Genetic, Immunohistochemical, and Clinical Features of Medullary Carcinoma of the Pancreas

A Newly Described and Characterized Entity

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**Medullary carcinomas of the pancreas are a recently
described, histologically distinct subset of poorly dif-
ferentiated adenocarcinomas that may have a unique
pathogenesis and clinical course. To further evaluate
these neoplasms, we studied genetic, pathological,
and clinical features of 13 newly identified medullary
carcinomas of the pancreas. Nine (69%) of these had
wild-type K-*ras* genes, and one had microsatellite
instability (MSI). This MSI medullary carcinoma,
along with three previously reported MSI medullary
carcinomas, were examined immunohistochemically
for Mlh1 and Msh2 expression, and all four expressed
Msh2 but did not express Mlh1. In contrast, all of the
medullary carcinomas without MSI expressed both
Msh2 and Mlh1. Remarkably, the MSI medullary car-
cinoma of the pancreas in the present series arose in
a patient with a synchronous but histologically dis-
tinct cecal carcinoma that also had MSI and did not
express Mlh1. The synchronous occurrence of two
MSI carcinomas suggests an inherited basis for the
development of these carcinomas. Indeed, the med-
ullary phenotype, irrespective of MSI, was highly as-
sociated with a family history of cancer in first-degree
relatives (*P* < 0.001). Finally, one medullary carci-
noma with lymphoepithelioma-like features con-
tained Epstein-Barr virus-encoded RNA-1 by *in situ*
hybridization. Therefore, because of medullary carci-
noma's special genetic, immunohistochemical, and
clinical features, recognition of the medullary variant
of pancreatic adenocarcinoma is important. Only
by classifying medullary carcinoma as special subset
of adenocarcinoma can we hope to further elucidate
its unique pathogenesis. (*Am J Pathol 2000,
156:1641–1651*)**

Most, but not all, poorly differentiated carcinomas arising
in the pancreas are conventional ductal adenocarcino-
mas.[1–3] Some poorly differentiated pancreatic carcino-
mas, however, are medullary carcinomas.[4] Although his-
torically grouped with poorly differentiated ductal
adenocarcinomas, medullary carcinomas are histologi-
cally distinct. As first described in 1998, they have poorly
defined cellular boundaries (syncytial growth pattern),
expanding tumor borders, and extensive necrosis, fea-
tures similar to their histological counterparts arising in
the colorectum (Figure 1).[4–8]

Medullary carcinomas may also be genetically distinct
from conventional ductal adenocarcinomas of the pan-
creas. Indeed, of five medullary carcinomas first de-
scribed by Goggins et al,[4] three (60%) had both micro-
satellite instability (MSI) and a wild-type K-*ras* gene. (One
of the six medullary carcinomas reported by Goggins et
al[4] was later determined to be a metastatic renal cell
carcinoma.) These data are atypical for nonmedullary
ductal adenocarcinomas of the pancreas, which nearly
universally harbor K-*ras* gene mutations and seldom if
ever have MSI.[4,9–13]

Although these preliminary findings favor a distinct
pathogenetic course for medullary pancreatic carcino-
mas, the small number of cases studied so far has re-
quired a cautious view. To further elucidate the clinical
and genetic features associated with the medullary mor-
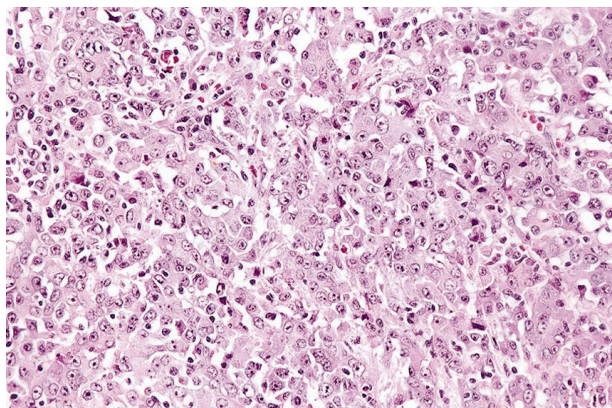phology, we studied a larger series of medullary pancre-

Figure 1. Histology of medullary carcinoma of the pancreas. The tumor has a syncytial growth pattern of the cells.

atic carcinomas. Specifically, our goals were to determine 1) the frequency of wild-type K-*ras* genes and MSI among medullary carcinomas; 2) whether immunohistochemistry for the DNA-repair gene products Mlh1 and Msh2 could identify the medullary carcinomas with MSI; and 3) whether the medullary carcinomas were associated with any other immunohistochemical, pathological, or clinical variables, including latent Epstein-Barr (EBV) infection or family history of cancer. Answering these three questions would help us to gauge the utility of classifying medullary carcinomas as a special subset of ductal adenocarcinomas of the pancreas.

Materials and Methods

Case Selection

The surgical pathology files of The Johns Hopkins Hospital and the Academic Medical Center (University of Amsterdam) were screened for pancreatic carcinomas with the medullary histological pattern.[4] A histological review of 450 randomly chosen pancreatic cancers revealed 18 (4.0%) possible medullary carcinomas, each originally diagnosed as a poorly differentiated ductal adenocarcinoma. Five of these cases were those originally identified by Goggins et al.[4] One of the medullary carcinomas also contained adenosquamous carcinoma comprising approximately half of the neoplasm. All 17 of the remaining medullary carcinomas were pure medullary carcinomas.

The 18 medullary carcinomas were then pooled by a reference pathologist (REW) with 17 other (nonmedullary) pancreatic carcinomas. The 17 nonmedullary carcinomas included 10 poorly differentiated ductal adenocarcinomas, four moderately differentiated ductal adenocarcinomas, two mucinous adenocarcinomas, and one adenosquamous carcinoma. One slide from each case was coded, and the set of slides was reviewed by three other pathologists (GJAO, RHH, and NVA), one of whom (NVA) was from an institution not associated with our original study on medullary carcinomas.[4] Each pathologist graded the cases according to criteria originally proposed by Goggins et al[4] (gland formation, invasion

pattern, syncytial growth pattern, necrosis). Each neoplasm then received an overall grade from 1 (most similar) to 5 (least similar), based on its similarity to the index medullary carcinoma case identified in the original study by Goggins et al.[4]

A neoplasm was considered a medullary carcinoma only when it met the following two criteria: first, an average grade less than 3; and second, at least two of the three pathologists providing a grade of 2 or lower. The three pathologists were said to disagree when any of the pathologists gave a score that was more than one point away from either of the other two pathologists.

Histological Analysis for Pancreatic Intraepithelial Neoplasias

When available, sections containing nonneoplastic pancreatic tissue surrounding each medullary carcinoma were histologically analyzed by the reference pathologist (REW) for associated pancreatic intraepithelial neoplasias (PanINs), the putative precursors of pancreatic ductal adenocarcinoma.[14–16] PanINs were identified and graded according to accepted criteria established at a National Cancer Institute-sponsored Pancreas Cancer Think Tank, held September, 1999, in Park City, Utah. These criteria are available on the Worldwide Web (http://pathology.jhu.edu/pancreas/panin).

In Situ *Hybridization for Epstein-Barr Virus*

Because they share some features with EBV-containing lymphoepitheliomas of the nasopharynx, all 18 medullary carcinomas were assayed by *in situ* hybridization for Epstein-Barr virus-encoded RNA-1. In addition, five of the 17 nonmedullary carcinomas were also studied for latent EBV infection. These five nonmedullary carcinomas included two poorly differentiated conventional adenocarcinomas, one moderately differentiated conventional adenocarcinoma, and two mucinous adenocarcinomas.

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and hydrated in graded ethanol. Sections were washed in water, digested with 10 μg/ml of proteinase K in 50 mmol/L Tris-HCl (pH 7.5) for 30 minutes at 37°C, and washed again in water. Sections were hybridized at 37°C for 16 to 18 hours with 20 μl of fluorescein isothiocyanate-labeled Epstein-Barr virus-encoded RNA riboprobe in hybridization solution (Novocastra, Newcastle on Tyne, UK). A parallel section was hybridized with a negative control probe. The sections were washed in Tris-buffered saline (pH 8.0) with 0.05% Tween 20 (TBS-T; Sigma Chemical Co., St. Louis, MO) and then immersed in 0.2× sodium chloride-sodium citrate/0.1% sodium dodecyl sulfate solution at 52°C for 10 minutes.

Sections were incubated in 10% normal rabbit serum for 30 minutes and then in a 1:200 dilution of anti-fluorescein isothiocyanate-alkaline phosphatase conjugate (*In Situ* Hybridization Detection Kit, Novocastra) in Tris-buffered saline (pH 8.0) with 0.05% Tween 20 for 30 minutes. After three washes, sections were placed in 1× alkaline-phosphatase buffer (100 mmol/L Tris-HCl, pH 9.5, 100

mmol/L NaCl, 50 mmol/L MgCl$_2$) for 5 minutes. Chromogenic detection was then performed with nitroblue tetrazolium and X-phosphate. Adequate color development occurred within 2 hours of incubation in the chromogen solution. The slides were rinsed in water, counterstained with hematoxylin, and mounted with aqueous mounting medium (GlycerGel; DAKO, Carpinteria, CA). An intense bluish-purple color indicated a positive hybridization signal.

Microdissection and DNA Extraction

The five medullary carcinomas originally identified by Goggins et al[4] had already been analyzed genetically. Therefore, 13 genetically unanalyzed medullary carcinomas remained. We also genetically analyzed five of the 17 nonmedullary pancreatic carcinomas with the same technique to ensure a sensitivity adequate to detect single base-pair genetic alterations. These five nonmedullary carcinomas included those also studied for EBV latent infection by *in situ* hybridization. Therefore, we microdissected and genetically analyzed 18 carcinomas (13 medullary and five nonmedullary) in this study.

Each of the 18 carcinomas to be genetically analyzed were microdissected from unstained 10-μm sections cut from archived paraffin blocks. The microdissected tissue was deparaffinized, digested with proteinase K, purified by phenol-chloroform extraction, and precipitated, as previously described.[9,17] The final product was reconstituted in 50 μl LoTE (3 mmol/L Tris, 0.1 mmol/L ethylenediaminetetraacetic acid, pH 7.5) and stored at −20°C. Because of the solid growth pattern of medullary carcinomas and microdissection with avoidance of lymphocytes, relatively pure samples of carcinoma were obtained. Samples containing at least 200 neoplastic cells were used for each polymerase chain reaction (PCR) below. Both the medullary and adenosquamous components of the mixed medullary carcinoma were separately microdissected.

K-ras Gene Analysis

Portions of exons 1 and 2 (encompassing codons 12, 13, and 61) of the K-*ras* gene (*KRAS2*) were amplified by PCR, as previously described.[9] Direct cycle sequencing (SequiTherm Excel II, Epicentre Technologies, Madison, WI) was performed using internal primers at an annealing temperature of 60°C. Products were separated on a 6% polyacrylamide gel and subjected to autoradiography.

Unusual K-*ras* genotypes were confirmed by direct sequencing of an independent PCR product and by sequencing of cloned PCR products (TOPO TA, Invitrogen, Carlsbad, CA). The N- and H-*ras* genes were not studied in this series, as we found previously that mutations in these two genes do not occur in pancreatic carcinomas, including medullary carcinomas.[18,19]

Microsatellite Instability Analysis

Evidence of MSI was sought in each of the 18 microdissected samples by: 1) length analysis of *BAT 25* and *BAT 26* markers; and by 2) direct sequencing of the polythymidine tract of the *TGFBR2* gene.[4,20–22] *BAT 25* and *BAT 26* sequences were amplified by PCR incorporating [α]-^{32}P-dCTP, and the products were resolved with 6% denaturing polyacrylamide gels, as previously described.[4] A portion of the *TGFBR2* gene was amplified by PCR, and the products were cycle sequenced and similarly resolved.[4] The three MSI carcinomas from the previous study of Goggins et al[4] were used as positive controls.

For each of these three loci, gain or loss of at least one nucleotide was regarded as a shift. Each carcinoma tested was found to have either shifts in at least two of the three loci (designated MSI), or shifts at no locus (designated microsatellite stable, MSS). Because carcinomas lacking a mononucleotide shift in at least one of these three markers generally do not have shifts in additional dinucleotide markers, additional markers were not used.[4,23]

Immunohistochemistry for Mlh1 and Msh2

Immunohistochemical labeling for the products of the *MLH1* and *MSH2* genes was performed on the current series of 13 medullary and five nonmedullary carcinomas, as well as on three MSI medullary carcinomas identified in the previous series collected by Goggins et al.[4] Unstained 6-μm sections were deparaffinized and rehydrated with xylene and graded alcohols. The slides were treated with sodium citrate buffer (10 mmol/L, pH 6.0) and then submitted to microwave antigen retrieval. Endogenous peroxidase was blocked by 20% hydrogen peroxide, and nonspecific binding was blocked by 20% Protein Blocker (Signet Laboratories, Dedham, MA) in buffered saline. After cooling for 5 minutes, the slides were labeled with mouse monoclonal antibodies to Mlh1 (1:50 dilution, clone G168–728; PharMingen, San Diego, CA) or to Msh2 (1:100 dilution, clone FE11; Oncogene Research Products, Cambridge, MA) and incubated overnight at room temperature. Each primary antibody was detected by biotinylated secondary antibodies, avidin-biotin complex, and 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin.[24]

Cancers interpreted to lack Mlh1 or Msh2 expression required a complete absence of labeling in the nuclei of neoplastic cells. Labeling of nonneoplastic epithelium, stromal cells, or lymphocytes served as an internal positive control in each of the sections.

Germline MLH1 Analysis

One patient in this study had synchronous pancreatic and colonic carcinomas. This patient's pancreatic carcinoma had a medullary phenotype with microglandular features and MSI, and his colonic carcinoma was an adenocarcinoma that also had MSI. In addition, as will be discussed later, neither carcinoma had immunodetectable *MLH1* gene product. Therefore, informed consent was obtained for germline *MLH1* analysis under a protocol approved by the institutional review board of The

Johns Hopkins Hospital. Conformation-sensitive gel electrophoresis was performed under clinical laboratory conditions at the University of Pennsylvania (Philadelphia, PA).[25–28] The results of the analysis were discussed with the patient, and he is currently undergoing genetic counseling.

Clinical and Pathological Data Collection

Clinical and pathological data for each of the patients were obtained from various sources, including the medical records of The Johns Hopkins Hospital and the Academic Medical Center, the surgical pathology databases of The Johns Hopkins Hospital and Academic Medical Center, and The Johns Hopkins Oncology Center's clinical information system. Specifically, family history was obtained from extensive review of these sources. Fourteen (78%) of the 18 patients with medullary carcinomas and 69 (90%) of the 77 patients with nonmedullary carcinomas had familial cancer pedigrees available. Data collected included tumor size, presence of lymph node metastases at presentation, age, gender, race, smoking history, alcohol history, comorbidities, presenting symptoms, and family history of cancer in a first-degree relative. Comorbidities included a history of myocardial infarction, peptic ulcer disease, peripheral vascular disease, hypertension, chronic or acute pancreatitis, and inflammatory bowel disease. Presenting symptoms included weight loss, abdominal pain, jaundice, nausea/vomiting, and fever/chills.

Statistical Methods

Data from the 18 microdissected tumors first described in this study and 77 xenografted tumors, many previously studied by Goggins et al[4] and by others in our laboratory,[9] were analyzed. Means were compared with a *t*-test or Wilcoxon rank sum test when the assumption of normality was not valid. Cross-tabulations were performed using Fisher exact tests.

The statistical endpoint of this study was survival status. Event-time distributions for this endpoint were estimated with the method of Kaplan and Meier[29] and compared using the log-rank statistic[30] or the proportional hazards regression model.[31] The simultaneous effect of two or more factors was studied using the multivariate proportional hazards regression model. Covariates that were marginally significant ($P < 0.19$) in univariate analyses were entered into the multivariate regression model, and nonsignificant effects were removed in a stepwise fashion.

All *P* values are two-sided. Computations were performed using the Statistical Analysis System[32] or EGRET.[33,34]

Results

Histology of the Medullary Carcinomas

The reference pathologist selected 35 cases for histological study. These included 18 cases classified as medullary by the reference pathologist and 17 cases classified as nonmedullary. The three other reviewing pathologists verified the diagnosis of medullary carcinoma in each of the 18 cases selected by the reference pathologist. The distribution of average grades for the similarity of the case in question to the index medullary carcinoma case was as follows: 1.0 (3 carcinomas); 1.2 (2 carcinomas); 1.8 (2 carcinomas); 2.0 (4 carcinomas); 2.1 (1 carcinoma); 2.2 (3 carcinomas); 2.3 (1 carcinoma);

Table 1. Selected Histological, Clinical, and Genetic Characteristics of 18 Medullary Carcinomas of the Pancreas

Case*	Average grade†	PanINs	EBV *in situ*	Special histologic features	Gender	Race	Age	Family history of cancer	Survival status	Survival (months)	K-*ras* codon 12	MMR phenotype
1	2.0	n/a	Neg	Squamoid features	M	W	72	Pos	Dec	0	CGT	MSS
2	2.2	n/a	Neg		F	W	74	Unk	Dec	8	Wt	MSS
3	2.0	n/a	Neg		M	W	79	Pos	Dec	5	GAT	MSS
4	2.2	Pres	Neg		F	W	53	Pos	Dec	45	Wt	MSS
5	2.3	Pres	Neg	Clear-cell features	M	W	44	Unk	Dec	11	Wt	MSS
6	2.2	Not	Neg		M	W	49	Unk	Dec	15	Wt	MSS
7	1.0	Pres	Neg		M	W	74	Pos	Dec	12	Wt	MSS
8	2.7	Pres	Neg		F	W	74	Unk	Dec	12	Wt	MSS
9	1.8	Not	Neg	Microglandular features	M	W	34	Pos	Alive	13	Wt	MSI
10	1.2	Not	Pos	Lymphoepithelioma-like features	M	W	33	Neg	Alive	126	Wt	MSS
11	2.0	Pres	Neg		F	W	67	Pos	Dec	15	TTT	MSS
12	2.1	Not	Neg	Clear-cell features	M	W	67	Pos	Dec	4	GAT	MSS
13	1.8	Not	Neg	Mixed adenosquamous carcinoma	F	AA	66	Pos	Dec	7	Wt	MSS
14	1.0	Not	Neg		M	W	71	Pos	Alive	67	Wt	MSI
15	1.2	Not	Neg		F	W	84	Pos	Dec	4	Wt	MSI
16	1.0	Not	Neg		M	W	72	Pos	Alive	24	Wt	MSI
17	2.0	n/a	Neg		M	W	85	Pos	Dec	40	GAT	MSS
18	2.7	n/a	Neg		M	W	65	Pos	Dec	9	GCT	MSS

Abbreviations: PanINs, pancreatic intraepithelial neoplasias; Pres, present; Not, not present; n/a, slides of non-neoplastic pancreas surrounding the medullary carcinoma were not available for review; EBV, Epstein-Barr Virus; Pos, positive; Neg, negative; M, male; F, female; W, white; AA, African-American; Unk, unknown family history; Dec, deceased; MMR, mismatch repair; MSS, microsatellite stable; MSI, microsatellite unstable.
*Cases 14 through 18 were first identified in the original study by Goggins et al.[4] Cases 1 through 13 were first identified in the present series.
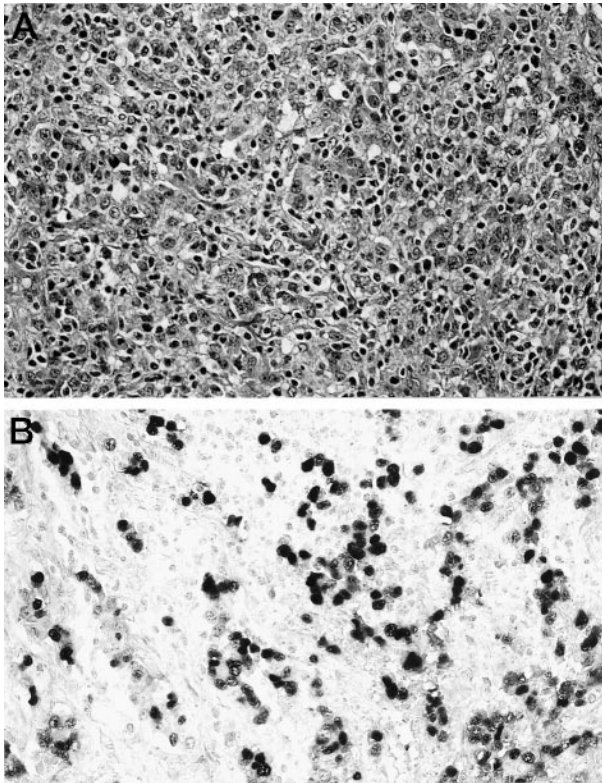†Average grade indicates similarity of the case to the index medullary carcinoma.

Figure 2. Histology of a medullary carcinoma of the pancreas with lympho-epithelioma-like features. **A:** The neoplasm has the characteristics of medullary carcinoma with the additional finding of an intense intratumoral lymphocytic infiltrate. **B:** *In situ* hybridization for Epstein-Barr virus-encoded RNA-1 is positive in the nuclei of the neoplastic cells.

and 2.7 (2 carcinomas) (Table 1). The medullary component from the case also containing adenosquamous carcinoma received an average grade of 1.8. The distribution of average scores for the 17 nonmedullary carcinomas was as follows: 5.0 (16 carcinomas) and 4.5 (1 carcinoma).

Among the three pathologists, there was disagreement in only three (two medullary carcinomas and one non-medullary carcinoma) of the 35 cases analyzed histologically. For each medullary carcinoma, two pathologists gave a score of 2, and one gave a score of 4 (average score, 2.7). For the nonmedullary carcinoma, two pathologists gave a score of 5, and one pathologist gave a score of 3.5 (average score, 4.5).

Whereas all of the medullary carcinomas had syncytial growth patterns, expanding tumor borders, and extensive necrosis, some had additional special features. For example, two medullary carcinomas had foci of clear

cells, one had a focal microglandular growth pattern, and one had areas of squamoid differentiation (Table 1). In addition, one medullary carcinoma had lymphoepithelioma-like features, with extensive intraepithelial and stromal infiltration by lymphocytes (Figure 2A). In general, pancreatic medullary carcinomas do not have a large number of intratumoral lymphocytes, as was seen in this latter neoplasm.[4]

Identification of Pancreatic Intraepithelial Neoplasia in Pancreata with Medullary Carcinoma

When available, slides of pancreatic parenchyma surrounding each of the medullary carcinomas were searched for PanIN. Slides of noncancerous tissue were available from 11 (61%) of the 18 medullary carcinomas (average of 7.2 slides per case; range, 2 to 25 slides). Five (45%) of these 11 cases had PanINs: case 4 had two PanINs-1B, case 5 had one PanIN-1B, case 7 had four PanINs-1A, case 8 had one PanIN-1B, and case 11 had two PanINs-1A and seven PanINs-1B. No higher grade duct lesions (PanINs-2 or -3) were identified.

In Situ *Hybridization for Epstein-Barr Virus RNA*

All 18 medullary carcinomas and five nonmedullary carcinomas were probed for latent EBV infection by *in situ* hybridization against Epstein-Barr virus-encoded RNA-1. The nuclei of the neoplastic cells in one (6%) of the 18 medullary carcinomas strongly labeled for the virus-encoded RNA (Figure 2B). The neoplasm demonstrating EBV infection was the one with lymphoepithelioma-like features. None of the other 17 medullary carcinomas and none of the five nonmedullary carcinomas contained EBV RNA.

Prevalence of K-ras Mutations

Each of the 18 microdissected carcinomas produced amplifiable K-*ras* PCR products. Of the 13 medullary carcinomas, nine (69%) had wild-type K-*ras* genes. This included the medullary carcinoma with an admixed adenosquamous carcinoma; both components in this single tumor had wild-type K-*ras* genes. In contrast, all five of the microdissected carcinomas without medullary histology had K-*ras* mutations ($P = 0.0294$). A group of 77 previously xenografted pancreatic carcinomas, many previously studied by Goggins et al[4] and by others in our

Table 2. K-*ras* Gene Status and Microsatellite Instability of Medullary and Nonmedullary Adenocarcinomas of the Pancreas

	No. with wild-type K-*ras* gene/no. studied	No. with MSI/no. studied
Microdissected medullary carcinomas	9/13 (69%)	1/13 (7.7%)
Xenografted medullary carcinomas	3/5 (60%)	3/5 (60%)
Microdissected nonmedullary carcinomas	0/5 (0%)	0/5 (0%)
Xenografted nonmedullary carcinomas	4/72 (5.6%)	0/72 (0%)
Total	16/95 (17%)	4/95 (4.2%)

Table 3. Distribution of K-*ras* Gene Mutations in Medullary and Nonmedullary Adenocarcinomas of the Pancreas

	GAT (codon 12)	GTT (codon 12)	CGT (codon 12)	GCT (codon 12)	AGT (codon 12)	TTT (codon 12)	GTTTGC (codons 12 and 13)	CTC (codon 13)	CAT (codon 61)	Wild type
Microdissected medullary carcinomas (*n* = 13)	2	0	1	0	0	1*	0	0	0	9
Xenografted medullary carcinomas (*n* = 5)	1	0	0	1	0	0	0	0	0	3
Microdissected non-medullary carcinomas (*n* = 5)	4	0	1	0	0	0	0	0	0	0
Xenografted non-medullary carcinomas (*n* = 72)	33	22	9	0	1	0	1[†]	1	1	4
Total (*n* = 95)	40	22	11	1	1	1	1	1	1	16

*One microdissected medullary carcinoma contained a dinucleotide mutation (GGT to TTT) at codon 12.
[†]One xenografted nonmedullary carcinoma contained a di-codon mutation of one allele, comprising a GTT mutation in codon 12 and a TGC mutation in codon 13.

laboratory, were also used as a control. Five (6%) of these 77 had the medullary phenotype, and 72 (94%) were conventional ductal adenocarcinomas. Three (60%) of the five medullary carcinomas had wild-type K-*ras* genes, whereas only four (5.6%) of the 72 conventional ductal adenocarcinomas had wild-type K-*ras* genes.[35] Thus, medullary pancreatic carcinomas are more often wild type at the K-*ras* gene than are conventional ductal adenocarcinomas (*P* = 0.0044). Table 2 summarizes these data.

All of the K-*ras* gene mutations detected in the microdissected medullary carcinomas were at codon 12. Two of the four were GGT (gly) to GAT (asp) transition mutations, and the third was a GGT to CGT (arg) transversion. The fourth medullary carcinoma had an unusual dinucleotide substitution, resulting in a TTT (phe) at codon 12. This mutation had not previously been seen in a human tumor. This latter mutation was therefore confirmed by repeated direct sequencing of an independent PCR product and by cloning and sequencing of the PCR products. All five of the microdissected, nonmedullary carcinomas had K-*ras* gene mutations at codon 12; four (80%) were GAT mutations, and the remaining tumor had a CGT mutation.

Sixty-eight (94%) of the 72 xenografted conventional ductal adenocarcinoma controls harbored K-*ras* gene mutations. Sixty-six of these were at codon 12, two were at codon 13, and one was at codon 61. One xenografted conventional adenocarcinoma harbored both a GTT (val) mutation at codon 12 and a TGC (cys) mutation at codon 13. This dicodon mutation also had not previously been seen in a human tumor. It was confirmed by repeat direct sequencing of an independent PCR product. Cloning and sequencing of the PCR products established that these two mutations were on the same allele. Two of the five medullary carcinomas in the previous series collected by Goggins et al[4] had K-*ras* gene mutations, and these were GAT (asp) and GCT (ala) changes at codon 12, respectively.

Table 3 summarizes the types of mutations occurring in medullary and nonmedullary carcinomas of the pan-
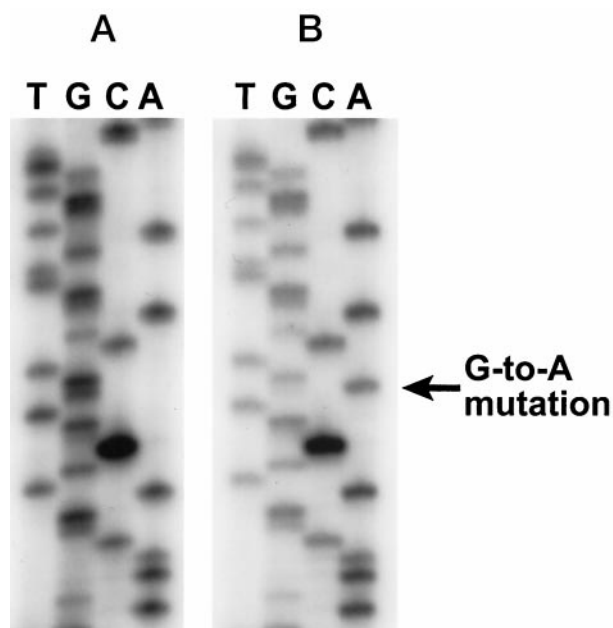

Figure 3. K-*ras* sequencing of codon 12 of a medullary carcinoma (**A**) and a poorly differentiated conventional adenocarcinoma (**B**). The medullary carcinoma has a wild-type K-*ras* gene, but the conventional adenocarcinoma has a GGT (gly) to GAT (asp) mutation.
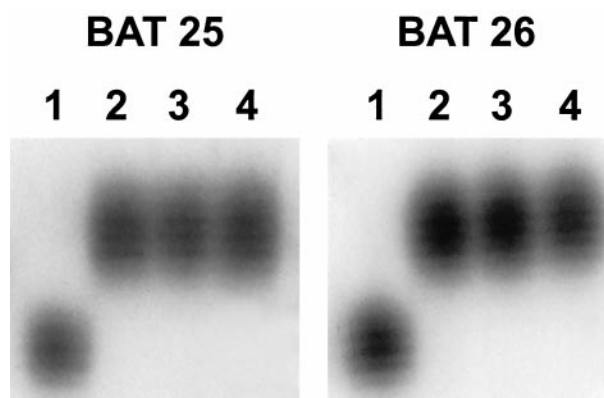

Figure 4. BAT 25 and BAT 26 PCR products for two medullary carcinomas, a conventional adenocarcinoma, and normal pancreas. For both the BAT 25 and BAT 26 PCR products: **lane 1,** pancreatic medullary carcinoma with a microsatellite shift; **lane 2,** normal pancreas from the same patient; **lane 3,** a different pancreatic medullary carcinoma without a shift; and **lane 4,** a conventional ductal adenocarcinoma without a shift. The shifting medullary carcinoma and the normal pancreas are both from the patient with synchronous pancreatic and colonic carcinomas.
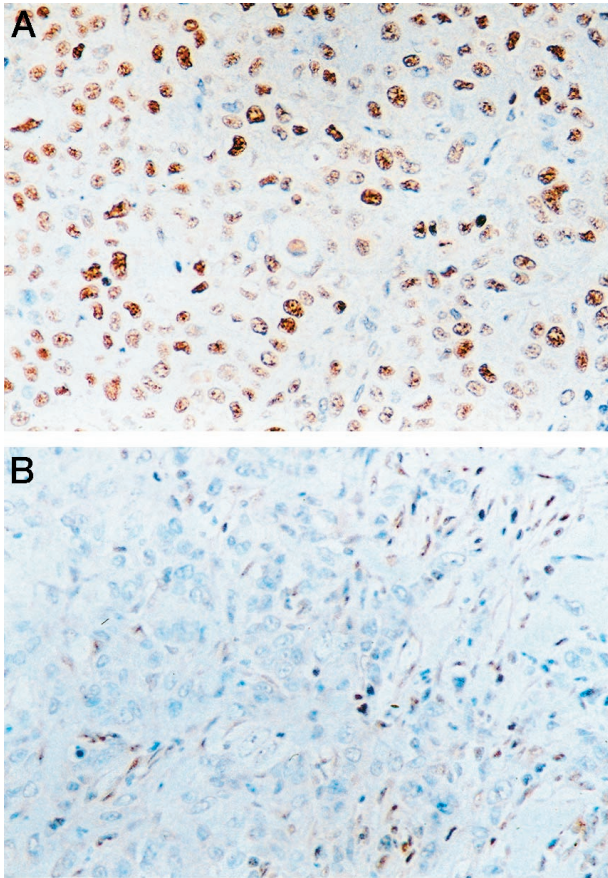
Figure 5. Immunohistochemistry for DNA repair-gene products in medullary carcinomas of the pancreas. **A:** This medullary carcinoma has intact nuclear labeling for the *MSH2* gene product. **B:** Another medullary carcinoma has no expression of Mlh1. Note that the nuclei of stromal cells do express Mlh1.

Figure 6. Histology of the Mlh1-deficient pancreatic (**A**) and colonic (**B**) carcinomas from the patient presumed to have HNPCC. Both carcinomas had MSI, and both had wild-type K-*ras* genes. Note that the histological patterns of the two cases are different: the pancreas has the medullary phenotype, whereas the colon cancer is gland-forming.

creas. Figure 3 presents examples of a wild-type and a mutant K-*ras* codon 12 in a medullary carcinoma and a conventional ductal adenocarcinoma, respectively.

Prevalence of MSI

Each of the 18 microdissected pancreatic carcinomas also produced amplifiable *BAT 25*, *BAT 26*, and *TGFB2* receptor gene products. One (7%) of the 13 microdissected medullary carcinomas had MSI. This carcinoma had shifts with both *BAT 25* and *BAT 26* markers and had only nine (*versus* the normal 10) thymidines in the poly-thymidine tract of its *TGFBR2* receptor gene. None of the other 17 microdissected carcinomas had shifts with any of the three markers. Also, three of the five previously xenografted medullary carcinomas originally reported by Goggins et al[4] were MSI. If we combine these two series of patients, a total of 4 (22%) of 18 xenografted and microdissected medullary carcinomas had MSI.

This contrasts with a combined total of 77 carcinomas with nonmedullary histologies, wherein none had MSI. The difference in MSI frequency between medullary carcinomas and nonmedullary carcinomas was significant ($P = 0.001$). Table 2 summarizes these microsatellite data. Figure 4 illustrates the *BAT 25* and *BAT 26* PCR
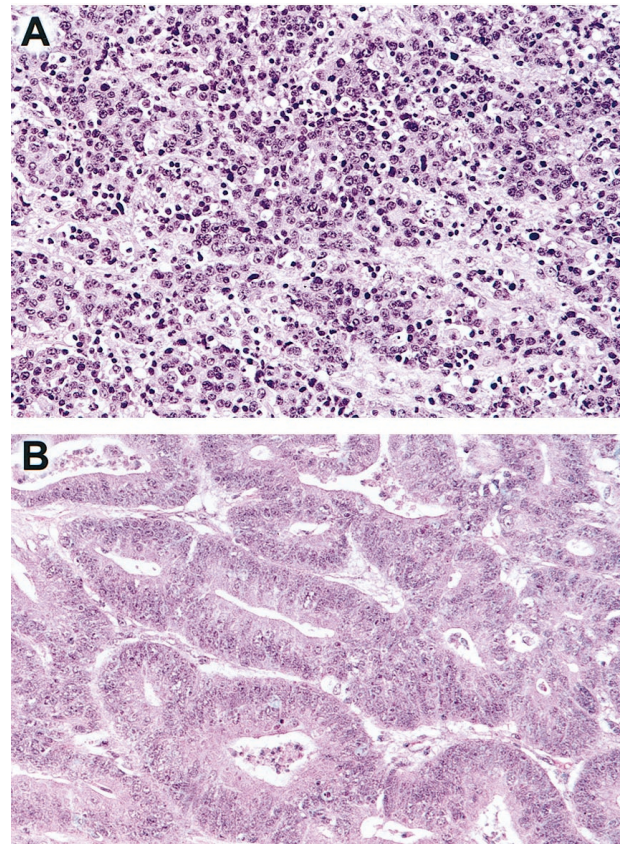
products of several tumors, including the microsatellite unstable tumor described in this study.

Immunohistochemistry for Mlh1 and Msh2

Immunohistochemistry for *MLH1* and *MSH2* gene products was performed on each of the 18 cases from this study and on the three MSI cases from the previous study of Goggins et al.[4] All four of the MSI cases from these two studies had absent Mlh1 expression and intact Msh2 expression (Figure 5). Expression of both Mlh1 and Msh2 was intact in all of the MSS cases. Therefore, the sensitivity and specificity of immunohistochemistry for DNA repair gene enzymes, in the identification of the genetically proven MSI tumors, were both 100%.

Features of the Newly Identified MSI Case

The microdissected MSI carcinoma with no Mlh1 expression in the current series originated in a 34-year-old patient with synchronous carcinomas of the pancreas and colon. Three lines of evidence suggest that these two carcinomas represented two independent primaries. First, the pancreatic carcinoma had a medullary pattern with focal microglandular features, whereas the colonic tumor was, at least partially, frankly gland-forming. Sec-

ond, the gross appearance of both tumors was typical of a primary carcinoma. Third, whereas the pancreatic primary was associated with multiple lymph node metastases to peripancreatic nodes, the colonic carcinoma had no lymph node metastases to surrounding mesenteric nodes. In addition, at the time of the operation to remove both primaries, no liver metastases were clinically present. Figure 6 compares the histology of both carcinomas.

This 34-year-old patient with simultaneous carcinomas of the pancreas and colon met the Bethesda criteria for hereditary nonpolyposis colorectal carcinoma (HNPCC) syndrome.[36,37] He therefore underwent clinical genetic counseling, and informed consent was obtained for microsatellite testing of his colonic carcinoma by a commercial laboratory (Thomas Jefferson University, Philadelphia, PA). MSI was identified. Our laboratory subsequently re-evaluated and compared the MSI status of both the pancreatic and the colonic carcinomas; nonidentical shifts of the *BAT 25, BAT 26,* and the *TGFBR2* gene markers were found. Both carcinomas also had wild-type K-*ras* genes.

Because both carcinomas were MSI, informed consent was also obtained for germline analysis of *MLH1*. Again, a commercial laboratory (University of Pennsylvania, Philadelphia, PA) performed this assay. Conformation-specific gel electrophoresis did not reveal an alteration in the germline copies of this patient's *MLH1* gene.[25–28] The patient and his family have undergone genetic counseling at The Johns Hopkins Oncology Center.

Analysis of Medullary Phenotype and Clinicopathological Variables

Multiple clinical, pathological, and genetic variables were analyzed for associations with the medullary phenotype. These analyses were performed using the combined total of 18 medullary carcinomas identified in this study and in the previous study of Goggins et al.[4] The medullary carcinomas were compared to the 77 well-characterized nonmedullary carcinomas (Table 2).

The only variables significantly associated with the medullary phenotype were a family history of any cancer (excluding basal cell and squamous cell of skin) in a first-degree relative ($P = 0.0004$), MSI ($P = 0.0010$), and a wild-type K-*ras* gene ($P < 0.0001$). Table 4 summarizes these data.

A variety of cancer types was seen in first-degree relatives of patients with pancreatic medullary carcinomas. The most common cancer in first-degree relatives was cancer primary to the breast ($n = 5$). Other cancers seen in family members included lung ($n = 4$), colon ($n = 3$), melanoma ($n = 2$), prostate ($n = 2$), pancreas ($n = 1$), liver ($n = 1$), and kidney cancers ($n = 1$). In two families the primary site of cancer in a first-degree relative was unknown.

We also examined the National Familial Pancreas Tumor Registry at The Johns Hopkins Hospital to determine whether any members of families with hereditary pancreas cancer syndromes had medullary cancers. These syndromes included the breast/pancreas cancer syndrome (associated with mutations in *BRCA2*),[38,39] familial atypical mole-malignant melanoma syndrome (associated with mutations in *p16*),[40] and Peutz-Jeghers syndrome (associated with mutations in *STK11/LKB1*).[41] No cases of medullary carcinoma were found among patients with these hereditary syndromes.[42]

The presence of the medullary phenotype, a mutated K-*ras* gene, or MSI did not impact survival significantly. Overall 2- and 5-year survival for the 18 patients with medullary carcinoma was 29% and 13%, respectively.

Discussion

Five significant conclusions about medullary carcinomas can be drawn from these studies. First, pancreatic medullary carcinomas have a genetic profile different from that of conventional pancreatic ductal adenocarcinomas. Medullary carcinomas usually have K-*ras* wild-type genes and sometimes are microsatellite unstable. When combined with the previous study of Goggins et al,[4] 12 (67%) of 18 medullary carcinomas reported to date have wild-type K-*ras* genes, and four (22%) of 18 have MSI. In contrast, conventional pancreatic ductal adenocarcinomas without the medullary phenotype almost always harbor K-*ras* gene mutations, and they virtually never have MSI.[4,9–13]

The difference in MSI prevalence between the original study by Goggins et al[4] and the current study might be explained by the difference in study designs. The original study was a retrospective study in which a separate set of xenografted carcinomas having MSI were identified and subsequently studied histologically. In contrast, the present study represents a prospective analysis of the genetic changes within 13 cases specifically selected for their medullary histological phenotypes.

Second, this present study demonstrates that immunohistochemistry for DNA repair enzymes can identify patients whose medullary pancreatic carcinomas have MSI. Therefore, it may be reasonable to use immunohistochemistry for Mlh1 and Msh2 as a first step to screen medullary pancreatic carcinomas for MSI. Those that have a loss of labeling could then be tested for MSI using microsatellite markers.

Third, medullary carcinomas represent the first instance in which pancreatic tumor histology may be used to identify an inherited susceptibility to cancer. This inherited susceptibility may occur in two ways. For example, we found that the medullary phenotype correlates significantly with family history of any cancer in a first-degree relative. Therefore, where appropriate, the identification of a medullary carcinoma of the pancreas should spur investigation of the cancer incidence among a patient's relatives. Also, one of the patients included in this study had synchronous MSI pancreatic and colonic cancers, suggesting that he has HNPCC. Indeed, Lynch et al[43] reported pancreatic cancer in some HNPCC kindreds. Although the patient in the present study met the clinical criteria for testing for HNPCC, conformation-specific gel electrophoresis studies have yet failed to identify a *MLH1* germline mutation. Conformation-spe-

Table 4. Associations between Medullary Phenotype and Pathological, Genetic, and Clinical Parameters

Factor	No. of medullary carcinomas	No. of nonmedullary carcinomas	P value
Family history of cancer			0.0004
Yes	13 (93%)	29 (42%)	
No	1 (7%)	40 (58%)	
K-*ras* gene			<0.0001
Mutant	6 (33%)	73 (95%)	
Wild type	12 (67%)	4 (5%)	
MMR status			0.0010
MSI	4 (22%)	0 (0%)	
MSS	14 (78%)	77 (100%)	
Race			>0.1
White	17 (94%)	70 (91%)	
Other*	1 (6%)	7 (9%)	
Gender			>0.1
Male	12 (67%)	40 (52%)	
Female	6 (33%)	37 (48%)	
Smoking history			>0.1
Yes	9 (50%)	32 (42%)	
No	9 (50%)	45 (58%)	
Alcohol history			>0.1
Yes	4 (22%)	18 (24%)	
No	14 (78%)	57 (76%)	
Nodal metastases			>0.1
Yes	15 (83%)	59 (77%)	
No	3 (17%)	18 (23%)	
Margin			>0.1
Positive	7 (39%)	22 (29%)	
Negative	11 (61%)	55 (71%)	
Age			>0.1
Over 60	13 (72%)	55 (71%)	
Under 60	5 (28%)	22 (29%)	
Tumor size			>0.1
Over 3.5 cm	10 (56%)	40 (52%)	
Under 3.5 cm	8 (44%)	37 (48%)	

Statistics in this table were analyzed using Fisher exact tests.
*Other includes African-Americans and patients of Hispanic, Asian, and Arabic descent.
Abbreviations: MMR, mismatch repair; MSI, microsatellite unstable; MSS, microsatellite stable.

cific gel electrophoresis, however, is not 100% sensitive, and this patient may have an unidentified germline *MLH1* mutation[25–28,44,45]. Sequencing of the entire *MLH1* gene and Southern blot analysis would presumably answer this question, but the patient has not requested such studies.

Fourth, medullary carcinomas may represent a subset of ductal adenocarcinomas, rather than a distinct tumor class. This classification is entertained because PanINs are found in nonneoplastic pancreas surrounding almost half of the medullary carcinomas. PanINs are believed to be the precursors to pancreatic ductal adenocarcinoma,[14–16] and their presence in tissue surrounding medullary carcinomas suggests that medullary carcinomas may be derived from these precursor lesions. However, no high-grade (and presumably more advanced) PanINs were found near any of the medullary carcinomas. Nor does the mere presence of such precursor lesions necessarily denote that the medullary carcinomas arose from these PanINs.

Fifth, we report here the first case of a pancreatic carcinoma with latent EBV, as demonstrated by *in situ* hybridization. This medullary carcinoma had lymphoepithelioma-like features, with infiltration of the neoplasm by large numbers of lymphocytes. This finding is not surprising, in that latent EBV infection is common in lymphomas, nasopharyngeal lymphoepitheliomas, and lymphoepithe-

lioma-like neoplasms in other sites, such as the stomach.[46–48] Therefore, medullary carcinomas may comprise two distinct subsets: those associated with and those not associated with EBV infection. A larger number of nonmedullary carcinomas will need to be studied to determine whether EBV infection also is associated with a minority of nonmedullary carcinomas as well.

In summary, we conclude that pathologists should distinguish medullary carcinomas from conventional ductal adenocarcinoma for two reasons. First, carcinomas with the medullary phenotype may have a distinct pathogenesis. Only by separating medullary carcinomas from conventional ductal adenocarcinomas will we be able to further define both the genetic changes and the role of EBV in these cancers. Second, medullary carcinomas can be a key clinical clue to the presence of an inherited cancer syndrome, including HNPCC. Further study of this familial association is justified. In the appropriate clinical setting, immunohistochemical studies, microsatellite analysis, germline testing, and genetic counseling may be warranted. In this scenario, immunohistochemical labeling for Mlh1 and Msh2 may help identify MSI in medullary carcinomas and characterize the specific underlying gene defect. More study will also be needed to see if the MSI subset of medullary carcinomas predicts a better

survival, as to this date the number of cases is too small to draw conclusions regarding prognosis.

Acknowledgments

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