**Predicting clinical outcome to 5-Fluorouracil-based chemotherapy for
colon cancer patients: is the CpG island methylator phenotype the
responsive subgroup?**

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Abstract

The CpG island methylator phenotype (CIMP+) of colorectal cancer occurs predominantly in the proximal colon and is characterized by frequent hypermethylation of gene promoter regions. In this review, we present evidence suggesting CIMP+ represents the subgroup of colon cancers that are responsive to 5-Fluorouracil (5FU)-based treatments. CIMP+ has been associated with survival benefit from 5FU in a clinical study of CRC, with additional evidence coming from studies on gastric cancer and tumour cell lines. Elevated concentrations of 5-10-methylene tetrahydrofolate (CH_2FH_4) occur in CIMP+ tumours and are probably due to low expression levels for γ -glutamyl hydrolase (GGH). Clinical and *in vitro* work has previously shown that high CH_2FH_4 and low GGH expression levels correlate with good response to 5FU. Methylation-induced silencing of dihydropyrimidine dehydrogenase, the rate limiting enzyme in 5FU degradation, may also provide a link between CIMP+ and good response to 5FU. The CIMP+-related phenotype referred to as microsatellite instability (MSI+) has been widely investigated as a predictive marker of response to 5FU, with contradictory results. The interpretation of these studies is likely to be confounded by the fact that some MSI+ tumours occur in the background of CIMP+ but a significant proportion of others do not. Further studies on tumours from randomized clinical trials are required to confirm the value of CIMP+ and associated molecular features for the prediction of clinical outcome to 5FU.

Key words: colon cancer, 5-fluorouracil, predictive, CIMP, MSI

Introduction

5-Fluorouracil (5-FU) is one of the most widely used anticancer agents and has been the mainstay of chemotherapy for many gastrointestinal malignancies including colorectal cancer (CRC). 5FU is converted into fluorodeoxyuridine monophosphate (FdUMP) and this molecule inhibits the activity of a key enzyme, thymidylate synthase (TS), required for DNA synthesis. Inhibition of TS by FdUMP is achieved through the formation of a ternary complex with the reduced folate 5,10-methylenetetrahydrofolate (CH_2FH_4). Experimental work has demonstrated that elevated cellular concentrations of CH_2FH_4 leads to stabilization of the ternary complex and stronger inhibition of TS.^{1,2} Consistent with this, the addition of folinic acid (leucovorin) to 5FU improves the response rates and survival of CRC patients.^{3,4} A 6-month course of 5FU/leucovorin is now standard chemotherapy for most stage III and many stage II colon cancer patients.

The aim of personalized medicine is to deliver the most effective drugs to individual patients while simultaneously minimizing the adverse side effects of treatment. This goal is especially important for the new generation of targeted but expensive treatments such as Cetuximab and Bevacizumab. While 5FU-based therapies are considerably less expensive, it would still be very helpful to identify colon cancer patients who are likely to respond. This is especially important for stage II cases where a survival benefit from 5FU adjuvant chemotherapy has yet to be firmly established.⁵

Despite considerable advances in our understanding of the mechanism of 5FU action, the identification of clinically useful molecular markers for predicting the response to 5FU remains elusive. In this review, we summarize the evidence suggesting that colon cancers with the CpG island methylator phenotype (CIMP) represent the 5FU-responsive subgroup.

The CpG island methylator phenotype

The notion of a methylator phenotype for CRC was first proposed in 1999 by Toyota *et al* to describe a subset of colorectal tumours with a very high frequency of DNA methylation at 'Type C' loci.⁶ These were defined as DNA loci that were methylated in cancer, but not in corresponding normal tissues. Subsequent studies found the CIMP+ trait to be associated with location in the proximal colon, female sex, older age, high tumour grade, mucinous histology, wild-type *TP53*, microsatellite instability and *BRAF* oncogene mutation.⁷⁻¹¹ Several marker panels have been proposed to standardize the classification of CIMP+, with the most widely adopted definition consisting of methylation in 3 or more of the 5 loci *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*.¹⁰ The CIMP+ subgroup accounts for 15-30% of CRC in most large studies of unselected CRC cohorts.⁷⁻¹¹ For many genes, including putative tumour suppressors such as *RUNX3*, *MLH1*, *TIMP3* and *P16*, hypermethylation of CpG islands within the promoter region has been associated with silencing of expression. This is believed to represent an alternative mechanism to mutation and LOH for the inactivation of tumour suppressor genes. Indeed, CIMP+ CRC is thought to develop from a different pre-malignant precursor to CIMP- tumours, referred to as the serrated adenoma or hyperplastic polyp.¹²

Older, female individuals are at greatest risk of developing CIMP+ tumours.⁷⁻⁹ It is interesting to note that older female patients also suffer the most toxicity to 5FU chemotherapy,^{13,14} raising the possibility that a common mechanism is responsible for the risks of developing CIMP+ tumours and of toxicity to 5FU/leucovorin. Although good experimental evidence is still lacking, this could involve increased levels of tissue folate in the colonic mucosa of older females, particularly in the proximal colon. Elevated concentrations of CH₂FH₄ and FH₄ have been observed in tumours from the proximal colon and in tumours from older patients.¹⁵ Moreover, gene hypermethylation was found to

be more frequent in the normal colonic mucosa of female and older CRC patients compared to male and younger patients, respectively.¹⁶ The same gender and age correlations are seen for expression of the DNA methyltransferase, DNMT3b, which was reported to be higher in the liver tissue of females and older individuals.¹⁷

Further investigation of tissue folate and DNA methylation levels in the normal colonic mucosa in relation to age, gender, anatomical site, dietary folate intake and genetic factors such as the *MTHFR* C677T polymorphism are important for two reasons. Firstly, such studies may lead to the identification of individuals who are at increased risk of developing CIMP+ colon cancers, thus allowing regular surveillance and perhaps also dietary modification to reduce the risk. Secondly, tissue folate and DNA methylation levels in normal colonic mucosa could have predictive value for toxicity to 5FU/leucovorin. The well established associations of sex and age with toxicity to 5FU^{13,14} could in fact be surrogate indicators for underlying biological factors such as tissue folate or DNA methylation levels. While both issues clearly merit further research attention, the present review will focus only on the predictive values of the CIMP+ and associated microsatellite instability (MSI+) markers for response to 5FU-based therapy.

CIMP+ and cellular folate levels

Several indirect lines of evidence suggest that CIMP+ tumours comprise the 5FU-responsive subgroup of colon cancers. These relate mainly to the link between CIMP+ and intracellular folate metabolism (Figure 1) and to the silencing of gene expression resulting from DNA methylation. Firstly, CIMP+ tumours have elevated levels of CH₂FH₄ and FH₄ compared to CIMP- tumours.¹⁵ Intracellular folate concentrations have critical importance in determining the response to 5FU.^{1,2,18,19} Despite an early study with head and neck cancers showing that elevated tumour CH₂FH₄ levels correlate with good response to 5FU-

based chemotherapy,²⁰ to our knowledge there are no comparable clinical studies published to date for CRC. This is likely to reflect the difficulties associated with collection of frozen human tissues and the subsequent biochemical analysis of CH₂FH₄ levels. Nevertheless, it is surprising these studies have not been carried out in view of the extensive *in vitro* and animal work showing the importance of intracellular folate levels for therapeutic efficacy of 5FU.^{18,19}

Expression levels for two of the key enzymes involved in regulating the intracellular folate concentrations have also shown predictive significance for response to 5FU. Folylpolylglutamate synthase (FPGS) converts intracellular folate to polyglutamated forms that are retained for longer in the cell, thus leading to increased levels. In contrast, γ -glutamyl hydrolase (GGH) removes glutamates thereby lowering the cellular folate level. Lower FPGS activity in the liver metastases of CRC correlates with 5FU resistance,²¹ as would be expected if this lead to decreased tumour folate levels. Results from several *in vitro* studies on tumour cell lines support this clinical finding.²²⁻²⁵ On the other hand, decreased GGH expression in colon cancer cell lines induced by siRNA resulted in increased sensitivity to 5FU, presumably due to the better retention of folate.²⁵ Low GGH expression was also reported recently to correlate with good response to 5FU-based therapy in a study of metastatic CRC.²⁶ CIMP+ tumours express significantly lower levels of GGH compared to CIMP- tumours,^{27,28} thus providing an explanation for the higher folate levels observed in these tumours¹⁵ and providing more indirect support for the prediction of better response to 5FU. FPGS levels were not significantly different however between CIMP+ and CIMP- tumours,²⁷ suggesting this enzyme may play a less important role than GGH in determining cellular folate concentrations in primary CRC.

Microarray analysis has revealed that a number of other genes that are differentially expressed between CIMP+ and CIMP- tumours.²⁸ Gene Ontology categories annotated to

the differentially expressed genes include several pathways involved in nucleotide metabolism, as well as folate and glutamine metabolism. The results also suggest that metabolic activity responsible for converting 5FU into the active metabolite FdUMP may be quite different between CIMP+ and CIMP- tumours, resulting in different chemosensitivity. In addition to the inhibition of TS activity, 5FU could also exert cytotoxic activity through the mis-incorporation of FUTP and FdUTP into RNA and DNA, respectively (Figure 1).²⁹ Further investigation of this mis-incorporation may provide novel insights into differences in chemosensitivity between CIMP+ and CIMP- tumours.

CIMP+ and gene hypermethylation

Remarkably, only one study has so far examined the predictive value of CIMP+ for response to 5FU in CRC.³⁰ This work involved 103 stage III CRC patients treated by surgery alone and another 103 cases that were matched for age, sex and tumour site and who also received adjuvant treatment with 5FU/leucovorin. CIMP+ patients showed a significant survival benefit from 5FU, but not those with CIMP- tumours. Further studies are anticipated using a standard panel of markers to define CIMP+¹⁰ and involving the analysis of tissues from randomized clinical trials. In support of the observations made in CRC, methylation of *p16* was reported to be predictive of survival benefit from 5FU in gastric cancer.³¹ Moreover, a recent *in vitro* study showed that cancer cell lines with methylated *TIMP3* were more sensitive to 5FU.³²

Aside from having high intracellular folate concentrations, another possible explanation for CIMP+ tumours showing better response to 5FU is because of the silencing of critical genes. One candidate for this is the methylation-induced silencing of dihydropyrimidine dehydrogenase (*DPYD*). DPD enzyme catalyses the rate-limiting step in 5FU degradation (Figure 1) and its activity is a major determinant of clinical resistance and

toxicity to this agent.³³ Methylation-induced silencing of *DPYD* has been implicated in the sensitivity of various tumour cell lines to 5FU.³⁴⁻³⁶ Down-regulation of DPD activity in association with *DPYD* methylation has also been reported in clinical samples, however this has yet to be correlated with tumour response to 5FU.³⁷

Several studies have investigated the prognostic significance of CIMP+ or gene methylation in CRC patients treated with 5FU. Methylation of *MGMT* was associated with low risk of recurrence in CRC patients treated with 5FU-based chemotherapy.³⁸ In contrast, two studies in advanced CRC reported that methylation of several genes was associated with worse survival in patients who received chemotherapy.^{39,40} A follow-up study by one of these groups found that CIMP+ colon cancer patients had good survival relative to CIMP- patients once adjustment was made for the presence of *BRAF* mutations.⁴¹ It must be emphasized, however, that such studies do not provide information on whether CIMP+ has predictive value for the response to 5FU. This requires comparison of clinical response rates or patient survival between CIMP+ patient groups treated with or without chemotherapy.

Microsatellite instability (MSI+) as a surrogate marker for CIMP+

Compared to CIMP+, considerably more work has been carried out on the MSI+ phenotype as a potential molecular predictive marker for response to 5FU in CRC.⁴²⁻⁵¹ MSI+ is characterized by frequent alterations in the size of nucleotide repeats due to a defective DNA mismatch repair system. The majority of sporadic MSI+ tumours arise because of methylation-induced silencing of the *MLH1* mismatch repair gene⁵² and hence MSI+ could be used as a surrogate marker for CIMP+, but with important qualifications as outlined below. Depending on the criteria used to define CIMP+, it has been estimated that 55-80% of MSI+ in non-selected CRC series are also CIMP+.^{7,8,11} The remaining MSI+

tumours presumably arise because of *MLH1* methylation in the absence of widespread methylation of other genes, or because of somatic or germline mutations that occur in *MLH1*, *MSH2* or other mismatch repair genes.

The underlying cause of MSI+ is likely to be a critical issue in determining the biological and clinical properties of MSI+ tumours, including the response to 5FU.^{53,54} For example, MSI+ tumours with *MLH1* methylation are far more frequent in older, female patients and have a high incidence of *BRAF* mutation compared to MSI+ tumours without *MLH1* methylation.^{55,56} Another illustration is that MSI+ tumours that arise in proximal colon have different gene expression profiles compared to those arising in the distal colon.⁵⁷ Such fundamental differences would be expected to influence the response of MSI+ tumours to 5FU and may explain the discordant results published to date for the predictive value of this marker. None of the studies so far has considered the potential impact of factors like patient age, tumour site or *MLH1* methylation status on the prognostic or predictive values of MSI+.

Several of the early studies on MSI+ showed associations with either excellent survival, or a survival benefit, for patients treated with 5FU.⁴²⁻⁴⁷ In contrast, other groups have reported a lack of survival benefit from 5FU for patients with MSI+ tumours,⁴⁸⁻⁵¹ leading some authors to go as far as proposing that MSI+ patients should not be treated with chemotherapy.^{58,59} There is evidence to suggest that MSI+ patients belonging to the hereditary bowel cancer condition known as Lynch syndrome do not benefit from 5FU-based chemotherapy.⁶⁰ However, until large-scale clinical studies are carried out on sporadic MSI+ tumours that specifically examine the effect of the underlying phenotype (eg. methylated or non-methylated *MLH1*, mutant or wildtype *BRAF*) on the predictive value of this marker, it would seem prudent to treat all eligible patients with adjuvant 5FU chemotherapy regardless of their MSI status. This is particularly important in light of a

recent meta-analysis showing that MSI+ patients actually gain more benefit from adjuvant chemotherapy than MSI- patients.⁶¹

Conclusions and future perspectives

CIMP+ tumours are a phenotypically distinct subgroup of CRC that comprise approximately one-third of all proximal colon cancers. They are most prevalent in older, female patients and have elevated levels of cellular folate and gene promoter methylation, but lower expression of GGH. Although there is still a paucity of information on the predictive value of CIMP+, the direct and indirect evidence published to date suggests this phenotype may be the 5FU-responsive subgroup of CRC. Clinical trials of early stage colon cancer that stratify patients according to CIMP status prior to randomization to 5FU-based treatments will be required to definitively address the question of whether CIMP+ has predictive value. More work is required to identify dietary and genetic risk factors for the development of CIMP+ and whether patients with these tumours are more likely to suffer toxicity from 5FU-based treatments. Possible associations between CIMP+ and other factors that are likely to influence the response to 5FU chemotherapy also require further investigation, including intracellular folate levels and the expression of thymidylate synthase and DPD. Finally, CIMP+ tumours show high densities of infiltrating lymphocytes independently of the MSI+ phenotype.⁶² This observation suggests that study of the anti-tumour immune response in CIMP+ patients before and after 5FU-based chemotherapy may also prove interesting.

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FIGURE LEGEND

Figure 1. Metabolic relationships amongst 5-FU, folate and DNA methylation. Colon cancers with frequent hypermethylation of CpG islands within gene promoter regions are referred to as having the CIMP+ phenotype. These tumours show elevated concentrations of Methylene-THF (CH_2FH_4) and decreased expression of GGH, both of which are predictive of better response to 5FU. Methylation-induced silencing of the 5FU degradation enzyme, DPD, may also contribute to better response of CIMP+ tumours to 5FU. DHF: dihydrofolate; DHFR: dihydrofolate reductase; DHFU: dihydrofluorouracil; DPD: dihydropyrimidine dehydrogenase; dUrd: deoxyuridine; F: fluoro; FBAL: fluoro- β alanine; FPGS: folylpolyglutamyl synthase; FR: folate receptor; FUPA: fluoroureidopropionic acid; GGH: γ -glutamyl hydrolase; Methylene-THF: 5, 10-methylene tetrahydrofolate (CH_2FH_4); MS: methionine synthase; MTHFR: methylenetetrahydrofolate reductase; OPRT: orotate phosphoribosyltransferase; RFC: reduced folate carrier; RR: ribonucleotide reductase; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; THF: tetrahydrofolate (FH_4); TK: thymidine kinase; TP: thymidine phosphorylase; TS: thymidylate synthase; UK: uridine kinase; UP: uridine phosphorylase; Urd: uridine