Polymorphisms of 5,10-Methylenetetrahydrofolate Reductase (MTHFR C677T) and Thymidylate Synthase Enhancer Region (TSER) as a Risk Factor of Cholangiocarcinoma in a Korean Population

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Abstract. Background: 5,10-Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism, plays a major role in the provision of methyl groups for DNA methylation; thymidylate synthase (TS) is a rate-limiting enzyme in the synthesis of dTMP and DNA repair. The clinical role of genetic polymorphisms of MTHFR and that of the TS enhancer region (TSER) were demonstrated in several clinical studies with colorectal, esophageal, gastric and breast cancer. However, there have never been any studies on the association between cholangiocarcinoma (CCC) and genetic polymorphisms of MTHFR and TSER. Therefore, the polymorphism of MTHFR and TSER, which share a common substrate, 5,10-methylenetetrahydrofolate, in CCC was examined, concurrently. The influence of these polymorphisms on plasma homocysteine levels was also investigated. Patients and Methods: Blood samples were obtained from 47 patients with CCC and 204 healthy control donors. Using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP), the C to T transition at position 677 of MTHFR and tandem repeat of 28bp in the enhancer region of TS gene were analyzed. Plasma homocysteine levels were also determined. Results: According to the logistic regression model, a combination of MTHFR 677CC with the TSER 2R(+) genotype had a relative risk of 5.38 (95% CI, 1.23-23.56) of developing CCC compared to MTHFR 677CC with TSER 2R(–) (p=0.0257). The level of homocysteine was lower in CCC patients than healthy controls without statistical significance (8.27 ± 4.17 vs. 9.40 ± 2.57, p=0.093). Conclusion: Our data suggest a role of MTHFR 677CC with the TSER 2R(+) genotype in increasing the risk of CCC. This study is the first to suggest an association between CCC and the polymorphisms of MTHFR and TSER.

Cholangiocarcinoma (CCC) is an increasing malignancy worldwide and is the 7th common cause of death among malignancies in Korea. Unfortunately, most CCC patients do not have a chance of curative resection. In addition, other treatment modalities, such as chemotherapy, radiotherapy and recently photodynamic therapy with metal stent insertion, did not improve the mortality rate. Furthermore, clinical tools for early diagnosis of CCC are not yet available. Recent advances in molecular investigations of CCC revealed a number of genetic alterations, including loss of heterozygosity at loci on chromosome 1p, 6q, 9p, 16q, and 17p (1), activating point mutations in the K-ras oncogene (2), and p53 mutations (3). Expression of some genes are high in CCC, such as matrix metalloproteinase (4), vascular endothelial growth factor (5), COX-2 and c-erbB-2 (6) and IL-6 (7) is also demonstrated. However, no definite cholangiocarcinogenesis has been suggested yet.

Single nucleotide polymorphism (SNP) emerges as a hot issue in the area of carcinogenesis of several malignancies, such as colorectal cancer, breast cancer and endometrial cancer. SNPs have been used as markers of cancer risk and several have been used to elucidate new biological pathways of cancer development. Recently, several SNPs have been introduced as important prognostic indicators for cancer patients and as promising pharmacogenetic determinants of response to

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cancer chemotherapy. Of which, methylenetetrahydrofolate reductase (MTHFR) was focused on as an ideal candidate for investigating the role of SNPs in the pharmacogenetics of cancer chemotherapy because of its biochemical effect on intracellular folate composition. The thymidylate synthase (TS) enhancer region (TSER) has also received attention because TS competes with the MTHFR for 5-methyltetrahydrofolate as substrate for thymidylate synthesis (8, 9).

Polymorphism of MTHFR in 677 C to T decreases the risk of colorectal cancer that may be modified by dietary habits and life style (10, 11), acute leukemia (12) and increases the risk of cervical intraepithelial neoplasia (13, 14), esophageal (15), endometrial (16), stomach (17), and breast cancer (18, 19). Hyperhomocystinemia as a result of MTHFR C677T, has been reported as a risk factor of carcinogenesis (20). Polymorphism of TSER is associated with an increased risk of colorectal cancer (21, 22) and the 2R2R, 2R3R genotypes of TSER show longer survival compared with 3R3R in gastric cancer (23).

However, there is no available data for the association between genetic polymorphism and risk of cholangiocarcinoma. Therefore, the association between polymorphism of MTHFR C677T, TSER and cholangiocarcinogenesis was investigated and also the plasma homocysteine levels were measured.

Patients and Methods

Study population. We enrolled forty seven CCC patients (mean age ± SD, 66.34 years ± 11.21 years; range 40 to 83 years) diagnosed at Bundang CHA General Hospital, Pochon CHA University, Seongnam, South Korea, from January 2001 to April 2004, and 204 healthy control donors (mean age ± SD, 46.13 years ± 14.64 years; range, 24 to 85 years). In 47 CCC patients, twenty had common bile duct cancer, fourteen Klatskin tumor, twelve peripheral CCC, and one gall bladder cancer. For control subjects, we selected healthy individuals from those presenting at our hospitals for a health examination, which included biochemistry and electrocardiogram; they also were free from a recent or past history of cerebrovascular disease, myocardial infarction, habitual abortion or malignancies. Baseline demographic data and a history of conventional vascular risk factors were obtained from each control subject. Exclusion criteria were the same as those used in the patient group, as mentioned above. The institutional review board (IRB) of Bundang CHA General Hospital approved this genetic study in December 2000. All the patients and controls were Korean and gave informed consent prior to enrollment in the study.

Molecular analyses. All samples were collected and processed using a standard protocol. Venous blood samples were collected in tubes containing trisodium EDTA and applied to genomic DNA extracting columns, according to the manufacturer's protocol. Genomic DNA was extracted using QIAamp blood kit (Extraction column, QIAamp blood kit, Qiagen, Hilden, Germany) following the manufacturer's instruction and MTHFR genotypes were determined using PCR-RFLP, as described previously (18, 24). Amplified fragments were digested using HinfI, which recognizes 677C to T substitution of MTHFR. This one nucleotide substitution corresponds to the conversion of alanine to valine in the C677T MTHFR encoding region. After electrophoresis on 3% agarose gel, the results were interpreted after staining with ethidium bromide.

Genotyping for the TSER polymorphism was carried out based on a method modified from that of Villarfanca et al. (21). Primers with the sequences 5'-GCC GGA CGG CGG AGA 3' (sense) and 5'-TCC GAG CCG GCC AGG CAT GCC GCG G 3' (antisense) were used in PCR reactions. PCR products were size-fractionated on 4% agarose gels. Total homocysteine levels were determined in patients and controls using fluorescence polarization immunoassay (FPIA) based on methods described previously (25, 26).

Statistical analyses. Relative risk of cholangiocarcinoma and 95% CI were calculated using conditional logistic regression. In order to examine influences of MTHFR C677T and TSER genotypes on plasma homocysteine level, ANOVA was used to calculate age adjusted geometric means for these biomarkers within each stratum of the MTHFR C677T and TSER genotypes, followed by comparison with a trend test.

Results

The distribution of MTHFR and TSER genotypes in both group is shown in Table I. No statistically significant differences were noted among MTHFR 677CC, CT, TT and TSER 2R2R, 2R3R, 3R3R genotypes. Because TS competes with MTHFR for 5-methyltetrahydrofolate as substrate, the association of combined MTHFR-TSER genotypes and plasma homocysteine levels was examined. When combined, MTHFR 677CC with TSER 2R(+) genotype (2R2R, 2R3R, 2R5R) had a relative risk of 5.38 (95% CI, 1.227-23.561) of developing CCC compared to MTHFR 677CT with TSER 2R(–) genotype (3R3R, 3R4R, 3R5R) (p=0.0257, Table II). No statistical difference was seen between healthy controls and CCC patients for genotypes of MTHFR and TSER.

The level of homocysteine was lower in CCC patients than in healthy controls without being statistically significant (8.27 μmol/L ± 4.17 μmol/L vs. 9.40 μmol/L ± 2.57 μmol/L, respectively, 0.093; Table III). But homocysteine levels were significantly lower in CCC patients with the 677CT genotype of MTHFR (7.58 μmol/L ± 2.61 μmol/L vs. 9.12 μmol/L ± 2.12 μmol/L, p=0.0145), 2R(–) genotype of TSER (8.21 μmol/L ± 3.66 μmol/L vs. 9.56 μmol/L ± 2.76 μmol/L, p=0.0461) and MTHFR 677CT with TSER 2R(+) (6.36 μmol/L ± 1.77 μmol/L vs. 8.7 μmol/L ± 2.0 μmol/L, p=0.0368).

Discussion

MTHFR plays a central role in DNA methylation, synthesis and repair. Polymorphism of the MTHFR gene is associated with changes in the cellular composition of folates critical for DNA synthesis, DNA repair and epigenetic regulation.
Low enzyme activity resulting from MTHFR C677T polymorphism may reduce the capacity for DNA methylation, and possibly reduce uracil misincorporation into DNA. The 677TT genotype has about 30% of the MTHFR enzyme activity of the wild-type (CC genotype), whereas the 677CT genotype has about 65% that of normal enzyme activity (27). Several malignances are reported to have an association between polymorphism of MTHFR C677T and risk and susceptibility to chemotherapy of cancers. Our results showed no definite association between MTHFR C677T polymorphism alone and risk of CCC.

The TS gene, located on chromosome 18p11.32, codes for a critical enzyme maintaining a balanced supply of deoxynucleotides required for DNA synthesis and repair (28). Several polymorphisms in the TS untranslated regions (UTRs), which may influence TS mRNA transcription, message stability or protein expression, have been described recently; one is a unique tandem repeat polymorphism identified in the 5'-UTR enhancer region of the thymidylate synthase (TS) promoter, which contains either triple (TS*3R) or double (TS*2R) repeats of 28bp sequence [29]. Polymorphism of TSER is associated with increased risk of colorectal cancer (21, 22). Ishida et al. (23) reported that the 2R2R and 2R3R genotypes are associated with longer survival in gastric cancer than the 3R3R genotype. A study with 52 colorectal cancer patients reported that triple repeat homozygous (3R3R) shows higher TS mRNA expression compared to double repeat homozygous (2R2R) (29). Recently, Obama et al. (30) reported 83% of CCC cases showed up-regulation of TS in microarray data, suggesting unfavorable response to 5-fluorouracil-based chemotherapy against CCC. The overexpression of TS mRNA may result from the TSER 3R3R genotype suggesting the possibility of TSER polymorphism as a risk factor in cholangiocarcinogenesis. However, our results showed no association between polymorphism of TSER alone and risk of CCC.

In the current study, we investigated whether common, functional polymorphisms in two critical genes involved in DNA synthesis and methylation are associated with cholangiocarcinoma risk. Few reports have been published about the association between carcinogenesis with polymorphism of MTHFR with TSER. Grieu et al. (31) reported no notable association between breast cancer and MTHFR and TSER polymorphism, respectively, like our data. However, our results showed a decreased risk of CCC in the TSER 2R(−) genotype in association with the MTHFR 677CC wild-type. This is the first report on the MTHFR C677T and TSER polymorphism with CCC patients. It was found that the occurrence of CCC was influenced by TSER polymorphism combined with the MTHFR wild-type. This study may provide a clue to the application of chemotherapeutic treatment modality based on further pharmacogenetic study for selected CCC patients. It is also expected that analysis of several polymorphisms in patients with CCC may yield a new tool for early diagnosis.

Hyperhomocystinemia, caused by decreased enzyme activity and gene polymorphism, is associated with

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<th>Table I. Comparison of MTHFR C677T and TSER genotypes between control and cholangiocarcinoma.</th>
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<td>MTHFR C677T genotype (%)</td>
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<tr>
<td>CC</td>
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<tr>
<td>Patients (n=47)</td>
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<td>Control (n=204)</td>
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*Including 2R5R, **Including 3R4R, 3R5R.

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<th>Table II. Association between TSER genotype and overall risk (OR) of cholangiocarcinoma stratified by MTHFR genotype.</th>
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<td>MTHFR C677T</td>
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<tr>
<td>Adjusted OR (95% CI)</td>
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<tr>
<td>CC</td>
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<td>CT</td>
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<td>TT</td>
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Adjusted odds ratio (adjusted by age and gender). *Including 2R2R and 2R3R; **p=0.0257.

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<th>Table III. Comparison of homocysteine level between control and cholangiocarcinoma according to MTHFR C677T and TSER polymorphism.</th>
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<td>Homocysteine (mean ± SD, μmol/L)</td>
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<td>p</td>
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<tr>
<td>Cholangiocarcinoma</td>
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<tr>
<td>Total</td>
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<tr>
<td>MTHFR 677CT</td>
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<td>TSER 2R(−)</td>
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<td>MTHFR 677CT/TSER 2R(+)</td>
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carcinogenesis of many malignancies (20). We expected hyperhomocysteinemia in cholangiocarcinoma patients, as well. Nevertheless, an unexpected trend towards hypohomocysteinemia without statistical significance was found in CCC patients compared to controls (8.27 μmol/L ± 4.17 μmol/L vs. 9.40 μmol/L ± 2.57 μmol/L, p=0.093; Table III). This unexpected data cannot be explained. Another study with hepatocellular carcinoma patients in our hospital also demonstrated, hypohomocysteinemia (unpublished data). This same trend for low levels of homocysteine leaves us another task for further study. A specific condition in the hepatobiliary system may result in the decrement of homocysteine. Folate deficiency due to hyperhomocysteinemia that ultimately suppresses DNA synthesis increases the risk of several malignancies (20). No data for folate level in association with risk of CCC is available.

Based on this study, large scale clinical research with folate and vitamin B12 is warranted. Furthermore, studies on the survival of cholangiocarcinoma patients based on the early diagnosis of CCC attributable to polymorphism are warranted, as are further studies on the role of phamacogenetics in CCC. Moreover, the survival of cholangiocarcinoma patients in association with MTHFR C677T and TSER polymorphisms is another task.

In conclusion, our data from a Korean population suggest that combined polymorphisms of MTHFR C677T and TSER are associated with a decreased risk of cholangiocarcinoma. Large scale clinical studies with folate level and other polymorphisms associated with CCC are warranted.

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References


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