Research Article

Kras, epidermal growth factor receptor, and p53, but not Nras or Braf, biomarkers are frequently altered in pancreatic adenocarcinoma and precursor lesions

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ABSTRACT

Aim: Pancreatic cancer is estimated to be the 12th most common cancer in men and the 11 most common in women worldwide. **Materials and Method:** As other cancers, pancreatic ductal adenocarcinoma (PDAC) accumulates genetic alterations in oncogenes and suppressor genes. In the present study, we analyzed five biomarkers status: Epidermal growth factor receptor (EGFR) and p53 using immunohistochemical labeling in 39 PDAC and Kras, Nras, and Braf, using pyrosequencing in 25 normal pancreatic tissues, 25 pancreatic intraductal neoplasia (PanIN), and 39 PDAC. **Result and Discussion:** Abnormal immunolabeling of p53 and EGFR was detected in 97.4% and 71.8%, respectively. There was a statistically significant progressive increment ($P < 0.001$) in the percentage of mutated cases through normal pancreas (8%), PanIN-1A (28.6%), PanIN-1B (33.3%), PanIN-2 (60%), and PanIN-3 (85.7%) to PDAC (94.8%). These mutations were arising in codons 12 and 61 of Kras and in codon 600 of Braf. Most frequent mutations were G12D (46%), G12A (23%), and G12V (18%) of Kras. No mutations were found in codons 13, 59, 117, and 146 of Kras or in codons 12, 13, 59, 61, 117, and 146 of Nras. **Conclusion:** We confirm that EGFR, Kras, and p53 are the most frequent altered biomarkers in PDAC with difference in predominated Kras mutations subtypes.

KEY WORDS: Epidermal growth factor receptor, Kras, Pancreatic intraductal neoplasia, Pancreatic ductal adenocarcinoma, p53

INTRODUCTION

Pancreatic cancer is estimated to be the 12th most common cancer in men and the 11th most common in women worldwide.[1] However, because of its high mortality, it forms the 7th cause of universal cancer deaths.[1] In fact, only 20% of patients have an operable tumor and the 5-year survival for patients after complete resection is in order of 15–25%,[2-5] while it is desperately low of 0.4–5% in patients with inoperable pancreatic cancer.[3,6,7]

Histologically, 90% of pancreatic tumors are pancreatic ductal adenocarcinoma (PDAC).[1] Conventional PDAC mainly originates from pancreatic intraepithelial neoplasia (PanIN).[8,9] PanIN has been further subdivided into 1A, 1B, 2, and 3 based on morphology and dysplasia grade.[10] Most attempts to better understand the characteristics of PDAC have focused on studying genes and proteins expression profiles of PDAC samples, PanIN, and tissues with normal histological appearance. In the present study, we analyzed three oncogenes status by pyrosequencing and two immunomarkers (EGFR and p53); in 39 PDAC, 25 PanIN and 25 normal pancreatic tissues. Consequently, we investigated their implications on clinicopathological parameters.[11]

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MATERIALS AND METHODS

Cases Selection

Formalin-fixed paraffin-embedded (FFPE) tissues from 39 patients with PDAC, resected in the period 2000–2016, were obtained from the archival tissue collection of pathology department. All specimens were fixed in 10% buffered formalin. Cases were revised by two experiment pathologists based on an evaluation of hematoxylin-eosin (HE) stained sections. Clinical, epidemiological, and prognosis analysis, including the following parameters: Age, sex, size, tumor location, TNM stage, differentiation, vascular emboli, and perineural invasion, was determined referring to patients' files. Survival status was available only for three patients.

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p53 and EGFR Immunohistochemistry

A total of 39 samples were immunostained using anti-p53 (Mouse monoclonal antibody, anti-human, NCL-L-p53-DO7, 1:800, Novocastra) and anti-EGFR (Mouse monoclonal antibody, anti-human, RTU-EGFR-384, ready to use, Novocastra). Immunohistochemical labeling was carried out using an automated Leica Bond-Max (Leica Microsystems, Germany). Briefly, 3 µm thick sections were prepared from each bloc and were dried at 60°C for 60 min. First, tissues were deparaffinized using xylene and pretreated with the Epitope Retrieval Solution (ER1, pH = 6) at 98°C for 20 min. After washing steps, peroxidase blocking (H2O2, 0.3%) was carried out for 5 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems, GmbH). Tissues were again washed and then incubated with primary antibody (anti-EGFR or anti-p53) for 20 min. Subsequently, tissues were incubated with polymer for 15 min and developed with DAB Chromogen for 5 min.[12-14]

p53 Immunohistochemical Evaluation

p53 expression was considered:

- Normal when scattered cells with nuclear labeling were presented like in the normal pancreatic tissue considered as a negative control.
- Underexpressed when a negative or weak immunolabeling was observed in <5% of neoplastic cells comparing with normal tissue.
- Overexpressed when a nuclear accumulation of immunolabeled protein was observed in plus than 30% of neoplastic cells.[2]

EGFR Immunohistochemical Evaluation

Immunohistochemical evaluation was performed for membranous and cytoplasmic expression separately. Membranous staining was divided into four scores as follows:

- Score 0: When staining, regardless of its intensity, was observed in <10% of tumor cells.
- Score 1+: When weak staining was detected in plus than 10% of neoplastic cells.
- Score 2+: When weak to modest staining was observed on entire membrane in plus than 10% of neoplastic cells.
- Score 3+: When robust staining was observed on whole membrane in plus than 10% of neoplastic cells.

Cytoplasmic staining was divided into three scores as follows:

- Score 0: When staining, regardless of its intensity, was observed in <10% of neoplastic cells.
- Score 1+: When weak staining was detected in plus than 10% of neoplastic cells.
- Score 2+: When moderate to strong staining was observed in plus than 10% of tumor cells.

Cytoplasmic and membranous overexpression was defined only for cases showing 2+ score and 2+ or 3+ scores, respectively.[3]

Molecular Analysis

Macrodissection and genomic DNA extraction

After HE reexamination, we selected 89 foci: 25 PanIN (7 PanIN-1A, 6 PanIN-1B, 5 PanIN-2, and 7 PanIN-3), 25 normal pancreatic tissues, and 39 tumor foci which contained at least 70% of tumor tissue. Macrodissection was performed by a 4 mm punch. The carrot was then transferred into a new recipient block. For each sample, 20 sections were used for DNA extraction. To minimize the cross-contamination risk, before each use, the microtome blade was renewed and the punch core was wiped by xylene followed by a DNA decontamination reagent (Thermo Fisher Scientific).

Genomic DNA extraction was performed according to Kit (QIAamp® DNA FFPE Tissue Qiagen) manufacturer's handbook. Briefly, sections were deparaffinized using xylene and resuspended in an appropriate amount of tissue lysis buffer and proteinase K, then incubated at 56°C for 48 h. The entire lysate was transferred to the QIAamp MinElute column. During centrifugation, the DNA binds to the membrane and contaminants flow through. Next, residual contaminants were eliminated with wash steps. After elution buffer addition, a full-speed centrifugation was performed to collect a pure and concentrated DNA. Quality control extraction was performed using a Nanodrop (IMPLEN, Thermo Fisher Scientific).

Mutations Analysis

Nine hotspot site mutations of Kras, Nras, and Braf were analyzed in this order: Kras: Codons 12 and 13, codons 59 and 61, codon 117 and codon 146, Nras: Codons 12 and 13, codons 59 and 61, codon 117 and codon 146, and Braf: Codon 600. After each pyrosequencing, the mutated samples were excluded and only wild-type samples can be amplified for following sequencing.

Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

Reverse primers were biotinylated to facilitate single-stranded DNA template isolation for the pyrosequencing reaction. Primers and PCR product size for each target are shown in Table 1.

PCR was performed in a LifeTouch thermal cycler (BIOER Technologies) in 30 µL final volume with 2 U of Taq polymerase (500U Taq polymerase, Agilent Technologies), 1× PCR buffer, 0.1 mM dNTPs, 30 pM of each primer, and 50 ng of genomic DNA. A wild-type genomic DNA (Qiagen) was used as a negative

control. Thermal program for each PCR is explained in Table 2. Successful and specific amplification was verified by visualizing PCR product on 2% agarose gel stained with ethidium bromide.

Single-Stranded DNA Template Preparation and Pyrosequencing

Preparation of template and sequencing reactions were performed according to manufacturer's directions. Briefly, biotinylated PCR products were immobilized onto streptavidin-coated beads (Streptavidin Sepharose High Performance, GE Healthcare) by mixing 15 µL of PCR product with l µL Streptavidin Sepharose suspension. To remove non-biotinylated DNA strand, samples were sequentially denatured using PyroMark Q24 Vacuum Prep Workstation Tool (Qiagen). Immobilized pure single-stranded DNA was then transferred to a microtiter plate containing 0.8 µL target-specific sequencing primer (100 pmol/L). Required volumes of substrates, enzymes, and nucleotides (Gold Reagent Kit, Qiagen) listed in the pre-run report were dispensed in a clean PyroMark Q24 Cartridge (Qiagen).

Real-time sequencing was performed using PyroMark Q24 pyrosequencing instrument and software according to the manufacturer's instructions (www.pyrosequencing.com). Table 3 summarizes sequencing primers, sequences to analyze and dispensation orders. Primers for ras and Braf were described, respectively, by (Andreas Jung, 2013) [18] and (Cynthia Spittle *et al.*, 2007)[19] and were all synthesized by Biomers (Biomers, The biopolymer factory, Germany).

Statistical Analysis

Data were processed using SPSS 20.0 statistical software (SPSS, Inc, USA). Patients' characteristics were analyzed using descriptive statistics. Qualitative and quantitative variables were analyzed, as appropriate, using Student's *t*-test, Pearson correlation test, ANOVA test, χ^2 test, or Fisher's exact test, with a statistically significant $P < 0.05$.

RESULTS

Clinicopathological and Histological Characteristics

A total of 20 (51%) patients were women and 19 (49%) were men. Median age at operation was 58 years (25–78 years). Mean tumor size was 3.5 cm (2–11 cm). Resection margins were positive in 15%. Tumor was well differentiated (46%), moderately differentiated (43%), and poorly differentiated (11%). T3 stage was observed in 52% of cases followed by T4 stage (8%) with N1 stage in 70% of cases. Perineural invasion and vascular emboli were present in 85% and 67% of cases, respectively. Vascular emboli were significantly associated with age and T stage ($P = 0.04$ and $P = 0.03$, respectively). N stage was significantly associated with perineural invasion and tumor size ($P = 0.001$ and $P = 0.03$, respectively) [Table 4].

Immunohistochemical Analysis

p53

Abnormal immunolabeling of p53 was detected in 38 (97.4%) cases. 29 (74.3%) PDAC showed virtual absence of p53 immunolabeling (underexpression) and 9 (23%) showed robust nuclear accumulation of immunolabeled p53 protein (overexpression) [Figure 1].

EGFR

EGFR expression was abnormal in 28 (71.8%) cases with cytoplasmic overexpression in 20 (51.3%) and membranous overexpression in 8 (20.5%) [Figure 2]. Details for EGFR immunohistochemical labeling are

Table 1: Primers set and amplicons' sizes

Target	Primers (5'→3')		Amplicon size (pb)
Codons 12 and 13 of Kras	Forward	AGGCCTGCTGAAAATGACTGAA	85
	Reverse	TTAGCTGTATCGTCAAGGCACTCT	
Codons 59 and 61 of Kras	Forward	AATTGATGGAGAAACCTGTCTCTT	86
	Reverse	TCCTCATGTACTGGTCCCTCATT	
Codon 117 of Kras	Forward	CTGAAGATGTACCTATGGTCCTAG	77
	Reverse	CTGAGCCTGTTTTGTGTCTACTG	
Codon 146 of Kras	Forward	GGCTCAGGACTTAGCAAGAAGTTA	114
	Reverse	AGTTATGATTTTGCAGAAAACAGA	
Codons 12 and 13 of Nras	Forward	CTTGCTGGTGTGAAATGACTGAG	79
	Reverse	TGGATTGTCAGTGCGCTTTT	
Codons 59 and 61 of Nras	Forward	AAACCTGTTTGTTGGACATACTG	67
	Reverse	TATTGGTCTCTCATGGCACTGT	
Codon 117 of Nras	Forward	ATGATGTACCTATGGTGCTAGTGG	93
	Reverse	CGTAACTCTTGGCCAGTTCG	
Codon 146 of Nras	Forward	CGAACTGGCCAAGAGTTACG	75
	Reverse	TGAAAGCTGTACCATACCTGTCTG	
Codon 600 of Braf	Forward	ATGCTTGCTCTGATAGGAA	228
	Reverse	GCATCTCAGGGCCAAA	

shown in Table 5. There was a significant association between EGFR cytoplasmic overexpression and advanced stage N1 [$P = 0.04$, Table 6].

Genetic alterations

In all 89 specimens, oncogenes mutations were present in 52 samples (58.4%) including 51 (57.3%) Kras mutated and only 1 (1.1%) Braf mutated. Figure 3 and Table 7 show, respectively, pyrograms of the most frequent mutation G12D compared with wild type and mutation

Table 2: PCR thermal programs

PCR	Thermal program
Kras 12–13	95°C×15 min; (95°C×30 s,
Kras 117	60°C×30 s, 72°C×30 s)×40 cycles;
Kras 146	72°C×2 min
Kras 59–61	95°C×15 min; (95°C×30 s,
Nras 12–13	60°C×30 s, 72°C×30 s)×50 cycles;
Nras 59–61	72°C×2 min
Nras 117	
Nras 146	
Braf 600	95°C×10 min; (95°C×30 s,
	54°C×30 s, 72°C×45 s)×35 cycles;
	72°C×2 min

PCR: Polymerase Chain Reaction

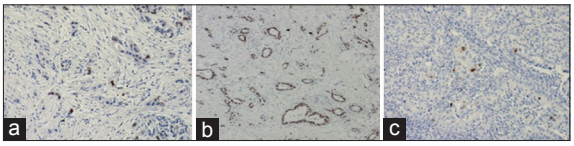

Figure 1: Typical immunohistochemical labeling profiles of p53 in pancreatic tissues. (A) PDAC showing a normal pattern of p53 immunohistochemical labeling. Scattered cells in the neoplastic glands with positive nuclear labeling are present. (B) PDAC with diffusely positive nuclear labeling for p53 (overexpression). (C) PDAC with loss of nuclear labeling (underexpression)

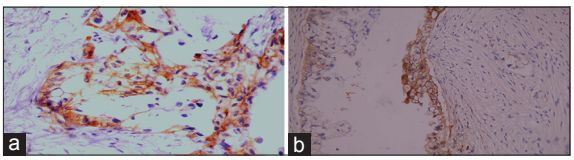

Figure 2: Typical immunohistochemicallabeling profiles of EGFR in pancreatic tissues. (A) Cytoplasmic EGFR overexpression score 2+: strong staining observed in plus than 10% of the tumor cells. (B) Membranous EGFR overexpression score 3+:robust and strong staining observed on the entire membrane in plus than 10% of neoplastic cells

frequencies with proportions of its various subtypes in our population. Most frequent mutations were G12D (46%), G12A (23%), and G12V (18%). There were no mutations in the codons 13, 59, 117, and 146 of Kras and 12, 13, 59, 61, 117, and 146 of Nras. There was a statistically significant progressive increase ($P < 0.001$) in the percentage of cases through normal pancreas (8%), PanIN1A (28.6%), PanIN1B (33.3%), PanIN2 (60%), and PanIN3 (85.7%) to PDAC (94.8%) [Figure 4].

DISCUSSION

Thanks to the development in molecular biology, there has been understanding progress in genetic cancer aspects, and it is recognized now that pancreatic carcinoma is a genetic disease produced by progressive mutations in cancer-related genes.[1] In 2008, 20,661 protein-coding genes have been analyzed in 24 PDAC; an average of 63 genetic alterations dominated by point mutations was found.[20] Furthermore, tissue samples from a patient diagnosed with liver metastatic PDAC were analyzed; 12 genes with higher allele frequencies of functional mutations were found including Kras and TP53.[21] Thus, we sought to explore molecular profile of pancreatic cancer using pyrosequencing of Kras, Nras, and Braf and immunohistochemical labeling of p53 and EGFR.

Transcription factor p53 regulates an essential growth checkpoint that protects against genomic rearrangement, mutations accumulation, and suppresses cellular transformation.[22] Its role in pancreatic carcinogenesis is well established.[16] In our study, p53 abnormal immunolabeling was detected in 97.4% of cases including 74.3% underexpression and 23% overexpression. Our frequencies were slightly higher than those of literature with p53 abnormal expression in 31–81% of PDAC.[2,4,15,23-29] Other studies have shown significative and progressive increment in p53 expression with lesion grade, suggesting that TP53 mutation is being an early event in pancreatic carcinogenesis.[10,25] Some IHC studies confirmed that patients with p53 overexpressed tumors had a poorer outcome[26,27] but others not.[23,28]

Gene amplification, overexpression, and activating mutations of EGFR have been reported in various

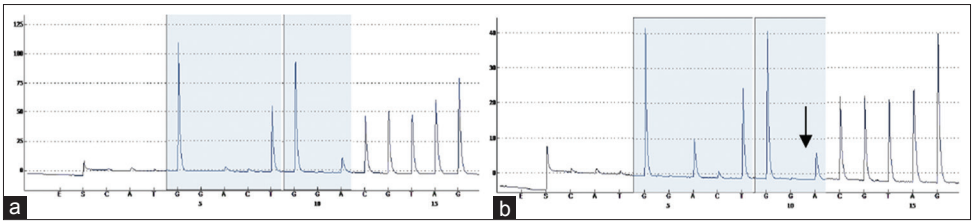

Fig 3: Pyrograms of Kras codons 12 and 13 in pancreatic DNA. (A) Wild-type GGT (Glycin). (B) c.35G>A - p.G12D (Aspartat)

Table 3: Sequencing primers (5'->3'), sequences to analyze and dispensation orders

Kras 12-13	Sequencing primer	TGTGGTAGTTGGAGCT
	Sequence to analyze	GNTGRCGTAGGCAA
	Dispensation order	CATGGACTGGACGTAG
Kras 59–61	Sequencing primer	TCTCTTGGATATTCTCGAC
	Sequence to analyze	AC/TAG/TCAGGA/TCAA/C/ TGAGGAGTACAGTGCA
	Dispensation order	GACAGCAGGTACGATCAGAGGAGTA
Kras 117	Sequencing primer	ACCTATGGTCCTAGTAGGAA
	Sequence to analyze	ATAAHTGTGATTTG
	Dispensation order	CAGTACTGTG
Kras 146	Sequencing primer	GAATTCCTTTTATTGAAAC
	Sequence to analyze	ATCAG/A/CC/TAAAGACAA
	Dispensation order	CATGCAGCTAGA
Nras 12–13	Sequencing primer	TGGTGGTGGTTGGAG
	Sequence to analyze	CAG/T/AGTG/CGTGTTGGGAAAAG
	Dispensation order	TCGATAGACTCGATGT
Nras 59–61	Sequencing primer	TTGTTGGACATACTGGAT
	Sequence to analyze	ACAGCTGGACA/T/GAG/CAGA
	Dispensation order	CACAGCTGAGCGTACGAG
Nras 117	Sequencing primer	TCCTTGTTGGCAAATC
	Sequence to analyze	ACACNTGTTTCCCACTAG
	Dispensation order	CACGACAGTGTC
Nras 146	Sequencing primer	TCCATTCATTGAAACCT
	Sequence to analyze	TCARCCAAGACCAG
	Dispensation order	CTGCAGCAG
Braf 600	Sequencing primer	GGTGATTTTGGTCTAGCTAC
	Sequence to analyze	AGWGAAATCT
	Dispensation order	GACGTAGAT

Table 4: Correlation between different clinicopathological parameters

Clinicopathological parameters	T stage		*P*	N stage		*P*
	T1+T2	T3+T4		N0	N1	
Age						
<58	7	11	1.00	8	10	0.08
>58	8	13		4	17	
Sex						
Male	6	13	0.38	5	14	0.55
Female	9	11		7	13	
Size *N* (mean)	15 (3.04)	24 (3.86)	0.56	12 (3.16)	27 (4.4)	0.03
Differentiation (#)						
Well #	2	1	0.69	1	2	0.87
Moderately #	6	11		6	11	
Poorly #	7	12		5	14	
Perineural invasion						
Present	12	21	0.65	7	26	0.001
Absent	3	3		5	1	
Vascular emboli						
Present	7	19	0.03	6	20	0.14
Absent	8	5		6	7	
Resection margins						
R0	14	19	0.37	9	24	0.34
R1	1	5		3	3	

human cancers including PDAC.[7] Others have corroborated that not only membranous overexpression but also cytoplasmic overexpression of EGFR is important for the acquisition of highly aggressive and metastatic properties of PDAC.[3] Thus, we had chosen to evaluate both cytoplasmic

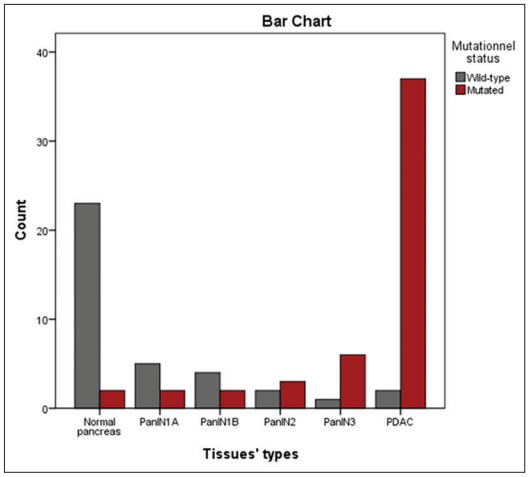

Figure 4: Progressive increment in mutations frequencies with tissues types

Table 5: EGFR immunohistochemical labeling

Membranous EGFR immunolabeling	Score 1+	6, 15.4%
	Score 2+	5, 12.8%
	Score 3+	3, 7.7%
Cytoplasmic EGFR immunolabeling	score 1+	8, 20.5%
	score 2+	20, 51.3%

EGFR: Epidermal growth factor receptor

and membranous expression of EGFR. EGFR expression was abnormal in 71.8% of PDAC with a cytoplasmic overexpression in 51.3% of cases and a membranous overexpression in 20.5% cases, consistent with other studies which have shown EGFR overexpression in 23–95% of PDAC.[3-5,24,30-33] EGFR overexpression was associated with tumor differentiation, size, perineural infiltration, and lymph node metastasis.[4] In our series, there was a significant correlation between cytoplasmic EGFR expression and advanced stage N1. A significant association between membranous EGFR overexpression and lymph node positivity, between cytoplasmic EGFR overexpression and positive margins,[5] and between membranous or cytoplasmic EGFR overexpression and histological grade were found.[33] EGFR expression was not associated with poor survival[31] while it has a significant negative association with survival in other studies.[5,33]

PDAC is also known to have the highest Kras mutation rate among all tumors.[34] The majority (94.8%) of our PDAC was Kras mutated. Mutations arose in 87.2% of cases in codon 12; G12D was detected in 46% of cases consisting with literature where mutations frequency ranges from 47–100%,[23,27,28,35-48] arising in the majority of cases in codon 12 with predominance of G12D mutation.[23,37,39,41-43,45,48] We found G12A in 23% of PDAC, unlike all other studies which detected G12V as the second most frequent mutation. [23,39,41-43,45,48,49] This difference might be attributed to the ethnic variation, environment factors, or geographical differences.

In our series, there was no mutation in codon 13 consisting with some studies.[37,42,43,45,50] Other teams have found mutations in codon 13 with rare frequencies ranging from 0.4% to 2.4%.[23,41,51] We have shown that codon 61 mutations were rare (4%) consisting with other studies which detected it in 0.7–2% of cases.[39,41,42] In addition, no mutations in Kras codon 146 were detected coherent with others.[44,52] No mutations in codon 59 or 117 of Kras were detected, events to the best of our knowledge, not studied in pancreatic cancer yet. Nras (codons 12, 13, 54, 61, 117, and 146) mutations were not present confirming literature data.[44,53,54] We have found only one sample Braf mutated. No Braf mutations were detected in three studies;[43-45] while, two others have found Braf mutations in 2.8% and 16% of PDAC.[35,46]

Moreover, Kras mutations are proven to be an early genetic event in pancreatic carcinogenesis as they are often seen in PanIN, increasing in frequency with cellular and architectural atypia, and even in pancreatic tissues with normal histological appearance. Eight percent of our normal pancreatic tissues harbored mutations in the codon 12 of Kras, event that was seen previously in 17–38% in two studies.[38,55] Furthermore, we have found a statistically significant increment ($P < 0.001$) in mutations frequency from normal pancreas (8%), PanIN1A (28.6%), PanIN1B (33.3%), PanIN2 (60%), and PanIN3 (85.7%) to PDAC (94.8%) confirming previous research.[17,22,52]

Many groups have investigated the influence of Kras mutations on PDAC prognosis. Significant negative correlation between Kras codon 12 mutations and survival time was proven.[23,39,41,42,47,49] G12D seems to be significantly associated with a poor prognosis. [23,45] Other studies showed that Kras mutations do not statistically influence survival.[27,37,43-46] In addition, 100% of our PDAC with R1 resection margins were Kras mutated. The presence of Kras mutations in resection margins could affect significantly the survival, suggesting that molecular evaluation of surgical margins should be considered to better define negative surgical margins.[56] In conclusion, we confirm that EGFR, Kras, and TP53 are the more frequent altered biomarkers in PDAC with slight difference in predominated Kras mutations subtypes. Obtained in a small series of Tunisian patients; these findings need to be confirmed in larger future studies with analysis of more prognostic factors and more detailed cancer-related genes analysis.

Table 6: Biomarkers alterations and clinicopathological parameters

The clinicopathological parameters	p53 expression				Cytoplasmic EGFR expression			Membranous EGFR expression			Kras		
	Normal	Over	Under	*P*	Normal	Over	*P*	Normal	Over	*P*	Wild type	Mutated	*P*
Sex													
Male	1	4	14	0.17	10	9	0.63	16	3	0.69	1	18	1.00
Female	0	9	11		9	11		15	5		2	18	
Age													
<58	1	6	11	0.85	6	12	0.07	14	4	1.00	2	16	0.58
>58	0	7	14		13	8		17	4		1	20	
Localization													
Head	0	12	17	0.04	15	14	0.71	21	8	0.08	3	26	0.55
Others	1	1	8		4	6		10	0		0	10	
Tumor size	1 (3.00)	13 (3.19)	25 (3.75)	0.62	19 (3.8)	20 (3.3)	0.38	31 (3.6)	8 (3.1)	0.42	3 (3)	36 (3.6)	0.57
Differentiation (#)													
Well#	0	1	2	0.77	2	1	1.00	1	2	0.80	0	3	1.00
Moderately#	0	7	10		8	9		13	4		1	16	
Poorly#	1	5	13		9	10		17	2		2	17	
Perineural invasion													
Present	1	11	21	1.00	15	4	0.40	26	7	1.00	2	31	0.40
Absent	0	2	4		18	2		5	1		1	5	
Vascular emboli													
Present	0	8	18	0.33	14	12	0.36	22	4	0.40	1	25	0.25
Absent	1	5	7		5	8		9	4		2	11	
Resection margins													
R0	1	12	20	0.69	16	17	1.00	28	5	0.08	3	30	1.00
R1	0	1	5		3	3		3	3		0	6	
T stage													
T1 + T2	1	4	10	0.46	6	9	0.38	12	3	1.00	1	14	1.00
T3 + T4	0	9	15		13	11		19	5		2	22	
N stage													
N0	0	3	9	0.64	9	3	0.04	8	4	0.22	1	11	1.00
N1	1	10	16		10	17		23	4		2	25	

EGFR: Epidermal growth factor receptor

Table 7: Mutations frequencies in different tissues

Tissues	Normal pancreas (*N*=25)	PanIN (*N*=25)	PanIN1A (*N*=7)	PanIN1B (*N*=6)	PanIN2 (*N*=5)	PanIN3 (*N*=7)	PDAC (*N*=39)	Total (*N*=89)
Kras, G12D	2; 8%	5; 20%	-	1; 16.6%	2; 40%	2; 28.5%	18; 46%	25; 28%
Kras, G12A	-	5; 20%	1; 14.3%	1; 16.6%	1; 20%	2; 28.5%	9; 23%	14; 15.7%
Kras, G12V	-	1; 4%	-	-	-	1; 14.3%	7; 18%	8; 8.9%
Kras, Q61L	-	2; 8%	1; 14.3%	-	-	1; 14.3%	1; 2.5%	3; 3.3%
Kras, Q61H	-	-	-	-	-	-	1; 2.5%	1; 1.1%
Kras, total	2; 8%	13; 52%	2; 28.6%	2; 33.3%	3; 60%	6; 85.7%	36; 92.3%	51; 57.3%
Braf, V600E	-	-	-	-	-	-	1; 2.5%	1; 1.1%
Total	2, 8%	13; 52%	2; 28.6%	2; 33.3%	3; 60%	6; 85.7%	37; 94.8%	52; 58.4%

PanIN: Pancreatic intraductal neoplasia, PDAC: Pancreatic ductal adenocarcinoma

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