

RESEARCH ARTICLE

One-carbon genetic variants and the role of *MTHFD1* 1958G>A in liver and colon cancer risk according to global DNA methylation

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Abstract

Several polymorphic gene variants within one-carbon metabolism, an essential pathway for nucleotide synthesis and methylation reactions, are related to cancer risk. An aberrant DNA methylation is a common feature in cancer but whether the link between one-carbon metabolism variants and cancer occurs through an altered DNA methylation is yet unclear. Aims of the study were to evaluate the frequency of one-carbon metabolism gene variants in hepatocellular-carcinoma, cholangiocarcinoma and colon cancer, and their relationship to cancer risk together with global DNA methylation status. Genotyping for *BHMT* 716A>G, *DHFR* 19bp ins/del, *MTHFD1* 1958G>A, *MTHFR* 677C>T, *MTR* 2756A>G, *MTRR* 66A>G, *RFC1* 80G>A, *SHMT1* 1420C>T, *TCII* 776C>G and *TS* 2rpt-3rpt was performed in 102 cancer patients and 363 cancer-free subjects. Methylcytosine (mCyt) content was measured by LC/MS/MS in peripheral blood mononuclear cells (PBMCs) DNA. The *MTHFD1* 1958AA genotype was significantly less frequent among cancer patients as compared to controls ($p = 0.007$) and related to 63% reduction of overall cancer risk ($p = 0.003$) and 75% of colon cancer risk ($p = 0.006$). When considering PBMCs mCyt content, carriers of the *MTHFD1* 1958GG genotype showed a lower DNA methylation as compared to carriers of the A allele ($p = 0.048$). No differences were highlighted by evaluating a possible relationship between the other polymorphisms analyzed with cancer risk and DNA methylation.

The *MTHFD1* 1958AA genotype is linked to a significantly reduced cancer risk. The 1958GG genotype is associated to PBMCs DNA hypomethylation as compared to the A allele carriership that may exert a protective effect for cancer risk by preserving from DNA hypomethylation.

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Introduction

One-carbon metabolism is a complex pathway involved both in the nucleotide synthesis and biological methylation reactions [1, 2]. For the implication of this metabolism in cellular development, proliferation and differentiation, polymorphic variants of genes encoding for enzymes related to one-carbon metabolism have been largely studied for their relationship with cancer disease [3–6]. The molecular mechanisms underlying such association are, however, not clearly defined yet, while it is known that one-carbon metabolism modulates a major epigenetic mechanism as DNA methylation [7, 8] that is strongly linked to cancer [6]. DNA methylation is the major and the most studied among the epigenetic mechanisms in mammalian cells consisting in the covalent binding of a methyl group to the 5' carbon position of cytosine, mostly occurring at CpG dinucleotide sequences with a function for gene expression regulation and maintainance of genomic stability [9]. DNA methylation is a heritable and reversible phenomenon with potential implications for the understanding of molecular mechanisms and disease prevention strategies in complex diseases such as cancer [6]. Both global and gene-specific DNA methylation show altered patterns in cancer disease and, in particular, a global DNA hypomethylation has been described as an almost universal finding in cancer cells [10]. There is evidence that global DNA hypomethylation in peripheral blood mononuclear cells (PBMCs) may be associated to an increased cancer risk and may be useful as a biomarker for cancer susceptibility [11]. Significantly reduced methylcytosine (mCyt) levels in PBMCs DNA from cancer patients were previously observed [8, 12, 13], where low levels of global DNA methylation was related to future cancer development and therefore might be considered as a potentially useful epigenetic marker for cancer risk [8]. Cancer is a major public health issue [14]. Among other types of cancer, primary liver cancers, i.e. hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC), and colon cancer, are common malignancies worldwide [14, 15]. In particular, the worldwide mortality rate of liver cancer is 14.6% in males and 5.7% in females, and the mortality of colon cancer is 9.7% in males and 7.0% in females [14].

A potential underlying mechanism in colon and liver carcinogenesis relates to one-carbon metabolism for its function in providing methyl groups for DNA methylation [2]. A research hypothesis is that functional genetic defects in one-carbon enzymes, by inducing an aberrant DNA methylation, may eventually lead to cancer development, though evidences in this regard are still limited (Fig 1) [16].

Aim of the present study was precisely to evaluate the possible association among the most common, functional genetic variants of one carbon-metabolism enzymes, global DNA methylation and their potential association with risk for primary liver and colon cancer.

Materials and methods

Study subjects

The study was approved by the Institutional Review Board Ethical Committee of the University of Verona School of Medicine Hospital (Verona, Italy). Written informed consent was obtained from each patient after a detailed explanation of the study.

Four hundred and sixty-five subjects were enrolled, precisely 102 cancer patients (38 HCC, 21 CC, and 43 colon cancer) and 363 cancer-free subjects [17]. The patients affected by cancer were enrolled from April 2009 to March 2013 among those referring to the Division of General and Hepatobiliary Surgery of the Verona University Hospital, for curative surgery intervention. The patients admitted to the study were ≥ 18 years old with the following surgical resectability criteria: preserved liver function, class A Child-Pugh score, absence of extrahepatic

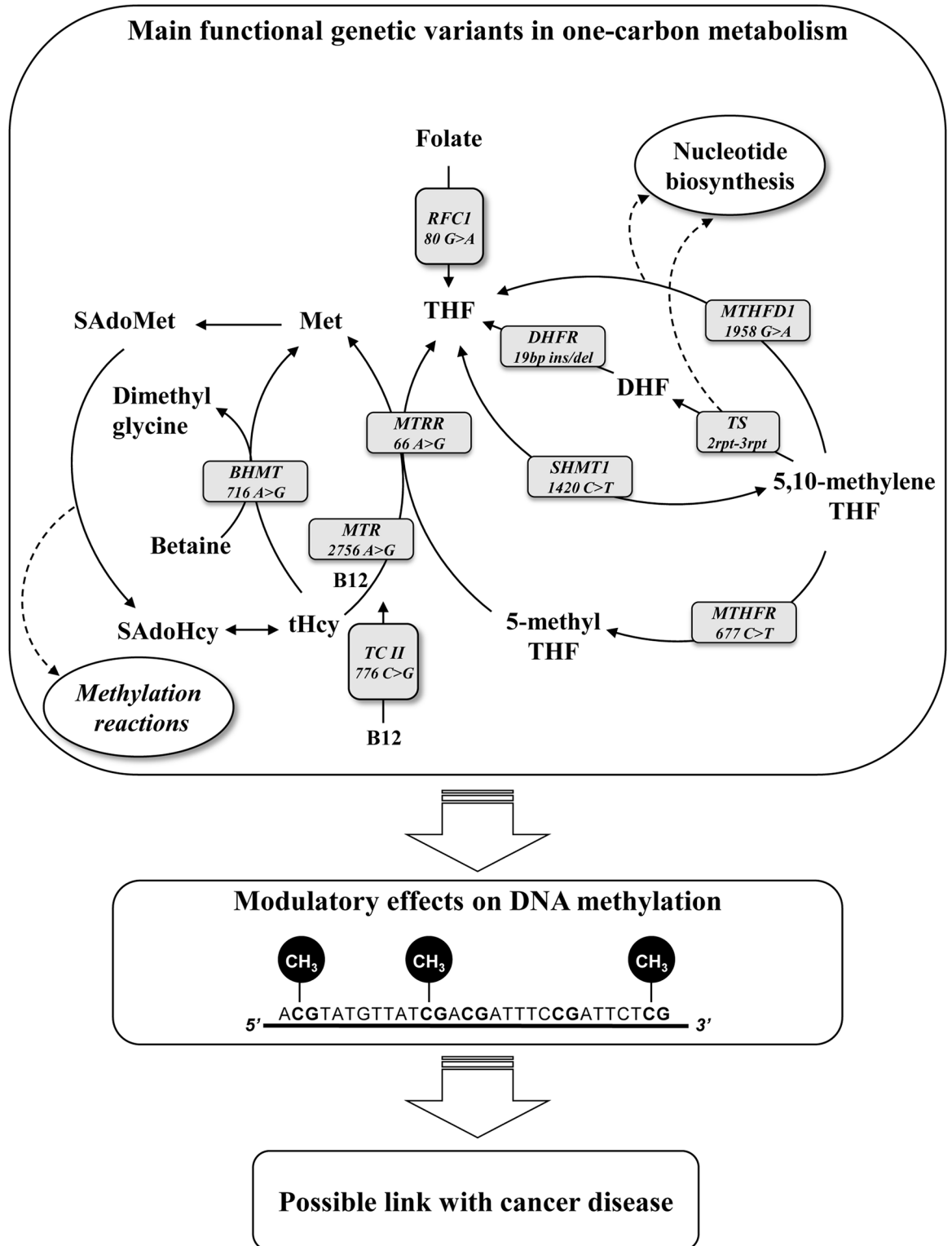


Fig 1. Hypothesis for a role of main functional genetic variants in one-carbon metabolism and cancer risk through DNA methylation. Polymorphic genetic variants in one-carbon enzymes can affect the balance between biological methylation and nucleic acids synthesis pathways inducing an aberrant DNA methylation and eventually leading to cancer development. BHMT, betaine-homocysteine S-methyltransferase; DHFR, dihydrofolate reductase; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-

homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; RFC1, reduced folate carrier 1; SHMT1, serine hydroxymethyltransferase 1; TCII, transcobalamin II; THF, tetrahydrofolate, and TS, thymidylate synthase.

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metastases. Exclusion criteria comprised: a coexisting human immunodeficiency (HIV), hepatitis B (HBV) or hepatitis C (HCV) viruses infection; presence of relevant concurrent medical conditions such as acute or chronic inflammatory diseases or haematological disorders, autoimmune liver diseases and hemochromatosis, decompensate liver cirrhosis (Child-Pugh B, C). A trained physician recorded a detailed clinical history data reporting also lifestyle habits. The cancer-free control group was of 363 age- and sex-matched subjects [17]. Key eligibility criteria were age ≥ 18 years, absence of neoplasia of any type, no history of viral infections (HBV, HCV) and absence of other relevant medical conditions. All subjects under B vitamins supplementation and/or using drugs known to interfere with folate-related one-carbon metabolism in the month before enrolment were excluded.

Biochemical analysis

Samples of venous blood were drawn from each subject after an overnight fasting for a complete blood count and determination of serum C-reactive protein (CRP), creatinine, aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyltranspeptidase (gGT), alkaline phosphatase (ALP), total bilirubin, albumin, glycemias, total cholesterol, triglycerides, prothrombin time-international ratio (PT-INR), ferritin, and tests for hepatitis B and C viruses serology, by using routine laboratory test analyses. Plasma folate and vitamin B12 were measured by an automated chemiluminescence method (ChironDiagnostics, East Walpole, MA) and total plasma homocysteine (tHcy) concentrations were determined by high-performance liquid chromatography (HPLC) with fluorescent detection [18].

Genotyping of one-carbon metabolism genes

From each subject venous blood was drawn into Vacutainer[®] tubes containing EDTA as anti-coagulant after an overnight fasting and DNA was extracted by Wizard Genomic DNA Purification Kit (Promega Corporation, Fitchburg, WI, USA). The one-carbon metabolism gene variants most commonly described with a functional effect were analyzed by different methods, as follows: *DHFR* 19bp ins/del (rs70991108)[19] and *TS* 2rpt-3rpt by PCR[20], *MTHFD1* 1958 G>A (rs2236225)[21], *MTHFR* 677 C>T (rs1801133)[22], *MTR* 2756A>G (rs12749581)[23], *MTRR* 66 A>G (rs1801394)[24], *RFC1* 80G>A (rs1051266)[25], and *SHMT1* 1420 C>T (rs1979277)[26] by PCR-restriction fragment length polymorphism assays, and the *BHMT* 716 A>G (rs3733890) and *TCII* 776 C>G (rs1801198) by allelic discrimination Real Time-PCR technology by using the C_11646606–20 assay and the C_325467–10 Taqman[®] SNP Genotyping assays, respectively (Applied Biosystems[™], ABI Prism 7500). The mean genotyping call rate among all the studied gene variants was 97.2%.

Global DNA methylation

Global DNA methylation was determined using a liquid chromatography/mass spectrometry (LC/MS/MS) method and mCyt levels expressed as percent (%)mCyt = [(mCyt)/(mCyt+Cyt)] x 100, as previously described [7, 27, 28]. The analysis was performed in the PBMC DNA of all the cancer-free subjects (n = 363) and in a subgroup of cancer patients (n = 62). Briefly, global DNA was extracted and hydrolyzed to nucleosides using 2 units of nuclease P1, 0.002 units of venom phosphodiesterase I and 0.5 units of alkaline phosphatase. Isotope-labeled internal

standards for deoxycytidine and 5-methyl-deoxycytidine were added to samples before running in a 3200 Q Trap MS-MS system coupled with an Agilent 1100 Series liquid chromatograph.

Statistical analysis

All the statistical computations were performed by using the IBM SPSS Statistics software version 22 for Windows (IBM Inc., Armonk, NY, USA). Continuous variables were expressed as mean values and standard deviations (SD), while those showing a skewed distribution were log-transformed and, thus, expressed as geometric means with 95% confidence interval (CI). Continuous variables were tested by Student's *t*-test or analysis of variance (ANOVA) with Tukey's post-hoc comparison when appropriate. Categorical variables were analyzed using a χ^2 -test. All genotype distributions were assessed according to Hardy-Weinberg equilibrium. The strength of association of gene variants with the risk of cancer was estimated by means of sex- and age-adjusted logistic regression and Odds Ratios (ORs) with 95% CI are reported. A value of $p < 0.05$ was considered significant.

Results

Clinical characteristics of the subjects

[Table 1](#) reports the main clinical and biochemical characteristics of the cancer patients and cancer-free subjects and, as shown, the two groups were age- and sex-matched. No differences were observed in terms of plasma folate and vitamin B12 concentrations between cases and controls while the groups differed for CRP, haemoglobin, platelets count, AST, ALT, ALP, gGT, homocysteine, albumin, total cholesterol, triglycerides, creatinine, and ferritin levels ([Table 1](#)). Serologic tests for HBV and HCV were negative for all patients according to the enrolment criteria, and indexes of hepatic function confirmed a compensated status of liver disease in all patients ([Table 1](#)).

One-carbon genetic variants and cancer risk

The genotype distribution of the gene polymorphic variants was in agreement with the Hardy-Weinberg equilibrium both among cases and controls, except for the distribution, in the cancer group, of the *MTRR* 66A>G that was, therefore, excluded from subsequent analysis. [Table 2](#) reports the distribution of polymorphic variants frequencies in cancer patients and in cancer-free subjects. As shown, there were no differences in the genotype distribution for all of the one-carbon pathway polymorphic variants analysed except for the *MTHFD1* 1958AA genotype that was significantly less frequent among cancer patients as compared to cancer-free subjects ($p = 0.008$) and such difference was confirmed even after adjustments for sex and age ($p = 0.007$). The association with risk of cancer was then evaluated by setting the *MTHFD1* 1958GG genotype subjects as the reference group (OR = 1). The presence of the 1958A allele was associated with a lower risk for cancer in a graded manner so that the heterozygous variant, i.e. the 1958GA, was associated with a 51% risk reduction (OR = 0.49, CI 0.28–0.86, $p = 0.012$) and the 1958AA homozygous genotype was associated to a 63% reduction of cancer risk (OR = 0.37, CI 0.19–0.71, $p = 0.003$) ([Fig 2](#)). The risk for cancer was then analysed taking into account the 1958A allele carriership and the presence of the 1958A allele was associated to a lower cancer risk (OR = 0.46, CI 0.28–0.78, $p = 0.005$).

The analysis for the distribution between cancer patients and cancer-free subjects did not detect any differences for the other polymorphic variants analysed.

Table 1. Clinical and biochemical characteristics of cancer patients and cancer-free subjects.

	Reference values	Cancer patients	Cancer-free subjects	p-value [°]
		n = 102	n = 363	
Age (years)		66.2 (10.9)	65.0 (5.9)	0.139
Gender (% males)		66.7%	70.5%	0.455 [§]
CRP (mg/L)	<5.0	7.79 (5.75–10.18)	2.89 (2.61–3.13)	< 0.0001
Hb (g/dL)	13.5–16.0	13.1 (1.72)	14.2 (1.27)	< 0.0001
MCV (fL)	86.0–98.0	91.2 (6.68)	91.2 (5.36)	0.969
WBC (10 ⁹ /L)	4.3–10.0	7.00 (2.77)	6.89 (1.70)	0.701
Platelet count (10 ⁹ /L)	150–400	254 (111)	212 (61)	< 0.0001
AST (U/L)*	8–50	40.9 (35.2–47.5)	21.3 (20.5–22.2)	< 0.0001
ALT (U/L)*	8–45	36.2 (30.0–43.4)	23.3 (22.2–24.5)	< 0.0001
ALP (U/L)*	30–130	98.5 (87.4–111.1)	74.4 (72.2–76.7)	< 0.0001
gGT (U/L)*	<50	73.7 (60.3–89.1)	30.9 (28.8–33.1)	< 0.0001
Total bilirubin (mg/dL)*	0.11–1.05	0.66 (0.59–0.74)	0.67 (0.64–0.70)	0.852
PT (INR)*	0.82–1.14	2.25 (1.05–1.12)	2.39 (1.06–1.12)	0.773
Albumin (g/L)	35–50	39.8 (6.00)	41.8 (3.59)	< 0.0001
Total cholesterol (mg/dL)	<200	173.4 (48.7)	231.3 (44.0)	< 0.0001
Triglycerides (mg/dL)*	<150	116.8 (107.8–126.5)	142.6 (135.6–148.4)	< 0.0001
Creatinine (mg/dL)*	0.59–1.29	0.85 (0.79–0.92)	1.09 (1.06–1.13)	< 0.0001
Glucose (mmol/L)	3.5–5.5	5.85 (1.41)	6.17 (1.94)	0.121
Folate (nmol/L)*	10.4–42.4	13.2 (10.9–15.8)	12.1 (11.6–12.7)	0.390
tHcy (µg/L)*	< 15.0	11.9 (10.2–13.9)	15.8 (15.2–16.4)	<0.0001
VitaminB12 (pmol/L)*	142–724	323.8 (290.0–361.4)	287.2 (275.9–301.9)	0.056
Ferritin (µg/L)*	30–400	98.5 (89.1–108.9)	164.0 (127.7–210.6)	< 0.0001

Continuous variables with normal distribution are expressed as mean (standard deviation).

*: log-transformed variables are shown as geometric mean with 95% confidence interval

°: by Student's t-test for comparison between cancer patients and cancer-free patients

§: χ^2 -squared test

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Table 2. Comparison of polymorphic variants frequencies between cancer patients and cancer-free subjects.

	Cancer patients N = 102			Cancer-free subjects N = 363			p-value
	w/w	w/m	m/m	w/w	w/m	m/m	
BHMT716 G>A	46.4	44.3	9.3	44.8	40.3	14.8	0.355
DHFR 19bp ins/del	41.2	42.3	16.5	35.3	49.4	15.3	0.180
MTHFD1 1958 G>A	29.6	49.0	21.4	16.2	52.9	30.9	0.007 *
MTHFR 677 C>T	41.2	39.2	19.6	34.5	49.6	15.9	0.189
MTR 2756 A>G	67.3	31.6	1.0	66.7	28.6	4.8	0.226
RFC1 80 G>A	25.5	56.1	18.4	25.1	51.0	23.9	0.486
SHMT1 1420 C>T	44.2	46.3	9.5	53.9	40.7	5.3	0.136
TCII 776 C>G	32.7	50.0	17.3	31.5	51.4	17.1	0.967
TS 2rpt/3rpt **	19.6	45.4	35.1	20.2	47.2	31.7	0.887

Abbreviations: w = major allele, m = minor allele, N.S. = not statistically significant;

* after adjustment for age and sex;

** 0.9% of cancer-free subjects were carriers of the 2rpt/4rpt and 3rpt/4rpt genotype.

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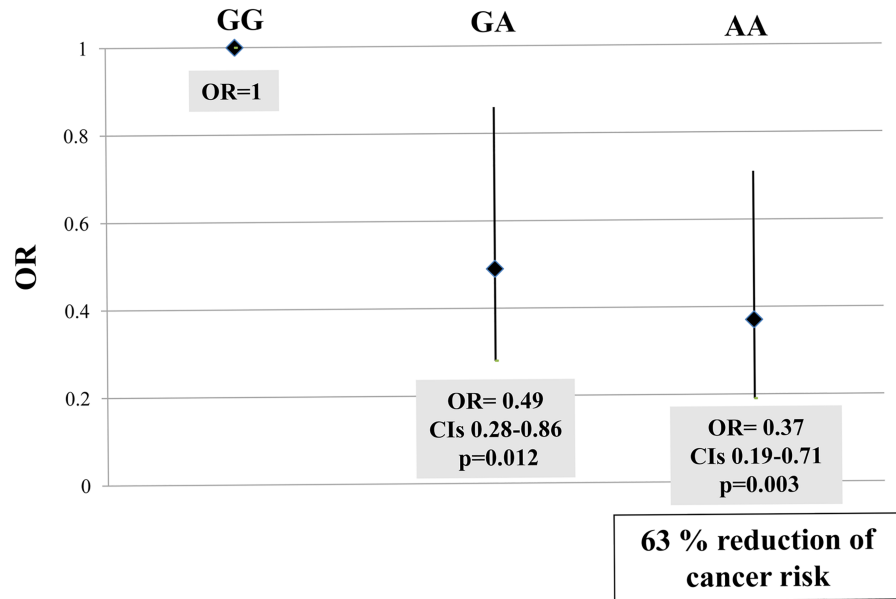


Fig 2. MTHFD1 1958G>A genotypes and cancer risk. The MTHFD1 1958A allele is associated to a lower cancer risk as expressed by means of Odds Ratio (OR) adjusted for sex and age.

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The MTHFD1 1958G>A genotypes according to cancer type and cancer risk

The MTHFD1 1958G>A genotype frequencies were then analysed according to the different types of cancer. Among HCC patients the frequencies were 24.3% (GG), 43.2% (GA) and 32.4% (AA); among CC patients the frequencies were 31.6% (GG), 57.9% (GA) and 10.5% (AA); as for the colon cancer patients group, the frequencies were 31.7% (GG), 52.2% (GA) and 17.1% (AA).

The MTHFD1 1958AA homozygous genotype was significantly less frequent among patients affected by colon cancer as compared to cancer-free subjects (17.1% versus 30.9%, $p = 0.025$) and this difference was confirmed when considering MTHFD1 1958A allele carrier-ship (68.3% in colon cancer versus 83.8% in controls, $p = 0.014$). In HCC and CC patients no statistically significant differences were observed in terms of distribution, as compared to cancer-free subjects. The association with risk of colon cancer was evaluated by setting the MTHFD1 1958GG subjects as the reference group (OR = 1) and the adjustment for sex and age was then performed. When the heterozygous MTHFD1 1958GA variant was evaluated according to cancer risk, the analysis did not reach statistical significance (OR = 0.48, CI 0.22–1.03, $p = 0.058$) whereas the homozygotic condition, i.e. 1958AA, was associated to a 75% reduction of colon cancer risk (OR = 0.25, CI 0.09–0.68, $p = 0.006$) (Fig 3). Moreover, the 1958A allele carriership was linked to a noticeable lower risk for colon cancer (OR = 0.45, CI 0.27–0.76, $p = 0.003$).

Global methylation in PBMCs DNA

Methylcytosine was measured in PBMCs of a subgroup of subjects and no significant differences were observed between cancer- (n = 62) and cancer-free subjects (n = 363) (5.34% versus 5.38%, $p = 0.38$). A similar level of mCyt content characterized the three groups affected by the different types of cancer (HCC 5.34%, CC 5.33%, colon cancer 5.35%, $p > 0.2$). The global

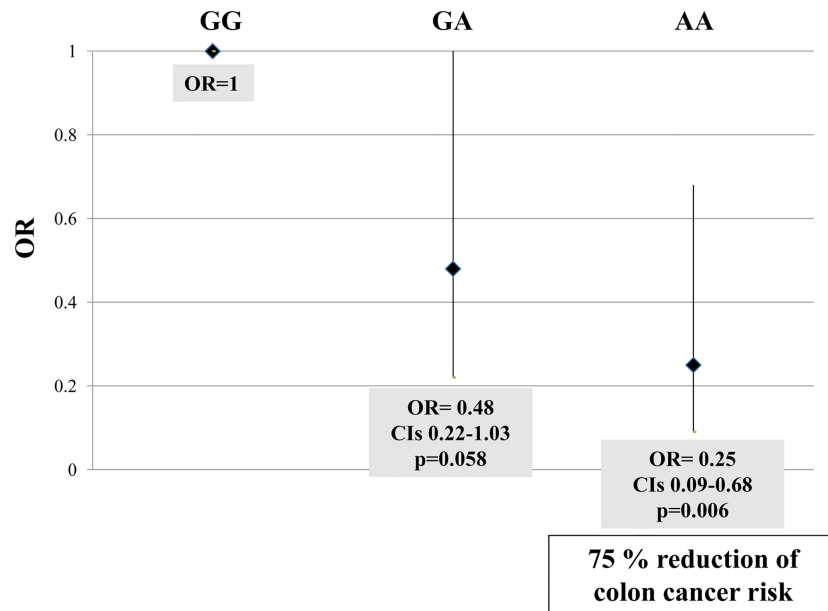


Fig 3. MTHFD1 1958G>A genotypes and colon cancer risk. The MTHFD11958AA genotype is associated to a significantly reduced colon cancer risk by means of Odds Ratio (OR) adjusted for sex and age.

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DNA methylation in PBMCs was then analysed according to the MTHFD1 1958G>A genotypes. When the cancer patients group was considered as a whole, no differences were associated to mCyt levels among the three genotypes, as compared to the cancer-free subjects ($p = 0.13$). Then the mCyt levels were analyzed according to the presence of the 1958A allele (A carriers versus GG genotype) in all the study subjects (cancer patients and cancer-free subjects). The A allele carriers and the GG genotype subjects varied significantly for mCyt levels (5.38% versus 5.31%, $p = 0.048$) (Fig 4). Then mCyt levels were analysed according to MTHFD1 1958G>A genotypes in the three different type of cancer. Among colon cancer patients ($n = 15$) mCyt levels resulted higher in MTHFD1 1958A carriers as compared with GG genotype, although the difference did not reach the statistical significance (5.42% versus 5.10% respectively, $p = 0.086$).

Discussion

Ten major polymorphisms related to one-carbon metabolism genes were analysed for the possible association with cancer risk, in particular HCC, CC and colon cancer risk. No differences in genotype distribution for all of the ten polymorphic variants analysed was, however, observed, neither there was an association with cancer risk, except for the MTHFD1 1958G>A. Furthermore, no differences were observed for folate and vitamin B12 levels between cancer and cancer-free subjects. This finding was, however, not surprising considering that both cancer and cancer-free subjects show folate and vitamin B12 status within a normality range.

This study demonstrates that the MTHFD1 1958GG genotype shows a higher frequency among cancer patients and it is associated, in all the study subjects, to PBMCs DNA hypomethylation as compared to the A allele carriers.

One-carbon metabolism is an essential crossroad in the balance between nucleotide synthesis and methylation reactions including that of DNA, known to be a key epigenetic mechanism

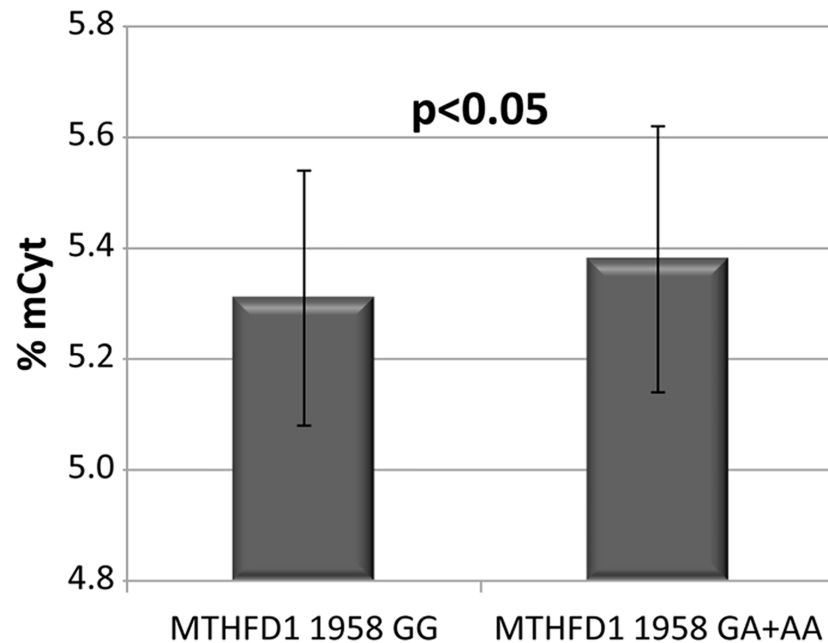


Fig 4. Global DNA methylation levels in PBMCs according to the *MTHFD1* 1958G>A genotypes. Global DNA methylation levels according to the *MTHFD1* 1958G>A genotypes in cancer patients and cancer-free subjects. GG: n = 72, GA+AA: n = 332. The error bar represents standard deviation (SD).

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in carcinogenesis [2, 12, 13]. Moreover, functional genetic variants of genes pertaining to one-carbon pathway are considered as potential risk factors for cancer disease (Fig 1) [5, 29]. Few data are available, so far concerning the mechanisms underlying the role of one-carbon metabolism variants in affecting cancer risk through the modulation of DNA methylation [8, 12, 13]. The MTHFD1 enzyme catalyzes three sequential and reversible reactions in the pathway of conversion of tetrahydrofolate (THF), the active form of folate, into substrates essential for the *de novo* purine and thymidylate synthesis [30] (Fig 5). Moreover, the MTHFD1 indirectly provides one-carbon units for methylation reactions by the synthesis of 5,10 methylene-THF [30]. Due to its role in nucleotide synthesis, the MTHFD1 enzyme may modulate cell division [31], it is therefore feasible that the functional alteration caused by the 1958G>A polymorphism may influence DNA synthesis reactions and cell development, eventually affecting carcinogenesis. The G>A substitution at position 1958 of the gene sequence leads to an arginine to glutamine substitution at 653 amino acid position in the enzyme synthase domain [21]. In a murine cell line model, the *MTHFD1* 1958AA genotype was associated to a 25% decreased purine synthesis [32] likely by affecting the supply of 10-formyl THF moieties for purine synthesis [33]. The function of the synthase domain was recently described to play an essential role in cellular proliferation by affecting nucleotide synthesis in rapidly dividing cells [34]. The *MTHFD1* 1958G>A variants have been mainly studied in relation to neural tube defects and embryonic development [31, 33, 35], but it has been also described as associated to cancer disease, although with not univocal results [3, 36, 37]. Wang and colleagues showed that the polymorphic 1958AA variant was linked to a significantly increased risk for gastric cancer as compared with the 1958GG or 1958AG genotypes [38], whereas most of the recent studies failed to demonstrate a significant association between the *MTHFD1* 1958 G>A polymorphic variants and different types of cancers [3, 36, 37]. As it refers to colon cancer disease, several studies failed to find a statistically significant association between *MTHFD1* 1958 G>A and either colon

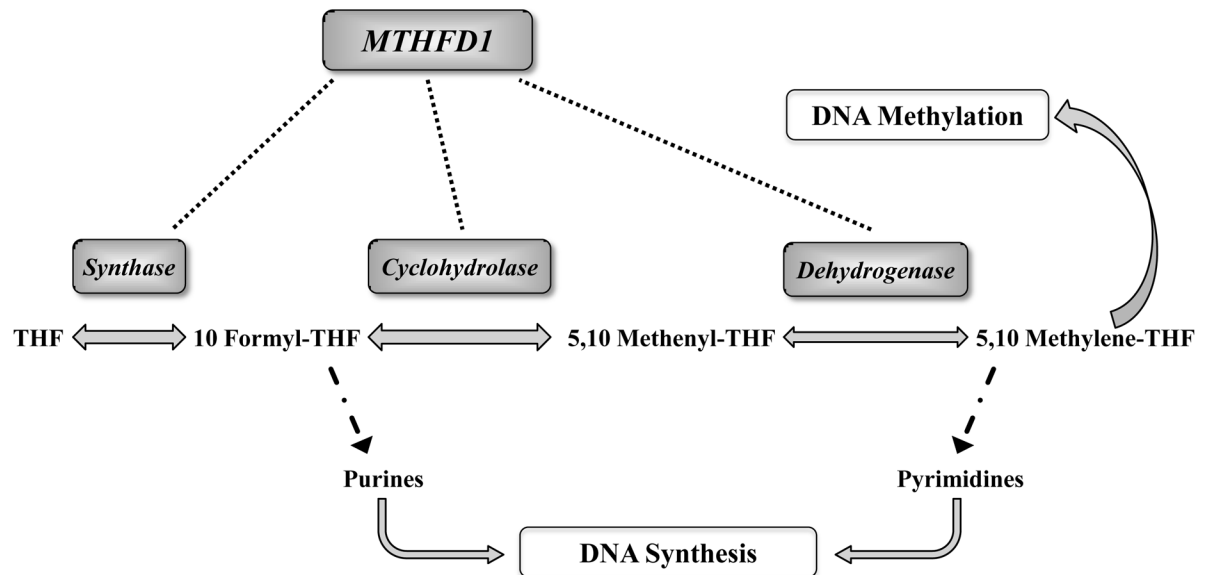


Fig 5. Pattern of reactions and functions of Methylene tetrahydrofolate dehydrogenase 1 (MTHFD1). The MTHFD1 enzyme catalyzes three sequential and reversible reactions in the pathway of conversion of tetrahydrofolate (THF), the active form of folate, into substrates essential for the *de novo* purine and thymidylate synthesis.

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cancer risk [5, 39, 40] or risk for colonic adenoma, a precursor lesion of colorectal cancer [41]. Interestingly, the present study showed a significantly different distribution of the *MTHFD1* 1958G>A genotypes between cancer and cancer-free subjects. In particular, there was a lower frequency of the *MTHFD1* 1958AA genotype among cancer patients as compared to cancer-free subjects and this difference was associated with a 63% reduction of the overall cancer risk. Even more intriguing was the significantly reduced *MTHFD1* 1958AA frequency in colon cancer patients as compared to controls and its association with a 75% reduction of colon cancer risk. Therefore our study demonstrates that the *MTHFD1* 1958AA is associated to a reduction for cancer risk, in particular for colon cancer. When global DNA methylation was analysed according to *MTHFD1* 1958G>A genotypes, the 1958GG genotype was either significantly more represented in patients affected by cancer, and associated with lower mCyt levels in PBMCs DNA. These results are in agreement with the previous observation of global DNA hypomethylation in cancer tissues [6, 12, 13] as well as in PBMCs DNA of patients affected by cancer [8]. In the subgroup of patients affected by colon cancer, the association between the presence of *MTHFD1* 1958GG genotype and DNA hypomethylation in PBMCs did not reach statistical significance probably due to the small sample size. The reported findings of the significantly lower frequency of the *MTHFD1* 1958AA genotype in patients affected by cancer disease and the association of this genotype with a lower risk for cancer appear suggestive for a protective role of this polymorphic variant, particularly in the development of colon cancer. The mechanism underlying this hypothesis may be a reduced synthase activity associated with the homozygous 1958AA genotype [32], which may slow down the cell proliferation processes and hinder the tumour initiation. Moreover, the present results suggest that both *MTHFD1* 1958AA and 1958GA genotypes could be protective for cancer through modulation of DNA methylation. In colon cancer patients the difference between mCyt levels in association with the 1958GG and the 1958 GA+AA genotypes respectively, may be due to the small sample size, thus further investigations are warranted.

In conclusion, the *MTHFD1* 1958AA genotype is less frequent among cancer patients and it is associated to a lower risk for cancer that is even more decreased specifically for colon cancer. Moreover, the association of A allele with the presence of higher DNA methylation levels in PBMCs suggests a possible protective role of this variant against DNA hypomethylation. On the other hand, the *MTHFD1* 1958GG genotype, more frequent among cancer patients, indicates a trend towards global PBMCs DNA hypomethylation as commonly observed in cancer. The link between the *MTHFD1* 1958AA genotype with a decreased cancer risk may be explained by its possible modulatory effect between DNA methylation and nucleotide synthesis. Further studies are certainly needed to better unravel the role of *MTHFD1* 1958 A>G in cancer risk.

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