

# Unfavourable prognosis associated with K-ras gene mutation in pancreatic cancer surgical margins

J Kim, H A Reber, S M Dry, D Elashoff, S L Chen, N Umetani, M Kitago, O J Hines, K K Kazanjian, S Hiramatsu, A J Bilchik, S Yong, M Shoup, D S B Hoon



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See end of article for authors' affiliations

Correspondence to:  
Dr D S B Hoon,  
Department of Molecular  
Oncology, John Wayne  
Cancer Institute, 2200  
Santa Monica Blvd, Santa  
Monica, CA 90404, USA;  
hoon@jwci.org

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**Background:** Despite intent to cure surgery with negative resection margins, locoregional recurrence is common in pancreatic cancer.

**Aims:** To determine whether detection of K-ras gene mutation in the histologically negative surgical margins of pancreatic cancer reflects unrecognised disease.

**Patients:** Seventy patients who underwent curative resection for pancreatic ductal adenocarcinoma were evaluated.

**Methods:** All patients had surgical resection margins (pancreatic transection and retroperitoneal) that were histologically free of invasive cancer. DNA was extracted from these paraffin embedded surgical margins and assessed by quantitative real time polymerase chain reaction to detect the K-ras gene mutation at codon 12. Detection of K-ras mutation was correlated with standard clinicopathological factors.

**Results:** K-ras mutation was detected in histologically negative surgical margins of 37 of 70 (53%) patients. A significant difference in overall survival was demonstrated between patients with margins that were K-ras mutation positive compared with negative (median 15 v 55 months, respectively;  $p=0.0008$ ). By univariate and multivariate analyses, detection of K-ras mutation in the margins was a significant prognostic factor for poor survival (hazard ratio (HR) 2.8 (95% confidence interval (CI) 1.5–5.3),  $p=0.0009$ ; and HR 2.8 (95% CI 1.4–5.5),  $p=0.004$ , respectively).

**Conclusions:** Detection of cells harbouring K-ras mutation in histologically negative surgical margins of pancreatic cancer may represent unrecognised disease and correlates with poor disease outcome. The study demonstrates that molecular-genetic evaluation of surgical resection margins can improve pathological staging and prognostic evaluation of patients with pancreatic ductal adenocarcinoma.

Pancreatic ductal adenocarcinoma has one of the worst five year survival rates of any cancer.<sup>1</sup> A contributing factor is frequent locoregional failure after curative resection.<sup>2–5</sup> The high rate of such failures may reflect inadequate removal of all preneoplastic cells or residual tumour (for example, occult cancer cells) despite histologically negative surgical resection margins. During the past decade, increasingly sophisticated molecular techniques have identified genetic, epigenetic, and molecular aberrancies in benign appearing cells in tissues surrounding primary tumours or at the surgical margins.<sup>6–7</sup> These aberrancies have been implicated in locoregional cancer recurrences.<sup>8–12</sup>

Genetic aberrancies may have an insidious role in cancer recurrence in pancreatic cancer because its genotypic features cannot be correctly assessed in surgical margins by conventional histological haematoxylin and eosin (H&E) and light microscopy techniques. Therefore, molecular-genetic assessment of surgical resection margins in pancreatic cancer would have particular clinical relevance. Accordingly, more sophisticated analysis of histologically negative surgical margins might improve pathological staging and prognostic evaluation of patients undergoing surgery for pancreatic cancer.

For this report, we examined the incidence and potential prognostic significance of K-ras gene mutation in the surgical margins of pancreatic cancer. Recently, Hingorani and colleagues directed endogenous expression of K-ras in a mouse model and recapitulated the entire progression of pancreatic cancer, demonstrating the key role of K-ras mutation in pancreatic cancer pathogenesis.<sup>13</sup> Since the

earliest reports of K-ras mutation in pancreatic cancer, innovative methods to utilise the mutation for diagnostic evaluation of blood, bile, or stool have been developed but have had little impact on disease outcome.<sup>14–20</sup> Here we hypothesised that detection of K-ras mutation in histologically negative surgical margins of pancreatic cancer correlates with less favourable clinical outcomes. Our findings indicate that K-ras mutation in surgical resection margins may be a clinically relevant surrogate for unrecognised disease.

## METHODS

### Patients with pancreatic cancer

Twenty three patients who underwent curative resection for pancreatic ductal adenocarcinoma from 1996 to 2004 were initially evaluated as a pilot cohort for this research study. Patient specimens were obtained from the John Wayne Cancer Institute (JWCI, Santa Monica, California, USA) and the David Geffen School of Medicine (UCLA, Los Angeles, California, USA). After analysis of the pilot cohort to ensure the feasibility of detection of K-ras gene mutation in paraffin embedded surgical margin tissues, 47 additional patients who underwent intent to cure surgery for pancreatic cancer were added to form a cohort of 70 patients for the study. Clinicopathological data were analysed for all 70 patients. Specimens from 30 patients, including the longest survivors,

**Abbreviations:** JWCI, John Wayne Cancer Institute; H&E, haematoxylin and eosin; PanIN, pancreatic intraepithelial neoplasia; IHC, immunohistochemistry; PNA, peptide nucleic acid; PCR, polymerase chain reaction; wt, wild-type; HR, hazard ratio

**Table 1** Patient demographics and clinicopathological factors

Clinicopathological factor	Patients (n (%))
Total No of patients	70
Males	35 (50%)
Females	35 (50%)
Age (y)	
Median (range)	67 (42–90)
Surgical procedures	
Pancreaticoduodenectomy	68 (97%)
Distal pancreatectomy	2 (3%)
UICC/TNM stage*	
pT	26 (37%)
pT <sub>1</sub>	44 (63%)
Primary tumour	
T <sub>1</sub>	16 (23%)
T <sub>2</sub>	46 (66%)
T <sub>3</sub>	8 (11%)
Lymph node metastasis	
N <sub>0</sub>	30 (43%)
N <sub>1</sub>	40 (57%)
Pathological grade	
Well	11 (16%)
Moderate	33 (47%)
Poor	26 (37%)
Tumour size (cm)	
0–2	20 (28%)
>2	50 (72%)
Perineural invasion	
Absent	24 (34%)
Present	46 (66%)
Lymphovascular invasion	
Absent	52 (74%)
Present	18 (26%)

\*UICC/TNM 6th edition.

†pT, pT<sub>1</sub>, pathological stage I and pathological stage II.

underwent independent pathologic re-review (by SY) and were reaffirmed to have histology consistent with pancreatic ductal adenocarcinoma. Only patients with an adequate follow up interval (that is,  $\geq 36$  months or until expiration if follow up was  $\leq 36$  months) were selected. Patients were excluded if the final permanent section of the pancreatic transection or retroperitoneal surgical resection margin was histologically positive by H&E (R1 resection; that is, microscopic evidence of invasive ductal adenocarcinoma), if a surgical margin was unavailable for analysis, or if the patient had expired within 30 postoperative days. Therefore, only R0 resections (that is, absence of microscopic invasive cancer cells in the margins) were included. None of the additional 47 patients had these exclusion criteria.

All patients, regardless of stage or lymph node status, were offered adjuvant radiotherapy and chemotherapy with various combinations of 5-fluorouracil, leucovorin, mitomycin C, dipyridamole, and gemcitabine at the discretion of their individual physicians. Institutional Review Board approval was obtained at the respective institutions (JWCI and UCLA) for the purposes of this study. Patient records, including radiographic films (when available), were reviewed. Patient demographics and clinicopathological factors are shown in table 1.

### Pancreatic cancer specimens

Uniform methods to assess and process surgical margins (pancreatic transection and retroperitoneal) were not implemented as standard practice. In the current study, the surgical margins were evaluated and processed by different methods at the two institutions (JWCI and UCLA). At JWCI, pancreatic transection and retroperitoneal margins were submitted as separate tissue specimens by the surgeons and analysed by frozen section analysis. Once analysis was

completed, the frozen sections were paraffin embedded and stored. At UCLA, only pancreatic transection margins were routinely evaluated by frozen section; retroperitoneal margins were evaluated only on final permanent section. Once the retroperitoneal margin was identified (by ink or suture or in relation to the location of the primary tumour), a perpendicular section to include the pancreatic tumour and margin was procured and paraffin embedded as a single retroperitoneal margin block. Each surgical margin was submitted in a separate block. In rare cases, more than one margin was submitted (for example, patients whose original transection margin was positive for invasive carcinoma). In these cases all blocks were clearly submitted and labelled, and only the final negative margin was analysed.

At both institutions, the tissue immediately adjacent to the superior mesenteric artery was generally considered the retroperitoneal margin. For the two patients with distal pancreatectomy, we defined the retroperitoneal margin as the pancreatic tissue radial from the primary tumour extending to the posterior peripancreatic soft tissues. Inclusion criteria for this study required the use of only paraffin embedded surgical margin tissues that were clearly marked and stored as the margins (except for the two patients who underwent distal pancreatectomy).

The archived H&E margin slide sections of the study cohort were reviewed under microscopy (by SMD) to confirm the absence of invasive carcinoma cells in the surgical margins (pancreatic transection and retroperitoneal). These margins were designated as “negative”. However, many surgical margins had evidence of low grade pancreatic intraepithelial neoplasia (PanIN; 1A and 1B) and inflammatory changes; it was generally not reported as a pathological finding in the surgical pathology reports. From available data, high grade PanIN (2 and 3) was identified in 10 patients (table 2). Detection of PanIN-3 was, by definition, not considered “positive” for invasive cancer in the margins; none of the patients underwent additional resection for its clearance. However, two patients did require additional resection for the presence of invasive cancer cells in the pancreatic transection margin. Additional pathological abnormalities were not characterised in the margin specimens. Immunohistochemistry (IHC) was not utilised to re-evaluate the surgical resection margins.

From the paraffin embedded primary tumour and margins, 30  $\mu\text{m}$  sections (10  $\mu\text{m} \times 3$ ) were cut from each block and collected in sterile microcentrifuge tubes (Eppendorf Biopur, Westbury, New York, USA). After procurement of paraffin sections for polymerase chain reaction (PCR) analysis, an additional section (5  $\mu\text{m}$ ) from the block was cut and stained by H&E and examined by a surgical pathologist to further confirm the absence of invasive carcinoma cells in the resection margins. The entire 30  $\mu\text{m}$  of sections cut from the paraffin embedded tissue blocks were first deparaffinised with xylene and then washed with 100% ethanol. DNA was extracted and purified from these paraffin sections using a modified assay (QIAamp DNA Mini Kit, Qiagen Inc., Valencia, California, USA), as previously described.<sup>21–22</sup> DNA was quantified by the PicoGreen assay. Due to the protocol for sampling retroperitoneal margins at UCLA, in some cases carcinoma was present within the pancreas but was not present at the inked retroperitoneal margin. In these cases, paraffin embedded sections (4 $\times$ 5  $\mu\text{m}$ ) were cut, placed on microscope slides, and histologically benign appearing tissue was microdissected with laser capture microdissection (Arcturus, Mountain View, California, USA), as previously described.<sup>23</sup> Due to the logistics of the study, IHC and PCR could not be performed on the same paraffin embedded sections. PCR analysis of all primary tumour and surgical resection margins was performed at JWCI.

**Table 2** Comparison of clinicopathological factors for patients with K-ras mutation negative and positive margins

Clinicopathological factor	K-ras mutation in margin		*p Value
	Negative (n = 33)	Positive (n = 37)	
Age (y)			0.075
Mean (SD)	65 (9)	69 (11)	
Sex			1.0
Female	17 (52%)	18 (49%)	
Male	16 (48%)	19 (51%)	
UICC/TNM stage†			0.33
pI‡	10 (30%)	16 (43%)	
pII‡	23 (70%)	21 (57%)	
Primary tumour			0.17
T <sub>1</sub>	11 (33%)	5 (14%)	
T <sub>2</sub>	19 (58%)	27 (72%)	
T <sub>3</sub>	3 (9%)	5 (14%)	
Lymph node metastasis			0.15
N <sub>0</sub>	11 (33%)	19 (51%)	
N <sub>1</sub>	22 (67%)	18 (49%)	
Tumour size (cm)			0.20
0–2	12 (36%)	8 (22%)	
>2	21 (64%)	29 (78%)	
Pathological grade			0.037
Well	3 (9%)	8 (22%)	
Moderate	21 (64%)	12 (32%)	
Poor	9 (27%)	17 (46%)	
Perineural invasion			0.024
Absent	16 (48%)	8 (22%)	
Present	17 (52%)	29 (78%)	
Lymphovascular invasion			0.027
Absent	29 (88%)	23 (62%)	
Present	4 (12%)	14 (38%)	
PanIN in margin¶			1.0
Absent	1 (7%)	2 (12.5%)	
Present	13 (93%)	14 (87.5%)	
1	9	8	
2–3	4	6	
K-ras mutation in tumour			0.36
Absent	8	25	
Present	5	32	

\*Comparison for age was performed by the Student's *t* test; the remaining clinical factors were compared by Fisher's exact test.

†UICC/TNM 6th edition.

‡pI and pII, pathological stage I and pathological stage II.

¶For patients with available pancreatic intraepithelial neoplasia data.

### K-ras mutation in pancreatic cancer cell lines

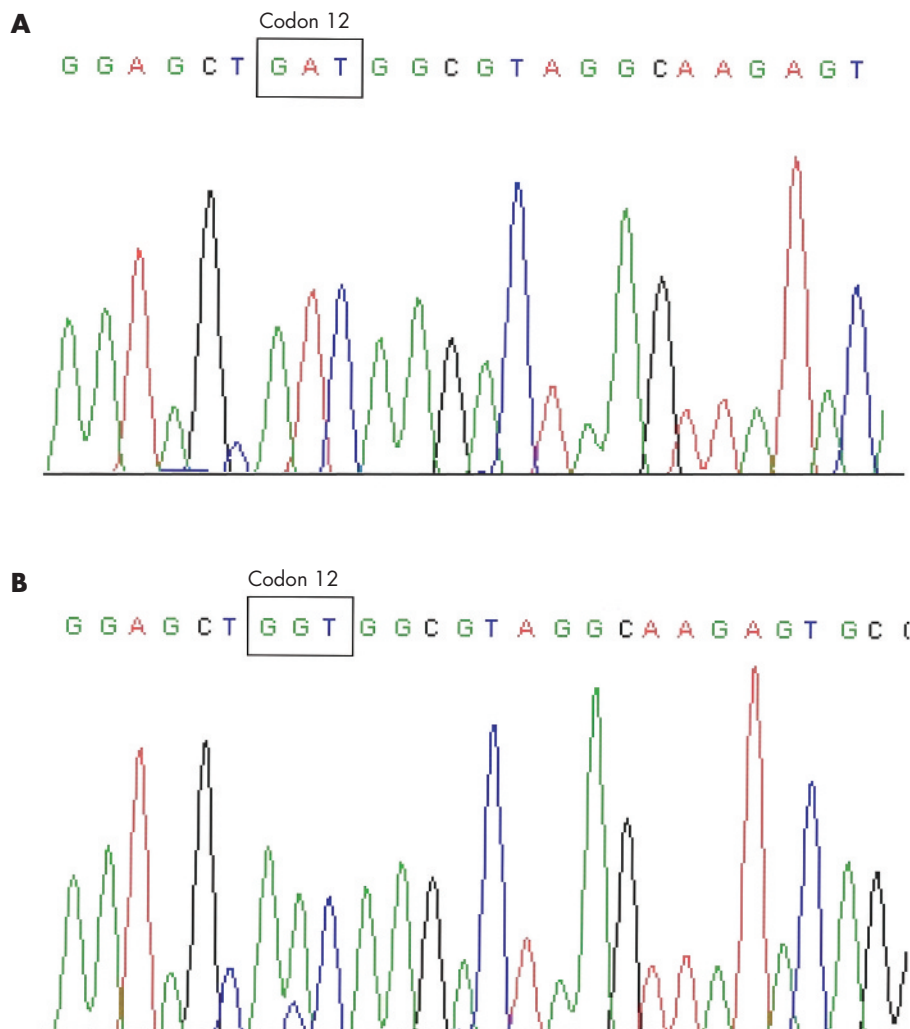
Established pancreatic cancer cell lines (MIA PaCa2, PANC-1, Hs 766T, and B×PC-3) were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and cultured as recommended. MIA PaCa2 and PANC-1 have K-ras gene mutations (GGT→TGT and GGT→GAT, respectively) at codon 12 whereas Hs 766T and B×PC-3 have wild-type (wt) K-ras. These cell lines were used as positive and negative cancer controls for the PCR assay. Mutations in codons 13 and 61 were not assessed because of the overwhelming predominance of codon 12 mutations in pancreatic cancer.<sup>24–25</sup> All cells were incubated at 37°C with 5% CO<sub>2</sub>. DNA from cell lines was extracted, isolated, and purified using DNAzol (Molecular Research Center Inc., Cincinnati, Ohio, USA) and then quantified as previously described.<sup>26</sup>

### Primers and probes and PCR assay

Because of the difficulties in detecting a small number of K-ras mutant copies among thousands of copies of wtK-ras, detection of the K-ras gene mutation was performed using a peptide nucleic acid (PNA) quantitative real time PCR assay which was previously established to specifically detect K-ras mutation at codon 12 in paraffin embedded tissue sections.<sup>27</sup> The PNA clamp, which has higher binding affinity for DNA than PCR primers, was designed for complementary hybridisation to the wtK-ras allele.<sup>28</sup> By hybridising to the wtDNA

template, it inhibits annealing of the partially overlapping reverse primer and thus inhibits the amplification of the wtK-ras. Because the PNA/DNA hybrid is unstable due to base-pair mismatch, it does not anneal to and inhibit the amplification of mutant K-ras. The high sensitivity of the PCR assay to detect micrometastases with K-ras mutation among normal cells bearing the wtK-ras allele has been previously demonstrated.<sup>27</sup> The PCR assay was analysed and expressed as binary (+/–) values.

Quantitative real time PCR was performed using the following primers: K-ras, 5'-CGC TCA CTG CGC TCA ACA C-3' (forward) and 5'-TCA GGC GGC CGC ACA CCT-3' (reverse); FRET probe, 5'-FAM-CAT TCT GTG CCG CTG AGC CG-BHQ-1-3'; PNA, 5'-TAC GCC ACC AGC TCC-3'. The PCR assay was performed with the iCycler iQ RealTime PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). Genomic DNA (20 ng) was amplified in a 20 µl reaction containing 1 µM of each primer, 1.75 µM PNA, 200 µM of each deoxynucleotide triphosphate, 4.0 mM MgCl<sub>2</sub>, 10× AmpliTaq Buffer, and 1 unit *AmpliTaq* Gold Polymerase (Applied Biosystems, Branchburg, New Jersey, USA). Each PCR reaction was subjected to 40 cycles at 94°C for 60 seconds, 70°C for 50 seconds, and 58°C for 50 seconds and 72°C for 60 seconds. PCR was also performed without PNA to amplify wtK-ras and verify DNA integrity. Each sample was assayed in triplicate with positive and negative PCR controls.



**Figure 1** Representative sequencing of pancreatic cancer tissues to validate polymerase chain reaction results. (A) Pancreatic cancer primary tumour with mutant K-ras sequence (GGT→GAT). (B) Pancreatic cancer surgical resection margin specimen with mutant K-ras sequence (GGT→GTT).

### K-ras sequencing

K-ras mutation was assessed initially on specimens using the PNA PCR assay. Representative K-ras mutation positive and negative specimens ( $n = 16$ ) were directly sequenced on both strands to confirm the accuracy of the PNA clamp method. A nested PCR assay was then performed. It was used to amplify K-ras mutation so that sequencing could be performed from the paraffin embedded tissue sections. This assay approach was specifically designed to detect occult cancer cells with K-ras mutation with minimal enrichment of mutant DNA. Then the following K-ras primers were used: 5'-GGT ACT GGT GGA GTA TTT GAT AGT G-3' (forward) and 5'-TGG ATC ATA TTC GTC CAC AAA A-3' (reverse). Each PCR reaction mixture was initially heated to 94°C for 10 minutes and was then subjected to 32–40 cycles at 94°C for 30 seconds, 64°C for 30 seconds, and 72°C for 7 minutes. PCR products were purified with QIAquick PCR Product Purification Kit (Qiagen) and direct sequenced using DTCS Quick Start kit (Beckman Coulter; Fullerton, California, USA) with an annealing temperature of 58°C. Dye terminated products were precipitated by ethanol and separated by capillary array electrophoresis on a CEQ8000XL automated sequencer (Beckman Coulter).

### Statistical analysis

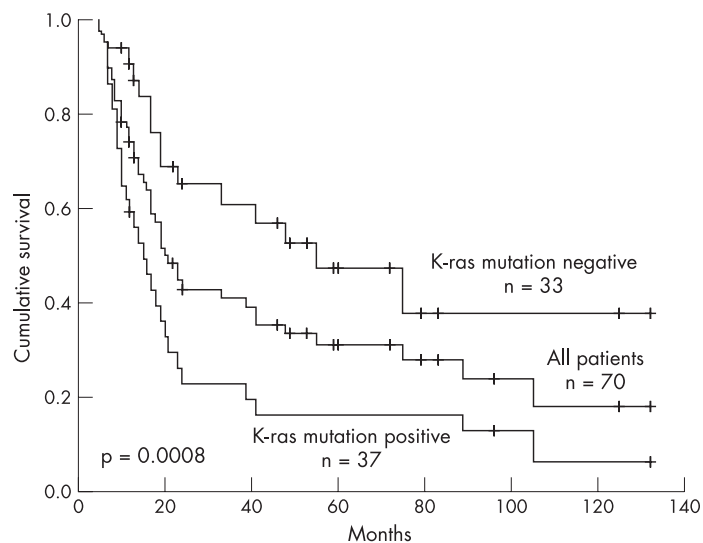
Patient characteristics and detection of K-ras mutation were summarised using mean, median, and frequency.

Clinicopathological factors of patients with positive or negative K-ras mutation were compared by Fisher's exact test and the Student's *t* test. Survival curves with respect to K-ras mutation were constructed using Kaplan-Meier's method. The log rank test was used to compare the equality of the two curves. Univariate analysis of prognostic factors included age, sex, stage, tumour extent, lymph node status, grade, tumour size, perineural invasion, and lymphovascular invasion. The presence of PanIN in the surgical margins was also assessed. The Cox proportional hazard regression model was used to evaluate the prognostic significance of K-ras mutation when clinical prognostic factors were adjusted. A stepwise method was chosen for covariate selection. All analyses were performed using SAS (SAS/STAT User's Guide, version 8; SAS Institute Inc, Cary, North Carolina, USA) and tests were two sided and were considered significant when  $p$  values were  $\leq 0.05$ .

## RESULTS

### Validation of PCR assay

The accuracy and sensitivity of the PNA clamp method for detection of K-ras mutation have been previously established.<sup>27</sup> To validate the accuracy of the PNA quantitative real time PCR assay in our current study, we analysed 16 representative K-ras mutation positive ( $n = 8$ ) and negative ( $n = 8$ ) paraffin embedded pancreatic cancer specimens. Representative sequences of K-ras mutation are presented



**Figure 2** Kaplan-Meier curves comparing overall survival between patients with K-ras mutation positive and negative surgical margins.

Patients at risk

K-ras mutation negative	33	33	19	15	8	3	2	2
K-ras mutation positive	37	37	11	16	5	5	2	1

in fig 1. The wild-type K-ras DNA sequence at codon 12 is GGT.

### K-ras mutation in patients with pancreatic cancer

Twenty three patients with pancreatic cancer were initially analysed as the pilot cohort. K-ras mutation was detected in 19 of 23 (83%) primary tumours and in 11 of 23 (48%) surgical resection margins (pancreatic transection and/or retroperitoneal). The pancreatic transection and retroperitoneal margins were positive for K-ras mutation in four and eight patients, respectively; both margins were positive in one patient. After analysis of this pilot cohort, an additional 47 patients were accrued and assessed. In assessment of all 70 patients, median survival was 21 months at a median follow up of 17 months. The five year overall survival rate was 19%. Patients were treated with pancreaticoduodenectomy ( $n = 68$ ) or distal pancreatectomy ( $n = 2$ ); no patient underwent total resection of the pancreas. One patient had segmental resection of the superior mesenteric-portal vein confluence. At the time of analysis, 45 of 70 (64%) patients had succumbed to disease.

Overall, 57 of 70 (81%) patients had K-ras gene mutation in the primary tumour. The mutation was detected in either margin (pancreatic transection and/or retroperitoneal) in 37 of 70 (53%) patients. K-ras mutation was detected in the pancreatic transection ( $n = 17$ ), retroperitoneal ( $n = 27$ ), or both ( $n = 7$ ) margins. Comparison of patients based on K-ras mutation status of the surgical margins revealed a higher rate of perineural invasion, lymphovascular invasion, and poorly differentiated tumours, when K-ras mutation was detected in the margin. There was no significant difference in the presence of K-ras mutation in the margin by TNM classification, tumour size, age, sex, or presence of PanIN (table 2). Kaplan-Meier curves showed a significant difference in overall survival for patients with K-ras mutation positive versus negative surgical margins (median 15 v 55 months, respectively; log rank,  $p = 0.0008$ ) (fig 2).

By univariate analysis, K-ras mutation in the surgical margins, grade, and perineural invasion were significant factors for disease outcome (table 3). When clinicopathological factors were adjusted, multivariate analysis identified K-ras mutation in the surgical margins as a significant

prognostic factor for poor survival (hazard ratio (HR) 2.8 (95% confidence interval (CI) 1.4–5.5);  $p = 0.004$ ) (table 3). Tumour grade and perineural invasion were also significant for poor survival (HR 6.7 (95% CI 2.4–18.5),  $p = 0.0001$ ; and HR 2.1 (95% CI 1.0–4.2),  $p = 0.04$ , respectively).

### DISCUSSION

The value of obtaining histologically negative surgical resection margins by H&E has been established for pancreatic cancer.<sup>29,30</sup> Neoptolemos *et al* identified a six month survival advantage for patients with negative compared with positive surgical margins.<sup>29</sup> Our study delineates further this subgroup of patients with H&E negative surgical margins into those with PCR negative and positive surgical margins. Using a quantitative PNA PCR approach, we detected K-ras mutation in the surgical resection margins of 37 of 70 (53%) patients, all of whom had negative margins by H&E. Ohigashi *et al* have also detected K-ras mutation in retroperitoneal tissues of patients with pancreatic cancer.<sup>31</sup> Here we correlated K-ras margin status with disease outcome and demonstrated a significant difference in overall survival (fig 2). These findings therefore suggest that the current techniques to determine the adequacy of surgical margins may not be sensitive to identify relevant genetic aberrancies which may be the surrogates of unrecognized disease. Molecular-genetic aberrancies in the surgical margins may be indicative of field cancerisation or occult cancer cells, both of which appear benign under the microscope.<sup>6–12,32</sup> Further investigation will be necessary to identify the specific occult cells harbouring these aberrancies.

To correlate PCR detection of K-ras mutation with clinical cancer recurrence, we reviewed radiographic reports and interventional studies for patients with positive K-ras mutation in the margins and poor survival. Because most patients were referred from outside institutions, radiographic data was, for the most part, unavailable and we were unable to determine disease free intervals for the entire cohort. However, in nine patients with positive K-ras mutations in the surgical margins and poor survival, radiographic imaging studies and peritoneal cytology revealed local recurrence ( $n = 6$ ) or malignant ascites ( $n = 3$ ), respectively. In studies of patients with head and neck cancers, Brennan *et al* have

**Table 3** Univariate and multivariate analyses

	Death/n	Univariate analysis		Multivariate analysis	
		HR (95% CI)	p Value	HR (95% CI)	p Value
Age (y)			0.41		NS
≤70	27/41 (66%)	1.0			
>70	18/29 (62%)	1.3 (0.7–2.4)			
Sex			0.63		NS
Female	22/35 (63%)	1.0			
Male	23/35 (66%)	1.2 (0.6–2.1)			
UICC/TNM stage*			0.59		NS
pI†	17/26 (65%)	1.0			
pII†	28/44 (64%)	1.2 (0.6–2.2)			
Tumour extent			0.28		NS
T <sub>1</sub>	9/16 (56%)	1.0			
T <sub>2</sub>	32/46 (70%)	1.5 (0.7–3.2)			
T <sub>3</sub>	4/8 (50%)	0.8 (0.2–2.6)			
Lymph node disease			0.23		NS
No	18/30 (60%)	1.0			
N <sub>1</sub>	27/40 (68%)	1.5 (0.8–2.7)			
Tumour size (cm)			0.47		NS
0–2	12/20 (60%)	1.0			
>2	33/50 (66%)	1.3 (0.7–2.5)			
Tumour grade			0.0001		0.0001
Well	6/11 (55%)	1.0		1.0	
Moderate	16/33 (48%)	1.4 (0.5–3.5)		2.6 (0.9–7.3)	
Poor	23/26 (88%)	5.0 (1.9–13.0)		6.7 (2.4–18.5)	
Perineural invasion			0.03		0.04
No	13/24 (54%)	1.00		1.0	
Yes	32/46 (70%)	2.2 (1.1–4.2)		2.1 (1.0–4.2)	
Lymphovascular invasion			NS		NS
No	33/52 (63%)	1.0			
Yes	12/18 (67%)	1.5 (0.8–2.9)			
K-ras in margins			0.0013		0.004
No	15/33 (45%)	1.00		1.0	
Yes	30/37 (81%)	2.8 (1.5–5.3)		2.8 (1.4–5.5)	
PanIN in margins‡			0.67		NS
No	1/3	1.0			
Yes	11/27	0.72 (0.16–3.2)			
K-ras in tumour			0.15		NS
No	11/13 (85%)	1.0			
Yes	34/57 (60%)	.60 (.30–1.2)			

\*UICC/TNM 6th edition.

†pI and pII, pathological stage I and pathological stage II.

‡For patients with available pancreatic intraepithelial neoplasia data. HR (95% CI), hazard ratio (95% confidence interval).

provided evidence to support the implications of our study.<sup>8</sup> They found that patients whose histologically negative surgical margins harboured p53 mutation had recurrence at those margins and had worse disease outcome than patients without such genetic aberrancies.

K-ras mutation was chosen for this study because of its frequent occurrence in pancreatic cancer and its potential pathogenetic role.<sup>13–16</sup> Although recent evidence demonstrates that K-ras gene mutation may be an essential precursor of pancreatic malignancy, there are reports of K-ras mutation in benign pancreatic disorders.<sup>13 33 34</sup> Moreover, K-ras mutation has been detected in PanIN lesions, which were mostly present in the surgical margins of this study cohort.<sup>35</sup> It is therefore feasible that detection of K-ras mutation may be a surrogate feature of PanIN. However, there are no current reports that propose or identify a clinical significance for PanIN in the surgical margins. At the participating institutions of this study, detection of any grade PanIN in a surgical margin was designated as a histopathologically “negative” margin; however, detection of PanIN-3 generally prompted consideration for further surgical resection.

K-ras mutation in the surgical margins could be a reflection of tumours with more aggressive biology. The prognostic significance of grade and perineural invasion by multivariate analysis correlates with the high number of PCR positive retroperitoneal margins (table 3). Differences in tumour grade, perineural invasion, and K-ras mutation in the

margins were manifest as a 40 month survival advantage in the K-ras mutation negative group. Median survival of 55 months for the K-ras mutation negative patients may seem indiscriminately high; however, this survival figure is derived from the entire cohort which had an overall median survival of 21 months and a five year overall survival rate of 19%. These outcomes are consistent with survival data from large prospective and retrospective studies.<sup>29 30 36</sup> Furthermore, prolonged survival is not uncommon for a small percentage of patients with pancreatic cancer. This has been reported from both large national cancer registries and smaller retrospective reviews.<sup>1 37–39</sup> Our findings specifically relate to PCR positivity and negativity in R0 margins. We acknowledge that the power of our study appears to be limited inasmuch as univariate and multivariate analyses did not find lymph node metastasis and tumour size to be prognostic factors for survival, despite the fact that 40 of 70 (57%) patients had lymph node disease and mean tumour size was >2 cm. These factors have been found to be significant predictors of prognosis in other studies.<sup>29 30 36</sup>

Although unrecognised disease in the surgical margins may occur in many cancers, the anatomical limitations of the pancreas make it particularly problematic to determine whether wider PCR negative surgical margins could affect outcome.<sup>6 7</sup> Even when total pancreatectomy has been performed for pancreatic cancer, survival data are no better, an outcome which, perhaps, can be explained by our high

number of K-ras mutation positive retroperitoneal margins.<sup>40</sup> Adjuvant therapy may appear attractive when surgical margins are PCR positive but a recent large randomised controlled trial demonstrated no survival advantage in patients receiving radiation therapy.<sup>41</sup> However, a meta-analysis of chemotherapy has demonstrated a survival advantage.<sup>42</sup> Furthermore, the development of promising new therapeutic agents provides a potential avenue of treatment for high risk patients, as defined by the detection of genetic mutation in surgical resection margins.

Expansion of genotypically altered benign or malignant cells has important clinical consequences. We have demonstrated in patients with pancreatic cancer that detection of K-ras mutation in the surgical margins may represent unrecognised disease and correlates strongly with clinical outcomes. We are currently investigating whether additional genetic or epigenetic aberrancies are present in the margin tissues. We have relied on PCR assays to detect such defects because sensitive and efficient diagnostic antibodies are still lacking. A recent study by Bogoevski *et al* further demonstrates the importance of molecular techniques in identifying occult spread of cancer cells in pancreatic cancer.<sup>43</sup> Here we present an argument to implement measures to assess surgical resection margins beyond the standard H&E techniques. Our study findings have direct and immediate clinical implications for pathological staging and prognostic evaluation of patients undergoing surgery for pancreatic cancer. We suggest that molecular-genetic evaluation of surgical margins should be considered to better define "negative" surgical margins. Moreover, such characterisation of surgical resection margins can provide valuable data that may potentially lead to treatment strategies to alter outcomes in patients undergoing surgery for pancreatic cancer.

#### Authors' affiliations

**J Kim**, Department of Molecular Oncology and Division of Surgical

Oncology, John Wayne Cancer Institute, Santa Monica, California, USA

**H A Reber**, **O J Hines**, **K K Kazanjian**, Department of Surgery, David Geffen School of Medicine, University of California-Los Angeles, Los Angeles, California, USA

**S M Dry**, Department of Pathology, David Geffen School of Medicine,

University of California-Los Angeles, Los Angeles, California, USA

**D Elashoff**, Department of Biostatistics, University of California-Los

Angeles, Los Angeles, California, USA

**S L Chen**, **A J Bilchik**, Division of Surgical Oncology, John Wayne

Cancer Institute, Santa Monica, California, USA

**N Umetani**, **M Kitago**, **S Hiramatsu**, **D S B Hoon**, Department of

Molecular Oncology, John Wayne Cancer Institute, Santa Monica,

California, USA

**S Yong**, Department of Pathology, Loyola University Chicago Stritch

School of Medicine, Maywood, Illinois, USA

**M Shoup**, Department of Surgery, Loyola University Chicago Stritch

School of Medicine, Maywood, Illinois, USA

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## EDITOR'S QUIZ: GI SNAPSHOT .....

### Bleeding terminal ileal ulcer

#### Clinical presentation

A 24 years old woman of Hong Kong origin but resident in the UK for the past 14 years presented with a four week history of abdominal pain, watery diarrhoea, and vomiting on a background history of adult onset Still's disease. She was found to be pyrexial with a raised erythrocyte sedimentation rate and an albumin of 25. Gastroscopy was normal but at colonoscopy she was found to have a solitary bleeding terminal ileal ulcer (fig 1).

Histology of the ulcer showed necrotising granuloma (fig 2). Following a presumptive diagnosis of Crohn's disease, she was started on 40 mg/day of oral prednisolone with an initial good clinical response but her symptoms returned along with large volume ascites four weeks after cessation of steroids. At this point she also developed a pancytopenia with a further fall in albumin (<16). Computed tomography of the chest and abdomen (fig 3) revealed small bilateral pleural effusions and small pericardial effusion with atelectasis of both lung bases along with large volume ascites and multiple distended oedematous small as well as large bowel loops. Subsequent laparoscopy and peritoneal biopsies were normal.

#### Question

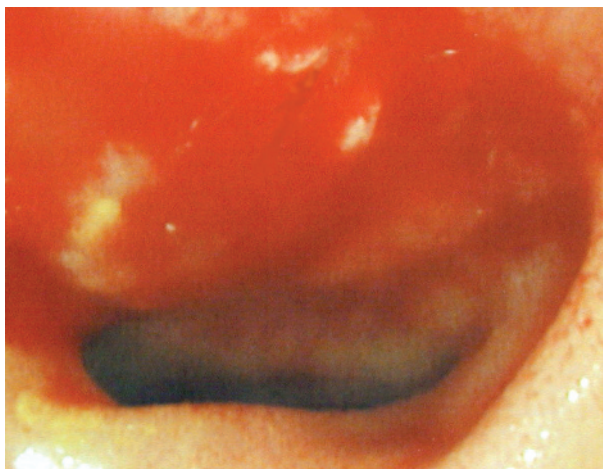
What is the diagnosis?

See page 1630 for answer

This case is submitted by:

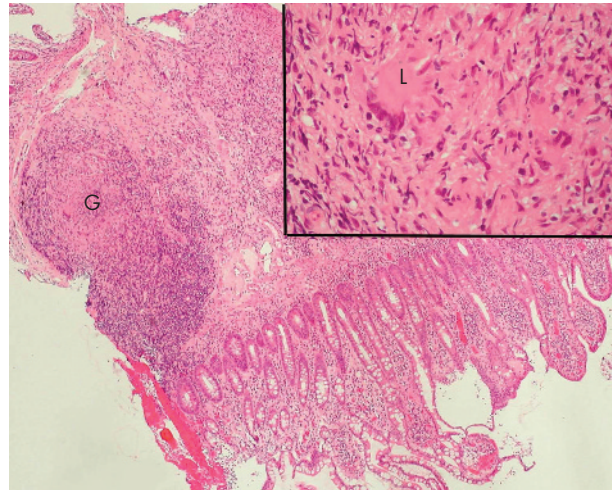
**A Sarkar**  
**K Basu**  
**S S Johal**

Gastroenterology Department, Northern General Hospital, Sheffield, UK

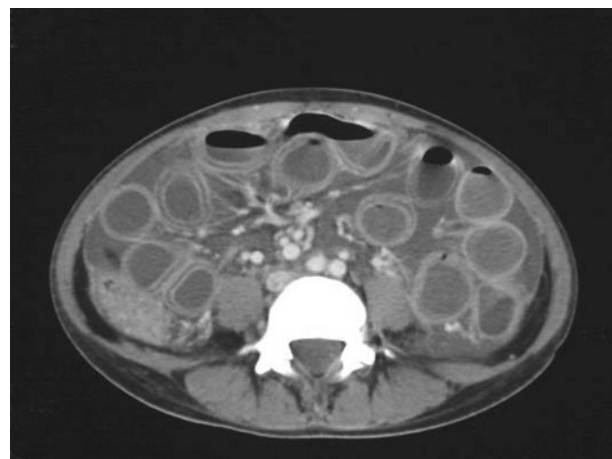


**Figure 1** Solitary bleeding terminal ileal ulcer.

**Robin Spiller**, Editor



**Figure 2** Histology showing necrotising granuloma. G, granuloma with surrounding lymphocyte infiltration; L, Langhans' giant cell.



**Figure 3** Computed tomography showing diffuse oedema of the bowel wall (target appearance).

**K Suvarna**  
Department of Pathology, Northern General Hospital, Sheffield, UK  
**K Clout**  
Department of Radiology, Northern General Hospital, Sheffield, UK

Correspondence to: Dr A Sarkar, Gastroenterology Department, Northern General Hospital, Herries Rd, Sheffield S57 4AU, UK; sarkar00001@hotmail.com

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