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A candidate gene study of one-carbon metabolism pathway genes and colorectal cancer risk

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Abstract

The risk of colorectal cancer (CRC) may be influenced by aberrant DNA methylation and altered nucleotide synthesis and repair, possibly caused by impaired dietary folate intake as well as by polymorphic variants in one-carbon metabolism genes. A case–control study using seventy-one CRC patients and eighty unrelated healthy controls was carried out to assess the genetic association of fifteen SNP and one insertion in nine genes belonging to the folate pathway. Polymorphism selection was based on literature data, and included those which have a known or suspected functional impact on cancer and missense polymorphisms that are most likely to alter protein function. Genotyping was performed by real-time PCR and PCR followed by restriction analysis. The likelihood ratio statistic indicated that most of the polymorphisms were not associated with the risk of CRC. However, an increased risk of CRC was observed for two variant alleles of SNP mapping on the transcobalamin 2 gene (*TCN2*): C776G (rs1801198) and c.1026-394T > G (rs7286680). Considering the crucial biological function played by one-carbon metabolism genes, further investigations with larger cohorts of CRC patients are needed in order to confirm our preliminary results. These preliminary results indicate that TCN2 polymorphisms can be a susceptibility factor for CRC.

Key words: Colorectal cancer: Folate: Polymorphisms: Transcobalamin 2

Folate is a water-soluble B vitamin which is essential for DNA synthesis, repair and methylation. Epidemiological analyses have shown that regular consumption of fruit and vegetables – natural sources of folate – correlates with a decreased risk of cancer[1,2]. A pregnant woman's intake of one-carbon nutrients can modify her child's risk of incurring tumours, even decades after birth[3]. Perturbation of different steps in the folate pathway may induce and accelerate carcinogenesis, as a consequence of DNA instability. There is extensive literature which correlates genetic polymorphisms of folate pathway genes with the occurrence of several diseases and malformations (e.g. neural tube defects, cleft lip/palate, etc.). It is believed that these polymorphisms could alter gene and/or protein functions; however, little is known about the functional impact of each polymorphism and the possible perturbation effect of the overall one-carbon biochemical pathway.

Colorectal cancer (CRC) is currently considered to be the second cause of morbidity and death in the Western world (WHO).

Specific epidemiological and experimental studies have been performed to evaluate the implication of low folate intake in the risk of CRC[4,5].

Some genetic studies have assessed the association between certain nucleotide polymorphisms (SNP) of genes belonging to the folate pathway.

The allelic associations between CRC and the functional C677T (rs1801133) polymorphism of the methylenetetrahydrofolate reductase (*MTHFR*) gene (which codes for a key enzyme for homocysteine remethylation to methionine) have been extensively studied. The enzyme coded by the 677T MTHFR variant allele shows a 60% activity reduction *in vitro*[6]. In four meta-analysis studies, a modest but statistically significant decrease in the risk of CRC (15–18%) has been reported for 677TT homozygotes[7–10]. The data were recently confirmed in an independent study[11].

Methionine synthase (MTR) catalyses the vitamin B_{12}-mediated transfer of a methyl group to convert homocysteine to methionine. Common *MTR* variants have been reported as

Abbreviations: BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; CRC, colorectal cancer; CUBN, cubilin; DHFR, dihydrofolate reductase; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; RFC1, reduced folate carrier 1; TCN2, transcobalamin 2.

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being associated with a decrease in CRC risk among non-users of multivitamin supplements[12]. The homocysteine remethylation to methionine process also needs betaine-homocysteine methyltransferase (BHMT): a Zn-dependent cytosolic enzyme that utilises betaine as a methyl donor. A slight increased risk of CRC has been associated with the G716A (rs3733890) variant allele of *BHMT*[13].

Homocysteine trans-sulfuration (the alternative path for homocysteine clearance) is initially catalysed by cystathionine β-synthase (CBS), a vitamin B_6-dependent lyase that generates cystathionine from the condensation of homocysteine with serine. The *CBS* 844ins68 variant would appear to have a protective effect against CRC because it was less frequently found in patients who developed proximal tumours than in normal control subjects[14].

Dihydrofolate reductase (DHFR) reduces dihydrofolate to tetrahydrofolate, thus allowing its use in methyl group transfers. In patients who do not take multivitamin supplements, two common variants of *DHFR* SNP have been suggested to be associated with a decreased risk of CRC[12].

Transcobalamin 2 (TCN2) is a plasma transport protein for cobalamin (vitamin B_{12}) and determines vitamin B_{12} cellular availability. Hazra *et al.*[15] observed a modest increased risk among carriers of the *TCN2* C776G (rs1801198) variant in homo- or heterozygosis for colorectal adenoma.

Although other genes involved in folate uptake, metabolism or distribution have been investigated, no association with CRC risk has been found. Examples are as follows: reduced folate carrier 1 (RFC1/SLC19A1), which is involved in folate uptake[16]; methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate-cyclohydrolase/formyltetrahydrofolate synthetase 1 (MTHFD1), a trifunctional enzyme which catalyses sequential interconversion of tetrahydrofolate derivatives required for purine, methionine and thymidylate synthesis[12]; the cubilin gene (*CUBN*) that encodes for the intrinsic factor-cobalamin receptor, a transmembrane protein important for the intestinal absorption of cobalamin[12].

In the present study, we investigated fifteen genetic polymorphisms and one insertion mapping in nine genes in order to verify their genetic association with CRC. These nine genes belonging to folic acid and methionine pathways are involved in nucleotide synthesis, methionine metabolism, uptake and distribution of vitamin B_{12}.

Table 1. Patients and control subjects

(Mean values, standard deviations and ranges, or number of subjects)

Characteristics	Patients	Controls	*P*
Age (years)			
Mean	69	58	0·64
SD	13·3	12·6	
Range	29–92	22–83	
Sex (*n*)			0·51
Female	29	37	
Male	42	43	
Primary surgery for CRC (*n*)			
Yes	71	0	
No	0	80	
Anatomic site (*n*)			
Colon	36	–	
Rectosigmoideum	16	–	
Rectum	19	–	
Stage* (*n*)			
I	6	–	
II	28	–	
III	22	–	
IV	15	–	

CRC, colorectal cancer.
* Staging according to the American Joint Committee on Cancer and International Union against Cancer.

Methods

Sample study

For the present Italian population-based study, seventy-one consecutively ascertained unrelated CRC patients with confirmed diagnoses were enlisted. The subjects were recruited from the Department of General Surgery at the S. Orsola-Malpighi Polyclinic, Bologna University. The control sample consisted of eighty unrelated volunteers with a healthy general clinical and biochemical assessment. All patient and control information is summarised in Table 1. The study was approved by the Sant'Orsola-Malpighi General Hospital ethical committee and complied with the Ethical Principles for Medical Research Involving Human Subjects of the Helsinki Declaration. Written informed consent was obtained from all patients and healthy control subjects before being enrolled in the study. DNA extraction from the peripheral whole blood, collected before primary surgery, was performed as described previously[17].

Polymorphisms

In order to investigate nine folate pathway genes, fifteen SNP and one insertion polymorphism were selected. Polymorphism selection was based on literature data and included those with a known or suspected functional impact, such as SNP associated with cancer or other diseases and missense polymorphisms. Our preference was for those with a minor allele frequency >0·1 or a moderate degree of linkage disequilibrium between each other.

Genotyping was performed by real-time PCR, using TaqMan assays for *BHMT*, *CBS* and *CUBN* SNP, following the manufacturer's protocol. PCR followed by analysis and separation of fragments by 10 % PAGE stained with ethidium bromide was carried out to investigate *MTHFD1*, *MTR*, *TCN2*, *RFC1*, *MTHFR* and *DHFR* SNP. The presence of the *CBS* 68 bp insertion (844ins68) was assessed by electrophoresis run on 2·5 % agarose of amplicons. To assess the accuracy of the genotyping outputs, 30 % of the random genotypes were tested in a blind check by a second operator.

The information regarding markers is summarised in Table 2.

Statistical analysis

The distribution of genotypes in the patient and control groups was tested for deviations from the Hardy–Weinberg equilibrium using Pearson's χ^2 test. Case–control association tests were performed using Unphased software version

Table 2. SNP characteristics, allele count and allelic association test

(Odds ratios and 95 % confidence intervals)

	Polymorphism characteristics			Cases				Controls				Allelic association	a	
Gene and polymorphism ID	Nucleotide change*	Location or amino acid substitution	Assay ID/restriction site	A	%	a	%	A	%	a	%	*P*	OR	95 % CI
BHMT														
rs3733890	G/A	Arg239Gln	C_11646606_20	88	62	54	38	103	64	57	36	0·67	1·11	0·69, 1·77
CBS														
rs234713	G/A	Intron	C_1605453_1	104	73	38	27	115	72	45	28	0·79	0·93	0·56, 1·55
rs4920037	G/A	Intron	C_1605440_1	104	79	28	21	125	78	35	22	0·89	0·96	0·55, 1·69
844ins68	–	Exon 8		135	95	7	05	152	95	8	05	0·98	0·99	0·35, 2·79
CUBN														
rs1907362	G/A	Intron	C_11640657_10	131	95	7	05	145	91	15	09	0·15	0·52	0·20, 1·31
rs1801231	C/T	Pro1559Ser	C_26740615_10	124	87	18	13	143	89	17	11	0·58	1·22	0·60, 2·47
rs1801222	C/T	Phe253Ser	C_2822674_10	119	85	21	15	131	82	29	18	0·47	0·82	0·40, 1·66
MTHFD1														
rs2236225	G/A	Arg653Gln	*Msp*I	72	51	70	49	81	51	79	49	0·99	1·00	0·63, 1·57
rs1950902	G/A	Arg134Lys	*BsmA*I	129	91	13	09	141	88	19	12	0·44	0·75	0·36, 1·58
MTR														
rs1805087	A/G	Asp919Gly	*Hae*III	122	86	20	14	124	78	36	23	0·06	0·56	0·31, 1·03
TCN2														
rs1801198	C/G	Pro259Arg	*Bst*NI	61	43	81	57	90	56	72	44	0·03	1·66	1·05, 2·62
rs7286680	T/G	Intron	*Hae*III	73	51	69	49	106	65	56	35	0·01	1·79	1·13, 2·84
rs10418	C/T	3′-UTR	*Ava*II†	109	77	33	23	133	83	27	17	0·17	1·49	0·84, 2·63
RFC1														
rs1051266	A/G	His27Arg	*Hae*II	76	54	66	46	84	53	76	48	0·86	0·96	0·61, 1·51
MTHFR														
rs1801133	C/T	Ala222Val	*Hinf*I	83	58	59	42	84	52	78	48	0·25	0·77	0·49, 1·21
DHFR														
rs1643659	A/G	Intron	*Bst*NI	106	75	36	25	109	68	51	32	0·21	0·73	0·44, 1·20

BHMT, betaine-homocysteine methyltransferase; *CBS*, cystathionine β-synthase; *CUBN*, cubilin; *MTHFD1*, methylenetetrahydrofolate dehydrogenase 1; *MTR*, methionine synthase; *TCN2*, transcobalamin 2; *RFC1*, reduced folate carrier 1; *MTHFR*, methylenetetrahydrofolate reductase; *DHFR*, dihydrofolate reductase.

* Major allele/minor.

† An artificial restriction site for *Ava*II was created by using a mutagenic sense primer with a mismatch in the nucleotide sequence.

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3.1.5 within a Windows Vista operative system[18]. Genetic association was investigated using both allelic and genotypic tests with a likelihood ratio approach, and statistical significance was assessed by *P* value. The heterozygote and homozygote OR for the risk allele were calculated in the case of a significant overall genotypic association.

Haplotype association was performed when two or three polymorphisms within a single gene were analysed. First, we carried out a global test to check for any haplotype association, and then we carried out a test on each single haplotype identified.

Results

A high success rate was obtained during the genotyping stage; in fact, almost 100 % of the genotypes were obtained with the different assays. Genotype distribution was in agreement with the Hardy–Weinberg law.

The allelic association test revealed that allele frequencies in the case study and control groups were similar, with the exception of two polymorphisms in the *TCN2* gene (Table 2). Indeed, the variant allele at both C776G (rs1801198) and c.1026-394T>G (rs7286680) loci was more frequent among those in the case study group. The calculated OR were 1·66 (95 % CI 1·05, 2·62; *P* 0·03) and 1·79 (95 % CI 1·13, 2·84; *P* 0·01), respectively. However, with regard to the multiple testing issue, none of the association tests exceeded the Bonferroni corrected threshold for statistical significance ($P = 0{\cdot}05/16 = 0{\cdot}003$).

The likelihood ratio statistic used to test genotypic association provided similar results (Table 3). The polymorphic variant rs1801198 showed a significant overall genotypic association ($P = 0{\cdot}01$) and an increased risk for the homozygote carrier of the rare allele (OR 2·9, 95 % CI 1·1, 7·6). A significant genotypic association was also detected at rs7286680 ($P = 0{\cdot}04$), again with an increased risk for homozygotes (OR 3·1, 95 % CI 1·2, 8·2).

The haplotype association study confirmed the lack of an association with *CBS*, *CUBN* and *MTHFD1*, while it confirmed the association between CRC and polymorphisms at the *TCN2* locus. In fact, the overall statistics provided evidence of the association between three marker haplotypes ($P = 0{\cdot}03$). The estimated OR for each relevant haplotype is reported in Table 4. The haplotypes that included the variant allele at each of the three SNP loci demonstrated a significant increase in the risk of CRC (OR 2·1, 95 % CI 1·1, 3·9).

Discussion

CRC is among the major causes of morbidity and death in Italy. Both genetic and environmental factors are strongly involved in its aetiology. Epidemiological studies have highlighted the importance of a correct diet and lifestyle, estimating that inappropriate nutrition can be the cause of more than one-third of cancer deaths[1].

Folates are vitamins responsible for a number of processes including DNA stability, synthesis, repair and, not least, methylation. Folate pathway genetic polymorphisms may cause inactivation of the tumour suppressor genes. The hypothesis that a perturbation of the folate pathway could induce carcinogenesis[2] has encouraged researchers to investigate the different enzymes and cofactors involved in this crucial metabolic process as possible cancer susceptibility factors.

For the present investigation, we considered fifteen genetic polymorphisms and one insertion mapping in nine genes of the folate pathway for their possible involvement in colorectal carcinogenesis in the Italian population. Non-synonymous polymorphisms were preferentially selected for the candidate gene investigation because they are more likely to alter protein function. Biochemical data supporting this hypothesis were reported in some cases but in others, no functional data were available.

With the exception of *TCN2* polymorphisms, no evidence of an association was found between SNP alleles and the

Table 3. Genotype distribution and overall genotypic association (Odds ratios and 95 % confidence intervals)

		Case			Control			Association	Heterozygote		Homozygote	
Gene	Polymorphism ID	AA	Aa	aa	AA	Aa	aa	*P*	OR	95 % CI	OR	95 % CI
BHMT	rs3733890	29	30	12	32	39	9	0·54	1·47	0·54, 4·00	0·85	0·42, 1·70
CBS	rs234713	41	22	8	42	31	7	0·58	0·73	0·36, 1·46	1·17	0·39, 3·52
CBS	rs4920037	42	20	4	52	21	7	0·75	1·18	0·57, 2·46	0·71	0·19, 2·58
CBS	844ins68	64	7	0	73	6	1	0·62	1·33	0·43, 4·17		–
CUBN	rs1907362	62	7	0	67	11	2	0·46	0·69	0·25, 1·89		–
CUBN	rs1801231	53	18	0	63	17	0	0·55	1·26	0·59, 2·68		–
CUBN	rs1801222	52	15	3	53	25	2	0·36	0·61	0·29, 1·29	1·53	0·25, 9·53
MTHFD1	rs2236225	16	40	15	21	39	20	0·65	1·35	0·61, 2·95	0·98	0·39, 2·50
MTHFD1	rs1950902	60	9	2	61	19	0	0·09	0·48	0·20, 1·15		–
MTR	rs1805087	53	16	2	50	24	6	0·20	0·63	0·30, 1·32	0·31	0·06, 1·63
TCN2	rs1801198	15	31	25	21	48	12	0·01	0·90	0·41, 2·02	2·92	1·12, 7·58
TCN2	rs7286680	18	37	16	35	36	10	0·04	2·00	0·96, 4·15	3·11	1·18, 8·24
TCN2	rs10418	41	27	3	56	21	3	0·28	1·76	0·87, 3·53	1·37	0·26, 7·11
RFC1	rs1051266	22	32	17	19	46	15	0·31	0·60	0·28, 1·29	0·98	0·39, 2·47
MTHFR	rs1801133	25	33	13	21	42	18	0·45	0·66	0·32, 1·38	0·61	0·24, 1·52
DHFR	rs1643659	39	28	4	35	39	6	0·39	0·64	0·33, 1·25	0·60	0·16, 2·30

BHMT, betaine-homocysteine methyltransferase; *CBS*, cystathionine β-synthase; *CUBN*, cubilin; *MTHFD1*, methylenetetrahydrofolate dehydrogenase 1; *MTR*, methionine synthase; *TCN2*, transcobalamin 2; *RFC1*, reduced folate carrier 1; *MTHFR*, methylenetetrahydrofolate reductase; *DHFR*, dihydrofolate reductase.

Table 4. Haplotype association analysis between transcobalamin 2 polymorphisms and colorectal cancer*

(Odds ratios and 95 % confidence intervals)

Haplotype	Cases	Controls	Ca-Freq	Co-Freq	OR	95 % CI
C-T-C	57	81	0·41	0·55	Ref.	
G-T-C	14	21	0·10	0·14	0·95	0·44, 2·02
G-G-C	35	24	0·25	0·16	2·07	1·12, 3·85
G-G-T	32	22	0·23	0·15	2·07	1·09, 3·92

Ca-Freq, case frequency; Co-Freq, control frequency; Ref., reference.
* Haplotypes are combinations of rs1801198, rs7286680 and rs10418 alleles.

occurrence of CRC. Of the three investigated polymorphisms at *TCN2*, two showed a significantly higher frequency of the variant allele. A significantly increased risk of colon cancer was found in homozygote subjects for the variant alleles, and the OR were 2·9 (95 % CI 1·1, 7·6) for C776G (rs1801198) and 3·1 (95 % CI 1·2, 8·2) for c.1026-394T > G (rs7286680).

TCN2 is an essential carrier for the cellular uptake of vitamin B_{12}, a critical cofactor in the remethylation of homocysteine to methionine in mammals[19]. Functional studies on *TCN2* C776G (rs1801198) have provided evidence that the variant allele may be able to reduce the affinity of TCN2 to vitamin B_{12}, thereby lowering the delivery of vitamin B_{12} to the tissues[20,21], even if further studies are required to confirm such findings.

Previous investigations have reported conflicting results. Indeed, the *TCN2* 776G (259Arg) variant allele was found to be relevant to adenoma development[15], while it was not seen to be significantly associated with advanced colorectal adenoma or cancer[13].

In the present study, the functional *TCN2* C776G (rs1801198) was not the only *TCN2* polymorphism associated with CRC. The intronic SNP c.1026-394T > G (rs7286680) showed a degree of association and OR close to those observed for C776G. Linkage disequilibrium levels between these two polymorphisms were not very close ($D = 0{\cdot}8$, r^2 0·4 in controls), and this led us to suppose that both of them were independently associated with CRC. However, we cannot exclude the possibility that an unrecognised CRC susceptibility allele(s) could explain the observed data.

The sample size of the present investigation could be considered too small to offer conclusive results regarding the link between the analysed SNP and CRC susceptibility. On the other hand, this is the first significant data to have supported the hypothesis that *TCN2* alleles can alter the risk of developing CRC. Although there is only preliminary biochemical evidence available in relation to the functional effects of variant alleles, genetic data support a relationship between *TCN2* and CRC. Since CRC continues to be a serious health issue, we strongly believe that further investigations are essential, both to confirm the present findings and to identify possible interactions with the diet and other genes.

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