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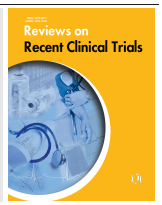

What is the Current Role of Immunotherapy for Colon Cancer?

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Abstract: Colon cancer is a leading cause of cancer related mortality. Until very recently the only existing options that medical oncologists had to treat metastatic colon cancer were a combination of chemotherapy, anti-EGFR and anti-angiogenic agents. We currently have the first proof that immune therapies could be an effective approach to battle colorectal cancers that carry a mismatch repair machinery deficient phenotype. It is expected that as our knowledge of the different mechanisms of immune-resistance grows, this therapeutic modality might soon be applicable to all patients. However, due to the continuous increase in the cost of oncological drugs, some treatment overheads may soon become prohibitive for many. In this review we will examine the current evidence related to this topic with the objective to provide the reader with concise but practical information about the potential role of immunotherapy in CRC.

Keywords: Colon cancer, lynch syndrome, microsatellite instability, PD-1 blockage, pembrolizumab.

INTRODUCTION

Colorectal Cancer (CRC) is within the top three leading causes of cancer-related deaths in the United States, irrespective of gender [1]. Regardless of the recent significant improvement in the number of options available to treat metastatic disease (mCRC), stage IV colon cancer remains incurable in the vast majority of cases. Until very recently the only existing options that medical oncologists had to treat mCRC were a combination of chemotherapy, anti-EGFR and anti-angiogenic agents [2]. At least for a proportion of patients, this situation has changed dramatically.

Research efforts over the past three decades in molecular genetics have profoundly influenced our understanding of CRC etiology and pathogenesis. Inheritable forms and familial syndromes of colon malignancies have been known for decades. Hereditary nonpolyposis colorectal cancer, previously known as Lynch syndrome, is probably the best understood and one of the most extensively studied ones. The molecular basis for this genetic predisposition to develop different types of cancers is a germline non-functional mutation in one of the many genes that constitute the DNA Mismatch Repair (MMR) machinery [3]. Deficient MMR has been established as a good prognostic factor in localized disease. However, it is a poor prognostic feature in metastatic CRC and seems to be related to B-RAF mutation. MMR-deficient tumors are prone to developing large numbers of somatic mutations, especially in regions of small repetitive tandems of DNA called microsatellites. This type of genomic instability reflects the inherent difficulty encountered by the DNA polymerase machinery to replicate these regions, occasionally "stuttering" in response to interstrand slippage.

The proportion of CRC patients with a hereditary form of MMR deficiency is relatively small (~3%) [4]. However, 10-15% of sporadic CRCs seem to have, as a consequence of random mutagenesis or gene silencing, some sort of defect in the MMR genes that results in Microsatellite Instability (MSI) phenotype [5]. These non-hereditary CRCs behave clinically and molecularly as their hereditary counterparts.

MSI tumors have unique anatomopathologic as well as clinical features that distinguish them from the classical ones. They tend to locate proximally, have a mucinous histology with a large number of infiltrating lymphocytes, and are less sensitive to fluoropyrimidines [6]. MSI has been associated with better survival compared with microsatellite stable (MSS) tumors. In a retrospective multivariate analysis by Gryfe *et al.*, analyzing more than 600 young patients with CRC, the presence of high levels of microsatellite instability correlated with a lower risk of nodal involvement, distant metastases and overall survival [7]. Hazard Ratios (HR) ranged from 0.33 to 0.50 arguing that MSI clearly grants some type of protection against those poor outcomes. The effect could be estimated to range from one-half to a third of the risk associated with MSS tumors. Previous investigations have attributed this discrepancy in survival to intrinsic molecular differences, such as the relatively higher prevalence of the type II TGF-β1 mutation receptor. The presence of the wild type allele conferred a higher risk of relapse and death after adjuvant chemotherapy [8]. However, recent evidence coming from the oncoimmunology field has

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broadened our understanding of this particular subtype of CRC, suggesting that the immune system might play a much bigger role than previously thought.

This propensity to evoke a stronger response by the host immune system could certainly explain some of the clinical characteristics mentioned above. Hyperreactive immune response to colon cancer cells, for example, has long been suggested not only to correlate with higher odds of retrieving more than 12 lymph nodes in pathology samples, but also with less chances of finding nodal metastases presumably due to the destruction of tumoral cells [9]. However, and beyond the potential insight that this could provide to cancer pathophysiology, with the advent of new and revolutionary immunotherapies there is now a clear and tangible practical application [10].

We currently have the first proof that immune therapies could be an effective approach to fight this particular subtype of CRC. In the subsequent sections of this review, we will focus our attention on examining the current knowledge related to this topic with the objective to provide the reader with concise but practical information about the potential role of immunotherapy in CRC.

CORRELATION BETWEEN GENETIC INSTABILITY AND IMMUNE RESPONSE

In a collaborative study from the Colorectal Cancer Subtyping Consortium, an integrated analysis of 4,562 CRC samples from different databases was able to identify four different consensus molecular subtypes and an additional one that corresponds to the unclassifiable samples [11]. The first subtype, named CMS-1, represented 13% of the samples and consisted of tumors with high levels of MSI (MSI-H) that also showed high frequency of BRAF mutations, TGF-β2 dysregulation, immune activation and infiltration.

The inherent inability of MSI tumors to efficiently proofread the DNA polymerase product generates numerous insertions and deletions of 1-2 pairs of DNA bases throughout the newly replicated DNA strand. When this phenomenon occurs within the coding portions of the transcribed genes, it may randomly create a change in the reading frames which will ultimate result in the translation of a mutated protein (nonsilent mutations). These mutated proteins might carry peptide combinations which were previously unseen by the host immune system, constituting real "neoantigens" that will promote a strong immune reaction. By simple probability, tumors with more mutations are likely to harbor more neoantigens. Multiple investigations support the concept that MSI is associated with recurrent somatic frame shift mutations in multiple genes explaining an unusually elevated proportion of neoantigens.

Upon exploring the different patterns of somatic mutations in human cancers, Greenman *et al*. found that MSI-H tumors harbored 32 mutations per megabase compared with 1.2 in MSS colorectal carcinoma [12]. In a specific analysis between MSI-H and MSS colorectal cancers performed using next generation sequencing, 1,304 somatic variants were found in MSI-H tumors compared with 198 in the MSS counterparts [13]. These variants were principally missense mutations, capable of generating neoantigens and eliciting specific immune responses. Moreover, the Cancer Genome Atlas Network performed a genome scale analysis where CRC was divided into hypermutated and non-hypermutated phenotype groups based on the frequency of mutations per megabase [14]. Hypermutated CRC comprised 16% of the samples. Most of the hypermutated tumors contained high levels of MSI, either due to germline mutations in MMR genes or, more frequently, sporadic silencing of MLH-1 gene by promoter methylation. Recurrent specific somatic mutations were found in different frequencies between the two groups. Among non-hypermutated tumors, frequent mutations were found in the following genes: APC (81%), TP53 (60%), KRAS (43%), PIK3CA (18%), FBXW7 (11%), SMAD4 (10%), TCF7L2 (9%) and NRAS (9%). By contrast, in hypermutated tumors, ACVR2A (63%), APC (51%), TGFBR2 (51%), BRAF (46%), MSH3 (40%), MSH6 (40%), and TCF7L2 (31%) were genes frequently altered. Overall, the most recurrently dysregulated pathway in CRC is the Wnt-β-catenin pathway, altered in 93% of all tumors. TGF-β signaling is altered preferentially in hypermutated tumors (87%) with the consequent activation of MYC. PI3K pathway signals are equally activated in 50% of all tumors, and RAS-MAPK pathway is differentially activated in both groups. In MSI-H tumors there is frequent activation of BRAF, as opposed to higher mutation rates in KRAS among non-hypermutated CRC. This could have a clinical implication because preliminary studies suggest an inverse correlation between KRAS mutation status and PD-1/PD-L1 expression in lymphocytes and tumoral cells [15].

The presence of a robust immune response to MSI-H colorectal cancer has been previously described. Jass *et al*. found 33% of MSI-H colorectal cancers with Tumor Infiltrating Lymphocytes (TILs) [16]. Numerous investigations have linked TILs with better prognosis. In a recent meta-analysis, the presence of TILs correlated with significant longer overall survival and disease-free survival (HR = 0.59 and 0.72, respectively) [17]. French investigators found that specific subtypes of immune cells were correlated with lower rates of early metastatic findings - lymphovascular invasion and perineural infiltration - and better survival. By means of quantitative real time PCR and tissue microarrays they found that the presence of effector memory T cells within the tumor, defined as CD3+/CD8+ and CD45RO+ cells, was associated with better survival [18, 19]. In another study, an "inmunoscore" based on the relative densities of CD8+ and CD45RO+ cells in the tumor center and the invasive margin could better predict the recurrence rate of localized CRC than the standard TNM classification [20].

Moreover, and importantly, this relationship between high mutation frequencies and stronger immune response goes far beyond the area of tumors with microsatellite instability. In a seminal study, Alexandrov and colleagues analyzed mutational catalogues of approximately 7,000 primary cancers [21]. The prevalence of somatic mutations - measured as number of mutations per Mb - was higher in melanoma and squamous cell lung cancer. These two tumors are known to be caused by chronic exposure to two of the strongest carcinogenic agents, namely ultraviolet radiation

and tobacco. It is precisely in these two cancers where immunotherapy has the most solid evidence of clinical activity. However, the same concept is also applicable in other malignancies. Within the field of gynecological malignancies, for example, investigators have recently proven that polymutated, non-MSI, endometrial cancers harbor high numbers of neoantigens with elevated levels of TILs as well as PD-1 and PD-L1 expression [22, 23]. Similar discoveries were reported in BRCA mutated ovarian cancers [24]. Furthermore, through the utilization of a different approach, scientists have been trying for a long time to find appropriate tumor vaccines based on these mutated peptides [25].

PRACTICE-CHANGING CLINICAL EVIDENCE

The hypothesis outlined in the preceding paragraphs was clinically tested in a recently published phase II clinical trial. In this study, Le *et al*. aimed to establish the clinical efficacy of the immune checkpoint inhibitor pembrolizumab - an anti PD1 IgG4 monoclonal antibody - in MSI-H and MSS colorectal cancers as well as in MSI-H non-colorectal tumors [26]. This trial consisted in 3 cohorts: cohort A comprised of 11 MSI-H mCRC patients, cohort B with 21 MSS mCRC patients, and cohort C with 9 metastatic non-MSI-H patients. Almost every patient had received two or more previous therapies. All groups were treated with pembrolizumab at 10 mg/kg IV every 2 weeks. The co-primary end points were immune related ORR and PFS at 20 weeks. While the study enrolled a very limited number of patients (N = 41), its importance lies in the fact that it could be seen as a proof of principle.

In the primary analysis, the reported immune related Objective Response Rate (irORR) was 40% and 71% for colorectal and non-colorectal MMR deficient cancer patients, respectively. The time to response was significantly faster among non-colorectal MSI cancer patients. The immune related Progression Free Survival (irPFS) at 20 weeks among MSI-H CRC and non-CRC patients was 78% and 67%, respectively. Remarkably, no responses were observed in the MSS CRC cohort, and this correlated with a dismal immune related Progression Free Survival (irPFS) measured at 20 weeks. For CRC patients, eight out nine MSS patients had progressed by that time period in comparison with only two in the MSI-H cohort. When the clinical response was analyzed using the classical RECIST criteria no substantial differences were observed. The Disease Control Rate (DCR) was 90% for MSI-H CRC patients compared with 11% in the MSS CRC cohort. Only limited information, perhaps just exploratory in nature, could be mentioned about hard endpoints such as median PFS and Overall Survival (OS). Since only two patients in the MSI-H CRC and one in the non-CRC cohorts died, the median OS was not reached. Similar scenario is relevant for the PFS estimates, some of which are statistically possible to be calculated but clearly immature considering the relative high proportion of censored observations. For MSS patients the median PFS and OS were poor (two and five months, respectively) and such patients should be thought of as only receiving palliative care. Authors claimed that the conclusions of the clinical study were validated by adjusting some variables and performing multivariate analyses. However, once again, given the few number of events in each cohort this should be considered with caution.

Interestingly, all six patients with sporadic MSI cancers had an objective response compared to only one-third of the 11 patients with Lynch syndrome. New clinical trials comparing germline and sporadic MSI-H tumors will be needed to confirm this observation. Meanwhile, we could hypothesize that the presence of an MMR defect since birth could prime the immune system to a “more tolerant” state.

Since the fundamental hypothesis of the authors was that MSI-H status results in hypermutated, immunogenic tumors with increased responsiveness to immune checkpoint inhibitors, probably one of the most interesting aspects of this clinical trial was in reference to the biomarkers and correlative analyses. The density of CD8+ lymphocytes in MSI-H tumors was almost double than in MMR proficient tumors, and PD-L1 was expressed in tumor-associated lymphocytes and macrophages only in MSI-H tumors. However, no clear association with clinical outcomes was evident. On the other hand, researchers compared the number of somatic mutations according to mismatch repair status. As it might have been anticipated based on what was described in the previous section of this review, the mean number of somatic mutations in the nine patients with MSI-H was almost 25 times higher than that observed in the six mismatch repair proficient tumors (1,782 *vs.* 73; P = 0.007). Further analysis of these mutations was done using a prediction algorithm for potential MHC class I binding peptides based on the individual HLA haplotype. The mean number of these mutations associated with neoantigens was again 27 times higher in the MMR deficient compared with MMR proficient tumors. Lastly, a drop in the Carcinoembryonic Antigen (CEA) levels seemed to precede the radiographic response of immune checkpoint inhibitors. This may become a useful and promising tool for evaluating the benefit of treatment early in its course.

FUTURE DIRECTIONS

In spite of the fact that the previously described phase II clinical trial findings will have to be validated in larger studies, this trial reveals an important therapeutic tool for MSI-H metastatic CRC and sheds some light over the possible immune escape mechanisms of the large majority of MSS CRC. Hereafter, it would be expected that much of the incoming preclinical research on this topic would be focused on expanding the applicability of immune therapy in all CRC.

The virtual lack of response of MSS colorectal tumors indeed contrasts with the fact that much of the pioneer work relating the inflammatory microenvironment with oncogenesis was done in colorectal cancer models. The importance of immune infiltrates and their prognostic value discussed earlier was evaluated in colorectal cancer cohorts not stratified by MMR status [18, 20]. Thus, the explanation for the poor response rate of MSS CRC remains speculative. In a recent work by Angelova *et al*., the use of large scale cancer genomic data helped to identify different immune

escape mechanisms between MSI-H and MSS colorectal cancer [27]. In the hypermutated group, there was a relative depletion of immuno-inhibitory cells such as regulatory T lymphocytes (T_{regs}) and myeloid-derived suppressor cells (MDSCs), with upregulation of inhibitory molecules like CTLA-4 and IDO1. MSI-H tumors were specially enriched in PD1 and PD-L1. As for MSS tumors, there was a down-regulation of inhibitory molecules and MHC proteins, with enrichment of T_{regs} and MDSCs. Different mechanisms of T_{regs} accumulation have been proposed [28]. Conversion from T CD4+ cells into T_{regs} in response to various signals, such as TGF-β; recruitment of T_{reg} cells through specific chemokines; and secretion of VEGF-A resulting in inhibition of dendritic cell maturation have all been implied.

The prognostic value of infiltrating T_{reg} cells is controversial. In a retrospective analysis, high FoxP3+/CD4+ and FoxP3+/CD8+ ratios were independent predictors of shorter survival [29]. In contrast, Salama *et al*. found that high densities of T_{reg} cells in tumor tissue was associated with better survival, whereas infiltration of FoxP3+ cells in normal mucosa had a worse prognosis [30].

The expression of MHC class I molecules has been proposed as a prognostic indicator in colorectal cancer [31]. Specifically, the down-regulation of MHC-I molecules by tumor cells was associated with worse prognosis reflecting a potential immune escape mechanism, whereas the absence of expression did not confer any survival difference probably because of the elimination of MHC absent cells by natural killer cells. In a Spanish study by Coca *et al*., CRC patients with low to moderate NK cell infiltration in their tumors had worse survival than those with high degree of infiltration ($P<0.01$) [32]. These observations show differential mechanisms of immune escape between genetic subtypes of CRC, and could partially explain the inefficacy of checkpoint inhibitors in MSS tumors. In addition, the relative importance of the colonic microbiota has to be clarified. It plays a fundamental role in intestine immune surveillance and in the production of pro-inflammatory environments highly associated with western dietary and cultural habits.

Moreover, in BRAF-mutated MSI-H CRC, results from a recent phase I/II trial of dabrafenib, trametinib and panitumumab showed ORR of 26%, with a mean PFS of 4.1 months [33]. These results, combined with the toxicity of EGFR/BRAF/MEK inhibitor combinations, make pembrolizumab a promising new therapeutic option. Other advances in the field of colorectal cancer immunotherapy are being orchestrated through various means. From new checkpoint inhibitors to adoptive cell therapy, there is a growing awareness about immune evasion mechanisms gathered from malignant transformation, and promising research is being carried out.

Adoptive T-cell transfer refers to the infusion of specific anti-tumor T cells to the oncological patient. These cells are identified and expanded *ex vivo*, and can elicit strong specific responses to tumors. This approach has been partially successful in melanoma and renal cell cancer; but its role in CRC remains in a preclinical stage. An Italian group has recently presented a method to select specific antitumor T cells by isolating tumor dendritic cells loaded with neo-antigens and selecting specific reactive lymphocytes [34]. Burga *et al*. recently reported results from a phase I trial investigating a Chimeric Antigen Receptor-modified (CAR) T cells for CEA positive liver metastasis from CRC [35]. In that report, the intra-arterial infusion of CAR T cells was safe and associated with declining CEA levels and necrosis of liver metastasis.

Cancer vaccines have been designed using Tumor Associated Antigens (TAA), often derived from embryonal peptides that are expressed by different types of cancer cells, and using tumor specific antigens, also known as neo-antigens, derived from mutations in coding sequences of the genome. A German group from the Heidelberg University Hospital reported the preliminary results from a phase I/II trial evaluating vaccination of MSI-H CRC patients with frameshift peptide (FSP) antigens. The FSP vaccine (Micoryx) was generated using three coding MSI-FSP antigens shared by the majority of colorectal cancers. In a preliminary report, the vaccine successfully induced cytotoxic and humoral responses in all 22 patients [36].

Viral vector vaccines are directed to enhance antigen presentation of tumor epitopes. TAA are most commonly used in these vaccines because they are shared by a large number of different cancer patients. Gabitzsch *et al*. recently reported a phase I/II trial of the agent ETBX-011, a recombinant adenovirus serotype 5, carrying the tumor associated antigen CEA in heavily pretreated patients with metastatic CRC [37]. The median OS was 11 months, and at 29 months of follow up, over 20% of the population remained alive. Tecemotide, an active MUC-1 specific immunotherapy, is currently being evaluated as adjuvant treatment for patients with MUC-1 positive metastatic CRC with R0/R1 resected liver metastasis. MUC-1 expression is present in nearly 88% of colorectal tumors.

Dendritic cell vaccination offers several advantages over peptide or vector vaccination. These cells in their immature state are proficient in capturing and processing antigens. When mature, they express high levels of MHC I/II complexes, costimulatory molecules and IL-12, which makes them a strong stimulus to naïve T cells. Rodriguez *et al*. are conducting a phase II study evaluating the effect of dendritic cell immunotherapy loaded with self-tumor antigens in completely resected CRC patients with liver metastasis after adjuvant chemotherapy [38].

CONCLUSION

It is evident that the use of immunotherapy to treat CRC represents a major clinical accomplishment. It is expected that as our understanding of the different mechanisms of immune-resistance grows, this therapeutic modality might soon be applicable to all patients with CRC. In that situation, we would be seeing just the tip of the iceberg of a new and exciting era in the treatment of CRC. However, some other aspects should not be ignored. On a pharmaco-economic analysis, with the doses used in the trial discussed in this review (10 mg/kg) an average-weight American would face a yearly cost of around 1 million dollars. This is almost 5 times higher than the dose used in many other pembrolizumab

trials. Being a frequent cause of cancer deaths, its use only in MSI-H tumors would still mean an almost impossible financial burden for most of the world's health systems. Scientifically exciting; financially concerning.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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