Pancreaticobiliary Cancers With Deficient Methylenetetrahydrofolate Reductase Genotypes

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Background & Aims: **Methyl group deficiency might promote carcinogenesis by inducing DNA breaks and DNA hypomethylation. We hypothesized that deficient methylenetetrahydrofolate reductase (*MTHFR*) genotypes could promote pancreatic cancer development.** ***Methods:*** **First, we performed a case-control study of germline *MTHFR* polymorphisms (C677T, A1298C) in 303 patients with pancreatic cancer and 305 matched control subjects. Pancreatic neoplasms frequently lose an *MTHFR* allele during tumorigenesis; we hypothesized that such loss could promote carcinogenesis. We therefore evaluated the cancer *MTHFR* genotypes of 82 patients with pancreaticobiliary cancers and correlated them to genome-wide measures of chromosomal deletion by using 386 microsatellite markers. Finally, *MTHFR* genotypes were correlated with global DNA methylation in 68 cancer cell lines.** ***Results:*** **Germline *MTHFR* polymorphisms were not associated with an increased likelihood of having pancreatic cancer. Fractional allelic loss (a measure of chromosomal loss) trended higher in cancers with 677T genotypes than in cancers with other genotypes ($P = .055$). Among cancers with loss of an *MTHFR* allele, cancers with 677T *MTHFR* alleles had more deletions at folate-sensitive fragile sites (36.9%) and at tumor suppressor gene loci (68.5%) than 677C cancers (28.7% and 47.8%, $P = .079$ and .014, respectively). LINE1 methylation was lower in cancers with less functional 677T/TT genotypes (24.4%) than in those with 677CT (26.0%) and CC/C genotypes (32.5%) ($P = .014$).** ***Conclusion:*** **Cancers with defective *MTHFR* genotypes have more DNA hypomethylation and more chromosomal losses. Deficient MTHFR function due to loss of an *MTHFR* allele by an evolving neoplasm might, by promoting chromosomal losses, accelerate cancer development.**

Pancreatic ductal adenocarcinoma remains one of the deadliest cancers. The molecular pathogenesis of pancreatic cancer is increasingly well characterized. Many genetic and epigenetic alterations arise during pancreatic tumorigenesis including oncogene activation by mutation (K-*ras*,[1] *BRAF*), amplification (*c-myc, AKT*[2]), and inactivation of suppressor genes (*p16*,[3] *p53*,[4] *SMAD4*, *BRCA2*,[5] *STK11*, *hCDC4*, *MKK4*, and *Fanconi anemia genes*[6]).[2] In addition, many genes undergo silencing in pancreatic neoplasms by methylation.[2,7–9] Most pancreatic carcinomas harbor chromosomal instability[2,10,11]; only a few harbor microsatellite instability.[12] Pancreatic adenocarcinomas exhibit high levels of allelic loss, a feature that has been independently associated with poor histologic differentiation[11] and poor survival.[13]

Smoking, aging, obesity, and diabetes mellitus are well-known risk factors of pancreatic cancer,[14] and nutritional factors such as methyl group availability might also influence pancreatic carcinogenesis.[14–17] Folate deficiency lowers the concentration of *S*-adenosylmethionine, reducing global DNA methylation as well as synthesis of thymidine from uracil. Uracil misincorporation in place of thymidine leads to an imbalanced nucleotide pool and increased occurrence of DNA strand breaks,[18] which increases genomic instability[19] and is thought to contribute to cancer development. Epidemiologic studies have implicated low folate status with the development of other cancers, but this relationship is less studied in the pancreas.[14,15,20,21]

Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme in folate metabolism that converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.[22] The latter form of folate is used for the re-methylation of homo-

Abbreviations used in this paper: CHLC, Cooperative Human Linkage Center; CI, confidence interval; COBRA, combined bisulfite restriction analysis; FAL, fractional allelic loss; LOH, loss of heterozygosity; MTHFR, methylenetetrahydrofolate reductase; OR, odds ratio; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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cysteine to methionine, catalyzed by vitamin B_{12}-dependent methionine synthase. If not reduced to 5-methyltetrahydrofolate by MTHFR, 5,10-methylenetetrahydrofolate can transfer its methylene group to deoxyuridine monophosphate in the synthesis of deoxythymidine monophosphate, or it might contribute to purine synthesis. The *MTHFR* gene is polymorphic, with single nucleotide variants at nucleotide 677 (C→T) and nucleotide 1298 (A→C). The 677T polymorphism encodes a thermolabile enzyme with reduced activity[23,24] (more reduced than 1298C), decreasing enzyme activity by ~50%.[25] Individuals with 677TT alleles have higher serum homocysteine concentrations than 1298CC allele carriers and have homocysteine levels more sensitive to serum vitamin B_{12} and folate.[26] Thus, individuals with 677TT genotypes retain ~25%–33%[23,27] of MTHFR function relative to individuals with 677CC genotypes. Reduced MTHFR function leads to a shift in the distribution of forms of folate at the expense of 5-methyltetrahydrofolate,[28] elevated plasma homocysteine,[22,23,29] decreased serum folate,[29] and global DNA methylation.[30] Thus, for a given folate level, the *MTHFR* 677T allele reduces methyl group availability but increases thymidine synthesis. Approximately 50%–60% of the US population are 677CC, ~30%–40% are 677CT, ~10% are 677TT,[24,27,29,31–33] and 6%–~12% of the population have the 1298C polymorphism.[27,31,33]

Several studies have reported that carriers of germline *MTHFR* 677TT genotypes have altered cancer risks. Some studies have found an increase in cancer risk among 677TT carriers; others have found a decreased risk or no effect. For example, 677TT carriers have an increased risk of cervical neoplasia but a reduced risk of colorectal cancer[20] and acute lymphocytic leukemia.[31,34] The apparently contradicting increased or decreased risk of different cancers with *MTHFR* genotypes is thought to be related to the relative importance of ensuring sufficient methyl group availability versus optimal thymidine production in that cell type and also on dietary methyl group availability. The cancer risk associated with *MTHFR* genotype is influenced by folate and vitamin B_{12} status,[29,35] as well as age,[22] smoking,[29] and alcohol consumption, with smoking and alcohol able to directly adversely affect folate metabolism probably by multiple mechanisms.[22,35]

We carried out a case-control analysis to determine whether defective *MTHFR* genotypes are more frequent in individuals who develop pancreatic cancer than in non-cancer control subjects. In addition, we hypothesized that pancreatic neoplasms with loss of heterozygosity at *MTHFR*, and particularly those retaining only a 677T allele, would exhibit less methylation of DNA and greater chromosomal losses than cancers that had retained both *MTHFR* alleles. We also investigated the relationship between the *MTHFR* status and genome-wide measurements of chromosomal losses in pancreaticobiliary cancers and determined if there was any relationship between *MTHFR* genotype and the level of global DNA methylation in a series of cancer cell lines.

Materials and Methods

Case-Control Study

Cases were 333 patients who underwent pancreaticoduodenectomy for treatment of primary pancreatic adenocarcinoma at Johns Hopkins Hospital from 1993–1999. Control subjects (identified by using the Johns Hopkins Hospital Surgical-Pathology Database, n = 333) had undergone cholecystectomy for benign gallbladder disease at Johns Hopkins Hospital through 1999. Control subjects were frequency-matched to cases by gender, self-reported ethnicity, and 5-year age group. Diabetes status was recorded for all control subjects and 270 cases and smoking status from 302 control subjects and 268 cases. Cases and control subjects with a history of cancer (except non-melanoma skin cancer) were excluded. This study was approved by the Johns Hopkins Committee for Clinical Investigation.

The presurgical anesthesiologists' assessments in the medical record provided information on diabetes, cigarette smoking, and cancer history. The duration of diabetes before pancreatic cancer diagnosis was grouped as 2 or more years, <2 years, and uncertain date of onset. Cigarette smoking quantity (packs per day), duration (years), and time since quitting were recorded. Those who reported quitting within 12 months of their surgery were considered current smokers. We alternatively categorized cigarette smoking history into 2 categories, "ever smoker" and "never smoker." Frozen or paraffin-embedded specimens of tumor-free duodenum provided DNA for case genotypes. Control genotypes were ascertained by using DNA from paraffin-embedded gallbladder samples.

Pancreaticobiliary Cancers Analyzed for Their Genetic Losses and *MTHFR* Status

Cancer xenograft DNA[36] and corresponding germline DNA (from normal duodenum) were analyzed for *MTHFR* genotypes from 82 patients with pancreatic or biliary cancer. These patients had their cancer surgically resected with curative intent between 1992 and 1997 as described elsewhere.[37] The proportion of the cancer genome with deletions (the % of markers deleted/markers analyzed, also known as the fractional allelic loss [FAL]) was determined from each cancer by using 386 microsatellite markers (modified CHLC version 9 marker set, average spacing of 10 centimorgans) as previously described in collaboration with the Center for Inherited Disease Research.[38] Seventy-four of the 82 cancers were of pancreatic origin, and 8 were of biliary origin. Sixty-one of these 74 patients were included in the population described above