Germ Line Fanconi Anemia Complementation Group C Mutations and Pancreatic Cancer

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Abstract

Biallelic mutations in Fanconi anemia complementation group genes disrupt DNA repair and result in the complex Fanconi anemia phenotype. In addition, germ line mutations in the BRCA2/FANCD1 Fanconi anemia complementation group gene have also been implicated in predisposition to a number of cancers including pancreatic cancer. The recent identification of FANCC and FANCG mutations in resected pancreatic tumors selected for loss of heterozygosity on chromosome 9, some of which were present in the germ line DNA, suggests that inactivation of these and other Fanconi complementation group genes may contribute to pancreatic cancer. To further assess the relevance of FANCC and FANCG mutations to pancreatic cancer we conducted a mutation screen of these genes in DNA from blood of 421 sequentially collected pancreatic cancer cases diagnosed at the Mayo Clinic. Two truncating FANCC mutations but no truncating FANCG mutations were identified in young onset (<55 years) pancreatic cancer cases with no family history of pancreatic cancer. Both mutations were associated with loss of heterozygosity of the wild-type allele in corresponding pancreatic tumors. In addition, no truncating mutations were identified in germ line DNA from blood of 658 control individuals undergoing routine colonoscopy. Taken together these data support the assertion that inherited mutations in FANCC can predispose to pancreatic cancer. (Cancer Res 2005; 65(2): 383-6)

Introduction

Familial clustering or a family history of pancreatic cancer is a significant risk factor for the disease (1, 2). It has been estimated that 16% of patients with pancreatic cancer have a family history and about 4% have a hereditary predisposition to this disease. Hereditary pancreatic cancer has been associated with germ line mutations in p16 (familial atypical mole syndrome), STK11 (Peutz-Jeghers syndrome), mismatch repair genes, and genes that predispose to the development of chronic pancreatitis. In addition, germ line mutations in the BRCA2 breast and ovarian cancer predisposition gene have been identified in individuals with pancreatic cancer (3, 4), in 17% of pancreatic cancer cases with three or more first-degree relatives with pancreatic cancer (5), and in 12% of cases with two or more first-degree relatives with pancreatic cancer (6). Howlett and colleagues recently identified biallelic BRCA2 mutations in patients from the FANCD1 Fanconi anemia complementation group (7). Because of the known role of BRCA2 mutations in pancreatic cancer development, their findings suggest that disruption of the Fanconi anemia functional pathway can contribute to pancreatic cancer. Thus, it is possible that inactivation of any of the 10 other Fanconi anemia complementation group genes might also be involved in the etiology of pancreatic cancer. Evidence in support of this comes from the recent identification of somatic and inherited mutations of FANCC and somatic mutations in FANCG in a small collection of pancreatic tumors and cell lines with loss of heterozygosity (LOH) at the FANCC and FANCG loci (8). Subsequent analysis of DNA from individuals with pancreatic cancer in families with multiple pancreatic cancers did not identify FANCC and FANCG mutations. This finding suggests that if inherited mutations in FANCC and FANCG predispose to pancreatic cancer, then they are not necessarily associated with a high penetrance for the disease. Interestingly, the same is true for BRCA2 mutations, which are often found in the germ line DNA of patients with pancreatic cancer who lack a family history of cancer.

In this study we sought to clarify the frequency of inherited FANCC and FANCG mutations among patients with pancreatic cancer and to provide genetic data in support of a role for inactivated forms of these genes in predisposition to pancreatic cancer. Our rationale was that identification and characterization of familial pancreatic cancer susceptibility genes is important because individuals at risk in these families may have an elevated lifetime risk of developing this particularly lethal form of cancer and because the hypersensitivity of cells that are deficient in Fanconi anemia pathway function to mitomycin C and other similar DNA-damaging agents (9) may prove useful as a form of therapy for pancreatic cancer.

Materials and Methods

Sample Collection and Processing. Pancreatic cancer cases for this study were identified through an Institutional Review Board–approved Ultrarapid Patient Registry at the Mayo Clinic. Patients were identified and consented to the study through pancreatology and oncology clinics prior to diagnosis and were asked to provide blood samples, to complete risk factor questionnaires, and to provide access to medical records and archived tumor tissues. Approximately 76% of patients with subsequently confirmed pancreatic adenocarcinoma were consented to the registry. Of these, 35% were male, 95% were Caucasian, and 45% were nonsmokers. For this study, we utilized 421 sequentially collected cases, including 389 with adenocarcinoma and 32 with intraductal papillary mucinous neoplasia (Table 1). The average age of diagnosis of these cases was 65.6 years and patients ranged in age from 38 to 88 years. Genomic DNA was extracted from the blood samples by standard techniques in the biospecimen accession and processing core of the Mayo Clinic Comprehensive Cancer Center. In addition, blood samples were collected from 654 control individuals undergoing routine colonoscopy at the...
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Mayo Clinic. These individuals had no personal history of colon or pancreatic cancer and had a mean age of 59 years at screening (Table 1). Genomic DNA was extracted as described above.

Mutational Analysis. Coding regions and exon/intron boundaries of the FANCC and FANCG genes were PCR amplified using previously described primers (8), and an additional primer set for exon 12 of FANCC. Briefly, 20 ng of genomic DNA template from each of the 421 pancreatic patients and 654 normal colonoscopy controls, and 1.25 units of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA) was used in 20 µL PCR reactions on an MJ Research PTC-200 thermocycler (MJ Research, Waltham, MA) according to the manufacturer’s instructions. PCR products were denatured for 5 min at 95°C, reannealed at 65°C, and heteroduplexes were evaluated for alterations by WAVE denaturing high-performance liquid chromatography analysis (dHPLC, Transgenomics Inc., Carpentaria, CA) using PCR product specific melting temperatures and solvent gradients. Samples showing abnormal elution profiles were reamplified from genomic DNA and the products were sequenced in the Mayo Clinic DNA Sequencing Facility. As the sensitivity of the dHPLC technique is not 100%, it is possible that mutations in the FANCC and FANCG genes may have been missed. However, studies of other genes have placed the sensitivity of dHPLC at about 95%, so we approximate that 95% of FANCC and FANCG mutations were identified in this study.

LOH Analysis of Pancreatic Tumors. Paraffin block specimens of the pancreatic tumors from the patients with inherited truncating FANCC mutations were available from the Mayo Clinic Pancreatic Specialized Programs of Research Excellence (SPORE) tumor registry. Use of these samples was approved by the Mayo Clinic Institutional Review Board. Following H&E analysis, 0.6-mm cores of tumor were taken from the paraffin blocks to enrich for tumor cells and genomic DNA was prepared by the biospecimens accession and processing core of the Mayo Clinic Comprehensive Cancer Center. Tumor DNA samples were PCR amplified using primers flanking the exons containing the mutations in the corresponding germ line DNA samples. Products were separated both by gel electrophoresis and by dHPLC using standard methods and conditions and the absence of the wild-type allele in the tumor DNA relative to the germ line DNA were noted.

Results

FANCC and FANCG Mutation Analysis. As noted above, DNA samples from 421 sequentially collected pancreatic cancer cases were screened for the presence of mutations in the entire coding regions and the flanking splice sites of the FANCC and FANCG genes by dHPLC. Two truncating mutations in the FANCC gene (322delG and 1246delAAGC) were identified (Table 2). These were associated with an early age of pancreatic cancer onset of 40 and 55 years, respectively. Neither patient reported a family history of pancreatic or other cancer. Eight independent missense mutations were detected, six of which were found only once in the 421 samples (Table 2). The influence of these mutations on FANCC function is not known. No truncating mutations were detected in the FANCG gene in the 421 samples. A single T297K missense mutation was also detected. This mutation alters the same residue as the T297I variant, which has been identified in a patient with Fanconi anemia, but is not thought to inactivate FANCG because it was found on the same allele as a truncating mutation (10).

FANCC Mutation Analysis in Controls. The entire FANCC gene was screened for mutations in 654 DNA samples from individuals undergoing routine colonoscopy in an effort to determine the frequency of FANCC truncating mutations in the general population. No truncating mutations were identified. Five missense mutations were detected and four of these corresponded to missense mutations detected in the patients with pancreatic cancer. Thus, four missense mutations in the cases were not detected in controls. The controls were not screened for FANCG mutations because of the absence of FANCG truncating mutations in the cases.

FANCC LOH Analysis in Pancreatic Tumors. Pancreatic tumor paraffin block specimens from the patients with the truncating FANCC mutations were obtained from the Mayo Clinic Pancreatic SPORE Tumor Registry. Cores of tumor tissue were removed from the blocks and extracted genomic DNA was used as template for amplification of exons 1 and 9 that contain the observed truncating mutations. Analysis by dHPLC showed that the normal alleles were absent in both cases, indicating the presence of LOH in the tumors.

Discussion

Two truncating mutations within the FANCC gene were identified in DNA from blood samples from a cohort of 421 sequentially collected cases of pancreatic cancer. Both mutations inactivate the FANCC protein. Of these mutations, the 1246delAAGC mutation has never been reported before, whereas the 322delG mutation has been frequently observed in patients with Fanconi anemia. This mutation is associated with a lower number of somatic abnormalities in cells and a lesser clinical severity than other Fanconi anemia or FANCC mutations (11). Whereas the truncating mutations were in the heterozygous form in the DNA from the patient’s blood, the corresponding tumors displayed hemizygosity due to LOH. The biallelic inactivation of

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pancreatic cancer cases (n = 421)</th>
<th>Normal colonoscopy controls (n = 654)</th>
</tr>
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<tbody>
<tr>
<td>Gender (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>58</td>
<td>53</td>
</tr>
<tr>
<td>Female</td>
<td>42</td>
<td>47</td>
</tr>
<tr>
<td>Mean age of onset (y)</td>
<td>65.6</td>
<td>NA</td>
</tr>
<tr>
<td>Mean age of ascertainment (y)</td>
<td>65.6</td>
<td>59</td>
</tr>
<tr>
<td>Tumor histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>389</td>
<td>NA</td>
</tr>
<tr>
<td>IPMN</td>
<td>32</td>
<td>NA</td>
</tr>
</tbody>
</table>

NOTE: Abbreviations: NA, not applicable; IPMN, intraductal papillary mucinous neoplasia.
FANCC in these tumors supports the assertion by van der Heijden and colleagues (8) that mutations in FANCC can predispose to pancreatic cancer. Further support comes from our inability to detect truncating mutations in 654 control individuals with no personal history of cancer undergoing routine colonoscopy at the Mayo Clinic. This is consistent with the estimation that only 1 in 3000 control individuals should carry a FANCC truncating mutation, because 1 in 600 alleles in the general population are thought to carry Fanconi anemia mutations (12), and mutations in FANCC have been associated with 10% of all Fanconi anemia complementation groups (11, 13). Given that we identified two mutations in 421 individuals with pancreatic cancer, the frequency of observed mutations is considerably higher than the frequency of expected mutations. The combination of the mutation data from the cases and controls and the LOH in the tumors strongly suggests an association between germ line controls and the LOH in the tumors strongly suggests an association between germ line FANCC mutations and susceptibility to pancreatic cancer, although the number of events is too low to statistically confirm this finding. However, if one accepts this evidence, it is clear that only a small proportion of pancreatic cancers may arise due to inherited FANCC mutations.

In the course of the study we also identified four missense mutations in the FANCC gene in pancreatic cancer cases that were not present in controls. Corresponding pancreatic tumor specimens were not available for assessment of LOH. Each variant was only detected once in the cases. Two of these (E85G and G139E) were previously detected in patients with Fanconi anemia (14) and have been excluded as FANCC-inactivating mutations. However, no information was available for the remaining missense variants (F275L and G401R). In the future, it may be possible to establish the relevance of these mutations to pancreatic cancer and Fanconi anemia using functional assays of the wild-type and mutant FANCC protein. Several intronic variants were also identified. None were predicted to alter consensus splice sites or to generate cryptic splice sites, suggesting no influence on the FANCC protein.

Unlike with FANCC, we failed to identify FANCG mutations in the pancreatic cases in this study, suggesting that predisposition to pancreatic cancer is rarely if ever associated with FANCG mutations. Indeed, the one FANCG mutation that has previously been associated with pancreatic cancer is present in the Hs766T cell line (8) and may represent a somatic rather than an inherited mutation. Thus, FANCG mutations do not seem to predispose to pancreatic cancer, but it remains possible that somatic FANCG mutations may contribute to pancreatic tumor progression.

Although individuals with Fanconi anemia have not been shown to have an increased frequency of pancreatic cancer (9, 15), it remains possible that the association has just not been noticed due to the apparent low penetrance of FANCC and BRCA2 mutations. Furthermore, the late age of onset of pancreatic cancer suggests that most patients with Fanconi anemia may not survive the other malignancies associated with their disease to an age when pancreatic cancer can become prevalent.

Importantly, neither truncating mutation detected in this study was associated with a family history of pancreatic or other cancers, suggesting that these mutations are associated with a low penetrance of disease. This is in keeping with previous observations showing that individuals with pancreatic tumors that carry germ line BRCA2 mutations rarely have a family history of pancreatic, breast, or ovarian cancer (3), and that individuals from high-risk pancreatic cancer families do not seem to carry FANCC and FANCG mutations (16). In addition, both truncating mutations were associated with young-onset pancreatic cancer (<55 years). In fact, these mutations were found in 2 of the 50 patients in the cohort with young-onset pancreatic cancer. As predisposition to pancreatic cancer due to mutations in BRCA2, CDKN4, and HNPCC genes is associated with early onset disease, the finding that the individuals with the FANCC truncating mutations also developed early-onset disease further implicates these mutations in predisposition to this form of cancer. Furthermore, when considering the early age of onset

<table>
<thead>
<tr>
<th>FANCC exon</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>No. of cases (n = 421)</th>
<th>No. of controls (n = 654)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1</td>
<td>322delG</td>
<td>L45X</td>
<td>1</td>
<td>0</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>332C&gt;T</td>
<td>S26F</td>
<td>4</td>
<td>5</td>
<td>(18)</td>
</tr>
<tr>
<td></td>
<td>191C&gt;T</td>
<td>S' UTR</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>433G&gt;A</td>
<td>V60I</td>
<td>2</td>
<td>5</td>
<td>(19)</td>
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<tr>
<td>3</td>
<td>509A&gt;G</td>
<td>E85G</td>
<td>1</td>
<td>0</td>
<td>(14)</td>
</tr>
<tr>
<td>4</td>
<td>671G&gt;A</td>
<td>G139E</td>
<td>1</td>
<td>0</td>
<td>(14)</td>
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<tr>
<td></td>
<td>IVS3-47 T&gt;C</td>
<td></td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>960C&gt;T</td>
<td>M235M</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1022A&gt;G</td>
<td>F256R</td>
<td>0</td>
<td>1</td>
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<tr>
<td></td>
<td>1071C&gt;T</td>
<td>E272I</td>
<td>0</td>
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<td>(19)</td>
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<tr>
<td></td>
<td>1072G&gt;A</td>
<td>E273K</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1078T&gt;C</td>
<td>F275L</td>
<td>1</td>
<td>0</td>
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<tr>
<td></td>
<td>IVS7 + 4 C&gt;T</td>
<td></td>
<td>1</td>
<td>0</td>
<td>(20)</td>
</tr>
<tr>
<td>8</td>
<td>IVS8 + 6</td>
<td></td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1246delAAGC</td>
<td>L368X</td>
<td>1</td>
<td>0</td>
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<td>10</td>
<td>IVS9-3 C&gt;T</td>
<td></td>
<td>0</td>
<td>1</td>
<td></td>
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<tr>
<td>12</td>
<td>1456G&gt;A</td>
<td>G401R</td>
<td>1</td>
<td>0</td>
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<tr>
<td>13</td>
<td>1597G&gt;A</td>
<td>V488M</td>
<td>1</td>
<td>1</td>
<td>(19)</td>
</tr>
</tbody>
</table>

NOTE: Abbreviation: UTR, untranslated region.
of pancreatic cancer caused by inherited $BRCA2$ mutations (4), it seems likely that the inherited mutations in the Fanconi anemia functional pathway account for a significant proportion of unselected young-onset cases of this disease. Thus, it is reasonable to suggest that additional studies may identify mutations in other Fanconi anemia genes and further clarify the role of the Fanconi anemia pathway in predisposition to pancreatic cancer. As all Fanconi anemia cells including $BRCA2$/$FANCD1$-deficient cells are hypersensitive to DNA cross-linking agents such as mitomycin C and cisplatin, it may also be possible to treat pancreatic tumors associated with $BRCA2$, $FANCC$, or other Fanconi anemia mutations with these or other DNA-damaging agents (17).

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