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KRAS MUTATIONS IN PANCREATIC ADENOCARCINOMA IN CORRELATION WITH CLINICAL AND PATHOLOGICAL CHARACTERISTICS

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Abstract

Pancreatic adenocarcinoma is the seventh cause of death of all malignant tumors worldwide and has the worst prognosis of all solid tumors. In Europe, it is the sixth most common cause of cancer related death and in United States it is the fifth cause of death after lung cancer, prostate cancer, breast and colorectal cancer. Numerous molecular studies have analyzed genetic and epigenetic changes as responsible for the histological variants of this cancer, their correlation with family predisposition, and opportunities for better treatment and survival. This study included 42 patients with pancreatic adenocarcinoma. Tumor tissue samples obtained from surgical specimen were histopathologicaly examined and genetic mutations were determinate. Prior to surgery, patients were diagnosed by imaging modalities (abdominal ultrasound and/or CT), clinical and laboratory examinations. Histopathological analyses included: T category, grade of tumor differentiation, vascular invasion, lymph node involvement and metastasis. We obtained the KRAS and EGFR gene mutations on the Randox investigator diagnostic platform.

The aim of the study was to determine the frequency of KRAS and EGFR mutations in pancreatic adenocarcinoma and their correlation with multiple tumor characteristics. No one patient had EGFR mutation.

The results showed that more of the patients with KRAS genetic mutations are frequently associated with advanced disease stage and worse prognosis, although the difference was not statistically significant in comparison to patients without KRAS mutations.

Key words: pancreatic adenocarcinoma, KRAS, EGFR

Introduction

Pancreatic cancer has the worst prognosis of all solid tumors despite that is the seventh cause of all malignant tumors. It is the sixth most common cause of cancer related death in Europe, and in United States it is the fifth cause of death after lung cancer, prostate cancer, breast and colorectal cancer [1]. It is expected to be the second leading cause of cancer deaths by 2030 [2]. Five years survival rate in patients with pancreatic adenocarcinoma does not exceed 5% [3,4] and this is unchanged statistics for the last 50 years [5], while metastatic disease has a 2% annual survival. More than 80% of pancreatic cancers are advanced at the time of diagnosis. Mutation in RAS genes (HRAS, KRAS, NRAS) occur in nearly 30% in tumor oncogenesis (pancreatic, colorectal, breast, etc.), and therefore the development of targeted RAS therapy has research priority [6.] The incidence of KRAS mutations of all found mutation in pancreatic adenocarcinomas is up to 90%.[7].

KRAS is the predominant mutated RAS gene (84% of all RAS mutations), followed by NRAS (12%) and rarely mutated HRAS (4%) in patient with pancreatic adenocarcinoma. KRAS proteins function as binary molecular switches. When they bound to guanosine triphosphate (GTP), interact with signaling molecules that regulate cell activity, such as proliferation, differentiation, apoptosis or migration. Many growth factor receptors, cytokines, hormones, neurotransmitters and other regulators can activate RAS by directly or indirectly enhancing the access of guanine nucleotide exchange factors. KRAS has a central role in controlling tumor metabolism through a number of metabolic changes including stimulation of glucose uptake, differential channeling of glucose by-products, reprogrammed glutamine metabolism, increased autophagy and macropinocytosis. Investigating these processes are key for discovering new treatments for pancreatic adenocarcinoma [8-11].

EGFR (Epidermal Growth Factor Receptor) is a transmembrane glycoprotein or also known as a tyrosine kinase-bound protein, which is a member of the type I family of growth factors [12]. Epidermal growth factor (EGF) and its receptor, the EGF-EGFR, is a proto-oncogene with increased expression in pancreatic adenocarcinoma and it indicates advanced malignant disease with the presence of metastases and poor prognosis. Inhibition of the EGFR represents a new therapeutic approach for pancreatic adenocarcinoma and combined with chemotherapy they affect tumor angiogenesis and apoptosis [12,13]. Erlotinib as a tyrosine kinase inhibitor has been approved by FDA, for advanced or metastatic pancreatic adenocarcinoma treatment and combination with gemcitabine have better success than gemcitabine therapy alone [14-18].

Objectives

The aim of this study was to determine the presence of KRAS gene mutation, the frequency and codon mutation types, as well as EGFR mutation in pancreatic adenocarcinoma.

In addition the correlation between gene mutation and histopathological tumor characteristics that include: T category, grade of differentiation, vascular invasion, lymph nodes enlargement and distant metastasis, as well as the association between KRAS gene mutation and survival rate in pancreatic adenocarcinoma patients, were made.

Material and Methods

The study included 42 patients with pancreatic adenocarcinoma. Tumor tissue samples obtained from surgical specimen were histopathologicaly examined and genetic mutations were determined. Prior to surgery, patients were diagnosed by imaging techniques (abdominal ultrasound and/or CT), clinical and laboratory examinations. Histopathological analyses included: T category, grade of tumor differentiation, vascular invasion, lymph node involvement and metastasis. Tumor classification was made according to World Health Organization recommendations and UICC pTNM staging system.

Tumor KRAS and EGFR mutation were analyzed by polymerase chain reaction (PCR):

1. **DNA isolation**

DNA form paraffin embedded tissue (FFPE) was extracted using Cobas®DNA Sample Preparation Kit (Roche Diagnostics). DNA concentration was measured using ScanDrop2 (AnalyticJenna) spectrophotometer and dilution of isolated DNA was performed according to protocol for detecting mutations.

2. **Detection of KRAS and EGFR mutations**

Cobas® EGFR Mutation Test V2 (Roche Diagnostics) was used for detecting mutations in exon 18-21 of EGFR gene. According to protocol, 2ng/uL isolated DNA was used for detecting mutations with this assay on Cobas Z480 IVD RealTime PCR Machine. The Cobas®EGFR Test is designed to detect the following mutations:

Exon 18: G719X (G719A, G719C, and G719S)
Exon 19: deletions and complex mutations
Exon 20: S768I, T790M, and insertions
Exon 21: L858R and L861Q

A mutant control and negative control are included in each run to confirm the validity of the run. The Cobas® EGFR Test can detect mutations with at least 5% mutation level using the standard input of 50 ng per reaction well.

After isolation of DNA from FFPE samples, short multiplex PCR and hybridization, and microarray technology was used to detect present mutations using Randox Evidence Investigator System

With this microarray assay can be detected, 20 point mutations in codons 12, 13, 61, and 146 of KRAS gene,

Statistical analysis: The data were analysed with SPSS v. 23.0 statistical software, tabulated and graphically presented. The qualitative data were made with relative and absolute numbers, and the quantitative data with average and median values. Parametric (Student t-test, Mann-Whitney test), and

nonparametric tests (Chi-square test, Fisher exact test) were used to compare KRAS negative (-) and KRAS positive (+) tumours. Significant results were with p <0.05 value.

Results

The study included 42 patients with pancreatic adenocarcinoma. Forty (95.2%) patients had pancreas head tumor localization and 2 (4.7%) patients had pancreas tail tumor localization. Patients age was from 48-72 years, with a mean age of 63.3 ± 8.7 years. Twenty seven out of 42 patients were males.

Tumor genetic mutations revealed that 52.4% (22) patients were KRAS positive (+) and the remaining 47.6% (20) were KRAS negative (-).

None of 42 patients had EGFR mutation.

The KRAS positive and the KRAS negative patients groups were similar in age 63.6 ± 9.15 years and 63.6 ± 8.5 years, respectively (p = 0.99).

KRAS positive patients had a shorter average and median survival time than KRAS negative patients, mean time of 10.2 ± 4.8 vs. 11.25 ± 5.5 months, and median time of 9 and 11 months, that was statistically confirmed as nonsignificant (p=0.5). Table 1.

Table 1.

Variable	Descriptive statistics				p-level
	N	mean ± SD	min-max	median (IQR)	
Age					
KRAS-	20	63.6 ± 8.5	47 – 78		t=0.013
KRAS+	22	63.64 ± 9.2	42 – 77		p=0.9 ns
Survival in months					
KRAS-	20	11.25 ± 5.5	2 – 19	11 (7 – 15.5)	Z=0.72
KRAS+	22	10.18 ± 4.8	4 – 22	9 (7 – 13)	p=0.47 ns

t (Student t-test), Z (Mann-Whitney test)

Correlation between tumor characteristics and KRAS mutation positive/negative groups are presented in Table 2. The results showed a statistically insignificant difference between KRAS positive group and KRAS negative group, in respect to T category (p=0.7), lymph node involvement (p=0.1) and distant metastases (p=0.2).

In terms of T category, the results showed that both, KRAS positive 54.55% (12) and KRAS negative 65% (13) patients most commonly presented in T2 tumor stage.

KRAS positive tumors, more often than KRAS negative tumors, had N1 status; that is, with metastasis to regional lymph nodes in 40.9% (9) versus 15% (3) patients.

Distant metastases had 4 out of 22 KRAS positive patients and only one KRAS negative patient (p = 0,2).

Table 2.

	N	KRAS-	KRAS+	p-level
T- stage				
1	5	3 (15)	2 (9,09)	Fisher exact
2	25	13 (65)	12 (54,55)	P =0.7ns
3	11	4 (20)	7 (31,82)	
4	1	0	1 (4,55)	
N- limph nodes invasion				
N0	19	12 (60)	7 (31.82)	$\chi^2 = 4.52$
N1	12	3 (15)	9 (40.91)	p = 0.1 ns
N2	10	4 (20)	6 (27.27)	
Nx	1	1 (5)	0	
M-distant metastases				
M0	37	19 (95)	18 (81.82)	Fisher exact P = 0.2 ns
M1	5	1 (5)	4 (18.18)	

$\chi 2$ (Pearson Chi-square)

Concerning disease stage, patients with KRAS negative tumors were most commonly diagnosed in stage IIA, (6-30%) and patients with KRAS positive tumors in stage III, (9-40.9%). There was no statistically significant difference between KRAS- and KRAS + tumors in relation to the stage (p = 0.61). (Table 3)

Table 3.

Stage	N	KRAS-	KRAS+	p-level
I A	5	3 (15)	2 (9.09)	P = 0.61 ns
I B	4	3 (15)	1 (4.55)	
II A	11	6 (30)	5 (22.73)	
II B	8	3 (15)	5 (22.73)	
III	14	5 (25)	9 (40.91)	

p (Fisher exact test)

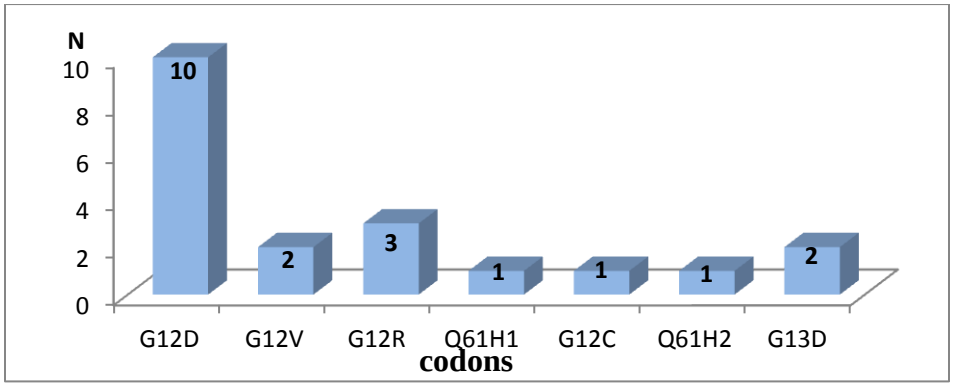
Vascular invasion was more frequent in KRAS+ tumors with 50% (11) patients vs. KRAS negative in 35% (7) patients, (p = 0.33). KRAS positive tumors were more frequently classified as moderate or poor differentiated tumors in 50% (11) and 45.45% (10) patients (grade 2 and grade 3) respectively. KRAS negative tumors were classified as grade 3 in 30% (6) patients (p = 0.67). (Table 4).

Table 4.

	N	KRAS-	KRAS+	p-level
Vascular invasion				
No	24	13 (65)	11 (50)	$\chi^2 = 0.96$
Yess	18	7 (35)	11 (50)	p = 0.33 ns
Differentiation				
G1 - good	2	1 (5)	1 (4.55)	Fisher exact
G2 - modarate	24	13 (65)	11 (50)	P = 0.67 ns
G3 – poor	16	6 (30)	10 (45.45)	

$\chi 2$ (Pearson Chi-square)

Mutation were determined in 20 out of 42 KRAS patients,. The most common KRAS mutation was G12D found in 10 patients, G12R mutation was detected in 3 patients, 2 patients had G12V mutation, and G13D mutation were found in 2 patients. Q61H1, G12C, and Q61H2 mutation have been found, separately in 3 patients.. Figure 1.



Fugure 1.

Results of the correlation between patients with codon G12D mutation and histopathology are presented in Table 5.

Table 5.

Variable	G12D
Age (mean±SD)	65.8 ± 8.05
Survival in months (mean±SD)	9.8 ± 3.76
T – Category	
1	1 (100)
2	7 (70)
3	2 (20)
4	0
N- Limph nodes invasion	
N0	4 (40)
N1	5 (50)
N2	1 (10)
M-Distant metastases	
M0	9 (90)
M1	1 (10)
G- Grade	
G2	6 (60)
G3	4 (40)
Stage	
I A	1 (10)
I B	1 (10)
II A	3 (30)
II B	3 (30)
III	2 (20)
Vascular invasion	
Yes	5 (50)
No	5 (50)

Discussion

KRAS is the most frequently mutated gene in pancreatic adenocarcinoma In this study, 22 (52.4%) out of 42 patients with pancreatic adenocarcinoma revealed KRAS mutations. Several signalling pathways are activated by mutated KRAS genes. Of these, mitogen-activated protein kinases (MAPK) and PI3K pathways are the most widely studied. The MAPK pathway is a kinase cascade that involves activation of RAF kinase by KRAS and eventual activation of MEK1/2. MEK kinases further activate ERK1/2 through phosphorylation. This signalling is active in pancreatic intraepithelial neoplasia (PaIN) lesions as well as invasive pancreatic adenocarcinomas [24-27]. Presence of KRAS and EGFR gene mutations in patients with pancreatic adenocarcinoma has particular importance because of new therapeutic modalities and the use of targeted gene therapy.

These patients have limited therapeutic options with poor response to current therapy. Development of gene therapy will enable better prognosis and quality of life. The growth factor receptor, or any of these molecules, may be affected by very specific mutations, resulting in abnormally constant activation of a specific signalling pathway. Alternatively, over expression may either be due to gene amplification at the level of genomic DNA or may result from increased gene transcription or protein transfer. Patients with present EGFR mutation have the potential for new treatments with Erlotinib, a

small molecule inhibitor, recently approved in combination with gemcitabine for metastatic disease therapy. Mutations of EGFR pancreatic adenocarcinoma are rare [19, 28]. In our study we did not identify any mutation of EGFR. Some clinical studies outcomes the positive correlation of EGFR mutations with tumour stage and lymph node metastasis. The presence of EGFR mutations was reported that has no significant association with patient survival rate [29].

KRAS mutation was not effective in predicting response to both anti-epidermal growth factor receptor and/or chemotherapy treatments. Gene therapy combined with standard chemotherapy is most commonly used [20]. The present study showed KRAS mutation in 22 out of 42 patients with pancreatic adenocarcinoma, Lemstrova et al. explain the failure of KRAS-targeted therapies in pancreatic adenocarcinoma [21]. Frequency of mutation was higher in patients with advanced disease stage. KRAS negative tumors were most commonly diagnosed in stage II A, and KRAS positive tumors in stage III. Vascular invasion was more common in patients with KRAS positivity than KRAS negative 50% versus 35% patients, as well as more frequently showed moderately or poor cell differentiation 45.45% and 30% patients, respectively. These data are similar with some studies in the literature [22]. Presence of KRAS mutations can be detected at an earlier tumour stage, but there is no significance in grade determination [23]. Concerning codons mutation, the present study showed that the most common was the G12D mutation which is similar with another reports [30, 31]. G12R, G12V, G13D, Q61H1, G12C and Q61H2 mutations were rarely detected. Some previous studies have shown that more than 90% of pancreatic adenocarcinomas had mutation codon 12 of KRAS gene [32], whereas in many other studies the prevalence was reduced to about 75% [33]. G12C mutation is rare in pancreatic adenocarcinoma (1% of all KRAS mutations [34].

Until now, it has been assumed that different mutations cause substantially identical effects on RAS function [35]. Thus, a shift has emerged from the long-standing "all RAS mutations are created equal" paradigm, and recent studies are looking for specific RAS mutants, a selective mutation that can be used in different therapeutic strategies. Mutation specific inhibitors have been developed as a target therapy in the G12C-mutant KRAS in lung adenocarcinoma [36]. There is limited evidence that specificity of mutants also plays an important role in the prognosis of pancreatic cancer. One study suggests that KRAS mutations are associated with more aggressive cancers [37]. Another studies have found that KRAS Q61 mutations are associated with better outcome compared to patients with KRAS G12 mutation [38].

Conclusion

This study was design to determine the correlation between genetic mutations and clinical and pathological characteristics of pancreatic adenocarcinoma. Although there was no significant difference between two groups of KRAS positive and KRAS negative patients with pancreatic adenocarcinoma in relation to survival and tumor characteristics. The results showed that more of the patients with KRAS genetic mutations are frequently associated with advanced disease stage and worse prognosis, although the difference was not statistically significant in comparison to patients without KRAS mutations. This study should be extended to large number of patients because has many different mutations in KRAS and other genes included in this study. Therefore, development of new treatment options related to these genetic mutations are expected to improve the survival rate and prognosis in patient with pancreatic adenocarcinoma.

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