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SURVIVAL IN AMPULLARY CANCER: POTENTIAL ROLE OF DIFFERENT KRAS MUTATIONS

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Abstract

Objective—The prognosis of ampullary adenocarcinoma (AA) is usually favorable; however, a subset of AA have poor biology and outcomes similar to pancreatic cancer. Patients in this subset will have early recurrence and death usually within two years. To date there are no genetic markers to identify these patients. This study identifies the high-risk subset of AA, and evaluates the mutational status of KRAS in predicting poor outcome.

Methods—The tumor registry of an academic center was reviewed for data on surgically managed patients with AA. KRAS genotypes were determined for these patients using a PCR-based assay on clinical specimens. ANOVA and χ^2 tests were used to categorize continuous and categorical variables. Univariate and multivariate survival analyses were performed using Kaplan-Meier (KM) and Cox methods, respectively.

Results—146 patients were identified with AA between 1982 and 2008. After stringent pathologic review, 97 patients were confirmed with AA of which 75 had available tissue specimens for analysis. Genotyping revealed, 67% were wild - type (KRAS^{WT}) and 33% were mutant for KRAS. Patients with KRAS^{G12D}, the most common mutational genotype, had significantly poorer median survival (62 months, mo) compared to KRAS^{non-G12D} mutants (median survival not reached, mean 145 mo) and KRAS^{WT} patients (155 mo), $p = 0.05$. Patients with survival ≤ 30 mo were labeled 'high-risk'. Of patients with KRAS^{G12D}, 55.6% were in this high-risk subset, compared to only 18% of KRAS^{WT} ($p = 0.02$) and 31.3% of KRAS^{non-G12D} ($p >$

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0.05) populations. Patients with KRAS^{G12D} were also more likely to present with advanced-T stage.

Conclusions—The KRAS^{G12D} mutation identifies a subset of AA patients with poor prognoses and, may be used to identify patients at risk of early recurrence and poorer survival, who may benefit from adjuvant therapy.

Introduction

Ampullary adenocarcinoma is the second most common periampullary tumor, representing 0.5% of all GI malignancies. Compared to biliary and pancreatic cancers, ampullary cancer has significantly better biologic behavior and outcome, with five-year survivals ranging between 37% and 68% (1–6). However, a subset of patients with ampullary cancer will die much sooner. This subset behaves more like pancreatic cancer, with early recurrence and significantly poorer five-year survival. To date, this high-risk subset has not been clearly defined. Previously published literature focused on pathologic features that correlated with poor prognosis, such as advanced stage (2) (7), lymph node involvement (8), pancreatobiliary morphology (2), and vascular invasion (9); however, these parameters are unable to accurately identify this high-risk subset.

Mutations in KRAS are an early event in carcinogenesis (10) and are found in 30% to 40% of patients with ampullary adenocarcinoma (11–13). However, the correlation between a mutation in KRAS and outcome is not well understood. Analyses with non-small-cell lung cancer (14–17), colorectal cancer (18,19), pancreatic cancer (20), and ampullary cancer (12) have resulted in highly variable and often contradictory results with regard to the association between a KRAS mutation and poor survival, even within the same type of neoplasm. Therefore, the objectives of the current study are to (1) identify the high-risk subset in patients with ampullary adenocarcinoma, (2) determine the frequency of various KRAS mutations, and (3) evaluate whether the mutational status of KRAS may be used to stratify patients into risk groups based on outcome.

Materials and Methods

The tumor registry of the Massachusetts General Hospital (MGH) was queried for patients who underwent an R0 resection for an ampullary tumor between 1982 and 2008. A manual review of patient medical records was conducted, including operative notes, pathology reports, patient notes, and discharge summaries. From this retrospective review, age at diagnosis, gender, race and ethnicity, presenting symptoms, diagnostic tests employed, and pathologic variables such as tumor stage (AJCC, 7th edition), lymph node, vascular, and peri-neural invasion, and epithelial phenotype were abstracted onto a clinical database. Finally, specific dates of first contact, most definite surgery, last follow-up, and date of death were added.

Pathology slides for the patients in the above database were reviewed by a blinded GI pathologist (MMK) to determine those cases that were true ampullary adenocarcinoma, and a subset of these were further reviewed by a second GI pathologist (RM) who was blinded to the original report. During this histopathological classification, both pathologists were

blinded to the results of the KRAS genotyping analysis. Using stringent guidelines (4), a tumor was included in this study as a primary ampullary adenocarcinoma if its epicenter was located in the ampulla of Vater (the major papilla), and there was involvement of the papilla - Vater mucosa. All tumors were excluded if more than 75% of the tumor was located outside the ampulla. Also excluded were those distal common bile duct tumors that formed a plaque under the papilla and involved the common channel without involving the mucosa of the papilla. These criteria ensured that all neoplasms of the pancreas, biliary tree, and duodenum, or neoplasms with an ambiguous origin, were excluded from this study. Ampullary cancer can be of two distinct histologic subtypes; pancreatobiliary, and intestinal. A third mixed category has also been used. A 10% cut off was used by the GI pathologist in deciding the histological type. If more than 10% of the tumor had one of the histological types then it was classified as that type. In our population of AA, only 4 cases were of the mixed type of epithelium where the tumor cells in excess of 10% for each pathological type, intestinal and pancreatobiliary, were present.

Genotyping of KRAS

The mutational status of KRAS was determined using a peptide nucleic acid (PNA) and PCR-based assay similar to one described previously (21). DNA was extracted from paraffin-embedded samples using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany). The extracted genomic DNA was amplified by PCR using 0.5 U *Taq* DNA Polymerase and PCR buffer (Invitrogen, Carlsbad, CA, USA) with 5 mM dNTPs, 1.5 pM KRAS forward primer (5'-AGGCCTGCTGAAAATGACTG-3', Invitrogen), 1.5 pM KRAS reverse primer (5'-CGTCAAGGCACTCTTGCCTAC-3', Invitrogen), 31.25 mM MgCl₂, 200 pM PNA (H₂N-TACGCCACCACCAGCTCC-CON₂N, BioSynthesis Inc, Dallas, TX, USA), and 75 ng of genomic DNA. After an initial incubation for 10 minutes at 95°C, PCR thermo cycling was performed as follows: 95°C for 60 sec, 70°C for 60 sec, 58°C for 30 sec, and 72°C for 30 sec, for 40 cycles. This was followed by a 10-minute extension at 72°C. PCR products were resolved by 4% agarose gel electrophoresis. PCR products in the presence of the PNA (KRAS^{mutant}) were extracted using the MinElute Gel extraction kit (QIAGEN, Hilden, Germany), cloned into the pGEM®-T Easy vector system (Promega, Fitchburg, WI). Initially three clones each were selected from three tissue specimens in this dataset, and all three clones from a given specimen yielded identical KRAS mutations, subsequently one clone for every patient specimen was sequenced (GENEWIZ, South Plainfield, NJ, USA).

Statistical analysis

The variables in our dataset were categorized as either continuous or categorical. Age of the patient and size of tumor were identified as continuous variables, whereas the presence of symptoms, diagnostic tests, tumor stage, epithelial type, degree of differentiation, perineural invasion, vascular invasion, and lymphatic invasion were categorical. Age of the patient, and tumor size were subdivided in two groups (mean age as cut point; 70 years, and < 70 years, median tumor size as cut point; 20 mm, and < 20 mm). Continuous variables were compared between two groups using the T test of means. When comparing between more than two groups, the ANOVA test and Tukey's test were used. Categorical variables were compared using the χ^2 test or Fisher's exact test as appropriate. A review of

the medical records was used to determine time of recurrence; the institutional Research Patient Data Registry (RPDR) was used to calculate vital status and survival time for every patient. Univariate survival analyses were performed using the Kaplan-Meier method and the log rank test. Variables considered for the univariate analysis included tumor size, tumor stage, lymph node invasion, peri-neural invasion, and epithelial phenotype. Multivariate analyses were performed using the Cox proportional hazards method and hazard ratios (HR) and corresponding 95% confidence intervals (CI) were calculated. Cox modeling was performed in multiple steps with the clinical covariates analyzed first and the KRAS covariate added in successive steps in order to determine the best model fit. The alpha for all statistical tests was set at 0.05, with $p = 0.05$ required for significance and $0.05 < p < 0.09$ for borderline significance. All data were analyzed using SPSS version 15.0 software (SPSS, Inc., Chicago, IL). This study was approved by the institutional review board of the Massachusetts General Hospital.

Results

Patient and tumor characteristics

Between 1982 and 2008, 146 patients underwent an R0 resection for ampullary adenocarcinoma at the Massachusetts General Hospital. There were two deaths in the immediate postoperative period (< 30 days) and these patients were excluded from our study. Review of histopathology slides using the criteria previously outlined reduced this number to 97 cases. Of these, only 75 patients had tissue blocks containing adequate tumor regions and these were included in the final analysis. The median follow up time was 97 months.

The study population revealed (Table 1) a predominance of males in the older age group. Fifty-seven percent of patients were male, with a median age of 69 years at time of diagnosis (range: 34 to 92 years). In accordance with the strategic location of ampullary adenocarcinoma, a vast majority (88.9%) were symptomatic at presentation. Jaundice was the most common symptom (66.7%), followed by abdominal pain (24%), anorexia (13.3%), GI bleeding (5.3%), and malabsorption (4.0%). While the diagnostic work up of these patients frequently included a CT scan (in 62.7%) and ERCP (in 41.3%), a small number of patients also underwent EUS (25.3%) and MR (6.7%).

Consistent with the earlier detection of this disease, our dataset showed that ampullary adenocarcinoma was usually found as a small tumor; median tumor diameter at the time of diagnosis was 20.2 mm. In spite of presenting with a small tumor size, tumors were most commonly found in the AJCC T3 (38.6%) stage, lymph node positivity was noted in 34.6%, and perineural invasion was seen in 24% of the patient population. Ampullary adenocarcinomas have an epithelial morphology that may resemble intestinal, pancreatobiliary or mixed types. In this series, the dominant (72%) epithelial type reported was intestinal; 23% were of the pancreatobiliary type. All patients received a pancreaticoduodenectomy (Whipple). Thirty one percent received adjuvant chemotherapy with either Gemcitabine or 5FU based regimens.

Patients with ampullary adenocarcinoma exhibit two distinct patterns of survival

In our dataset, patients with ampullary adenocarcinoma had a long median survival overall; however, evaluation of the Kaplan-Meier survival curves identified two distinct survival groups. The univariate survival curve demonstrated that the median overall survival for patients was 101 months (Figure 1); however, a subset of patients (25%, 19/75) had recurrence or died within the first 30 months after diagnosis (Population A, Figure 1). This caused an inflection point in rate of death (hazard) at 30 months, as seen on the Kaplan-Meier curve. After this time, the overall risk of death was significantly lower (Population B, Figure 1). In Population B, the percentage of patients reaching three-year survival was not significantly different from the percentage reaching five-year survival (overall 3-year survival was 70% and 5-year survival was 66%), indicating that patients who survived the initial period of high mortality (up to 30 months) were likely to survive the intervening period and attain long-term survival. Univariate survival analysis (Table 2) also demonstrated that advanced overall stage (IIB – III), T stage (T3 and T4), lymph node positivity, perineural invasion, vascular invasion, pancreatobiliary epithelial morphology, and larger tumors (≥ 20 mm) were associated with poorer survival and that a markedly higher percentage of patients in Population A were associated with the above poor prognostic factors.

Proper pathologic characterization is crucial for this present study; and a second independent pathologic analysis was performed in order to exclude the possibility of pancreatic and biliary tumors in the “poor prognostic” group. To ensure that all of our “poor prognosis” patients were properly categorized, these cases ($n = 19$) were re-reviewed by a second GI pathologist (RM) who was blinded to both the original pathology report and the results of the genotyping. This second independent review confirmed the results of the original pathological classification as true ampullary cancers.

The KRAS^{G12D} genotype predicts greater likelihood of early recurrence and shorter survival

Using a peptide nucleic acid-based PCR assay, our patient population was evaluated for the presence of KRAS mutations on codons 12 and 13. This genotyping revealed 25 patients (33%) with KRAS mutations. Kaplan-Meier univariate survival analysis demonstrated that there was no significant difference in median overall survival ($p = 0.407$) between patients with mutant and wild-type (WT) KRAS (Figure 2a). Although the presence of mutations in KRAS did not correlate with survival, sequence analysis of the mutant KRAS population revealed that patients with a specific KRAS genotype exhibited a poorer overall survival. The KRAS mutant population comprised a variety of mutations in codons 12 and 13 (Figure 2b). The most common mutation was an aspartate (D) for glycine (G) substitution at codon 12 (KRAS^{G12D}), accounting for 36% ($n = 9$) of the total mutant KRAS population. Patients were grouped into three independent subsets, KRAS^{G12D}, KRAS^{non-G12D} ($n = 16$), and KRAS^{WT} ($n = 50$), for subsequent statistical analysis. Univariate survival analysis (Figure 2c) revealed that there was no difference in survival between the KRAS^{non-G12D} and KRAS^{WT} sub-populations ($p = 0.248$). The median survival for KRAS^{WT} was 155 months, while KRAS^{non-G12D} did not reach median survival; their mean survival was 145 months.

However, patients with KRAS^{G12D} were associated with significantly poorer overall survival. Median survival for KRAS^{G12D} was 62 months (^{G12D} vs. ^{WT} $p = 0.05$).

Patients with KRAS^{G12D} have poorer survival (Figure 2c) and, as evidenced from the shape of the survival curves, this difference in median overall survival was due to the early mortality of the patients that were KRAS^{G12D} (being at or before 30 months). Of patients with KRAS^{G12D}, 55.6% presented within Population A ($p = 0.02$) compared to 31.3% for KRAS^{non-G12D} and 18% for KRAS^{WT} (Figure 2d). Therefore, survival analysis revealed that a mutation in KRAS did not represent a homogeneous patient population; rather, a specific genotype, KRAS^{G12D}, conferred significantly higher overall mortality and resulted in earlier recurrence of disease. Interestingly, patients with mutations other than the KRAS^{G12D} did not appear to be any different from those that were KRAS^{WT}.

Multivariate survival analysis

The Cox multivariate proportional hazards analysis (Table 3) demonstrated that advanced tumor stage (IIB – IIIB) was associated with worse survival when adjusted for factors such as tumor stage, peri-neural invasion, vascular invasion, and epithelial type. Advanced tumor stage (IIB – IIIB) was associated with a 176% elevated risk of death, with a hazard ratio of 2.76 (95% confidence interval, 1.13 – 5.83), $p = 0.04$. When adjusted for other clinical covariates, patients with KRAS^{G12D} were associated with a 96% higher risk of death than patients that were KRAS^{WT}. The hazard ratio for KRAS^{G12D} was 1.96 (95% confidence interval, 0.93 – 6.59), with $p = 0.07$. The KRAS^{non-G12D} sub-population did not predict a worse survival compared with the KRAS^{WT} patients, $p = 0.416$.

Patients with the KRAS^{G12D} mutation present with advanced T stage

Analysis of the patient population in subgroups based on KRAS mutational status (Table 4) revealed that patients with the KRAS^{G12D} mutation were associated with more advanced clinical and pathologic characteristics. Among the KRAS^{G12D} patients, a significantly higher percentage had advanced T stage: 55.6% were T4, compared with 12.5% among the KRAS^{non-G12D} and 8% among the KRAS^{WT}. Additionally, patients with KRAS^{G12D} showed a trend towards associating with pathologic factors such as peri-neural invasion, larger tumor size, and pancreatobiliary morphology compared to the patients with KRAS^{WT}.

The median number of lymph nodes in this dataset was 13. However, there was no correlation of the lymph node status with KRAS status (table 4). Among patients with the KRAS^{G12D}, 33% were lymph node positive compared to 44% of the KRAS^{non-G12D} and 32% of the KRAS^{Wild-Type}, $p > 0.05$. Furthermore, it was advanced overall stage, rather than lymph node involvement, and lymph node ratios that were associated with significantly poorer overall survival.

Discussion

Between 80% and 90% of patients with ampullary adenocarcinoma are resectable at diagnosis (7), and a majority of patients who undergo a pancreaticoduodenectomy with negative lymph nodes will reach long-term survival and be deemed cured (22). In this population of pathologically confirmed cases of ampullary adenocarcinoma, the median

overall survival was greater than 100 months. In the context of this generally favorable outcome, patients with ampullary adenocarcinoma have a unique pattern of mortality based on the Kaplan-Meier survival curve and fall into two distinct prognostic groups, good and poor. While most patients fall into the 'good prognosis' category, one in four patients will have a 'poor prognosis', which can be defined by our survival analysis as recurrence or death prior to 30 months. Patients with a KRAS^{G12D} genotype were more likely to fall into this poor prognosis group associated with poor survival, suggesting that differential KRAS activation variably affects the biology and behavior of this cancer.

Previous attempts to identify poor prognostic features in AA have resulted in inconsistent and often contradictory results (4,6,7,23,24). Studies have identified histological grade, histological type, presence of coexisting adenoma, perineural invasion, and overall stage as strong prognostic factors (6, 23). Others have variously identified tumor budding, pancreatobiliary epithelial subtype (4), and lymph node invasion (25). Given the relative rarity of this disease and the resulting small numbers of patients in most institutional datasets, it is not surprising that different studies are unable to agree on the relevant predictors of survival. Although stage is one of the most consistently mentioned predictors of survival, the sensitivity and specificity of tumor stage in classifying patients into good and poor prognosis groups in our dataset is 75% and 70% respectively. This suggests that classification based on stage does not accurately prognosticate patients with ampullary cancer into good and poor outcome groups, and that molecular and genetic factors may play a deeper role in determining outcome.

Of the patients in this dataset, 33% had a mutation in the KRAS gene. This figure, along with the percentage of patients with the KRAS^{G12D} genotype, is similar to what has been previously described (26), (27), (12); however, this study adds to the literature published so far by deriving a specific cut-point that identifies a high-risk subset of patients. This analysis, then, not only compares overall survival between the subgroups of KRAS genotypes, but also correlates the presence of a KRAS^{G12D} genotype with this high-risk population.

Survival analyses of patients with other GI and non-GI malignancies have demonstrated a similar association between KRAS mutational status and survival. A recent study demonstrated a negative association between the presence of a KRAS mutation and survival for ampullary adenocarcinoma (28) and pancreatic adenocarcinoma (29). Studies in colorectal carcinoma generally indicate that a mutation in KRAS predicts a poorer outcome. Cerottini et al. (18) evaluated KRAS as a prognostic factor in a dataset of 98 patients with colorectal cancer. The authors determined that patients with a KRAS mutation had a poorer outcome; specifically, codon 12 substitution of glycine to arginine, and codon 13 substitution of glycine to aspartate were both associated with poorer survival. In another study of patients with colorectal cancer (30), aspartate and serine mutations on codon 12 had a negative impact on survival.

KRAS has also been shown to predict survival in non-small-cell lung cancer. As with colon cancer, a study by Nelson and colleagues demonstrated an association between a KRAS mutation and lower survival, and that this association was limited to patients with stage I

disease (16). However, different studies draw slightly different conclusions concerning the specific mutational genotype of KRAS associated with poorer survival. Retrospective studies have identified valine (14), arginine (14,17) aspartate (15,17), serine (15), and cystine (17) mutations on codon 12 as those which predict poor overall survival compared with other KRAS mutant and wild-type KRAS populations. In one study on patients with advanced staged lung adenocarcinoma, however, the presence of a valine mutation on codon 12 was not associated worse outcome. (31) Non-small cell lung cancer comprises a heterogeneous group of tumors that vary widely in outcome and tumor biology; this may account for the varying results that have been reported.

An interesting observation in the studies discussed above is that overall mutant KRAS status (compared to wild-type) correlated with poorer survival only in some studies (15,18). On the other hand, the correlation of a specific genotype of KRAS with survival was far more consistent. The presence of a higher proportion of patients with the specific genotype associated with poorer outcome likely governs whether the overall mutational status of KRAS is associated with decreased patient survival. This observation supports an emphasis on genotyping these neoplasms as opposed to a limited analysis of mutant or wild type KRAS. In this paper, the differences in survival are statistically significant but we acknowledge that the number of observations is small. This study would benefit from a larger sample size. Additional numbers of patients may be required before the association of KRAS and poor survival can be better demonstrated in a multivariate model of survival. Nevertheless, the common theme underlying the above studies and our conclusions is that different mutational genotypes of KRAS correlate with very different biological behaviors and outcome, some performing very poorly while others show no difference compared to their wild-type counterparts.

The presence of a genetic marker of poor outcome such as KRAS^{G12D} may allow us to stratify patients with AA more appropriately into different risk groups, predicting which patients may benefit from adjuvant treatment. Currently, patients with advanced clinical features are more likely to be treated with adjuvant chemotherapy (5,32,33). In this dataset, among the high-risk patients that were lymph-node-negative or those with early stage, two were positive for KRAS^{G12D}. The identification of a unique subset of patients based on a biomarker may help to shift the clinical practice paradigm and enable us to determine accurately, criteria beyond clinical parameters for inclusion of patients into adjuvant treatment regimens.

The success of different chemotherapies may also be dependent on the identification of a unique genotypic pattern. Recently, the KRAS mutation phenotype has been used to predict resistance to certain chemotherapeutics. In colorectal cancer, specific mutational genotypes of KRAS have been shown to reliably predict which patients will not respond to an EGFR inhibitor (34). In that study, patients with the KRAS^{G13D} genotype of KRAS were associated with better outcome after being treated with Cetuximab. Studies in lung cancer have also shown that patients with mutations in KRAS are less likely to respond to small-molecule EGFR inhibitors such as gefitinib and erlotinib (35,36). However, in personalizing care for individual patients, tumor heterogeneity may confound chemotherapeutic strategies (31). One of the limitations of our study is that we are unable to address this question of

tumor heterogeneity using our samples. Tissue blocks available to us contained limited areas with high tumor cellularity and only a single area of each tumor was chosen for analysis. Nevertheless, it appears that a specific mutation may be important in the biology of a cancer, but its effect on chemotherapy and resistance remains to be determined in ampullary cancer.

Conclusion

Patients with ampullary adenocarcinoma represent a heterogeneous population. A survival time of less than 30 months identifies a high-risk population that shares clinical markers of poor outcome. KRAS is mutated in fewer than half of patients with ampullary adenocarcinoma, but not all genotypes of the KRAS mutation portend the same biologic behavior. The presence of the KRAS^{G12D} mutation predicts a greater likelihood of shortened survival and strongly correlates with a high-risk subset, potentially allowing the use of KRAS genotyping to identify patients at risk of early recurrence and death.

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Figure 1.

Kaplan-Meier survival curve for the population in this study showing an inflection rate in the rate of death at 30 months (arrow) allowing us to classify patients into a poor prognosis group (population A) and those that survived the initial period of high mortality to attain longer term survival (population B).

Figure 2.

(A) Kaplan-Meier survival analysis comparing patients with mutant and wild-type KRAS.

(B) Figure showing the various mutational genotypes of KRAS that were observed. (C) KM survival curves of patients with KRAS^{G12D}, KRAS^{non-G12D}, and KRAS^{WT}. (D) Figure showing the proportion of patients among each of the KRAS populations (G12D, non-G12D, WT) that presented with early (< 30 months) recurrence or death.

Table 1**Patient and tumor characteristics (n = 75)**

Mean age at presentation, (Range)	69.3, (34 – 92)
Sex, male, n (%)	43, (57.3)
Symptomatic at diagnosis, yes, n (%)	65 (88.9)
Presenting symptoms, n (%)	
Abdominal pain	18 (24)
Anorexia	10 (13.3)
GI Bleeding	4 (5.3)
Jaundice	50 (66.7)
Malabsorption	3 (4.0)
Diagnostic tests employed	
CT	47 (62.7)
ERCP	31 (41.3)
EUS	19 (25.3)
MR	5 (6.7)
Median tumor diameter, mm, (95% CI)	20.2 (16.4 – 24.1)
T stage, n (%)	
T1	21 (28.0)
T2	14 (18.6)
T3	29 (38.6)
T4	11 (14.6)
Node positive, n (%)	26 (34.6)
Perineural invasion present, n (%)	18 (24)
Epithelial Type, n (%)	
Intestinal	54 (72)
Mixed	4 (5.3)
Pancreatobiliary	17 (22.7)

Table 2

Univariate Kaplan-Meier survival across different pathological factors

	Survival (%)		Median survival (Months)	p
	3-Year	5-Year		
T Stage				
T1 and T2	82	78	-	0.01**
T3 and T4	60	60	68	
N Stage				
N0	78	76	155	0.008**
N1	54	54	31	
Perineural Invasion				
Absent	79	76	155	0.005**
Present	39	39	31	
Vascular Invasion				
Absent	74	72	155	0.06*
Present	46	46	29	
Epithelial type				
Intestinal	74	73	-	0.02**
Pancreatobiliary	56	56	59	
Size of Tumor				
20 mm	82	78.5	-	0.01**
< 20 mm	52.5	52.5	59	

** statistically significant,

* borderline significant

Table 3

Cox multivariate survival analyzing the contribution of KRAS-G12D with respect to AJCC stage of the tumor

Covariate	Reference categories	Hazard Ratio	95% CI	p
Peri-neural invasion	Present vs. absent	1.29	0.49 – 3.39	0.59
Vascular invasion	Present vs. absent	1.64	0.61 – 4.43	0.32
Epithelial type	PB vs. Intestinal	1.02	0.39 – 2.70	0.96
Binned tumor size	Tumor size (≥ 20 mm vs. < 20 mm)	2.11	0.91 – 4.90	0.08*
Stage	IIB – IIIB vs. IA – IIA	2.76	1.13 – 5.83	0.04**
KRAS mutation	KRAS ^{G12D} vs. KRAS ^{WT}	1.96	.933 – 6.59	0.07*

G12D = Asp for Gly substitution on codon 12, WT = wild type, PB = pancreatobiliary,

** statistically significant,

* borderline significant

Table 4
Patient and tumor characteristics between Mutant and Wild Type KRAS patient populations

	KRAS ^{G12D} n = 9	KRAS ^{non-G12D} n = 16	KRAS ^{WT} n = 50	P Value	
				G12 D vs. nonG12 D	G12D vs. WT
Mean age at presentation, (Range)	70 (58 – 92)	66 (34 – 84)	70 (41 – 86)		
Symptomatic at diagnosis, n (%)	9 (100)	16 (100)	31 (81.6)	Constant	0.019**
Median tumor diameter, mm, (95% CI)	25.1 (13.4 – 36.8)	23 (16.9 – 29.1)	18.4 (13.4 – 23.4)	0.677	0.258
T stage, n (%)					
T1	0 (0)	6 (37.5)	15 (30)		
T2	3 (33.3)	1 (6.3)	10 (20)		
T3	1 (11.1)	7 (43.8)	21 (42)	0.011**	0.012**
T4	5 (55.6)	2 (12.5)	4 (8.0)		
Node Status					
Node positive, n (%)	3 (33.3)	7 (43.8)	16 (32)	0.768	0.825
Overall stage, n (%)					
I – IIA	4 (44.4)	9 (56.3)	32 (64)	0.572	0.271
IIB – IIIB	5 (55.6)	7 (43.7)	18 (36)		
Perineural invasion					
Present, n (%)	3 (33.3)	5 (31.3)	10 (20)	0.772	0.312
Vascular invasion					
Present, n (%)	2 (22.2)	3 (18.8)	6 (12)	0.835	0.410
Epithelial Type, n (%)					
Intestinal	5 (55.6)	12 (75)	37 (74)		
Pancreatobiliary	4 (44.4)	3 (18.8)	10 (20)	0.193	0.206
Mixed	0	1 (6.2)	3 (6)		

G12D = Asp for Gly substitution on codon 12, WT = wild type,

*** statistically significant,

* borderline significant