Determinants of Homocysteine Levels in Colorectal and Breast Cancer Patients

P. FERRONI[1], R. PALMIROTTA[1], F. MARTINI[1], S. RIONDINO[1], A. SAVONAROLA[1], A. SPILA[1], F. CIATTI[1], V. SINI[2], S. MARIOTTI[2], G. DEL MONTE[3], M. ROSELLI[2] and F. GUADAGNI[1]

[1]*Department of Laboratory Medicine and Advanced Biotechnologies, IRCCS San Raffaele Pisana, 00163, Rome;*
[2]*Department of Internal Medicine, Medical Oncology, University of Rome "Tor Vergata", Tor Vergata Clinical Center, 00133, Rome;*
[3]*San Raffaele Rocca di Papa, 00040, Rocca di Papa, Rome, Italy*

Abstract. *Background: Homocysteinemia has been associated with oncogenic risk. This study was designed to investigate the homocysteine (Hcy) genotype/phenotype interactions together with the inflammatory and nutritional status of cancer patients. Patients and Methods: The Hcy levels were analyzed in 47 cancer patients in association with methylenetetrahydrofolate reductase (MTHFR) polymorphisms, folate and inflammatory markers. Results: The MTHFR C677T and A1298C genotype distributions did not differ from those predicted by the Hardy-Weinberg distribution. Conversely, the Hcy levels were higher in the cancer patients (p=0.04), who were also characterized by low-grade inflammation. The Hcy levels correlated with the interleukin-6 (IL-6) (p=0.001), tumor necrosis factor-α (TNF-α) (p=0.042) and folate (p<0.0001) levels of the patients. Multivariate analysis showed that TNF-α (p=0.014) and folate (p=0.019) were independent predictors of elevated Hcy levels in the cancer patients. Conclusion: The MTHFR polymorphisms do not significantly contribute to tHcy (total Hcy) levels in cancer patients, and cancer-related inflammation may be associated with elevated tHcy levels, possibly involving a TNF-α mediated pathway.*

Homocysteine (Hcy) is a non-protein-forming, sulfur-containing amino acid, formed exclusively by demethylation of methionine and degraded by remethylation and transsulfuration (1). Hcy plasma levels are influenced by genetic polymorphisms of key enzymes of its metabolism (1). Among these, methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in Hcy metabolism being responsible for intracellular folate pool regulation for the synthesis and methylation of DNA. The allele variant C677T of the *MTHFR* gene, has been associated with reduced enzyme activity (up to 60-70% in the 677T homozygous carriers) and increased total Hcy (tHcy) plasma levels (2), mostly in patients with folate deficiency (3).

Recent studies have shown that low folate status, with hyperhomocysteinemia as a consequence, is associated with oncogenic risk in patients with inflammatory bowel disease (4), probably due to hypomethylation (5). Aberrant methylation of DNA is frequently found in tumor cells (6). Global hypomethylation can result in chromosome instability (7), whereas region-specific hypermethylation has been associated with the inactivation of tumor suppressor genes (6).

Hcy influences the DNA methylation level, as its remethylation produces methionine, the precursor of the *S*-adenosylmethionine (SAM) methyl group donor involved in DNA transmethylation reactions (6). Additional pathways may involve folate-dependent reactions leading to changes in the availability of nucleotides, such as thymidylate, for DNA synthesis and repair (8). Finally, hyperhomocysteinemia may exert its pathogenic effects through the metabolic accumulation of *S*-adenosyl-L-homocysteine, a strong non-competitive inhibitor of the catechol-O-methyltransferase (COMT)-mediated methylation metabolism of various catechol substrates, such as catechol estrogens (CEs) (9). Methylation of CEs in target organs is ultimately responsible for decreased formation of 2-methoxyestradiol, a strong antiangiogenic and anticancer agent and increased accumulation of the procarcinogenic 4-hydroxyestradiol, thus leading to the development of estrogen-induced hormonal cancer in the target organs (9).

Correspondence to: Patrizia Ferroni, MD, Ph.D., Department of Laboratory Medicine and Advanced Biotechnologies, IRCCS San Raffaele, Via della Pisana 235, 00163, Rome, Italy. Tel: +39 0666130425, Fax: +39 0666130407, e-mail: patrizia.ferroni@sanraffaele.it

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One final mechanism by which Hcy might predispose to cancer is the activation of proinflammatory genes due to region-specific hypomethylation. Results of *in vitro* and *in vivo* experiments have suggested that homocysteine might provoke intestinal mucosal injury by modulating tumor necrosis factor alpha (TNF-α)-mediated cytotoxicity (10). Indeed, plasma tHcy has been regarded as a determinant of TNF-α in pathological conditions characterized by low-grade inflammation (11) and targeting the TNF pathway can significantly reduce tHcy (12), suggesting a role for this cytokine in Hcy metabolism.

For all these reasons, hyperhomocysteinemia has been regarded as a risk factor for cancer and tHcy levels have been proposed as a new tumor marker since they not only accurately reflect the proliferation rates of tumor cells but also respond to tumor cell death. (13). Thus, the aim of this study was to analyze the possible associations between tHcy levels, MTHFR polymorphisms, folate status and inflammatory markers in patients with breast or colorectal cancer.

Patients and Methods

Patients. Forty-seven consecutive patients (15 males, mean age 59±14 years) with breast (n=18) or colorectal cancer (n=29) entered into the study. Out of these, 35 had primary cancer, whereas the remaining 12 had relapsing cancer. All the patients were followed-up at the Medical Oncology of "Tor Vergata" Clinical Center. Forty-seven healthy subjects (15 males, mean age 43±12 years) from the same geographical area were recruited for the control group.

Exclusion criteria were: no informed consent to the study, acute inflammatory disease, impaired liver (bilirubin level >1.5 mg/dl) or renal (creatinine level >1.5 mg/dl) function, Karnofsky performance status lower than 90% , concurrent therapy with anti-folate drugs, or medications known to influence homocysteine levels (thus interfering with the genotype/phenotype correlation study), such as current or recent use of a folate or vitamin B12 supplement or of any multivitamin preparation; current or recent use of drugs interfering with homocysteine levels (*i.e.* anticonvulsants, methotrexate and penicillamine).

Informed consent was obtained from all the subjects. The study was performed under the appropriate institutional ethics approvals and in accordance with the principles embodied in the Declaration of Helsinki.

Sample collection and laboratory analysis. Blood samples from the cancer patients were drawn within 1 week before surgery, or prior to neoadjuvant chemotherapy and/or irradiation. All the patients attended the clinic on an outpatient basis at 8:00 a.m. After an overnight fast and a rest period of at least 20 min, the blood samples were drawn without stasis, from the antecubital vein using a 20 G needle and either non-anti-coagulated, for serum recovery, or anti-coagulated in Na citrate 3.8% (1:9, v:v) or in ethylenediaminetetraacetic acid (EDTA), for plasma separation. The EDTA-containing vacuum tubes were kept on ice and in the dark for tHcy (which refers to the sum of homocysteine, homocystine, and homocysteine-cysteine mixed disulfide, free and protein bound) analysis. The EDTA-anti-coagulated whole blood samples for the DNA analyses were immediately frozen at –80˚C until processing; EDTA plasma was separated within 90 min.

For serum preparation the blood was allowed to clot and then centrifuged at 2000 g for 10 min at 4˚C. The serum samples were either immediately analyzed for vitamin B12 and folate levels or aliquoted, coded and stored at –80˚C for batch analysis for interleukin-6 (IL-6), TNF-α and high-sensitivity C reactive protein (hs-CRP). Citrated plasma was obtained by centrifugation at 2000 xg for 10 min at 4˚C and immediately analyzed for fibrinogen levels. The storage conditions were carefully maintained and all the aliquots were limited to one freeze-thaw cycle, thus ensuring no decline of antigen values due to long-time storage as previously demonstrated (14).

The *MTHFR* 677C→T and 1298A→C polymorphisms were investigated in DNA samples extracted from the whole blood by a MagNA Pure LC instrument and total DNA isolation kit I (Roche Diagnostics, Mannheim, Germany) using real-time polymerase chain reaction-based assay kits LC DNA Master Hybridization Probes and LC Fast Start DNA Master Hybridization Probes (Roche Diagnostics) performed on a LightCycler 2.0 (Roche Diagnostics).

Quantitative vitamin B12 and plasma folate levels were measured by chemiluminescent microparticle immunoassays on an Architect i2000 instrument (Abbott Laboratories, Abbott Park, IL, USA). The hs-CRP levels were assayed by quantitative immunoturbidimetric ultrasensitive determination on an Architect c8000 instrument (Abbott Laboratories). The plasma tHcy and fibrinogen levels were assayed on an ACL TOP automated coagulometer (Instrumentation Laboratory (IL) Co, Lexington, MA, USA). The serum proinflammatory cytokine (IL-6 and TNF-α) levels were measured by enzyme-immunometric assays (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions.

All the samples were assayed in duplicate and those showing values above the standard curve were re-tested with appropriate dilutions. Both the biochemical and mutation analyses were conducted blind as to whether a sample came from a cancer or cancer-free subject.

Statistical analysis. The allelic frequencies were estimated by gene counting and the genotypes were scored. The observed numbers of each *MTHFR* genotype were compared with that expected for a population in Hardy-Weinberg equilibrium. Differences between percentages were assessed by Chi-square test. Student's unpaired *t*-test, Anova test and Pearson Product moment correlation analysis were used for the normally distributed continuous variables. Appropriate non-parametric tests (Mann Whitney *U*-test, Kruskal-Wallis ANOVA and median test and Spearman rank correlation test) were employed for all the other variables. Multiple linear regression analyses were performed to further quantify the relationship between the clinical and biochemical variables. Only two-tailed probabilities were used for testing statistical significance. The data are presented as mean±SD, or median and interquartile ranges (IQR). Only *p*-values lower than 0.05 were regarded as statistically significant. All the calculations were made using computer software packages (Statistica 8.0, StatSoft Inc., Tulsa, OK, USA).

Results

Based on the molecular studies, all the cancer and control subjects were divided into three genotypes of the *MTHFR* gene: CC, CT and TT for the C677T mutation and AA, AC and CC for the A1298C mutation. The genotype distribution

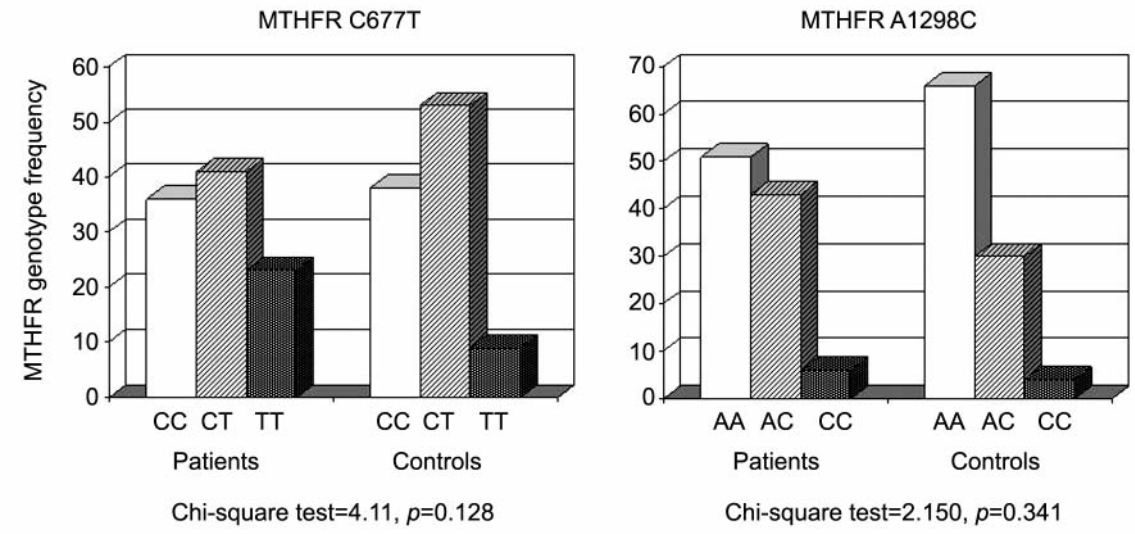

Figure 1. *Frequency distribution of MTHFR C677T and A1298C genotypes between cancer patients and healthy subjects.*

Table I. *Laboratory variables of cancer patients and controls.*

		Control subjects (N=47)	Cancer patients (N=47)			
			P-value*	Breast cancer (N=18)	*P*-value	Colorectal cancer (N=29)
Vitamin B12 (pg/ml)	Median (IQR)	299 (267-340)	0.999	311 (234-473)	0.651	310 (224-369)
Folate (ng/ml)	Median (IQR)	5.5 (4.2-8.6)	0.438	5.1 (3.5-8.1)	0.073	7.3 (5.0-13.0)
Fibrinogen (mg/dl)	Mean±SD	348±86	0.258	348±104	0.108	390±94
hs-CRP (mg/l)	Median (IQR)	0.11 (0.05-0.51)	0.004	0.26 (0.09-1.28)	0.068	0.86 (0.23-2.38)
Homocysteine (μM)	Median (IQR)	6.4 (5.8-8.8)	0.040	8.3 (6.8-10.2)	0.370	8.0 (5.2-9.9)
IL-6 (pg/ml)	Median (IQR)	0.8 (0.4-1.4)	<0.001	2.3 (1.1-1.6)	0.743	2.3 (1.2-3.7)
TNF-α (pg/ml)	Median (IQR)	1.9 (0.4-1.6)	0.042	1.5 (1.0-1.8)	0.547	1.6 (1.0-3.0)

hs-CRP, High sensitive C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α, IQR, interquartile range; *All cancer patients *vs.* controls.

of *MTHFR* C677T in the cancer patients (36%, 41% and 23%, respectively) and controls (38%, 53% and 9%, respectively) did not differ significantly from those predicted by the Hardy-Weinberg distribution (HW probability test=1.491 for patients and 1.317 for controls). Additionally, the distribution of the *MTHFR* A1298C genotypes of the cancer patients (51%, 43% and 6%, respectively) and controls (66%, 30% and 4%, respectively) did not differ significantly from those predicted by the Hardy-Weinberg distribution (HW probability test=0.189 for patients and 0.068 for controls). The frequency distribution of the *MTHFR* genotypes between cancer patients and healthy subjects is depicted in Figure 1. The frequencies of the C677T and A1298C alleles did not significantly differ between the breast cancer and colorectal cancer patients and no association was found between the allele frequencies and the clinicopathological variables of the cancer patients (data not shown).

On the other hand, the plasma tHcy levels were higher in the cancer patients (Mann Whitney test for median (IQR): 8.0 μM (6.0-10.0), p=0.04) compared to the controls (6.4 μM (5.8-8.8)) (Table I). The tHcy levels were then categorized into low (≤11.5 μM) or high (>11.5 μM) on the basis of the mean +2SD of the values observed in the controls. The associations between the tHcy and the clinicopathological variables were analyzed after categorization. As shown in Table II, no significant correlation was observed between tHcy and site, stage, tumor size or lymph node involvement of the cancer patients, which only showed a positive trend, while high plasma tHcy levels were associated with metastatic disease (Chi-square analysis: p<0.01).

Table II. *Association between clinicopathological variables and plasma total homocysteine (tHcy) level in cancer patients.*

		Plasma tHcy level*		
Variable	N	≤11.5 μM	>11.5 μM	*P*-value
Gender				
Male	15	11 (73)	4 (27)	
Female	32	27 (84)	5 (16)	0.37
Diagnosis				
Colorectal	29	23 (79)	6 (21)	
Breast	18	15 (83)	3 (17)	0.73
Stage of disease				
Early	22	20 (91)	2 (9)	
Advanced	25	18 (72)	7 (28)	0.10
Tumor size**				
T1-2	17	14 (82)	3 (18)	
T3-4	18	15 (83)	3 (17)	0.94
Lymph node involvement**				
N0	23	21 (91)	2 (9)	
N+	12	8 (67)	4 (33)	0.07
Distant metastases				
No	28	26 (93)	2 (7)	
Yes	19	12 (63)	7 (37)	0.01

*Categorized on the basis of the mean +2SD of the values observed in healthy subjects. **Including 35 primary carcinomas.

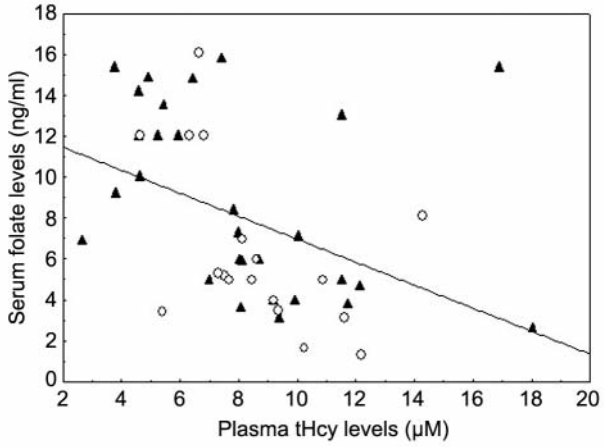

Figure 2. *Correlation of total homocysteine (tHcy) and folate serum levels in breast (open circles) and colorectal (closed triangles) cancer patients. Spearman rank correlation: Rho=0.567, p<0.0001.*

The ANOVA test showed that the plasma tHcy levels were not associated with either *MTHFR* 677C→T (F=0.216, p=0.81) or 1298A→C (F=0.243, p=0.79), but they were inversely correlated to the folate status of the patients (Spearman rank correlation: Rho=0.567, p<0.0001) (Figure 2). Further analysis of the plasma tHcy distribution in the patients with different *MTHFR* C677T genotypes and low or high folate levels (categorized on the basis of the upper quartile of the values observed in all the recruited subjects, *i.e.* 12 ng/ml) showed that the highest tHcy levels were found in the patients with the TT genotype and low folate levels (F=3.34, p=0.045) (Figure 3).

The nutritional and inflammatory status of the cancer patients was further analyzed by determination of vitamin B12, hs-CRP, fibrinogen, IL-6 and TNF-α levels. The results are summarized in Table I. As expected, the cancer patients had serum hs-CRP (p=0.004) IL-6 (p=0.001) and TNF-α (p=0.04) levels higher than the controls, whereas the vitamin B12 and fibrinogen levels were similar to the control subjects. No significant differences were observed between the breast and colorectal cancer patients.

Univariate correlation analysis performed by Spearman rank test demonstrated that the tHcy levels significantly correlated with both IL-6 (Rho=0.483, p=0.001) and TNF-α (Rho=0.298, p=0.042) both in the breast and colorectal cancer patients (Figure 4). Therefore, to further assess the possible determinants of the tHcy levels among the clinical and laboratory features of the cancer patients, a multivariate linear regression analysis was performed including tHcy as the dependent variable and sex, diagnosis, tumor stage, vitamin B12, folate, fibrinogen, hs-CRP, IL-6, TNF-α and the allele frequencies of the *MTHFR* C677T polymorphism as the predictor variables. The final model by forward stepping showed that TNF-α (beta=0.45, p=0.014) and folate levels (beta=–0.44, p=0.019) were the only independent predictors of elevated tHcy levels (Table III). TNF-α, in turn, was significantly associated with elevated IL-6 levels (beta=0.52, p=0.003) (Table III).

Discussion

In the present study, no association between the breast or colorectal cancer patients and the *MTHFR* alleles or genotypes was identified. These results corroborated recent findings of a lack of association, especially for the TT genotype of the *MTHFR* gene, with increased cancer risk (15-19), but disagree with others showing that the *MTHFR* 677T/T genotype was associated with a protective effect (20-27). Indeed, there are conflicting data regarding the role of the *MTHFR* C677T variants among cancer patients, and an inverse effect (28-30) or the need for predisposing factors (31, 32) has been advocated by some authors (Table IV).

An increased risk of tumor formation associated with the wild-type genotypes might be explained by aberrant methylation of DNA (6) leading to chromosome instability in the case of hypomethylation (7), or inactivation of tumor suppressor genes in the case of region-specific hypermethylation (6). On the other hand, the association of the mutant genotype with increased cancer risk has been

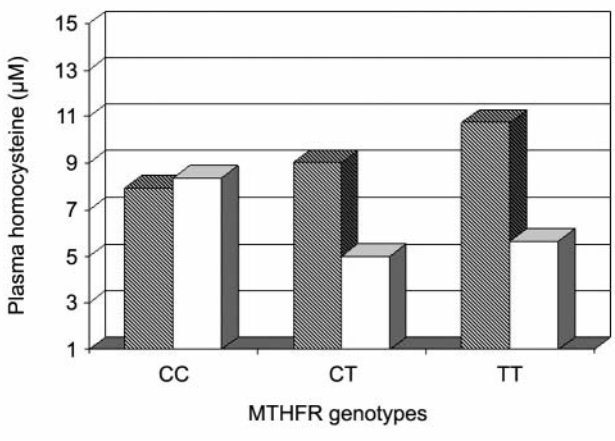

Figure 3. *Total homocysteine (tHcy) serum levels for genotypes of the 677C→T MTHFR mutation, stratified by low (below 12 ng/ml, dashed columns) and high (above 12 ng/ml, open columns) serum folate levels, in the cancer study population.*

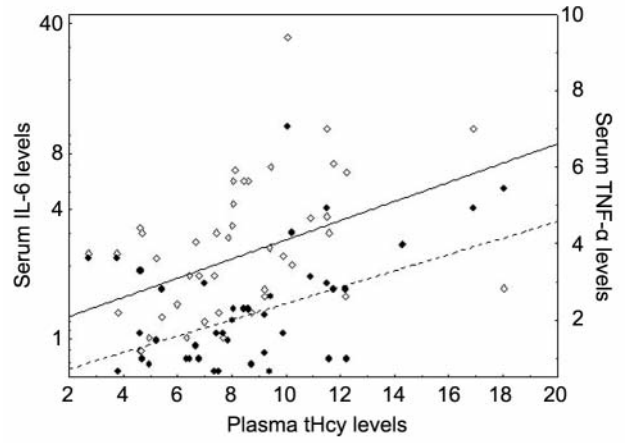

Figure 4. *Correlation of total homocysteine (tHcy) and IL-6 (left Y, open diamonds) or TNF-α (right Y, closed circles) serum levels in cancer patients. Spearman rank correlation for IL-6 (solid line): Rho=0.483, p=0.001. Spearman rank correlation for TNF-α (dashed line): Rho=0.298, p=0.042.*

Table III. *Multiple regression analysis of variables associated with homocysteine or TNF-α in cancer patients.*

Entire model

Predictor variable	Plasma tHcy level			Serum TNF-α level		
	Regression coefficient	Standard error	*p*-Value	Regression coefficient	Standard error	*p*-Value
Gender	0.183	0.245	0.466	0.048	0.232	0.839
Diagnosis	0.034	0.240	0.891	0.059	0.224	0.795
Stage of disease	–0.147	0.222	0.518	0.023	0.210	0.896
Folate	–0.500	0.241	0.056	0.134	0.253	0.603
Vitamin B12	0.101	0.209	0.637	–0.178	0.191	0.365
Fibrinogen	0.125	0.251	0.625	–0.141	0.233	0.553
hs-CRP	–0.616	0.476	0.215	0.009	0.468	0.985
Homocysteine	-----	-----	-----	0.405	0.217	0.082
IL-6	0.361	0.462	0.447	0.577	0.414	0.184
TNF-α	0.464	0.249	0.082	-----	-----	
T allele	0.088	0.234	0.713	0.078	0.218	0.726
C allele	0.396	0.244	0.125	–0.210	0.241	0.398
Forward stepwise method						
TNF-α	0.445	0.166	0.014	-----	-----	-----
Folate	–0.446	0.176	0.019	-----	-----	-----
IL-6	-----	-----	-----	0.524	0.159	0.004
Homocysteine	-----	-----	-----	0.304	0.158	0.068

hs-CRP, High sensitive C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

related to an augmented production of Hcy. Individuals who are severely deficient in MTHFR activity due to the presence of the T variant at nucleotide 677 show excessive plasma levels of Hcy. The latter influences DNA methylation level (6) and, due to folate-dependent reactions, may lead to changes in the availability of nucleotides for DNA synthesis and repair (8). However, *MTHFR*-knockout mice show the typical phenotypes seen in patients carrying *MTHFR* germline mutations such as neuronal tube defects, hyperhomocysteinemia or decreased methylation capacity, but no increased tumor incidence has been reported in this animal model (24).

Table IV. *Studies of the MTHFR 677 TT genotype in breast and colorectal cancer.*

	Population	Cases	Controls	OR	Adjustment	Effects
Breast cancer						
Gershoni-Baruch *et al*., 2000 (28)	Israeli	491	---	---	None	Associated with bilateral cancer
Campbell *et al*., 2002 (29)	Australian	335	233	1.43	None	Increased risk of early onset
Shrubsole *et al*., 2005 (31)	Shanghai	1144	1236	2.51	Folate intake	Modify association with folate intake
Le Marchand *et al*., 2004 (21)	Multiethnic	1189	2414	0.62	HRT	Protective
Beilby *et al*., 2004 (22)	Australian	141	109	0.08	Folate intake	Protective
Justenhoven *et al*., 2005 (15)	German	688	724	0.69	HRT	No association
Chen *et al*., 2005 (30)	USA	1481	1518	1.37	Folate intake	Increased risk
Martin *et al*., 2006 (23)	Afroamerican	143	---	0.65	none	Survival improvement
	Caucasian	105	---			
Reljic *et al*., 2007 (16)	Croatian	93	102	1.52	None	No association
Colorectal cancer						
Slattery *et al*., 1999 (24)	USA	1467	1821	0.9	Age, BMI, smoking, physical activity	Protective
Shannon *et al*., 2002 (32)	Australian	501	1207	1.03	None	Predisposing when microsatellite positive
Keku *et al*., 2002 (25)	USA	552	868	0.8	Age, sex, ethnicity	Slightly protective with high folate intake
Plaschke *et al*. 1, 2003 (17)	German	287	346	1.13	None	No association
Curtin *et al*., 2004 (18)	USA	1608	1972	0.8	HRT	No association
Ulrich *et al*., 2005 (26)	USA	1600	1962	---	Genotype interactions	Low risk when low TS expression
Le Marchand *et al*., 2005 (27)	Multiethnic	822	2021	0.77	Folate and alcohol intake	Protective
Battistelli *et al*., 2006 (19)	Italian	93	100	0.8	HRT	No association

HRT, Hormone replacement therapy; OR, odds ratio.

One of the objectives of this study was to correlate the distribution of plasma homocysteine levels with the *MTHFR* genotypes in the cancer patients. The results demonstrated that the tHcy levels were significantly higher in the patients with cancer compared with the controls. Moreover, the genotype correlation in the cancer population confirmed that the T variant at nucleotide 677 of the *MTHFR* gene was a major determinant of plasma tHcy levels, but only in the patients with low folate status, in agreement with a previously published study performed in other subsets of patients (3).

Apart from being responsible for faulty DNA methylation, hyperhomocysteinemia may promote inflammatory processes through oxidative stress (9). Homocysteine-induced damage related to cell adhesion molecules, cytokines and chemokines may therefore contribute to the biology of cancer. An inflammatory condition, as evidenced by increased proinflammatory cytokines (IL-6 and TNF-α) and hs-CRP, was found in the present population, closely related to the increased levels of tHcy. Indeed, multivariate analysis of the cancer patients demonstrated that, besides the association with folate status, TNF-α levels were an independent predictor of increased tHcy.

It is well known that tumor cells and/or tumor-associated leukocytes may produce inflammatory cytokines, such as IL-6 and TNF-α (33). Circulating levels of these cytokines have been associated with the disease status of cancer patients (34-41) and it has been suggested that IL-6 might represent an independent negative prognostic marker of survival in colorectal cancer (35, 41). We are aware that, given the multiple pathophysiological changes known to be associated with cancer, statistical correlation does not necessarily indicate biological relationship, and we cannot conclusively define the pathophysiological significance of TNF-α and tHcy in cancer. Nonetheless, the present findings that the *MTHFR* polymorphisms did not significantly contribute to the tHcy levels in breast and colorectal cancer, but TNF-α was independently associated with the tHcy in patients with breast or colorectal cancer suggested that cancer-related inflammation may be associated with elevated tHcy levels, possibly involving a TNF-α mediated pathway.

Despite the limited number of patients evaluated in the present study, this is the first report, to our knowledge, that correlated TNF-α and tHcy levels in cancer patients. It is hoped that new studies will be undertaken to fully elucidate the role of these molecules in cancer-related vascular inflammation. Better knowledge of the interplay between Hcy and TNF-α will help to improve understanding of the pathophysiological mechanisms involved in cancer initiation and progression.

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