Published in final edited form as:

Transl Res. 2018 June ; 196: 62–70. doi:10.1016/j.trsl.2018.02.001.

Genotypic and Phenotypic Signatures to Predict Immune Checkpoint Blockade Therapy Response in Colorectal Cancer Patients

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Abstract

Immune checkpoint blockade therapy (ICBT) has resulted in extended overall survival for some patients with certain types of cancer, most prominently including colorectal cancer (CRC) patients associated with microsatellite instability (MSI). However, most patients with CRC whose phenotypes have microsatellite stability (MSS) are unresponsive to ICBT. In efforts to understand the responsiveness of CRC tumors to ICBT, genotypic and phenotypic signatures of CRC tumors are now being investigated. The MSI and MSS classification has been clinically validated as helpful in predicting response vs. nonresponse to ICBT in CRC patients. Other potential predictive markers include mutational and neoantigen loads, T-cell receptor diversity, and the immune score system, all of which have mechanistic connections to ICBT response. These novel predictive signatures could provide unprecedented insights into patients with CRC associated with MSS. Clinical trials or prospective cohort studies using standardized methodologies for biomarker quantification should be illuminating. Further validation of these novel predictive signatures will be essential to tailoring treatment of patients whose CRC is most likely to respond to ICBT.

Keywords

colorectal cancer; immune checkpoints; microsatellite instability; combination therapy; biomarker

Introduction

Most cancer treatment regimens have targeted tumor cells through radiotherapy, chemotherapy, and surgical procedures. But recently, novel therapies have shifted from targeting tumor cells to enhancing the antitumor capabilities of the host immune system. Immunotherapy works with host immune cells, particularly the tumor-infiltrating lymphocytes (TILs), to attack tumor cells that were previously recognized as “self” antigens.

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Conflict of interest

The authors declare no conflicts of interest.

Examples of immunotherapy include immune checkpoint blockade therapy (ICBT), antibody-based oncoprotein targeted therapy, cancer vaccines, and adoptive immune cell transfer [1].

As compared with other varieties of immunotherapy, ICBT (which targets immune checkpoint receptors and their ligands) has demonstrated the most responses among patients with various cancers [2–5]. The immune checkpoints possessed by immune cells are either inhibitory or stimulatory pathways, which either limit or enhance the immune response [6]. In its current clinical application, ICBT targets 2 major immune checkpoint pathways: the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) pathway and the programmed death 1 (PD-1) pathway [2–5, 7–9]. CTLA-4 is a receptor protein expressed on T cells that are upregulated during T-cell activation. This protein competes with CD28 to bind with the 2 ligands that they share, CD80 and CD86 [6, 10]. When the T-cell receptor (TCR) is engaged by peptide-major histocompatibility complex (MHC) binding, receptor protein CD28 binds to CD80 and/or CD86, thereby enhancing TCR signaling and serving as a major secondary stimulatory pathway [10, 11]. CTLA-4 has a higher binding affinity for ligand CD80 and CD86, as compared with receptor protein CD28.

The mechanisms by which CTLA-4 inhibits T-cell function remain somewhat controversial. One biochemical study suggested that CTLA-4 recruits a phosphatase to the TCR, thus attenuating the signal [12]. Other studies have suggested that CTLA-4 functions by capturing and removing CD80 and CD86 from the membranes of antigen-presenting cells (APCs) and by preventing the formation of firm contact between T cells and APCs [13–15]. Thus, expression of CTLA-4 on activated T cells functions to decrease T-cell priming and proliferation.

The other major inhibitory immune checkpoint pathway involves PD-1 and its ligands, PD-L1 and PD-L2 [16, 17]. PD-1 is expressed on activated T cells, B lymphocytes, and natural killer cells [18–20]. PD-L1 is widely expressed by tumor cells and tumor stromal cells, such as macrophages, fibroblasts, and T cells. When the PD-1 receptor binds its ligand (i.e., either PD-L1 or PD-L2), the result is the recruitment of SH2 domains containing tyrosine phosphatases, which work to suppress T-cell activation and proliferation [21]. More detailed explanations of immune checkpoint mechanisms have been summarized in several reviews [22–24].

In patients with various types of cancer, blockade of both PD-1 and CTLA-4 receptors has yielded positive results [2–5, 22]. This claim is supported by a pooled analysis of long-term survival data of Ipilimumab (anti-CTLA-4) treated unresectable or metastatic melanoma patients [25]. Of the patient pool, 22% were alive after 3 years; even more impressively, results with ipilimumab were consistent in both untreated and previously treated melanoma patients [25]. A comparable positive clinical benefit was observed in patients with various types of cancer who were on nivolumab (anti-PD-1) [26–28].

ICBT in colorectal cancer treatment

In both men and women, CRC) is the third most common type of cancer [29]. Although the clinical activity of ICBT is consistent in some forms of cancer, there is a clear dichotomy of clinical benefit in patients with metastatic CRC [30]. The sentinel trial using PD-1 blockade was a phase 1 trial that enrolled 296 patients with heavily pretreated cancer; 19 of them had chemorefractory CRC. Objective responses were seen in patients with melanoma, non-small cell lung cancer, and renal carcinoma, but no objective responses were observed in any of these 19 CRC patients [3]. A separate phase I study reported objective response in 1 of 14 cases of heavily pre-treated CRC [31]. Based on this sentinel response (1 of 33 total CRC cases), investigators hypothesized that tumors in the exceptional responder harbored MSI disease. A phase II trial was planned to assess the efficacy of the PD-1 inhibitor pembrolizumab in 41 patients with heavily pretreated carcinomas, 32 of whom had cancers of the colon or rectum. Of these 32 cases, 11 harbored microsatellite instability (MSI), and 21 were microsatellite stable (MSS). The MMR deficient cohorts included patients with inherited germline MMR deficiency (Lynch syndrome) as well as patients with sporadic MMR deficient tumors. When the response was assessed, 40% of the MSI cases showed a partial response, and none of the 21 MSS tumors showed objective response [32]. The median progression-free survival (PFS) and overall survival (OS) were 2.2 and 5.0 months, respectively, in the patients with MSS colorectal tumors. However, the median PFS and OS were not yet determined for the cohort with MSI colorectal tumors [32]. Based primarily on these findings, in May 2017 the U.S. Food and Drug Administration (FDA) approved the use of pembrolizumab in patients with heavily pretreated forms of MSI-metastatic carcinoma. In the case of CRC specifically, the approval specifies that patients must have been treated and become refractory to standard-of-care chemotherapeutic drugs 5-fluorouracil, oxaliplatin, and irinotecan, or the equivalent.

In the ongoing phase 2 clinical trial CheckMate-142, the combination of Nivolumab and Ipilimumab was tolerable for most MSI and MSS CRC patients [33]. The median progression-free survival (PFS) of MSI tumors was 5.3 months, compared to a median PFS of 1.4 months for patients with MSS CRC [33]. The response was independent of baseline PD-L1 expression level, and BRAF or KRAS mutation status [34].

The rationale of using MSI and MSS phenotype as a predictive marker in ICBT sensitivity of CRC

Genomic instability in CRC

Genomic instability is a hallmark of tumors, and mutations can accumulate because of increased exposure to DNA damage and/or decreased DNA repair activity [35]. The 3 major avenues for CRC pathogenesis involve chromosomal instability (CIN), the CpG island methylator phenotype (CIMP), and MSI [36–38]. CIN accounts for a majority of the sporadic type of CRC [39], resulting in DNA aneuploidy and various mutations in proto-oncogenes and tumor-suppressor genes [40]. Mutations in tumor suppressors such as APC, SMAD4, and TP53—along with mutations in the proto-oncogene KRAS—are commonly identified in patients with CRC expressing CIN [41]. CIMP represents the global genome

hypermethylation condition, resulting in the inactivation of several tumor suppressor genes or other tumor-related genes [37]. MSI, which is prevalent in about 15% of patients with CRC [38, 42], is caused by the loss of DNA MMR activity. The MMR system consists of a family of enzymes that are encoded by the MMR genes, such as MLH1, MSH2, MSH6, and PMS2. The MMR system detects and repairs DNA replication errors produced in the S phase. As compared with other sections of the DNA strand, DNA polymerases are more likely to make errors in long repetitive DNA sequences, such as microsatellites, during replication. MMR pathway deficiencies due to mutations in MMR genes and/or to hypermethylation of the promoter region of MMR genes result in the accumulation of single base-pair mismatches and an insertion-deletion loop (IDL) in the repetitive sequence. Consequently, a large number of truncated, nonfunctional proteins are produced in MMR-deficient cells [35, 38].

MMR deficiency and neoantigen burden in CRC

The accumulation of somatic mutations is a hallmark of tumors and thus the foundation of cancer immunotherapy, which targets tumor-specific antigens. MMR deficiency mechanisms in CRC result in a hypermutable phenotype. Although the CRC mutation load widely ranges in different reports, from dozens to thousands [32, 43–45], the number of mutations is commonly related to the MSI vs. MSS classification; in general, patients with CRC associated with MSI (vs. MSS) have a higher average mutation load.

While the total mutation load represents the potential of inducing a tumor-specific immune response, it is the mutations that are transferred to neoantigens can elicit an immune response. Neoantigens are foreign epitopes (resulting from nonsynonymous somatic mutations within tumor cells) that can be presented by the MHC [46]. Many bioinformatics algorithms have been developed to predict the development of tumor neoantigens according to predicted T-cell receptor binding, MHC class I molecule binding, and the patient's human leukocyte antigen (HLA) type [12, 47, 48]. In most clinical studies that measured the neoantigen load, it was proportional to the total overall mutation burden [49–51]. In patients with CRC associated with MSI (vs. MSS), the tumors have a higher number of total somatic mutations, as well as more neoantigens, on average [32, 43, 44].

To date, evidence suggests that the vast majority of predicted neoantigens are generated by tumor-specific mutations, but not by typically known oncogene mutations [46]. This fact implies that the tumor-driving mutations might not have strong immunogenicity. Give that the MSS and MSI CRC tumors have a similar relative burden in the major tumor-driving mutations, it is appropriate to infer that most neoantigens in CRC are derived from passenger mutations that have accumulated in MMR-deficient tumors. These mechanisms provide the basis for the response to ICBT of CRC associated with MSI.

Neoantigen load and ICBT sensitivity

Similar to the principle that a small number of genetic alterations in oncogenes and tumor suppressor genes are responsible for tumorigenesis, there is growing evidence to show that it is the small amount of tumor-specific neoantigens that determine ICBT sensitivity [46]. Recent clinical studies in patients with non-small cell lung cancer showed that tumors with

high levels of clonal neoantigens responded robustly to ICBT. However, the neoantigens can be lost through the elimination of tumor subclones or deletion of chromosomal regions containing truncal alterations in ICBT-responsive tumors [52, 53]. Loss of 7 to 18 neoantigens that were able to elicit clonal T-cell expansion in autologous T-cell cultures was associated with decreases in tumor-infiltrating T-cell receptor clonality and with resistance to ICBT [52]. Those studies were conducted in small cohorts of patients, but provide insight into the dynamics of neoantigen load during ICBT and have implications for the possible use of neoantigen load as a predictive marker of response to ICBT. But in patients with CRC, no reports have yet indicated the association between neoantigen load and response to ICBT.

Tumor-specific neoantigens provide the basis of an antitumor immune response. However, intratumor immune cells are what eliminate the tumors. Tumors with a higher (vs. lower) neoantigen load also have a higher number of TILs (46). In patients with CRC, more CD4⁺ and CD8⁺ T cells have been observed in tumors associated with MSI (vs. MSS) [54]. Also, higher expression of interferon (IFN)- γ , granzyme B, and perforin in CD8⁺ T cells has been observed in patients with CRC associated with MSI (vs. MSS) [54, 55], indicating that their T cells are more functional and reactive. Furthermore, tumor-infiltrating dendritic cells in patients with CRC associated with MSI have been shown to express higher levels of costimulatory molecules [56]. The number of TILs, both before and during treatment, has shown validated value in predicting response to ICBT in patients with solid tumors [57]. The underlying mechanisms of how neoantigen load regulates intratumor immune infiltration have not been fully characterized, but the finding that neoantigen-enriched tumors have better immune infiltration is notable: it strengthens the idea that neoantigen load determines response to ICBT.

***POLE* mutation in MSS CRC tumor represents a hypermutated phenotype**

An emerging body of data supports a role for mutations in DNA polymerase epsilon (known as Pol ϵ or, usually, *POLE*), which is involved in DNA replication and repair and might help drive a hypermutated phenotype in tumors [58, 59]. The *POLE* mutation has been found in patients with CRC associated with MSS [58]. In 1 patient, treatment-refractory metastatic CRC associated not only with MSS but also with the *POLE* mutation responded to anti-PD-L1 therapy [60]. That patient's tumor (unlike most CRC tumors associated with MSS) represented a hypermutated phenotype. Evaluation of that tumor tissue revealed a large number of CD8⁺ tumor-infiltrating T cells in the tumor microenvironment, with > 90% of them expressing PD-1. In addition, PD-L1 was also expressed in that tumor microenvironment. This body of data suggests that the *POLE* mutation could be a novel genomic biomarker in predicting response to ICBT in patients with CRC.

Potential predictive markers in DNA damage and repair-based therapies and ICBT combinations

Need for combination immunotherapy in CRC patients

Over the past 2 decades, options for patients with metastatic CRC have significantly broadened, regarding the number of approved drugs (chemotherapeutic and biologic) as well

as the number of validated combinations that can be used safely as first-line treatment and beyond. Cytotoxic chemotherapy based on 5-fluorouracil remains the standard of care, combining 5-fluorouracil with either irinotecan or oxaliplatin. Such doublet combinations can be used in concert with biologic agents, including those targeting angiogenesis (bevacizumab, aflibercept) as well as targeted therapy using monoclonal antibodies directed toward epidermal growth factor receptors (cetuximab, panitumumab) in patients whose tumors do not harbor mutated forms of RAS [61, 62]. When CRC failed to respond further to the above forms of therapy, remaining options either lacked clinically meaningful or significant biologic activity or included clinical trials, when available, for those patients whose performance status had not been compromised after months or years of ongoing treatment. Data supporting ICBT, leading to FDA approval of this class of drugs for pre-treated refractory metastatic CRC, represents a major advance in treating patients with this disease. Still, the initial studies have shown that only *some* patients with CRC (typically, those whose tumors are associated with MSI) have, so far, benefited from ICBT. The identification of MSI in patients most likely to benefit was an important step, providing clinicians with a tangible predictive biomarker of potential response. However, MSI is detected in no more than 15% of all patients with CRC. Future trials must investigate combination strategies that could strength antitumor immunity so that the much larger MSS cohort of patients can also benefit from ICBT.

The mechanisms of DNA damaging cancer therapies mediated antitumor immunity

Traditionally, conventional chemotherapy—including regimens using DNA-damaging agents—has been considered immunosuppressive, because of its lymphopenic toxicity. But an increasing number of experimental studies have suggested that DNA-damaging chemotherapy regimens can promote antitumor immunity and can be combined with ICBT. Unlike apoptosis, which kills cells in a nonimmunogenic way, chemotherapy can result in cell killing with enhanced release of tumor cell antigens [63]. Primarily, the underlying mechanisms involve the expression and secretion (because of the cellular stress caused by chemotherapy) of danger-associated molecular patterns (DAMPs), such as cytosolic DNA, high-mobility group box 1 (HMGB1), calreticulin, hyaluronan, and heat shock proteins. The activation of DAMPs promotes secretion of type I interferon and other chemokines by cancer and stromal cells to facilitate dendritic cell-mediated antigen presentation and T-cell priming [63]. The second mechanism by which DNA damaging chemotherapies cancer elicit tumor-specific immunity involved in modifying the tumor microenvironment. It has been shown that drugs such as gemcitabine, paclitaxel, cyclophosphamide, and 5-fluorouracil suppress the functions of regulatory T-cell and myeloid-derived suppressor cells [64–67].

The immunoregulatory function of ionizing radiation that can induce numerous types of DNA damage, including double-strand breaks, has been appreciated for decades [68]. Mechanistically, radiation induces upregulation of MHC class I molecules and costimulatory signaling molecules, including CD86 (B7-2) and CD70, on dendritic cells, thereby facilitating tumor antigen cross-presentation [69]. Additionally, radiation stimulates the release of DAMPs, such as HMGB1, from tumor cells [70]. The so-called “abscopal” effect (meaning a systemic response to focal radiation) suggests the distant immunoregulatory impact. In patients treated by localized radiation and ICBT, shrinkage of nonirradiated

lesions has been documented [71]. Radiation therapy might have distinctive features in transforming nonimmunogenic tumors to immunogenic tumors.

The evidence is also emerging to support the strategy of combining ICBT with DNA repair agents, such as poly(adenosine diphosphate [ADP]–ribose) polymerase (PARP) inhibitors, as a potential way to overcome resistance to ICBT. Studies had shown that, when a PARP inhibitor was administered, T-cell infiltration and production of IFN- γ and tumor necrosis factor (TNF)- α increased in *BRCA-1*-deficient tumors [72]. Thus, combining a PARP inhibitor with ICBT has promise. A recent review elegantly summarized the ongoing clinical trials involving the combination of ICBT and DNA-damaging agents [73].

Need for predictive markers in the combinatory treatment

As a predictive marker of response to IGBT, the traditional MSI and MSS classification has been highly useful in pretreated tumors, but it might not apply to combination therapy. The mutations in MMR genes in tumors are not changed by DNA-damaging agents. Because multiple DNA-damaging agents have routinely been used in patients with CRC, it is critical for clinicians to have predictive markers that can help evaluate the feasibility of combining ICBT and DNA-damaging agents. No direct evidence is available yet in patients with CRC, but several potential predictive markers have been investigated in several other types of tumors—markers that could be used to dynamically evaluate the activity of ICBT.

As mentioned above, the number of neoantigens has been correlated with response to ICBT in patients with non–small cell lung cancer and patients with melanoma. Moreover, intratumor heterogeneity in the neoantigen landscape also influences sensitivity to ICBT and has potential predictive value [52, 53]. In addition to determining a tumor’s neoantigen load, analyzing T-cell receptor (TCR) sequences have been established as a more direct method for determining tumor-specific immune response. The diversity of TCRs and expansion of tumor-specific T-cell provide the basis of tumor-specific immune response: TILs with different TCRs are expected to be efficient in eliminating tumor cells with high heterogeneity. Next-generation sequencing of TCRs promises to provide insights into the complexity and heterogeneity of intratumor T-cell infiltration from patient to patient. In patients with non–small cell lung cancer, a decrease in clonality of cytotoxic TCR clonotypes reflects tumor immune evasion at the time resistance to ICBT emerges [52]. All of these valuable clinical results shed light on TCR diversity as an indicator of response to ICBT.

A recent study compared TCRs from TILs of CRC tumors vs. T cells found in adjacent healthy mucosal tissue. Sequencing revealed a higher number of unique TCR clones in the tumor tissue than in the healthy mucosal tissue [74]. Another study characterized the T-cell repertoire of patients with CRC, before vs. after treatment with pembrolizumab [75]. The TCR-seq of patients samples responding to pembrolizumab revealed that tumor-specific T-cell clones were selectively expanded in the periphery blood after treatment initiation [75]. Moreover, the radiologic responses were observed after the peak of tumor-specific T-cell expansion. These observations suggested the strong correlation between tumor-specific T-cell expansion and ICBT response.

Other potential predictive markers that have been investigated include some tumor-infiltrated immune cells and immune checkpoints, such as PD-L1. One study evaluated the immune landscape in patients with melanoma, before vs. after treatment [57]. A beneficial response was demonstrated in patients who had the high expression, before treatment, of CD8⁺ T cells in the invasive margin. During treatment, in those patients, expression of CD8⁺, PD-1⁺, and PD-L1⁺ cells increased [57]. Another clinical study regarding nivolumab in several solid tumors also suggested the value to assess the intratumoral PD-L1 protein expression to predict anti-PD-1 treatment response [31]. A number of large-scale clinical trials—in patients with non–small cell lung cancer, melanoma, renal clear cell carcinoma, squamous cell carcinoma of the head and neck, and bladder cancer—have recapitulated the finding that tumors with high expression of PD-L1 are more sensitive to anti-PD-1 and anti-PD-L1 treatment [50, 76]. But in patients with CRC treated by ICBT, the predictive value of immune infiltration and PD-L1 expression is not yet clear.

In patients with CRC, the so-called “immunoscore” system was designed to quantify the number of cytotoxic and memory T cells, both in the core of the tumor and in the surrounding invasive margin [42]. This immunoscore system requires the use of traditional immunohistochemistry to stain CD3⁺ and CD8⁺ cells, or CD45RO⁺ and CD8⁺ cells [77]. The high throughput RNA sequencing was also used to screen for immune-related gene expression, which functioned as a more integrative immunoscore system [42]. Patients with a low score had a worse prognosis and a higher likelihood of distant metastasis [68]. High PD-L1 expression in patients with MMR-proficient CRC was associated with an early T stage, the absence of lymph node metastases, a lower tumor grade, the absence of vascular invasion, and improved survival rates [78]. Clinical trials evaluating the predictive role of immunoscore system and PD-L1 expression and response to the combination of ICBT and DNA-damaging agents are urgently needed.

Conclusion and Perspectives

In some cancer patients, the clinical value of ICBT has already been demonstrated. But in a large subset of patients with CRC, especially if their tumors are associated with MSS, ICBT appears to be ineffective. Recent studies have investigated the predictive value of MSI/MSS phenotype in CRC patients' anti-PD-1 response. Meanwhile, the combination of DNA-damaging therapies showed promising potential to expand the use of ICBT in unresponsive patients. Studies in other cancers indicated several biomarkers, except MSI/MSS classification, have the potential to benefit CRC patients. However, several key challenges remain before we can translate our findings from bench to bedside.

First, most of the currently available evidence was derived from phase II studies involving a small number of patients with CRC who had already undergone a wide range of treatments before ICBT. Such previous treatments can induce immunogenic or immunosuppressive effects that influence response to ICBT and undermine the accuracy of biomarkers. To further validate the predictive value of specific biomarkers, future phase 2/3 clinical trials must control for the effects of pre-ICBT treatments. Notably, phase III studies are ongoing to compare the efficacy of the current standard-of-care with ICBT (or the current standard-of-care and ICBT combination) for MSI-high metastatic CRC patients (NCT02563002 and

NCT02997228). These well-controlled phase III studies will further validate the efficacy of ICBT in MSI-high CRC patients.

Second, unlike static predictive markers defined by DNA mutations directly linked to the response of targeted kinase inhibitors, the consistent-changing immune system poses unique challenges to predictive marker identification. Drug-resistant mechanisms can originate from many different aspects of the antitumor immunity process, such as the potential to initiate antitumor immunity, the ability to attract tumor-infiltrating immune cells, and the capability to keep immune cells alive in the tumor microenvironment. Meanwhile, it was known that different ICBT treatments have different mechanisms for stimulating antitumor immunity [79]. To fully understand the major resistant mechanisms of a specific tumor, we might need to combine multiple predictive models. For example, the MSS phenotype could indicate that weak stimulation of tumor-specific T-cell priming is the major reason for resistance to ICBT. Or, the MSI phenotype, especially in tandem with limited tumor-specific T-cell infiltration, might indicate that the barrier effects of tumor stromal cells prevent T-cell recruitment. Progress in these areas will drive the appropriate combination of DNA-damaging or immune microenvironment remodeling treatments with ICBT.

Third, the MSI phenotype is typically evaluated by identifying the size of selected microsatellites and/or by assessing protein expression of the major DNA MMR genes [80]. We also need such a standardized methodologic approach to evaluate the pertinent genomic and immune signatures. In one study, researchers recorded an average of 1,782 somatic mutations per tumor (in patients with CRC associated with MSI) and an average of 73 somatic mutations per tumor (MSS). Then, to calculate neoantigen load, they further analyzed the tumor genomes: they found a total of 578 potential mutation-associated neoantigens in tumors associated with MSI, but only 21 in tumors associated with MSS [32]. Another study found a similar number of mutation-associated neoantigens in the tumors associated with MSI [44]. Still, other studies found vastly different numbers of mutation-associated neoantigens in their MSI cohorts [43, 45]. These differences are due to inconsistencies in methodology. Some investigators evaluated exome mutation by comparing the entirety of the tumor exome sequence to a noncancerous sequence [32, 44], whereas others observed mutations from a selective gene panel [43, 45]. Before these genomic markers can be incorporated into clinical practice, a stable cutpoint must be established for high mutation load or high neoantigen number; the agreed-to evaluation procedure must standardize biopsy site positions, sample preparation, sequencing methods, read depth, and analysis algorithms.

Acknowledgments

SS is supported by research grants funded by the NIH/NCI grant R03CA219129 and Mezin-Koats Colon Cancer Research Fund; AM, by the Life Sciences Summer Undergraduate Research Program (LSSURP); and XZ, by a research fellowship from the Department of Surgery, University of Minnesota. Because of space restrictions, we cannot cite the many significant contributions made by numerous researchers and laboratories in this potentially important and rapidly progressing field. We thank Dr. Mary Knatterud for assisting in manuscript preparation.

The authors also declare no conflicts of interest and declare that all the authors have read the journal's policy of conflicts of interest. All the authors have read the journals authorship agreement.

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