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Advances in Brief

Microsatellite Instability and K-*ras* Mutations Associated with Pancreatic Adenocarcinoma and Pancreatitis[1]

Teresa A. Brentnall,[2] Ru Chen, John G. Lee, Michael B. Kimmey, Mary P. Bronner, Rodger C. Haggitt, Kris V. Kowdley, Lonny M. Hecker, and David R. Byrd

Departments of Medicine [T. A. B., M. B. K., R. C. H., K. V. K., L. M. H.], Pathology [M. P. B., R. C. H.], and Surgery [R. C., D. R. B.], University of Washington, Seattle, Washington 98195, and Department of Medicine, Portland Veterans Affairs Medical Center, Portland, Oregon [J. G. L.] 97207

Abstract

***ras* oncogene mutations and microsatellite instability (MIN) have been described in pancreatic cancer studies from paraffin blocks and fresh frozen tissue. We sought to determine whether they could be detected in endoscopic retrograde cholangiopancreatography-derived pancreatic juice. *ras* mutations were detected in the pancreatic juice of 40% (2 of 5) of patients with pancreatic cancer and 2 of 5 patients with pancreatitis. MIN was detected at a single locus in the pancreatic juice of 40% of pancreatic cancer patients and at ≥2 loci of 100% of pancreatitis patients. The finding of MIN in pancreatitis specimens was verified in studies performed on paraffin blocks. MIN was not detected in normal pancreas controls. All of the cancer patients who had *ras* mutations in their pancreatic juice also had evidence of MIN at one or more loci ($P \leq 0.05$), suggesting that MIN is associated with the development of a *ras* mutation. More importantly, the finding of MIN in pancreatitis specimens suggests that MIN can occur in nonneoplastic conditions of the pancreas and may represent the saturation of an intact mismatch repair system.**

Introduction

An enhanced understanding of the molecular genetic events that occur during neoplastic progression in the pancreas could lead to earlier diagnosis and improved survival rates. Mutations within the K-*ras* proto-oncogene are the most common genetic abnormality described in pancreatic adenocarcinoma; they have been detected in the majority of tissue specimens, as well as pancreatic juice specimens, obtained from patients with pancreatic cancer (1–4). Another molecular alteration that may be important in the development of pancreatic cancer is MIN.[3] Microsatellites are short, repetitive sequences of DNA that are stably inherited, vary from individual to individual, and have a relatively low inherent mutation rate. Instability within these sequences has been described in numerous tumor types, including pancreatic cancer; however, the frequency with which MIN has been found in pancreatic cancer is variable. Han *et al.* (5) reported MIN in 67% (6 of 9 tumors) of pancreatic cancers, whereas Seymour *et al.* (6) failed to detect MIN in 27 pancreatic cancers. We sought to determine whether the molecular abnormalities of K-*ras* mutation and microsatellite instability could be detected in pancreatic juice obtained at ERCP. In addition, microsatellite instability was evaluated in pancreatic tissues derived from paraffin blocks.

Materials and Methods

Patients. Seventeen patients were referred for ERCP for clinical indications. Approval for human subjects was obtained for molecular studies of the pancreatic juice, which was collected through a catheter placed selectively into the pancreatic duct. Five patients had pancreatic cancer proven by biopsy or fine-needle aspirate (patients PJ-1 thru PJ-5). Five patients had a clinical diagnosis of pancreatitis (PJ-6 thru PJ-10): one with acute pancreatitis secondary to gallstone impaction (PJ-6), one with probable acute and chronic pancreatitis (PJ-7), and three with chronic pancreatitis (2 due to alcoholism and one idiopathic; PJ-8 thru PJ-10). Two patients had biopsy-proven nonpancreatic neoplasms: one with ampullary adenoma associated with Gardner's Syndrome (PJ-11) and the other with cholangiocarcinoma (PJ-12). Two patients had positive family histories of pancreatic cancer and clinical presentations of steatorrhea (PJ-13) or interscapular back pain (PJ-14) but did not have a diagnosis of pancreatic adenocarcinoma. Pancreatic juice was collected during pancreatic sphincter manometry from three patients, who were referred for abdominal pain and no evidence of pancreatic disease (PJ-15 thru PJ-17). To further evaluate MIN in the pancreas, paraffin block studies were performed on surgical specimens from 21 additional patients: 8 patients with pancreatic cancer, 9 patients with chronic pancreatitis, and 4 patients with uninvolved normal pancreas distant to islet cell tumors (normal control).

***ras* Studies.** Two to 5 ml of pancreatic juice were collected during ERCP and diluted 1:6 with DNA extraction buffer [50 mM Tris (pH 8), 50 mM NaCl, 1 mM EDTA (pH 8), 0.5% SDS, and 1 mg/ml proteinase K] and incubated overnight at 55°C. DNA from matching constitutional tissue was obtained from peripheral blood lymphocytes. Exon 1 (which includes codons 12 and 13) of K-*ras* was PCR amplified using a cycle profile of 98°C (20 s) 55°C (1 min), and 72°C (40 s) for 37 cycles as described previously (7). Two hundred to 500 ng of template DNA were added to a 50-μl reaction mix containing 1× PCR buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 100 μM deoxynucleotide triphosphate, 50 ng of each oligomer, and 1 unit of Taq polymerase. Amplification yielded a 290-bp product, which was sequenced as described previously (7).

MIN Studies. MIN studies were performed on DNA extracted from ERCP-derived pancreatic juice (see above) and on DNA from paraffin blocks. The DNA from the paraffin blocks was extracted from pancreatic tissue microdissected from 5-μm sections on glass slides. Matching constitutional tissue for the pancreatic juice studies came from peripheral blood lymphocytes and for the paraffin block studies from lymph nodes, spleen, or small bowel. Microsatellite probes included *D2S123, D2S136, D3S1067, D5S107, D6S87, D8S255, D10S197, D11S904, D17S261, D17S361, D17S787,* and *D18S34* (8). PCR amplifications were performed with P^{32} end-labeled primers; products were denatured, electrophoresed in 8% polyacrylamide gels, and visualized by autoradiography. An average of 8 loci were tested per specimen. All samples were evaluated in a blinded fashion. Instability was defined by the presence of bands of DNA in the pancreatic specimen that were not visible in the matching constitutional DNA.

Statistical Methods. Fischer's Exact test was performed for statistical evaluation of data. Probability values were two tailed, with $P \leq .05$ regarded as statistically significant.

Results

K-*ras* Mutations: Pancreatic Juice. DNA was extracted and amplified from the pancreatic juice of 17 patients. Two of five patients with pancreatic cancer and two of five patients with pancreatitis had *ras* mutations (Table 1). None of the patients with a family history of pancreatic cancer, other nonpancreatic neoplasms, or normal controls

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[2] To whom requests for reprints should be addressed, at University of Washington, Division of Gastroenterology, Box 356424, Seattle, WA 98195.

[3] The abbreviations used are: MIN, microsatellite instability; ERCP, endoscopic retrograde cholangiopancreatography.

Table 1 *MIN and ras mutations in DNA from pancreatic juice*

	Ras[a] Mutation	MIN	Sites +[b] for MIN
Pancreatic cancer			
PJ-1	+	1/13	*D3S1067*
PJ-2	–	0/12	None
PJ-3	–	0/12	None
PJ-4	+	1/7	*D2S123*
PJ-5	–	0/4	None
Pancreatitis			
PJ-6	–	5/12	*D5S107, D6S87, D8S255, D10S197, D18S34*
PJ-7	+	2/5	*D8S255, D11S904*
PJ-8	–	4/11	*D2S123, D8S255, D17S787, D17S261*
PJ-9	–	2/11	*D8S255, D18S34*
PJ-10	+	1/12	*D17S26*
Other Neoplasms			
PJ-11 (Gardner's)	–	0/13	None
PJ-12 (Cholangio[c])	–	1/6	*D8S255*
Family history of pancreatic cancer			
PJ-13	–	0/12	None
PJ-14	–	2/10	*D2S123, D8S255*
Normal controls			
PJ-15	–	0/4	None
PJ-16	–	0/12	None
PJ-17	–	0/11	None

[a] *ras* mutations were located at codon 12, except for PJ-4, which had a mutation at codon 13.
[b] +, positive.
[c] Cholangio, cholangiocarcinoma.

Table 2 *Clinical data from patients with pancreatitis and patients with a family history of pancreatic cancer*

Patient	Age	Diagnosis	Family Hx of Pancr. CA[a]	F/U[b]	Ras	MIN
PJ-6	77	Pancreatitis	–	6 mo	–	5/12
PJ-7	66	Pancreatitis	–	3 mo	+	2/5
PJ-8	64	Pancreatitis	–	9 mo	–	4/11
PJ-9	38	Pancreatitis	–	36 mo	–	2/11
PJ-10	63	Pancreatitis	–	6 mo	+	1/12
PJ-13	44	h/o familial pancreatitis	+	6 mo	–	0/12
PJ-14	49	h/o familial pancr. cancer	+	4 mo	–	2/10

[a] Family history of pancreatic cancer; h/o, history of.
[b] Follow up.

undergoing manometry had evidence of a *ras* mutation. Clinical details of the patients with pancreatitis and with a family history of pancreatic cancer are presented in Table 2. All of the patients who had a *ras* mutation detected in their pancreatic juice also had evidence of MIN at one or more loci ($P \leq .05$; MIN data, see below; Tables 1 and 2).

MIN: Pancreatic Juice. MIN was detected in 40% (two of five) patients with pancreatic cancer (Table 1; Fig. 1). In each of these cases, MIN was detected at only one locus. All of the patients with pancreatitis had MIN, and in four of five patients, it was detected at 2 or more loci. One of the two patients with a nonpancreatic cancer (cholangiocarcinoma) had MIN at a single locus. One of the two patients with a family history of pancreatic cancer had MIN at two loci. None of the normal controls had MIN.

MIN: Paraffin Blocks. Twenty-one additional patients had surgical specimens microdissected from paraffin block sections to evaluate for MIN. MIN was detected in at least one locus in 75% (6 of 8) pancreatic cancer specimens derived from paraffin blocks and was detected at two or more loci in 50% (four of eight; Table 3). None of the four normal control pancreatic specimens had evidence of MIN. Eighty-eight % (eight of nine) of the pancreatitis specimens had MIN at one or more loci, and 44% (four of these nine) had MIN at two or more loci.

Discussion

We detected *ras* mutations in pancreatic juice from 2 of 5 (40%) patients with pancreatic cancer. This finding is consistent with previous reports in the literature (3, 4). We also detected *ras* mutations in two patients with pancreatitis (Table 1). *ras* mutations have been described in the premalignant pancreatic tissue surrounding pancreatic cancer, suggesting that it can be a relatively early event in pancreatic tumorigenesis (9). Furthermore, Berthélémy *et al.* (10) found *ras* mutations in the pancreatic juice of two patients who had no evidence of pancreatic cancer but who then developed pancreatic cancer at 18 months and 40 months, respectively, during follow-up (10). The follow-up period has been relatively short in our two patients who have *ras* mutations but who have not been diagnosed with pancreatic cancer. Surveillance will continue since these patients may have occult cancer.

MIN was detected at a single locus in 2 of 5 (40%) of the pancreatic juice specimens from patients with pancreatic cancer. Our studies of tissue from paraffin blocks revealed MIN at one or more loci in 75% (six of eight) of the cancers and at two or more loci in 50% (four of eight; Table 3). The more frequent detection of MIN in the paraffin blocks may reflect the greater percentage of neoplastic tissue that can be obtained by microdissection of tumor DNA from paraffin sections.

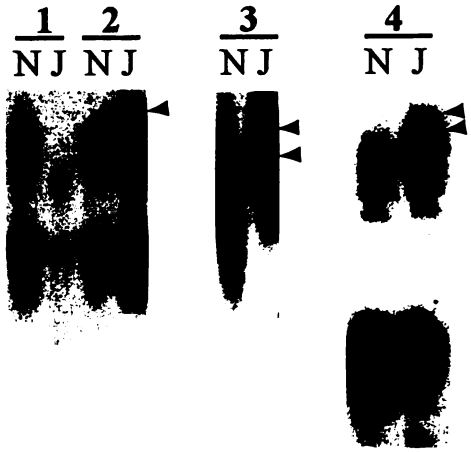

Fig. 1. MIN in DNA extracted from pancreatic juice. *n*, normal constitutional tissue; *J*, pancreatic juice. *Lanes 1, 3,* and *4*, pancreatitis patients; *Lane 2*, the patient with cholangiocarcinoma. *Lanes 1* and 2, probe *D8S255*; *Lane 3*, probe *D18S34*; *Lane 4*, probe *D5S107*.

Table 3 *MIN in DNA derived from paraffin blocks*

	MIN	Sites +[a] for MIN
Pancreatic cancer		
SU-1	0/6	None
SU-2	1/9	*D2S123*
SU-3	2/9	*D6S87, D18S34*
SU-4	1/8	*D2S123*
SU-5	4/9	*D2S123, D5S107, D6S87, D13S175*
SU-6	0/9	None
SU-7	2/12	*D6S87, D8S255*
SU-8	2/10	*D3S1067, D8S255*
Pancreatitis		
SU-9	1/11	*D3S1067*
SU-10	2/11	*D3S1067, D6S87*
SU-11	1/10	*D5S107*
SU-12	3/11	*D2S136, D5S107, D6S87*
SU-13	0/5	None
SU-14	2/8	*D10S197, D18S34*
SU-15	1/9	*D18S34*
SU-16	1/7	*D6S87*
SU-17	4/8	*D2S136, D5S107, D6S87, D11S904*

[a] +, positive.

DNA obtained from pancreatic juice could potentially be derived from a more heterogeneous population of cells.

To our knowledge, only two studies have examined MIN in pancreatic cancer, both of which were performed on tissue specimens and not pancreatic juice. Han and colleagues detected MIN in six of nine pancreatic tumors (5). Three of these MIN+ tumors (33%) were reported to have instability at two or more of the four loci tested. In contrast, Seymour *et al.* (6) found no evidence of MIN in any of the 27 pancreatic cancers they studied. Whether the discrepancies are due to differences in methods and interpretation, differences in patient population, or differences in numbers and location of the loci tested is unclear. Our data from our paraffin block studies appear to be more consistent with the findings of Han *et al.* (5); however, we were able to detect MIN at only a single locus in the pancreatic juice specimens. The relevance of instability at a single locus remains to be determined.

The most interesting finding in our study was the observation of MIN at two or more loci in the pancreatic juice from patients with pancreatitis. It is unlikely that this is an artifact of the methods used to obtain these specimens, because the control patients had no evidence of instability. Although it is possible that some of these patients may have a hidden neoplasm that could account for the MIN, it seems unlikely that all of them would have occult pancreatic cancer. In addition, tissues derived from paraffin blocks of resected specimens with pancreatitis also showed MIN (Table 3).

The implications of MIN in DNA from patients with pancreatitis may be far reaching. MIN is a marker of genome-wide mutations and, prior to this work, had been described only in sporadic neoplastic and premalignant tissues or in tissues from patients who had germline mutations in DNA mismatch repair genes. We theorize that MIN can develop in the presence of an intact DNA repair system. In the pancreatic juice study, all of the pancreatitis patients had MIN, and none of them had a personal or family history of pancreatic cancer (Table 2). We were also able to verify the finding of MIN in pancreatitis specimens from paraffin blocks. Thus, although it is possible that these patients could have acquired or inherited a defect in the mismatch repair genes, it seems unlikely. Rather, we speculate that MIN developed in the setting of pancreatitis because of saturation of an intact mismatch repair system.

Reactive oxygen species are generated early in the development of pancreatitis and appear to be important in its pathogenesis (11, 12). Both chronic and acute pancreatitis are associated with the production of reactive oxygen species, including hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), and superoxide radicals (O_2-). These species not only cause direct mutation of DNA but may also lead to mutagenic derivatives including lipid peroxides and malondialdehyde (13). Under normal cellular conditions, oxidative stress may be responsible for as many as 10,000 DNA-damaging events/cell/day (14). In the setting of pancreatitis, high levels of reactive oxygen species may cause enough DNA damage to saturate an intact DNA repair system and essentially overwhelm it. The hypothesis that an intact DNA repair mechanism is saturable is consistent with studies performed in *Escherichia coli*; the results presented here support this concept in humans (15).

Patients with pancreatitis are at increased risk for pancreatic cancer; the cumulative risk of pancreatic cancer in subjects with chronic pancreatitis is 1.8% at 10 years and 4% at 20 years of follow-up (16). It is possible that the process of pancreatic tumorigenesis in the setting of chronic pancreatitis may be caused by the following mechanism. Reactive oxygen species initiate DNA damage that saturates the DNA mismatch repair mechanism; during chronic inflammation, mutations escape repair and accumulate over time. In many patients, this DNA damage may remain occult, as evidenced by the MIN in our patients with pancreatitis. However, in some patients, secondary events might lead to entrance into the neoplastic pathway. Whether these secondary events could be due to modifying genes, hormonal influences, or environmental factors remains to be determined. For example, the p53 protein undergoes functional destabilization under oxidative conditions (17). This in turn may have an effect on cellular growth and proliferation in the pancreas. In addition, alterations in the *bcl-2* family of genes, or the ubiquitous *ras* oncogene, appear to be important genetic events in pancreatic cancer (18). It is interesting to note in our study that all of the patients with a *ras* mutation in their pancreatic juice also had evidence of MIN at one or more loci ($P \leq 0.05$). Since more patients have MIN than have *ras* mutations, and since all patients with a *ras* mutation have MIN, we theorize that MIN precedes the development of *ras* mutations and moreover, that the same events leading to MIN may eventually cause a *ras* mutation.

Other mitigating factors that might effect progression to pancreatic tumorigenesis include environmental influences. Dietary intake of antioxidants has been shown to improve pain and prevent relapse of pancreatitis, regardless of etiology or duration of disease (19). Furthermore, antioxidants have been found to have chemopreventive properties in experimental models of pancreatic cancer (20).

In summary, we have examined the pancreatic juice from patients with pancreatic cancer, pancreatitis, and normal control pancreases for the presence of *ras* mutations and microsatellite instability. Ras mutations were present in the pancreatic juice specimens of 40% of patients with pancreatic cancer and were also detected in chronic pancreatitis patients, who may in the future develop overt pancreatic cancer. Microsatellite instability was detected in patients with pancreatic cancer, but more importantly, also in patients with pancreatitis. This latter finding is particularly intriguing as it suggests that the DNA mismatch repair system may be saturated under certain conditions and that unsuspected genome-wide mutations may be present in nonneoplastic tissue. This is but one hypothesis; the elucidation of the true mechanism for MIN in pancreatitis represents an exciting area for future research.

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