

Biomarkers and Immunotherapy for Colorectal Cancer

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Abstract. Colorectal cancer (CRC) is the second major cause of mortality from cancer globally. Most CRCs are sporadic and may be classified into three main genetic pathways: the chromosomal instability (CIN) pathway, the microsatellite instability (MSI) pathway and the CpG island methylator phenotype (CIMP) pathway, which are associated with genetic mutations or epigenetic alterations and have the possibility to intersect, thus making the treatment of CRC challenging. Immunotherapy has offered some promising insights by inducing antitumor immune responses, but its effectiveness is restricted to certain groups of CRC patients with specific characteristics. Several biomarkers have demonstrated their potentials to predict the outcomes of immunotherapy in individual patients. Some of them include the extent of tumor mutations (MMR/MSI, POLE/POLD1, KRAS), PDL-1 expression, pre-existing immunity and gut microbial compositions. Immune checkpoint inhibitors (ICIs)-based immunotherapy is considered to be the relatively traditional immunotherapeutic strategy in the treatment of CRC. However, it mainly targets CRCs with defective mismatch repair (dMMR) mechanisms. The more recently developed immunotherapies include cancer vaccines (molecular-based, cell-based and vector-based vaccines) and adoptive cell therapy (ACT), which have the potential to further enhance the stimulation of antitumor immune responses. This review summarizes the predictive biomarkers that have the potential application in CRC treatment, and discusses the immunotherapeutic strategies targeting CRCs that have been developed or are currently under investigation.

Keywords: Colorectal cancer, Biomarker, Immunotherapy.

1. Introduction

Colorectal cancer (CRC) has an exceptionally high prevalence amongst cancers and is responsible for the second highest number of cancer-related deaths globally [1]. Its occurrence is also becoming more frequent amongst the young population [2]. CRC develops as a result of the gradual build-up of genetic mutations and epigenetic modifications that causes the normal epithelial cells of the colonic mucosa to become malignant cancerous cells. The incidence of CRC is sporadic instead of due to familial inheritance in approximately 75% of CRC patients. There are three major genetic pathways associated with sporadic CRCs. Around 70% of sporadic CRCs develop along the chromosomal instability (CIN) pathway, which involves alterations in the chromosome number or structure, and accumulations of mutations in tumor suppressor genes and oncogenes such as the Kristan RAS (KRAS) gene. Around 15% of sporadic CRCs develop along the microsatellite instability (MSI) pathway, which involves variations in the DNA mismatch repair (MMR) genes. The other pathway is the CpG island methylator phenotype (CIMP) pathway, which involves gene knockdown by CpG island methylation. These pathways are compatible and can interact with one another [3]. Immunotherapy has demonstrated some effectiveness in the treatment of CRCs. However, its efficacies are only limited to certain groups of CRC patients [2].

Cancer immunotherapy manipulates the immune system by utilizing immunologic agents that can stimulate anticancer immunity by enhancing the activation of effector cells and creating an inflammatory tumor microenvironment [4]. This review not only summarizes the biomarkers that can potentially be used to anticipate the adequacy of immunotherapy in different groups of CRC patients, but also discusses the current immunotherapies approved for CRC treatment and the recent progresses in the development of novel immunotherapeutic strategies.

2. Immunotherapy efficacy predictors (biomarkers)

Biomarkers are medical signs that can be used to predict a patient's responses towards immunotherapeutic treatments [5]. Four main types of biomarkers have been identified for CRC immunotherapies: tumor mutations, PDL-1 expression, pre-existing immunity and the gut microbiota [6].

2.1. Tumor mutations

2.1.1. MMR/MSI

MMR is an important mechanism that ensures DNA integrity [7]. CRCs can be either MMR-deficient (dMMR) or MMR-proficient (pMMR), depending on the amount of MMR proteins present in the patient. The MMR status determines the MSI status, with dMMR patients having microsatellite instability at high levels (dMMR-MSI-H) due to indels, and pMMR patients having microsatellite instability at low levels (pMMR-MSI-L) or being microsatellite stable (pMMR-MSS). The quantity of somatic mutations in a tumor genome's coding regions is known as tumor mutational burden (TMB). High TMB is often indicative of better responses towards cancer immunotherapies [6]. Since dMMR-MSI-H tumors have high TMB, they produce more neoantigens, which are mutated proteins produced by tumor cells that are not found in normal cells [8]. Upon T cells' recognition of the neoantigens presented by MHC class I molecules, antitumor immune responses would be induced. In particular, upon recognition of the neoantigens, CD8+ tumor infiltrating lymphocytes (TILs), T helper 1 (TH1) CD4+ TILs and macrophages infiltrate the tumor microenvironment and induce the release of type I interferons (IFN γ) (Fig. 1a).

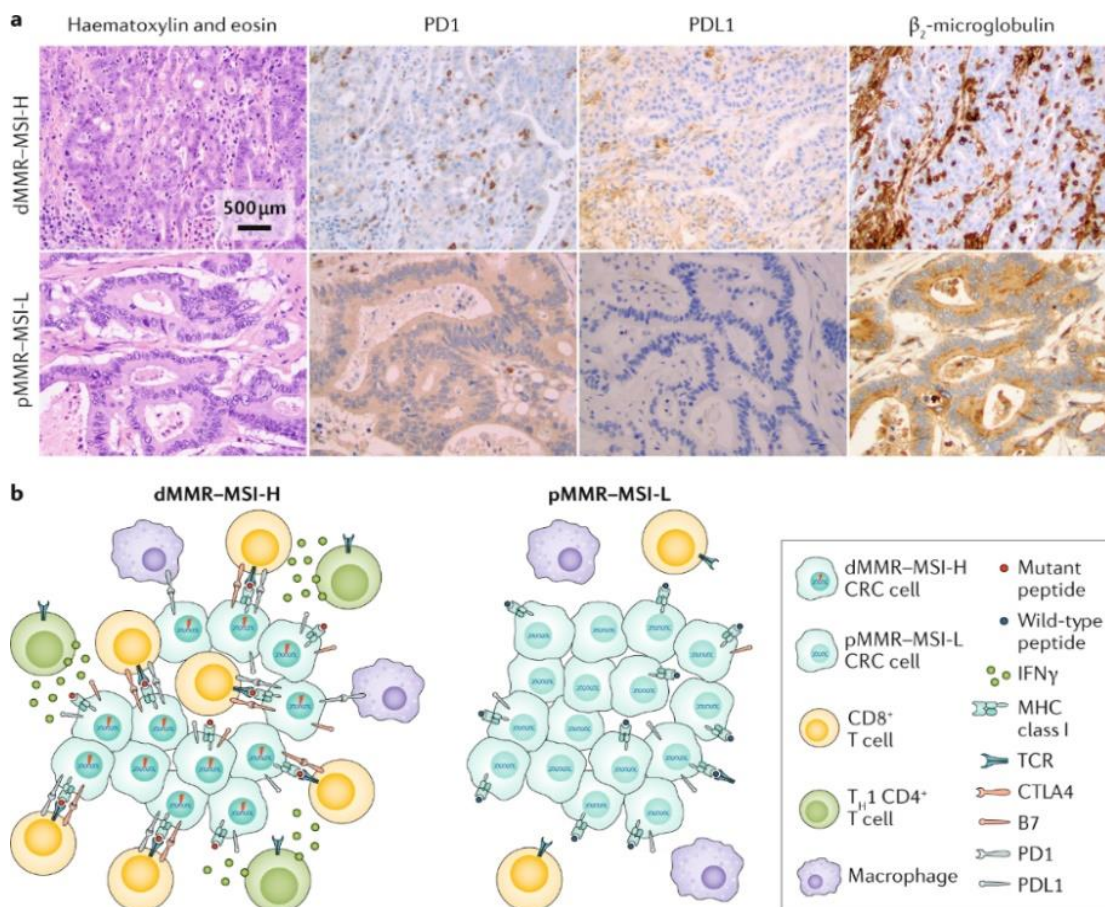


Figure 1. MMR/MSI biomarkers in colorectal cancers (CRCs)

- (a) Haematoxylin and eosin (HE) staining of pMMR-MSI-L tumor and dMMR-MSI-H tumor.
 (b) Antitumor immune responses in dMMR-MSI-H tumors and pMMR-MSI-L tumors [9].

To counteract this effect, the dMMR-MSI-H tumors express more programmed cell death 1 ligand 1 (PDL-1) on their cell surface, which can interact with the programmed cell death 1 (PD-1) proteins on T cells, to evade from immune surveillance (Fig. 1b) [6, 9]. Therefore, the MMR/MSI status is a prognostic biomarker that can prognosticate the efficacies of immune checkpoint inhibitors (ICIs)-based treatment, with the efficacies generally being higher in individuals with dMMR-MSI-H CRC (Fig. 2) [6, 10]. However, its accuracies as a biomarker may vary depending on the stage of CRC [6].

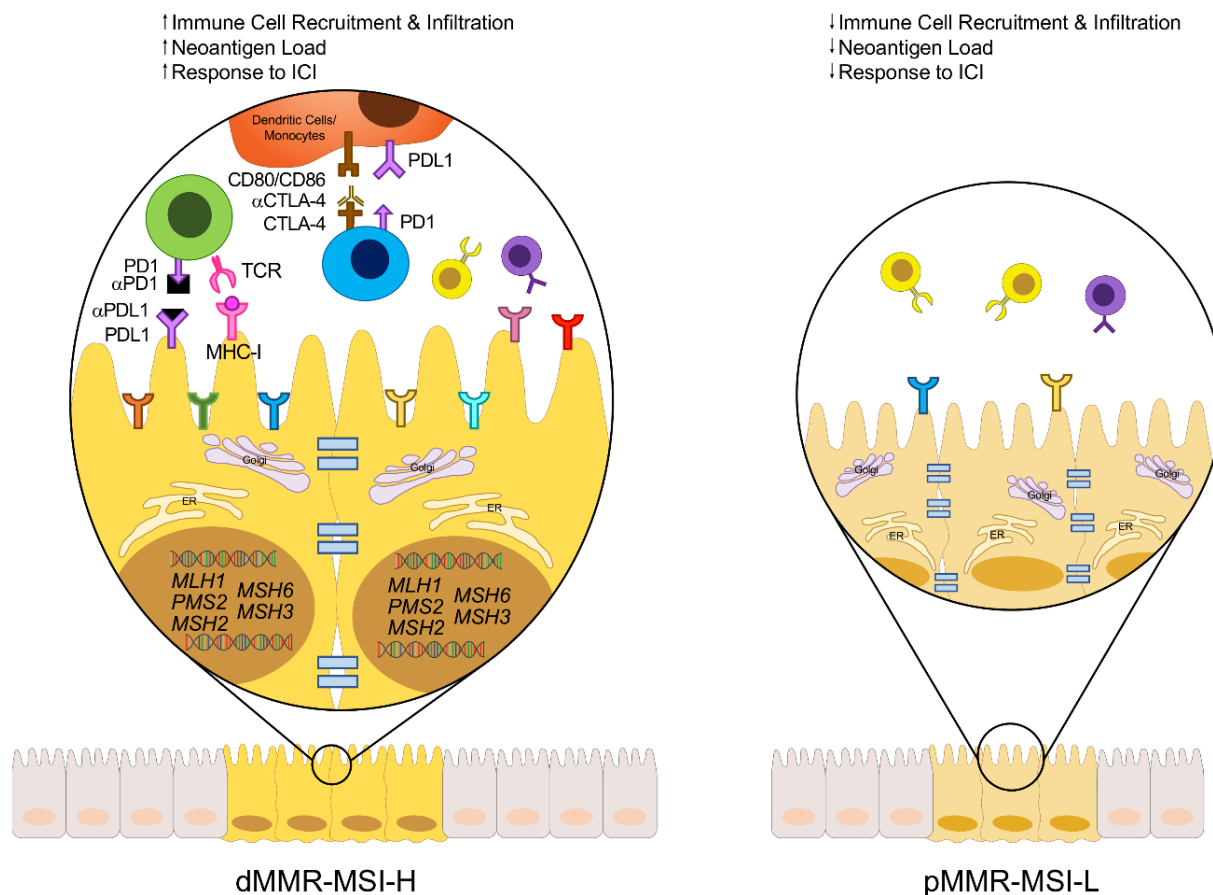


Figure 2. Effects of ICI in dMMR-MSI-H CRC tumors and pMMR-MSI-L tumors [10]

2.1.2. POLE/POLD1

POLE and POLD1 code for the proofreading exonuclease domains of DNA polymerases that ensure the accuracy of DNA replication. Both somatic and germline mutations in POLE and POLD1 have been observed in CRC tumors [11]. An estimate of 7.37% of CRC patients have POLE/POLD1 mutations, and the majority of them are MSS or have MSI-L [6]. Interestingly, tumor cells with POLE mutations have an upregulated expression of immunosuppressive checkpoints such as PDL-1 and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which are effective therapeutic targets for ICIs [11]. Thus, POLE/POLD1 mutations are potential biomarkers for non-MSI-H CRC patients receiving ICIs-based treatments.

2.1.3. KRAS

Kristen RAS (KRAS) is a proto-oncogene that is responsible for promoting normal cell division and proliferation. Point mutations in KRAS activate its encoded protein and turn it into an oncogene, which lead to cancer growth. Such mutated KRAS is observed in around 40% of CRC patients [12]. The activating mutations in KRAS render immunotherapies using monoclonal antibodies to block the epidermal growth factor receptors (EGFRs) ineffective [13]. EGFR is a type of tyrosine kinase receptor that takes part in controlling cell proliferation. An overexpression of EGFR often results in cancer [14]. Therefore, the presence of a KRAS mutation can be used to predict the effectiveness of anti-EGFR treatments.

2.2. PDL-1 expression

Since many CRC tumors overexpress PDL-1, especially MSI-H tumor cells, the idea of adopting ICIs-based immunotherapies to target PD1/PDL-1 is promising [15]. The expression level of PDL-1 in CRC tumors can thus be used as a prognostic tool to predict the benefits of ICIs-based immunotherapies for individual patients.

2.3. Pre-existing immunity

The immunoscore ranges from low to high depending on the amount of CD3+ and CD8+ T lymphocytes found at both the tumor center (TC) and the invasive margin (IM) [6]. Based on the immunoscore system, CRC tumors can be categorized into hot, altered, and cold tumors, with each of them corresponding to a high, intermediate, and low immunoscore respectively. As hot tumors are highly infiltrated by T cells, ICIs-based immunotherapy is a potential effective treatment by targeting CTLA-4 and PD-1 displayed by T cells [16]. Hence, a pre-existing immunity might be indicative of a more effective ICIs-based immunotherapy.

2.4. Gut microbiota

The gut microbiome is critical in the regulation of immunity. Certain species of bacteria (*Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium*) found in the gut microbiome enhance ICIs-based immunotherapy, while some other species (*Ruminococcus obeum* and *Roseburia intestinalis*) inhibit its effect [17]. The gut microbial composition would thus be a potential predictive indicator of the effectiveness of immunotherapy.

3. Immunotherapy for CRC

3.1. ICIs-based immunotherapy

The principle behind ICIs-based immunotherapy is to block the interaction between T cells and their negative regulators, leading to T cell activation (Fig. 3). ICIs-based immunotherapy has been proved to be beneficial for treating dMMR-MSI-H CRCs due to their high level of expression of checkpoint proteins such as PDL-1 [9].

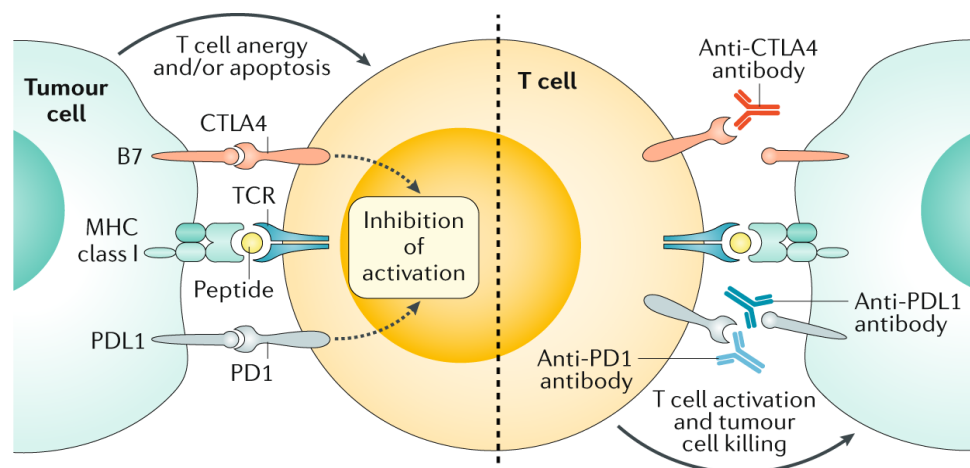


Figure 3. T cells activated by immune checkpoint inhibitors to block B7/CTLA4 and PD1/PDL-1 interactions for antitumor cytotoxicity [9]

Combinations of anti-PD-1/PDL-1 and anti-CTLA-4 antibodies have demonstrated efficacy in the treatment of CRCs. [6]. Durvalumab (PD-L1 inhibitor) and tremelimumab (CTLA-4 inhibitor) were coupled in CCTG CO.26, a phase II clinical study, with remarkable efficiency in treating patients with advanced refractory CRC (rCRC). In comparison to patients who only received best supportive care, those who were treated with combined immune checkpoint inhibitors had a longer overall survival (OS) of 2.5 months [18].

An upregulated expression of PDL-1 and CTLA-4 can lead to resistance against panitumumab treatment, which is a monoclonal antibody (mAb) that targets the EGFR to treat metastatic CRCs (mCRCs) without KRAS/NRAS/BRAF mutations. The phase II clinical trial LCCC1632 (NCT03442569) has shown that by combining nivolumab and ipilimumab with panitumumab, the resistance against anti-EGFR therapy among the MSS CRC patients was significantly reduced [6].

On the other hand, pMMR-MSI-L CRCs are mostly resistant against ICIs-based immunotherapy. This is mainly because of their low TMB which results in low antigenicity, making the recruitment of immune cells and T cell recognition difficult [9, 10]. ICIs-based immunotherapy alone would thus be insufficient to treat this CRC subtype. Combining ICIs with other therapies, such as chemotherapy and anti-angiogenic agents, might be beneficial to some pMMR-MSI-L CRC patients [6,9].

3.2. Cancer vaccines

There are mainly three types of therapeutic vaccines for CRC that are in development: molecular-based vaccines, cell-based vaccines and vector-based vaccines (Fig. 4) [19].

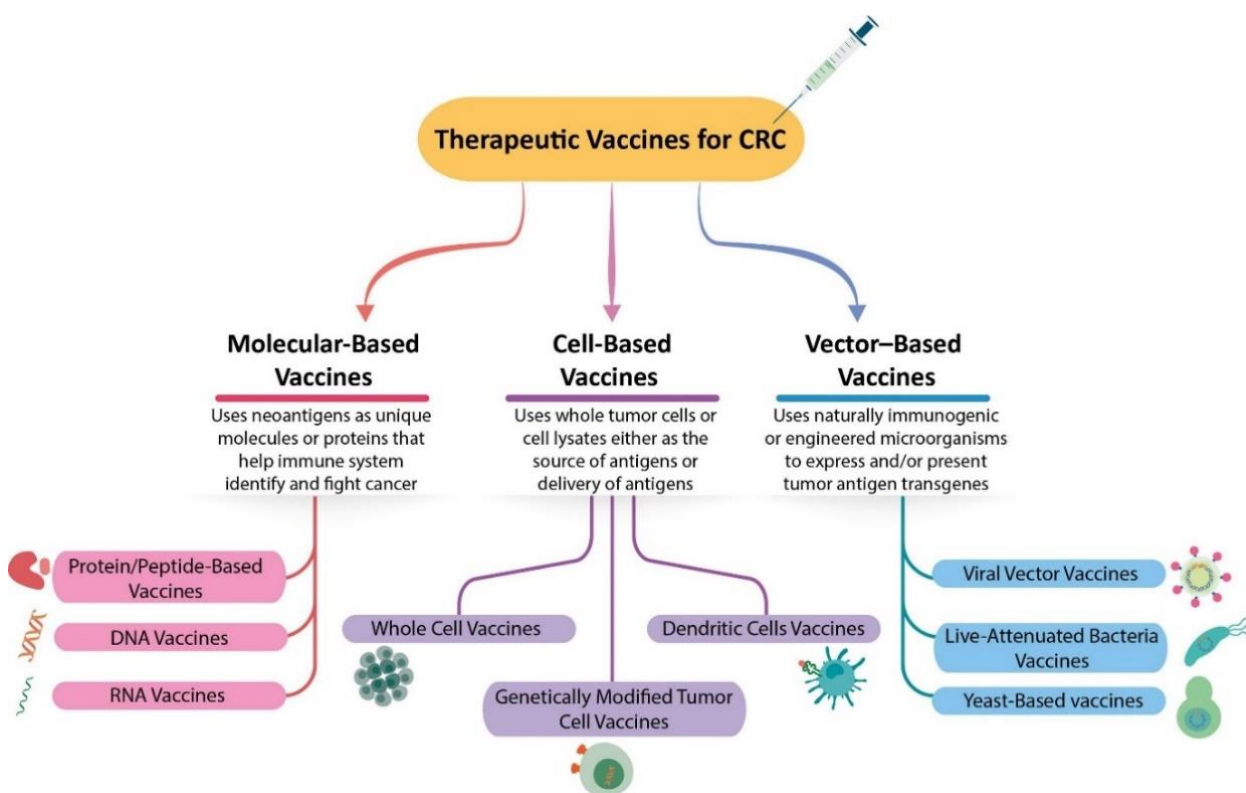


Figure 4. Types of therapeutic vaccines for CRC in clinical trials [19].

3.2.1. Molecular-based vaccines

Molecular-based vaccines for CRCs consist of full-length proteins, subunit peptide, DNA and RNA vaccines (Fig. 5) [19].

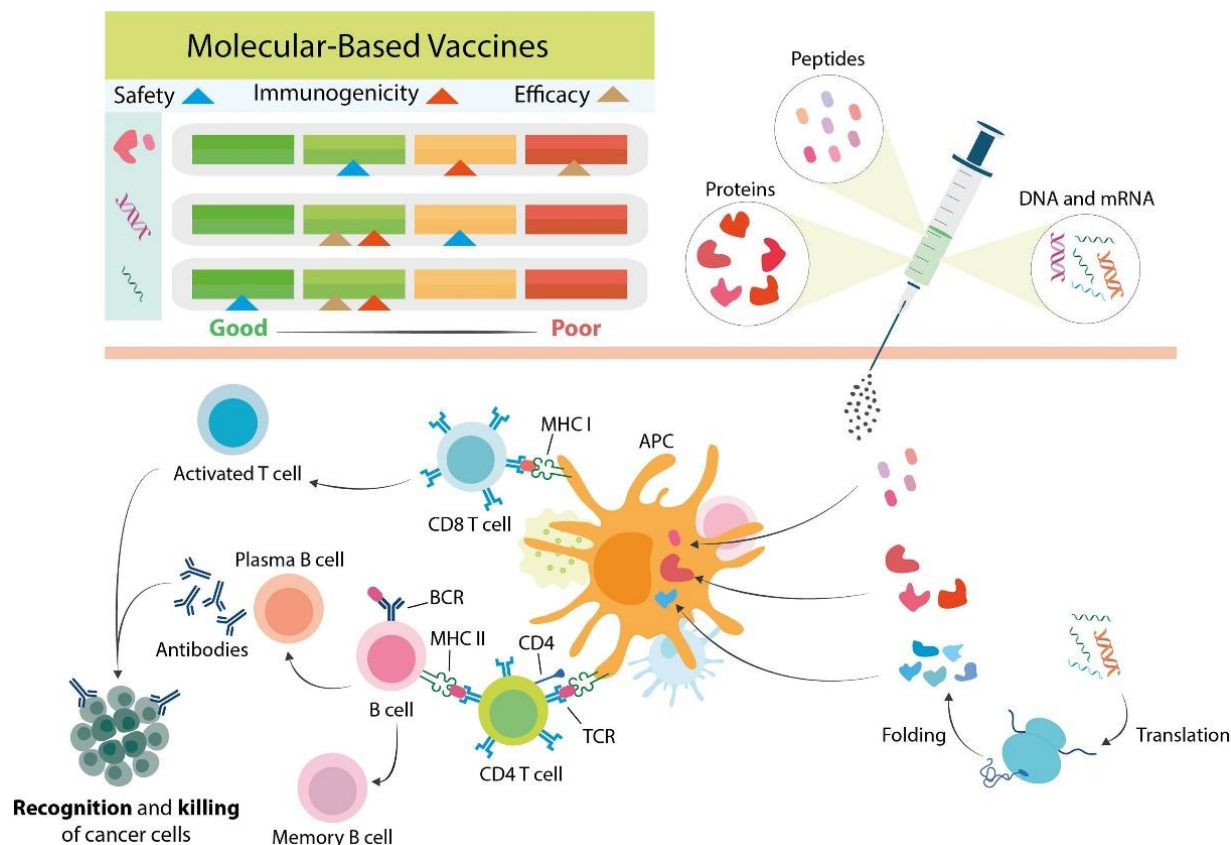


Figure 5. Summary of the degrees of safety, immunogenicity and effectiveness of protein/peptide-based, DNA and mRNA CRC vaccines [19].

1) Protein/peptide-based vaccines

The proteins or peptides used to design cancer vaccines contain antigens that are immunogenic and can be displayed by major histocompatibility complex (MHC) class I/II to induce T cell responses. This mainly includes tumor-associated antigens (TAA) and tumor-specific antigens (TSA). Protein/peptide-based vaccines are generally accompanied by adjuvants to enhance the stimulation of antitumor immune responses [19].

Protein/peptide-based vaccines are cheap to produce and store, and can induce specific immunogenicity against tumors. However, these vaccines have limited efficacies in treating CRCs. This is mainly due to their failure to induce high levels of antitumor immunity, the immune evasion of the tumor cells and the ability of the tumors to inhibit certain immune responses [19]. As a result, single-peptide vaccines usually fail to induce sufficient immunogenicity against tumors. Peptide-vaccines containing multiple epitopes are a potential solution to this problem [19, 20]. Cep55/c10orf3 derived peptide vaccine has shown improved feasibility to treat CRCs in comparison to single-peptide vaccines [20].

2) DNA vaccines

DNA vaccines involve the insertion of one or more genes encoding the tumor antigens into bacterial plasmids, which will then be introduced into the body. The antigens can be displayed by MHC class I/II when expressed, thereby stimulating adaptive immunity. The DNA structure can also be recognized by cytosolic DNA sensors, activating innate immunity [19, 21]. The overexpressed oncoprotein MYB is critical for CRC tumor progression, and MYB-based DNA vaccines have demonstrated considerable beneficial outcomes in CRCs [19]. However, there is a possibility of recombinant plasmid integration into the host genome, which may lead to insertional mutagenesis [21].

3) mRNA vaccine

mRNA vaccines involve synthesizing antigen-encoding mRNAs in vitro, which will then undergo translation within the cytoplasm of target cells to produce tumor antigens. Antigen-presenting cells (APCs) will display the tumor antigens in order to elicit potent T cell responses. Since mRNA vaccines

do not entail integration into the host genome like DNA vaccines do, they have less uncertainties than DNA vaccines. Moreover, mRNA vaccines are more effective, easier to modify and need less time for production [19, 21]. mRNA 5671 is a mRNA vaccine that has been developed by Moderna Therapeutics to treat cancers with KRAS mutations, including CRCs. mRNA encoding the four most common mutated peptides (G12D, G12V, G13D and G12C) from KRAS mutations were used, and the vaccine induced strong CD8+ T cell responses [19].

3.2.2. Cell-based vaccines

Cell-based vaccines for CRCs consist of autologous and allogeneic cancer cell vaccines, and dendritic cell (DC) vaccines (Fig. 6) [19].

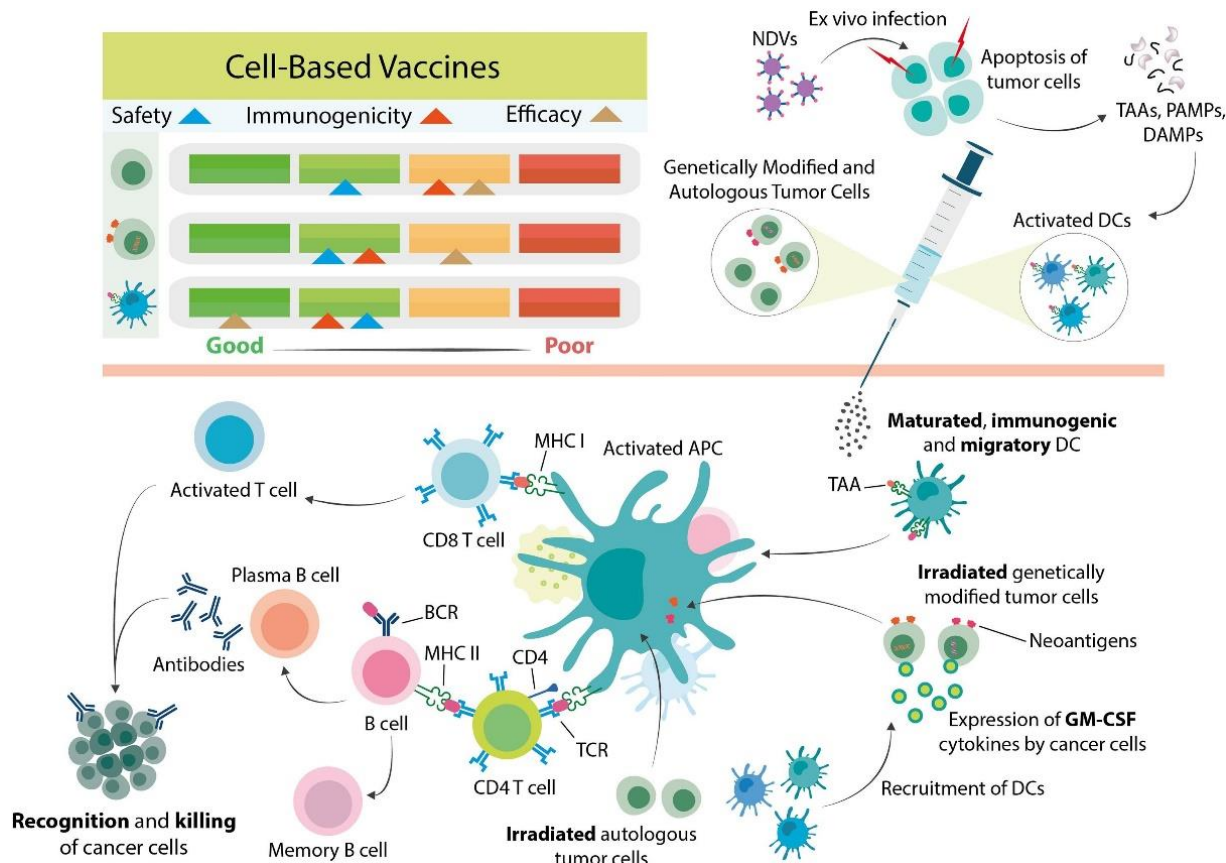


Figure 6. Summary of the degrees of safety, immunogenicity and effectiveness of autologous cancer cell, tumor cell with genetic modifications and activated dendritic cell (DC) vaccines [19]

1) Autologous cancer cell vaccines

Cancer cell vaccines can be created with either whole cancer cells or cancer cell lysates [21]. Autologous cancer cell vaccines use tumor cells derived from individual cancer patients, and hence comprise the full repertoire of tumor antigens specific to each patient. As compared to molecular-based vaccines, the inclusion of unknown TAAs reduces the probability of tumor immune escape [19, 21]. However, since tumor cells also express antigens that are found in normal cells, there is a possibility of inducing autoimmune responses [19]. Nonetheless, several autologous cancer cell vaccines have shown effectiveness in treating CRCs in clinical trials. Treatment of Stage II CRCs using tumor cells and bacillus Calmette-Guerin (BCG) demonstrated notable success [22].

2) Allogeneic cancer cell vaccines

The major difference between allogeneic and autologous cancer cell vaccines is that allogeneic cancer cell vaccines use genetically modified tumor cell lines that are grown in laboratories instead of from individual patients [23]. Although this type of vaccines is easier to produce, it does not contain patient-specific tumor antigens which may pose a limit to its effectiveness. Nevertheless, there have been indications that the allogeneic whole-cell vaccine GVAX, which was studied in a phase II clinical

trial for pMMR advanced CRCs, may have an impact on the regulation of antitumor immune responses [21].

3) DC vaccines

DCs are APCs that connect the innate and adaptive immune responses [19, 21]. The main principle behind most DC vaccines is to inject immature DCs that can present tumor antigens in vivo. It is also possible to load DCs from individual patients with anti-TAAs or mRNAs ex vivo before injecting DCs back into the host [19]. A recent progress in DC vaccines made use of a polymersomal nano-formulation (CCPS/HPPH/DOX) to recruit mature DCs and TAAs that can serve as self-adjuvants in situ, and this vaccine was capable of stimulating CD8+ T cell responses to inhibit MC38 CRC tumor progression [24]. Nevertheless, further research is necessary to improve and validate the efficacies of DC vaccines in CRC treatment [19, 21].

3.2.3. Vector-based vaccines

Vector-based vaccines comprise of viral vector vaccines, live-attenuated bacteria vaccines and yeast-based vaccines (Fig. 7) [19].

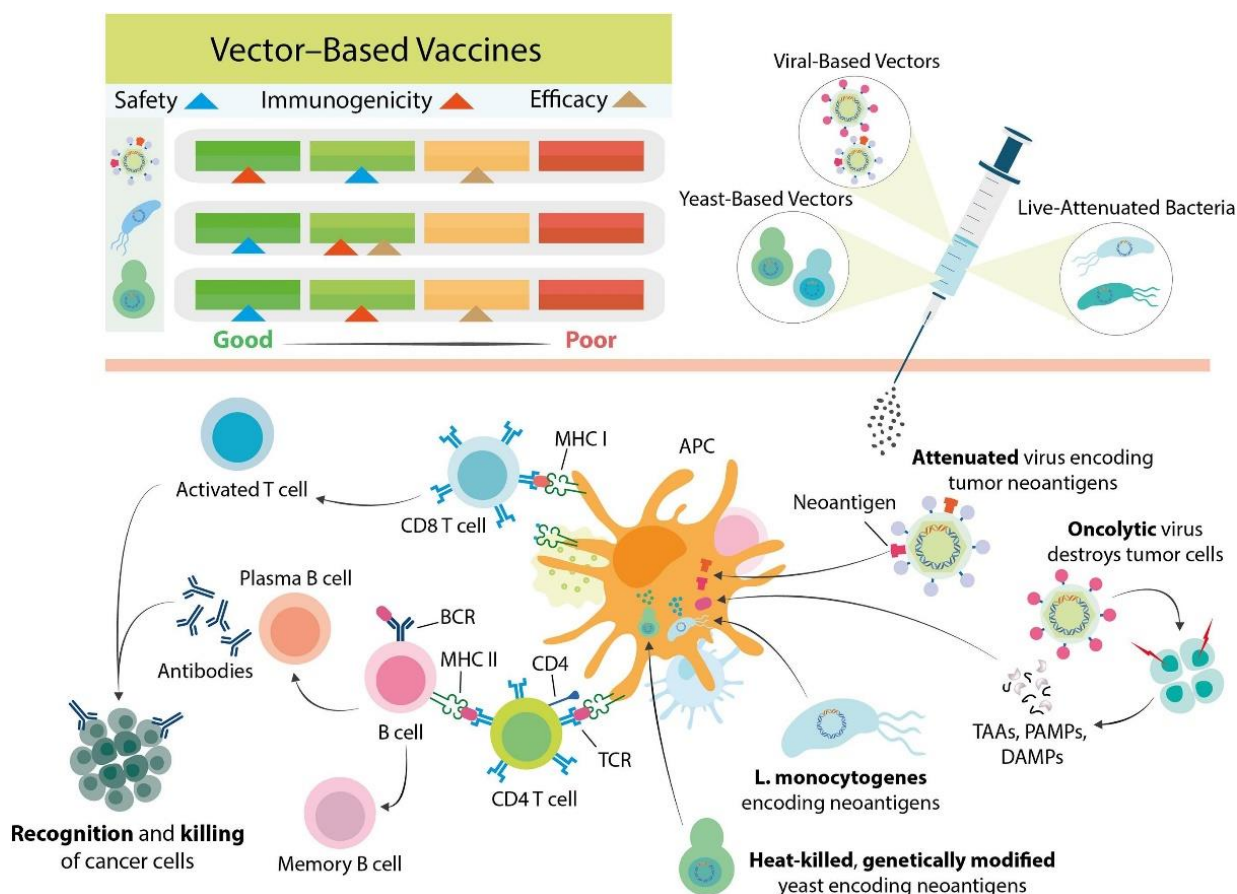


Figure 7. Summary of the degrees of safety, immunogenicity and effectiveness of viral vector, live-attenuated bacteria and yeast-based vaccines [19].

1) Viral vector vaccines

Viral vector vaccines involve genetically engineered recombinant viruses that express tumor antigens to induce immunogenicity. The pathogen-associated molecular patterns (PAMPs) found on the viral surfaces can also be recognised by pattern recognition receptors (PRRs) to enhance APC activation [19]. Common types of viruses that are being used to generate cancer vaccines include adenoviruses, retroviruses, lentiviruses and poxviruses. PANVAC is a viral vector vaccine that uses poxvirus as the platform to express carcinoembryonic antigen (CEA) and mucin-1 (MUC-1) proteins which are overexpressed in CRCs. It was assessed to be safe and able to promote T cell antitumor immunity in a pilot study [21]. However, viral vector vaccines are expensive to produce and the

probability for pathogenesis and insertional mutagenesis exists. Hence, the safety of viral vector vaccines needs to be further evaluated [19].

2) Live-attenuated bacteria vaccines

Live-attenuated bacteria can be genetically modified to express tumor neoantigens. The PAMPs on bacterial surfaces can be recognized by Toll-like receptors (TLRs) of the innate immune system to produce cytokines and recruit APCs. Live-attenuated bacteria vaccines can thus induce both innate and adaptive immune responses against tumor cells. The design of these vaccines generally depends on RNA interference mechanisms, inflammatory cytokines, anti-angiogenic agents, neoantigens and apoptosis inducers. *Salmonella typhimurium* has been used as the vector to introduce the endostatin gene, which is an anti-angiogenic agent, to suppress tumor growth in CRC models and the vaccine managed to induce antitumor immune responses. However, no live-attenuated bacterial vaccines have reached the clinical trial stage [19].

3) Yeast-based vaccines

Yeasts have also been used as a platform for the translation and/or presentation of tumor neoantigens to induce antitumor immune responses. MC38 CRC cell lysates and CpG were being loaded onto yeast-derived β -glucan particles to form a yeast-based vaccine which triggered significant antitumor immunity in murine models. The advantages of yeast-based vaccines include them being non-pathogenic, stable, easy and non-costly to manufacture, and their self-advanting nature, rendering them as a promising strategy to combat CRCs [19].

3.2.4. Nanovaccines

Nanovaccines are a rather new type of cancer vaccines that has been recently developed. They can be classified into lipid-based vaccines, polymeric vaccines, inorganic vector-based vaccines, and biologically derived vaccines (Fig. 8). BanNV is a polymeric nanovaccine that can self-assemble and contains the neoantigen Adpgk and two adjuvants. It can activate PD1 receptors, offering significant benefits to Adpgk-expressing mice models when combined with ICIs-based immunotherapy [21].

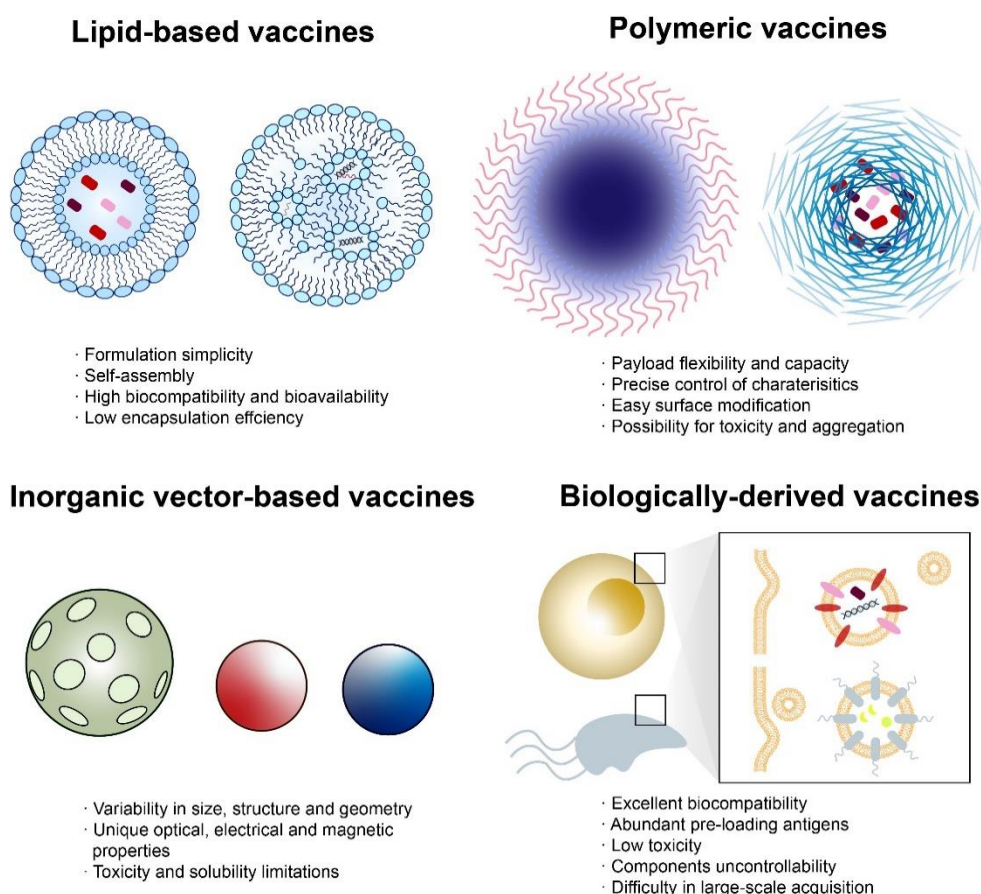


Figure 8. The characteristics of the 4 different classes of nanovaccines [21]

3.3. Adoptive Cell therapy (ACT)

ACT is a new and extremely personalized cancer treatment which involves injecting individual cancer patients with selected host immune cells with anti-tumor activities, or genetically engineered host cells with chimeric antigen receptors (CARs) or T cell receptors (TCRs) to stimulate anti-tumor immune responses [6]. There have been some successes of using selected T cells with specific TCRs to target CRCs with mutated KRAS G12D peptide [25]. Moreover, since CRC tumors express large amounts of CEA which is absent in normal cells, several clinical trials have used T cells engineered with CEA-specific CARs to treat CRCs [6]. Although the use of CARs-modified T cells did stabilize cancer progression in most patients [6], the side-effects associated with this therapy need to be further investigated [26].

4. Conclusions

With a large mutational signature and a complex TME, CRC is a disease with many facets and complexity. The treatments utilized to treat the illness are just as complicated as the illness itself. Indeed, remarkable advances has been achieved in the creation of immunotherapies for the treatment of CRCs, especially with the recent approval of pembrolizumab and nivolumab (with or without ipilimumab), which are ICIs, by FDA to treat dMMR-MSI-H CRC patients. However, difficulties remain in the invention of effective immunotherapeutic strategies targeting pMMR-MSI-L CRCs, which are most likely to be resistant against ICIs-based strategies. Combinations of ICIs-based immunotherapies and other types of therapies have been tested to enhance the stimulation of antitumor immune responses. Other types of immunotherapies, such as cancer vaccines and ACT, are also an area of robust research. In addition, biomarkers have the potential to be adopted as the criteria to identify the most suitable immunotherapy for individual CRC patients. As more individualized treatments arise, immunotherapy has a strong chance of dominating the field of CRC treatment in the near future.

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