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BRCA2 Is Inactivated Late in the Development of Pancreatic Intraepithelial Neoplasia

Evidence and Implications

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Patients harboring germline *BRCA2* mutations are at an increased risk of developing pancreatic cancer. We investigated the prevalence of biallelic inactivation of *BRCA2* in the presumed precursors to invasive pancreatic ductal carcinomas, pancreatic intraepithelial neoplasia (PanIN). Surgical resection specimens from three patients with germline *BRCA2* mutations who developed pancreatic ductal adenocarcinoma were studied. Fourteen PanINs were needle-microdissected from paraffin-embedded tissue. DNA was isolated from these microdissected tissues and amplified by primer-mediated pre-amplification. Loss of heterozygosity at the *BRCA2* locus was determined by polymerase chain reaction amplification and cycle sequencing. The presence of the wild-type alleles was evaluated at the nucleotide positions of the germline *BRCA2* mutations. The *K-ras* gene was sequenced at codon 12 and 13 to confirm the efficacy of microdissection. By histological evaluation the prevalence of PanINs in these patients was not notably elevated. Loss of the wild-type allele of *BRCA2* was present in one high-grade PanIN (PanIN 3), but in none of 13 low-grade PanINs (PanIN 1). In contrast, *K-ras* mutations were detectable in 7 of the 14 PanINs. These results suggest that biallelic inactivation of the *BRCA2* gene is a relatively late event in pancreatic tumorigenesis. In contrast to classical molecular progression models of tumorigenesis, the inactivation of the wild-type allele in a carrier of a recessive tumor susceptibility gene may not always be the first somatic event during the molecular evolution of a cancer. The necessity for earlier genetic alterations before biallelic inactivation of a recessive tumor susceptibility gene such as *BRCA2* may explain why affected carriers have normal numbers of neoplastic precursor lesions, a relatively low phenotypic penetrance, and late age of onset of pancreatic and other cancers. ***(Am J Pathol 2000, 156:1767–1771)***

Hereditary predisposition to adenocarcinoma of the pancreas is clinically evident in approximately 10% of patients who develop the disease.[1–3] Germline *BRCA2* mutations account for a portion of this group, and as many as 5 to 10% of patients with apparently sporadic pancreatic cancer harbor germline *BRCA2* mutations.[4,5] The lifetime risk of pancreatic cancer in carriers of a *BRCA2* germline mutation is, however, probably in the range of 5%.[4–8] The genetic and environmental influences that result in the low penetrance of pancreatic cancer in *BRCA2* mutant carriers are poorly understood. A critical genetic event that would be expected to influence penetrance of pancreatic cancer in these carriers is the occurrence and timing of inactivation of the wild-type *BRCA2* allele in pancreatic epithelium. For example, in patients with familial adenomatous polyposis (FAP), the inactivation of the wild-type allele of *APC* in colonic epithelial stem cells is probably the first genetic alteration (the gatekeeper) during the evolution of a neoplasm. Loss of the wild-type *APC* allele occurs in early adenomas both in FAP patients and the MIN mouse.[9–12] In breast and pancreatic cancers that occur in carriers with germline *BRCA2* mutations, the inactivation of the wild-type allele is usual.[4,13] Yet the timing of loss of the wild-type copy of *BRCA2* has not been studied in neoplastic precursor lesions that develop in patients with germline *BRCA2* mutations, and there are reasons to suspect that *BRCA2* does not follow the gatekeeper model.

To study the timing of *BRCA2* alterations in the development of pancreatic cancer we took advantage of the observation that multiple neoplastic precursor lesions are often present in the pancreata of patients with pancreatic ductal adenocarcinomas.[14–20] These lesions are called pancreatic intraepithelial neoplasia (PanIN; see http://www.path.jhu.edu/pancreas_panin). By determining the frequency of genetic alterations in the PanINs of varying histological severity, one can establish a progression model for the development of infiltrating adenocarcinoma of the pancreas. The histological and genetic analysis of

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Table 1. *BRCA2* Allelic Loss and *K-ras* Gene Mutation in Pancreatic Intraepithelial Neoplasia (PanIN)

Patient	PanIN grade	LOH at *BRCA2*	*K-ras* codon 12
PX101	3	Yes	GAT
	1	No	GAT
	1	No	GAT
	1	No	GGT
	1	No	GGT
	1	No	GGT
	1	No	GGT
PX66	1	No	GAT
	1	No	GGT
	1	No	GAT
	1	No	Not determined
PX182	1	No	GTT
	1	No	GGT
	1	No	GAT

The germline mutations of *BRCA2* in cases PX101, PX66, and PX182 were 2481insT, 6174delT, and 6158insT, respectively. PX101 and PX66 had LOH of *BRCA2* identified in the carcinoma samples.

such neoplastic precursors can provide insights into the genetic progression of pancreatic cancer in individuals with germline *BRCA2* mutations.

We investigated the timing of biallelic inactivation of the *BRCA2* gene in PanINs by analyzing DNA from a series of microdissected PanINs located in the pancreatic parenchyma adjacent to invasive pancreatic carcinomas resected from patients with germline *BRCA2* mutations. DNA from these PanINs was analyzed for loss of the wild-type allele at *BRCA2* and for the presence of *K-ras* gene mutations.

Materials and Methods

Patients with germline *BRCA2* mutations who developed pancreatic cancers were identified previously.[4] From the archives of The Johns Hopkins Hospital, slides of pancreatic resection specimens from these cases with germline *BRCA2* mutations were reviewed. Three cases were selected based on the presence of infiltrating pancreatic adenocarcinoma of the pancreas and the availability of archival material adequate for the study of associated PanINs (Table 1). PanINs were classified using criteria established at the National Cancer Institute-sponsored Pancreatic Cancer Think Tank in Park City, Utah (http://www.path.jhu.edu/pancreas_panin). Briefly, PanINs were classified as grade 1 when duct lesions lacked significant nuclear abnormalities, as grade 2 when duct lesions had moderate cytological and architectural atypia, and grade 3 when those lesions showed marked architectural or cytologic atypia. The histological features were graded by an experienced pathologist (R. H. H.) familiar with the Park City classification scheme before the molecular analysis.

Seven-micron sections from formalin-fixed, paraffin-embedded tissue blocks were stained with hematoxylin and eosin. PanINs, infiltrating carcinoma, and adjacent normal tissue were dissected under direct visualization using an inverted microscope and a glass needle attached to a micromanipulator.[21] Mock dissections were performed on slides lacking tissue, from which the tip of the glass needle was processed for DNA isolation and polymerase chain reaction (PCR) amplification to examine for and rule out contamination during the process of microdissection. DNA was extracted from microdissected tissue using 500 μg/ml of proteinase K and 0.5% NP40 and incubated overnight at 56°C. DNA samples were then subjected to whole genome amplification by primer-mediated pre-amplification (PEP) as previously described.[22] We have previously observed stochastic errors in PCR when amplifying paraffin-embedded DNA when the input DNA is less than ~50 cells, particularly if the PCR products are over 400 bp.[21] Hence, PEP was performed on 300 to 600 microdissected cells and amplified using a degenerate 15-mer for 50 cycles of 92° for 30 seconds, 37° for 2 minutes, and 55° for 4 minutes, ramping at 1° every 10 seconds. The reproducibility of PEP to amplify two alleles equally was determined by PEP amplification of DNA isolated from normal pancreatic acini and subsequently amplifying with PCR primers that specifically amplified the region of DNA spanning the patients' known germline *BRCA2* mutations. These results obtained using PEP were also confirmed by direct PCR amplification and sequencing of microdissected tissues in the absence of PEP. Sequencing of PCR products confirmed that PEP had amplified both *BRCA2* alleles from all 18 samples. PCR was also performed using primers to amplify across the region of the *K-ras* gene containing codons 12 and 13. PCR products were subsequently analyzed by DNA cycle sequencing as described.[23] Loss of heterozygosity (LOH) at the *BRCA2* locus was determined by the absence of the wild-type nucleotide sequence at the site of the germline mutation.

Results

The number and morphology of PanIN lesions were reviewed by obtaining all available hematoxylin-and-eosin-stained archival slides of the pancreatic carcinoma resection specimens from the three patients with known germline *BRCA2* mutations. All three patients had undergone a Whipple resection. There were no observable qualitative or quantitative differences in the number or morphology of the PanINs compared to that seen in patients without germline *BRCA2* mutations.

Three PanINs were selected from case PX182, four PanIN from case PX66, and seven PanIN from case PX101 for microdissection. The PX series comprises unique patients whose carcinomas were expanded by xenografting to allow genetic analysis. These 14 PanINs included 13 PanIN-1 and one PanIN-3. The germline mutations in the three cases were 6158insT, 6174delT, and 2481insT, respectively, as previously described.[4] Loss of the wild-type allele was evident in xenografts of the pancreatic adenocarcinoma in two of the three cases (6174delT and 2481insT).[4] LOH at the *BRCA2* gene locus was present in the single PanIN-3 (from PX101), but in none of 13 low grade duct lesions (PanIN-1; Figures 1 and 2). LOH was not detected in three normal ducts, nor

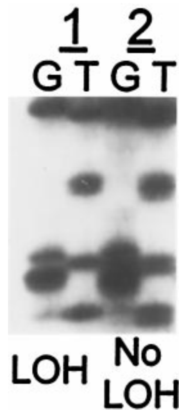

Figure 1. Sequence of the *BRCA2* gene upstream of the 2481insT germline mutation in two PanIN lesions from a patient with pancreas cancer (case PX101). The first duct lesion (lane pair 1, PanIN-3) shows the mutant sequence with loss of the wild-type *BRCA2* sequence, whereas the second lesion (lane pair 2, PanIN-1) shows the sequence of both the wild-type and mutant alleles (note the double bands for each nucleotide, best seen on comparison of the T lanes). G and T refer to deoxyguanosine and deoxythymidine termination reactions, respectively.

was it detected in multiple microdissections of pancreatic acini containing ~1000–2000 cells.

In contrast, distinct *K-ras* mutations were found by sequencing in 7 of the 14 PanINs. All of the *K-ras* mutations were at codon 12; six had GAT mutations, one was GTT. Results are summarized in Table 1. The high signal ratio of mutant to wild-type alleles confirmed the adequacy of the microdissection to exclude non-neoplastic cells. The variety of mutations confirmed the expectations that these lesions would represent independent neoplasms, each with an independent chance to suffer LOH during clonal progression. Thus, the lack of LOH in PX182 (potentially due to a subtle somatic mutation in the other allele) would not preclude the evolution of independent mutations in the PanIN lesions.

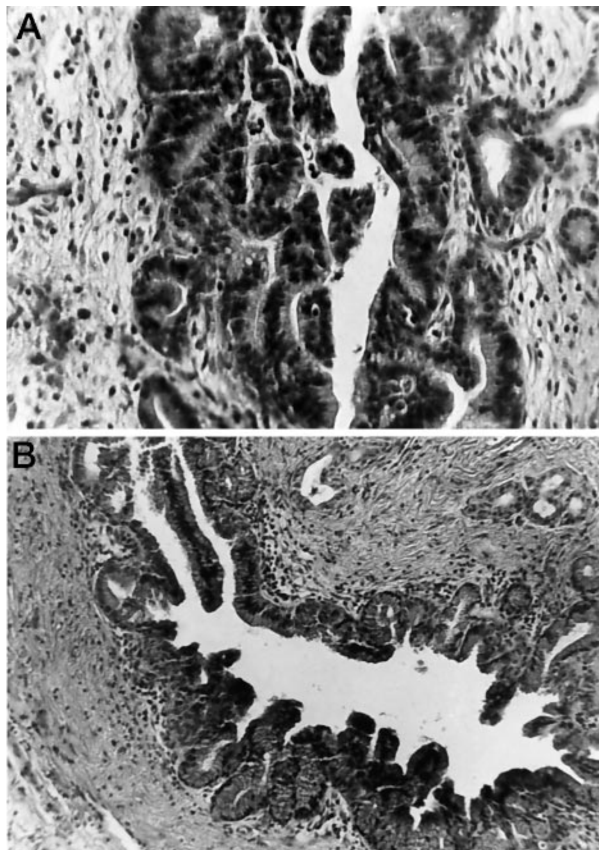

Figure 2. A: The PanIN-3 duct lesion that harbored LOH of the wild-type *BRCA2* gene. **B:** An example of a PanIN-1 duct lesion from patient PX66 that did not have LOH at *BRCA2*. Hematoxylin and eosin; original magnification, ×40.

Discussion

In this study we provide evidence for the biallelic inactivation of the *BRCA2* gene as a late event in the development of adenocarcinoma of the pancreas in patients with germline *BRCA2* mutations. Biallelic inactivation of the *BRCA2* gene was found in only one high-grade PanIN. This low second hit rate of LOH of *BRCA2* in such lesions contrasts with the relative frequency of *K-ras* mutation and *p16* inactivation.[16,24–29] From this data we conclude that *BRCA2* inactivation is generally not the first genetic alteration in PanINs from patients with germline *BRCA2* mutations.

There are a number of alternative conclusions that could be considered, although we do not find them compelling. First, if the PanIN lesions we studied were not clonal, then LOH would not be expected. Pancreatic cancer is thought to occur through the clonal evolution of precursor lesions. Yet the presence of *K-ras* mutations in approximately half of the PanINs analyzed supports the clonal nature of these lesions. The prior demonstration of the genetic inactivation of *p16* in a subset of PanINs also provided strong genetic evidence that PanINs are the precursor lesions for pancreatic adenocarcinoma and that they are clonal.[28,29] Second, the use of LOH as an indicator of biallelic inactivation does not take into account alternative mechanisms of gene inactivation such as intragenic mutation or promoter methylation. Indeed, some occasional cancers in carriers of *BRCA2* mutations lack LOH at *BRCA2*, and one such cancer is included in the current study.[4] Still, the available evidence points to LOH as the main mechanism of biallelic inactivation of the *BRCA2* gene in carcinomas that arise in carriers of *BRCA2* mutations.[13] LOH therefore should be present in the vast majority of PanIN if the early biallelic inactivation of *BRCA2* were critical to the carcinogenic mechanism. Third, one might consider the low rate of observed LOH in microdissected samples to reflect the contamination of samples with non-neoplastic DNA. This DNA could come from two sources; it could theoretically come from non-neoplastic tissues adjacent to the microdissected foci or cells or DNA other than from the patient. The first potential source of DNA contamination was controlled first by ensuring optimal microdissection using a needle micromanipulator with repeat analysis of all samples and then by confirmation of the expected high rate of *K-ras* mutations at the expected high allele ratio. The second potential source of DNA would be readily identified on sequencing as the allele ratios would exhibit a predominance of the

wild-type allele of *BRCA2*, yet this was not observed. Finally, an artifact could derive from the use of PEP, which, in the case of low template copy numbers, could introduce a biased amplification of one allele due to stochastic errors (as when the estimated DNA template number is <50 copies). We confirmed that PEP had amplified both alleles of the *BRCA2* gene equally among a large panel of samples; hence, an artifact of PEP is not a likely explanation for the single LOH event that we identified in a PanIN sample.

The rarity and apparently late onset of biallelic inactivation of *BRCA2* in PanIN contrasts with the timing of *APC* genetic alterations in colorectal adenomas. Biallelic inactivation of *APC* is almost certainly the first genetic event leading to the development of adenomas in carriers of a mutant *APC* gene.[9–12] Another notable difference between the *APC* gene and the *BRCA2* gene is that patients with familial adenomatous polyposis have a greatly increased number of precursor neoplasms; this does not appear to be the case for precursor neoplasms in patients with germline *BRCA2* mutations.[30] This suggests that unlike the *APC* gene in the colon, the *BRCA2* gene is not a gatekeeper for neoplasia. An attractive explanation for the rarity of LOH of *BRCA2* is that other genetic alterations are required before the biallelic inactivation of *BRCA2* can experience favorable selective advantages. In this regard, recent evidence in knockout mouse models suggests that inactivation of the p53 pathway may be such a prerequisite for the survival of embryos that have knockout of *BRCA2*.[31–34]

Two alternate explanations might be entertained. First, epigenetic changes might silence the remaining wild-type gene in some circumstances, Second, *BRCA2* may serve a caretaker rather than a strictly suppressor role by participating in chromosome maintenance functions.[35] For example, inherited mutations in caretakers such as the DNA mismatch repair genes produce few precursor lesions and can manifest the tumor phenotype in the absence of allelic deletions. Yet neither possibility is suggested by the accumulated data regarding *BRCA2* inactivation in carcinomas, wherein LOH is the prevailing (although not universal) means of inactivation of the remaining wild-type copy of *BRCA2*.

The relatively late onset of LOH at *BRCA2* represents an additional manifestation of the Knudson hypothesis. The fact that biallelic inactivation of a hereditary susceptibility gene is not always the first event in cancer progression may help explain the low penetrance and late age of onset of pancreatic cancer in carriers of *BRCA2* mutations[4,5] and the normal number of precursor lesions. Indeed, this paradigm may be a common, yet underappreciated, manifestation of the role of susceptibility genes in cancer predisposition.

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