Biologic Behavior of Microsatellite-Unstable Colorectal Cancer and Treatment with 5-Fluorouracil

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Abstract

Colorectal cancers develop as a consequence of genomic instability. Microsatellite instability is involved in the genesis of about 15% of sporadic colorectal cancers and in most hereditary non-polyposis cancers. High frequency MSI has been associated with a favorable prognosis, however it is not clear whether this is because MSI-H tumors are inherently less aggressive or because they are more sensitive to chemotherapy. Chemotherapy with a combination of 5-fluorouracil and leukovorin or levamizole has been the standard of care for high risk stage II and stage III CRC; it is also used in stage IV CRC. Several *in vitro* studies have shown that colon cancer cell lines displaying MSI-H are less responsive to fluorouracil than microsatellite-stable cell lines. Human studies, all of them retrospective, yielded conflicting results. The selection of patients with CRC for 5-FU treatment has been based so far on the stage of the tumor rather than the biology of the tumor. Although surgical staging is highly predictive of survival, there are indications that the form of genomic instability within a patient's colorectal tumor has clinical implications, with and without 5-FU treatment. This review suggests that patients with MSI-H colorectal tumors may not benefit from 5-FU-based chemotherapy and can avoid its potential side effects (nausea, diarrhea, stomatitis, dermatitis, alopecia, and neurologic symptoms) that occur in half the treated patients. If confirmed by future prospective randomized controlled studies, these findings would indicate that microsatellite-instability testing should be conducted routinely and the results used to direct rational adjuvant chemotherapy in colon cancer.

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Although surgical resection alone is potentially curative in colorectal cancer, local or distant recurrences develop in many patients. A number of cooperative group trials and analyses have shown that 5-fluorouracil-based systemic adjuvant chemotherapy improves survival in high risk patients [1,2]. Combination regimens of 5-FU and leukovorin or levamizole have now become standard care in patients with stage II (T3/N0/M0 or T4/N0/M0) and stage III CRC. Fluorouracil-based chemotherapy is also used in stage IV CRC.

CRCs develop as a consequence of genomic instability. Microsatellite instability is involved in the genesis of about 15% of sporadic CRCs and in most hereditary non-polyposis CRCs [3–5]. The multiple errors in repetitive DNA sequences (microsatellites) are due to a failure of the DNA mismatch repair system to edit errors that occur during DNA replication [6]. The DNA MMR system is inactivated either by hypermethylation of the promoter, which silences gene transcription of *hMLH1* (epigenetic phenomenon; sporadic CRC), or because of germ-line mutations in MMR genes *MLH1, MSH2, MSH6* and others (HNPCC) [7,8]. Tumors with high frequency MSI tend to be diploid, to possess a mucinous histology, and to have a surrounding lymphoid reaction. They are more prevalent in the proximal colon and have a fast (2–3 years) pass from polyp to cancer. Nevertheless, they are associated with longer survival than stage-matched low frequency MSI tumors or tumors with microsatellite stability [9–15]. It is still not clear whether this favorable prognosis is attributable to the inherently lower aggressiveness of MSI-H tumors or to their greater sensitivity to chemotherapy. Confounding factors, such as age and anatomic site of the tumor, may also be important and should be taken into consideration. Sporadic MSI-H tumors may occur in the elderly because of CpG island hypermethylation and gene silencing [7], and right-sided tumors were found to respond favorably to chemotherapy irrespective of MSI status [16]. Still, it is unlikely that tumors with these distinct pathways would respond similarly to chemotherapeutic agents that damage DNA. This article attempts to clarify this issue by reviewing the current literature on the biologic behavior and treatment of CRC tumors.

MSI = microsatellite instability
MSI-H - high frequency MSI
CRC = colorectal cancer
5-FU = 5-fluorouracil
MMR = mismatch repair
HNPCC = hereditary non-polyposis CRC

In vitro studies

Cell models may provide clues to the mechanism of colorectal tumor resistance or sensitivity to 5-FU. At 5-FU concentrations similar to *in vivo* tissue concentrations, ≥10% of 5-FU is incorporated into the DNA of CRC cells, indicating the potential of the

DNA MMR system to tolerate the altered nucleotide [17]. How the DNA MMR proteins recognize 5-FU is still unknown. Certainly the lack of MMR might allow incorporated 5-FU to adversely affect DNA synthesis and replication, but not necessarily to inhibit cell growth. A competent MMR system may trigger a cell death program, which is either additive to or independent of the effect of 5-FU on RNA metabolism and thymidylate synthetase activity, and might be operative in MSS colorectal tumors.

Merkelbach-Bruse and colleagues [18] investigated the association between MSI status and mRNA expression, as well as polymorphisms of the cellular target of 5-FU therapy, thymidylate synthase. Polymorphisms in the 3' and the 5'-UTR of the thymidylate synthase gene were determined by a polymerase chain reaction assay in 53 CRC tissue samples. No significant correlation was observed either between the polymorphisms in the *TS* gene and the MSI phenotype or between the mRNA expression and the MSI status.

Table 1. 5-FU-based chemotherapy by microsatellite status

Series	5FU-treated patients	Stage	No. of MSI-H tumors	No. of MSS/L tumors	Survival of MSI-H/ MSS/MSI-L	*P*
Carethers et al. 2004 [27]	66	II-III	10	56	80%/70%* 5 yrs	NS
de Vos et al. 2004 [28]	28	III HNPCC	28	0	70%/70% 5 yrs in 28 treated vs. 64 not treated	NS
Ribic et al. 2003 [29]	283	II-III	53	230	75%/70% 5 yrs	NS
Brueckl et al. 2003 [30]	43	IV	7	36	33 months/19 months median survival	0.021
Liang et al. 2002 [31]	169	IV	35	134	24 months/13 months median survival	0.0001
Hemminki et al. 2000 [26]	95	III	11	84	90%/43% 3 yrs	0.020
Elsaleh et al. 2000 [15]**	272	III	23	249	80%/35% 5 yrs	0.0007

* Estimated from survival curves.

** Only BAT26 examined, MSI-H more common in women.

Other *in vitro* studies have shown that colon cancer cell lines displaying MSI-H are less responsive to 5-FU than MSS cell lines [17,19–25]. One study using three different methods (cell enrichment assay, cell growth, and colony formation) found that 5-FU treatment killed MSS cells that were proficient in MMR but spared MSI-H colon cancer cells [17]. In another study, re-expression of *hMLH1* in a MSI-H cell line with hypermethylated *hMLH1* overcame resistance to treatment with 5-FU [19].

Human studies

The ability to treat CRC stage II and III has significant implications for survival and cost. Before initiating treatment it is of utmost importance to determine if MSI-H tumors are sensitive or resistant to chemotherapy. The main problems with the human studies conducted so far were their retrospective design, small number of MSI-H patients, and scarcity of direct comparisons of outcome of 5-FU treatment between patients with MSI-H and those with MSI-L tumors [12,14,16,17,26–31]. The studies listed in Table 1 show a direct comparison of prognosis between treated stage II-III MSI-H and MSS or MSI-L tumors. Furthermore, the studies that controlled for the effects of adjuvant therapy used small or non-randomized study populations harboring potential selection biases [14,16,17,26] and there was no clear distinction between sporadic CRC and HNPCC cases.

Carethers et al. [27] evaluated 204 patients with stage II and III CRC for whom pathologic material was available and the chemotherapeutic regimen known. Formalin-fixed, paraffin-embedded pathology blocks from each patient's tumor were cut into 5 µm slices and placed onto slides for DNA extraction. Using the panel of five microsatellite markers (BAT25, BAT26, D5S346, D2S123, and D17S250) recommended by the National Cancer Institute, the authors classified the tumor as MSI-H (two or more markers showing novel alleles compared with non-tumor tissue), MSI-L (one marker with a novel allele), and MSS (no marker with a novel allele). Owing to the similar features of the MSI-L and MSS tumors and their common absence of association with DNA MMR inactivation, they were grouped into a single category and compared with MSI-H tumors, which are associated with DNA MMR inactivation [33]. Polymerase chain reaction assay performed on the micro-dissected template DNA showed a significant overall survival benefit in the patients with CRC who were treated with 5-FU-based chemotherapy compared to those who were not. However, within the MSI-H group there was no difference in survival by 5-FU treatment, whereas in the non-MSI-H group the authors noted a significant difference in survival between patients treated with 5-FU and untreated patients. These findings indicate that the type of genomic instability within a colorectal tumor might dictate patient response to 5-FU-based chemotherapy.

High frequency microsatellite instability in colorectal cancer tissue is associated with a favorable prognosis

In a study of 298 patients with sporadic stage III CRC treated with 5-FU-based regimens, Watanabe and team [33] found 62 MSI-H tumors (21%), of which 38 (61%) had a mutation of

TS = thymidylate synthase
MSS = microsatellite stability
MSI-L = low frequency MSI

the gene controlling the type II receptor for transforming growth factor-beta 1. The 5 year survival rate was 74% in those with a mutated gene and 46% in those without this mutation (P = 0.03). Thus, in cancers with high levels of MSI, the presence of a mutation of the gene for the type II receptor for TGF-β1 is indicative of a favorable outcome for adjuvant chemotherapy. This finding was supported by Goel et al. [34] who described a significant association of *RUNX3* (a gene related to the RUNT transcription factor, an important target of the TGF-β superfamily proteins) promoter hypermethylation and MSI-H colon cancers. They suggested that *RUNX3* is a novel target of methylation, along with the *hMLH1* gene, in the evolution of MSI-H colorectal cancers.

By contrast, other large selected case series of patients with stage III colon cancer demonstrated a significant association between MSI-H and increased survival in patients receiving adjuvant chemotherapy [16,35]. MSI status was determined by screening for deletions in the BAT26 mononucleotide repeat only. Systemic adjuvant 5-FU-based chemotherapy was delivered to 266 patients. MSI-H status was predictive of excellent survival benefit from chemotherapy but was not associated with better prognosis for patients who did not receive treatment. However, the patients who did not receive chemotherapy were older than those who did, which may have introduced a bias into the study. Older age has been associated with poor outcome in CRC after adjustment for MSI status [26]. A significantly older mean age also implies that the presence of coexisting disease was an important reason for not offering adjuvant treatment to patients in this non-randomized sample [16].

Hemminki and co-workers [26] followed 95 patients with stage III cancer who had received adjuvant chemotherapy for 7–63 months (median 31 months). The 3 year recurrence-free survival rate was 90% in the MSI-H group (n=11) compared to 43% in the MSS group (n=84) (P = 0.020). In another retrospective study, de Vos and associates [28] noted a 70% 5 year survival rate in 92 patients with stage III CRC from HNPCC families regardless of treatment with adjuvant 5-FU.

Ribic and co-investigators [29] used specimens from patients with resected stage II or stage III colon cancer who were previously enrolled in prospective randomized trials of 5-FU-based chemotherapy. These included phase 3 studies with groups that received no treatment, thus permitting analysis of the true survival advantage for patients with MSI-H tumors untreated by adjuvant protocols. In addition, this design made it possible to analyze whether the phenotype of MSI-H was an independent predictor of benefit from 5-FU-based adjuvant chemotherapy. Of the 570 tumor samples tested, 16.7% were categorized as MSI-H, 10.5% as MSI-L, and 72.8% as MSS. Among the patients who had not received adjuvant chemotherapy, those with MSI-H tumors had longer overall survival and higher rates of 5 year disease-free survival than patients with MSI-L or MSS tumors. On multivariate analysis, controlling for disease stage and tumor grade, the MSI-H status in patients who did not receive 5-FU-based adjuvant chemotherapy was significantly and independently associated with a better survival. However, analysis of the patients who did receive adjuvant therapy did not yield significant differences in overall or disease-free survival according to MSI status. Patients with MSI-L or MSS tumors who received adjuvant chemotherapy had a significantly greater overall survival than patients who did not. Among the group with MSI-H tumors, treatment was associated with a worse outcome for both stage II and stage III cancer. Barratt et al. [36] found no improvement in survival among patients with MSI-H adjusted for tumor site.

These tumors may not be sensitive to 5-FU-based chemotherapy

One study in patients with stage IV CRC reported that patients with MSI-H tumors treated with 5-FU had a better survival than patients with non-MSI-H tumors [31]. This finding was confirmed in a study of 43 patients with stage IV CRC, of whom 7 had MSI-H tumors [30].

Possible mechanism

Fluorouracil is a fluoropyrimidine that is incorporated into RNA (messenger, ribosomal, and transfer RNA), and acts as an inhibitor of thymidylate synthetase, which catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) [37]. It is generally accepted that it is the incorporation of 5-FU into RNA (in which uracil is one of the four bases and replaces thymine) that is the important mechanism of 5-FU toxicity [37]. Under normal conditions, deoxyuridine triphosphatase prevents the incorporation of deoxyuridine triphosphate (dUTP) and 5-fluoro-deoxyuracil triphosphate (FdUTP) into DNA by dephosphorylating the nucleotides to dUMP and 5-fluoro-deoxyuracil monophosphate (FdUMP), respectively [38]. Fluorouracil, however, inhibits thymidylate synthetase, which prevents the synthesis of dTMP and deoxythymidine triphosphate. As a result, dUMP and FdUMP accumulate, thereby exhausting the ability of deoxyuridine triphosphatase to metabolize dUTP and FdUTP. As levels of dUTP and FdUTP increase and levels of thymidine triphosphate (TTP) decrease, dUTP and FdUTP replace TTP as substrates for DNA polymerases and are incorporated into DNA. This facilitates the incorporation of 5-FU into DNA. Although uracyl-*N*-glycosylase, an enzyme that removes uracil bases from DNA after the spontaneous deamination of deoxycytidine, will typically also remove the incorporated uracil bases, TTP is not available, and the DNA strand is repaired with dUTP or FdUTP as a substrate. Fluorouracil has been detected in cellular DNA in previous studies, but no correlation between 5-FU incorporation into DNA and cytotoxicity has been reported [39].

TGF-β1 = transforming growth factor-beta 1

It is not known whether the MMR system can recognize and respond to 5-FU incorporated into DNA. Unlike bulky intercalating adducts such as cisplatin, or incorporated purines, 5-FU may not physically distort the DNA double helix. Because it has a pyrimidine base (smaller than purine), 5-FU does not directly interfere with interstrand hydrogen bonding of DNA. It may be recognized by the position of attachment of chemical groups on the altered nucleotide or by the physical nature of the compound itself; the highly charged fluoropyrimidine may deform the DNA double strand enough to be recognized by MMR proteins. Another possibility is that the MMR system recognizes the strand distortion produced when uracil-*N*-glycosylase removes incorporated FdUTP or dUTP. These pyrimidines are eventually reincorporated in place of TTP (a pyrimidine) because thymidylate synthetase is inactivated.

Carethers and team [17] found neither cell cycle alterations in MMR-proficient cells after 5-FU treatment nor a G2/M cell cycle arrest within the first 24 hours after treatment, and this state was sustained for more than 5 days. Lack of cell cycle perturbations after 5-FU treatment suggests that 5-FU cytotoxicity induces an alternative pathway of response by the MMR system that does not trigger G2/M cycle arrest. The authors also reported a seven- to ninefold lesser DNA than RNA incorporation. The effect of the incorporation of 5-FU into DNA on subsequent cell divisions remains unclear. Moreover, it is not apparent that 5-FU would induce point mutations.

Conclusions

The finding that fluorouracil-based adjuvant chemotherapy does not always increase, and may potentially decrease, overall and disease-free survival among patients with tumors exhibiting high frequency microsatellite instability raises several challenging questions regarding postoperative management of stage II and stage III colon cancer. In future studies, identification of the form of genomic instability will be paramount for interpretation of the results. Microsatellite testing of colorectal tumors will need to be more commonplace to implement any findings, since a favorable outcome in MSI-H cases does not result from higher sensitivity to chemotherapy. Prospective randomized controlled studies are necessary to reach a final conclusion as to whether MSI-H CRC is sensitive or stabile to 5-FU-based chemotherapy.

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Don't worry about avoiding temptation ... As you grow older it avoids you.

Winston Churchill

Capsule

Toxoplasma infection and TLR

Mammalian Toll-like receptors (TLRs) are critical modulators of the immune response to pathogens. TLR recognition of bacteria and some viruses are well known, but there have been few examples of recognition of parasite ligands. Yarovinsky et al. describe the detection of a profilin-like protein derived from the protozoan parasite *Toxoplasma gondii* by a recently characterized mouse TLR, TLR11. The ligand induced the production of the pro-inflammatory cytokine interleukin-12 (IL-12) by engaging the TLR signaling pathway. In the absence of TLR11, loss of IL-12 production rendered mice susceptible to *T. gondii* infection. Similar detection of parasite proteins by TLRs may influence the course of immunity against a range of protozoan parasitic diseases.

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Eitan Israeli

Capsule

Fungal malaria control

There is a pressing need for alternatives to chemical insecticides for targeting adult mosquitoes, the vectors of malaria, owing to the development of resistance and worries about human toxicity. Blanford and co-researchers (*Science* 2005;308:1638) found that treating surfaces with a fungal pathogen of insects reduced the number of mosquitoes able to transmit malaria after an infectious blood meal by more than 100-fold. Fungal infection via contact with netting or solid surfaces was sufficient to cause more than 90% mortality. Scholte et al. (p. 1641) performed field-based research in rural African village houses, using a fungus in real-life conditions, to target wild mosquito vector populations. Large numbers of mosquitoes could be infected with the fungus, which could inhibit malaria parasite development. Even at moderate coverage rates, a dramatic fall in malaria transmission intensity should be achievable.

Eitan Israeli