Evaluation of *KRAS* Mutational Status in Pancreatic Ductal Adenocarcinoma

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies with a dismal prognostic. *KRAS* is a well-recognized driver gene in PDAC, detected in the earliest stages of pancreatic tumor transformation. To establish the frequency of *KRAS* mutations in a cohort of Romanian patients, we performed Sanger sequencing for exon 2 and exon 3 of the gene on 31 PDACs pairs. We further examined correlation of the mutational status with clinico-pathologic data of the patients and evaluated the prognostic implication of *KRAS* mutations. Ten out of 31 patients (32%) had *KRAS* missense mutations, all were detected in exon 2, and codon 12 only. Among patients with *KRAS* mutations, the majority (7/10, 70%) had c.35G>A (p.G12D) substitutions. Patients with p.G12D mutations had a marginally worse survival ($p=0.07$) and a shorter disease-free survival ($p=0.04$) when compared to *KRAS* wild-type patients. Correlation between patients age and recurrence was seen in *KRAS* wild-type cohort ($p=0.096$, $r=-0.6$). In summary, evaluation of *KRAS* mutational status is a predictor for PDAC patients' prognosis and further *KRAS* directed therapy might prove to be an efficient tool in patients having *KRAS* mutations.

Key words: pancreatic ductal adenocarcinoma, Sanger sequencing

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) remains the malignancy with one of the highest mortality rate among solid tumors (1). In the majority of cases (80-90%) the cancer is diagnosed in late stages while in the remaining, even the

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surgical intervention is a viable choice, local and systemic recurrence is often seen (2).

PDAC is characterized by an extensive stromal involvement and a variety of genetic and epigenetic events (3). Four genes are considered as driver gene or “mountains” (4) in PDAC: one oncogene, *KRAS*, and three tumor suppressor genes, *TP53*, *p16/CDKN2A* and *SMAD4* (5).

KRAS is a small GTPase, a switch between GTP-bound active and GDP inactive states. Mutation in *KRAS* found in more than 90% of PDAC cases, maintains a constitutive active state of the gene and a constant stimulation of down-stream pathways (6) like Raf/Mek/Erk, PI3K/Pdk1/Akt and the Ral guanine nucleotide exchange factor pathway (7, 8, 9, 10). This stimulation further leads to manifestation of the very well-known features of cancer: increased proliferation, suppression of apoptosis, alteration in the tumors microenvironment, evasion to chemotherapeutic drugs and metastasis (11).

The majority of *KRAS* mutations are point mutations, found in exon 2, codon 12 of the gene that determines a switch between amino acid glycine (G) with amino acid aspartic acid (D). Compared to other cancer (eg. colorectal cancer, non-small cell lung cancer), this type of activating mutation is found at a very high frequency in PDAC (12, 13).

Considering the high frequency of *KRAS* mutation reported so far in PDAC, we aimed in determining the incidence of mutations in a lot of Romanian patients and further to determine correlation with clinic-pathological features of the patients, overall survival and disease-free survival.

MATERIALS AND METHODS

Patients' samples

Based on an informed written consent, samples of pancreatic duodenal adenocarcinoma from 31 patients who underwent surgical resection were obtained from the Centre of Digestive Diseases and Liver Transplantation, Fundeni Clinical Institute. Histological diagnosis was established by a general pathologist.

Length of follow-up, time to recurrence, the time to death was calculated in months from the date of surgery for each patient.

DNA samples

Genomic DNA was extracted from pairs of normal and tumoral frozen tissue by means of Qiagen Blood

and Cell Culture Mini Kit (Hilden, Germany), a column based kit, according to manufacturer's instructions. The genomic DNA was bound to column, washed, eluted and quantified using spectrophotometry. Integrity was checked by running 200 ng of genomic DNA on 0.8% agarose gel.

KRAS mutations by Sanger sequencing

Genomic DNA was amplified with Illustra GenomPhi HY DNA Amplification Kit (GE healthcare, Buckinghamshire, UK) and Platinum *Taq* Polymerase (Life technologies, Carlsbad, USA) was used for PCR reaction using the following program: 95°C for 10 minute; 39 cycles of 95°C for 30seconds; 60°C for 30 seconds, 72°C for 1 minute and a final extension of 72°C for 10 min. Purified PCR products were sequenced with ABI BigDye Terminator v3.1 (Life technologies, Carlsbad, USA) following manufacturer's protocol and ran on an ABI 3730 genetic analyzer. The results were analyzed using Seqman II DNASTAR (v.5.05) (Madison, Wisconsin, USA) and Chromas 2.4.3 (South Brisbane, Australia). The primers used to perform PCR and Sanger sequencing are the ones previously described (14).

Statistical analysis

Dichotomous variables were evaluated using Fisher's exact test or Pearson's χ^2 . Differences between groups were evaluated by means of nonparametric Mann-Whitney or Kruskal-Wallis test. Survival analyses were performed using the Kaplan-Meier curves and the differences in survival curves were assessed by Log rank Mantel Cox test. A p-value ≤ 0.05 was considered statistically significant. All tests were performed with GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA).

RESULTS

Clinicopathologic characteristics of the patients

Clinico-pathologic characteristics of all patients are included in *table 1*. From the total of 31 patients, 13 (42%) received chemotherapy with gemcitabine alone, 13 (42%) received 5-fluorouracil (5-FU) or 5-fluorouracil and cisplatin and one patient (3.2%) received another chemotherapeutic regimen (for four patients the chemotherapeutic regimen received is unknown). Marginal association were observed when comparing overall survival between patients receiving gemcitabine or other chemotherapeutic regimen (median 18 months

Table 1 - Clinico-pathologic characteristics of the patients

Characteristics	n, (%)
Age at surgical intervention (yrs), median [range]	64 [46-77]
Gender	
Male	16 (51.6)
Female	15 (48.4)
pT	
T1	9 (29.0)
T2	16 (51.6)
T3	6 (19.4)
pN	
N0	12 (38.7)
N1	19 (61.3)
Tumor stage	
I	9 (29.0)
II	20 (64.5)
III	0 (0.0)
IV	2 (6.5)
Differentiation degree	
G1/ G1-G2	25 (80.7)
G2	5 (16.1)
G3	1 (3.2)
Tumor size, median [range]	2.5 [0.3-7]
Lymph-node ratio, LNR, median [range]	0.1 [0.0-0.75]
Type of resection	
R0	28 (90.3)
R1	2 (6.5)
Unknown	1 (3.2)
Recurrence	
Yes	20 (64.5)
No	6 (19.4)
Unknown	5 (16.1)
Status	
Alive	1 (3.2)
Deceased	30 (96.8)
Overall survival (months), median [range]	15 [2.0-109.0]

for gemcitabine versus 9 months for 5-FU/ 5-FU and cisplatin or other chemotherapeutic regimen, $p=0.09$, long-rank Mantel Cox test) and when comparing overall survival based on CA 19-9 level, using a cut off value of 1000 U/ml (median 18 months, for patients with CA 19-9 levels lower than 1000 U/ml versus 6.5 months, for patients with CA 19-9 levels higher than 1000 U/ml $p=0.06$, long-rank Mantel Cox test). The majority of patients had no associated diabetes at tumor diagnostic (23/31, 74.2%). A statistically significant difference was seen when comparing patients groups based on lymph-node ratio (LNR), when using a cut off value for LNR of 0.16 (median 21 months for patients with LNR lower than 0.16 versus median 11 months for patients with LNR higher than 0.16, $p=0.007$, long-rank Mantel Cox test).

KRAS p.G12D mutations shows a marginal association with worse overall-survival and disease-free survival

Detailed description of clinic-pathologic characteristics of patients with *KRAS* mutation versus *KRAS* wild type patients is included in table 2. Among the analyzed patients, 10/31 (32%) had *KRAS* mutations: seven had c.35G>A (p.G12D), two had c.35G>T (p.G12V) and one had c.34G>C (p.G12R) (fig. 1 a, b and c). All mutations were in exon2, codon 12 (fig. 1d). A marginal association with overall survival was seen when comparing patients with *KRAS* mutations and *KRAS* wild-type patients ($p=0.075$, median of 11.5 months for *KRAS* mutant patients versus median of 18 months for *KRAS* wild-type patients). The same trend, of marginal association, was seen when comparing patients with p.G12D mutations only and *KRAS* wild-type patients ($p=0.07$, median of 11 months for p.G12D mutations patients versus median of 18 months for *KRAS* wild-type patients, (fig. 1f). Analysis on disease-free survival, between patients with p.G12D mutations and *KRAS* wild-type patients showed a statistically significant difference ($p=0.04$, fig. 1f). Additional statistically significant correlations between clinic-pathologic features for patients with p.G12D mutations and *KRAS* wild-type patients are included in fig. 2 a and b.

DISCUSSIONS

PDAC is characterized by a complex mutational landscape (15) but is essentially a *KRAS* oncogene-driven solid tumor (16).

KRAS is the gene that is almost ubiquitously detected in pancreatic cancer whole exome and genome studies (17, 18).

It is a protein in the epidermal growth factor receptor (EGFR) signaling pathway whose importance in PDAC progression was shown by multiple mouse model (11, 19, 20, 21, and 22).

In our study we found the frequency of *KRAS* somatic mutation of 32%, much lower than the one previously reported for this cancer (23). One limitation of our current study is that the method of detection used has its own limits (24) which might led to a lower estimation of *KRAS* mutation frequency.

All detected *KRAS* point mutations were exclusively in exon 2, codon 12 with the majority (70%) being c.35G>A (p.G12D) substitutions.

We observed trends towards a worse overall survival when analyzing patients with *KRAS* mutations or p.G12D mutations exclusively and *KRAS* wild-type patients. A

Table 2 - Analysis of clinic-pathologic characteristics of the patients on *KRAS* mutational status

Characteristics ^a	<i>KRAS</i> mutant (n=10)	p.G12D <i>KRAS</i> (n=7)	<i>KRAS</i> wild-type (n=21)	p-value ^c	p-value ^d
Age at surgical intervention (yrs), median [range]	67 [46-77]	64 [46.0-72.0]	59 [50-74]	0.2	0.87
Gender, n (%)				0.7	1.0
Male	6	4	10		
Female	4	3	11		
pT, n (%)				0.24	0.13
T1	1	1	8		
T2	7	6	9		
T3	2	0	4		
pN, n (%)				1.0	1.0
N0	4	2	8		
N1	6	5	13		
Tumor stage, n (%)				0.84	0.69
I	3	2	6		
II	6	4	14		
III	0	0	0		
IV	1	1	1		
Differentiation degree, n (%)				0.64	0.5
G1/ G1-G2	6	5	19	0.098	0.19
G2	3	1	2		
G3	1	1	0		
Tumor size, median [range]	3 [1.5-6.0]	3 [1.5-3.0]	2.5 [0.3-7.0]	0.24	0.59
Lymph-node ratio (LNR), median [range]	0.11 [0.0-0.55]	0.13 [0.0-0.55]	0.0 [0.0-0.3]	0.36	0.38
				1.0	1.0
≤ 0.16	6	4	13		
>0.16	4	3	7		
Type of resection, n (%)				0.65	1.0
R0	9	6	19	1.0	0.45
R1	1	1	1		
Unknown ^b	0	0	1		
Recurrence, n (%)				0.38	1.0
Yes	8	5	12		
No	1	1	5		
Unknown ^b	1	1	4		
Status, n (%)				1.0	1.0
Alive	0	0	1		
Deceased	10	7	20		
CA 19-9 (U/ml) median [range]	108 [14.8-5560]	151.0 [21.9-5560]	108.4 [4.6-1179]	0.36	0.89

^ap-values between age and tumor size were calculated by means of Mann-Whitney test, differences in overall survival were calculated by means of Log-Rank Mantel Cox test; the rest were tested by Fisher exact t-test or Chi-square test

^bnot included in p-value calculation

^cp-values were calculated between *KRAS* mutant patients and *KRAS* wild-type patients

^dp-values were calculated between p.G12D mutant patients and *KRAS* wild-type patients

shorter overall survival was seen in patients with p.G12D mutations only compared to wild-type patients.

Further analysis in patients with p.G12D mutations and *KRAS* wild type patients showed correlation between overall survival and disease-free survival, stage and pN and LNR, disease-free survival and recurrence (in *KRAS* wild-type patients only) and age and recurrence (in patients with p.G12D mutations only). This is consis-

tent with previous studies showing a poor outcome in patients with p.G12D mutant tumors (25,26).

Transcriptomic characterization of PDAC, initially divided it into three types: quasi-mesenchymal, classic, and exocrine-like (9) and later, the classical subtype was sub-divided into pancreatic progenitor and immunogenic subtype (15). Interestingly *in vitro* and *in vivo* studies showed that the classical subtype depends on

Figure 1 - a) Graphic representation of *KRAS* gene and number of mutations; b) percentage of *KRAS* mutant and wild-type patients within the cohort; c) Type and number of patients with *KRAS* mutations; d) Representative chromatograms for cases with p.G12V, p.G12D and p.G12R mutations; e) Survival curve between patients with p.G12D and *KRAS* wild-type patients; f) Disease-free survival curve between patients with p.G12D and *KRAS* wild-type patients

Figure 2 - Correlation matrices between clinico-pathologic features of the patients with: a) p.G12D mutations and b) KRAS wild-type (OS-overall survival, DFS-disease-free survival, LNR-Lymph node ratio, G-differentiation degree); p-values are represented in a color-coded manner in the upper part of the matrices; Spearman R values are shown in the lower part of the matrices

KRAS, while those of the mesenchymal subtype are no (27). This studies, once again, shows the importance of evaluation of *KRAS* status in patients stratification and in precision medicine, respectively.

For long time *KRAS* mutations were considered undruggable, but today new methods of targeting *KRAS* mutations, with emphasis on p.G12D mutations, are being developed (eg. siG12D-LODER). A phase I/IIa clinical study using this approach in combination with FOLFIRINOX showed a potential efficacy in patients with locally advance pancreatic cancer (28).

The end results of this technological progress could be an effective treatment options for patients based on their tumor molecular blueprint, and in particular based on the *KRAS* mutational status.

Our results showed that a classification based on *KRAS* status has prognostic implications. Taken together with the literature data, these results highlight the importance of both *KRAS* genomic characterization of PDAC in treatment decision making.

CONCLUSIONS

Our study showed a lower *KRAS* mutation frequency in PDAC and confirmed the prognostic value of p.G12D mutations on the patients` outcome in this type of cancer. As research is directed into making *KRAS* to become therapeutically targetable, our results have implication in the field of personalized medicine. Moreover additional research will be needed to further characterize *KRAS* wild-type patients and to evaluate best therapy options for them.

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