Low expression of γ-glutamyl hydrolase mRNA in primary colorectal cancer with the CpG island methylator phenotype

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Running title: GGH expression and DNA methylation

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Abstract

The CpG island methylator phenotype (CIMP+) in colorectal cancer (CRC) is defined as concomitant and frequent hypermethylation of CpG islands within gene promoter regions. We previously demonstrated that CIMP+ was associated with elevated concentrations of folate intermediates in tumour tissues. In the present study we investigated whether CIMP+ was associated with a specific mRNA expression pattern for folate and nucleotide metabolizing enzymes. An exploratory study was conducted on 114 CRC samples from Australia. mRNA levels for 17 genes involved in folate and nucleotide metabolism were measured by real-time RT-PCR. CIMP+ was determined by real-time methylation specific PCR and compared to mRNA expression. Candidate genes showing association with CIMP+ were further investigated in a replication cohort of 150 CRC samples from Japan. In the exploratory study, low expression of gamma-glutamyl hydrolase (GGH) was strongly associated with CIMP+ and CIMP+–related clinicopathological and molecular features. Trends for inverse association between GGH expression and the concentration of folate intermediates were also observed. Analysis of the replication cohort confirmed that GGH expression was significantly lower in CIMP+ CRC. Promoter hypermethylation of GGH was observed in only 5.6% (1/18) CIMP+ tumours and could not account for the low expression level of this gene. CIMP+ CRC is associated with low expression of GGH, suggesting involvement of the folate pathway in the development and/or progression of this phenotype. Further studies of folate metabolism in CIMP+ CRC may help to elucidate the etiology of these tumours and to predict their response to anti-folates and 5-FU/leucovorin.

Keywords: CIMP; GGH; promoter methylation; colorectal cancer;

Introduction

Cancer is a disease with genetic and epigenetic abnormalities. Aberrant CpG island methylation is a common epigenetic alteration in a variety of malignancies (Jones, 2002). *De novo* methylation of CpG islands in promoter regions is believed to contribute to tumourigenesis by causing transcriptional silencing of tumour suppressor genes. Colorectal cancer (CRC) is one of the malignancies in which epigenetic changes have been extensively analyzed. Research on clinical samples has shown that a subgroup of CRC shows concurrent hypermethylation of a large number of CpG islands. These have been termed CIMP+, for CpG island methylator phenotype (Toyota et al., 1999). CIMP+ tumours occur more frequently in the proximal colon of older patients and are associated with the microsatellite instability phenotype, tumour-infiltrating lymphocytes (TILS) and mutations in the *BRAF* oncogene (Hawkins et al., 2002; Samowitz et al., 2005; van Rijnsoever et al., 2002). Quantitative DNA methylation analysis using real time techniques indicate that approximately 17% of CRC are CIMP+ (Iacopetta et al., 2007; Ogino et al., 2006; Weisenberger et al., 2006). A panel of five CpG island markers was recently proposed in order to standardize the definition of CIMP+ status (Weisenberger et al., 2006).

Although the existence of a CIMP+ CRC subgroup is evident, the etiology of this phenotype is not well understood. We previously reported that CIMP+ was associated with elevated concentrations of the folate intermediates CH_2FH_4 and FH_4 in CRC tissues (Kawakami et al., 2003). This suggests that folate metabolism may be an important factor in determining the DNA methylation status of primary CRC. Folate plays a major role in cellular homeostasis as a donor of one-carbon units for DNA methylation, protein methylation and nucleotide synthesis. Increased dietary folate

intake and serum levels of folate show correlations with increased global DNA methylation levels in epidemiological studies (Pufulete et al., 2005b), animal models (Sohn et al., 2003) and clinical intervention studies (Pufulete et al., 2005a). Associations between dietary folate intake (van Engeland et al., 2003) or genetic variants in folate metabolizing enzymes (Paz et al., 2002) and CpG island hypermethylation in CRC have also been reported, although other workers have found less evidence for this (Curtin et al., 2007; Slattery et al., 2007). These observations suggest that folate metabolism, at least in part, can influence CpG island methylation and may therefore be involved in the development of CIMP+ CRC, although firm evidence for this is still lacking.

Two key metabolic pathways for methyl donor / one-carbon transfer reactions are the synthesis of folate and nucleotides. In the present study we hypothesized that a specific expression pattern for folate and nucleotide metabolizing enzymes occurs in CIMP+ CRC. Our rationale was that a distinctive gene expression signature may be associated with the aberrant methyl group metabolism of CIMP+ tumours as evidenced by the frequent CpG island hypermethylation. To test this hypothesis, the mRNA expression level of 17 genes with important roles in folate and nucleotide metabolism were measured by real-time RT-PCR in two series of primary CRC in which the CIMP+ status was determined by methylation specific real-time PCR.

Materials and methods

Samples

For exploratory analysis of gene expression levels, tumour samples from a consecutive series of 114 CRC patients undergoing elective surgery at the Colorectal Unit of the Royal Adelaide Hospital in Australia were used. These were snap-frozen in liquid

nitrogen within 20–40 min of resection and stored at –70^{o}C. DNA was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was obtained from the corresponding formalin-fixed and paraffin embedded (FFPE) tissues. FFPE tissue blocks were reviewed for quality and tumour content and 5μm thickness sections were obtained. Sections were mounted on uncoated glass slides, deparaffinized in xylene, hydrated and stained with nuclear fast red (American MasterTech Scientific Inc., Lodi, CA). Tumour cells were isolated by laser capture microdissection (PALM Microsystem; Leica, Wetzlar, Germany) according to standard procedures (Bonner et al., 1997). RNA isolation after dissection was performed according to a proprietary procedure (Response Genetics, Inc., U.S. patent no. 6248535). We have previously measured the concentrations of the folate intermediates CH_2FH_4 and FH_4 (Kawakami et al., 2003) and analyzed for *BRAF* V600E mutation (Iacopetta et al., 2006) in this tumour series. Approval of this project was obtained from the IMVS Human Research Ethics Committee.

For the validation tumour set, 150 primary CRC from patients undergoing surgical treatment at Kanazawa University Hospital in Japan were used. Tumour was dissected manually from FFPE archival tissue sections of 10μm thickness. After deparaffinization using xylene and ethanol, genomic DNA was isolated using a QIAamp DNA mini kit. RNA was obtained from the manually dissected FFPE samples using the same method as for the Australian CRC series. Approval of this project was obtained from the Ethics Committee of the Kanazawa University School of Medicine.

Real-time RT-PCR and immunohistochemistry

Complementary DNA was prepared as described previously (Lord et al., 2000). Quantification of the genes of interest (Table 1) and an internal reference gene (ACTB)

was conducted using a fluorescence-based real-time detection method [ABI PRISM 7700 Sequence Detection System (TaqMan); Perkin-Elmer Applied Biosystems], as previously described (Dziadziuszko et al., 2006; Gibson et al., 1996). Gene expression values were expressed as ratios (differences between Ct values) between the gene of interest and an internal reference gene (ACTB). Primer and probe sequences used in this study are listed in supplementary Table 1.

For the validation study with Japanese CRC samples, different primer sets for ECGF1, GGH, RRM2 and ACTB (supplementary Table 1) were used with SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Japan) and following the protocol provided by the manufacturer using ABI PRISM 7700 Sequence Detection System. The quantity of mRNA was expressed as the ratio of the expression level between each test mRNA and ACTB mRNA.

Protein expression of GGH in tumor tissues was examined by immunohistochemistry for selected samples from the Japanese CRC cohort. The avidin-biotin-peroxidase complex (ABC) method with chicken polyclonal antibody (IgY) to human GGH (diluted 1:100; GenWay Biotech, San Diego, CA) and biotinylated rabbit anti-chicken IgY (diluted 1:200; Open Biosystems, Huntsville, AL) was used following microwave antigen retrieval of paraffin sections as described previously (Ougolkov et al., 2002).

Methylation analysis

Promoter methylation was evaluated for the CIMP panel of markers comprising *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*, where PMR (percentage methylated reference) values were derived using the ALU normalization control reaction (Weisenberger et al., 2006). Simultaneous hypermethylation (PMR ≥ 10) of 3

or more of these 5 markers was considered to represent CIMP+. Promoter hypermethylation of GGH was analyzed as previously described (Cheng et al., 2006). Sperm DNA and fully methylated DNA by SssI methylase (NewEngland Biolabs) were used as unmethylated and methylated control samples, respectively.

Statistics

Because mRNA expression levels did not show normal distribution, the results were expressed as median values (25th percentile – 75th percentile) in Tables or boxplots. Nonparametric models were used for univariate analyses. The Mann-Whitney U test was used to compare mRNA expression levels between two categorical variables. Correlations between mRNA expression and the concentration of folate intermediates were analyzed by Spearman's rank test. A multivariate stepwise logistic regression approach was used to select genes whose mRNA expression was significantly related to CIMP status. All *P* values shown are two tailed with $P < 0.05$ taken as significant.

Results

Associations between mRNA expression levels for folate and nucleotide metabolizing enzymes and CIMP+ or CIMP+–related features

RT-PCR assays were conducted for 17 genes in 114 colorectal tumour samples from Australia. The assays were performed in triplicate for RT samples and in a single assay for the non-RT controls, resulting in 1938 mRNA measurements by 7752 assays. The non-RT control reaction was positive in 31 measurements and the coefficient of variance was high among triplicate assays in 4 measurements. These were deemed as "no result". In all, 1903/1938 (98.2%) real-time RT-PCR measurements were successful

using RNA derived from laser capture microdissected FFPE tumour tissues. CIMP+ was found in 18/114 (15.8%) CRC samples.

Cluster analysis did not reveal a distinctive mRNA expression profile associated with CIMP+ (data not shown). In univariate analysis (Mann-Whitney U test), GGH expression was significantly lower in CIMP+ than CIMP- CRC, whereas the expression of DCK, DPYD, ECGF1, MTHFR and RRM2 were all higher in CIMP+ (Table 2). Multivariate analysis using a logistic regression model showed that GGH (odds ratio 0.70, 95%CI: 0.51 – 0.95, p = 0.023) and RRM2 expression (odds ratio 1.25, 95%CI: 1.04 – 1.49, p = 0.015) were associated with CIMP+ (p = 0.008). Univariate analysis (Mann-Whitney U test) showed that ECGF1 and GGH expression were strongly associated with the CIMP+ features of proximal tumour site, tumour-infiltrating lymphocytes (TILS) and *BRAF* mutation (Table 3, Supplementary Table2). GGH expression was lower, whereas ECGF1 was higher in tumour with these CIMP+ features. The analyses showed that low expression of GGH was consistently associated with CIMP+ and CIMP+–related features (Figure 1). The high expression levels of RRM2 and ECGF1 also showed strong associations with CIMP+ and CIMP+–related features, respectively.

Finally, mRNA expression was compared to the concentrations of the folate intermediates CH_2FH_4 and FH_4 in these CRC tissues (Table 4). None of the genes examined showed significant correlation with the concentrations of these intermediates, although high RFC1 expression was significantly correlated with low concentrations of FH_4 (Spearman's rho = -0.205, p = 0.046). Trends for negative correlation between GGH expression and CH_2FH_4 and FH_4 concentrations were observed (Spearman's rho = -0.200, p = 0.053 and Spearman's rho = -0.180, p = 0.083, respectively). The above exploratory analyses suggest that low GGH mRNA expression is a candidate CIMP+

molecular signature, possibly through its involvement in folate metabolism.

Validation of GGH down-regulation in CIMP+ CRC

A validation study was conducted using 150 primary CRC samples from a Japanese cohort of patients. GGH, ECGF1 and RRM2 were selected as candidates for further study because the expression of these genes was consistently associated with CIMP+ and/or CIMP+-related features in the Australian CRC series. Only 14/150 (9.3%) of the Japanese CRC samples were found to be CIMP+ compared to 15.8% of the Australian tumours ($p = 0.11$ in chi-square test). A random selection of CIMP- CRC ($n = 79$) and all 14 CIMP+ CRC from the Japanese cohort were subjected to RT-PCR analysis of GGH, ECGF1 and RRM2 expression. The results confirmed that GGH mRNA expression was again significantly lower in CIMP+ CRC from a separate tumour series ($p=0.0012$, Figure 2). No significant associations were observed between CIMP+ and either ECGF1 or RRM2 mRNA expression.

To further examine whether the mRNA level reflects GGH protein expression, selected paraffin tissues of Japanese CRC were immunostained using polyclonal antibody to human GGH. GGH protein was not detectable or was weakly expressed in 4 samples with low mRNA levels (0.01, 0.06, 0.28, and 0.32), whereas much stronger expression was observed in 5 samples with high mRNA levels (3.25, 3.50, 3.73, 6.33 and 8.29). Representative cases are shown in Figure 3. The results indicate an association between levels of GGH mRNA and its protein expression.

GGH promoter methylation is not a cause of GGH down-regulation in CIMP+ CRC

A recent study in leukemia found that hypermethylation of the GGH promoter was associated with silencing of GGH gene expression (Cheng et al., 2006). The above

exploratory analyses showing that CIMP+ CRC have low GGH mRNA expression levels also raise this possibility. Methylation of the GGH promoter was therefore analyzed in 18 CIMP+ tumours and in 20 randomly selected CIMP- tumours from the Australian CRC cohort. Only one CIMP+ tumour (5.6%) showed hypermethylation of the GGH promoter (Figure 4), indicating that it does not play a major role in down-regulating the mRNA expression of this gene in CRC.

Discussion

In this study we explored the possibility that genes involved in folate and nucleotide metabolism have a distinct mRNA expression signature in CIMP+ CRC. Although no clear expression pattern was found for the 17 genes analyzed, low GGH expression was observed in two independent series of CIMP+ CRC and could therefore play a role in the development of this phenotype. In the Australian CRC cohort, the mRNA expression levels for GGH and RRM2 were shown by univariate and multivariate analyses to be significantly associated with CIMP+ CRC. In addition, the mRNA expression of GGH and ECGF1 were associated with characteristic clinicopathological and molecular features of CIMP+ including proximal tumour site, TILS and *BRAF* mutation. Moreover, the concentrations of two folate intermediates, CH_2FH_4 and FH_4, showed trends for association with GGH mRNA expression. As might be predicted from the function of GGH in hydrolyzing glutamated folates and allowing escape from the cell (Figure 5), low GGH expression was associated with higher folate concentrations.

Overall, the results of the exploratory study on the Australian CRC cohort provided evidence that low expression of GGH mRNA was associated with CIMP+ and with CIMP+–related features. This led us to conduct a further study using an independent

cohort of primary CRC from Japan and in which we confirmed the relationship between low GGH mRNA expression and CIMP+ status. The frequency of CIMP+ was lower amongst Japanese CRC (9.3%) than Australian CRC (15.8%). Although this difference did not reach statistical significance, it suggests that dietary, environmental and genetic differences between these two populations could influence the frequency of the CIMP+ subgroup as a proportion of total CRC. Nevertheless, low expression of GGH mRNA was a consistent finding in both CIMP+ cohorts. While a recommended panel of markers was used here to define CIMP+ (Weisenberger et al., 2006), the GGH/CIMP+ association was also found using a different CpG island panel comprising of MLH1, P16 (INK4A), TIMP3 and P14 (ARF) (data not shown).

The present results suggest that low GGH mRNA expression may play a role in the development and/or progression of CIMP+ CRC. A possible explanation for this is the role played by GGH in regulating intracellular folate levels (Figure 5). Monoglutamyl folate is transported into mammalian cells mainly by FOLR1 and RFC1 (Matherly & Goldman, 2003). Intracellular monoglutamyl folate is converted to the polyglutamate form by FPGS (Qi et al., 1999) whereas the polyglutamate chains are removed by GGH (Schneider & Ryan, 2006). Polyglutamated forms of folate are more strongly retained within the cell and are a better substrate for intracellular folate-dependent enzymes than the monoglutamate form. Therefore, low GGH expression would be expected to lead to a higher concentration of polyglutamated folate because of better retention in the cell. In agreement with this, we observed trends for an inverse correlation between GGH expression and the concentrations of folate intermediates CH_2FH_4 and FH_4 (Table 4). FOLR1 and FPGS mRNA expression were not associated with the concentrations of these folate intermediates, however the increased expression of RFC1 was significantly correlated with low concentrations of FH_4. These results suggest that GGH expression

plays a role in regulating the intracellular folate level in CRC tissues, although other factors such as RFC1 expression are also likely to be involved.

We previously reported that frequent CpG promoter hypermethylation was associated with high folate levels in CRC (Kawakami et al., 2003). A recent study also found that the level of p16 (INK4A) promoter methylation in the normal colonic mucosa of older mice increased following folate-supplementation (Keyes et al., 2007). Together, the above results suggest that low GGH expression may be linked to increased promoter methylation in CIMP+ tumours by causing elevation of the folate concentration. An alternate explanation involving transcriptional silencing of GGH by promoter methylation was excluded by the finding that only 5.6% of CIMP+ tumours showed GGH hypermethylation (Figure 4). It is unknown whether low GGH expression and its link with high tissue folate concentrations play a causal or even supportive role in the development of CIMP+ CRC. Further studies are required in which GGH expression, folate status and CpG island methylation are evaluated in normal colonic tissue as well as in the proposed precursor lesion for CIMP+, the so-called serrated adenoma or hyperplastic polyp (Jass, 2006). The mechanism(s) by which GGH expression is regulated in both normal and malignant colorectal tissue also warrants further investigation. Apart from two studies that found GGH expression was increased in CRC compared to adjacent normal colonic mucosa (Kidd et al., 2005; Odin et al., 2003), no other work has been published in this area.

In addition to possible implications for the etiology of CIMP+ CRC, the current findings are also relevant for the response of CRC to inhibitors of dihydrofolate reductase and thymidylate synthase (TS), both of which are key enzymes in nucleotide synthesis. The growth inhibitory effect of anti-folates such as methotrexate (MTX) and raltitrexed depends upon the polyglutamylation state of these agents (Barnes et al.,

1999). MTX is transported into cells using the same mechanism as that for folates and is also better retained following polyglutamylation. High GGH activity has been associated with the resistance of tumour cell lines to MTX via shortening of polyglutamate chains and consequently a lower intracellular drug concentration and less inhibition of dihydrofolate reductase and TS (Barnes et al., 1999; Rhee et al., 1993). Raltitrexed, a specific inhibitor of TS, is also polyglutamylated and its anti-tumour activity correlates with the amount of polyglutamylated drug inside the cells (Takemura et al., 1996). The importance of polyglutamylation in the anti-tumour activity suggests that CIMP+ CRC might have higher sensitivity to these anti-folates because of low GGH expression in this subtype of CRC. Neither MTX nor Raltitrexed is widely used in chemotherapy for CRC. However, these anti-folates might be of clinical use for tailored chemotherapy.

In contrast to above-mentioned anti-folates, 5-fluorouracil (5-FU) and leucovorin have been key drugs for the chemotherapy of CRC. 5-FU is thought to exert its major cytotoxic activity by inhibiting TS. It does this by forming a stable ternary complex between 5,10-methylenetetrahydrofolate, TS and fluoro-dUMP, the metabolite of 5-FU (Longley et al., 2003). Leucovorin, also known as folinic acid, increases the activity of 5-FU by raising the intracellular levels of 5,10-methylenetetrahydrofolate and thereby prolonging the inhibition of TS. 5,10-methylenetetrahydrofolate is also better retained following polyglutamylation (Radparvar et al., 1989) and this is critical for the anti-tumor activity of 5-FU even when this folate intermediate is present at relatively high concentrations (Romanini et al., 1991). Therefore, supplementation of 5-FU with leucovorin may be more effective in CIMP+ compared to CIMP- CRC because the low GGH levels would better allow the retention and modulatory action of 5,10-methylenetetrahydrofolate. An earlier study did indeed find that adjuvant treatment

with 5-FU/leucovorin conferred more benefit to CIMP+ tumours in stage III CRC (Van Rijnsoever et al., 2003). Two more recent studies reported that CIMP+ CRC was associated with poor survival in advanced CRC treated with 5-FU-based chemotherapy (Ogino et al., 2007; Shen et al., 2007). This may however be a reflection of the prognostic rather than predictive value of CIMP+. Moreover, the regimens used in the two studies of advanced CRC included 5-FU alone but also in combination with other chemotheraputic agents such as IFNalpha-2a and irinotecan. Further prospective studies are needed to test whether CIMP+ is a predictive marker for response to 5-FU/leucovorin in CRC. These may allow chemotherapy regimens to be tailored according to CIMP+ status, leading to more effective cancer treatment.

The present study investigated 17 genes involved in folate and nucleotide metabolism. Low expression of GGH was one of the features associated with CIMP+ CRC, however it was not a specific marker for this phenotype because many CIMP- tumours also showed low expression of this gene. The aberrant promoter methylation observed in CIMP+ CRC is likely to be a multi-step phenomenon that involves many factors in addition to folate metabolism and could include for example the expression levels of methyltransferases and histone deacetylase. Some of these factors may be revealed by array-based transcriptome analysis of CIMP+ and CIMP- CRC tissues. Although no study to date has addressed this issue, a few reports have described the mRNA expression profile of the closely associated microsatellite instability (MSI+) phenotype in CRC (Banerjea et al., 2004; di Pietro et al., 2005; Lanza et al., 2007). Interestingly, each of these studies showed that GGH mRNA expression was lower in MSI+ compared to MSI- CRC. Because of the strong concordance between MSI+ and CIMP+ in population-based CRC cohorts (Hawkins et al., 2002; Ogino et al., 2006; Samowitz et al., 2005; van Rijnsoever et al., 2002; Weisenberger et al., 2006), the

results from these independent, array-based studies confirm the current results obtained using RT-PCR and two separate CIMP+ CRC series. Together, the studies provide strong evidences of low GGH expression in MSI+ and/or CIMP+ CRC. The three array-based studies did not show consistent association between expression of the other genes analyzed in current study with MSI status. However, two of them (Banerjea et al., 2004; di Pietro et al., 2005) demonstrated higher expression of TYMS in MSI+ compared to MSI- CRC. Our result on TYMS expression between CIMP+ and CIMP- CRC did not support this association ($p=0.099$, Table 2). TYMS might differently express in MSI+ compared to CIMP+ CRC, requiring further study as to molecular difference between MSI+ and CIMP+ CRC.

In conclusion, the present study is the first to investigate the expression of genes involved in folate and nucleotide metabolism in relation to CIMP+ CRC. This tumour phenotype is associated with low expression of GGH, suggesting involvement of the folate pathway in its development and/or growth. Further studies of folate metabolism in CIMP+ CRC, premalignant precursors and normal colonic mucosa may help to elucidate the etiology of these tumours. A better understanding of the role of folate metabolism in DNA methylation may also lead to tailored chemotherapy that employs anti-folates, 5-FU/leucovorin and the use of CIMP+ markers.

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Table 1. Folate and nucleotide metabolizing genes analyzed in this study

Gene symbol	Gene name	Genbank Acc.
CDA	cytidine deaminase	NM_001785
DCK	deoxycytidine kinase	XM_003471
DCTD	dCMP deaminase	NM_001921
DHFR	dihydrofolate reductase	NM_000791
DPYD	dihydropyrimidine dehydrogenase	NM_000110
DUT	dUTP pyrophosphatase	U90223
	/ deoxyuridine triphosphate nucleotidohydrolase	
ECGF1	endotherial cell growth factor 1 (platelet-derived)	M63193
	/ thymidine phosphorylase	
FOLR1	folate receptor 1 / folate receptor alpha	NM_016730
FPGS	folyypolyglutamate synthetase	M98045
GGH	gamma-glutamyl hydrolase	NM_003878
MTHFD1	methylene tetrahydrofolate dehydrogenase 1	NM_005956
MTHFR	methylene tetrahydrofolate reductase	NM_005957
RFC1	reduced folate carrier 1	NM_003056
RRM1	ribonucleotide reductase M1 subunit	X59543
RRM2	ribonucleotide reductase M2 subunit	NM_001034
TYMS	thymidylate synthase	NM_001071
UMPS	uridine monophosphate synthetase	XM_050552
	/ orotate phosphoribosyl transferase	

Gene symbol is based on HUGO Gene Nomenclature Committee

(http://www.genenames.org/index.html)

Table 2. Associations between mRNA expression and CIMP status in CRC from an Australian cohort

	mRNA expression level		
Gene symbol	CIMP+	CIMP–	p-value
CDA	3.44 (1.79 – 5.05)	2.08 (0.92 – 4.28)	0.131
DCK	2.78 (2.57 – 3.22)	2.49 (1.67 – 3.00)	0.025
DCTD	4.21 (3.52 – 5.21)	4.04 (3.00 – 5.24)	0.403
DHFR	4.67 (3.52 – 5.37)	3.72 (2.84 – 5.38)	0.129
DPYD	0.48 (0.32 – 0.76)	0.32 (0.24 – 0.48)	0.025
DUT	123.8 (53.6 – 196.8)	116.2 (68.1 – 166.2)	0.828
ECGF1	4.76 (3.16 – 7.08)	2.71 (1.93 – 4.01)	0.001
FOLR1	0.00 (0.00 – 0.07)	0.10 (0.00 – 0.43)	0.060
FPGS	0.77 (0.56 – 0.95)	0.70 (0.52 – 0.85)	0.458
GGH	1.97 (1.04 – 3.06)	3.31 (1.98 – 5.69)	0.013
MTHFD1	4.46 (3.76 – 5.11)	3.84 (2.90 – 5.08)	0.197
MTHFR	1.20 (0.97 – 1.40)	0.91 (0.65 – 1.34)	0.044
RFC1	2.97 (2.29 – 4.30)	2.91 (1.99 – 3.92)	0.923
RRM1	1.02 (0.84 – 1.29)	0.96 (0.65 – 1.27)	0.265
RRM2	6.96 (4.60 – 7.70)	3.51 (2.27 – 6.03)	0.004
TYMS	3.32 (2.48 – 5.81)	2.99 (1.91 – 4.24)	0.099
UMPS	1.17 (1.07 – 1.40)	1.29 (0.96 – 1.73)	0.660

mRNA expression levels are shown as median (25^{th} percentile – 75^{th} percentile).

The Mann-Whitney U test was used for statistical analysis.

Table 3. Associations between mRNA expression and clinicopathological and molecular features in CRC from an Australian cohort

	tumour site		TILS		*BRAF* mutation	
Gene symbol	Proximal	Distal	present	absent	present	absent
CDA	2.08	2.42	2.06	2.21	3.81	2.12
DCK	2.55	2.59	2.90	2.53 *	2.70	2.54
DCTD	3.97	4.28	4.39	4.07	4.01	4.08
DHFR	3.74	4.39	4.52	3.78	4.75	3.90
DPYD	0.36	0.30	0.62	0.32 *	0.59	0.32
DUT	123.2	117.2	166.3	112.7	126.3	114.6
ECGF1	3.44	2.50 *	5.29	2.85 **	6.73	2.96 **
FOLR1	0.00	0.16 **	0.15	0.08	0.00	0.08
FPGS	0.67	0.73	0.76	0.70	0.73	0.70
GGH	2.44	3.91 *	1.38	3.35 **	1.38	3.33 **
MTHFD1	3.94	4.06	4.15	3.98	4.68	3.87
MTHFR	1.12	0.85	1.28	0.94	1.32	0.94 *
RFC1	2.68	3.16 *	2.70	3.00	2.70	3.02
RRM1	0.94	0.99	0.99	0.95	1.07	0.96
RRM2	4.36	4.28	5.33	3.91	7.01	3.91 *
TYMS	3.35	2.69	4.74	2.64 **	4.48	2.99 *
UMPS	1.14	1.36 *	1.13	1.29	1.13	1.30

Median mRNA expression levels are shown.

The Mann-Whitney U test was used for statistical analysis. * $p < 0.05$; ** $p < 0.01$

Data presented with median (25^{th} percentile – 75^{th} percentile) and p-value is available in supplementary Table 2.

Table 4. Associations between mRNA expression level and the concentration of folate intermediates in CRC from an Australian cohort

	CH_2FH_4		FH_4	
Gene symbol	Spearman's rho	p-value	Spearman's rho	p-value
CDA	0.205	0.063	0.154	0.163
DCK	0.058	0.583	0.056	0.599
DCTD	-0.042	0.684	0.024	0.817
DHFR	-0.081	0.448	-0.145	0.174
DPYD	0.020	0.845	0.010	0.924
DUT	-0.022	0.834	-0.022	0.834
ECGF1	0.062	0.559	0.043	0.686
FOLR1	-0.136	0.190	-0.073	0.481
FPGS	-0.025	0.807	0.023	0.822
GGH	-0.200	0.053	-0.180	0.083
MTHFD1	-0.014	0.856	-0.033	0.752
MTHFR	-0.028	0.784	0.037	0.720
RFC1	-0.159	0.123	-0.205	0.046
RRM1	0.039	0.707	0.006	0.952
RRM2	0.107	0.303	0.046	0.661
TYMS	0.076	0.471	-0.018	0.864
UMPS	-0.121	0.246	-0.050	0.630

Spearman's rank correlation test was used for analyses.

Spearman's Rho and p-value is presented for each analysis of correlations.

Figure Legends

Fig. 1. GGH mRNA expression according to CIMP status, tumour site, TILS and *BRAF* mutation status in an Australian CRC cohort was shown by boxplot. The level of GGH mRNA expression was significantly different between all dichotomized variables (Mann-Whitney U test; CIMP, p = 0.013; tumor site, p = 0.021; TILS, p = 0.001; *BRAF* mutation, p = 0.002)

Fig. 2. GGH mRNA expression according to CIMP status in a Japanese CRC cohort used for validation was shown by boxplot. Lower GGH expression in CIMP+ compared to CIMP- CRC was confirmed (Mann-Whitney U test; p = 0.0012).

Fig. 3. Immunohistochemical staining of GGH in CRC tissues. GGH protein was not detectable in tumor cells in case No. 5209 and 2426, in which the GGH mRNA levels were 0.01 and 0.06, respectively. Expression of GGH was observed in tumor cells in case No. 4040 and 616, in which the GGH mRNA levels were 3.73 and 8.29, respectively.

Fig. 4. Methylation specific PCR analysis of the GGH promoter. Promoter methylation of GGH was analyzed using unmethylated DNA specific primer sets (U) and methylated DNA specific primer sets (M). Representative results using 18 samples of CIMP+ and 20 randomly selected samples of CIMP- tumours are shown. Only one sample, a CIMP+ tumour, showed GGH promoter hypermethylation. Sperm DNA and fully methylated sperm DNA produced with SssI methylase were used for unmethylated control (UC) and methylated control (MC), respectively. DNA from the colon cancer

cell line WiDr was also used as a positive control.

Fig. 5. Simplified representation of folate transport and polyglutamylation reactions within the cell. RFC1 is ubiquitously expressed in epithelial cells and play a role as a major transport system for folates. FOLR1 is anchored to cell membranes and transport folates via an endocytotic process. Intracellular monoglutamyl folate is converted to the polyglutamate form by FPGS whereas the polyglutamate chains are removed by GGH.

Supplementary Table 1. Primers used for real-time RT-PCR

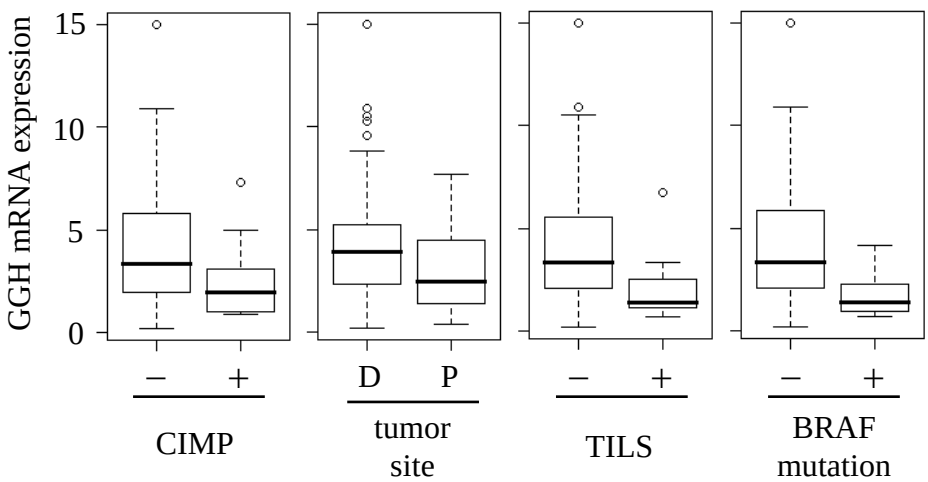
Genes	forward primer	probe	reverse primer
for Australian CRC series			
CDA	AAAGGGTGCAACATAGAAAATGC	TGCTACCCGCTGGGCATCTGTG	CTGGATAGCGGTCCGTTCA
DCK	GACTGGCATGACTGGATGAA	TCCATCCAATTCAAGGCTTTGGCC	TCTCTGGAGTGGCTTGAAGA
DCTD	GCAGAGAATAAGCTGGACACCAA	TACCCGTACGTGTGCCATGCGGA	TTTTTGTTCATGATGGCATTCAG
DHFR	GTCCTCCCGCTGCTGTCA	TTCGCTAAACTGCATCGTCGCTGTGTC	GCCGATGCCCATGTTCTG
DPYD	AGGACGCAAGGAGGGTTTG	CAGTGCCTACAGTCTCGAGTCTGCCAGTG	GTCCGCCGAGTCCTTACTGA
DUT	GTCTCCTCGCTCGCCTTCT	TTCAGAGCAGGGCATGGCAGAGC	GGTGAAATGGCGGGTGTCT
ECGF1	CCTGCGGACGGAATCCT	CAGCCAGAGATGTGACAGCCACCGT	GCTGTGATGAGTGGCAGGCT
FOLR1	GAAGATTGTCGCACCTCCTACAC	CCCTTGTGCCAGTTGCTCTTGCA	CGCACTTGTTAAACCCTGAAGTC
FPGS	GGCTGGAGGAGACCAAGGAT	CAGCTGTGTCTCCATGCCCCCCTAC	CATGAGTGTCAGGAAGCGGA
GGH	GCGAGCCTCGAGCTGTCTA	ACCCCACGGCGACACCGC	AATATTCCGATGATGGGCTTCTT
MTHFD1	CGTGGGCAGCGGACTAA	CGCCAGCAGAAATCCTGAACGG	CCTTATTTGCGCGGAGATCT
MTHFR	CGGGTTAATTACCACCTTGTCAA	TGAAGGGTGAAAACATCACCAATGCCC	GCATTCGGCTGCAGTTCA
RFC1	CATCGCCACCTTTCAGATT	CCCGAAGACCAGGGCACAGA	TGGCAAAGAACGTGTTGAC
RRM1	ACTAAGCACCCTGACTATGCTATCC	CAGCCAGGATCGCTGTCTCTAACTTGCA	CTTCCATCACATCACTGAACACTTT
RRM2	ACCGCGAGGAGGATCT	TTTCGGCTCCGTGGGCTCCT	TCAGCAGCGGCTCATC
TYMS	GCCTCGGTGTGCCTTTCA	TCGCCAGCTACGCCCTGCTCA	CCCGTGATGTGCGCAAT
UMPS	TAGTGTTTTGGAAACTGTTGAGGTT	TGGCATCAGTGACCTTCAAGCCCTCCT	CTTGCCTCCCTGCTCTCTGT
ACTB	GAGCGCGGCTACAGCTT	ACCACCACGGCCGAGCGG	TCCTTAATGTCACGCACGATTT
for Japanese CRC series			
ECGF1	GGATTCAATGTCATCCAGAG	no probe	CCTCCACGAGTTTCTTACTG
GGH	AACCTCTGACTGCCAATTTCCATAA	no probe	TCTCTGGATGCCACTGGACAC
RRM2	CCCGCTGTTTCTATGGCTTC	no probe	CCCAGTCTGCCTTCTTCTTG
ACTB	ATTGCCGACAGGATGCAGA	no probe	GAGTACTTGCGCTCAGGAGGA

Supplementary Table 2. Associations between mRNA expression and clinicopathological features in the Australian CRC series

	Tumor site			TILS			*BRAF* mutation		
Genes	Proximal	Distal	p-value	present	absent	p-value	present	absent	p-value
CDA	2.08 (1.04 – 3.75)	2.42 (0.89 – 4.80)	0.685	2.06 (1.10 – 3.07)	2.21 (0.97 – 5.27)	0.479	3.81 (1.41 – 4.75)	2.12 (0.97 – 4.20)	0.426
DCK	2.55 (1.73 – 3.07)	2.59 (1.80 – 3.01)	0.945	2.90 (2.55 – 4.16)	2.53 (1.69 – 2.96)	0.013	2.70 (2.64 – 3.63)	2.54 (1.71 – 3.05)	0.088
DCTD	3.97 (2.96 – 5.02)	4.28 (3.27 – 5.63)	0.160	4.39 (3.92 – 4.95)	4.07 (3.05 – 5.29)	0.648	4.01 (3.36 – 4.77)	4.08 (3.01 – 5.33)	0.731
DHFR	3.74 (2.82 – 4.74)	4.39 (2.95 – 5.70)	0.335	4.52 (3.24 – 5.95)	3.78 (2.89 – 5.16)	0.181	4.75 (3.68 – 6.87)	3.90 (2.92 – 5.37)	0.151
DPYD	0.36 (0.29 – 0.59)	0.30 (0.23 – 0.46)	0.086	0.62 (0.30 – 0.94)	0.32 (0.25 – 0.47)	0.036	0.59 (0.30 – 1.07)	0.32 (0.25 – 0.49)	0.136
DUT	123.2 (67.6 – 169.0)	117.2 (68.0 – 166.4)	0.744	166.3 (87.1 – 198.6)	112.7 (67.8 – 162.2)	0.151	126.3 (100.7 – 198.6)	114.6 (64.4 – 166.1)	0.171
ECGF1	3.44 (2.58 – 4.94)	2.50 (1.85 – 4.03)	0.014	5.29 (3.46 – 7.38)	2.85 (1.96 – 4.17)	0.002	6.73 (3.99 – 8.17)	2.96 (2.04 – 4.17)	0.001
FOLR1	0.00 (0.00 –0.22)	0.16 (0.00 – 0.75)	0.008	0.15 (0.00 – 0.55)	0.08 (0.00 – 0.36)	0.612	0.00 (0.00 – 0.19)	0.08 (0.00 – 0.48)	0.422
FPGS	0.67 (0.53 – 0.84)	0.73 (0.50 – 0.95)	0.715	0.76 (0.50 – 0.88)	0.70 (0.52 – 0.87)	0.691	0.73 (0.60 – 0.86)	0.70 (0.52 – 0.89)	0.855
GGH	2.44 (1.38 – 4.45)	3.91 (2.32 – 5.22)	0.021	1.38 (1.16 – 2.52)	3.35 (2.08 – 5.55)	0.001	1.38 (0.94 – 2.27)	3.33 (2.08 – 5.84)	0.002
MTHFD1	3.94 (2.96 – 5.21)	4.06 (3.06 – 5.23)	0.623	4.15 (3.66 – 5.18)	3.98 (2.93 – 5.04)	0.485	4.68 (3.96 – 5.41)	3.87 (2.61 – 5.17)	0.136
MTHFR	1.12 (0.73 – 1.39)	0.85 (0.65 – 1.25)	0.189	1.28 (0.94 – 1.36)	0.94 (0.65 – 1.35)	0.081	1.32 (1.07 – 1.41)	0.94 (0.67 – 1.32)	0.027
RFC1	2.68 (1.93 – 3.43)	3.16 (2.14 – 4.38)	0.045	2.70 (2.14 – 3.65)	3.00 (92.00 – 4.05)	0.736	2.70 (1.82 – 4.09)	3.02 (2.12 – 4.05)	0.626
RRM1	0.94 (0.57 – 1.13)	0.99 (0.66 – 1.30)	0.295	0.99 (0.81 – 1.26)	0.95 (0.65 – 1.27)	0.407	1.07 (0.85 – 1.29)	0.96 (0.62 – 1.28)	0.204
RRM2	4.36 (2.23 – 6.53)	4.28 (2.39 – 6.80)	0.788	5.33 (3.69 – 7.27)	3.91 (2.28 – 6.39)	0.174	7.01 (4.70 – 7.650	3.91 (2.23 – 6.23)	0.030
TYMS	3.35 (2.27 – 4.28)	2.69 (1.95 – 4.29)	0.350	4.74 (3.71 – 5.87)	2.64 (1.92 – 4.00)	0.0002	4.48 (2.83 – 6.07)	2.99 (1.94 – 4.24)	0.029
UMPS	1.14 (0.90 – 1.52)	1.36 (1.05 – 1.98)	0.021	1.13 (0.85 – 1.59)	1.29 (1.02 – 1.72)	0.340	1.13 (1.09 – 1.44)	1.30 (0.99 – 1.750)	0.597

mRNA expression levels are presented with median (25th percentile – 75th percentile).

Mann-Whitney's U test was used for statistical analyses.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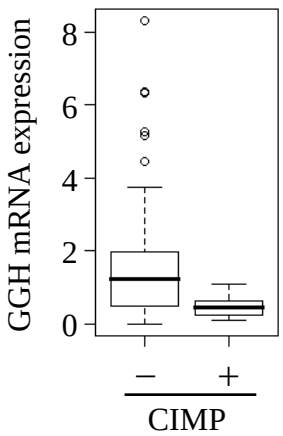
BRAF mutation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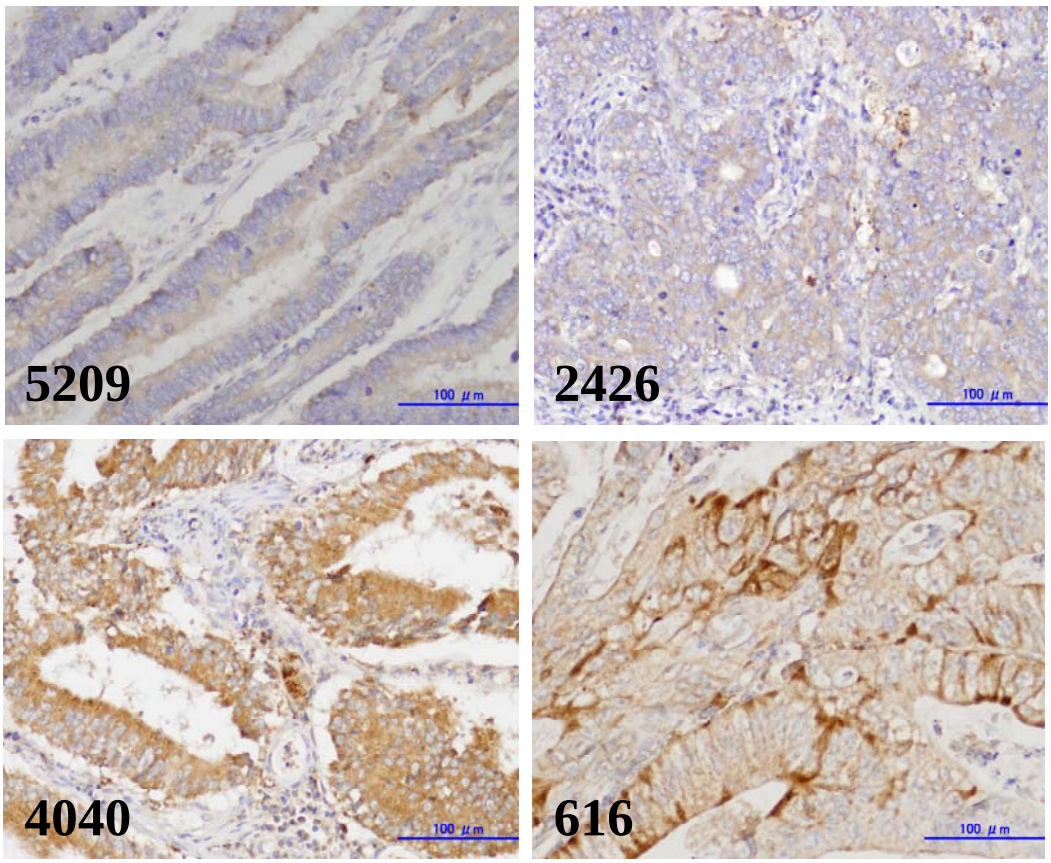
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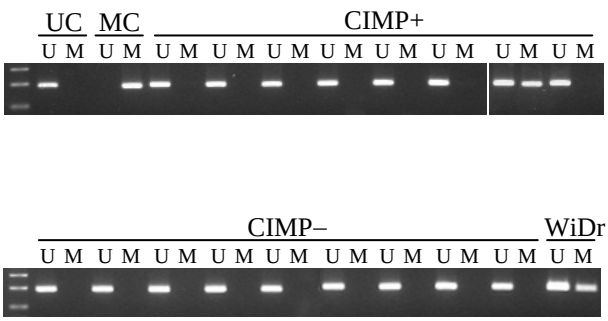
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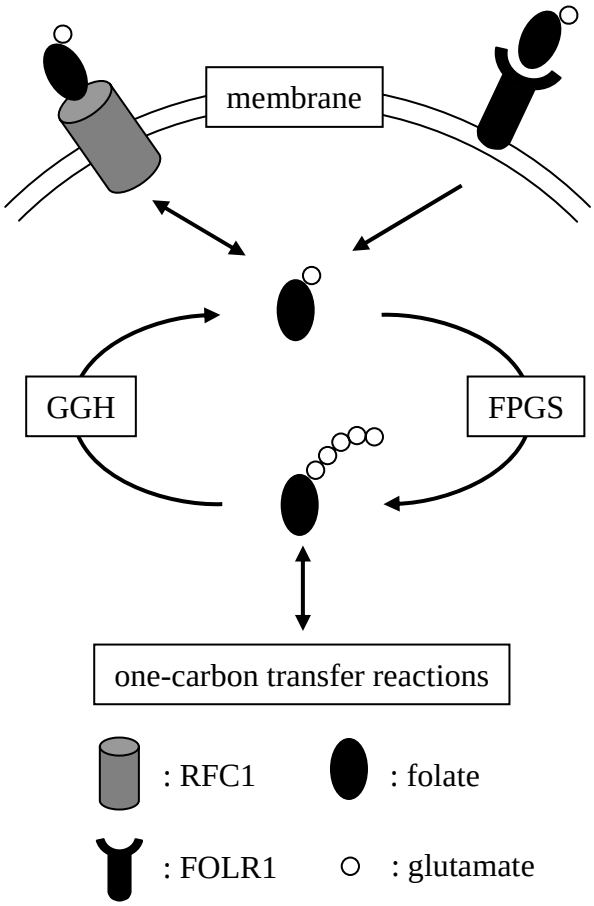
membrane
GGH
FPGS
one-carbon transfer reactions
: RFC1
: folate
: FOLR1
: glutamate