Gastroenterology and Hepatology From Bed to Bench. ***2008;1(2):57-63***

ORIGINAL ARTICLE

Reverse association between *MTHFR* polymorphism (C677T) with sporadic colorectal cancer

Mahdi Montazer Haghighi[1], Seyed Reza Mohebbi[1], Fatemeh Khatami[1], Somaye Ghiasi[1],Faramarz Derakhshan[1], Hamid Atarian[2], Mohammad Reza Zali[1].

[1] *Research Institute for Gastroenterology and Liver Disease, Shahid Beheshti University, M.C., Tehran, Iran*

[2] *Departments of Oncology, Taleghani Hospital, Shahid Beheshti University, M.C., Tehran, Iran*

ABSTRACT

Aim: To investigate association between MTHFR gene polymorphism with colorectal cancer.

Background: The enzyme 5,10-methylene-tetrahydrofolate reductase (MTHFR) is linked to DNA methylation, synthesis and repair. One of the most important polymorphisms that has been identified in the MTHFR gene is C677T. The single nucleotide polymorphism C677T has been found to be associated with decreased enzyme activity and decreased plasma folate. Thus it might play an important role in the etiology of colorectal neoplasia.

Patients and methods: Using pyrosequencing, we analyzed the MTHFR genotypes in 234 colorectal cancer patients and 257 normal matched controls.

Results: Whereas the CC, CT and TT genotypes of *MTHFR* among the colorectal cancer patients were 50%, 29% and 21% respectively, we found 36.6% of 677CC, 31.1% of 677CT and 32.3% of 677TT in the normal controls. We observed a decreased risk of colon cancer when folate intake was high for participants with wild type genotype. This association was stronger at higher levels of folate intake.

Conclusion: Our study corroborates previous findings of an inverse association of the MTHFR 677TT genotype with colorectal cancer, especially at high levels of folate.

Keywords: *sporadic colorectal cancer, MTHFR, Pyrosequencing, Polymorphism.*

(Gastroenterology and Hepatology From Bed to Bench 2008;1(2):57-63).

INTRODUCTION

Colon cancer is a complex disease influenced by multiple genetic and environmental factors. It is a prevalent cancer in the United States and other developed countries. Among environmental factors, diet has received a great deal of attention. (1).

Risk of development of colorectal cancer has been linked to diets that are low in the methyl donors, folate and methionine and high in alcohol, a methyl group antagonist (1). Dietary methyl group availability may influence cancer risk by altering DNA methylation or by influencing the rate of DNA mutation. Selective growth and transformation of cells can result from DNA hypomethylation of protooncogenes (2) or hypermethylation of tumor suppressor genes (3) in their promoter regions. In contrast to these mechanisms, in which aberrant DNA methylation influences gene expression, the mutation mediated hypothesis proposes that the oncogenic process is affected by a disproportionately high rate of CpG to TpG transitions, such as those frequently observed in the p53 gene in colorectal tumors (4-6), potentially due to deamination of

Received: 6 September 2007 *Accepted*: 11 December 2007
Reprint or Correspondence: Mahdi Montazer Haghighi
Research Institue for Gastroenterology and Liver Diseases, Shahid Beheshti University, M.C., Iran.
E-mail: mah_haghighi@hotmail.com

5-methylcytosine. Finally, methyl deficient diets may cause imbalances in the pools of nucleotide precursors leading to DNA strand breakages and mutations (7, 8).

Some colon cancers exhibit aberrant DNA methylation or CpG island methylator phenotype, which involves inactivation or silencing of genes by hypermethylation of promoter cytosine-guanosine (CpG) residues. Evidence from epidemiologic studies suggests that adequate folate intake is associated with decreased risk of colorectal cancer (9-11), although not all studies support this association (12, 13).

The 5,10 methylenetetrahydrofolate reductase (MTHFR) enzyme plays an important role in folate metabolism and determines the balance between the different forms of folate for DNA synthesis and DNA methylation (14). MTHFR is a critical enzyme regulating the metabolism of folate and methionine, both of which are important factors in DNA methylation and synthesis. MTHFR irreversibly converts 5,10-methylenetetrahydro-folate to 5-methyltetrahydrofolate, which is the primary methyl donor for the remethylation of homocysteine to methionine. A common C677T (Ala →Val) mutation of the gene was found to enhance malfunction of the enzyme (14, 15).

Altered folate metabolism leads to shifts in the balance between availability of 5-methyltetra-hydrofolate for methylation reactions and 5,10-methylenetetrahydrofolate for DNA synthesis and repair (16, 17), which may influence colorectal cancer risk. As a matter of fact, polymorphisms in genes related to folate metabolism, specifically MTHFR, are thought to play an important role in carcinogenesis of the large bowel. One polymorphism in the *MTHFR* gene that affects the efficiency of folate metabolism has been described (18-20). The *MTHFR* 677 C>T transition in exon 4 is associated with reduced enzyme activity resulting in slower folate metabolism. Individuals with the variant *MTHFR* 677*TT* genotype show about 30% of the enzyme activity found among those with the wild-type (*CC*) enzyme (21). Subjects who are heterozygous for the mutation (*CT*) have about 65% of wild-type enzyme activity (21).

Among 677*TT* (val/val) individuals, the MTHFR enzyme is less efficient in converting 5,10-methylenetetrahydrofolate to 5-methyltetra-hydrofolate, thus potentially preventing depletion of 5,l0-methylenetetrahydrofolate, a cofactor for de novo DNA synthesis, especially dTMP. As a result, cells may be less prone to "dNTP stress" which has been shown to promote cancer-associated genetic alterations due to alterations in the pool of nucleotide precursors available for DNA synthesis. Alteration of these precursor pools induced by methyl (folate) deficiency significantly increases the uracil content and the frequency of chromosome breakages in human leukocyte DNA (22-23).

Individuals with the *TT* genotype, particularly if combined with a low folate diet, have elevated plasma homocysteine levels, which illustrates the physiological importance of this genotype (23–25).

In this study, we investigated the association of *MTHFR* polymorphism, codon 677 C>T, among colon cancer cases. We hypothesized that *MTHFR* polymorphisms are linked with reduced MTHFR enzyme activity, and that the association would be modified by folate status.

PATIENTS and METHODS

Blood samples were collected from 234 unrelated Iranian patients with non familial colon cancer and 257 healthy individuals as controls. All subjects were genetically-unrelated ethnic Iranian patients with histopathologically confirmed incident sporadic colorectal cancers and were recruited between September 2003 and December 2007 at the Research Institute for Gastroenterology and Liver Disease (RIGLD). Cancer-free controls were randomly selected from individuals referred to Taleghani hospital during the same time when

the cases were being recruited. These control subjects had no history of cancer and were frequency-matched to the cases by age within five years and sex.

Questionnaires included information on dietary intake, smoking habits, medical information, and family history of polyps and cancer (especially history of colon, breast, endometrial, or ovarian cancers). In case that data were incomplete, study staff would follow up participants.

Briefly, fasting blood of 234 cases and 257 controls was sampled in the morning. RBC (Red Blood Cells), plasma, and whole blood folate were measured in both groups. Plasma folate was measured by an automated chemiluminescence method (Chiron Diagnostics, East Walpole, MA). For the RBC folate assay, RBCs were isolated by centrifugation at 36,000 rpm for 15 min within 1 h of collection, washed with 9 g/L of sodium chloride solution, and immediately stored at -70°C, until folate analysis. RBC folate was analyzed by affinity chromatography followed by reversed-phase chromatography with electrochemical detection, as previously described (26).

Genomic DNA was isolated from peripheral-blood lymphocytes according to standard procedures (27). All samples (patients and controls) were analyzed for C677T SNP by PCR/pyrosequencing technique and our results were confirmed by direct sequencing of 12 randomly selected samples.

Pyrosequencing is based on the detection of released pyrophosphate (PPi) during DNA synthesis. In a cascade of enzymatic reactions, visible light proportional to the number of incorporated nucleotides is generated.

We designed three primers (forward: 5'-GAGGCTGACCTGAAGCACTTGA-3', reverse: 5'-ATGCCTTCACAAAGCGGAAGA-3' and sequencing: 5'-CGTGATGATGAAATCG-3') of which either forward or reverse was biotinylated. Forward and reverse primers were used for PCR and sequencing primer for running pyrosequencing (Assay Design software v1.0.6). The reaction conditions were as follows: 1µl of genomic DNA solution (10 ng/µl), 20µM of each primer, 0.2 mmol/l of each dNTP, and 2.0 mmol/l of MgCl2 and 0.02 U/µl of Taq DNA polymerase (Fermentase, Germany) in 50µl total volume. Thermal cycling was performed as follows: 94^{o}C for 5 min followed by 35 cycles of 95^{o}C for 30s, annealing temperature for 45s and 72^{o}C for 40s, followed by 72^{o}C for 10 min. The biotinylated products of the single PCR were immobilized on streptavidin-coated paramagnetic beads (Magnetic Biosolutions), and the strands were separated using 0.10 mol/l NaOH. This ssDNA were genotyped in polymorphism locus by sequencing primer and pyrosequencer (PSQ 96MA, Uppsala, Sweden).

Direct sequencing was done for 12 randomly selected subjects to confirm our data. Sequencing results were analyzed by DNASIS MAX software v2.6 (Hitachi Software Engineering Co.).

Standard techniques of matched case-control studies were used. Odds ratios (OD) and 95% confidence intervals were estimated by logistic regression analysis. Exposure was defined as homozygosity for the Valine substitution (*TT*). Homozygous wild-type individuals (*CC*) were combined with heterozygotes (*CT*) as a single "unexposed" group, to increase statistical power in stratified analyses. RBC and plasma folate and other stratification variables were categorized into quartiles. Category boundaries were determined from the exposure distribution of the entire sample.

The association between *MTHFR* genotype and colorectal cancer was estimated in the entire studied population. We used t- tests to compare mean plasma and RBC folate between levels of genotype and Pearson correlation coefficients were used to determine correlations between the different measures of folate status (using the computer software SPSS for Windows v14.0). All *P*- values were two-sided; *P*-values <0.05 were considered statistically significant.

RESULTS

During the accrual period, we identified 263 cases and 268 controls that were potentially eligible; of these, 29 cases and 11 controls refused to interview, thus we analyzed 234 cases and 257 controls by pyrosequencing (Fig. 1). Table 1 represents characteristics of cases and controls in studied population.

Table 1. Characteristics of cases and controls in studied population

Risk factor	Cases (n = 234)	Controls (n = 257)
Gender		
Male	129	144
Female	105	113
Age	63.3±7.1	57.1±6.3
Folates		
Total ((µg/day)	446.1±252.1	448.5±260.3
RBC (ng/ml)	258.4±166.7	269.5±143.6
Plasma (ng/ml	10.8±7.6	14.1±9.1
Smoking status		
Current	21	24
Former	18	55
Never	79	110

Forty-nine cases and 83 controls were homozygous for the TT genotype. Allele frequencies in cases were T=35.5% and C=64.5%, while among the controls were T= 48% and C= 52%. Table 2 presents the inverse association between TT genotype and colorectal cancer in this population. The frequencies of 677TT (val/val), C677T (ala/val), and CC677 (ala/ala) genotypes among the cases were 21, 29 and 50%, respectively (Table 2). The frequency of val/val genotype among the cases was lower than controls.

Table 2. MTHFR C677T genotype prevalence and main effects in Iranian patients with colorectal cancer

Genotype	Cases (%)	Controls (%)	OR* (95% CI)
TT	49(20.9)	83 (32.3)	0.47(0.03 - 0.76)
CT	68 (29.1)	80 (31.1)	0.68(0.44 - 1.06)
CC	117 (50)	94 (36.6)	1.00
TT/CT + CC	49/185	83/174	0.56(0.36 - 0.85)

* Odds Ratio

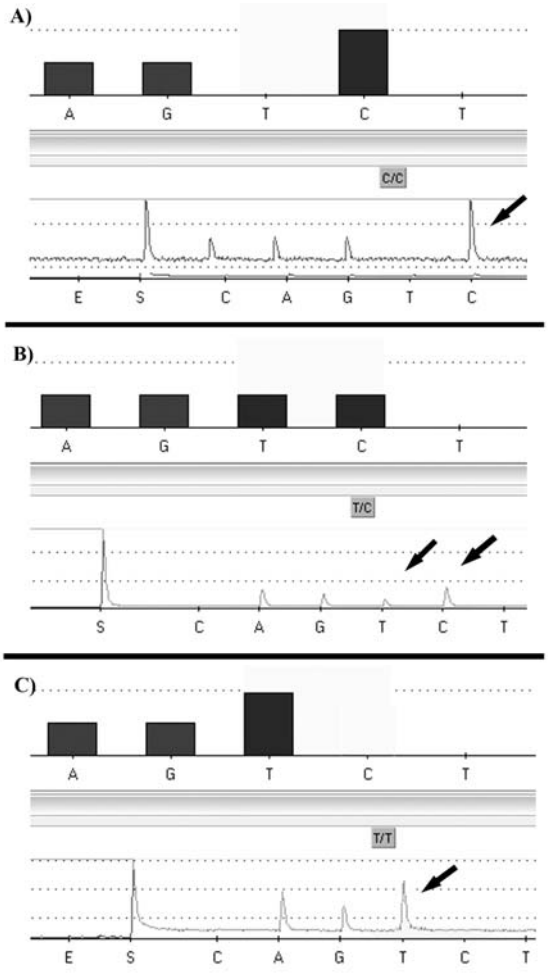

Fig 1. Pyrosequencing analysis of DNA from colorectal cancer patients containing MTHFR C677T sequence
A: CC wild type genotype; B: TC heterozygote genotype; C: TT homozygote genotype

Table 3 shows the joint effect of *MTHFR* genotype and folate on adenoma risk. For those with RBC folate levels in the lowest quartile (<165 ng/ml), subjects with the *TT* genotype had approximately twice the adenoma risk of those with at least one wild-type allele. At the highest folate levels, adenoma risk was <1.0 for both *TT* homozygotes and those with a wild-type allele.
We observed that the association with the *TT* genotype was limited to advanced colon tumors. Our results bring additional evidence for an inverse

Table 3. ORs and 95% CI for colon cancer in relation to MTHFR C677T genotypes, total folate intake, and supplement use in Iranian population

Genotype	Intake*	Food folate		Total folate	
		Case/control	OR (95% CI)	Case/control	OR (95% CI)
CC+CT	≤Median	96/98	1.00	104/91	1.00
CC+CT	>Median	89/76	1.2(0.77-1.85)	81/83	0.44(0.28-1.25)
TT	<Median	22/47	0.48(0.26-0.89)	31/41	0.98(0.50-1.37)
TT	>Median	27/36	0.77(0.41-1.41)	18/42	0.38(0.19-0.73)

*The median intake was 320μg/d for folate from foods, 450μg/d for total folate

association between the *MTHFR* 677*TT* genotype and colorectal cancer.

DISCUSSION

We investigated the association of *MTHFR* genotypes and colon cancer in a population-based case-control study of Iranian patients. The effect of *MTHFR* codon 677 was evaluated in relation to total folate intake.

This association was similar in both sexes, stronger at high levels of folate intake. There was a statistically significant trend toward the protective effect of food folate (P= 0.01) among those with the *TT* genotype, compared with the total plasma folate.

Our results revealed an inverse association between the *MTHFR* 677*TT* genotype and colorectal cancer. This association was first reported in two male Harvard cohorts [28] and was reproduced in five of eight case-control studies to date (29). Four of five past studies suggested interactions between folate and the *TT* genotype, with the inverse association being greatest among persons with the highest intake or plasma levels of folate (28, 30-32). Our results are remarkably consistent with those findings.

Colon cancer is difficult to cure when the disease has spread outside the large intestine. Moreover, if the sub-site specificity of the association can be replicated, it will be interesting to see whether the folic acid fortification of the diet initiated in the United States in 1998 will result in a greater decrease in rates, if any, for colon as compared to rectal cancer (33-35). Interestingly, the lower frequency of the T allele in African Americans and Native Hawaiians and the finding of a specificity of its protective effect against advanced colorectal cancer are consistent with the late-stage presentation and poorer survival observed for the disease in these ethnic groups (33-35).

There are several limitations to our study. First, we were unable to distinguish between intake of vitamin B-6, B-12, folate and methyonin in the form of supplements, thus our findings for supplement use could be attributable to folate, vitamin B6, vitamin B12, or other compounds found in dietary supplements. Because dietary intake of folate and B vitamins are often highly correlated (36), some or all of the effect of total folate intake in our study could be attributable to vitamin B-6 or B-12. Second, since alcohol consumption is forbidden in our country, we could not collect clear data from our patients and control subjects. Moreover, since alcohol consumption is not recommended in our culture thereby, we could not gather correct data in this field in order to examine the effect of it on cancer as well.

In conclusion, these data corroborate previous findings of fine inverse association of the *MTHFR* 677*TT* genotype with colorectal cancer, especially at high levels of folate. It is also suggested that this effect may be specific to advanced colon cancer.

Acknowledgments

We are indebted to the patients for their cooperation. This research was supported by grants from the Research Institute for Gastroenterology and Liver Diseases, Shaheed Beheshti Medical University.

REFERENCES

1. Giovannucci E, Rimm EB, Ascherio A, Stampfer MJ, Colditz GA, Willett WC. Alcohol, low-methionine-low-folate diets, and risk of colon cancer in men. J Nat Cancer Inst 1990;87:265-73.

2. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759-67.

3. Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links aging and neoplasia in human colon. Nat Genet 1994 ;7:536-40.

4. Greenblau MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994;54:4855-78.

5. James SJ, Basnakian AG, Miller BJ. In vitro folate deficiency induces deoxynucleotide pool imbalance, apoptosis, and mutagenesis in Chinese hamster ovary cells. Cancer Res 1994;54:5075-80.

6. Blount BC, Ames BN. DNA damage in folate deficiency. Baillieres Clin Haematol 1995;8:461-78.

7. Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Rüschoff J. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res 1997;57:4749–56.

8. Thibodeau SN, French AJ, Cunningham JM, Tester D, Burgart LJ, Roche PC, et al. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. Cancer Res 1998;58:1713–1 8.

9. Konings EJ, Goldbohm RA, Brants HA, Saris WH, van den Brandt PA. Intake of dietary folate vitamers and risk of colorectal carcinoma: results from The Netherlands Cohort Study. Cancer 2002;95:1421–33.

10. Slattery ML, Potter JD, Samowitz W, Schaffer D, Leppert M. Methylenetetrahydrofolate reductase, diet, and risk of colon cancer. Cancer Epidemiol Biomarkers Prev 1999;8:513–18.

11. Su LJ, Arab L. Nutritional status of folate and colon cancer risk: evidence from NHANES I epidemiologic follow-up study. Ann Epidemiol 2001;11:65–72.

12. Levi F, Pasche C, Lucchini F, La Vecchia C. Selected micronutrients and colorectal cancer. a case-control study from the canton of Vaud, Switzerland. Eur J Cancer 2000;36:2115–19.

13. Harnack L, Jacobs DR Jr, Nicodemus K, Lazovich D, Anderson K, Folsom AR, et al Relationship of folate, vitamin B-6, vitamin B-12, and methionine intake to incidence of colorectal cancers. Nutr Cancer 2002;43:152–58.

14. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111-13.

15. Libbus BL, Borman LS, Ventrone CH, Branda RF. Nutritional folatedeficiency in Chinese hamster ovary cells. Chromosomal abnormalities associated with perturbations in nucleic acid precursors. Cancer Genet Cytogenet 1990;46:231–42.

16. Choi SW, Mason JB. Folate and carcinogenesis: an integrated scheme. J Nutr 2000;130:129–32.

17. Duthie SJ. Folic acid deficiency and cancer: mechanisms of DNA instability. Br Med Bull 1999;55:578–92.

18. Toffoli G, Gafà R, Russo A, Lanza G, Dolcetti R, Sartor F, et al .Methylenetetrahydrofolate Reductase 677 C→T Polymorphism and Risk of Proximal Colon Cancer in North Italy. Clin Cancer Res 2003;9:743-48.

19. Lievers KJ, Boers GH, Verhoef P, den Heijer M, Kluijtmans LA, van der Put NM, et al. A second common variant in the methylenetetrahydrofolate reductase (MTHFR) gene and its relationship to MTHFR enzyme activity, homocysteine, and cardiovascular disease risk. J Mol Med 2001;79:522–28.

20. van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998;62:1044–51.

21. Song C, Xing D, Tan W, Wei Q, Lin D. Methylenetetrahydrofolate reductase polymorphisms increase risk of esophageal squamous cell carcinoma in a Chinese population. Cancer Res 2001;8:3272–75.

22. Deloughery TG, Evans A, Sadeghi A, McWilliams J, Henner WD, Taylor LM Jr, et al. Common mutation

in methylenetetrahydrofolate reductase. Correlation with homocysteine metabolism and late-onset vascular disease. Circulation 1996;94:3074–78.

23. Ma J, Stampfer MJ, Hennekens CH, Frosst P, Selhub J, Horsford J, et al. Methylenetetrahydrofolate reductase polymorphism, plasma folate, homocysteine, and risk of myocardial infarction in US physicians. Circulation 1996;94:2410–16.

24. Harmon DL, Woodside JV, Yarnell JW, McMaster D, Young IS, McCrum EE, et al The common "thermolabile" variant of methylene tetrahydrofolate reductase is a major determinant of mild hyperhomocysteinaemia. Q J Med 1996;89:571–77.

25. Malinow MR, Nieto FJ, Kruger WD, Duell PB, Hess DL, Gluckman RA, et al The effects of folic acid supplementation on plasma total homocysteine are modulated by multivitamin use and methylenetetrahydrofolate reductase genotypes. Arterioscler Thromb Vasc Biol 1997;17:1157–62.

26. Hoffbrand AV, Newcombe FA, Mollin DL. Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. J Clin Pathol 1996;19:17–28.

27. Chen J, Giovannucci E, Kelsey K, Rimm EB, Stampfer MJ, Colditz GA, et al. A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. Cancer Res 1996;56:4862 –64.

28. Ma J, Stampfer MJ, Giovannucci E, Artigas C, Hunter DJ, Fuchs C, et al . Methylenetetrahydrofolate reductase polymorphism, dietary interactions and risk of colorectal cancer. Cancer Res 1997;57:1098–102.

29. Weisberg I, Tran P, Christensen B, Sibani R, Rozen R. A second polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. Mol Genet Metab 1998;64:169–72.

30. Le Marchand L, Donlon T, Hankin JH, Kolonel LN, Wilkens LR, Seifried A. B-vitamin intake, metabolic genes and colorectal cancer risk. Cancer Causes Control 2002;13:239– 48.

31. Slattery ML, Potter JD, Samowitz W, Schaffer D, Leppert M. Methylenetetrahydrofolate reductase, diet, and risk of colon cancer. Cancer Epidemiol Biomarkers Prev 1999;8:513–18.

32. Keku T, Millikan R, Worley K, Winkel S, Eaton A, Biscocho L, et al. 5,10-Methylenetetrahydrofolate reductase codon 677 and 1298 polymorphisms and colon cancer in African Americans and whites. Cancer Epidemiol Biomarkers Prev 2002;11:1611–21.

33. Chen VW, Fenoglio-Preiser CM, Wu XC, Coates RJ, Reynolds P, Wickerham DL, et al. Aggressiveness of colon carcinoma in blacks and whites. Cancer Epidemiol Biomarkers Prev 1997;6:1087–93.

34. Wegner EL, Kolonel LN, Nomura AMY, Lee J. Racial and socioeconomic status differences in survival of colorectal cancer patients in Hawaii. Cancer 1982;49:2208–16.

35. Pagano IS, Morita SY, Dhakal S, Hundahl SA, Maskarinec G. Time dependent ethnic convergence in colorectal cancer survival in Hawaii. BMC Cancer 2003;25:5.

36. Poirier L, Wise C, Delongchamp R, Sinha R. Blood determinations of S-adenosylmethionine. S-adenosylhomocysteine, and homocysteine: correlations with diet. Cancer Epidemiol Biomark Prev 2002;10:649–55.