# Polymorphisms in genes involved in folate metabolism and plasma DNA methylation in colorectal cancer patients

JONG WOO KIM[1*], HYE MI PARK[2*], YOUNG-KOOK CHOI[2*], SO YOUNG CHONG[3],

DOYEUN OH[2,3] and NAM KEUN KIM[2,3]

[1]Department of Surgery, [2]Institute for Clinical Research, and [3]Department of Internal Medicine,
School of Medicine, CHA University, Seongnam 463-712, Republic of Korea

**Abstract.** Aberrant methylation of promoter regions associated with gene silencing is one of the major mechanisms underlying the inactivation of tumor suppressor genes in carcinogenesis. Previous studies have proposed that methylated DNA from tumor cells is released into the circulation and might be widely used as a marker for non-invasive tumor detection. Catalytic activities of folate metabolism-related enzymes and adequate synthesis of methyl donors are important elements for the cellular methylation reaction. In the present study, we sought to determine the following: i) genotype frequencies of *MTHFR* and *MTR* involved in folate metabolism in cases and cancer-free controls; and ii) the methylation status of three candidate genes (*p16INK4A*, *p73* and *hMLH1*) in plasma related to the folate and homocysteine levels. From genotype frequency analysis, individuals homozygous for the *MTHFR* 677TT genotype had a significantly reduced risk of developing colorectal cancer compared with those harboring the *MTHFR* 677CC genotype (OR, 0.206; 95% CI, 0.070-0.604; P=0.005), and had a lower plasma folate concentration than those with the *MTHFR* 677CC+CT genotype (P<0.05). Using methylation-specific PCR, *p73* was shown to be more frequently methylated in the high folate group [50% (8 of 16)] than in the medium [16.7% (3 of 18)] or low folate subgroups [11.1% (2 of 18)]. In conclusion, subjects with the variant *MTHFR* 677TT genotype appeared to have a significantly lower risk for colorectal cancer than those with the *MTHFR* 677CC genotype, and the methylation status of circulating *p73* genomic DNA was substantially altered by the plasma folate level.

*Correspondence to:* Dr Nam Keun Kim, Institute for Clinical Research, School of Medicine, CHA University, 351, Yatap-dong, Bundang-gu, Seongnam 463-712, Republic of Korea
E-mail: nkkim@cha.ac.kr or namkkim@naver.com

*Contributed equally

## Introduction

Methylation of the promoter-associated CpG islands is a well-documented epigenetic modification, acting as a mechanism to regulate gene expression associated with the development of cancer (1,2). Aberrant methylation of the tumor suppressor or DNA repair gene promoter has been detected in many different types of cancers (3-6). Tumor DNA is released into the circulation and enriches the plasma and serum (7), which enables new approaches to detection, diagnosis, and monitoring (8,9).

Folate is essential for the synthesis of S-adenosyl-methionine, which is the methyl donor required for all methylation reactions in cells (10). Homocysteine (Hcy) is an intermediary product in methionine metabolism and elevated plasma Hcy is a sensitive biomarker of an imbalance in the integrated pathways of one-carbon metabolism (11). The folate-metabolizing enzyme, methylenetetrahydrofolate reductase (MTHFR), reduces 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which is then utilized to convert Hcy to methionine by methionine synthase; methionine synthase is encoded by the *MTR* gene. The polymorphisms of *MTHFR* (677C→T and 1298A→C) and *MTR* (2756A→G) associated with decreased enzymatic activity may contribute to the development of many cancers due to an imbalance of folate and Hcy in plasma (10-12).

p16INK4A is a cyclin-dependent kinase inhibitor that prevents the phosphorylation of retinoblastoma protein, and thereby impedes mitosis at the G1-S transition of the cell cycle pathway (13). This gene is inactivated by mutations, homozygous deletions, or gene methylation in many tumors of diverse origin (14). p73, a structural and functional homologue of p53, exists in two major forms (full-length TA-p73 and N-terminal truncated ΔN-p73). TA-p73 shares some functional characteristics with p53 and functions as a tumor suppressor. Whereas ΔN-p73 exhibits anti-apoptotic activities, the expression of ΔN-p73 is up-regulated in many human cancers (15). The hMLH1 protein, a mismatch repair enzyme, maintains the fidelity of the genome during cellular proliferation (16). The function of DNA mismatch repair (MMR) is diminished by aberrant methylation and transcriptional silencing of the *hMLH1* gene promoter (17-19).

Although a series of studies has shown genomic DNA methylation through an interaction with folate status in

tumors, the effect of folate and Hcy on circulating DNA methylation, such as *p16^{INK4A}*, *p73* and *hMLH1*, in colorectal cancer patients has not been evaluated. We postulate that polymorphisms of *MTHFR* (677C→T and 1298A→C) and *MTR* (2756A→G) associated with decreased enzymatic activity could result in an imbalance between the folate and Hcy levels that may play a role in the etiology of colorectal cancer through effects on DNA methylation (20). Thus, the purpose of the current study was to determine the following: i) the effect of *MTHFR* and *MTR* polymorphisms on the incidence of colorectal cancer; and ii) the effect of folate and Hcy on *p16^{INK4A}*, *p73*, and *hMLH1* promoter DNA methylation in plasma.

**Materials and methods**

*Study subjects*. We recruited 67 patients from CHA Bundang Medical Center at the CHA University with newly diagnosed and histologically-confirmed colorectal cancer. The cancer-free controls included 53 healthy, hospital-based patients. Control participants with a history of cancer, previous radiotherapy or chemotherapy, heart disease, diabetes, and thrombosis were excluded from this study. Genomic DNA was extracted from white blood cells (WBC) by phenol/chloroform extraction, and used for genotyping of *MTHFR* and *MTR*. *MTHFR* 677C→T, 1298A→C, and *MTR* 2756A→G genotypes were analyzed by PCR-RFLP, as described earlier (21,22). The Institutional Review Board of CHA Bundang Medical Center approved our study, and written informed consent was obtained from all participants who were genetically-unrelated ethnic Koreans.

*Genetic analysis*. Blood samples were collected in a tube containing anticoagulant from 67 colorectal cancer patients (35 males and 32 females) and 53 healthy controls (23 males and 30 females) 12 h after the subject's last meal. WBC and plasma were separated from 10 ml of blood in trisodium EDTA-treated blood collection tubes. The tube was centrifuged for 15 min at 1000 x g to separate the plasma. The concentrations of folate and Hcy in plasma were measured by chemiluminescent immunoassay (ACS-180; Bayer Diagnostics, Tarrytown, NY, USA). For the isolation of circulating DNA from plasma, 700 $\mu$l of plasma was extracted with TRIzol™ LS reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's recommendation. The clear upper layer from the extraction was precipitated after overnight incubation at -20˚C by adding 0.1 volume of 7.5 M ammonium acetate, 2 volumes of 100% ethanol, and 5 $\mu$l of glycogen (5 mg/ml stock solution). The resulting pellet was washed with 1 ml of 70% ethanol, then dissolved in 40 $\mu$l of nuclease-free water. DNA samples were subjected to sodium bisulfite conversion using the CpGenome™ DNA Modification Kit (Chemicon International Inc., Temecula, CA, USA). Treatment of DNA samples with bisulfite converts all unmethylated cytosines to uracils, but does not affect methylated cytosines. We performed methylation-specific PCR (MSP) using modified DNA. For the methylated reaction, the primer sequences were as follows: 5'-GGA CGT AGC GAA ATC GGG GTT C-3' (sense) and 5'-ACC CCG AAC ATC GAC GTC CG-3' (antisense) for *p73*; 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3' (sense) and 5'-GAC

Table I. Characteristics of the control subjects and patients.

|  | Controls (%) | Cases (%) |
|---|---|---|
| Gender |  |  |
| Male | 23 (43.4) | 35 (52.2) |
| Female | 30 (56.6) | 32 (47.8) |
| Age |  |  |
| Mean ± SD | 58.68±15.8 | 61.78±11.46 |
| Stage |  |  |
| 0, I, II |  | 33 (49.3) |
| III, IV |  | 34 (50.7) |
| Location |  |  |
| Distal |  | 47 (70.1) |
| Proximal |  | 20 (29.9) |

CCC GAA CCG CGA CCG TAA-3' (antisense) for *p16*; and 5'-ACG TAG ACG TTT TAT TAG GGT CGC-3' (sense) and 5'-CCT CAT CGT AAC TAC CCG CG-3' (antisense) for *hMLH1*. For the unmethylated reaction, the primer sequences were as follows: 5'-AGG GGA TGT AGT GAA ATT GGG GTT T-3' (sense) and 5'-ATC ACA ACC CCA AAC ATC AAC ATC CA-3' (antisense) for *p73*; 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3' (sense) and 5'-CAA CCC CAA ACC ACA ACC ATA A-3' (antisense) for *p16*; 5'-TTT TGA TGT AGA TGT TTT ATT AGG GTT GT-3' (sense) and 5'-ACC ACC TCA TCA TAA CTA CCC ACA-3' (antisense) for *hMLH1*. Thermal cycling was initiated at 95˚C for 5 min, followed by 40 cycles at 94˚C for 30 sec, 67˚C for 30 sec (*p16*) or 62˚C for 30 sec (*p73* and *hMLH1*), and 72˚C for 30 sec, and final extension at 72˚C for 2 min. The PCR products were separated by 3% agarose gel and visualized by ethidium bromide staining. We determined the methylation status as positive or negative based on the PCR results. The presence of an amplification band in the methylated reaction or in the methylated and unmethylated reactions was designated 'methylation negative' or 'methylation positive', respectively.

*Statistical analysis*. Comparisons of SNP allele frequencies and the methylation status in the case and control groups were performed using Fisher's exact test. The concentrations of plasma folate and Hcy in patients were expressed as the mean ± SD values. Statistical significance was accepted at the P≤0.05. All statistical analyses were performed using SPSS for Windows (version 11.0; SPSS Inc., Chicago, IL, USA).

**Results**

Sixty-seven colorectal cancer patients (mean age, 61.78±11.46 years; males, 52.2%) and 53 cancer-free subjects (mean age, 58.68±15.82 years; males, 43.4%) were recruited. The clinical characteristics of the patients are given in Table I. Thirty-three cases (49.3%) were classified as stages 0-II, and 34 cases (50.7%) were classified as stages III and IV. Forty-seven patients (70.1%) had distally-located tumors, whereas 20 (29.9%) had proximally-located tumors.

Table II. Genotype distribution of *MTHFR* 677C→T, 1298A→C, *MTR* 2756A→G polymorphisms in colorectal cancer patients.

| Genotype | Control (%) | Cases (%) | OR (95% CI) | P-value |
|---|---|---|---|---|
| *MTHFR* 677 | | | | |
| CC | 15 (28.3) | 30 (44.8) | 1.000 (Reference) | |
| CT | 21 (39.6) | 30 (44.8) | 0.714 (0.310-1.645) | 0.527 |
| TT | 17 (32.1) | 7 (10.4) | 0.206 (0.070-0.604) | 0.005 |
| *MTHFR* 1298 | | | | |
| AA | 36 (67.9) | 44 (65.7) | 1.000 (Reference) | |
| AC | 16 (30.2) | 22 (32.8) | 1.125 (0.516-2.455) | 0.844 |
| CC | 1 (1.9) | 1 (1.5) | 0.818 (0.050-13.55) | 1.000 |
| *MTR* 2756 | | | | |
| AA | 42 (79.2) | 51 (76.1) | 1.000 (Reference) | |
| AG | 9 (17.0) | 16 (23.9) | 1.464 (0.587-3.469) | 0.498 |
| GG | 2 (3.8) | 0 (0.0) | 0.165 (0.007-3.535) | 0.212 |

Table III. Methylation status of $p16^{INK4A}$, *p73* and *hMLH1* genes in colorectal cancer patients according to folate level.

| Folate level (ng/ml) | Methylation status | | OR (95% CI) | P-value |
|---|---|---|---|---|
| | *p16 (-)* | *p16 (+)* | | |
| <4.1 | 13 (72.2) | 5 (27.8) | 1.000 (Reference) | |
| ≥4.1 and <6.7 | 14 (77.8) | 4 (22.2) | 0.743 (0.163-3.385) | 1.000 |
| ≥6.7 | 15 (93.8) | 1 (6.2) | 0.173 (0.018-1.682) | 0.180 |
| | *p73 (-)* | *p73 (+)* | | |
| <4.1 | 16 (88.9) | 2 (11.1) | 1.000 (Reference) | |
| ≥4.1 and <6.7 | 15 (83.3) | 3 (16.7) | 1.600 (0.234-10.950) | 1.000 |
| ≥6.7 | 8 (50.0) | 8 (50.0) | 8.000 (1.367-46.830) | 0.023 |
| | *hMLH1 (-)* | *hMLH1 (+)* | | |
| <4.1 | 11 (61.1) | 6 (33.3) | 1.000 (Reference) | |
| ≥4.1 and <6.7 | 14 (77.8) | 4 (22.2) | 0.524 (0.118-2.328) | 0.471 |
| ≥6.7 | 11 (68.8) | 5 (31.2) | 0.833 (0.195-3.559) | 1.000 |

The cases were divided into low (<4.1 ng/ml), medium (≥4.1 and <6.7 ng/ml) and high (≤6.7 ng/ml) level groups according to their plasma folate level. In methylation status, (-) and (+) indicate unmethylated and methylated, respectively.

In the cases and controls, we first assessed the association of polymorphisms in the *MTHFR* and *MTR* genes that play important roles maintaining the level of folate and Hcy. The *MTHFR* 677C→T polymorphism frequency was significantly different between controls and colorectal cancer patients. The frequencies of the *MTHFR* 677CC, *MTHFR* 677CT, and *MTHFR* 677TT genotypes were 44.8, 44.8, and 10.4%, respectively, for the cases, and 28.3, 39.6, and 32.1% for the controls, respectively (Table II). When the *MTHFR* 677CC genotype was used as the reference group, individuals who harbored the *MTHFR* 677TT appeared to have a significantly lower risk for colorectal cancer (OR, 0.206; 95% CI, 0.070-0.604; P=0.005). However, the frequencies of *MTHFR* 1298A→C and *MTR* 2756A→G genotypes did not differ between the cases and controls.

When we next analyzed the relationships between the plasma levels of Hcy and folate among patients, the plasma folate levels were inversely associated with the plasma concen-tration of Hcy (r=-0.486, P=0.0003; Fig. 1A). *MTHFR* 677TT homozygotes have significantly lower plasma folate levels than those with the other two genotypes (TT vs. CC+CT, P<0.05). Even though the plasma levels of Hcy in patients with the *MTHFR* 677TT genotype have a tendency to be higher than those with the 677CC+CT genotypes, no statistically significant differences exist (Fig. 1B and C).

We then investigated changes in the status of circulating DNA methylation among patients according to the plasma folate level. The cases were divided into the following 3 sub-groups according to the plasma folate level: low (<4.1 ng/ml), medium (≥4.1 and <6.7 ng/ml), and high (≤6.7 ng/ml). The methylation rate of the *p73* gene was increased in the high folate level group compared to the low or medium folate level groups (P=0.023; Table III). However, when colorectal cancer patients were divided into 3 subgroups according to the plasma Hcy levels [low (<7.4 $\mu$mol/l), medium (≥7.4 and <10.8 $\mu$mol/l), and high (≤10.8 $\mu$mol/l)], there was
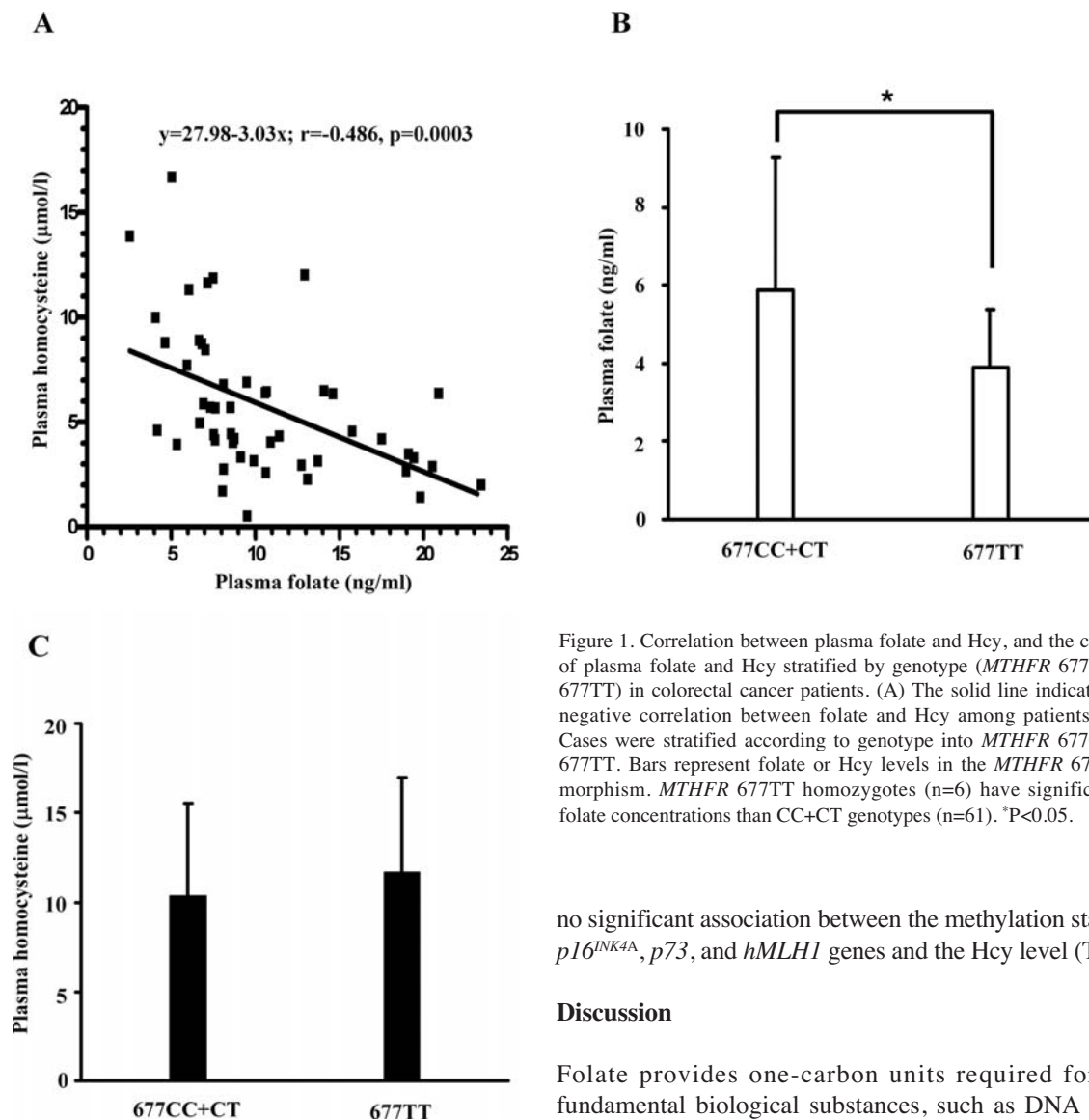
Figure 1. Correlation between plasma folate and Hcy, and the concentration of plasma folate and Hcy stratified by genotype (*MTHFR* 677CC+CT and 677TT) in colorectal cancer patients. (A) The solid line indicates the mean negative correlation between folate and Hcy among patients. (B and C) Cases were stratified according to genotype into *MTHFR* 677CC+CT and 677TT. Bars represent folate or Hcy levels in the *MTHFR* 677C→T polymorphism. *MTHFR* 677TT homozygotes (n=6) have significantly lower folate concentrations than CC+CT genotypes (n=61). *P<0.05.

no significant association between the methylation status of the *p16*[INK4A], *p73*, and *hMLH1* genes and the Hcy level (Table IV).

**Discussion**

Folate provides one-carbon units required for various fundamental biological substances, such as DNA synthesis,

Table IV. Methylation status of *p16*[INK4A], *p73* and *hMLH1* genes in colorectal cancer patients according to homocysteine level.

| Homocysteine level ($\mu$mol/l) | Methylation status | | OR (95% CI) | P-value |
|---|---|---|---|---|
| | *p16 (-)* | *p16 (+)* | | |
| <7.4 | 15 (88.2) | 2 (11.8) | 1.000 (Reference) | |
| ≥7.4 and <10.8 | 13 (72.2) | 5 (27.8) | 2.885 (0.477-17.46) | 0.402 |
| ≥10.8 | 14 (82.4) | 3 (17.6) | 1.607 (0.233-11.10) | 1.000 |
| | *p73 (-)* | *p73 (+)* | | |
| <7.4 | 12 (70.6) | 5 (29.4) | 1.000 (Reference) | |
| ≥7.4 and <10.8 | 12 (66.7) | 6 (33.3) | 1.200 (0.287-5.023) | 1.000 |
| ≥10.8 | 16 (94.1) | 1 (5.9) | 0.150 (0.015-1.458) | 0.175 |
| | *hMLH1 (-)* | *hMLH1 (+)* | | |
| <7.4 | 11 (64.7) | 6 (35.3) | 1.000 (Reference) | |
| ≥7.4 and <10.8 | 16 (88.9) | 2 (11.1) | 0.229 (0.039-1.353) | 0.121 |
| ≥10.8 | 9 (52.9) | 7 (41.2) | 1.426 (0.351-5.795) | 0.728 |

Colorectal cancer patients were divided into three groups, low (<7.4 $\mu$mol/l), medium (≥7.4 and <10.8 $\mu$mol/l) and high (≤10.8 $\mu$mol/l) level groups according to their plasma homocysteine level. In methylation status, (-) and (+) indicate unmethylated and methylated, respectively.

repair, and methylation. *MTHFR* and *MTR* are important enzymes in one-carbon metabolism, and their polymorphisms influence the plasma folate and Hcy levels have been implicated with the risk and the progression of colorectal cancer. Methylation of genomic DNA relies on an adequate supply of folate, and inappropriate folate status may increase the risk for colorectal carcinogenesis by inducing aberrant DNA methylation, DNA instability, and alteration of gene expression (23-25).

In the current study we found that subjects homozygous for the *MTHFR* 677TT genotype had a reduced risk of developing colorectal cancer compared with the other genotypes, *MTHFR* 677CC and 677CT; our observation is consistent with previous reports (26). An inverse association was observed in the current study between plasma folate and Hcy levels among patients, which is in good agreement with previous studies (27,28). *MTHFR* 677TT homozygotes with reduced enzymatic activity usually have lower plasma folate concentrations than CT and CC genotypes, and are also associated with hypomethylation (29). Although it has been suggested that folate helps prevent cancer, the relationship between folate and colorectal cancer is complicated because folic acid has been shown to have a dual role in cancer development; specifically, low folate ingestion protects against early carcinogenesis, but high folate intake promotes carcinogenesis (30). It is possible that the reduced enzymatic activity of the *MTHFR* 677TT genotype may be advantageous in terms of DNA synthesis and DNA repair due to the increased availability of 5,10-methylenetetrahydrofolate in the presence of high folate levels, whereas reduced enzymatic activity may be disadvantageous in terms of the methylation reaction because lower levels of 5-methylenetetrahydrofolate may result in DNA hypomethylation due to reduced methionine synthesis in the presence of low folate levels (31). However, it still remains to be determined whether or not these genetic-nutrient intake interactions play as a causative or a preventive factor for the development of colorectal cancer (32,33).

The subsequent analysis of the relationship between promoter methylation and plasma folate levels among patients showed that a high folate level (≥6.7 ng/ml) greatly increased the methylation rates of *p73* DNA, whereas patients with low or medium levels of folate had a much lower level of *p73* DNA methylation. We did not observe the change in methylation status on *p16INK4A* and *hMLH1* DNA according to folate levels. This observation raises the possibility that the effect of folate levels on DNA methylation may not be global hypo- or hypermethylation, rather may be site- and gene-specific. In fact, Sohn *et al* (34) reported that rats with severe folate deficiency produced significant hypomethylation within exons 6-7, but not in exon 8 of the *p53* tumor suppressor genes, in spite of a 56% increase in genomic DNA methylation. However, there are no conclusive data supporting whether or not folate levels induce global changes of DNA methylation or site- or gene-specific methylation in colorectal cancer.

The *p73* gene encodes two major forms (TA-p73 and ΔN-p73) and is located on chromosome 1p36.2-3. The expression of the *p73* gene is augmented in diverse tumor types. TA-p73 generated from the first promoter (TA promoter) can transactivate p53 genes and possesses tumor suppressive properties, whereas ΔN-p73 lacks the transactivation domain generated from an alternative promoter in intron 3 and can exhibit anti-apoptotic properties. Recently, another N-terminal splice variant, ΔN'-p73, has been identified which is generated from the TA promoter. Pützer *et al* (35) reported that the E2F1-regulated ΔN'-p73 transcripts are the predominant source of potentially oncogenic p73 proteins. We assessed the methylation status of the TA promoter region, but did not measure the differential expression of TA-p73 and ΔN'-p73 in tumor tissues. Regarding this issue, it should be investigated how the different p73 isoforms are regulated through CpG island methylation in different folate levels.

Although there is a report that the methylation pattern in primary tumor and serum was concordant in matched tissue and serum samples of the cases with gliomas (36), whether or not the methylation pattern in tumor tissues or in plasma DNA is similar at different folate levels remains to be determined. To answer this question, further research is required that critically evaluates the methylation pattern of circulating DNA and tumor DNA according to different folate levels.

Although our data propose that in colorectal cancer patients, plasma folate is an important determinant regulating *p73* promoter methylation, there were some limitations to the present study. We did not ascertain the effect of the *MTHFR* genotype on *p73* methylation due to a limited number of patients. In addition, *p73* methylation status was not compared between cases and controls according to the concentration of plasma folate. Finally, it should be noted that a single assessment of plasma folate at one time point may not be a meaningful indicator of long-term folate intake. In summary, our observations suggest that promoter methylation of *p73* may be a unique marker regulated by plasma folate in colorectal cancer; further research is clearly warranted to confirm both the relationships between promoter methylation of *p73* and folate levels, and the roles of *p73* variants in the development of colorectal cancer development.

## References

1. Robertson KD and Wolffe AP: DNA methylation in health and disease. Nat Rev Genet 1: 11-19, 2000.
2. Jones PA and Laird PW: Cancer epigenetics comes of age. Nat Genet 21: 163-167, 1999.
3. Esteller M, Tortola S, Toyota M, *et al*: Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res 60: 129-133, 2000.
4. Benassi MS, Molendini L, Gamberi G, *et al*: Involvement of INK4A gene products in the pathogenesis and development of human osteosarcoma. Cancer 92: 3062-3067, 2001.
5. Waki T, Tamura G, Tsuchiya T, Sato K, Nishizuka S and Motoyama T: Promoter methylation status of E-cadherin, hMLH1, and p16 genes in non-neoplastic gastric epithelia. Am J Pathol 161: 399-403, 2002.

6. Tycko B: Epigenetic gene silencing in cancer. J Clin Invest 105: 401-407, 2000.
7. Lopez M, Aguirre JM, Cuevas N, *et al*: Gene promoter hypermethylation in oral rinses of leukoplakia patients, a diagnostic and/or prognostic tool? Eur J Cancer 39: 2306-2309, 2003.
8. Anker P and Stroun M: Tumor-related alterations in circulating DNA, potential for diagnosis, prognosis and detection of minimal residual disease. Leukemia 15: 289-291, 2001.
9. Hibi K, Robinson CR, Booker S, *et al*: Molecular detection of genetic alterations in the serum of colorectal cancer patients. Cancer Res 58: 1405-1407, 1998.
10. Wasson GR, McGlynn AP, McNulty H, *et al*: Global DNA and p53 region-specific hypomethylation in human colorectalic cells is induced by folate depletion and reversed by folate supplementation. J Nutr 136: 2748-2753, 2006.
11. Chou YC, Lee MS, Wu MH, *et al*: Plasma homocysteine as a metabolic risk factor for breast cancer: findings from a case-control study in Taiwan. Breast Cancer Res Treat 101: 199-205, 2007.
12. Fowler B: Homocysteine: overview of biochemistry, molecular biology, and role in disease processes. Semin Vasc Med 5: 77-86, 2005.
13. Serrano M, Hannon GJ and Beach D: A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. Nature 366: 704-707, 1993.
14. Ruas M and Peters G: The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378: F115-F177, 1998.
15. Zaika AI, Slade N, Erster SH, *et al*: DeltaNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. J Exp Med 196: 765-780, 2002.
16. Nan HM, Song YJ, Yun HY, Park JS and Kim H: Effects of dietary intake and genetic factors on hypermethylation of the hMLH1 gene promoter in gastric cancer. World J Gastroenterol 11: 3834-3841, 2005.
17. Kane MF, Loda M, Gaida GM, *et al*: Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colorectal tumors and mismatch repair-defective human tumor cell lines. Cancer Res 57: 808-811, 1997.
18. Herman JG, Umar A, Polyak K, *et al*: Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95: 6870-6875, 1998.
19. Veigl ML, Kasturi L, Olechnowicz J, *et al*: Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci USA 95: 8698-8702, 1998.
20. Ma J, Stampfer MJ, Giovannucci E, *et al*: Methylene-tetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. Cancer Res 57: 1098-1102, 1997.
21. Reyes-Engel A, Muñoz E, Gaitan MJ, *et al*: Implications on human fertility of the 677C—>T and 1298A—>C polymorphisms of the MTHFR. Mol Hum Reprod 8: 952-957, 2002.
22. Zhu H, Wicker NJ, Shaw GM, *et al*: Homocysteine remethylation enzyme polymorphisms and increased risks for neural tube defects. Mol Genet Metab 78: 216-221, 2003.
23. Hayashi I, Sohn KJ, Stempak JM, Croxford R and Kim YI: Folate deficiency induces cell-specific changes in the steady-state transcript levels of genes involved in folate metabolism and 1-carbon transfer reactions in human colonic epithelial cells. J Nutr 137: 607-613, 2007.
24. Duthie SJ: Folic acid deficiency and cancer: mechanisms of DNA instability. Br Med Bull 55: 578-592, 1999.
25. Duthie SJ, Narayanan S, Blum S, Pirie L and Brand GM: Folate deficiency in vitro induces uracil misincorporation and DNA hypomethylation and inhibits DNA excision repair in immortalized normal human colon epithelial cells. Nutr Cancer 37: 245-251, 2000.
26. Hubner RA and Houlston RS: MTHFR C677T and colorectal cancer risk: a meta-analysis of 25 populations. Int J Cancer 120: 1027-1035, 2007.
27. Botto N, Andreassi MG, Manfredi S, *et al*: Genetic polymorphisms in folate and homocysteine metabolism as risk factors for DNA damage. Eur J Hum Genet 11: 671-678, 2003.
28. Venn BJ, Mann JI, Williams SM, *et al*: Assessment of three levels of folic acid on serum folate and plasma homocysteine: a randomised placebo-controlled double-blind dietary intervention trial. Eur J Clin Nutr 56: 748-754, 2002.
29. Castro R, Rivera I, Ravasco P, *et al*: 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T and 1298A>C mutations are associated with DNA hypomethylation. J Med Genet 41: 454-458, 2004.
30. Kim YI: Role of folate in colon cancer development and progression. J Nutr 133: S3731-S3739, 2003.
31. Marugame T, Tsuji E, Kiyohara C, *et al*: Relation of plasma folate and methylene-tetrahydrofolate reductase C677T polymorphism to colorectal adenomas. Int J Epidemiol 32: 64-66, 2003.
32. Du W, Li WY, Lu R and Fang JY: Folate and fiber in the prevention of colorectal cancer: between shadows and the light. World J Gastroenterol 16: 921-926, 2010.
33. Mason JB: Folate, cancer risk, and the Greek god, Proteus: a tale of two chameleons. Nutr Rev 67: 206-212, 2009.
34. Sohn KJ, Stempak JM, Reid S, Shirwadkar S, Mason JB and Kim YI: The effect of dietary folate on genomic and p53-specific DNA methylation in rat colon. Carcinogenesis 24: 81-90, 2003.
35. Pützer BM, Tuve S, Tannapfel A and Stiewe T: Increased DeltaN-p73 expression in tumors by upregulation of the E2F1-regulated, TA-promoter-derived DeltaN'-p73 transcript. Cell Death Differ 10: 612-614, 2003.
36. Zheng C, Ji S, Gong F, *et al*: Aberrantly methylated MGMT, hMLH1 and hMSH2 in tumor and serum DNA of gliomas patients. Clin Oncol Cancer Res 6: 42-46, 2009.