analyzed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Our study showed that K-ras mutation is present with a high frequency (66%) in the pancreatic tissue of patients with pancreatic cancer.

Key words: Pancreatic cancer, K-ras codon 12 mutation, molecular marker

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INTRODUCTION

Pancreatic cancer is one of the most aggressive human malignancies and the fourth leading cause of cancer death in Europe and North America (Klapman and Malafa, 2008). Patients with this tumor have a median survival time of 6 months after initial diagnosis and a 5-year survival rate of only 2% (Tomaino et al., 2007). Despite considerable development in sophisticated imaging techniques and cytological examination, pancreatic cancer is most often diagnosed late in the course of the disease, after local spread and distant metastases have occurred. 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. Furthermore, surgical therapy is frequently not curative. These facts make pancreatic cancer extremely difficult to treat. An understanding of the molecular basis of transformation into a malignant tumor of the pancreas may provide a basis for the development of more effective strategies for the prevention, diagnosis, and treatment of this cancer.

It has been suggested that ras oncogenes play a central role in the development of certain malignancies (Dunn et al., 2005). The family of ras protooncogenes consists of three genes: K-ras, N-ras, and H-ras. While wildtype cellular proteins in humans play a vital role in normal tissue signalling, mutated genes are potent oncogenes that play a role in many human cancers. Among ras genes, K-ras is the most common target of mutations, while defects in N-ras and H-ras are found less frequently.

The K-ras gene encodes the human cellular homolog of a transforming gene isolated from the Kirsten rat sarcoma virus. It is one of the most activated oncogenes, with 17% to 25% of all human tumors harboring an activating K-ras mutation (Dunn et al., 2005). Critical regions of the K-ras gene for oncogenic activation include codons 12, 13, 59, 61, and 63. These activating mutations cause protein to accumulate in the active GTP-bound state by impairing intrinsic GTPase activity and conferring resistance to GTPase-activating proteins. The mutations therefore result in the expression of

altered protein products that are capable of transforming cells into a malignant phenotype through maintaining of the constitutive transmission of a positive signal for cell growth.

Mutations in the K-ras gene were found to be related to the development of pancreatic cancer and are considered to be early molecular events in pancreatic tumorigenesis (Hezel et al., 2006). The K-ras mutations are detected with a frequency of 75 to 100% in pancreatic cancer tissue (Mu et al., 2004). Codon 12 of the K-ras gene is by far the most common site of ras activation in human malignancies, with the highest incidence found in ductal adenocarcinoma of the pancreas (Moore et al., 2001). The high prevalence of point mutations in the K-ras gene in pancreatic tumors suggests that K-ras mutation detection might be used in future screening protocols for pancreatic cancer. Additionally, activation of K-ras has been recognized in microdissected foci of pancreatic intraepithelial neoplasia, the precursor lesion of pancreatic cancer. Mutations in the K-ras gene are also associated with more aggressive, recurrent, and metastatic tumors; they therefore seem to be an important target for novel anti-cancer therapies (Morioka et al., 2004; Kim et al., 2006).

The aim of this study was to analyze the status of K-ras codon 12 mutation in the pancreatic tissue of Serbian patients with pancreatic cancer, investigate the correlation between the presence of the mutation and histopathological findings, and assess whether the presence of the mutation might be used as a molecular marker in future screening protocols for pancreatic cancer.

MATERIALS AND METHODS

The study encompassed 40 patients (24 male and 16 female, age 42-67 years) who underwent partial duodenopancreatectomy for ductal adenocarcinoma of the pancreas at the First Surgical Clinic of the Clinical Center of Serbia. Written informed consent was obtained from all study patients and the investigation was approved by the hospital's ethical committee. Prior to surgical procedure, clinical diagnosis of pancreatic cancer in all patients was made based on detailed clinical examinations, abdominal

ultrasound examination, and spiral computed tomography (CT) scan. The stage of the cancer was determined using TNM Classification of Malignant Tumors, which is based on criteria proposed by the American Joint Committee on Cancer (AJCC) (Greene et al., 2002).

The tissue samples used in this study were obtained during the surgical procedure and were available as fresh tissue samples in 20 cases and as paraffin-embedded tissue sections in 20 cases. The fresh tissue samples were obtained on surgery and kept in isotonic sodium chloride solution prior to DNA extraction. The paraffin-embedded tissue sections were cut into slices, the blade being replaced after each block was cut to prevent carryover of DNA between sections.

Phenol/chloroform extraction of DNA was performed on both paraffin-embedded tissue sections and fresh tissue slices. Prior to extraction, the sections of paraffin-embedded tissue were deparaffinized through washing, twice with 1 mL of xylol, twice with 1 mL of absolute ethanol, and once with 1 mL of 70% ethanol. After each wash, the mixture was subjected to vortexing, centrifugation at 13000 rpm for 5 min, and removal of the supernatant. The final pellets of deparaffinized tissues and slices of fresh tissues (50-100 mg) were diluted in 50 mmol/L Tris-HCl, pH 8.5, and subjected to overnight digestion at 55°C by proteinase K (20 mg/mL). After overnight digestion, 2.4 mL of phenol was added and the mixture centrifuged for 5 min at 3000 rpm at 10°C. After transferring the supernatant to a new tube, 2.4 mL of phenol/chloroform/isoamyl alcohol (25: 24: 1) solution was added and the mixture centrifuged for 5 min at 3000 rpm at 10°C. The supernatant was then transferred to a new tube, after which 2.4 mL of chloroform/isoamyl alcohol (24: 1) solution was added and the mixture centrifuged for 5 min at 3000 rpm at 10°C. After again transferring the supernatant to a new tube, 25 µL of 3 M sodium acetate and 5 mL of ethanol were added and the mixture incubated at -20°C overnight. The mixture was then washed in 2 mL of 70% ethanol and the pellet dried. The obtained DNA pellets were resuspended in 100 µL of sterile water.



Fig. 1. Design of the PCR-RFLP method for detection of K-ras codon 12 mutation.

Detection of K-ras codon 12 mutation was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The PCR amplification of the part of the K-ras gene that contains codon 12 was performed with the primers 5'-TCAAAGAATGGTCCTGG*ACC-3' and 5'-ACTGAATATAAAGCTTGTGGTAGTTGGAC*CT-3' (*-mismatch nucleotide) to produce a 157 bp-long fragment. Both primers introduce the recognition site for BstNI endonuclease, while the presence of K-ras codon 12 mutation abolishes the recognition site created by the second primer (Fig. 1). The amplification procedure was performed in the following reaction mixture: 15 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each dNTP, 10 pmol of each primer, 1 U of Taq polymerase FIREPol (Solis BioDyne), and approximately 250 ng of DNA. Amplification was performed according to the following program: initial denaturation at 94°C for 5 min; 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and final elongation at 72°C for 10 min.

The fragments obtained by PCR amplification were subjected to reamplification according to the



Fig. 2. Analysis by PCR-RFLP of K-ras codon 12 mutation on 10% polyacrylamide gels. 1 - PCR product; 2, 3, 5, 8, 9, 12 - carriers of normal K-ras codon 12 allele; 4, 6, 7, 10, 11, 13 - carriers of mutant K-ras codon 12 allele.

same protocol, but with 1 µL of the PCR product used as a template instead of DNA. Both amplified and reamplified fragments were subjected to restriction enzyme cleavage by endonuclease BstNI (New England BioLabs) at 60°C overnight. Digestion products were analyzed by electrophoresis on 10% polyacrylamide gels, followed by silver staining of the gels (Radojković and Kušić, 2000). Upon visualization of bands on polyacrylamide gels, the 143-bp band depicts the presence of the mutant allele, while the 114-bp band depicts the presence of the normal allele (Fig. 2).

RESULTS

After histopathological analysis, a clinical diagnosis of pancreatic cancer was confirmed in 38 of 40 patients included in the study, while in two patients a diagnosis of chronic pancreatitis was established (Table 1).

Detection of K-ras codon 12 mutation by PCR-RFLP analysis was successfully performed for all tissue samples. Of 38 analyzed tumor samples, K-ras codon 12 mutation was present in 25 (66%), while

Table 1. Histopathological findings and distribution of K-ras codon 12 mutation.

	number (%)	k-ras codon 12 mutation-positive (%)	k-ras codon 12 mutation-negative (%)
subjects	40 (100)	25 (62)	15 (38)
histopathological finding			
pancreatic carcinoma	38 (95)	25 (66)	13 (34)
chronic pancreatitis	2 (5)		2 (100)

Table 2. Characteristics of patients and distribution of K-ras codon 12 mutation.

	number (%)	k-ras codon 12 mutation-positive (%)	k-ras codon 12 mutation-negative (%)
subjects	38 (100)	25 (66)	13 (34)
age			
≤ 60	10 (26)	7 (70)	3 (30)
> 60	28 (74)	18 (64)	10 (36)
gender			
male	23 (61)	16 (70)	7 (30)
female	15 (39)	9 (60)	6 (40)
Tumor stage			
T1	1 (3)		1 (100)
T2	4 (10)	4 (100)	
T3	32 (84)	20 (62)	12 (38)
T4	1 (3)	1 (100)	
Regional lymph nodes			
N0	8 (21)	4 (50)	4 (50)
N1	30 (79)	21(70)	9 (30)

in 13 (34%) only the normal allele was detected. The mutation was not detected in either of the patients with chronic pancreatitis. The distribution of K-ras codon 12 mutation did not differ among patients in respect to age or gender (Table 2). Carriers of K-ras codon 12 mutation were more frequent among patients with advanced tumor stages T3 and T4 (64%), as well as among patients with regional lymph node metastasis (70%).

DISCUSSION

In order to reduce mortality and improve survival in the management of pancreatic cancer, it is crucial to identify sensitive and specific diagnostic markers for detection of the disease at a surgically resectable stage. The preoperative assessment algorithm most commonly consists of imaging analysis, cytological analysis, and determination of biomarker levels in the serum. Since there is no definite consensus on

the best approach, the choice of diagnostic techniques and biomarkers, as well as the criteria that are used to stratify patients, are quite variable. The most commonly used serum markers for pancreatic cancer are carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), but they are used only in combination with imaging techniques and cytological analysis for diagnostic purposes (FreeLove and Walling, 2006). Although the development of modern imaging techniques with improved resolution has allowed for the better preoperative staging of patients with pancreatic cancer, existing biomarkers are not adequate as early detection markers for this disease due to low sensitivity and/or specificity. Numerous new molecular markers (DNA-, RNA-, and protein-based) are currently being investigated as potential diagnostic tools for pancreatic cancer, but they need validation in large scale studies before they can be used in clinical settings (Liang et al., 2009).

In spite of extensive research efforts, the potential value of K-ras codon 12 mutation as a diagnostic tool in clinical practice remains uncertain. The presence of K-ras mutation in the pancreatic tissue usually indicates either the presence of a premalignant lesion or early stage pancreatic cancer. It has therefore been suggested that this marker might be potentially valuable in early detection of pancreatic malignancy (Welsch et al., 2007). The aim of our study was to investigate the potential use of this molecular marker in Serbian patients with pancreatic cancer. The mutation was detected in 66% of patients with pancreatic cancer, which is slightly lower than in most studies, where K-ras mutation incidence in pancreatic cancers was estimated to be 75 to 100% (Hruban et al., 1993; Kubrusly et al., 2002; Liu et al., 2003; Ren et al., 2004). However, considering that all patients included in our study were diagnosed with pancreatic cancer at a surgically resectable tumor stage and that the mutation was present in two thirds of them, introduction of K-ras codon 12 mutation analysis as part of diagnostic procedures in pancreatic cancer should be seriously considered.

The crucial limitation in detection of K-ras codon 12 mutation in pancreatic tissue is sensitivity of the method used for its analysis. Since K-ras mutation is usually present in a relatively small number of cells in precancerous lesions or early tumor stages, its presence in DNA extracted from tissue sections is detectable only in traces. Sensitivity of the method can additionally be affected by the quantity and quality of DNA extracted from tissue sections. In general, DNA extracted from fresh tissue sections is of better yield and higher quality than DNA extracted from paraffin-embedded tissue sections. On the other hand, paraffin-embedded tissue sections can be kept for long periods of time and are valuable as a source of DNA for retrospective studies.

In this study, the presence of mutation was detected by the PCR-RFLP method, whose sensitivity was increased by reamplification of PCR fragments prior to RFLP analysis. As codon 12 mutation encompasses different nucleotide changes at this position in the K-ras gene, the PCR-RFLP method

was designed for detection of any nucleotide change within the given nucleotide triplet, which eliminates the influence of codon 12 variability and existing ethnic differences (Hayashi et al., 1996). In addition to reamplification, increased sensitivity and specificity of PCR-based methods used for the detection of K-ras codon 12 mutation in pancreatic tissue could also be achieved by application of nested PCR or performing a whole genome amplification prior to PCR. Although highly specific and reliable, the PCR-RFLP method is not sufficiently sensitive for diagnostic detection of K-ras codon 12 mutation, and the possibility remains that in some patients the amount of mutated DNA may be not be detectable by this method. More sensitive techniques (such as DNA sequencing) should therefore be applied for K-ras codon 12 mutation detection in future diagnostic protocols for pancreatic cancer.

Since the presence of mutation in the K-ras gene indicates a preneoplastic condition or cancer at an early stage, this test might be used to detect early pancreatic cancer and differentiate pancreatic cancer from chronic pancreatitis. In this study, the mutation was not present in either of the two patients with chronic pancreatitis, who unnecessarily underwent surgery. However, chronic pancreatitis can be positive for the mutation in the absence of cancer, while pancreatic cancer does not necessarily have it, as was the case with 34% of patients with pancreatic cancer in this study (Pugliese et al., 2001; Mu et al., 2004; Ren et al., 2004; Marchese et al., 2006). It follows that detection of K-ras codon 12 mutation in chronic pancreatitis and pancreatic cancer is not informative enough to differentiate between the two diseases, but could serve as one of the parameters in setting the diagnosis of pancreatic cancer.

Molecular detection of K-ras mutation in pancreatic cancer management can also be used for staging of the disease (Lohr et al., 2005; Kim et al., 2006). In this study, a higher frequency of K-ras codon 12 mutation was observed in patients with advanced tumor stages and regional lymph node metastases, which correlates with the finding that K-ras mutations are associated with more aggressive, recurrent, and metastatic tumors (Morioka et al., 2004; Kim et al., 2006).

In addition to analysis of the presence of K-ras gene mutation in pancreatic tissue, this molecular marker can also be detected in pancreatic juice and blood plasma, since small amounts of DNA are released from tumor cells due to necrosis (Castells et al., 1999; Sorenson, 2000; Trumper et al., 2002; Jiao et al., 2007). Although its sensitivity is relatively low, this approach to molecular monitoring of pancreatic cancer is highly specific and has potential for future application in diagnostic and prognostic clinical practice (Castells et al., 1999; Sorenson, 2000; Jiao et al., 2007).

Our study confirms that K-ras mutation is present with a high frequency in patients with pancreatic cancer, especially at advanced tumor stages. Although the finding of K-ras point mutation in cytology or biopsy samples from pancreatic mass does not specifically confirm the diagnosis of pancreatic cancer, it can increase the sensitivity of existing techniques and hence be used as an adjunct to imaging and cytological analyses in pancreatic cancer diagnosis. Further studies are needed to define the value of K-ras mutation screening in patients with other evidence suggesting the presence of pancreatic cancer. The ultimate goal is to detect pancreatic cancer at an early stage and avoid unnecessary duodenopancreatectomies for chronic pancreatitis.

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АНАЛИЗА МУТАЦИЈЕ У КОДОНУ 12 ГЕНА К-RAS У ПАНКРЕАСНОМ ТКИВУ ПАЦИЈЕНАТА СА КАРЦИНОМОМ ПАНКРЕАСА

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Ово истраживање је имало за циљ да се анализира присуство мутације у кодону 12 гена К-ras у панкреасном ткиву пацијената са карциномом панкреаса и да се процени да ли ова мутација представља потенцијални молекуларни маркер за карцином панкреаса у српској популацији. Истраживање је обухватило анализу 40 узорака ткива панкреаса пацијената

са клиничком дијагнозом карцинома панкреаса. Присуство мутације у кодону 12 гена К-ras анализирано је методом PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism). Резултати истраживања указују да је мутација у кодону 12 гена К-ras у ткиву панкреаса присутна са високом учесталošћу (66 %) код пацијената са карциномом панкреаса.