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# Pancreatic Adenocarcinomas with DNA Replication Errors (RER$^+$) Are Associated with Wild-Type *K-ras* and Characteristic Histopathology

## *Poor Differentiation, a Syncytial Growth Pattern, and Pushing Borders Suggest RER$^+$*

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**The clinical and pathological features of carcinomas of the pancreas with DNA replication errors (RER$^+$) have not been characterized. Eighty-two xenografted carcinomas of the pancreas were screened for DNA replication errors using polymerase chain reaction amplification of microsatellite markers. Cases with microsatellite instability in at least two markers of a minimum of five tested were considered RER$^+$. RER status was correlated with histological appearance, karyotype of the carcinomas when available, *K-ras* mutational status, and patient outcome. Three (3.7%) of the eighty-two carcinomas were RER$^+$. In contrast to typical gland-forming adenocarcinomas of the pancreas, all three RER$^+$ carcinomas were poorly differentiated and had expanding borders and a prominent syncytial growth pattern. Neither a Crohn's-like lymphoid infiltrate nor extracellular mucin production were prominent. Ductal adenocarcinomas of the pancreas typically contain a mutant *K-ras* gene, yet all three RER$^+$ carcinomas had wild-type *K-ras*. One of the three RER$^+$ carcinomas was karyotyped and showed a near diploid pattern. All three of the RER$^+$ tumors were removed via Whipple resection. One of the three patients is free of disease 16 months after pancreaticoduodenectomy, one is alive and free of tumor at 52 months but developed two colon carcinomas during this period, and the third died of pancreatic cancer at 4 months. None of the three patients had a family history of colorectal carcinoma. A review of the *K-ras* wild-type carcinomas in a previously characterized series of pancreatic carcinomas with known *K-ras* mutational status identified two additional cancers with poor differentiation, a syncytial growth pattern, and pushing borders. Both of the cancers were diploid and both patients were long-term survivors (over 5 years). The inclusion of such patients in previous prognostic studies of pancreas cancer may explain the failure of histological grade to be a predictor of prognosis. These data suggest that DNA replication errors occur in a small percentage of resected carcinomas of the pancreas and that wild-type *K-ras* gene status and a medullary phenotype characterized by poor differentiation, an expanding pattern of invasion, and syncytial growth should suggest the possibility of DNA replication errors in carcinomas of the pancreas. *(Am J Pathol 1998, 152:1501–1507)***

The establishment of well defined criteria (the Amsterdam criteria) for the identification of kindreds with the hereditary nonpolyposis colon cancer syndrome (HNPCC, or Lynch syndrome)[1–4] was instrumental in the discovery that tumors from patients with this syndrome typically have microsatellite instability (RER$^+$) suggestive of a mismatch repair defect.[5] This observation was, in turn, pivotal to the discovery of the genes responsible for mismatch repair in humans. Six human genes have been identified to date and they include *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH6/GTBP*, and *hMSH3*.[6–11] The products of these genes are mismatch repair enzymes that function as heterodimers to repair single base pair changes and small insertion/deletions that occur during

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DNA replication. Microsatellite instability has been demonstrated in a wide variety of cancers, including colorectal, endometrial, stomach, urothelial, and lung cancer. Ten to fifteen percent of colorectal carcinomas have microsatellite instability (predominantly due to biallelic inactivation of *hMSH2* and/or *hMLH1*),[4,12–14] whereas a lower rate is typical of most other types of cancer.[14–21] Cancers with microsatellite instability have been shown to have both alleles inactivated of one or more of the mismatch repair genes. Patients with HNPCC inherit an inactive copy of one of these genes, and the second copy is somatically inactivated, whereas patients with sporadic $RER^+$ cancers somatically inactivate both copies. Once both copies of a mismatch repair gene are inactivated, *de novo* mutations are not repaired appropriately and carcinomas evolve relatively rapidly. This altered pathway of tumorigenesis may account for the clinicopathological differences between $RER^+$ and $RER^-$ cancers.[22–24] For example, probably as a result of the rapid accumulation of mutations of cellular antigens, $RER^+$ cancers have been found to elicit a more vigorous peritumor immune response than do $RER^-$ cancers.[22,23]

Colorectal carcinomas occurring in patients with the HNPCC (or Lynch) syndrome are notable for a young age of onset, for occurring predominantly in the right side of the colon, for a high rate of second cancers in the absence of subtotal colectomy, and for a characteristic histological appearance.[1–4,22,23] Although most sporadic colorectal carcinomas are aneuploid, those from patients with HNPCC are typically diploid.[24] The same morphological features found in colorectal carcinomas from patients with HNPCC also occur in sporadic colorectal carcinomas with microsatellite instability[23]; these tumors can show a distinct pattern of genetic alterations when compared with other colorectal tumors.[25,26]

HNPCC families are also at risk for a variety of other cancers, including endometrial, ovarian, urothelial, stomach, intestinal, skin, and pancreas cancers.[1–4,27] For most of the HNPCC-associated extracolonic cancers, studies have not yet been done to determine whether morphological features can be used to distinguish them from sporadic cancers. The prevalence of the mutator phenotype in carcinomas of the pancreas is thought to be low,[28] and the clinicopathological features of $RER^+$ pancreatic adenocarcinomas have not been described. We therefore examined a large series of pancreatic cancers for DNA replication errors. We then correlated the RER status with the histology and karyotype of the cancers, the *K-ras* mutational status of the tumors, and the patient's clinical outcome to determine whether $RER^+$ carcinomas of the pancreas are clinically or pathologically distinct from those without the mutator phenotype.

## Materials and Methods

### Patient Population and Tissue Samples

Normal and tumor specimens were obtained from pancreatic exocrine adenocarcinomas resected at The Johns Hopkins Hospital. The institutional review committee on clinical investigation reviewed and approved the collection of the tissue samples for genetic analysis. The 82 patients had a mean age of 64.5 years. There were 46 males and 36 females. Metastases to regional lymph nodes were identified in 65 of the 82 resected specimens.

Pancreatic carcinoma xenografts were established from the primary cancers as previously described, and carcinoma and normal tissues were stored at −70°C.[29]

### Microsatellite Analysis

Cases were considered to have the mutator phenotype ($RER^+$) when the size of at least two or more of five or more microsatellite loci were altered in the carcinomas compared with constitutional DNA. All cases were screened with the BAT26 microsatellite marker and at least four $(CA)_n$ microsatellite markers, including the D10S579, D10S541 D9S272, D9S258, and D9S1809 markers, to determine RER status as has been previously described.[28]

### Histopathological Review

Three of the eighty-two carcinomas were found to be $RER^+$, and an initial review of the histological features of these three cases by one of us (R.H. Hruban) revealed that all three had a similar histological appearance. We therefore performed a blinded histological review of all 82 cases. All available hematoxylin and eosin (H&E)-stained histological slides were graded by one of us (G.J.A. Offerhaus) without knowledge of the RER status, the *K-ras* mutational status, or the karyotype of the carcinomas. The following parameters were evaluated as has been described.[23] Differentiation was graded based on the formation of glands: in well differentiated cases, more than 90% of tumor cells formed glands; moderately differentiated, 30% to 90%; poorly differentiated, less than 30%. The grading of mucin production was adapted from that used in the colon by Wiggers et al[30]: absent, no extracellular mucin; focal, less than 50% extracellular mucin by area; predominant, greater than 50% of extracellular mucin by area. The invasion pattern was analyzed using modified Jass's criteria for colorectal cancer[31] as infiltrating, expanding, or mixed types. The Crohn's-like lymphoid reaction with discrete lymphoid aggregates, some with germinal centers, and surrounding fibrosis was classified as intense, occasional, or absent according to the criteria of Graham and Appelman.[32] Intratumor lymphocytic infiltration and necrosis were classified by the amount of involved area: absent, no areas of involvement; moderate, 10% to 50% involved; severe, more than 50% involved. In addition, the degree of syncytial growth was graded from 1 (absent) to 4 (uniformly diffuse). Finally, the reviewing pathologist was shown one of the three $RER^+$ cases, the case was replaced, and all 82 cases were graded based on the overall histological similarity of the case being evaluated to the index case from 1 (identical) to 5 (highly dissimilar).

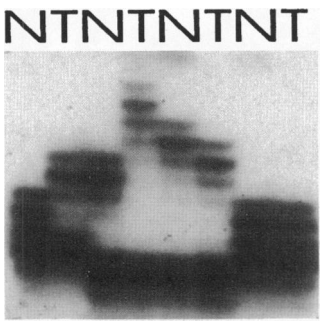


**Figure 1.** Microsatellite instability in adenocarcinoma of the pancreas. The D9S1809 $(CA)_n$ microsatellite marker was resolved by electrophoresis and autoradiography after amplification by PCR in four pairs of normal (N) and tumor (T) DNA. The first three N/T pairs (from left to right) demonstrate microsatellite instability in their tumor relative to their normal DNA and represent $RER^+$ adenocarcinomas of the pancreas. The fourth N/T pair is from a patient with an $RER^-$ adenocarcinoma of the pancreas.

### *Immunohistochemical Staining*

Immunohistochemical studies for cytokeratin (AE1/AE3, Boehringer Mannheim, Indianapolis, IN), for lipase (Chemicon, Temecula, CA), and for chromagranin (Boehringer Mannheim) were performed as previously described.[33]

### *Karyotyping*

Karyotyping was performed as described.[34]

### K-ras *Genotype*

The pancreas carcinomas identified as histologically similar to the index case were analyzed for *K-ras* mutations by cycle sequencing of polymerase chain reaction (PCR) products as previously described.[35]

### *Clinical Follow-Up*

Clinical follow-up was obtained from the Johns Hopkins Pancreas Cancer database. Follow-up was available for all cases.

### *Statistics*

Fisher's exact test was used to compare the occurrences of $RER^+$ or $RER^-$ cases in the pathological groups of pancreas cancer.

## *Results*

### *Microsatellite Analysis*

Three (3.7%) of the eighty-two cases met the criteria for microsatellite instability ($RER^+$). All three cases that were $RER^+$ had microsatellite instability in at least four of six loci (Figure 1).

### *Clinical Findings*

The three $RER^+$ cases (Table 1) included a 71-year-old Caucasian man with a pancreatic adenocarcinoma (5 cm tumor diameter, metastatic to regional lymph nodes) who underwent a Whipple procedure in 1993. His mother had developed kidney and lung cancer; otherwise, the family cancer history was negative. The patient developed two separate colon cancers that were resected in 1996, and he is alive and free of cancer 52 months after his Whipple procedure. Histological examination of this patient's colon carcinomas revealed both to be infiltrating moderately differentiated adenocarcinomas, and both involved the left side of the colon. There were no histological features associated with $RER^+$ colon carcinomas. We attempted to determine the RER status of these colon carcinomas; however, the DNA could not be PCR amplified. The second patient was an 84-year-old Caucasian woman with a 2-cm pancreatic adenocarcinoma (metastatic to regional lymph nodes). She underwent a Whipple procedure in 1995 and died of cancer 4 months after the procedure. Her mother had developed breast cancer at age 42 and a son and a brother had developed lung cancer. Neither the patient nor any of her immediate family had a history of colorectal cancer. The third patient with an $RER^+$ pancreatic carcinoma was a 72-year-old man. His carci-

**Table 1.** Clinical Features of the $RER^+$ and $RER^-$ Pancreatic Carcinomas

| Case | Age (years) | Sex | Tumor size (cm) | Metastasis to nodes* | Survival (months/status) |
|---|---|---|---|---|---|
| Cases resembling the index case | | | | | |
| $RER^+$ cases | | | | | |
| 1 | 71 | Male | 5 | + | 52/ASD |
| 2 | 84 | Female | 2 | + | 4/DOD |
| 3 | 72 | Male | 2.5 | − | 16/ASD |
| $RER^-$ cases | | | | | |
| 4 | 65 | Male | 7 | − | 13/DOD |
| 5 | 55 | Male | 4 | + | 12/ASD |
| 6 | 85 | Male | 8 | − | 7/DOD |
| Cases with typical histology | | | | | |
| $RER^-$ cases ($n$ = 76) | [Mode, 65] Range, 36–85 | | Range, 1.5–14 | | |
| mode | ~65 | 53% Male | | +† | 11‡ |

$RER^+$, microsatellite instability present; $RER^-$, microsatellite instability absent; ASD, alive without disease; DOD, died of disease.
*+, metastasis identified at the time of surgery; −, metastasis not identified.
†Sixty-four $RER^-$ cases had lymph node metastases.
‡Inclusive of only those cases ($n$ = 60) with a minimum follow-up of 1 year.

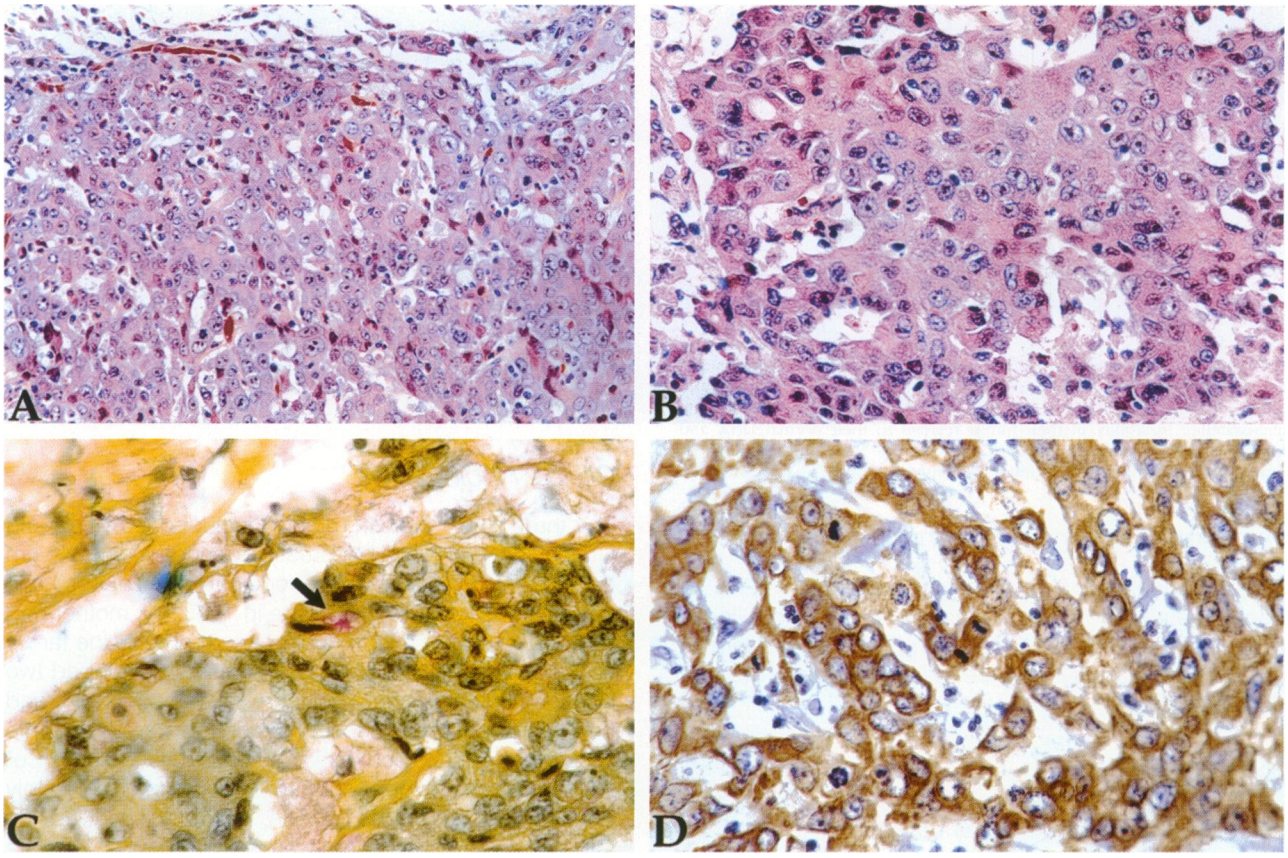


**Figure 2.** Histological appearance of RER$^+$ adenocarcinomas of the pancreas. Note the pushing borders (**A**) and the syncytial growth pattern and poor differentiation (**B**). In two cases, focal mucin production was noted (**C**, **arrow**) whereas in the third it was more extensive. All three cases expressed cytokeratin as detected by immunohistochemistry (**D**). H&E (**A** and **B**); mucicarmine stain for mucin (**C**); immunohistochemical localization of cytokeratin (**D**). All of the figures are from the index case, apart from **D**, which is from patient 3.

noma measured 2.5 cm in diameter, and no metastases were found in resected lymph nodes. He underwent a Whipple procedure in 1996 and has no evidence of disease 16 months after the operation. His sister had breast cancer, but again there was no family history of colorectal cancer.

## *Description of the Tumors*

All three RER$^+$ carcinomas had a similar histological appearance (Figure 2). All three were poorly differentiated and did not form distinct glands. Instead, the neoplastic cells grew as sheets, often with an expanding rather than an infiltrating border (Figure 2A). At higher magnification, the cells were remarkable for a syncytial growth pattern, in which the cell borders were not well defined (Figure 2B). The cytoplasm of the neoplastic cells was basophilic and abundant, and the nuclei showed marked pleomorphism and prominent nucleoli. Necrosis was often extensive. Scattered intraepithelial lymphocytes were observed in all three cases. A mucicarmine stain for mucin was positive in all three cases, in two cases very focally (Figure 2C) and in one case more diffusely. The neoplastic cells expressed cytokeratin as detected by immunohistochemical studies (Figure 2D). In contrast, lipase and chromagranin expression were not detected.

## *Histology*

Of the histological parameters analyzed, those most specific for RER$^+$ were the syncytial growth pattern (7/82, 8.5% of all cases, 100% of RER$^+$ cancers, and 4.8% of RER$^-$ cancers) and an expanding invasion pattern (4/82, 12.2% of all cases, 100% of RER$^+$ cancers, and 1% of RER$^-$ cancers) (Table 2). The single case having an expanding invasion pattern but lacking RER$^+$ was an adenocarcinoma arising from an intraductal papillary adenoma (intraductal papillary mucinous neoplasm). Finally, all 3 of the RER$^+$ carcinomas were classified as closely resembling the index case, whereas only 3 of 79 cases without RER$^+$ were similarly classified ($P = 0.004$, Fisher exact test). This suggests that RER$^+$ carcinomas of the pancreas could be identified on histological grounds with a sensitivity of 100% and a specificity of 50%. A summary of the histological and clinical features of the three RER$^+$ pancreas carcinomas and the remaining RER$^-$ cancers is shown in Table 1.

## *Other Mutations*

The six carcinomas classified as closely resembling the index case were analyzed for *K-ras* mutations, and all results were replicated with independent assays. Two of the six cases had mutant *K-ras*; all three RER$^+$ cases had

**Table 2.** RER$^+$ and RER$^-$ Pancreatic Carcinomas: Pathological Features and *K-ras* Genotype

| Case | % gland formation | Invasion pattern | Syncytial growth (grade 1 to 4)* | % mucin | Lymphoid nodules | % TILs | % necrosis | *K-ras* status | Similarity to index case (1 to 5)† |
|---|---|---|---|---|---|---|---|---|---|
| Cases resembling the index case | | | | | | | | | |
| RER$^+$ cases | | | | | | | | | |
| 1 | <30 | Expanding | 3 | Absent | Absent | <10 | Extensive | Wild type | 1 |
| 2 | <30 | Expanding | 3 | <50 | Absent | <10 | Extensive | Wild type | 2 |
| 3 | <30 | Expanding | 3 | Absent | Occasional | <10 | Extensive | Wild type | 3 |
| RER$^-$ cases | | | | | | | | | |
| 4 | 30–90 | Infiltrating | 2 | <50 | Occasional | 10–50% | Occasional | Mutated | 3 |
| 5 | <30 | Mixed | 3 | Absent | Occasional | <10 | Extensive | Wild type | 2 |
| 6 | <30 | Mixed | 3 | Absent | Intense | 10–50% | Occasional | Mutated | 3 |
| Cases with typical histology | | | | | | | | | |
| RER$^-$ cases ($n$ = 76), mode‡ | 90 | Infiltrating | 1 | <50 | Occasional | 10–50% | Occasional | Mutated | 5 |

RER$^+$, microsatellite instability present; RER$^-$, microsatellite instability absent; TILs; tumor-infiltrating lymphocytes.
*Grade 1, absent; grade 4, extensive.
†Grade 1, identical to index case; grade 5, no similarities to index case.
‡The frequency of RER$^-$ cases with the modal parameter: 90% gland formation ($n$ = 40), infiltrating ($n$ = 69), grade 1 synctial growth ($n$ = 53), percentage mucin <50% ($n$ = 51), occasional lymphoid nodules ($n$ = 44), percent TILs ($n$ = 50), occasional necrosis ($n$ = 49), mutant *K-ras* (90%), similarity to index case 5 (completely dissimilar; $n$ = 65).

wild-type *K-ras* sequences at *K-ras* codons 12 and 13. This prompted us to review a previously published series of pancreas carcinomas from our institution that had been analyzed for *K-ras* mutations.[36] The carcinomas in this series that were wild-type for *K-ras* were reviewed, and among the *K-ras* wild-type cancers were two additional cases closely resembling the index case. Both of these cancers were diploid,[37] and at the time of most recent follow-up, one of the two patients was alive at 10 years after the Whipple operation and the other patient died 6 years after surgery.

One of the three cases in the present series that was RER$^+$ had also been previously analyzed for the presence of *p53*, *DPC-4*, and *p16* genetic alterations.[38] No additional mutations or deletions were identified, an unusual genetic profile for an adenocarcinoma of the pancreas.[38] This tumor was previously reported in error to have a *K-ras* gene mutation.[38]

### *Karyotyping*

One of the three RER$^+$ carcinomas had been karyotyped. The majority of the population was near diploid (composite karyotype 47–51, XY, +7, +11, +12, +21, mar 1, mar 2).

## *Discussion*

Adenocarcinomas of the colon with the mutator phenotype (RER$^+$) are characterized by a distinctive morphology, better patient survival, and a diploid karyotype. Three (3.7%) of the eighty-two adenocarcinomas of the pancreas that we examined were RER$^+$. Remarkably, all three of these carcinomas were poorly differentiated, all had a syncytial growth pattern, and all grew with an expanding rather than an infiltrating border. This distinctive (medullary) histological appearance is rare in RER$^-$ adenocarcinomas of the pancreas[39–41] and resembles that often seen in RER$^+$ colorectal cancers. RER$^+$ colorectal cancers are characterized by poor differentiation, occasional neuroendocrine changes, prominent mucin production, a Crohn's disease-like lymphoid infiltrate, and a medullary growth pattern.[22,23] Unlike RER$^+$ colorectal carcinomas,[22,23] extracellular mucin production and a marked host lymphocytic response were neither prominent nor specific for the RER$^+$ pancreatic cancers. In contrast, gastric cancers with microsatellite instability display prominent T lymphocyte infiltration, intestinal-type morphology, and diploid karyotype and are associated with an improved prognosis.[15–17] Characteristic morphological differences have not been noted in RER$^+$ endometrial,[18,19] urothelial,[20] or lung[21] cancers.

The morphological variant of pancreatic cancer associated with RER$^+$ is not recognized as a distinct entity in the current pathological classification systems of pancreatic neoplasms.[39–41] Recognition of a histologically distinct subgroup of pancreatic cancers associated with a specific molecular change has several practical clinical implications. First, it suggests that this specific phenotype can be used to identify patients with germline mutations in DNA mismatch repair genes. Once identified, such patients could receive the benefit of genetic counseling and testing, and affected carriers could be offered appropriate screening for other HNPCC-associated cancers.[42] Second, the identification of an RER$^+$ carcinoma might affect the type of adjuvant therapy a patient receives, as RER$^+$ cell lines have been shown to be resistant to alkylating agents.[43] Finally, RER status may have an impact on prognosis. Despite the poor differentiation, patients with HNPCC-related colon cancers are thought to have a better stage-specific prognosis than are patients with sporadic colorectal cancer.[44] Similarly, ampullary carcinomas with microsatellite instability have been shown to have a better prognosis than those without microsatellite instability.[45] It is also possible that RER$^+$ pancreatic cancers might be associated with a better prognosis. Indeed, one of the three patients with an RER$^+$ cancer in this series is a long-term survivor (52 months after the Whipple procedure at last follow-up), and the two patients whose medullary carcinomas were

identified by their wild-type *K-ras* status were both long-term survivors (one patient lived 6 years and the second patient is still alive at 10 years after surgery). The good outcome for patients with poorly differentiated medullary carcinomas of the pancreas may explain why previous studies have failed to find a correlation between histological differentiation and prognosis for pancreas cancer. The inclusion of long-surviving, poorly differentiated $RER^+$ pancreatic carcinomas in such studies could have obscured a potentially significant correlation. The reason for the improved prognosis in $RER^+$ colorectal cancers[12,14,46] is not yet known. Decreased vascularity, diploid karyotype, vigorous immune response, and a relative infrequency of p53 mutations might play a role.[23,46]

*K-ras* mutations are present in 80 to 95% of ductal adenocarcinomas of the pancreas.[34,47–49] The absence of *K-ras* mutations in the three $RER^+$ pancreas adenocarcinomas in the present series suggests that *K-ras* is not typically involved in the tumorigenic pathway of $RER^+$ pancreatic cancers. Indeed, by reviewing a previously reported series of pancreatic carcinomas for cases having wild-type *K-ras*, we were able to identify two additional diploid carcinomas with the medullary phenotype.

In summary, DNA replication errors occur in approximately 4% of carcinomas of the pancreas. Poor differentiation and a syncytial growth pattern with pushing borders should suggest the possibility of DNA replication errors in pancreatic cancers. Carcinomas of the pancreas with what we term medullary histology are frequently $RER^+$ and diploid and have wild-type *K-ras* genes, suggesting that these carcinomas arise through a genetic pathway distinct from the more typical ductal carcinomas of the pancreas.

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