Diagnostics and Therapeutic Advances in GI Malignancies
Series Editor: Ganji Purnachandra Nagaraju

Ramakrishna Vadde
Ganji Purnachandra Nagaraju *Editors*

Immunotherapy for Gastrointestinal Malignancies

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Diagnostics and Therapeutic Advances in GI Malignancies

This series will highlight the recent innovations in the diagnostics and therapeutic strategies for different Gastrointestinal (GI) cancers.

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This book is dedicated to our families, teachers, contributors, and friends.

Preface

Gastrointestinal malignancies are the most common cancers diagnosed worldwide, with increasing incidence and mortality rate every year. The traditional therapeutic strategies that include surgery, chemotherapy, and radiotherapy are found effective. However, the prognosis percentage always remains poor. Therefore, novel approaches for gastrointestinal malignancies therapy are essential. Immunotherapy is a novel therapeutic strategy at present and it could be a better therapeutic option for cancer.

Immunological therapy was carried out for the first time by Coley in the year 1891, against a malignant patient using a bacterial immunotoxin. Burnet in the year 1970, presented the concept of immunological surveillance and van der Bruggen et al. in the year 1991 finally reported cytolytic T lymphocytes recognizing antigen on human melanoma. This field of immunology progressed highly with the discovery of novel immune-based targets that are based on understanding the tumor microenvironment and tumor immunology. Varied types of immunomodulatory treatments including dendritic cell vaccines, IL-2 activated lymphocytes, tumor-associated antigen-derived peptides, and tumor-specific reactive $CD8^+$ T lymphocytes are demonstrated for the therapy of gastrointestinal malignancies. However, the confidence of immunotherapy is built only with the initiation of immune checkpoint inhibitors and was widely encouraged as the "Breakthrough of the year 2013" by Science. Thus, immunotherapy is the current mainstream for gastrointestinal malignancies.

This book focuses on the novel immunotherapeutic strategies including immune checkpoint inhibitors, peptide vaccines, and adoptive cell transfer therapy against gastrointestinal malignancies including esophageal cancer, gastric cancer, and colorectal cancer. The authors in the book summarized types and drivers for heterogeneity in respect to their biological and clinical importance with respect to tumor evolution. This knowledge will allow to understand heterogeneity in determining the cause for cancer patients not responding to the therapy. The book also clarifies about the cytokines involved in gastrointestinal malignancy therapy and application with the review of meta-analysis to determine the association between gene

polymorphism and predicting the risk for the cause of cancer. It will also focus on the immunomarkers that play a crucial role in predicting the malignant behavior of the cancer cells and help clinicians for early diagnosis and employing them as therapeutic targets for therapy of gastrointestinal malignancies. Lastly, the book explores the diverse facts of computational biology for the diagnosis and therapy of gastrointestinal malignancies. Finally, it explores how these novel advances integrate into a precision and personalized medicine approach that eventually enhances patient care.

It is our pleasure to present this comprehensive summary of novel fields to the science community for a better understanding of the future advances in the field of immunotherapeutic application toward gastrointestinal malignancies. We hope this book reflects the novel research ideas for better innovation and ultimate benefit to patients and their families.

Kadapa, Andhra Pradesh, India — Ramakrishna Vadde
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Chapter 1
Tumor Heterogeneity: Challenges and Perspectives for Gastrointestinal Cancer Therapy

Manoj Kumar Gupta, Gayatri Gouda, Ravindra Donde, and Ramakrishna Vadde

Abstract Cancer is clinically characterized via the uncontrolled proliferation of cells. Several studies have reported that tumor heterogeneity is the main reason for the low treatment response rate in cancer patients. Thus, there is always a quest to understand the tumor heterogeneity in any cancer type. In this chapter, the authors attempted to understand the types and drivers for tumor heterogeneity, especially in gastrointestinal cancers, and discussed their biological as well as clinical importance with respect to tumor evolution. Obtained information revealed that tumor heterogeneity can be either at inter- (amongst diverse tumors from diverse patients or within the same patients) or intra- (amongst diverse cells in the same tumor) level. Nevertheless, the main reason for inter-tumor heterogeneity is the intra-tumor heterogeneity. To understand this heterogeneity various high throughput sequencing approaches, for instance, single-cell RNA sequencing, and models, for instance, the "Clonal evolution" model and "big bang" model, have been developed to date. However, the complete mechanism associated with tumor heterogeneity remains elusive to date. Authors believe that by integrating information obtained from various disciplines, including pathology, clinical-radiology, genetic and molecular biology, we can unravel the mechanism comprehensively associated with tumor heterogeneity. In the near future, the information present in this chapter will be highly useful for the early detection and prevention of gastrointestinal cancer in humans.

Keywords Cancer · Heterogeneity · Clonal evolution model · Cancer stem cell model · Tumor

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1.1 Introduction

Recent advancements in high throughput sequencing technologies have provided us an extraordinary insight into the cancer genome and its evolution (Jamal-Hanjani et al. 2015; Gupta et al. 2017, 2019a; Gupta and Vadde 2019a). Cancer is clinically characterized via the uncontrolled proliferation of cells, which is often referred to as a tumor. As cancer can develop from any cell type, there are more than 100 cancer forms, including gastrointestinal (GI) cancer (Cooper 2000). A tumor may be either benign or malignant. A benign tumor remains restrained to its site of origin and neither invades nearby normal tissue nor disperses to distal body sites. On the contrary, malignant tumor invades nearby normal tissue as well as dispersed throughout the body either via the lymphatic or via circulatory systems. While benign tumors can be removed surgically; malignant tumors are often hard for treatment due to their metastatic nature (Cooper 2000; Gupta et al. 2017, 2019b). Earlier studies have suggested that benign tumor converts into malignant form through series of events, i.e., avoidance of cell death and growth suppression related signals and angiogenesis stimulation, which in turn activates nearby tissue invasion as well as metastasis. Thus, the stochastic nature of cancer suggests that cancer initiation and formation do not follow a fixed path but rather a complex mechanism comprised of numerous critical cellular process. Even after attaining the metastatic phase, it remains active and evolves continuously. This ongoing evolution might produce heterogeneous tumors composed of distinct cancer cells with a unique molecular signature which respond distinctly with anti-cancer therapies (Dagogo-Jack and Shaw 2018). Thus, tumor heterogeneity is the main reason for the low treatment response rate in cancer patients. Additionally, the presence of specific traits, for instance, point mutations responsible for enhancing drug resistance, is high. Thus, intra-tumor heterogeneity is like an "arsenal" that shields cancer against cancer therapies. Nevertheless, the environment of this arsenal changes continuously due to the aggregation of novel mutations. Besides this, the non-cancerous cell in tumor and nearby tissue is also changing continuously, which augments diversification in the arsenal, thereby making the situation more tough for anti-cancer therapies (Janiszewska 2020). Thus, it is highly required to exploit and understand the tumor at the cellular level. Considering this, in this chapter, we summarized the types and drivers for tumor heterogeneity especially in gastrointestinal cancer and discussed their biological as well as clinical importance with respect to tumor evolution. In the near future, the information present in this chapter will be highly useful for the early detection and prevention of gastrointestinal cancer in humans.

1.2 Heterogeneity Type

Heterogeneity is a Greek word, which means "different kinds," thereby representing a dissimilar mass composition instead of a uniform mass. Tumor heterogeneity may be either inter-tumor (amongst diverse tumors from diverse patients or within the same patients) or intra-tumor (amongst diverse cells in the same tumor) (Liu et al. 2018; Lin and Lin 2019).

1.2.1 Inter-Tumor Heterogeneity

Inter-tumor heterogeneity refers to the alteration in the phenotype as well as genotype within different or same cancer patients that are induced via various environmental as well as etiological factors (Liu et al. 2018). In 2003, Ribic and the team suggested that fluorouracil-based adjuvant chemotherapy enables early detection of stage II and stage III colon carcinoma with microsatellite-stable tumors and tumors displaying low-frequency microsatellite instability, respectively. However, it fails to detect tumors displaying high-frequency microsatellite instability (Ribic et al. 2003). This was plausibly the first inter-tumor heterogeneity that was clearly applicable to clinicians in practice (Liu et al. 2018). Since then, cancer has been classified into numerous subtypes using different markers, for instance, in lung cancer (e.g., ALK fusion+ and EGFR+), gastric cancer (e.g., microsatellite unstable and Epstein–Barr virus+), and breast cancer (e.g., basal-like, luminal, and Her2+). Irrespective of all significant findings, detailed insight into inter-tumoral heterogeneity in several cancer forms remains elusive to date (Liu et al. 2018).

Recently, in 2017, The Cancer Genome Atlas (TCGA) consortium categorized 90 esophageal squamous cell carcinoma (ESCC) specimens into three subgroups, namely, ESCC1–3 (Cancer Genome Atlas Research Network et al. 2017). ESCC1 and ESCC3 mainly belong to Asian and North America samples, respectively. ESCC2 belongs to Eastern European and South American samples. ESCC1 harbors modification in the NRF2 pathway (*KEAP1, NFE2L2, ATG7,* and *CUL3*) and *SOX2* and/or TP63 amplification. ESCC2 is clinically characterized via enhanced rates of *ZNF750N* and *NOTCH1* mutations, *KDM2D, PIK3R1, KDM6A,* and *PTEN* inactivation, and CDK6 amplification. Because of the small population size, no definitive clinical features have been established with ESCC3 (Liu et al. 2018). In the same study, it was stated that gastric cancer (GC) heterogeneity has a negative impact on the response to therapies directed to *FGFR*, *EGFR*, and *HER2*, and possibly to *CCND1* and *MYC*. It also proposed four GC subtypes with (1) microsatellite instability (MSI-high), (2) chromosomal instability (CIN), (3) Epstein–Barr infection (EBV+), and (4) genomic stability (GS). EBV + GC (EBVaGC) demonstrates a distinct profile recognized in various studies and well established in Asian and Western populations. The key feature of this subtype is the proximal location, male predominance, *CDKN2A* (p16) promoter hyper-methylation, and extreme

CpG island methylator phenotype, mutation of *PIK3CA, ARID1A,* and *BCOR,* and *PD-L1*, *JAK2,* and *PD-L2* amplification (Alsina et al. 2017). Another study reported that mutations in *ARID1A* are more common in the Epstein–Barr virus-infected and microsatellite instability types of gastric cancer (Wang et al. 2011). In 2012, Zang and the team observed recurrent mutations in *PIK3CA*, *TP53*, and *ARID1A* within 15 gastric cancer samples. They also reported genetic abnormalities within the chromatin remodeling genes, namely, *MLL3*, *MLL*, and *ARID1A*, and the E-cadherin family gene, namely, *FAT4,* amongst 110 gastric cancer samples. Inactivation of *ARID1A* and *FAT4* was found to be associated with malignant characteristics of gastric cancer, for instance, cellular proliferation, migration, and invasion (Zang et al. 2012).

Cases reporting both intra- and inter-tumoral heterogeneity in colorectal cancer are very less (Jeantet et al. 2016). To date, numerous mutations in *BRAF, NRAS,* and *KRAS* that are responsible for causing colorectal cancer have been detected. However, as most of these previous studies employed sequencing methods with low sensitivity, no significant inter-heterogeneity between a primary and metastatic lesion in metastatic colorectal cancer was detected. Moreover, the concordance between *BRAF* and *KRAS* status is found to be above 95% (Santini et al. 2008; Baldus et al. 2010; Brannon et al. 2014). In 2016, Jeantet and the team investigated the intra- and inter-tumoral heterogeneity of *BRAF* and *RAS* mutations within 60 tumor regions from 18 colorectal cancer cases employing pyrosequencing (Jeantet et al. 2016). Results obtained revealed that, in the primary tumors, intra-tumor heterogeneity associated with RAS mutation was detected in 33% of the cases. However, inter-tumor heterogeneity associated with RAS mutation amongst metastatic lymph nodes and primary tumors was detected in 36% of the cases (Jeantet et al. 2016). However, in 2015, Sanborn and the team reveal that the main reason for inter-tumor heterogeneity is intra-tumor heterogeneity (Sanborn et al. 2015).

1.2.2 Intra-Heterogeneity

Intra-tumor heterogeneity denotes the intrinsic temporal–spatial variances amongst distinct tumor cells sub-populations in the same tumor at both epigenetic and genetic levels (Welch 2016). Recently developed high throughput sequencing technologies enable detection of various regions with distinct epigenetic and genetic characteristics features within the same tumor (Gerlinger et al. 2012). Additionally, non-cancerous cells, namely, infiltrating immune cells, stromal cells, etc., interact with nearby cancer cells and form a distinct microenvironment. Subsequently, factors associated with each microenvironment respond distinctly to chemotherapeutics, which in turn make the system complex and, hence, more difficult for treatment (Gao et al. 2018). To date, four important models, namely, the clonal evolution model, the cancer stem cell, the plasticity model, and the big bang model have been proposed to understand the intra-tumor heterogeneity in cancer (Fig. 1.1).

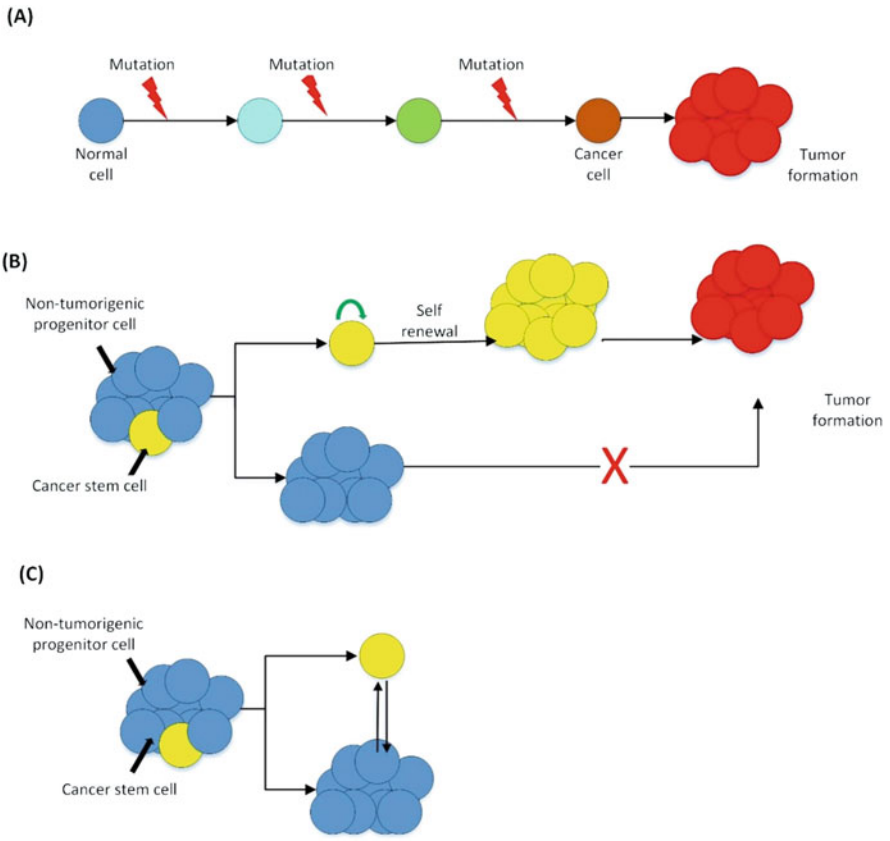

Fig. 1.1 Depicting the hypothesis associated with (**a**) the clonal evolution model, (**b**) the cancer stem cell, (**c**) the plasticity model to understand the intra-tumor heterogeneity in cancer

1.2.2.1 The Clonal Evolution Model

This model hypothesizes that a normal cell undergoes "neoplastic proliferation" after experiencing either spontaneous or induced genetic modification (Nowell 1976). Consequently, random genetic modifications in these neoplastic cells generate novel mutant cells with flexible fitness, and the cellular population endures selection. The majority of these genetic variants are deleterious and thus are eliminated through the immune system cells of the host. Few of these are advantageous to a tumor cell and may generate dominant sub-population (Ding et al. 2013). This works similarly to natural selection (Gupta and Vadde 2019b). The successive selection and diversification make the tumor malignancy more severe (Ding et al. 2013).

Since four decades several studies supporting this theory in numerous cancer types have been conducted by employing cytogenetic tools, molecular genetics, and high throughput sequencing approaches (Navin et al. 2010; Snuderl et al. 2011; Xu et al. 2012; Gerlinger et al. 2012; Welch et al. 2012). In 2011, Snuderl and the team

reported amplification of three distinct receptor tyrosine kinases, namely, EGFR, PDGFRA, and MET, within different cells of a single tumor in a mutually exclusive manner. Each distinct sub-population was actively dividing, and co-existing sub--population experiences mutual primary genetic modification, thereby suggesting their origin from individual precursor cells (Snuderl et al. 2011). In the same year, another study examined the clonal makeup of gastric glands in the stomach of humans. Results obtained revealed that metaplastic organs originate from the same clone and all lineages shared a mutual mtDNA mutation. They also reported that dysplasia originates from metaplasia (Gutierrez–Gonzalez et al. 2011).

As it is widely accepted that cancer is a micro-evolutionary process and develops from a single cell, recognizing cancer phylogeny may help us in identifying mutations associated with the branch, trunk, as well as a private branch (Hanahan and Weinberg 2011; Vormehr et al. 2016). Since trunk mutations denote the genomic variation amongst normal and the cancer cells, they serve as an essential biomarker toward early cancer detection. In 2017, Zhou and the team reported that non-trunk mutations have lower variant allele frequencies (VAFs) in comparison to trunk mutations. Trunk mutation present in the protein-coding regions may produce mutant proteins that are plausibly tumor-causing neo-antigens within cancer cells (Zhou et al. 2017). Yachida and the team observed two distinct categories of mutations in pancreatic cancer and their associated metastases. Trunk mutation, which denotes early tumorigenesis, was found to be present in a large amount (64%) in both primary and secondary metastatic tumors (Yachida et al. 2010). Zhou and the team have reported that small-scale multiregional sampling and subsequent screening of low VAF somatic mutations might be a cost-effective approach for identifying the majority of trunk mutations in gastric carcinoma (Zhou et al. 2017). Other studies have also reported about the clonal evolution concept in colorectal cancer as well as a few breast cancer (Navin et al. 2010; Kim et al. 2015).

1.2.2.2 The Cancer Stem Cell Model

Amongst the early studies on heterogeneity in cancer, for the first time, Virchow and Cohnheim hypothesized the involvement of cancer stem cells in tumor development. They believed that these cancer stem cells originate from "activation of dormant embryonic tissue remnants" (Huntly and Gilliland 2005). For the first time, cancer stem cells were isolated from acute myeloid leukemia by Bonnet and Dick (Bonnet and Dick 1997). However, information about their definitive properties and functions in various tumors remains elusive to date. Unlike the clonal evolution model, the cancer stem cells model hypothesizes that few stem cells present in the tumor are capable of self-renew as well as differentiation into a various cell types with distinct capabilities as well as phenotypes (Michor and Polyak 2010; Gerdes et al. 2014; Plaks et al. 2015). Few other studies have also proposed that development process associated with normal tissue organization, to a certain extent, may also be associated with cancer initiation in small cell lung carcinoma (Baylin et al. 1978),

mammary carcinoma (Hager et al. 1981), and teratocarcinoma (Pierce et al. 1960). These studies reported that various differentiated cells of tumors originate from tumor "stem" cells, as like normal differentiated tissues that develop from normal tissue stem cells. Hence, tumors can be considered as a caricature of normal tissue renewal or embryogenesis (Pierce and Speers 1988). Like normal tissue-specific stem cells, there are also quiescent sub-population of "cancer stem cells." Additionally, in these cancer stem cells, anti-apoptotic proteins and cellular efflux pumps are highly expressed and reactive oxygen species are suppressed. These cancer stem cells also play key role in DNA damage repair. Thus, cancer stem cells are more resistant to radio- and chemo-therapies and, are the main reason for cancer reoccurrence (Allan et al. 2006; Bao et al. 2006; Todaro et al. 2007; Li and Clevers 2010).

Earlier several studies have also reported that most of the leukemia blasts are post-mitotic and required replacement via a small amount of highly proliferative cells (Kreso and Dick 2014). In pancreatic cancer of humans, the CXCR4 portion of the CD133+ cancer stem cells is only capable of metastasis (Hermann et al. 2007). In colorectal cancer, CD26+ sub-population of cancer stem cells is only capable of metastasis and their presence indicates successive metastasis within the liver of primary colon cancer patients. Recently, several studies have suggested the presence of cancer stem cells in gastric cancer. Since cancer stem cells are generally produced from tissue-specific stem cells, there is always a debate if gastric cancer develops from cancerous gastric stem cells (Zhao et al. 2015).

For the first time, villins were detected as a biomarker for gastric stem cells. Villins are calcium-modulated actin-binding protein present on epithelial cell and are associated with regulating the re-organization of microvillar actin filaments (Nomura et al. 1998). Unlike extremely proliferative putative gastric stem cells present within the isthmus, the villin promoter-marked gastric stem cells (V-GSCs) are quiescent and situated nearby the lower-third of the antral glands (Qiao et al. 2007). As V-GSCs are mostly present in the antrum's lesser curvature (Qiao et al. 2007), the primary site of origin of human gastric cancer (Odze 2005), numerous studies have hypothesized that V-GSCs modification may cause gastric cancer. Another study has also reported that the down-regulation of Klf4 is responsible for developing gastric cancer in humans (Wei et al. 2005). Klf4 can also restrict cell proliferation via activating the cyclin-dependent kinase-inhibitors' expression (Katz et al. 2005; Wei et al. 2008). Klf4 deletion may also enhance expression of the FoxM1, pro-proliferative factor, in the gastric tissue (LI et al. 2012), which in turn may modify V-GSCs, thereby causing gastric cancer. Few researchers have also reported that the Lgr5+ stem cells present within the intestine and stomach could be responsible for initiating tumors (Zhao et al. 2015).

1.2.2.3 The Plasticity Model

The plasticity model hypothesizes that the processes and stimuli associated with inherent tumor cells may cause them to behave like normal stem cells. On the

contrary, these processes may also influence cancer stem cells to differentiate into non-stem cancer cells. In general, cancer cells experience higher plasticity than normal cells and this plasticity is associated with modulation of the epithelial–mesenchymal transition process (Rich 2016). Studies have suggested that various form of T cells, including Th17, Th1 and Th1, shows the outstanding amount of developmental plasticity via epigenetic mechanisms that are essential for preserving homeostasis specifically within the gastrointestinal region (Rezalotfi et al. 2019). Few studies have also reported that, under certain inflammatory conditions, normal function of $FOXP3^+$ Treg cells may become disrupted and behave like an effector $CD4^+$ T cells (Sakaguchi et al. 2013). Additionally, loss of FoxP3 expression may cause Treg cells to behave like an IL-17-secreting cell. Furthermore, in response to IL-12 under in vitro condition, Treg cells can also generate IFN-γ (Muranski and Restifo 2013).

1.2.2.4 The Big Bang Model

Sottoriva and the team proposed the temporal aspect of tumor mutations that may lead to heterogeneity during cancer (Sottoriva et al. 2015). The "big bang model" proposes that the mutations associated with tumor development as well as progression occur at early stage of colorectal cancer. Hence, the tumor behavior is determined at an early stage of cancer. That is why some tumor metastasize at any early stage while some never metastasize. To understand this model, single gland investigation in diverse tumor regions was employed for mapping the regional spreading of genetic modifications in colorectal cancer. Obtained result revealed that merging sub-clones are the characteristic of invasive carcinomas. Separated sub-clones are the characteristic of adenomas. This temporal and spatial analysis would support a "single clonal expansion" concept. Instead of dominant sub-clones spatially overgrowing others, Sottoriva and the team also detected large amount of mixed sub-clones that are driven by bystander mutations instead of Darwinian selection of the "fittest" sub-clone that causes spatial dominance (Sottoriva et al. 2015; Blank et al. 2018).

1.3 Approaches to Explore Tumor Heterogeneity

In recent years, genomic studies have suggested that tumor heterogeneity is the main reason for ineffective cancer treatment as well as personalized medicine. Hence, there is an urgent need to understand tumor heterogeneity in the early onset of any cancer, which in turn may improvise the outcomes of this killer disease. To date, several experimental approaches, for instance, "next-generation sequencing" approaches, have been developed to elucidate tumor heterogeneity, which may provide biomarkers in the prevention or curing of any cancer type. After employing "next-generation sequencing" approaches, in 2016, Clavé and the team suggested

that *ROS1* deletions and amplifications are heterogeneous in non-small-cell lung cancer but have no impact on overall survival of the patient (Clavé et al. 2016). Other studies have reported about *LRP2, ATM,* and *APC* deletion during gastric cancer. This finding is in accordance with the Cancer Genome Atlas (Cai et al. 2019). Zehir and the team investigated the mutational landscape of ~10,000 pan-cancer patients employing a hybridization capture-based NGS panel, namely, "MSK-IMPACT" (Zehir et al. 2017). In another study, Chen and the team did whole-exome sequencing on 78 gastric cancer patients within the Northern Province of China, namely, Tianjin, and differentiated gastric cancer into two subtypes, namely, high-clonality or low-clonality (Chen et al. 2015).

Though "next-generation sequencing" approaches enable somatic mutation identification in cancer, they are incapable of detecting rare mutations because of the errors that generate during library preparation as well as genome amplification at the time of the sequencing process (Etchings 2017). To overcome these problems, two approaches, namely, safe sequencing and duplex sequencing, have been developed (Gupta and Somer 2017). Though these two technologies effectively detect sub-clonal mutations, they are only beneficial for investigating small genomic subjects because of their low genome coverage and short read lengths problems (Gupta and Somer 2017). To overcome this problem, another technology, namely, circle-sequencing technology has been developed. In circle sequencing, genomic DNA is fragmented and subsequently circularized via ligating terminal region of the fragment, and amplified employing a rolling-circle polymerase (Lou et al. 2013). Later analyzing blood-based solid tumors employing intact circulating tumor cells, as well as cell-free ctDNA, provided a unique way of investigating temporal intra-tumor heterogeneity. However, these techniques are limited to analysis of structural rearrangements, DNA methylation changes, single-nucleotide variants, and copy number alterations (Gupta and Somer 2017).

However, most of these approaches to study tumor heterogeneity in cancer were mainly designed upon bulk-cell analysis, which in turn provides little information about the population of cells. For the first time, in 2009, transcriptomic data was estimated at the single-cell level by Tang and the team (Tang et al. 2009). Since then, the technique of single-cell RNA sequencing (scRNA-seq) has experienced an explosive development in the past 10 years. In comparison to bulk-based techniques, scRNA-seq provides more detailed insights into cellular heterogeneity and brings important discoveries in biology (Tang et al. 2011; Zeisel et al. 2015). For instance, Deng and the team identified the stochastic expression of monoallelic genes in mammalian cells (Deng et al. 2014). Earlier Xin and team (Xin et al. 2016) and Segerstolpe and team (Segerstolpe et al. 2016) reported the expression heterogeneity of human islet cells (for instance, β-cells, α-cells, and δ-cells). They also investigated the modifications in patterns of gene expression and the enriched signaling pathways in T2D in comparison with healthy people. Hence, the single-cell RNA-sequencing technology can provide detailed insight about the molecular mechanisms associated with any disease or trait, including gastrointestinal cancer, by capturing gene expression at the inter-cell level.

Bockerstett and the team employed scRNA-seq and identified a population of "Mucin 6 (*Muc6*)+gastric intrinsic factor (*Gif*)+ cells" within the healthy tissue. However, these cells do not play important role in "spasmolytic polypeptide-expressing metaplasia" in gastric mucosa, the regenerative lesion that is the plausible precursor for "intestinal metaplasia/gastric adenocarcinoma" (Bockerstett et al. 2019). In another study, Yang and the team performed analysis on 218 scRNA-seq libraries and identified the wide range of gene expression in "esophageal squamous cell carcinoma" cells. Additionally, they also reported that genes, namely, *ITGB4*, *LAMA5*, *SDC4, CFLAR,* and *ITGA6*, and pathways, namely, cell differentiation and proliferation pathways tumor cell migration, PI3K-AKT pathway, invasion pathways, pathways evading apoptosis, are responsible for radio-resistance development (Yang et al. 2019). Chen and the team analyzed ~30,000 single cells retrieved from eight gastric tumors and identified heterogeneous fibroblasts (*COLA1*+, *THY1*+, and *ACTA2*+), endothelial cells (*PECAM1*+ and *VWF*+), epithelial cells (*TFF1*+, *CDH1*+, *PGC*+, *EPCAM*+, and *MUC5AC*+), and various immune cells including CD4 T cells (*CD4D*+ and *CD3*+), M1 and M2 macrophages (*IL1B*+, *CD68*+, *MARCO*+, *TNF*+, *IL1A*+, and *MSR1*+), Gamma Delta T cells (*TRDC*+ and *TRGC2*+), B cells (*CD79*+), CD8 T cells (*CD8A*+), and plasma cells (*CD20*+, *CD19*+, and IgG+) (Chen et al. 2019). Component of both non-immune, namely, CD45-, and immune, namely, CD45+, cell is found to be highly heterogeneous in human gastric cancers (82). Irrespective of the benefits of the scRNA-seq technologies over technologies, information about tumor heterogeneity remain a topic of debate to date. Thus, there is always a scope for developing more effective strategies and approaches that may help to understand the complete mechanism associated with tumor heterogeneity in a more comprehensive way.

1.4 Conclusion

In conclusion, tumor heterogeneity is the main reason for the low treatment response rate in cancer patients. Tumor heterogeneity can be either at inter- or intra-level. Though to date, several approaches have been developed, there is still scope for development for more effective strategies and approaches towards understanding tumor heterogeneity in cancer. Authors believe that tumor heterogeneity can be investigated effectively by integrating information obtained from various disciplines, including pathology, clinical-radiology, genetic and molecular biology. Authors also believe that employing deep learning techniques, for instance, Tensor Flow (https://www.tensorflow.org/), in clinical-pathological diagnostic cases may help in developing various algorithms that may aid in early detection of cancer. In the near future, the information present in this chapter will be highly useful for the early detection and prevention of gastrointestinal cancer in humans.

Conflict of Interests None.

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Chapter 2
Immunocomposition of Gastrointestinal Tract of Gut

Mekapogu Madakka, Nambi Rajesh, and Jinka Rajeswari

Abstract The human gastrointestinal tract (GI tract) is a distinctive organ occupied by a series of commensal microorganisms, while also being showed to an overwhelming load of antigens in the form of dietary antigens on a daily basis. The GI tract has played dual role in the body, in that it performs uptake of nutrients and digestion while also performing out the complex and principal task of maintaining immune homeostasis, i.e., maintaining the balance between the good and the bad. It is equally important that we protect ourselves from reacting against the good, meaning that we reside tolerant to harmless food, commensal bacteria and self-antigens, as well as react with force against the bad, meaning induction of immune responses against harmful microorganisms. This complex task is achieved through the presence of a highly efficient mucosal barrier and a specialised multifaceted immune system, made up of a large population of scattered immune cells and organised lymphoid tissues termed the gut-associated lymphoid tissue (GALT). This book chapter provides an overview of the primary components of the human mucosal immune system and how the immune responses in the GI tract are coordinated and induced.

Keywords Lamina propria · GALT · Mucosal immunity · Mucosal tolerance · Immune homeostasis · Gut microbiota

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2.1 Introduction

2.1.1 Gastrointestinal Tract Architecture

Gastrointestinal (GI) tract plays an important role in uptaking nutrients, digestion. GI tract has large surface area due to multiple folds which allows absorbing nutrients, and gastrointestinal tract facilitates dual roles i.e., uptake of nutrients and defence against potentially pathogenic organisms (PPOs) (O'Leary and Sweeney 1987) followed by maintaining immune homeostasis. GI tract has several levels of invaginations at the tissue (Kerckring folds), cellular (villi) and membrane levels (microvilli). At the cellular level, intestinal epithelial cells (IECs) lined up the villi region which has microvilli to absorb nutrients released during digestion and housing for large number of immune cells in the body. Filamentous brush border glycocalyx (FBBG) present at the tip of microvillus is composed of a layer of membrane anchored glycoproteins that allows nutrients to cross, while restricting entry of whole bacteria. Antimicrobial peptides, mucins and trefoil peptides also act to restrict pathogen access to mucosal surfaces (Mestecky 2005; Brown et al. 1990).

2.2 Immune System of Gut

Immune system of mucosal gut comprises intestinal epithelial barrier, lamina propria and GALT—Gut-associated lymphoid tissue. The components of mucosal immune system of gut includes intestinal epithelial barrier, the lamina propria and the gut (GALT). GALT is the leading organ further categorised into Peyer's patches (PP), isolated lymphoid follicles (ILF) and mesenteric lymph nodes (MLN) (Mestecky 2005; O'Leary and Sweeney 1987) (Fig. 2.1).

GALT is again differentiated into Peyer's patches (PP), both form the biggest lymphatic organ. Defence function, carried out by two sites includes inductive and effector sites. Antigens from the mucosal surface, in the inductive sites activate naive and memory T and B lymphocytes, consist of PP, ILF and MLN which are organised nodes of lymphoid follicles (Mestecky 2005; Bhide et al. 2001; Heel et al. 1997). Effector sites include epithelium and lamina propria, site of lymphatic scattered (Mestecky 2005; O'Leary and Sweeney 1987; Bhide et al. 2001; Heel et al. 1997).

The mucosa-associated lymphoid tissue (MALT) is the largest immune organ in the body containing more plasma B cells than the lymphoid nodes, spleen and bone marrow combined, its role is to defend the mucosal surfaces from pathogenic organisms. The MALT can be divided into two morphologically distinct regions: (1) diffuse lymphoid tissue, where loosely ordered clusters of lymphoid cells are scattered in the lamina propria of the mucosae and (2) well-organised lymphoid tissue, where lymphoid cells are grouped together forming aggregates in the submucosa (Mestecky 2005).

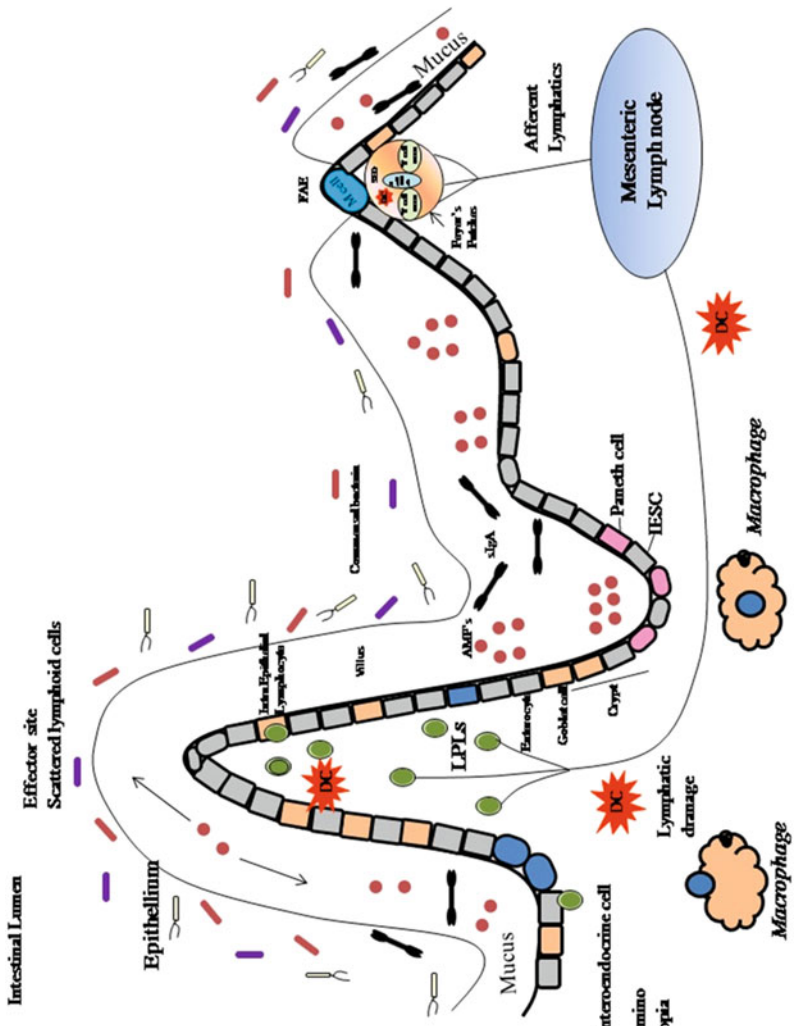

Fig. 2.1 Representation of mucosal immune system of gastrointestinal tract. Intestinal epithelial cells and the mucous layer form a biochemical and physical barrier that maintains segregation between the gut lumen and the mucosal immune system

The mucosal membrane of the GIT comprised of single layer of absorptive epithelial cells enterocytes. Enterocytes not only allow for the absorption of nutrients from the lumen but they also function as a protective barrier preventing the adherence and entry of microorganisms. Gut-associated lymphoid tissue (GALT) provides immunological defence for the GIT. The GALT must discriminate pathogens where a protective immune response is induced from commensals and dietary antigens, where homeostasis is preserved by maintaining a state of non-responsiveness. Johannes Conrad Peyer, a Swiss anatomist is credited with identifying PPs in 1673 (and first publishing his observations in 1677). PPs are mainly located on the antimesenteric wall of the ileum but can also be found in the duodenum and jejunum. In humans, the greatest number of individual follicles are found in the large intestine and in the appendix and caecum where there is a high number of commensals (O'Leary and Sweeney 1987). Human PPs develop in association with the ileum, where PPs are first seen at the 15th week of gestation (Bhide et al. 2001). However, the typical PP structure is not seen until after birth, where the germinal centre develops rapidly following antigenic stimulation by the gut luminal bacteria (Heel et al. 1997). Morphologically, the PP is divided into four main domains: the lymphoid follicle, the interfollicular region (IFR), the sub-epithelium dome (SED) and the follicle-associated epithelium. The lymphoid follicle contains a germinal centre consisting of proliferating B cells with a small population of $CD4^+$ T cells and follicular dendritic cells that present antigens to B cells. The germinal centre contains a large B cell population with a small population of $CD4^+$ T cells and follicular dendritic cells. The IFR is a T cell-rich area containing mainly $CD4^+$ T cells, macrophages, dendritic cells and high endothelial venules. The SED lies above the follicle and contains B cells, T cells, dendritic cells and macrophages. M cells are found in the follicle-associated epithelium overlaying the PP (Neutra and Kozlowski 2006).

2.3 Intestinal Epithelial Barrier

Intestinal epithelium consists of intestinal epithelial cells (IECs) further modified into crypts and villi. Hence this is the body's largest mucosal surface, intestinal epithelium comprised of specialised cells includes immune response and defence antigens, toxins, pathogens and enteric microbiota and absorbs selectively by nutrients, electrolytes and water (Bevins 2006; Frey et al. 1996). Inductive site consists of lymphoid follicle nodes, PP, ILF and MLN, where antigens from mucosal surface activate naïve and memory T and B lymphocytes, effector site consists of epithelium and lamina propria. The lymphocytes are scattered throughout the tissue in which effector cells after extravasation, retention and differentiation perform this action. Crypts contain pluripotent epithelial stem cells at the base, which are renewed continually in the epithelium. IECs forms the segregates the gut lumen and its contents from the cells in lamina propria found underneath tight junctions, seals the intercellular spaces by linking adjacent epithelial cells, by forming physical and

biochemical barrier (Brown et al. 1990; Owen and Bhalla 1983). To maintain integrity and permeability the expression of the junctional proteins was highly regulated. If any alterations in intestinal permeability and mucosal defect barriers leads to inflammatory bowel disease and irritable bowel syndrome (Bevins 2006; Frey et al. 1996).

2.4 Specialised Secretory IECs

Goblet cells and Paneth cells are specialised cells that secrete mucins and antimicrobial proteins (AMPs) to establish barrier functions (Kraehenbuhl and Neutra 1992) and the organisation of the intestinal mucous layer (Kraehenbuhl and Neutra 1992; Bjerke and Brandtzaeg 1988). The Paneth cells secrete AMPs, which include defensins, cathelicidins, secretory phospholipase A2 and lysosomes, further form the barrier and selectively descriptive cell wall and surface membranes of bacteria (Kraehenbuhl and Neutra 1992; Neutra et al. 1996; Giannasca et al. 1994).

2.5 Lamina Propria

Lamina propria is the layer of loosely packed connective tissue that lies below the epithelium (Mestecky 2005; Sharma et al. 1996). It majorly consists of intestinal immune cells, the blood supply, the lymphatic drainage system and the nervous supply for the mucosa (Jang et al. 2004) and the major site of intestinal immune response. Its role is to prevent the entry, spread and destruction of pathogens across the gut mucosa (Sharma et al. 1996; Mooseker 1985).

2.6 Peyer's Patches

Peyer's patches are well organised and primary inductive site for mucosal immune response usually found in the distal ileum. It consists of large B cell follicles with intervening smaller T cell areas known as subepithelial dome (SED) due to its dome shape and single layered follicle-associated epithelium (FAE) separates the intestinal lumen (Heel et al. 1997; Iwasaki and Kelsall 2000). The FAE contains M cells (specialised IECs), mediates primary step in initiating a mucosal immune response by attacking luminal antigens and intact microbes by transporting to DCs, lying within the SED (Sharma et al. 1996; Regoli et al. 1995). PPs contain B cells, CD4þ T cells and CD8þT cells (Wolf et al. 1983), dendritic cells and macrophages (Jepson et al. 1993).

2.7 Follicle-Associated Epithelium (FAE)

The FAE of the PP differs greatly from the surrounding villus epithelium. The majority of the villus epithelium consists of absorptive enterocytes that are formed in the crypts of Lieberkuhn from stem cells. Enterocytes migrate out of the crypts into the villi, where they differentiate into mature absorptive cells that take up nutrients and aid indigestion. Stem cells in the crypts can also give rise to goblet cells, enteroendocrine cells and Paneth cells (Mestecky 2005). They provide defence against microbes by secreting a number of antimicrobial molecules such as defensins, lysozyme and phospholipase A_2 in response to microbial stimulation (Bevins 2006) In contrast to the villus epithelium, the FAE contains enterocytes and specialised antigen sampling M cells, but there are few or no enteroendocrine or goblet cells (Mestecky 2005) Therefore, there is less mucin produced by the FAE allowing greater access for luminal antigens to the FAE. Enterocytes of the FAE are morphologically similar to villus enterocytes; they are polarised columnar cells with a well-defined brush border with a thick glycocalyx (Frey et al. 1996). However, enterocytes of the FAE express less membrane (apical) bound digestive enzymes such as sucrase, isomaltase and alkaline phosphatase (Brown et al. 1990; Owen and Bhalla 1983; Smith 1985). Polymeric immunoglobulin receptor (p IgR) that mediates the basolateral to apical transport, and secretion of immunoglobulin A (IgA) is absent from the basolateral membrane of the FAE (Bjerke and Brandtzaeg 1988; Pappo and Owen 1988). The glycosylation patterns of the FAE differ from the villus, even within the FAE, the glycosylation pattern of the follicle enterocytes differs from the patterns seen on the M cells (Clark et al. 1995; Giannasca et al. 1994; Sharma et al. 1996). The FAE lacks subepithelial myofibroblasts found in the villus. One the most notable differences between the FAE and the villus epithelium is seen in the basement membrane composition. Perlecan and laminin a2 are absent from the basement membrane of the FAE (Sierro et al. 2000). The difference in basement membrane composition is thought to influence the proliferation and differentiation of the FAE as well as forming a more porous basal lamina, thus allowing for easier migration of lymphocytes and dendritic cells from the antigenic sampling M cells to the SED (Mc Clugage et al. 1996). M cells were once believed to be a unique feature of FAE; however, recent studies have shown that M cells may also be found on the intestinal villi (Jang et al. 2004; Nochi et al. 2007; Terahara et al. 2008). Villus M cells are found at a higher density towards the end of the ileum than throughout the small intestine. Villus M cells share all the features and functions of PP M cells; however, they exist independent of the PP (Jang et al. 2004).

It should be noted that the villus M cells were induced with cholera toxin. Therefore, the villus M cells may more readily represent an intermediate state in the conversion of M cells from villus enterocytes (discussed in the development of M cells). M cells are specialised polarised epithelial cells that are found in the FAE. M cells were first described in 1965 by Schmedtje who, using immune histochemistry, noted the presence of lymphoepithelial cells in the appendix of rabbits. The involvement of M cells in the transport of antigens was reported in 1977 when Owen

showed that M cells transport horseradish peroxidase (Owen 1977). The thick glycocalyx that overlays the microvilli of enterocytes is much reduced over M cells (Frey et al. 1996). M cells lack membrane hydrolytic enzymes such as alkaline phosphatase and sucrase isomaltase (Brown et al. 1990; Smith 1985). Brush border assembly requires the recruitment of actin binding proteins such as villin to the apical membrane (Mooseker 1985). In M cells, villin is not associated with the apical membrane and is instead found in the cytoplasm (Kanaya et al. 2007; Kerneis et al. 1996). The apical cytoplasm is rich in mitochondria and vesicles involved in transcytosis (Wolf et al. 1983). The M cell cytoplasm generally contains fewer lysosomes. The reduction in lysosomes suggests that the M cell delivers antigenic material unchanged to the lymphoid follicle (Owen et al. 1986a). Similar to enterocytes, the Golgi apparatus and the endoplasmic reticulum (ER) are located above the nucleus. The nucleus of the M cell is displaced basally because the basolateral membrane of the M cells is invaginated and forms a pocket that contains lymphocytes and dendritic cells (Mestecky 2005; Iwasaki and Kelsall 2000; Regoli et al. 1995). The primary function of M cells is to sample antigens in the lumen, to take them up and deliver them to the underlying follicle. The structure of M cells allows for the effective uptake and swift delivery of antigens. The lack of tightly packed microvilli and a thick glycocalyx enables, the deep invagination of the basolateral contained in the M cell pocket (Jepson et al. 1993).

M cells take up bacteria and large molecules by phagocytosis, where the M cell is seen to engulf the bacteria. Although studies have reported on the adherence and transcytosis of many microbes, there is still much to be investigated. Many of the studies with M cells are done in mice and it is known that M cells differ greatly between species. Therefore, M cells in mice differ greatly from M cells in humans. The development of the in vitro M cell model by Kerneis and the subsequent modification by Gullberg have provided a human M cell model to allow a greater understanding of "human" M cells to be elucidated (Gullberg et al. 2000; Kerneis et al. 1997). The model has been used to study the morphology, interaction with commensal and pathogenic organisms, expression of cell-specific apical receptors, drug absorption and novel vaccine targeting of M cells. Although the in vitro M cell model has allowed us to generate a greater understanding of humanised M cells, it is a simplified system, where the interaction of signalling factors from other immune cells, especially those needed for proper PP function (T cells and dendritic cells), is not taken into account.

The in vitro M cell model was designed by Kerneis and adapted by Gullberg et al. (Gullberg et al. 2000) and Kerneis et al. (Kerneis et al. 1997). Caco-2 cells are seeded on to a semi-permeable membrane and cultured until fully polarised (21 days). Either PP lymphocytes or Raji lymphocytes (B cells) are added to the basolateral chamber. Cells are cultured for 3 days to allow phenotypic M cells (M-like cells) to develop within the polarised Caco-2 monolayer.

2.8 Mesenteric Lymph Nodes

Mesenteric lymph nodes are the largest lymph nodes in the body comprised of paracortex, the cortex and the medulla with more number of lymphocytes (Jensen et al. 1998; Neutra et al. 1987). Its main role is to filter intestine lymph and attack incoming antigens and initiate immune responses in either in free form nor bring them to MLN by DCs. MLNs also consist of macrophages and APCs and are an efficient location for the interaction of naive or primed lymphocytes with APCs to undergo further differentiation (Jensen et al. 1998; Neutra et al. 1987).

2.9 Antigen Sampling by M Cells

The ability of M cells to take up antigens from the lumen and transport them across the epithelial barrier to the underlying follicle is enhanced by the structural characteristics of the M cells. The reduced brush border, glycocalyx and amount of hydrolytic enzymes on the apical membrane allow for greater interaction between lumen antigens and the M cells. The deep invagination of the basolateral pocket means that the distance from the apical membrane to the basolateral membrane is shortened allowing endocytosed antigens to be delivered to the pocket lymphocytes in as little as 15 min (Neutra et al. 1987; Ouzilou et al. 2002). Many pathogens, both bacteria and viruses, survive the transcytosis process mediated by M cells, even though the endosomal vesicles are acidified and can proceed to cause infection of the mucosae (Allan and Trier 1991; Phalipon and Sansonetti 1999). M cells have been shown to transcytose a diverse array of infectious agents across the epithelial barrier, including bacteria, viruses, parasites and prions (Ouzilou et al. 2002; Heppner et al. 2001; Owen et al. 1986b). This surveillance function can be exploited by invasive pathogens to yield an entry route into the underlying tissue.

Infection with poliovirus (PV), a member of picornaviridae and the causative agent of poliomyelitis, occurs by ingestion of contaminated material via the gastrointestinal mucosal surfaces. Initial viral replication occurs in the FAE of PPs (Bodian 1955, 1956). PV has been shown to adhere to, and be transcytosed by M cells from the lumen to the underlying lymphoid tissue (Ouzilou et al. 2002; Sicinski et al. 1990). Following replication of PV in the PPs, most PV infections result in asymptomatic transient viraemia with 4–8% of cases developing major viraemia and $<1\%$ develop neurological symptoms (Melnick 1996; Nathanson and Martin 1979; Sabin 1956). Members of the Retroviridae family, in particular human immunodeficiency virus (HIV) and mouse mammary tumour virus (MMTV), have been shown to be transcytosed by M cells. HIV-1 has been shown to adhere to M cells in rabbits and mice and was transcytosed by M cells to the underling lymphoid follicle to infect $CD4^+$ T cells (Amerongen et al. 1991). Further studies showed that transcytosis of HIV-1 by M cells was receptor mediated (Fotopoulos et al. 2002). Using the in vitro M cell model, a lymphotropic strain of HIV-1 was transcytosed by M cells, mediated

by lactosyl cerebroside and CXCR4 receptors that are expressed on the apical surface of Caco-2 and M cells50. A monotropic HIV-1 strain is unable to cross in vitro M cell monolayers.

However, transfection of the Caco-2 cells with CCR5 resulted in transcytosis of the virus50 MMTV is transmitted vertically via milk from mother to pup. The virus is transcytosed by M cells, where it infects the pup's macrophages and then lymphocytes (Moore et al. 1979; Weltzin et al. 1989). Reovirus type 1, known to be a cause of systemic and intestinal disease in mice, is transmitted via the faecal, oral route. Reovirus type 1 has been shown to selectively adhere to and be transcytosed by M cells in the PP where replication occurs (Wolf et al. 1981). During cell entry, the reovirus capsid undergoes a series of disassembly steps (native virions to ISVPs-intermediate sub-viral particles) to activate its membrane-penetration machinery for delivery of particles into the cytoplasm (Bodkin et al. 1989). Studies have shown that conversion of native reovirus to ISVPs is a prerequisite for M cell adherence, where either sigma 1 or mu 1 (capsid proteins) mediate interaction of virus with M cell apical membranes (Amerongen et al. 1994). The transport of reovirus has been shown to be receptor mediated, where reovirus sigma 1 protein (of ISVP) binds to glycoconjugates containing a(2-3) sialic acid that are accessible to viral particles only on M cell apical surfaces (Helander et al. 2003). Various bacteria are transcytosed by M cells such as *Vibrio cholerae*, *Yersinia spp*, *Salmonella spp*, *Shigella spp*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Escherichia coli*, *Brucella abortus*, *Haemophilus influenzae*, *Streptococcus pyogenes* and *Campylobacter jejuni* (Jensen et al. 1998; Owen et al. 1986b; Ackermann et al. 1988; Walker et al. 1988). Pathogenic bacteria such as *Salmonella spp*, *Yersinia spp* and *Shigella spp* can cause direct infection of M cells, damaging the M cells and spreading to neighbouring enterocytes (Jones et al. 1994). For example, *Salmonella typhimurium* is selectively transcytosed by M cells, where it is associated with extensive ruffling of the apical surface of the M cells and damage to the FAE (Jensen et al. 1998; Sansonetti and Phalipon 1999). *S. typhimurium* infection in mice is used as a model for S. typhi (the causative agent of typhoid fever) in humans. *S. typhimurium* adhesion to M cells induces cytoskeleton rearrangement with ruffling of the apical membrane and actin polymerisation resulting in engulfment of the bacteria (Jones et al. 1994). In calves, *S. typhimurium* was taken up by M cells within 5 min of exposure (Frost et al. 1997). Thirty minutes after infection, the majority of M cells infected in the FAE have been sloughed off, after 1 h the cells had been destroyed (Frost et al. 1997; Watson et al. 1995). The destruction allows entry of the bacteria into the host and infection of neighbouring enterocytes (Phalipon and Sansonetti 1999). Studies have identified both Salmonella pathogenicity island (SPI) and the long polar fimbria (LFP) operon as having a role in the adherence of *Salmonella* to M cells as both SPI and LFP mutants show reduced virulence (Baumler et al. 1996, 1997; Clark et al. 1998). Recently, a study by Lim and colleagues has shown that *S. typhimurium* associates with caveolae in the apical membrane in the in vitro M cell model and that expression of caveolin-1 (a marker for caveolae) mediates transcytosis of *S. typhimurium* (Lim et al. 2009). In vitro M cells in which the expression of caveolin-1 had been down-regulated by siRNA did

not transcytose *S. typhimurium* (Lim et al. 2009). Caveolae (a type of lipid raft) are flask-shaped invaginations in the plasma membrane that are cholesterol and sphingolipid rich (Brown and London 1998). Caveolin-1 was not expressed by Caco-2 cells but was expressed in M-like cells and was seen to localise with sialyl Lewis A antigen (Lim et al. 2009). This also suggests that M cells may be identified/isolated from the surrounding FAE and enterocytes based on lipid rafts present in the apical membrane. Hase and colleagues have recently found that glycoprotein 2 that is preferentially expressed by M cells binds FimH (component of type-I-pili) and mediates transcytosis of the bacteria as glycoprotein 2 was also present in the endocytosed vesicles containing the bacteria (Hase et al. 2009) *S. typhimurium, S. enteritidis* and *E. coli* all express type-I-pili and their transcytosis by M cells was mediated by glycoprotein 2 (Hase et al. 2009). Enteropathogenic *E. coli* are non-invasive but cause disease by colonising the mucosal surface. M cells have been shown to transcytose enteropathogenic *E. coli* that leads to the production of secretory Ig A that limits the duration and severity of the disease (Levine et al. 1987). Early studies identified expression of AF/R1 pili as necessary for attachment to M cells (Inman and Cantey 1983; Sansonetti et al. 1996). Hase and colleagues have now shown that the FimH sub-unit of the type-I-pili mediates transcytosis of *E. coli* by binding glycoprotein 2 (Hase et al. 2009). *V. cholerae* a non-invasive pathogen adheres to M cells inducing actin rearrangement and phagocytosis of the bacteria by extensions of the plasma membrane fusing around the bacteria to form a phagosome (Owen et al. 1986b). A recent study by Blanco and DiRita reported that transcytosis of *V. cholerae* by M cells is mediated by bacteria-associated cholera toxin-binding ganglioside receptor GM1 (Blanco and Dirita 2006b). Transcytosis of *V. cholerae* by M cells stimulates the production and secretion of s IgA (Apter et al. 1993; Winner 3rd et al. 1991). *V. Cholerae*-bound s IgA promotes M cell-mediated transcytosis of *V. cholerae (*Blanco and Dirita 2006*)*.

2.10 Mucosal Vaccination

There is great interest in exploiting the transcytotic activity of M cells in the development of oral mucosal vaccines and oral drug therapies. The lack of a universal M cell marker or a human M cell marker has impeded M cell characterisation and targeting for vaccine development. Oral vaccination offers advantages over parenteral routes of vaccination. Mucosal vaccination induces both mucosal and systemic immune responses (Belyakov et al. 1998). The mucosal immune system is a critical part of the defence against infectious disease, as to first cause infection many pathogens must gain access to the host by crossing the mucosae and thus many infections are initiated at mucosal sites. The induction of specific immune responses (both humoral and cellular) at the mucosal sites of entry could limit infections at the point of entry. Oral vaccination does not require trained medical personnel to administer the vaccine, thus making vaccination campaigns in the developing world easier, where access to medical staff is often limited. Oral vaccines

are easier and safer to administer as they do not require sterile needles and syringes. There are a limited number of oral vaccines that have been approved for use. Oral polio vaccine (OPV) or Sabin vaccine was the most successful oral vaccine. Its use has resulted in a dramatic reduction in paralytic poliomyelitis (Kimman and Boot 2006). However, polio vaccination strategies have changed as the attenuated oral vaccine was found to revert to the wild-type virus giving rise to vaccine-derived poliovirus and persistence of the virus within populations (Fine and Carneiro 1999). Successful oral vaccines must overcome the innate immune barriers such as peristalsis, mucus, secreted antibodies, stomach acid, proteases, nucleases and the epithelia glycocalyx in the GIT. Mucosal pathogens themselves are one of the most obvious choices for vaccine design as they have evolved to overcome the mucosal barriers to cause disease. Currently, the most successful oral vaccines are live attenuated *S. typhi* Ty21a and the previously mentioned OPV both of which are known to target M cells through selective binding and are transcytosed by M cells to the underlying lymphoid tissue (Sicinski et al. 1990; Jones et al. 1994; Hase et al. 2009). Currently, recombinant or attenuated strains of *V. cholerae, Salmonella, Shigella, E. coli, Yersinia and L. monocytogenes* are being investigated as oral vaccine vectors to deliver antigens to the mucosal immune system as they have been shown to be preferentially transcytosed by M cells in the follicle of the PP (Bowe et al. 2003; Liang et al. 2009). The use of live attenuated microbes as vaccine vectors offers a number of advantages, they specifically bind to and are transcytosed by M cells, they are stable in the GIT, dose size is low due to replication and they are commercially cheaper to produce. However, pre-existing immunity to these attenuated organisms may prevent them acting as vaccines. It is estimated that one out of ten million epithelial cells in the intestinal tract is an M cell (Bye et al. 1984; Kuolee and Chen 2008). Due to the low numbers of M cells present, it is necessary to target vaccines to M cells to ensure that they are transcytosed. The transcriptome of the FAE and M cells has been investigated using DNA microarrays in order to determine novel M cells markers and the elusive universal M cell marker (Terahara et al. 2008; Hase et al. 2005; Verbrugghe et al. 2006). Terahara and colleagues produced the first study that isolated M cells from the surrounding FAE and isolated villus M cells from the surrounding villus epithelium in mice. They used the selective binding of UEA-1 and NKM 16-2-4 mAb (a newly proposed M cell-specific antibody) to M cells to sort the labelled population (M cells) using flow cytometry (Terahara et al. 2008). They reported the preferential expression of both glycoprotein 2 and myristoylated alanine-rich C kinase substrate (MARCKS)-like protein by M cells in the PP (Terahara et al. 2008). Glycoprotein 2 has been shown to preferentially bind type-I-pili and mediate transcytosis of the bacteria suggesting that designing oral vaccines to glycoprotein 2 may aid M cell targeting (Hase et al. 2009). A recent study by Nakato and colleagues reported that PrPC is highly expressed on the apical surface of murine M cells (Nakato et al. 2009). PrPC is highly expressed not only by M cells but also by follicular DCs, mature myeloid cells and activated T cells (Thielen et al. 2001). This cellular distribution suggests that PrPC may be of interest as a target for future vaccine design. M cells have been shown to not only transcytose microorganisms but also to transcytose particles (up to 1 mm in size) that adhere to

their apical surface (Frey et al. 1996; Frey and Neutra 1997). This has led to the development of vaccine strategies based on attachment of antigens to latex or poly-DL-lactide-co-glycolide (PLG) microspheres. The microspheres are not degraded in the GIT and are preferentially transcytosed by M cells (Jepson et al. 1993; Foster et al. 1998; Pappo and Ermak 1989). However, targeting of the microspheres to M cells results in greater induction of an immune response UEA-1-coated PLG microparticles expressing HIV peptides were found to preferentially adhere to M cells and generate both a mucosal and systemic immune response in mice (Manocha et al. 2005). The vaccine was found to be more immunogenic when administered mucosally than when it was administered systemically (Manocha et al. 2005). UEA-1 has also been used to target killed *Helicobacter pylori* and *C. jejuni* (inactivated vaccines) to M cells to induce immune responses against challenge with the live bacteria (Chionh et al. 2009). PLG microspheres containing a surface antigen from enterotoxigenic *E. coli* were used in a human trial where a modest increase in sIgA and IgG serum levels was observed (Katz et al. 2003) Although the vaccine itself was not overly effective, the method of vaccination did not produce any adverse effects in the five human test subjects (Katz et al. 2003). The size of the microspheres used in M cell targeting is an important factor for particle uptake and thus for initiation of an immune response. Reovirus adhesion protein sigma 1 that has been shown to mediate adherence of reovirus to apical membranes of M cells has also been used to target M cells to induce uptake of vaccines such as an HIV DNA vaccine (Amerongen et al. 1994; Wang et al. 2003). PV is also being investigated as a potential vaccine vector (Andino et al. 1994). Studies have shown that PV vectors induce both humoral and cell-specific immune responses (Mandl et al. 1998). A great deal of recent vaccine research has been targeted towards stimulation of pattern recognition receptors (PRR), in particular Toll-like receptors (TLR).

2.11 Pattern Recognition Receptors

PRR play a central role in the innate immune response by recognising conserved PAMPs in microorganisms. A class of PRR called the TLR family has the ability to recognise pathogens and pathogen-derived products and initiate signalling events that lead to the activation of the innate immune system.

After the discovery of TLRs, several classes of cytoplasmic PRRs, including retinoic acid-inducible gene I (RIG-I)*like helicases (RLH) and nucleotide-binding oligomerisation domain (Nod), were identified. Nod proteins have been shown to play a pivotal role in the detection of bacterial cell wall components within epithelial cells. Nod1 and Nod2 recognise peptides derived from the degradation of peptidoglycan (PGN) and when stimulated produce pro-inflammatory cytokines through the recruitment of NFkB (Girardin et al. 2003a, b). The RLR family consists of three members, RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (Lgp2) that are involved in the detection of viral RNA (Kang et al. 2002; Yoneyama et al. 2004). Detection of viral RNA by RLH

leads to the production of type 1 interferons (IFNa/b) that are essential for the development of an anti-viral immune response (Kawai et al. 2005). Viral RNA can also be detected by RNA dependent protein kinase R that is a cytoplasmic serine kinase which recognises short-stem loop structures within RNA in a RLH independent manner (Jacobs and Langland 1996). Recently, several groups identified another cytoplasmic PRR family, PYHIN proteins (pyrin an HIN domain containing protein) that detect dsDNA (Burckstummer et al. 2009; Schroder et al. 2009).

2.12 Toll-Like Receptors

TLRs are evolutionarily conserved type 1 transmembrane proteins with leucine-rich repeats responsible for binding various PAMP and an intracellular Toll/interleukin 1 domain responsible for initializing signalling. TLRs have been shown to be expressed on cells of the innate immune system, such as dendritic cells, macrophages and antigen-presenting cells, and have been shown to be involved in phagocytosis and in the development of a pro-inflammatory immune response (Blander and Medzhitov 2004).

Stimulation of the Toll/Interleukin 1 domain of the TLR by its PAMP is responsible for initialising signalling, leading to dimerisation and activation of a signalling cascade through recruitment of adaptor molecules such as MyD88 (Myeloid differentiation primary response gene 88), TRIF (TIR-domain-containing adapter-inducing interferon-b), TRAM (TICAM1) (Toll-like receptor adaptor molecule 1) and TIRAP (Toll-interleukin 1 receptor (TIR) domain containing adaptor protein). Early studies showed that signalling through TLR3 and TLR4 generated both type I interferon and inflammatory cytokine responses, whereas stimulation of TLR1/TLR2, TLR2/TLR6 and TLR5 mainly generated inflammatory cytokines that lead to the realisation that TLRs activated distinct signalling events through adaptor proteins mediating specific immune responses. The TLR signalling pathways can be broadly divided into two, one the MyD88-dependent pathway that drives the induction of inflammatory cytokines in early phase activation of NFkB via the IL-1R-associated kinase (IRAK) pathway, and two, the TRIF-dependent pathway that induces type I interferon as well as inflammatory cytokines through the interferon regulatory factor (IRF) three pathway that results in a later phase activation of NFkB. MyD88 is used by all TLRs bar TLR3 to activate the MyD88-dependent pathway (Brikos and O'Neill 2008). TRIF is used by TLR3 and TLR4 and induces the TRIF-dependent pathway. TLR4 is the only TLR molecule that can use all four adaptor molecules and can activate either the MyD88-dependent or the TRIF-dependent pathways. TRAM recruits TRIF to TLR4 and TIRAP recruits MyD88 to TLR2 and TLR4 to initiate signalling (Doyle and ONeill 2006). When the MyD88-dependent signalling pathway is stimulated, MyD88 recruits members of the IRAK family. IRAK4 is initially activated and it in turn activates IRAK1 and IRAK2 (Kawagoe et al. 2008).

2.13 Nod1 and Nod2

The Nod-like receptors (NLR) proteins are structurally similar to R proteins that are found in plants and are involved in disease resistance (Jones and Dangl 2006). NLRs represent a large family (over 20 members) of PRRs that respond to various stimuli that include PAMPs and cellular stresses (Brodsky and Monack 2009). NLRs recognise microbial products, thereby initiating host defence pathways through the activation of the NFkB (Brodsky and Monack 2009). Activation of NLRs has also been shown to have a role in the activation of autophagy and cell death (Suzuki et al. 2007; Willingham et al. 2007). Nod1 and Nod2 are the best studied members of the NLR family; they represent an intracellular pathogen-sensing system (Harton et al. 2002). The Nod1 and Nod2 proteins can structurally be divided into three regions, the first, a carboxy terminal ligand recognition domain containing leucine-rich repeats, and the second a central nucleotide-binding domain (also known as a NACHT domain). Nod1 and Nod2 differ from each other in the third domain, the amino terminal. Nod1 contains one caspase activating and recruitment domain (CARD), whereas Nod2 contains two CARD domains. Studies have found that Nod1 is stimulated by g-D-glutamyl-meso-diamino-pimelic acid (iE-DAP) that is mainly derived from the PGN of Gram-negative bacteria (Chamaillard et al. 2003).

Nod2 senses muramyl dipeptide (MDP), another PGN derivative present in both Gram-positive and Gram-negative PGN (Mc Clugage et al. 1996). Stimulation of Nod by its ligand results in homo oligomerisation of Nod proteins, resulting in the recruitment of adaptor protein Rip2 that mediates both Nod1- and Nod2-dependent activation of NFkB and MAPK signalling. Although a cytosolic protein, studies have shown that for Nod2 to be stimulated by MDP, it is necessary for Nod2 and Rip2 to localise to the plasma membrane (Barnich et al. 2005; Lecine et al. 2007). Nod2 is crucial for PP homeostasis (Barreau et al. 2007). Nod2 knockout mice have an increased number of larger PPs. These PPs also had an increased number of M cells present within the FAE. An increase in the translocation/permeability of bacteria across the FAE was observed. However, it is unclear whether this increase was, as a result of the increased number of M cells or, through the loss of epithelial integrity by the loss of tight junctions within the PP or through some as of yet undefined action of Nod2. Stimulation by the normal gut microflora of the PP in Nod2 knockout mice leads to an increase in the production of Th1 pro-inflammatory cytokines. Mutations in CARD15, the gene that encodes Nod2, have been associated with the development of Crohn's disease and Blau's syndrome (Inohara et al. 2003; Rose et al. 2005). Mutations in CARD4 (the gene that encodes Nod1) have been associated with an increased risk of developing asthma and atopic eczema (Hysi et al. 2005; Weidinger et al. 2005).

2.14 RIG-Like Helicases

RLH are a family of cytoplasmic sensors that detect viral nucleic acids. To date, three members of the RHL family have been identified, RIG-1, MDA5 and Lgp2 (Kang et al. 2002; Yoneyama et al. 2004). Detection of viral RNA by RLH leads to the production of type 1 IFNs that are essential for the development of an anti-viral immune response (Kawai et al. 2005). Structurally, they contain two CARD domains at the amino terminal and a central ATPase and helicase domain (Yoneyama et al. 2005). RIG-I contains a repressor/regulatory domain at the carboxyl terminal that inhibits signalling (Saito et al. 2007). MDA5 contains a carboxyl terminal that is similar to RIG-I; however, it is not known if it acts as a repressor domain (Yoneyama et al. 2005). Lgp2 lacks a CARD domain but has a helicase and repressor domain. It was originally suggested that Lgp2 was a negative regulatory of RIG-1 as the Lgp2 repressor domain binds the RIG-I repressor domain and thus suppresses (Rothenfusser et al. 2005). However, a recent study has shown that Lgp2 was required for both RIG-I and MDA5 signalling. It was also reported that Lgp2 knockout mice were more susceptible to infection with encephalomyocarditis virus (a picornavirus) as plasmacytoid dendritic cells showed impaired production of type I IFN (Satoh et al. 2010). Originally, it was thought that both RIG-I and MDA5 were functionally redundant and detected poly I:C (Kang et al. 2002; Brikos and O'Neill 2008). However, the development of RIG-1 and MDA5 knockout mice allowed the individual function of each RLH to be elucidated. In RIG-I knockout mice, the production of IFN was reduced in dendritic cells infected with virus and vesicular stomatitis virus (Kato et al. 2005). The same study also reported that IFN production in plasmacytoid dendritic cells was not dependent on RIG-1 but on TLRs. Studies in MDA5 knockout mice have shown that MDA5 mediates IFN responses to picornaviruses and poly I:C (Gitlin et al. 2006; Kato et al. 2006). RIG-I and MDA5 have been shown to detect different lengths of dsRNA. In virus-infected cells, long dsRNA (I1 kb) induces IFN via MDA5, whereas short dsRNA (B1 kb) induces IFN via RIG-I (Kato et al. 2008). RIG-I has also been shown to detect 5′-triphosphate RNA that is generated during the replication of some viruses, this 5′ modification also allows RIG-I to distinguish cellular RNA from viral RNA (Hornung et al. 2009; Pichlmair et al. 2006). A recent in vitro assay has shown that a recombinant RIG-I protein bound to short (25 bp) dsRNA with a 5′ or 3′ phosphate group as well as ssRNA with a 5′-triphosphate group (Kawai et al. 2005; Takahasi et al. 2008). IPS-1 consists of an amino terminal CARD domain, a central region that is proline rich and a carboxyl terminal containing a transmembrane domain that anchors IPS-1 to the mitochondrial membrane. IPS-1 activates IKKi and TBK1 (Seth et al. 2005). These kinases phosphorylate NFkB, IRF3 and inflammatory cytokines that RIG-I associates with the actin cytoskeleton through the CARD domain, where its expression is localised to membrane ruffles in non-polarised Caco-2 cells. In polarised cells, RIG-I expression was localised to the apico-lateral cell junctions (Mukherjee et al. 2009). Actin depolymerisation resulted in RIG-1 activation and the induction of IRF3 and NFkB (Mukherjee

et al. 2009). MDA5 was expressed in the cytoplasm and was not found to associate with actin (Mukherjee et al. 2009).

2.15 Gastrointestinal Immune System and Inflammatory Bowel Disease

Inflammatory bowel diseases (both Crohn's disease and Ulcerative Colitis) are chronic idiopathic, inflammatory, immune-mediated disorders of the intestine characterised by diarrhoea, rectal bleeding, abdominal pain, fever and weight loss. The average age of onset is late teens to early twenties. Lesions are characterised histologically as immune-mediated pathology with large numbers of infiltrating polymorphonuclear leukocytes, monocytes and activated lymphocytes. It is generally believed that the gut inflammation is driven through a dysregulated immune response to commensal or "normal" (non-pathogenic) flora (Blumberg 2006; Kaser et al. 2010). What triggers this dysregulated immune response after 20 years of normal regulation? We cannot rule out a de novo gastrointestinal infection as a trigger. It is clear that the maintenance of the integrity of the mucosal barrier is essential for the prevention of dissemination of gastrointestinal pathogens and normal commensal flora to systemic sites. Several subsets of T cells in the gut have an important barrier-promoting role. These include gamma delta T cells, Th17, T cells, natural killer NK and NK T cells. These cells produce a range of cytokines, including IL17A, IL17F, IL22 and IL26 that induce antimicrobial proteins, e.g., defensins and chemokines strengthening the mucosal barrier (Blaschitz and Raffatellu 2010). However, when intra-vascular T cells are induced to traffic to the GALT or mesenteric lymph nodes subsequent to invasive infection, they undergo antigen priming and activation. They become polarised and expand yielding effector cells to destroy invading microorganisms in an inflammatory milieu. Critically, this response must be effectively down-regulated on elimination of the infection to prevent these antigen induced effector T cells from maintaining and promoting chronic intestinal inflammation. Once naive-T cells are polarised to their Th1/Th17 phenotype in the absence of down-regulation, they are capable of perpetuating inflammatory bowel disease (Koboziev et al. 2010). Probiotics have been shown to engage both the innate and adaptive immune responses in in vitro and in animal models in a strain-specific manner (Petrof 2009; OMahony et al. 2001) and link this effect to cytokine balance (Mc Carthy et al. 2003). *Lactobacillus* UCC118 was also shown to attenuate arthritis in a murine model (Sheil et al. 2004). Feeding mice with the probiotic *Bifidobacterium infantis* 35624 mediated profound inhibition of salmonella infection and LPS-induced NFkB activity. *B. infantis* increased $CH4^{+}$ $CD25^{+}$ $Foxp3^{+}$ regulatory T cell numbers in the mucosa and spleen of conventionally colonised mice (O'Mahony et al. 2008). Mazmanian also demonstrated the ability of a commensal *B. fragilis* to induce Treys in germ-free mice. This finding demonstrates the ability of a highly selected probiotic when consumed orally

to engage and modify both the mucosal and systemic immune system (spleen) in a fully immunocompetent animal. In human trials, probiotic consumption has also been shown to modulate both the mucosal and systemic immune systems. Humans consuming L. *salivarius* UCC118 showed significantly increased granulocyte phagocytic activity when compared to baseline levels and placebo-fed controls. Subjects also produced an increase in mucosal IgA antibodies to the *Lactobacillus* UCC118 strain (Dunne et al. 1999). Other probiotic preparations, e.g., VSLH3 *E. coli* Nissle 1917 and *Lactobacillus reuteri*, have also been shown to attenuate colitis in humans and in murine models (Petrof 2009). It is clear that oral consumption of highly selected probiotic strains can engage even in the presence of a normal microbiota. How could oral probiotic consumption be immunologically perceived in the presence of the overwhelming numbers of the commensal microbiota. Oral consumption of a bolus of 1010 probiotic bacteria that can survive gastric acid and bile would provide a dominant microbiota in the almost sterile small bowel that houses a significant amount of the human immune system with multiple sampling sites.

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Chapter 3
Immunomarkers for Detection of GI Malignancies

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Abstract Gastrointestinal (GI) cancers are related to several diseases of the GI tract including adenocarcinomas of the esophagus, stomach, colon, and rectum, which are among the leading cause of morality worldwide. In spite of rapid development in molecular and genomic techniques, prognosis of the malignant potential of GI cancers is challenging. Immunomarkers may play an important role in the prediction of malignant behavior of these cancers. In this chapter, we have made an attempt to provide a comprehensive review on immunomarkers which are discovered recently and used in the detection of GI malignancies. These immunomarkers can help clinicians in the early diagnosis and as therapeutic targets to treat GI cancers.

Keywords Immunomarkers · Esophageal cancer · Gastric cancer · Colorectal cancer · Carcinoembryonic antigen · Vascular endothelial growth factor

3.1 Introduction

Gastrointestinal (GI) cancers are considered to be an overwhelming global health issue, and are among the principal cause of morbidity and deaths worldwide. GI cancers are associated with malignancies emerging in the esophagus, stomach, intestine, colon, and rectum. The two primary GI malignancies are gastric and colorectal cancers, which affect around 1.4 million and 9.5 lakh deaths, respectively, per year (Vedeld et al. 2018). Epigenetic alterations are found to be responsible for cancer initiation and progression in GI cancers. DNA methylation is the most

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common unusual epigenetic change involved in these tumors (Portela and Esteller 2010). They include both benign as well as malignant forms and represent an array of malignant potential projected primarily by the size and mitotic activity. Computed tomography, endoscopy, and colonoscopy are the gold standards for early detection of these cancers. However, these methods are invasive, inconvenient, expensive, and are hindered by low compliance rates (Taylor et al. 2011). Non-invasive diagnostics such as blood-dependent assays have the ability to improvise the patient amenability in relation to invasive methods. Immunomarkers are disease-specific and biologically related to the pathophysiological development process of the disease. Further, exclusive diagnostic molecular techniques and skilled labor are avoided, and there is no need for tissue processing when compared to genetic markers. In this chapter, we have emphasized the significance of promising immunomarkers for the initial detection and prevention of GI malignancies, as represented in Table 3.1.

3.2 Esophageal Cancer Immunomarkers

Esophageal cancer (EC) is the fourth most evident cancer in males with a high mortality rate worldwide (Chava et al. 2012). The histological subtypes of EC found globally are adenocarcinoma and squamous cell carcinoma, which may vary depending on lifestyle, genetic susceptibility, and various environmental factors (Di Pardo et al. 2016). EC is normally diagnosed in the middle-late situation, thereby extending treatment duration resulting in reduced survival rate. Recent evidence suggests that EC tissues abnormally express different molecules that help in the detection, diagnosis, and treatment of EC.

Epidermal growth factor receptor (EGFR) is a membrane protein exhibiting tyrosine kinase activity. Enhanced EGFR levels have been reported in EC, which is associated with disease progression, tumor metastases, and can be used to forecast patient prognosis. HER2, belonging to the EGFR family, also performs a crucial role in the treatment of EC. Chan et al. (2012) reported a decreased survival rate with positive expression of HER2. Besides these receptors, E-cadherin (cell adhesion molecule), α-catenin, and β-catenin (cytoskeleton linking molecules) are the three important proteins having promising prognostic importance in EC. Increased expression of these proteins is directly proportional to the increased survival of patients. The ACTN-4 (α-actinins) are actin-binding proteins that contribute a significant role in cell–cell adhesion, fiber formation, and maintaining cell structure. Laminin is a glycoprotein of the basement membrane, which mediates several biological functions that are facilitated via interaction with cell-specific membrane receptors. 67 kDa laminin receptor has been reported to be overexpressed by cytokines, extracellular matrix proteins, and inflammatory agents. Fu et al. (2007) demonstrated that the upregulation of these two proteins could be employed for predicting the stage of tumor and patient prognosis in esophageal squamous cell carcinomas (ESCC).

Table 3.1 Immunomarkers for gastrointestinal cancers

Cancer type	Biomarkers	Specimen	Methodology
Esophageal cancer	EGFR	Tissue	IHC
	ACTN-4	Tissue	2-DE
	Cox-2	Tissue	IHC
	VEGF	Tissue	IHC
	Laminin	Tissue	2-DE
	TGF-β receptors	Tissue	IHC
	Cyclin D1	Tissue	IHC
	Cyclin E1	Tissue	IHC
	MCM4	Tissue	IHC
	MCM7	Tissue	IHC
Gastric cancer	HER	Tissue	IHC
	VEGF	Tissue	IHC
	YB-1	Tissue	IHC
	SAT-B2	Tissue	WB
	IL-1b, IL-8, and TFF-α	Blood sample	ELISA
	CEA, CA19-9, and CA72-4	Tissue	IHC
	bcl-2 and bax	Tissue	IHC
	DKK3	Tissue	IHC
Colorectal cancer	CEA	Tissue	RT-PCR
	CYRA 21-1	Serum	ELISA
	Osteoprotegerin	Tissue	IHC
	TIMP	Serum	ELISA
	ER-β	Tissue	IHC
	IGF	Tissue	IHC
	P^{53}	Tissue	IHC
	Ki 67	Tissue	IHC
	Cyclin D1	Tissue	IHC
	IL6	Serum	ELISA

IHC Immunohistochemistry, *2-DE* Two-Dimensional Gel Electrophoresis, *WB* Western Blotting

Angiogenic factors, like vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (Cox-2) are potential prognostic indicators in patients with EC. Studies have reported an association between enhanced VEGF and COX-2 expression with patient survival and cancer stage (Kulke et al. 2004; Prins et al. 2012).

Besides these two angiogenic factors, TGF-β receptors also perform a vital function in the progression of tumor by inhibiting epithelial cell proliferation. Decreased expression of these receptors in ESCC is related to enhanced tumor growth, metastasis of lymph node, and poor prediction (Fukai et al. 2003).

The p53 protein is a DNA-binding protein which plays a vital role in tumorigenesis as it regulates cell growth, apoptosis, and angiogenesis. The reduced expression of p53 protein is linked with a better prognosis, as reported by Ikeguchi et al. (2000).

Survivin, an inhibitor of apoptosis, is proven as an efficient marker for the detection of cancer, its diagnosis and prediction of outcome. Studies by Rosato et al. (2006) discovered that the expression of surviving could be a predictive factor only in ESCC.

Cyclin D1 and E1 are vital proteins that play a major function in cell cycle, and the increased levels of these two proteins have been reported in various tumor tissues (Zhao et al. 2015). The upregulation of cyclin D1 protein may offer vital prognostic evidence and for determining the optimal therapeutic approach for EC (Nagasawa et al. 2001).

The minichromosomal maintenance (MCM) protein family comprises six proteins playing a vital part in DNA replication (Frigola et al. 2013). Findings of Choy et al. (2016) showed increased expression of MCM4 and MCM7 in EC. These two proteins serve as significant proliferation markers for the assessment of EC.

3.3 Gastric Cancer Immunomarkers

Gastric cancer (GC), a global health issue, is one of the prominent causes of cancer-associated death globally (Wu et al. 2015). Presently, the tumor staging system and histologic cataloging are used for routine prognosis and treatment among GC patients but lacks substantial prognostic value (Jiang et al. 2018).

The human epidermal growth factor receptor (HER) family includes four different receptors, HER 1,2,3, and 4. HER2 is a well-established marker for GC, and these receptors work together in the maintenance of various functions like cell division, differentiation, and survival. This receptor family is involved in the progression of diverse tumor types and is documented targets for multiple cancers therapy (Lastraioli et al. 2012).

The vascular endothelial growth factor (VEGF) family serves a pivotal role in processes including inflammation, vascular regeneration, and angiogenesis. This family comprises of VEGF-A, B, C, D and E, among which VEGF-A has long been recognized as an important regulator in tumor angiogenesis (Ferrara et al. 2007). Although this family has been regarded to effect tumor-linked angiogenesis, the prognostic implication of VEGF expression is still debatable in GC.

Y-box binding protein-1 (YB-1) is a versatile protein associated with angiogenesis, proliferation, and aggressiveness of cancer cells. Its upregulation in different cancers was connected with adverse patient diagnosis. Studies have demonstrated that YB-1 might be an essential biomarker for the management of GC patients (Wu et al. 2015).

Special AT-rich sequence-binding protein 2 (SATB2) is a nuclear transcription factor involved in transcription and chromatin remodeling. Wu et al. (2016) reported that SATB2 expression was reduced in GC tissues. The decreased expression of SATB2 is correlated with a shortened lifespan of GC patients.

The most widely used serum-based tumor markers in GC are carcinoembryonic antigen (CEA), CA 72-4, and cancer-related antigen 19-9 (CA 19-9). Majority of the

studies suggested that CA 72-4, a glycoprotein present on the tumor cell surface, is the most potential marker for detecting GC. Other important serum markers for GC detection are alpha-fetoprotein, CA125, and sialyl Tn antigens (Shimada et al. 2014). Apart from traditional serum markers, studies have shown that interleukin 1b (IL-1b), IL-8 and tumor necrosis factor-α (TNF-α), may be regarded as reliable markers in screening and prognostic assessment of gastric carcinoma (Macrì et al. 2006).

Cyclooxygenase-2 (COX-2) performs an essential function in carcinogenesis and inflammation. Studies suggest that they are involved in angiogenesis, metastasis, invasion, proliferation, and apoptosis (Wang and Du Bois 2006). Mrena et al. (2010) reported that COX-2 expression could be used as a valuable indicator for aggressive tumor growth in GC.

bcl-2 family comprises bcl-2 and bax that play a remarkable role in apoptotic regulatory mechanism. The bcl-2 expression can serve as one of the important biomarkers for predicting the prognosis of GC patients and may be a candidate for detecting the different stages of cancer apart from conventional sources (Liu et al. 2011).

Dickkopf-related protein 3 (DKK3) may act as a tumor inhibitor, and its expression is reduced in different cancer types. Park et al. (2015) reported decreased DKK3 protein level in GC patients, which can be employed as a prognostic indicator.

3.4 Colorectal Cancer Immunomarkers

Colorectal cancer (CRC) is a common cancer worldwide affecting both men and women, accounting for around 700,000 deaths annually (Arnold et al. 2017; Guo et al. 2018). It is a heterogeneous disease varying in clinical presentation and molecular characteristics (Linnekamp et al. 2018). The well-established tools for screening include colonoscopy, flexible sigmoidoscopy, and fecal occult blood analysis (Kolligs 2016). Biochemical indicators for CRC are potentially helpful in screening, diagnosis, and prognosis of the disease. Although numerous markers have been defined for CRC, confusion persists with regard to the usefulness of these markers (Duffy et al. 2003).

Carcinoembryonic antigen (CEA) has been regularly employed as a serum-based marker to identify CRC metastasis/recurrences. Moreover, its detection as a marker in tumor samples with prognostic evidence has not been well established. Apart from CEA, sialyl Lewis antigen (sLex), a glycoprotein found on the cell surface may act as a ligand for endothelial leucocyte adhesion protein facilitating the interaction between tumor cells and endothelial cells. The upregulation of this glycoprotein is related to augmented tumor cell progression (McLeod and Murray 1999; Crawford et al. 2003). Cytokeratin 19 fragment (CYRA 21-1) is also an established marker that exhibited a better sensitivity in stage IV when compared to the early stages of CRC (Wild et al. 2010).

Tissue inhibitor of metalloproteinase 1 (TIMP-1), a glycoprotein that triggers cell growth, inhibits apoptosis, and suppresses metalloprotease activity (Duffy et al. 2003). The TIMP-1 content was found to be elevated significantly in CRC (Holten-Andersen et al. 2002). Studies by Birgisson et al. (2018) analyzed 92 plasma proteins in CRC patients, among which osteoprotegerin was found to be a potent predictive marker and can be a possible target for disease management.

The level of estrogen receptor β (ER-β) is related to patient prognosis and as an important marker of tumor development and a possible target for chemoprevention in CRC patients (Filho et al. 2018). Insulin-like growth factor (IGF), 1, 2, and 1R regulate cell metabolism and play a remarkable role in apoptosis and proliferation. Recent evidence revealed enhanced levels of IGF-1 and 2 in the initial identification of CRC pathogenesis (Peters et al. 2003).

Interleukin-6 (IL-6) is a pro-inflammatory cytokine involved in many biological functions. It is synthesized by various cell types and performs a principal role in immunity, metabolism, hematopoiesis, angiogenesis, neuronal development and inflammation. Xu et al. (2016) reported the prognostic and diagnostic significance of serum IL-6 in CRC and reported that IL-6 could be used as a valuable biomarker that could distinguish the CRC patients from healthy persons (Vainer et al. 2018).

p53, a tumor suppressor gene, codes for a transcription factor that governs the expression of regulatory genes related to angiogenesis, apoptosis, and cell cycle. It has been extensively used as an indicative and prognostic therapeutic marker for CRC (Duffy et al. 2007). bcl-2 is an antiapoptotic protein that arrests cell death in normal and tumor cells. Many investigators have found a strong correlation between bcl-2 expression and CRC prognosis (Bosari et al. 1995). Ki-67 and Cyclin D1 are the critical proteins associated with cell cycle regulation. However, there is no correlation between the upregulation of these proteins with prognosis in CRC (Hilska et al. 2005).

Transforming growth factor (TGF) family comprises α and β subgroups, which promotes the growth and proliferation of colon cancer cells. In addition to TGF, EGFR expression also has a prognostic significance in CRC.

Apart from above-discussed markers, recent evidence has suggested that hormones also act as an important biomarker for cancer detection. Adipocytokines, such as resistin, visfatin, leptin, and adiponectin are adipocyte-secreted hormones associated with colorectal malignancies. Studies by Nakajima et al. (2010) documented that resistin, visfatin, and adiponectin levels may be good indicators of CRC stage progression. Glycoprotein hormone hCG beta upregulation has been documented in CRC patients (Lundin et al. 2000). Studies by Pressler et al. (2011) reported that organic anion-transporting proteins are significantly elevated in colon cancer and can be used as a biomarker for chemotherapy management. Prolactin, a pituitary gland hormone, is overexpressed in CRC. Soroush et al. (2004) demonstrated that prolactin could be a better tumor indicator than CEA in CRC patients.

3.5 Concluding Remarks

In conclusion, the identification of novel tumor markers is one of the thrust areas of cancer research. The overall review of the biomarkers in this chapter could throw light on the recognition of efficient biomarkers for GI cancers, at different stages of developmental and extent of malignancy. Further, it is essential to know about clinical aspects of these biomarkers to have a better understanding of their physiological, pathophysiological, and biochemical aspects. However, knowledge in relation to tumor markers has to be constantly updated with respect to advances in clinical manifestation and medicine.

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Conflicts of Interest The authors declare no conflict of interest.

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Chapter 4
Immunotherapeutics of Gastrointestinal Malignancies

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Abstract Gastrointestinal (GI) malignancies in humans are the most widespread cancers worldwide. Surgical resection and radio or chemotherapy offer the primary line of the treatment strategy for patients suffering from GI malignancies. However, the majority of the patient population receives only palliative care due to a lack of poor or timely diagnosis. Thus it is imperative to establish novel treatment strategies for patients suffering from advanced metastasis or GI malignancies. Immunotherapy evolved as one of the most efficient strategies for the treatment of various cancers including GI malignancies. This chapter discusses the critical checkpoints of tumor escape from the immune system with an emphasis on novel immunotherapeutic strategies and potential drug target markers of the immune system for GI malignancies.

Keywords Gastrointestinal cancers · Immune checkpoint inhibitors · Humanized antibodies · Vaccines · Adoptive T-cell transfer · Clinical trials · Immunotherapy

Abbreviations

ACT	Adoptive T-cell transfer
APCs	Antigen-presenting cells
CAF	Cancer-associated fibroblast
CAR T-cells	Chimeric antigen receptor expressing T-cells
CD	Cluster differentiation
CRC	Colorectal cancer
CRISPR/Cas9	(Clustered regularly interspaced short palindromic repeats/ CRISPR associated protein 9)
CTLA4	Cytotoxic T-lymphocyte associated protein-4

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DCs	Dendritic cells
dMMR	DNA mismatch repair deficient
FDA	Food and Drug Administration, USA
GC	Gastric cancer
GEJC	Gastroesophageal junction cancer
GI	Gastrointestinal
HCC	Hepatocellular carcinoma
HLA	Human leukocyte antigen
IL-2	Interleukin-2
kg	Kilogram
MAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cell
mg	Milligram
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK cells	Natural killer cells
ORR	Objective response rate
PD-1	Programmed cell death receptor-1
PD-L1	Programmed cell death receptor-1 ligand
TCR	T-cell receptor
TGF	Transforming growth factor
TILs	Tumor-infiltrating lymphocytes
VEGF	Vascular endothelial growth factor

4.1 Background

4.1.1 Tumor Escape: A Rate Limiting Step in GI Malignancies

Certain molecules of the immune system serve as critical checkpoints or biomarkers for tumor microenvironment. Increased expression of immune checkpoint markers such as receptor for programmed cell death protein-1 (PD-1) or its ligand PD-L1/2 (CD274/CD273), and cytotoxic T-lymphocyte associated protein-4 (CTLA4) either suppresses the immune system or induces tolerance that results in cancer cells to be recognized as self and to escape from the host immune system (Yaghoubi et al. 2019). Thus tumors develop various strategies to invade the host immune system. A precise understanding of the molecular mechanisms underlying tumor escape may help to identify potential targets for developing immune therapy against GI malignancies. Usually, foreign antigen peptides are presented on the T-cell surface by

antigen-presenting cells (APCs) with the help of major histocompatibility complex to discriminate self vs. non-self. Of note, this phenomenon is negatively regulated by co-inhibitor molecules such as PD-1 and CTLA4 in the case of cancer tumors (Yaghoubi et al. 2019; Harris and Drake 2013; Goode and Smyth 2016).

CTLA-4 binds a cluster of differentiation (CD) 80/86 on APCs by competing with CD28 to inhibit the activation of T-cells (Harris and Drake 2013). Specific expression of PD-1 on activated CD8+T-cells interacts with PDL-1/2 on the APCs, for example, dendritic cells (DCs) and macrophages of tumor targets. An increase in PD-1 expression results in exhaustion of T-cell that curtails the primary line of defense mechanism against tumors (Kamphorst et al. 2017). Therefore, pharmacological inhibition of the PD-1 receptor might help to provoke the second line of immune response beyond the lymphoid tissue. Overall, CTLA-4 serves as a key checkpoint inhibitor in lymphoid tissue, whereas PD-1/PDL-1 as a peripheral checkpoint (Yaghoubi et al. 2019).

4.2 Immunotherapy Against GI Malignancies

4.2.1 Pharmacological Manipulation of PD-1 and CTLA-4 and Its Clinical Significance

The efficacy of immune therapy by targeting pathways such as CTLA4, PD-1/PD-L1 in various cancers extensively investigated in clinical settings (Myint and Goel 2017; Pardoll 2012). For example, in patients subjected to PD-1 therapy (sample size about 300), the tumor size reduced significantly in melanoma, kidney, and lung cancers by 31%, 29%, and 17%, respectively (Topalian et al. 2012). In the current chapter, we highlighted some of the important outcomes of clinical studies aiming for PD-1 and CTLA4 pathway in various GI malignancies (Table 4.1). Colorectal cancer (CRC) stands the fourth leading cause of cancer deaths worldwide (Dekker et al. 2019; Overman et al. 2017). Dysfunction of DNA mismatch repair (dMMR) system associated with microsatellite instability serves as a good biomarker for prognosis of early CRC but not in patients with advanced or metastatic CRC (Overman et al. 2017; Zhao et al. 2019). Of note, blocking PD-1 in such patients with a monoclonal antibody (MAb) nivolumab (pretreatment@3 mg/kg) provides robust disease control against metastatic CRC and dMMR (Overman et al. 2017). Combination treatment with nivolumab and ipilimumab (dosage at 1 and 3 mg/kg respectively) in gastric cancers (GC) showed a good response (~24%), particularly in PDL-1 negative patients (Checkmate 032 study) (Janjigian et al. 2016). Further, humanized MAbs against PDL-1 such as avelumab showed durability of response condition when administered as primary therapy in the advanced stage and as supportive treatment followed by first-line chemotherapy in GC (Chung et al. 2016). Consistent with the

Table 4.1 Approved immune checkpoint and other inhibitors for clinical use in GI cancers

Drug target	Drug used	Type of GI malignancy	Clinical Outcome/ Trial	Reference	Current status
PD-1	Pembrolizumab (MAb)	GCs; solid tumors with dMMR associated microsatellite instability-high	Manageable toxicity; Antitumor activity (KEYNOTE-059)	Le et al. (2017), Fuchs et al. (2017)	FDA approved
PD-1	Nivolumab (MAb)	HCC; CRC with dMMR associated microsatellite instability-high	Manageable safety profile; durable ORR and disease control (CheckMate 142; 040)	Overman et al. (2017), Khoueiry et al. (2017)	FDA approved
PD-1	Nivolumab (MAb)	GC or GEJC	Survival benefits in patients previously undergone heavy chemotherapy (ONO-4538-12, ATTRACTION-2)	Kang et al. (2017)	JAPAN approved
VEGF	Bevacizumab (MAb)	CRC	Survival in combination with chemotherapy	Hurwitz et al. (2004), Nienhüser and Schmidt (2017)	FDA approved
Multi-kinase inhibitors	Sorafenib (Nexavar) Lenvatinib	HCC (unresectable)	Longer survival (REFLECT for Lenvatinib)	Lang (2008), Personeni et al. (2019)	FDA approved
VEGF receptor 2	Ramucirumab (MAb)	GC	Second line of treatment for advanced GC (REGARD and RAINBOW)	Nienhüser and Schmidt (2017)	FDA approved

up-regulation of PDL-1, a phase 1b clinical trial (KEYNOTE-012) reported the efficacy and safety of pembrolizumab (anti-PD-1 MAb) that showed antitumor activity and improved outcome in PDL-1 positive patients with recurrent/metastatic adenocarcinoma of the gastroesophageal junction (Muro et al. 2016; Joshi et al. 2018). The overall response of PD-1 MAb treatment seems promising when compared to PDL1 MAb therapy in patients with GC.

Sorafenib, a multi-kinase inhibitor is the only drug of choice approved for treating advanced hepatocellular carcinoma (HCC), however with poor outcomes in clinical settings (Llovet et al. 2008; Khoueiry et al. 2017). CheckMate 040 (phase 1/2 clinical trial) evaluated the efficacy of nivolumab (MAb that blocks PD-1 activity)

in advanced HCC patients (Khoueiry et al. 2017). Administration of nivolumab@3 mg/kg showed promising results in terms of safety and the addition of new complications in patients with advanced HCC. Durable objective responses conferred the potential of nivolumab for the treatment of HCC (Khoueiry et al. 2017). Of note, the use of nivolumab has been approved by the FDA for clinical use in patients with HCC subjected to sorafenib treatment earlier (Hazama et al. 2018). Thus PD-1 appears as a potential drug target to establish novel therapeutic strategies for GI malignancies.

Further, tremelimumab (MAb that blocks CTLA-4) assessed for its efficacy in a clinical trial (phase-II) that included patients ($n = 18$) with metastatic gastric and esophageal adenocarcinomas (Ralph et al. 2010). Tremelimumab administered as a secondary treatment once every three months until the disease progression, but only one individual who benefited from the treatment group with significant durability. Thus it suggests further studies are required to establish CTLA-4 targeted immunotherapy, perhaps in combination with other drugs. Targeting immune checkpoints leads to immune suppression by secreting cytokines, e.g., transforming growth factor β, interleukin-6, prostaglandins, and regulatory T-cells that induce T-cells with antitumor activity in the lymph node. Thus altering tumor microenvironment helps to establish successful immunotherapy against GI malignancies (Hazama et al. 2018). Tumors with massive infiltration of CTLs (known as "hot tumors") may have a very good response against immune checkpoint inhibitors alone, without any immune suppression. While tumors with high immunogenicity and suppressive immunity (known as "dark tumors") and tumors with low immunogenicity with suppressive immunity and immune exhaustion (known as "cold tumors") require a combinatorial therapeutic approach to regulate immunosuppressive mechanism and to augment the immunogenicity of the tumor microenvironment.

4.3 Adoptive T-cell Transfer (ACT)

Earlier, in 1984 Mule et al. suggested that administration of lymphokine-activated killer cells in the combination of recombinant interleukin-2 at higher dose resulted in the poor outcome against metastatic GI cancer (Mule et al. 1984). Later the concept of ACT has become a promising immunotherapy approach for cancer treatment. The ACT includes TILs (tumor-infiltrating lymphocytes), TCRs (T-cell receptor T-cells), and CAR T-cells (chimeric antigen receptor expressing T-cells), which are now being commercialized by some pharmaceutical and biotechnology companies (June et al. 2018).

Both the TILs and natural killer (NK) cells have got prognostic relevance in GC (Dolcetti et al. 2018). Treatment with TILs isolated from a metastatic lymph node in combination with recombinant interleukin-2 in unresectable advanced GC patients ($n = 23$) showed a complete reduction of tumor focus in three patients (i.e., 13.0%) and partial reduction in five patients (i.e., 21.7%), while the others with no response to the treatment (Xu et al. 1995). The expansion of NK cells in combination with

OK432 (produced from Streptococcus pyogenes of human origin) and IL-2 showed a better outcome in advanced GC and unresectable patients (Dolcetti et al. 2018). In the case of TCRs, the infiltrating capacity of TCR repertoires of gastric pancreatic lesions gradually increased during gastric malignant transformation (Kuang et al. 2017).

The role of CAR T-cells demonstrated in chronic lymphoid leukemia (CLL), which has shown durable effects. Targeting CD19 elicited a specific immune response in the bone marrow through cytokine release, ablation of CLL cells with concomitant infiltration of CAR T-cells (Porter et al. 2011). To date, very limited clinical data is available on the role of CAR-T therapy related to GI cancers. The phase I clinical trial (dose escalation) of CAR-T therapy showed better tolerance in patients with metastatic CRC administered in high doses (Zhang et al. 2017). Similarly, a phase I clinical trial on CAR-T immunotherapy has a promising outcome in biliary tract and pancreatic cancer patients positive for human epidermal growth factor receptor 2 (Feng et al. 2018). Thus CAR-T immunotherapy emphasizes that there is an emerging need for precise investigation on the clinical efficacy of CAR-T immunotherapy aiming GI malignancies including other solid tumors.

4.4 Vaccines

Conventional chemotherapy and radiotherapy show partial efficacy and high toxicity in cancer patients. Therefore, alternative therapeutic strategies such as immunotherapy have been explored, which showed good efficacy and tolerance against various cancers including GI malignancies. Increased understanding of the molecular basis of tumor biology in the recent past has prompted the development of vaccines against GI cancers (Hazama et al. 2018).

Cancer vaccines stimulate humoral (antibody-mediated) or cell-mediated immune responses against tumor cells. The tumor antigens must be processed and presented in the form of peptides by the APCs to induce T-cell response (Rahma and Khleif 2011). Therefore, antigens can be administered by vector-mediated (viruses and nucleic acids) in the form of peptides, whole proteins, and recombinant proteins. DCs are potent APCs in generating specific primary T-cell responses that can be used as adoptive immunotherapy. As combination therapy for pancreatic cancer, adoptive T-cell therapy tested using matured DCs transfected with either mucin-1 mRNA or peptide and CTLs along with gemcitabine (promotes antitumor activity) drug treatment proved to be effective clinical settings (Shindo et al. 2014). Further, treating HCC patients (viral infection-related) with heat shock protein 70 mRNA transfected DCs showed better efficacy without any significant side effects (Maeda et al. 2015).

Peptide-based vaccines seem to play a critical role in modulating advanced GCs. For instance, the OTSGC-A24 peptide vaccine clinically tested in GC patients particularly in those positive for HLA-A*24:02 haplotype (Sundar et al. 2018). Administration of OTSGC-A24 combined vaccine (sub-cutaneous @1 mg dose

every 2 weeks) showed significant CTL responses and better tolerance (Sundar et al. 2018). Existing clinical data suggests that blockade of immune checkpoints may have beneficial outcomes based on immunogenicity and inflammatory responses. Thus, combination treatment with cancer vaccine and immune checkpoint inhibitors helps to develop novel effective therapeutics for GI cancers.

4.5 Conclusions and Future Perspective

Immunotherapeutic strategies emerged as most promising in the treatment of various cancers including GI malignancies (Fig. 4.1). The possible basis for successful immunotherapy is based on the fact, i.e., not to include patients with poor responses subjected to immunotherapy. Both the PD-1 and CTLA-4 negatively regulate immune response that helps tumor antigens escape from the host line defense mechanism and causes immune tolerance. So far, monotherapy with the humanized antibody pembrolizumab (targets PD-1 receptor) showed the most promising result in patients with advanced GC. Similarly, nivolumab plus ipilimumab combination

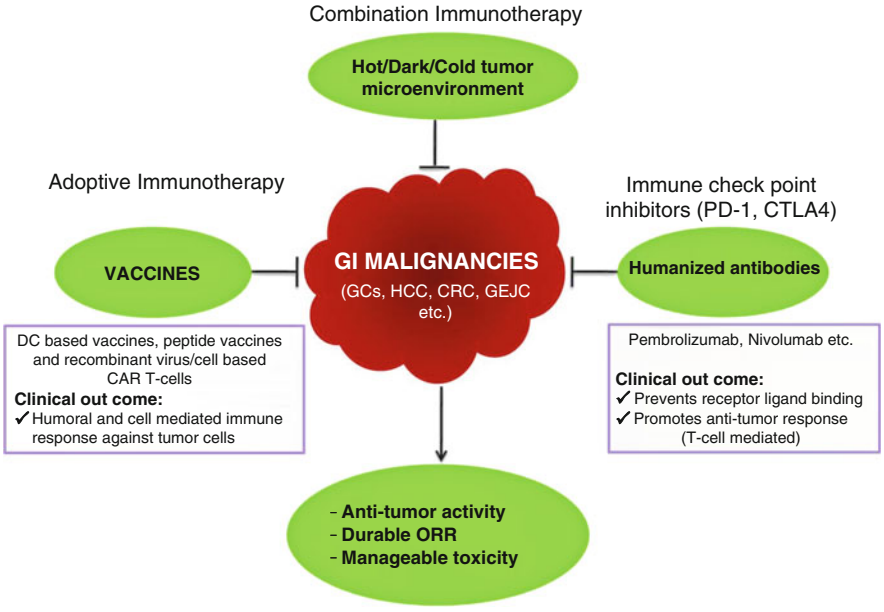

Fig. 4.1 Illustrate strategies of immunotherapy for various GI malignancies. Immune checkpoint inhibitors help to curtail tumor evasion from the host line of immunity and to recognize non-self-antigens. However, a combinatorial immunotherapeutic strategy seems to be instrumental depending on the tumor microenvironment. Adoptive cell immunotherapy (passive immunization with tumor-specific T-cells), DC-based vaccines for proper presentation of tumor antigens, enhancing NK cell activation, etc. are very promising for developing newer and effective therapeutic strategies for GI malignancies

also demonstrated a good response against CRC in clinical settings. Thus pharmacological regulation of immune checkpoint inhibitors seems promising drug targets to develop immune therapies against GI malignancies. However, some early phase clinical trials showed unsatisfactory results possibly due to tumor heterogeneity or the lack of effective host immune response. Altering the tumor microenvironment by inducing CTL (CD8+) infiltration without suppressing the immune system might respond well to immune checkpoint inhibitors. Suppressing the host immune response makes the tumors low immunogenic that eventually promotes tumor survival. Thus the cautious use of immune-suppressive drugs is warranted for developing immunotherapy against GI cancers. The partial improvement observed even after using immune checkpoint inhibitors in individuals with dMMR associated microsatellite instability-high possibly influenced by several factors within the tumor microenvironment such as insufficient newly formed antigens, increased burden of tumors, suppressed immune system, etc. The suppressive immunity caused by regulatory T-cells, IL-6, TGF-β, MDSC, and CAF can be regulated by using specific inhibitors (Hazama et al. 2018). Nonetheless, in-depth sequencing analysis of whole-exome/protein-coding regions would be helpful to develop precise immunotherapeutics for GI malignancies. A recent report suggests that TCR recognizes and kills most of the cancer cells via class I MHC related protein (Crowther et al. 2020). Thus future studies to establish novel immunotherapies for GI malignancies should also aim towards screening CRISPR/Cas9 based genome-wide screening.

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Chapter 5
Immune Cell Therapy Against Gastrointestinal Tract Cancers

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Abstract Gastrointestinal (GI) cancers are responsible for major cancer-related mortality around the world. It has imposed a substantial burden and pressure on the healthcare sector across the globe. Recently advancements in high throughput techniques provide us with a unique opportunity to detect biomarkers and treat various diseases, including GI cancer, more comprehensively. However, most of these approaches are ineffective for treating patients with advanced or metastatic stages. Additionally, these treatments have severe side effects on cancer patients. Thus, there is an urgent requirement to identify new drugs and innovative immune therapies for the treatment of GI malignancies. Considering this, recently developed immune cell therapy provides a unique opportunity for early detection and treatment of various cancers, including GI cancer. It controls cancer either by activating or suppressing the immune system of cancer patients. Recently, immune checkpoints approaches have also been employed in the treatment and prevention of cancer. However, various studies have reported that few of these therapies have side effects. Thus, these therapies must be employed with utter caution. Recently several studies have also proposed that the personalized immunotherapy approach can also be used for therapeutic cancer treatment with fewer side effects. Authors believe that by employing classical and advanced immunotherapeutic techniques together, we can easily diagnose and treat GI cancer in a more comprehensive way. In the near future, the information present in this chapter will be highly useful for the early detection and treatment of various cancers, including GI cancer.

Keywords Gastrointestinal cancers · Immune cell therapy · Personalized immunotherapy · Immune checkpoints · Monoclonal antibody

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Abbreviations

APCs	Antigen-presenting cells
ATCT	Adoptive T-cell therapy
BCG	Bacillus Calmette–Guérin
CTL	Cytotoxic T lymphocytes
CAR	Chimeric antigen receptor
CpG	Cytosine-phosphate-guanosine
G-CSF	Granulocyte colony-stimulating factor
GI	Gastrointestinal tract
GvHD	Graft versus host disease
NK	Natural killer
SIRPα	Signal regulatory protein alpha
TCR	Transgenic T-cell receptor

5.1 Introduction

Gastrointestinal tract (GI) cancer is one of the most important cancer types that are responsible for cancer-related mortality worldwide. The most common gastrointestinal tract malignancies are esophageal cancer, gastric cancer, colorectal cancer, liver cancer, and pancreatic cancer (Pourhoseingholi et al. 2015). At the initial stage, symptoms associated with GI cancers mostly remain unknown. However, in the case of a few cancers, namely, esophagus and stomach cancers, patients experience struggle in swallowing, abnormal bleeding, and digestive system associated problems. To date, several cancers treatment approaches have been designed for the early detection and prevention of GI malignancies. These approaches mainly comprised of surgery, chemotherapy, radiotherapy, and molecularly targeted therapy (Gupta et al. 2017, 2019a, b; Mallepalli et al. 2019). The accurate diagnosis of cancers generally requires endoscopy and followed by biopsy for suspicious tissues and cell identification. The tumor's location and the cancer cell type decide which approach may be employed for treatment. However, most of these approaches are ineffective for treating patients with advanced or metastatic stages. Additionally, these treatments have severe side effects on the health of cancer patients. Thus, there is an urgent requirement to identify new drugs and innovative immune therapies for the treatment of GI malignancies (Rao et al. 2019). Considering this, recently developed immune cell therapy provides a unique opportunity for early detection and treatment of various cancers, including GI cancer. It controls cancer either by activating or suppressing the immune system of cancer patients. In 2001, Masihi and the team reported that immunomodulatory therapy often has fewer side effects in comparison to existing chemo as well as drug therapy (Masihi 2001). The immune cell therapy works along with various immune effector cells, including lymphocytes, macrophages, "cytotoxic T lymphocytes" (CTL), "natural killer" (NK) cell, and dendritic

cells. Together they protect the body against various cancer types via targeting antigens that are expressed on the tumor cells surface. Several studies have also reported that immune therapies have been used effectively against several cancer biomarkers, for instance, interferons, and "granulocyte colony-stimulating factor" (G-CSF) (Rao et al. 2019; Moehler et al. 2016; Hazama et al. 2018; Zappasodi et al. 2018). Additionally, few other biomarkers, namely, IL-12, IL-7, IL-2, synthetic cytosine-phosphate-guanosine (CpG) oligodeoxynucleotides, several chemokines, as well as glucans were also employed in both pre-clinical and clinical studies (Moehler et al. 2016; Hazama et al. 2018; Hendry et al. 2017). In 2015, Fuge and the team reported that the "Bacillus Calmette–Guérin" (BCG) vaccine, which is widely employed against tuberculosis, can also be used for treating bladder cancer (Fuge et al. 2015). Similarly, monoclonal antibody rituximab, an anti-CD20, can also be used for treating various cancers (Pento 2017). In 2019, Tannapfel and Reinacher-Schick reported that cytokines, for instance, interleukin-2 or interferon-alpha, are widely used for treating various cancers (Tannapfel and Reinacher-Schick 2019). In 2018, James P. Allison and Tasuku Honjo had been awarded Nobel Prizes in Physiology or Medicine, "for their discovery of cancer therapy by inhibition of negative immune regulation" (Guo 2018). Considering this, in the present chapter, authors attempted to discuss the importance of different immune cell therapy and how these approaches can be used for the treatment of GI cancer. In the near future, immunotherapy may be the key answer for treating gastrointestinal tract cancers.

5.2 Immunotherapy Approaches

Cellular immunotherapy comprised of both active and passive immunotherapy approaches. Active immune cell therapy directly uses the immune system of the patient for attacking tumor cells by targeting tumor antigens. Passive immune cell therapies increase the existing anti-tumor responses of immune cells as well as tissues by using monoclonal antibodies, lymphocytes, and cytokines. Active cellular immunotherapies include the removal of cancer immune cells from cancer patient's bodies. These tumor-specific immune cells are subsequently grown within the culture and later injected to the cancer patient, where they attack tumor cells. Otherwise, the genetically engineering approach can also be employed for generating immune cells, which in turn can be used for expressing tumor-specific receptors. The genetically engineered tumor cells are cultured and later injected to the patient, which in turn acts against the tumor cells. Cell types that work against the cancer cells are NK cells, lymphokine-activated killer cells, dendritic cells, and cytotoxic T-cells (Hazama et al. 2018; Zappasodi et al. 2018). Passive immune cell therapy involves the introduction of either cytotoxic T lymphocytes or antibodies. Antibodies may function either in a specific or non-specific manner (Baxter 2014). Passive immune cell therapy usually targets the receptors present on the cell surface, including CD20, CD274, and CD279 antibodies. These antibodies, when attached with a cancer antigen, experience configurational changes, which in turn stimulate

"antibody-dependent cell-mediated cytotoxicity" and trigger the "complement system." This, in turn, inhibits the interaction between the receptor and its ligand and causes cell death (Espinoza-Sánchez and Götte 2019).

The most widely used immune cell types that are being employed in immunotherapies during various cancers, including gastric cancer, are dendritic cells, lymphocytes, myeloid-derived suppressor cells, natural killers, neutrophils, macrophages. These cells modulate the tumor microenvironment through the production of chemokines and cytokines. Unlike chemical drugs, cytokines can inhibit specific proteins that are responsible for inducing the inflammatory process. Additionally, with the advancement of high throughput technologies, it is relatively cheaper and faster to express and isolate highly purified recombinant proteins, e.g., cytokine. Thus, recently, cytokine therapy has also been utilized for increasing immunity against tumors (Rider et al. 2016). Two forms of cytokines, namely, interferons and interleukins, are commonly used in cancer treatment (Dranoff 2004). Interferons are encoded by the immune system, where they are generally involved in the anti-viral response. Interferons are also used against tumors. The interferons can be broadly classified into three groups, i.e., "type I (IFNα and IFNβ), type II (IFNγ), and type III (IFNλ)." The IFNα has been approved as a drug against AIDS-related Kaposi's sarcoma, hairy-cell leukemia, chronic myeloid leukemia, follicular lymphoma, and melanoma. Nowadays, researchers are also employing type I and II IFNs as the anti-tumor immune system, but the only type I IFNs have shown clinically useful (Rider et al. 2016; Lai and Dong 2016; Hegner et al. 2018; Lambertsen et al. 2019). Few studies have also reported that the type III IFNλ is potentially used for its anti-tumor response in animal models (Dunn et al. 2006; Lasfar et al. 2011). However, type II IFNs, namely, interferon gamma, show effective immune response only in those patients having bladder carcinoma and melanoma cancers (Razaghi et al. 2016). The most effective immune response was achieved when the patients were having ovarian carcinoma identified at second or third stages. In 2016 Razaghi and the team reported that anti-proliferative activity of IFN-gamma causes cell death or growth inhibition. This is generally induced through autophagy and apoptosis (Razaghi et al. 2016). Another study reported that interleukins have an array of immune system effects. Hence, interleukin-2 is usually used in renal cell carcinoma and malignant melanoma treatment (Dranoff 2004; Coventry and Ashdown 2012).

5.3 The Cell in Immunotherapy Approaches

As stated above, the most widely used immune cell types that are being employed in immunotherapies during various cancers, including gastric cancer are macrophages, dendritic cells, adoptive T-cells, neutrophils.

5.3.1 Macrophages

Macrophages are mononuclear phagocytic cells that play a key role in pro-inflammatory, homeostatic, and immune regulatory responses within tissues (Pahl et al. 2014). Based on signals, macrophages may be classified as either classical or alternative activation. While classical macrophages have anti-tumor activity, alternative macrophages have low tumoricidal activity. "Tumor-associated macrophages" (TAMs) are mostly situated within the tumor mass and thus play a key role in the intra-tumoral activity (Eyileten et al. 2016). Few authors proposed that TAM modulated "switch" among non-canonical and canonical Wnt signaling pathways may help in controlling cancers. Inhibition of canonical Wnt signaling pathway via proteins secreted by macrophages inhibits cancer. Nevertheless, it activates the non-canonical Wnt pathway, which in turn promotes cancer cell motility, invasiveness, and epithelial–mesenchymal transition. These authors also proposed that, in any way, we can modulate both canonical and non-canonical Wnt pathway, we can effectively control the formation of various cancers (Eyileten et al. 2016). In one study, Tseng and the team employed macrophages for enhancing immune response via T-cells (Tseng et al. 2013). Anti-CD47 antibody-modulated phagocytosis of cancer cells via macrophages enhances and reduces priming of $CD8^+$ and $CD4^+$ cells, respectively. This, in turn, reduces the level of regulatory T-cells. This causes reduced tumor mass in animals. The result obtained from that study also suggests that anti-CD47 antibody treatment is capable of both macrophage phagocytosis of cancer cells and initiation of anti-tumor cytotoxic T-cell immune response (Tseng et al. 2013). In another study, Pahl and the team tried to initiate anti-tumor activity of macrophages via modifying macrophage phenotype via IFN-γ and liposomal muramyl tripeptide (Pahl et al. 2014).

Since several studies have reported that M1-activates are clinically safe, M1-activated macrophages are also used as a delivery system (Griffiths et al. 2000; Muthana et al. 2011, 2013). For instance, Griffiths and the team reported that *CYP2B6* delivery via macrophage under the constitutive human cytomegalovirus promoter initiates a killing of tumor cells in the presence of cyclophosphamide (Griffiths et al. 2000). In another study, Muthana and the team developed a novel system that employed the infiltration of classically activated macrophages and restricted tumor growth (Muthana et al. 2011, 2013). Seo and the team developed stable macrophages of RAW264.7 cell line via genetically engineering approaches (Seo et al. 2012). Seo and the team employed these macrophages for delivering the prodrug-activating enzyme to the lung melanomas (Seo et al. 2012).

5.3.2 Dendritic Cell (DC)

In 1973, for the first time, Steinman reported that antigen-presenting cells play a key role in activating the adaptive immune system (Steinman and Cohn 1973). DCs are

the most potent APCs and can be generated from monocytoid or myeloid precursor cells present within bone marrow or peripheral blood (Okur and Brenner 2010). DCs, which are present throughout the body, keep continuous monitoring of antigens and harmful signals. Once activated, they experience maturation and travel to lymphoid organs, where they stimulate numerous effector immune cells, specifically B-cells and T-cells (Banchereau and Steinman 1998). Thus, DCs are very crucial for immunosurveillance, which in turn provides protection against pathogens and cancerous cells (Wirth et al. 2010). Nevertheless, this immunosurveillance sometimes fails to detect cancer cells at the initial stage. DC vaccination can correct this failure effectively via reversing the ignorance of the immune system towards malignant cells (Wirth et al. 2010). The main objective of the DC vaccination is to destroy tumor cells via the generation of functional antigen-specific T-cells (Draube et al. 2011). For enhancing maturation as well as activation of DCs, "cocktails" of various cytokines like *GM-CSF, IL-6, IL-1ß, TNF-α,* and *IL4* have been employed without or with prostaglandin E2 (Okur and Brenner 2010). With the help of these agents, monocytoid/myeloid DCs take up as well as present APCs more effectively, which in turn enhances expression of co-stimulatory bio-molecules, for instance, CD86, CD54, CD40, and CD80. Subsequently, they polarize the resultant immune response towards a T effector phenotype (Okur and Brenner 2010).

Production of DC vaccination follows a few basic principles. At first, natural circulating DC or monocytes are isolated from "autologous peripheral blood mononuclear cells." Monocytes undergo ex vivo differentiation to form DC. Later, DC-derived from monocytes as well circulating DC undergoes maturation, which in turn highly required for the activation of T-cell. After maturation, DC show increased expression of co-stimulatory molecules, MHC complexes I and II, and enhanced cytokine production capability. These processes are highly required for inducing immunity. During the manufacturing of this vaccine, DC is laden with appropriate tumor antigen(s) for producing a tumor-specific immune response within any patient. Subsequent to quality control, the vaccine is later introduced in the patient (van Willigen et al. 2018). However, this underlying protocol may vary during the process of manufacturing the DC vaccination. These variations may be in the culture methods, maturation methods, utility of DC subsets, used antigens, approaches of loading antigen, and administration route (van Willigen et al. 2018).

5.3.3 Adoptive T-cell

ATC is a form of "passive immunization therapy." In this therapy, transfusion of adoptive T-cells takes place from blood and tissues. They generally get activated when they come in contact with foreign protein or pathogens. These activated T-cells are called either "antigen-presenting cells" (APCs) or infected cells. They are present in both normal tissues and in tumor tissue. Within the tumor, this ATC is known as "tumor-infiltrating lymphocytes" (TILs). In 2012 Restifo and the team reported these cells could attack tumor cells. But within the tumor environment, they are highly

immune-suppressive and prevent immune-mediated tumor death (Restifo et al. 2012). Patients with cancer experience multiple ways to produce anti-tumor targeted T-cells. The tumor antigen-specific T-cells can be either discarded from blood or tumor samples. Subsequently, the initiation, as well as culture, is carried out via the ex vivo approach, with the results re-infused. Several researchers have also reported the T-cells initiation can also be carried out using gene therapy approaches and exposing T-cells to tumor-specific antigens. As of 2014, several ATC clinical trials were underway (Carroll 2013). Recently one important study revealed that "clinical responses can be obtained in patients with metastatic melanoma resistant to multiple previous immunotherapies" (Andersen et al. 2018). In 2017, the first two ATC, namely, axicabtagene ciloleucel and tisagenlecleucel, were approved via the FDA. Furthermore, the adoptive transfer of NK cells and haploidentical γδ T-cells from a healthy donor can also be employed in another approach. The main benefit of employing this therapy is that these cells do not cause "Graft versus host disease" (GvHD). However, this approach is often associated with abnormal function of the transferred cells (Wilhelm et al. 2014).

To date, several adoptive T-cell therapies, namely, TIL treatment and therapy with "chimeric antigen receptor" (CAR-T) and "transgenic T cell receptor" (TCR)-modified T-cells, have been utilized for the treatment of cancer. Out of all approaches, CAR-T is more effective (Magalhaes et al. 2019). In this immunotherapy, the T-cells are modified so that they can recognize cancer cells more efficiently and abolish them. T-cells are harvested from cancer-affected patients, and subsequently, CAR added to them using genetically engineered approaches. This, in turn, makes T-cells to recognize cancer cells more quickly and destroy them. For the first time, CART-T-cell therapy was used for treating advanced follicular lymphoma. Since then, CART-T therapy has emerged as a promising adoptive T-cell therapy (Magalhaes et al. 2019; Almåsbak et al. 2016; Miliotou and Papadopoulou 2018). In 2017, "Tisagenlecleucel (Kymriah)," a CAR-T therapy, was approved for treating "acute lymphoblastic leukemia" (Commissioner O 2018). However, to date, CAR-T-cell therapy is in the initial phase. Thus, its usage has been restricted to only small clinical trials comprised of patients with advanced blood cancers (https://www.cancer.gov).

5.3.4 Neutrophils

Neutrophils provide the first line of defense against entering pathogens via emitting activating cytokines and reactive oxygen species. Additionally, they also play a key role in inhibiting tumor development. Nevertheless, their impact on tumor microenvironment is still a topic of debate (Eyileten et al. 2016). Few studies claim that neutrophils in tumor may promote tumor formation (Mócsai 2013). Neutrophils promote tumor formation via emitting various factors. For instance, oncostatin M is a cytokine and belongs to interleukin-6 (IL-6) family (Grenier et al. 2001). Reactive oxygen species emitted via neutrophils also play a key role in tumor

development. Güngör and the team also suggested that major "neutrophilic oxidant hypochlorous acid" stimulates three distinct forms of DNA damage as well as mutagenicity within alveolar epithelial cells in human lung (Güngör et al. 2010). Additionally, one study has also reported that proteinase of neutrophil elastase encoded via TANs stimulates tumor cell proliferation within both mouse and human lung adenocarcinomas (Houghton et al. 2010).

On the contrary, few studies have also reported that neutrophils inhibit tumor formation (Chee et al. 1978; Dvorak et al. 1978). For the first time, two independent groups, namely, Godleski and the team (Godleski et al. 1970) and Bubeník and the team (Bubeník et al. 1970), separately, reported that neutrophil may inhibit rat mammary gland carcinosarcoma and human bladder tumors, respectively. Later, Pickaver and the team (Pickaver et al. 1972) confirm the neutrophils inhibit tumor cells. Another study suggested that proteases, defensins, and ROS produced via neutrophils (Reeves et al. 2002) can directly inhibit targeted tumors cells (Reeves et al. 2002; Stuart and Ezekowitz 2005). Dallegri and the team suggested that apoptosis as well as necrosis in tumor cell is mainly because of the enhanced secretion of HOCl via neutrophils (Dallegri et al. 1991). Additionally, the inhibition of tumor cells via neutrophils can be enhanced through target-specific antibodies (Di Carlo et al. 2001; Scott et al. 2012). Repp and the team suggested that neutrophils retrieved from patients that have been treated with recombinant human G-CSF expressed FcγRI receptor, which is a high affinity receptor for IgG (Repp et al. 1991).

Few researchers also employed live bacteria and bacterial products, *Mycobacterium bovis* (Hanna et al. 1973), *Clostridium novyi* (Agrawal et al. 2004), *Salmonella choleraesuis* (Lee et al. 2008), *Corynebacterium parvum* (Lichtenstein et al. 1984), and *Salmonella typhimurium* (Avogadri et al. 2005) for inducing neutrophil infiltrations within the tumor microenvironment. Lee and the team subjected *S. choleraesuis* within the mouse experiencing orthotopic hepatocellular carcinoma for stimulating a plausible inflammatory response. This in turn inhibited intratumoral micro-vessel density and enhances neutrophils infiltration. This results in increased death of cancer cell, thereby increasing the survival rate of the patient (Lee et al. 2008). Since 1970, BCG vaccine has been widely employed for treating bladder cancer patients after surgery. BCG administration enhances neutrophil infiltration within the bladder (de Boer et al. 1991).

5.4 Other Strategies Employed in Immune Cell Therapy

5.4.1 Monoclonal Antibodies

The monoclonal **a**ntibodies are a fundamental constituent of adaptive immune therapy. It plays a key role in the identification of foreign antigens and stimulating immune cell responses in tumor cells (Rao et al. 2019; Moehler et al. 2016; Mody et al. 2019). Monoclonal antibodies are produced by fusing an immortalized cell line

with antibody-producing cells, which in turn results in a cell line known as "hybridoma." Since "hybridoma" is "immortal," we can generate the exact antibody for several years (Corthell 2014). To date, several monoclonal antibodies have been produced for the treatment of various diseases, including cancer (Rajewsky 2019). The 2018 "Nobel Prize in Physiology or Medicine" was awarded for the "discovery of cancer therapy by [antibody-mediated] inhibition of negative immune regulation" (Rajewsky 2019). To date, only two monoclonal antibodies, namely, ramucirumab and trastuzumab, have been approved for the treatment of cytotoxics. Trastuzumab, a HER2 monoclonal antibody, inhibits cell-cycle at the G1 phase. It also has anti-cancer activity within HER2 overexpressed gastric cancer cells (Kim et al. 2008). Ramucirumab specifically binds with "VEGF receptor-2" and restricts the binding of "VEGF receptor ligands," namely, VEGF-D, VEGF-C, and VEGF-A. This, in turn, inhibits ligand-induced proliferation as well as the migration of endothelial cells in humans (Fala 2015). While other antibodies, namely, cetuximab, panitumumab, rilotumumab, and bevacizumab showed conflicting results during clinical trial studies (Sibertin-Blanc et al. 2016).

5.4.2 Polysaccharide-K

In the 1980s, for the first time, polysaccharide-K as immunotherapy was used and approved by Japan. The drug polysaccharide-K is extracted from mushroom, known as *Coriolus versicolor*. It can up-regulate the immune system and have anti-cancer properties. It stimulates the immune system's response against cancer patients that were undergoing chemotherapy. This drug is given to the patients through orally as a dietary supplement in the USA and other jurisdictions (http://www.cancer.org).

5.4.3 Anti-CD47 Therapy

Anti-CD47 therapy is widely used against tumor cells. Many tumor cells generally overexpress CD47 to escape immunosurveillance of the host immune system. In 2010 Jaiswal and the team reported that CD47 binds to its receptor "Signal Regulatory Protein Alpha" (SIRPα), which in turn causes downregulation of phagocytosis of tumor cell (Jaiswal et al. 2010). Therefore, the main objective of the anti-CD47 therapy is to restore the clearance of tumor cells and increase phagocytosis of tumor cells. Additionally, few studies have also reported the application of tumor antigen-specific T-cells in anti-CD47 therapy (Matlung et al. 2017; Weiskopf 2017). To date, several therapeutic approaches have been developed, such as engineered decoy receptors, anti-CD47 antibodies, bispecific agents, and anti-SIRPα antibodies (Weiskopf 2017).

5.4.4 *Anti-GD2 Antibodies*

The anti-GD2 therapy is also widely used to treat cancer where carbohydrate antigens are present on the cells surface; therefore, carbohydrates are widely used as targets for immunotherapy. GD2 is a ganglioside present on the cancer cell surface, including retinoblastoma, neuroblastoma, melanoma, brain tumors, rhabdomyosarcoma, small cell lung cancer, osteosarcoma, Ewing's sarcoma, fibrosarcoma, leiomyosarcoma, liposarcoma, and other soft tissue sarcomas. In 2014, Roth and the team reported that it is usually expressed only on the cancer tissue surface, which makes it a good target for immunotherapy (Roth et al. 2014).

5.5 Immune Checkpoints Used as Biomarkers—New Concept

In the body, there are numerous immune checkpoints that help cancer cells to protect from immune systems. Therefore, immune checkpoints affect immune system function and can have a stimulatory or inhibitory role. To date, several immune checkpoints biomarkers, such as host genomic factors, immune-regulating factors, as well as tumor-infiltrating immune cells, have been used in immunotherapy for cancer treatment. These checkpoints are continuously used by tumor cells for protecting themselves from immune system attacks. Recently several approaches have been developed for blocking inhibitory checkpoint receptors. However, reliable immunotherapy biomarkers are fewer due to our limited knowledge of the human immune system. Some of the biomarkers are used as both prognostic and predictive markers. For instance, MSI- H and PD-L1 serve as a sensible immune checkpoint biomarker (Marin-Acevedo et al. 2018; Darvin et al. 2018; Tundo et al. 2019; Qin et al. 2019). In 2016, Moehler and the team showed that "a stromal gene expression signature as well as the ITS proportion quantified by morphometry in tissue sections of patient samples was correlated and could both serve as potential prognostic markers" (Moehler et al. 2016). Few studies have also reported that "gastric cancer patients with high ITS were found to have poorer cancer-specific survival compared to patients with low ITS proportion" (Moehler et al. 2016). In 2012 Pardoll and the team reported that blocking of negative feedback signaling to immune cells enhances the immune response against tumors (Pardoll 2012). Another author reported that when the ligand PD-L1 binds to PD1 cell surface of an immune cell, it inhibits immune cell response. PD-L1 on cancer cells can also inhibit interferon- and FAS-dependent apoptosis, which in turn protect cells from cytotoxic molecules generated via T-cells (Dong et al. 2016; Alsaab et al. 2017; Wu et al. 2019).

In 2011, the FDA approved ipilimumab for the treatment of melanoma cancer (Cameron et al. 2011). This immune checkpoint blockade blocks the immune checkpoint molecule CTLA-4. Several clinical trials have been shown some benefits of anti-CTLA-4 therapy on lung cancer and pancreatic cancer, specifically in

combination treatment with other drug molecules (Lynch et al. 2012; Le et al. 2013). Furthermore, clinical trials of the combination treatment of CTLA-4 blockade with PD-1 or PD-L1 inhibitors were also tested on different types of cancer (https://www.clinicaltrials.gov/show/NCT01928394). In 2017, Hooren and the team reported that patients treated with the combination of checkpoint blocking antibodies therapy like CTLA-4 blocking antibodies + PD-1 or PD-L1 also suffer from immune-related side effects, for example, endocrine, gastrointestinal, dermatologic, or hepatic autoimmune reactions (Hooren et al. 2017). These are most likely because of the breadth of the induced T-cell activation when anti-CTLA-4 antibodies are administered by injection in the bloodstream. In this context, Hooren and team have used a mouse model with bladder cancer and found that a local injection of a low dose anti-CTLA-4 in the tumor area had the same tumor-inhibiting capacity as when the antibody was delivered in the blood. At the same time, the levels of circulating antibodies were lower, thereby suggesting that local administration of the anti-CTLA-4 therapy might result in fewer adverse events (van Hooren et al. 2017).

Another IgG4 PD1 antibody, namely, nivolumab, has also been approved for the treatment of several cancers like melanoma, lung cancer, kidney cancer, bladder cancer, head and neck cancer, and Hodgkin's lymphoma (Rao et al. 2019; Moehler et al. 2016; Hazama et al. 2018; Myint and Goel 2017; Cui et al. 2019). However, in 2016, a clinical trial for non-small cell lung cancer failed to meet its primary endpoint for treatment in the first-line setting. FDA has also approved another PD1 inhibitor, namely, pembrolizumab, for the treatment of various melanoma and lung cancers (Borrie and Maleki Vareki 2018; Ratermann et al. 2018; Patel and Liu 2019)

In May 2016, a PD-L1 inhibitor, namely, atezolizumab (www.roche.com/investor) antibody, was approved for the treatment of bladder cancer. At present, the anti-PD-L1 antibody is in the development stage (Hendry et al. 2017; Wang et al. 2007; Pallin et al. 2018). There are also several types of enhancing adoptive immunotherapy available. It includes targeting intrinsic checkpoint blockades, e.g., CISH. Hazama and team have reported that some cancer patients do not respond to immune checkpoint blockade because the introduction of immune checkpoint inhibitors was not substantial (Hazama et al. 2018). This response rate may be improved in some patients by combined treatment of immune checkpoint blockade with additional sensibly selected anti-cancer therapies. For example, targeted therapies such as radiotherapy, vasculature targeting agents, and immunogenic chemotherapy (Pfirschke et al. 2016) can improve immune checkpoint blockade response in the animal. Thus, information retrieved from literature published to date suggests that immunotherapy may be the key answer to gastrointestinal tract cancers.

5.6 Future Perspective

Recently developed immune cell therapy provides a unique opportunity for early detection and treatment of various cancers, including GI cancer. However, multiple studies have reported that a few of these therapies have side effects. Thus, these therapies must be employed with utter caution. Recently several studies have also proposed that the personalized immunotherapy approach can be used for therapeutic cancer treatment. In this therapy, the drug molecules are truly custom-made for every single individual. In general, the human immune system can recognize tumor cells and kill cancer cells, but this ability of the immune system is insufficient to cure cancer. In this context, it is an urgent need of the time to increase human immune systems by harnessing and potentiating the ability of the immune system to fight cancer and to prevent continuous spreading of cancer cells (Tran et al. 2015). Furthermore, due to the higher heterogeneity present in cancer cells, each tumor has its genetic fingerprint. Thus it is also highly required to understand the tumor environment at the single-cell level. Therefore, it is an urgent need to identify specific drug and individualized cancer vaccination therapy that target specific cancer cells (Alsina et al. 2017; Sahin and Türeci 2018). Authors believe that by employing classical and advanced techniques, like immune cell therapy, together, we can quickly diagnose and treat GI cancer in a more comprehensive way.

5.7 Conclusion

In conclusion, recently developed immune cell therapy controls cancer either by activating or suppressing the immune system of cancer patients. The most widely used immune cell therapies towards the treatment of various cancers, including GI cancers, are cellular immunotherapy, monoclonal antibodies, cytokine therapy, polysaccharide-K, anti-CD47 therapy, anti-GD2 antibodies. The most widely used immune cell types that are being employed in immunotherapies during various cancers, including gastric cancer, are macrophages, dendritic cells, adoptive T-cells, neutrophils. Recently, immune checkpoints are also employed in the treatment and prevention of cancer. However, various studies have reported that few therapies have side effects, and thus, these therapies must be employed with utter caution. Recently, few researchers have also proposed that by applying personalized immunotherapy and single-cell approaches, we can also treat various cancers, including GI cancer, more effectively. Authors believe that by employing classical and advanced techniques, like immune cell therapy, together, we can quickly diagnose and treat GI cancer in a more comprehensive way. In the near future, the information present in the chapter will be highly useful for medical practitioners and researchers working in the field of cancer.

Conflict of Interest None.

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Chapter 6
Immune Checkpoint Inhibitors in Gastrointestinal Malignancies

Padmaraju Vasudevaraju and Malla Rama Rao

Abstract Gastrointestinal (GI) malignancies like esophageal, gastric, colorectal cancers, etc. are responsible for 22% cancer deaths and are the third most cause of cancer related deaths. GI cancers are treated by surgery, radiotherapy, and chemotherapy. The overall survival rate (5 years of survival) after the surgery is very poor in patients with GI cancers. To increase the survival rate adjuvant therapies like chemotherapy and radiotherapy were performed. As GI cancers are diagnosed at the advanced stages by the limitations in the diagnosis methods, always there is a need to find the improved diagnostic criteria and effective combination treatments for GI cancers. Immunotherapy is used in combination with other therapies to treat GI cancers. Specifically use of immune checkpoint inhibitors (ICIs) in treating the different GI cancers is investigated by the researchers in clinical studies. Generally, cancer cells are recognized by patients own immune system as foreign and can be eliminated. But cancer cells escape the immune system by expressing the inhibitory immune checkpoint (IC) signal target receptor molecules for PD-1, CTLA4, A2AR, etc., and immune cells cannot recognize these cancer cells as foreign for attack. GI cancer cells also express these inhibitory signals especially PD-1 and interact with PD-L1 on natural killer cells and escape from its action. Immune checkpoint inhibitors like nivolumab, pembrolizumab, atezolizumab, ipilimumab are used to inhibit the inhibitory signals and activate the immune system to eliminate the cancer cells. The use of immune checkpoint inhibitors will be beneficial to treat recurrent malignancy as combination therapy.

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Keywords Gastrointestinal malignancies · Immunotherapy · Immune checkpoint inhibitors · Tumor microenvironment

Abbreviations

A2AR	Adenosine receptor 2
AFP	Alpha-fetoprotein
BTLA	B and T-lymphocyte attenuator
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	CX-C: chemokine receptor type 4
CEA	Carcinoembryonic antigen
CA	19-9
CD	Cluster of differentiation
CTL4	Cytotoxic T-lymphocyte associated protein 4
CRC	Colorectal cancer
CSF-1	Colony stimulating factor-1
EBV	Epstein–Barr virus
ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2
ESCC	Esophageal squamous cell carcinoma
EMT	Epithelial to mesenchymal transition
FAP	Fibroblast activation protein
FAK	Focal adhesion kinase
GI	Gastrointestinal
GITR	Glucocorticoid-induced tumor necrosis factor receptor-related protein
HLA	Human leukocyte antigen
HAT1	Histone acetyltransferase 1
HIF-1	Hypoxia inducible factor-1
HER2	Human epidermal growth factor receptor 2
ICOS	Inducible Co-Stimulator
IDO	Indoleamine-2,3-dioxygenase
ICIs	Immune checkpoint inhibitors
IL-6	Interleukin-6
LAG3	Lymphocyte activating gene 3
ICIPI	Induced pancreatic injury
IRE	Irreversible electroporation
KIR	Killer cell immunoglobulin-like receptors
MSS	Microsatellite stable
MDSC	Myeloid-derived suppressor cells
MSI	Microsatellite instability
NOX2	NADPH oxidase isoform 2
NK	Natural killer cells
NE	Neuroendocrine
OPN	Osteopontin 40
PD-1	Programmed cell death protein 1

PD-L1	Programmed cell death protein 1 ligand
PDAC	Pancreatic ductal adenocarcinoma
SRCC	Signet-ring cell carcinoma
TME	Tumor microenvironment
TIM-3	T-cell immunoglobulin and mucin domain 3
TMITs	Tumor microenvironment immune types
TAA	Tumor-associated antigen
TIL	Tumor infiltrating lymphocytes
TAMs	Tumor-associated macrophages
VEGFR2	Vascular endothelial growth factor receptor 2
VISTA	V-domain immunoglobulin suppressor of T-cell activation

6.1 Introduction

Gastrointestinal (GI) malignancies have a colossal impact on cancer-related mortality. They include esophageal, gastric, and colorectal cancers, which are responsible for 22% of cancer deaths. This is due to poor dietary intake, use of tobacco, irrational consumption of alcohol, obesity, and some pathogens (Sonnenberg 2017). Traditionally, GI cancers are being treated by surgery, radiotherapy, and chemotherapy. Despite, prognosis and treatment of metastatic GI cancers is still dismal. They can be diagnosed by routine serological biomarkers such as CEA, CA 19-9, and AFP (Posner and Mayer 1994). In addition, recent advances in molecular mechanisms of GI cancers lead to shaping of the therapeutic approaches including immunotherapy, in which immune system of patient's is reprogrammed to selectively target tumor (Rao et al. 2019). The immune system presents an initial recognition and targeting in hide and seek manner within the tumor microenvironment (TME). Immune checkpoints are regulators of self- and non-self antigen discrimination and auto-immunity suppression. They can also involve in immune escape of cancer cells via genetic and epigenetic manipulation of formation, presentation, and processing of neoantigen by modulating PD-1/PD-L1 and CTLA4 pathways (Pitt et al. 2016). Therefore, immune evasion can be targeted by blocking checkpoints using immune checkpoint inhibitors. Three complex systems that exist in cancer are tumor cell, tissue microenvironment, and immune response. Understanding these three systems and their interactions between them in specific cancer type is very important in treating the tumors. In gastrointestinal malignancies there is a link between infection, chronic inflammation, and malignancy development. *Helicobacter pylori* infection is one such example of infection leading to tumor development and *H. pylori* regarded as class I antigen (Murphy and Kelly 2015). An antitumor mechanism of immune system involves identifying the cancer cells as non-self by immune cells through regulating the checkpoint control. This strategy becomes an important phenomenon in establishing potential therapies for treating different GI cancers. Immune checkpoints suppress the cytotoxic action of immune

cells on cancer cells and cancer cells escape the destruction by immune action. Immune checkpoints are of stimulatory and inhibitory by their action, stimulatory signals recognize target molecules as non-self and execute the cytotoxic action and inhibitory signals recognize the target molecules as self and prevent them from cytotoxic action of immune cells. The stimulatory checkpoint molecules include CD27, CD28, CD40, CD122, CD137, OX40, GITR, ICOS, etc. The inhibitory checkpoint molecules include A2AR, B7-H3, B7-H4, BTLA, CTLA4, IDO, KIR, LAG3, NOX2, PD-1, TIM-3, VISTA, etc. Immune checkpoint inhibitors enable the immune cells to effectively kill the cancer cells by inhibiting the inhibitory signals. The cancer cells express high levels of inhibitory target molecules on their surfaces which interact with inhibitory signals and escape the cytotoxic effects of immune cells. ICI increases the immunomodulating ability of natural killer (NK) cells and enhances the effects of anticancer activity. Along with immune checkpoint molecules like PD-L1, other components in the tumor microenvironment like tumor cell-intrinsic osteopontin (OPN) and the expansion of tumor-associated macrophages (TAMs) drive the immune escape. Immune checkpoint inhibitor (ICI) strategy may have the potential to induce an abscopal effect in treating the malignancy. In this effect, treatment of tumor with radiation therapy at one site in combination with ICI shows response to ICI at another site providing the high beneficial effect in treating the metastatic tumors (Cecchini et al. 2015). In this chapter, the role of ICI in preventing the different gastric malignancies and their mechanisms are discussed (Fig. 6.1).

6.2 Immune Checkpoint Blockade in Esophagus Malignancies

Immunotherapy is emerging as a potential therapy for esophageal cancers and immune checkpoint inhibitors are under clinical trials (Tanaka et al. 2017; Kojima and Doi 2017). Esophageal squamous cell carcinoma (ESCC) patient's population showed a heterogeneous expression of PD-L1 (programmed cell death protein 1 ligand) with high level of amplification in programmed cell death protein 1 ligand precursor (CD274) protein (Guo et al. 2018). Blocking the immune checkpoint protein PD-1 interaction with PD-L1 can induce the action of immune system on cancer cells and can act as a potential method of treating the ESCC patients. The clinical study conducted by Chen et al. also showed the higher expression of immune checkpoint target molecule, PD-L1 in the epithelial to mesenchymal transition (EMT) positive subgroup of human esophageal cancer (Chen et al. 2017a). This indicates the immune suppression is mediated by the inhibitory checkpoint molecule PD-1 in esophageal cancer. In the study by Jang et al., also showed that the low risk ESCC group exhibits the PD-L1 expression and immune checkpoint inhibitor treatment is suggested to treat ESCC patients (Jang and Lee 2017). Increased expression of PD-1 in natural killer cells (NK cells) has also been observed in

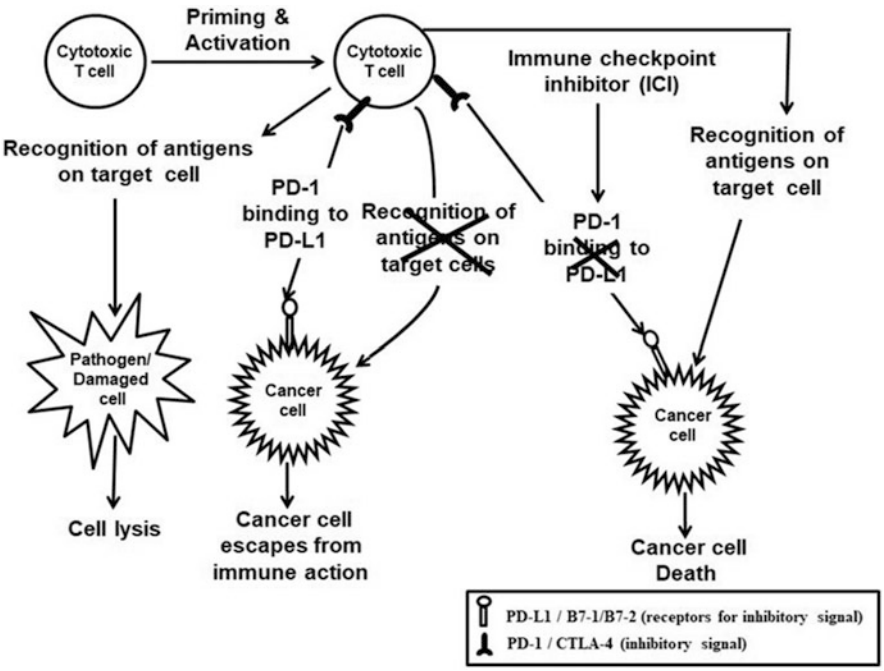

Fig. 6.1 General mechanism of immune escape of cancer cell and role of ICIs in enhancing anticancer Immune therapy. Cancer cell expresses immune inhibitor protein molecules like PD-L1 or B7-1/B7-2 and during priming cytotoxic T-cells (CTLs) in cancer tissue inhibitor checkpoints like PD-1/CTLA-4 are expressed at high levels. The interaction of inhibitory signals on CTLs with their receptors on cancer cells results in the escape of cancer cells from cytotoxic action of CTLs. ICIs block the interaction between inhibitor signals with their receptors allowing the attack of CTLs on cancer cells and killing them

gastrointestinal cancers indicating the role of immune checkpoint alteration in these cancers (Liu et al. 2017a). This positive sensitization of immune cells to express more inhibitory molecules is triggered by the cancer cells and becomes important to know the mechanism by which the cancer cells can program immune cells to survival. Tumor infiltrating lymphocytes (TIL)—a positive group of patients have shown good cancer specific survival in one of the clinical studies (Sudo et al. 2017). The tumor infiltrating lymphocytes are the immune cells that are infiltrated into tumor from blood and fight with the cancer cells. Tumor infiltrated immune cells are used in treatment of cancers by isolating the TILs from patients and improving their ability to attack the cancer cells by in vitro methods in research labs. Then the activated TIL cells which are prepared in research labs are injected to patients for treatment. The study by Zheng et al. suggested the prognostic role of TIL subtypes in esophageal carcinoma patient can be used as prognostic biomarkers in treating cancer (Zheng et al. 2018). Identification of esophageal cancer subtype and markers is important to select the treatment module.

Impaired DNA mismatch repair leads to a condition of genetic hyper mutability termed as microsatellite instability (MSI). Yu Imamura et al. reviewed that microsatellite markers BAT25, BAT26, BAT40, D2S123, D5S346, and D17S250 were high in surgically resected esophagogastric junction (EGJ) adenocarcinoma in Japanese patients. They suggested that the MSI status is highly beneficial in selecting immune checkpoint inhibitors treatment for EGJ adenocarcinoma (Imamura et al. 2019). The MSI status is suggested to be a prognostic biomarker for the patients undergoing chemotherapy treatment among EGJ adenocarcinoma patients (Haag et al. 2019). MSI positive phenotype groups are predicted to have improved response to PD-1 inhibitors in treatment module (Lin et al. 2018). Another potential biomarker identified to monitor the prognosis and treatment response is the elevation of T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) in ESCC with nivolumab therapy (Kato et al. 2018). Monoclonal antibody directed against PD-1 (pembrolizumab) is approved by FDA as first and second line therapy in combination with chemotherapy for EGJ adenocarcinoma (Joshi et al. 2018). Based on the above studies and hypothesis the immunotherapy using immune checkpoints is a promising strategy in treating esophageal cancers. Especially antibodies and compounds targeting PD-1/PD-L1 are going to be a promising method for esophageal cancers.

6.3 Immune Checkpoint Blockade in Stomach Malignancies

Gastric cancer is one of the most cancer related cause of death. Gastric cancer has a very poor survival rate after the conventional treatment of surgery (Abozeid et al. 2017). Gastric cancer is an aggressive type and majority of them have unresectable disease and distant metastasis. Gastric cancer is detected generally in the advanced stage and the treatment of advanced gastric cancer is a challenging task (Jou and Rajdev 2016). The clinical stage I gastric adenocarcinoma is treated surgically and class II, III stages treated with a multidisciplinary approach along with surgical intervention. The clinical stage IV is an advanced stage and has a survival period of around 9–10 months (Ajani et al. 2017). First line of therapy for the treatment of advanced adenocarcinoma is chemotherapy. An effective line of treatment emerging to treat gastric cancer is by identifying the molecular drivers of different biological targets. This has led to the identification of human epidermal growth factor receptor 2 (HER2) and vascular endothelial growth factor receptor 2 (VEGF-R2) as biological targets. Immunotherapy in combination with the other treatments is gaining importance in gastric cancer treatment (Lazar et al. 2018). Along with these targets, another new line of biological targets called immune checkpoints becoming the target molecules and inhibition of these immune checkpoint targets by ICIs can be used for effective treatment in gastric cancers (Sun and Yan 2016). In one of the gastric cancer subtype tumor positive for Epstein–Barr virus (EBV), CD274

(PD-L1) and PD-L2 expression was elevated indicating the intervention of immune checkpoint inhibitors in treatment strategy (Cancer Genome Atlas Research Network 2014). The PD-L1 expression is induced by the inhibition of autophagy in gastric cancer cell lines (Wang et al. 2019). The use of ICIs in the treatment has to be supported with patient expression data of immune checkpoint targets. The use of ICIs in unselected population may lead to failure of response to the treatment (Abdel-Rahman 2016). The blockade of PD1/PDL1 using the antibodies along with other treatments of cancer like chemotherapy, radiotherapy, and other immunotherapies becomes an effective strategy to combat gastrointestinal cancers (Lote et al. 2015). Different immune modulating agents are in clinical trials to find molecules for immunotherapy and one such molecule is pembrolizumab. Pembrolizumab is an anti-programmed death 1 receptor antibody that is under clinical trial (Davidson et al. 2015).

The study by Thompson et al. showed the expression of PD-L1 in the cell membranes of gastric adenocarcinoma tumor cells (around 12%) and immune stromal cells (around 44%). In this study they also observed the CD8+ T-cell densities correlating with that of PD-L1 expression in both tumor cells and stromal cells (Thompson et al. 2017). Modification of tumor microenvironment with ICIs and matrix metalloproteinase-9 inhibitors also gave better treatment results (Lordick et al. 2017). Fibroblast activation protein-a (FAP) is expressed in cancer associated fibroblasts and targeting the FAP+ cancer associated fibroblasts enhanced the immune checkpoint inhibitor effects (Wen et al. 2017). In gastric signet-ring cell carcinoma (SRCC) there is a correlation between PD-1, PD-L1 expression and CD3+ T-cell infiltration. In advanced gastric cancer a positive correlation between PD-L1 and CD8+ T-cell infiltration was observed in patients (Wang et al. 2018a). The combination of these changes can be evaluated as potential biomarkers for SRCC cancers and the combination therapies including the immune checkpoint inhibitors emerge as potential treatment (Jin et al. 2017).

Tumor microenvironment (TME) is composed of cellular and non-cellular components such as fibroblasts, adipose cells, neuroendocrine (NE) cells, immune inflammatory cells, blood, lymphatic networks, myofibroblasts, extracellular matrix, etc. (Wang et al. 2017). Based on PD-L1 and CD8 antigen/cytolytic activity (CYT) the tumor microenvironment immune types (TMITs) are classified into four types (Table 6.1). The stomach cancers fall into type I category having high PD-L1 and CD8A expression and identifying the immune type helps in selecting the treatment strategy (Chen et al. 2017b). Cancer Genome Atlas gastric cancer data analysis showed that the EBV positive tumors are microsatellite stable (MSS) group and have higher expression of PD-L1 and TILs with low mutation burden. This suggests that EBV positive-MSS gastric cancers can be treated with immune checkpoint inhibitors (Panda et al. 2018). Apart from that the potential biomarkers like PD-L1 expression, TILs, CD8A expression other markers like MSI status and DNA mismatch repair (MMR) status is used in selecting the immunotherapy for treatment. Gastric cancers with MSI are categorized into a new subgroup having different prognostic value and need different treatment strategy (Ratti et al. 2018). The gastric cancers showed increased MMR deficiency

Table 6.1 Mechanism of immune escape and role of ICIs in immunotherapy of different GI malignancies

Type of GI cancer	Mechanism of immune escape	Possible ICI intervention
Esophageal cancer	• Heterogeneous expression of PD-L1 and its precursor CD247 • Heterogeneous level of tumor infiltrating lymphocytes (TILs)	• Biomarker identification of molecules like BAT25, DS123, Tim-3, etc., to identify subtype • ICIs are used to suppress the inhibitory signal PD-1 • TILs isolation from patient and activated TILs in labs and then injected again • Combination of other therapies along with ICIs
Stomach cancer	• Epstein–Barr virus (EBV) subtype expresses PD-L1 and PD-L2 • Both PD-L1 and CD8+ T-cell densities elevated • Cancer associated fibroblasts express fibroblast activation protein-A	• Identification gastric cancer subtype and other biomarkers like MSI status, TILs, etc. • Use of ICIs to inhibit the PD-1 binding to PD-L1 • Combination therapy along with the ICIs like anti-VEGF-R2 therapy
Pancreatic cancer	• Excessive stromal matrix and hyper vasculature • Decreased level of ICI target molecules like PD-L1, etc. • Poor response to immunotherapy • Decreased CTL infiltration • Increased immune inhibitory cells	• Vaccine inducing T-cell response along with ICIs • Targeting pathways like FAK inhibition, IL-6, macrophage derived granulin which increase T-cell infiltration • Selection of treatment with monitoring responses • ICI treatment enhances other treatment efficiency and vice versa
Liver cancer	• Decreased CTL infiltration • Increased PD-L1 • Hypoxia increased HIF-1 and CXCR4 • Increased IL-6	• Treatment with ICIs like anti-PDl1 and anti-CTLA4 • Inhibition of *myc* gene and Tim-3 along with ICIs • Other combination therapies increase the efficiency of ICIs
Colorectal cancer	• Increased immunosuppressive cells and signals	• Identification of colorectal cancer subtype • Induction of T-cell recruitment • Blocking the immunosuppressive signals with ICIs and immunosuppressive cells

and MSI status compared to esophageal cancers. While gastric cancers are positive for EBV when compared to esophageal cancers, MSI and MRR are not indicating the importance of characterizing the cancer biomarkers in selecting the immune checkpoint inhibitor strategy (Hewitt et al. 2018).

Immunotherapy using the immune checkpoint inhibitors for the advanced stages of gastric cancer has promising results in treatment. The targets of ICIs are PD-1, PD-L1 and these target levels serve as biomarkers in identifying the stages of gastric cancer (Tran et al. 2017). Pinto MP et al. hypothesized that combination therapy

using angiogenic inhibitors along with immunotherapy using immune checkpoint inhibitors will be a good treatment model for gastric cancers (Pinto et al. 2017). PD-1 and PD-L1 expression is frequent in neuroendocrine malignancies of digestive system and targeting PD-1and PD-L1 using checkpoint blockade has the potential in treating these carcinomas (Roberts et al. 2017). FDA (US) approved drug pembrolizumab is suggested for the first and second line therapy with a combination of other therapies in treating gastric adenocarcinoma (Joshi et al. 2018). Other drugs like nivolumab, avelumab, atezolizumab are the antibodies developed to block the PD-1/PD-L1 blocking are showing promising results in clinical trials for gastric cancer treatment. These drugs along with the combination of other target candidates like anti-CTLA4 and anti-VEGF-R2 therapy are suggested for the treatment of adenocarcinoma. Cytotoxic T-Lymphocyte associated protein 4 (CTL4 or CD152) is a protein receptor involved in the inhibition of cytotoxic T-Lymphocytes and VEGFR-2 is involved in the signaling pathway of immunosuppression (Smyth and Thuss-patience 2018).

6.4 Immune Checkpoint Blockade in Pancreatic Malignancies

Tumor microenvironment plays a critical role in treating pancreatic malignancies. A unique TME exists in pancreatic cancer having an excessive stromal matrix and hyper vasculature creating the immune barrier for CTL infiltration. Other resistant factors like pancreatic stroma, genetic predisposition, immune inhibitory cells, cytokines, and epigenetics pose difficulty in treatment. Pancreatic cancers are regarded as non-immunogenic cancers and have a very poor response to immunotherapy. Pancreatic cancer has only a 9% survival (5 year survival) rate and novel strategies have to be identified to combat this type of cancer. In a mouse model of pancreatic ductal adenocarcinoma (PDAC), the inhibition of myeloid growth factor receptor (CSF1R) increased the antigen presentation and T-cell antitumor responses to ICIs (Zhu et al. 2014). Immune checkpoint inhibitors targeting CTLA-4, PD-1, and PD-L1 did not show good promising treatment effects in PDAC when used alone. But when ICIs are used together with other treatment strategies like vaccine inducing T-cell response they have shown some good results. This response is mainly due to the increased expression of ICI targets with vaccine treatment (Soares et al. 2015). In this type of strategy, the vaccine induces T-cell to kill pancreatic cancer cells, while other treatments used in combination with ICIs facilitates the trafficking of T-cells to the site of cancer in cell culture model of PDAC the combination of vaccine therapy, indoleamine-2,3-dioxygenase (IDO1) inhibitor and PD-L1 showed no positive correlation in comparison of vaccine therapy and IDO1 inhibitors. This indicates that the selection of combination therapy along with ICI is essential in attempting the treatment (Blair et al. 2019). In general, the cancer cells exposed to constant antigenic exposure lead to T-cell transformation from

active state to inactive state. This causes T-cell exhaustion and T-cell proliferation and activation are needed to treat this stage in pancreatic cancers (Bauer et al. 2016). Jiang et al. showed that the focal adhesion kinase (FAK) inhibition increases the cytotoxic CD8+ T-cell infiltration and increases the ICI responses in pancreatic cancers (Jiang and Hegde 2016). In another study it has been shown that targeting macrophage derived granulin restored the CD8+ T-cell infiltration in metastatic PDAC and increased response to ICIs (Quaranta et al. 2018). The targeted inhibition of interleukin IL-6 also increased the response of ICI treatment in the preclinical trials (Mace et al. 2018). Receptor-interacting serine/threonine protein kinase 1 (RIP1) is upregulated in tumor-associated macrophages (TAMs) in PDAC and the inhibition of RIP1 in combination with ICIs benefits greatly in treating PDAC with immunotherapy (Wang et al. 2018b). In pancreatic cancer, histone acetyltransferase 1 (HAT1) is upregulated and PD-L1 is linked to the regulation of HAT1 expression. The HAT1 expression may be used as a prognostic biomarker for the treatment of PDAC (Fan et al. 2019). The combination therapies along with immune checkpoint inhibitors used to treat pancreatic malignancies are vaccination, tumor targeted oncolytic viruses, whole cell immunotherapy, CD40 agonists to promote APC maturation, MEK inhibitors, cytokine inhibitors, etc.

The above studies indicated that the effectiveness of ICIs is enhanced by the other combination therapies in treating the pancreatic tumors. In some cases the blockade of PD-L1 enhanced the effectiveness of the radiotherapy in PDAC model (Azad et al. 2017). Also the PD-L1 treatment sensitizes the antiangiogenic therapy and in turn the antiangiogenic therapy increases response to ICIs and CTL infiltration (Allen and Jabouille 2017). However, the PDAC patients show poor prognosis with combination therapies and it is very challenging to find effective treatments to PDAC treatment. A complete tumor genotyping and gene expression analysis may provide novel targets and improves ICI effective usage in treatments. The biomarker driven approach for finding the target molecules may give additional strength to treat this complex PDAC patients (Zhen et al. 2018). The usage of ICIs may lead to immune checkpoint inhibitor induced pancreatic injury (ICIPI) in some of the patients emphasizing the use of ICIs with caution (Abu-Sbeih et al. 2019). Recently the study by Zhao et al. using irreversible electroporation (IRE) technique in combination with ICI treatment showed promising approach in treating the PDAC (Zhao et al. 2019). In the cell line models the use of chemotherapy agent gemcitabine and a novel programmed death-ligand 1 (PD-L1) inhibitor (MN-siPDL1) showed a 90% reduction in tumor growth and increased survival (Yoo et al. 2019). Gemcitabine is a chemical compound which is incorporated into DNA during DNA synthesis and halts DNA synthesis leading to cell death. These recent strategies show some good promising results and suggest the use of immune checkpoint inhibitors in treating pancreatic cancers.

6.5 Immune Checkpoint Blockade in Liver Malignancies

Hepatocellular carcinoma (HCC) incidence is increasing and identifying the targets for the treatment is becoming a challenge. In the first line therapy sorafenib is approved for the systemic therapy and second line therapy molecules like regorafenib, lenvatinib, cabozantinib, and ramucirumab are shown to improve the survival. To increase the median survival periods, other line of therapies like ICIs is being incorporated in the treatment (Llovet et al. 2018). Chronic inflammation is reported in hepatocellular carcinoma patients indicating the potential immune based therapies in treating HCC patients. HCC models showed decreased infiltration of cytotoxic CD8+ T-cells to the site of tumor. In the HCC tumor environment hypoxia develops and this condition stabilizes the hypoxia inducible factor-1 (HIF-1) which stimulates the expression of ectonucleoside triphosphate diphosphohydrolase 2 (ENTPD2/CD39L1). The ENTPD2 enzyme converts extracellular ATP to 5′-AMP and the 5′-AMP inhibits the myeloid-derived suppressor cells (MDSCs) differentiation. The MDSCs exert immunosuppressive actions and cancer cells escape the action of immune system to eliminate the cancer cells (Chiu et al. 2017). In HCC models the use of systemic therapy molecule, sorafenib (tyrosine kinase inhibitor) increases hypoxia which in turn activates the immunosuppressive actions. The hypoxia condition increases the expression of stromal cell derived 1 alpha receptor (CXCR4) and CXCR4 may be responsible for immunosuppressive action (Chen et al. 2015). The hypoxia condition in HCC also induces increased expression of PD-L1 along with CXCL12 (Semaan et al. 2017). The prevalence of the above conditions makes the tumor more immunocompromised. The immune checkpoint inhibitor treatment with antibody against PD1 receptor along with the sorafenib and anti-CXCR4 molecule AMD3100 has better results in treating the HCC. In one of the case study with metastatic hepatocellular carcinoma pembrolizumab (immune checkpoint inhibitor) treatment after failure of using sorafenib showed good response by decreasing the tumor size (Truong et al. 2016). In HCC, interleukin-6 (IL-6) is secreted by tumor-associated fibroblast and recruits immunosuppressive cells like myeloid-derived suppressive cells inducing the immunosuppressive mechanisms. The interleukin-6 is also involved in the expression of immune checkpoints which inhibit the immune action against cancer cells. In the mouse models of HCC, inhibition or targeting the IL-6 expression increased the ability of the anti-PD-L1 treatment (Liu et al. 2017b). Radiation therapy also showed improved response to the ICIs indicating the combination therapies are effective methods in the treatment of cancer. Radiation therapy increased the PD-L1 expression which resulted in effective response to anti-PD-L1 inhibitors (Kim et al. 2017). Patients treated with radiotherapy showed increased soluble PD-L1 (sPD-L1) in blood and this sPD-L1 can act as a predictive biomarker for the combined therapy of radiotherapy and ICIs (Kim et al. 2018). Along with the PD-L1, other molecules like CTLA-4, lymphocyte activating gene 3 (LAG3), and hepatitis A virus cellular receptor 2 (TIM3) are highly expressed on tumor-associated antigen (TAA) specific-CD8+ TIL cells in HCC patients. This strongly

suggests the combination therapy with multiple ICIs will have additive effects in HCC treatment (Zhou et al. 2017). Treatment of HCC patients with anti-CTLA4 and tremelimumab activated the T-cell responses in HCC patients. In the treated patients, $CD4^+$-HLA-DR^+, $CD4^+$ PD-1^+, $CD8^+$ HLA-DR^+, $CD8^+$ PD-1^+, $CD4^+$ $ICOS^+$, and $CD8^+$ $ICOS^+$ T-cells are increased in the peripheral blood. Among these patients, patients having high frequency of $CD4^+$ PD-1^+ respond more to treatment indicating that $CD4^+$ PD-1^+ may act as potential biomarker for treatment (Agdashian et al. 2019). The oncogene *myc* inhibition in HCC induces the expression of interferon-γ (INF-γ) which upregulates the PD-L1 levels. In lymphomas *myc* gene upregulates PD-L1 in contrast to its effects in HCC, indicating different tumor environments regulate immune checkpoint molecules differently in different types of cancers. The identification of *myc* gene behavior in HCC suggests the potential of combination therapy of inhibiting the *myc* gene and the use of ICIs (Zou et al. 2018). In the mouse models of HCC, the tumors with high expression of tumor cell-intrinsic osteopontin (OPN) in the tumor microenvironment decreased the expression of PD-L1 and expansion of tumor-associated macrophages (TAMs). The decrease in these molecules is mediated by the stimulation of the colony stimulating factor-1 (CSF1) and CSF1 receptor (CSF1R). This study suggests the use of the ICIs along with the inhibition of CSF1/CSF1R, OPN levels are established in the HCC patients (Zhu et al. 2019). Some patients with HCC showed resistance to anti-PD-1 therapies and transgenic mouse models showing the exogenous expression of antigens in *myc; Trp53*$^{-/-}$ HCCs escaped from immune actions. In this model the HCCs escaped the immune system by upregulating the β-catenin (CTNNB1) pathway. This identification of β-catenin induced immune escape makes to develop new strategies to treat the anti-PD1 resistant HCCs (Ruiz de Galarreta et al. 2019; Berraondo and Ochoa 2019).

6.6 Immune Checkpoint Blockade in Colorectal Cancer

Colorectal cancer (CRC) is the third prominent cause of death in both males and females worldwide. In the recent past, all survival (OS) of CRC patients have notably enhanced due to advancement in chemotherapy as well as immunotherapy. A large body of literature showed the importance of anti-PD-1 therapy for CRC subtypes (Yaghoubi et al. 2019). Immune checkpoint inhibitors have showed promising results in metastatic CRCs (mCRCs) (Kamatham et al. 2019). For example, FDA approved combination of nivolumab and ipilimumab has significantly benefited mCRC patients (Morse et al. 2020). The efficacy of pembrolizumab against mCRCs is under clinical trial at phase 2 multicenters (Le et al. 2015). The studies on efficacy of anti-PD/PD-L1 agents, durvalumab and atezolizumab are in progress. Alternative strategy for targeting CRCs is peptide vaccine which is in clinical trial. In this strategy, specific neoantigen is detected using next-generation sequencing on tumor tissue, specific peptides which can combine with human leukocyte antigen (HLA) and coding for the neoantigen are synthesized (Ghiringhelli and Fumet 2019). The strategies that enhance immunogenicity by

using oncolytic vaccines are currently under evaluation. They can be exploited to induce a local immune response against cancer cells. Currently, FOLFOX plus bevacizumab with or without an oncolytic reovirus in RAS mutated colon cancer is in phase II trial. This strategy showed an improved response with shorter median duration of response (Jonker et al. 2018). Another strategy for treatment of CRCs is induction of T-cell recruitment using T-cell bispecific antibodies. In this strategy, bispecific antibody which can bind to CD3 and tumor specific antigen (e.g. TCB-CEA) can able to induce T-cell activation and forces them to detect and kill cancer cells (Argilés et al. 2017). The removal of immunosuppressive cells or molecules is also another strategy especially targeting MDSC and immunosuppressive macrophages. The inhibitors of CSF1R and anti PD-1/PDL1 are currently in development for targeting MDSC and immunosuppressive macrophages. Adenosine is an important immunosuppressive molecule produced by both MDSC and Tregs (Arab and Hadjati 2019). This molecule is generated by CD73 and CD39 molecules, which degrade extracellular ATP. Therefore, combination of CD39 or CD73 inhibitors with checkpoints to reduce immunosuppression might be relevant (Perrot et al. 2019). Clinical trials with anti-PD1/PDL1 and anti-CD73 or anti-adenosine receptor are ongoing. The strategies targeting immune checkpoints using inhibitors, vaccine, bispecific mAbs as well as drugs targeting immunosuppression will probably change the face of CRCs treatments.

6.7 Adverse Effects of ICI Usage

The use of ICIs sometimes leads to immune related adverse effects (irAEs) and are different from other therapies. The commonly observed toxicities are gastrointestinal, skin, liver, endocrine, eyes, pancreas, kidney, lung, and nervous system. These toxicities are relieved by withdrawal of ICI treatment and sometimes suppressing the immune response using steroids (Kottschade et al. 2016). The analysis of different immune checkpoint inhibitors in treating cancer patients showed hepatotoxicity and the high risk is noted with CTLA-4 treatment compared to PD-L1 treatment. Recent study reported that ICI treatment lead to the development of insulin-dependent diabetes in patients (Harsch and Konturek 2018).

6.8 Conclusion

Gastrointestinal (GI) tumors including esophageal, gastric, and colorectal cancers have immense impact on cancer-related mortality and account for 22% of cancer deaths. GI malignancies are traditionally treated with surgery, radiotherapy, and chemotherapy, but their prognosis is still dismal due to poor dietary intake, use of tobacco, irrational consumption of alcohol, obesity, and some pathogens. Recently, immunotherapeutic approaches such as immune checkpoint inhibitors (ICIs) are

gaining advances for targeting GI cancers, which reprogram immune system of patient to selectively target tumor. Even though, ICIs reported to show some toxicities, nivolumab, pembrolizumab, atezolizumab, ipilimumab showed potential to inhibit the inhibitory signals and activating the immune system to eliminate the cancer cells. Therefore, use of ICIs will be beneficial to treat recurrent malignancy as combination therapy.

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Conflict of Interest The authors declare that there are no conflicts of interest.

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Chapter 7
Monoclonal Antibody Therapy Against Gastrointestinal Tract Cancers

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Abstract Gastrointestinal (GI) cancer is one of the leading causes of cancer death across the globe. To date, numerous techniques have been developed for the removal or destruction of cancer cells via surgery, radiation, or chemotherapy. However, these techniques have various side effects on the human body. In comparison to other techniques, recently developed monoclonal antibodies have fewer side effects. Thus their usage in cancer treatment has increased recently. Considering above, in this chapter, the authors attempted to understand the molecular feature associated with monoclonal antibodies and how they can be employed for the treatment of GI cancer. Information obtained revealed that the two most widespread techniques used for producing monoclonal antibodies are hybridoma and phage display. Since 1986, various monoclonal antibodies have been developed against numerous receptors/genes, namely epidermal growth factor receptor (EGFR), human epidermal growth factor 2 (HER2), HER4, VEGF, CD20, CD30, tumor necrosis factor member11, PD1 and IL4, that play a key role in causing GI cancer at different stages. For instance, panitumumab in combination with epirubicin, oxaliplatin, and capecitabine can be used for treating advanced esophageal gastro adenocarcinoma. Tremelimumab, a monoclonal antibody, works against anti-CTL4 and can be used for the treatment of gastro cancer, colon cancer, and melanoma. However, few studies have reported that these monoclonal antibodies have side effects. For instance, mucositis was observed for the cetuximab antibody. Thus, the monoclonal antibody should be used carefully under the provision of the medical practitioner. In the near future, the information present in this chapter will be highly useful for treatment in GI cancer.

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Abbreviations

CTL4	Cytotoxic T lymphocyte-associated antigen 4
EBV	Epstein–Barr virus
EGFR	Epidermal growth factor receptor
FDA	Food and Drug Administration
GI	Gastrointestinal
HER2	Human epidermal growth factor 2
IDO1	Indolemine-2,3-dioxygenase
mAbs	Monoclonal antibodies
PD1	Programmed cell death protein 1
PI3K	Phosphatidylinositol 3-kinase
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VH	Variable heavy chain
VL	Variable light chain

7.1 Introduction

Gastrointestinal (GI) cancer is a deadly disease that occurs in the gastrointestinal tract (Bijlsma et al. 2017; Gupta et al. 2019). The GI tract begins at the mouth and terminates at the anus. The main function of the GI tract is to digest food, absorb nutrients from the food, protect the body against pathogens, and excrete feces (Bruneau 2017). GI cancers denote the primary cause of cancer death globally (Somi et al. 2019). As GI cancer is detected at the malignant state, the death rate associated with gastric cancer is higher in comparison to other cancer types (Bijlsma et al. 2017). Recently, the incidence of GI cancer is reported to increase globally, which is mainly due to adaptation to the western lifestyle, including food habits (www.cancer.org). Other well-known risk factors associated with GI cancer formation are smoking, exposure to polycyclic aromatic hydrocarbons (PAH), *H. pylori* infection, higher usage of biomass, and opium (Roshandel et al. 2019). In various studies, it was found that *Helicobacter pylori* causes gastric ulcers. Additionally, Epstein–Barr virus (EBV) was also found to be associated with gastric cancer (Derks et al. 2016). EBV was found within the epithelial cell in the malignant state of the gastro cancer (Singh and Jha 2017). *CDK2NA* hyper-methylation and *CIMP* methylation occur in EBV infected cells to develop a tumor in the GI tract of the human body (Birdwell et al. 2014). In addition, EBV-infected tumors are associated with high PD-L1 expression (Derks et al. 2016). Various mutations in *TP53*, *PIK3CA*, and *ARID1A* genes are also reported to be associated with the gastric cancer

development. Three nonsense mutations that occur at the genomic level of *ARID1A* gene are also reported to initiate tumors formation (Zang et al. 2012). The *ARID1A* gene could also help to maintain pluripotency in the stem cell. Zhang et al. reported that *ARID1A* and *PIK3CA* together involved in gastric cancer formation (Zang et al. 2012).

To date, numerous techniques have been developed for the removal or destruction of cancer cells via surgery, radiation, or chemotherapy. Though radiation and surgery are highly useful for a benign tumor, chemotherapy works effectively in the case of metastatic cancer. Nevertheless, chemotherapy may also cause various side effects, for instance, removal of rapidly dividing healthy tissues, e.g., cells lining the GI tract and blood cell (Pento 2017). Development of hybridoma technology along with serological techniques and various tools like monoclonal antibodies (mAbs) provided a unique way for recognizing cancer cell-associated cell surface receptors, which in turn revolutionized the field of cancer research (Pento 2017). Monoclonal antibodies affect cancer cells either by neutralizing the expression of proteins or by modifying the ligand binding to block or modify the expression of cancer causing genes (Redman et al. 2015). Additionally, due to high specificity, monoclonal antibodies have fewer side effects, and thus their usage in cancer treatment has increased recently (Lu et al. 2020). Considering this, in the present chapter, the authors attempted to understand the molecular feature associated with monoclonal antibodies and how they can be employed for the treatment of GI cancer.

7.2 Monoclonal Antibody

Antibodies are proteins of the immune system that identify and bind tightly with foreign particles. Antibodies may be either monoclonal (a single antibody clone is produced) or polyclonal (various antibodies with distinct features are produced). Because of the high specificity towards target molecules, recently monoclonal antibodies have demonstrated as promising candidates for the treatment of various diseases, including cancer (Shimasaki 2014). Monoclonal antibodies are single antibodies that are produced by fusing an immortalized cell line with antibody-producing cells, which in turn produce a new form of the cell line, namely, hybridoma. As hybridoma is "immortal," we can generate the exact antibody from them for several years. Nevertheless, few studies have also reported that we must re-test these antibodies after a few years to ensure that no new mutation has been introduced in these cell lines. Initially, monoclonal antibodies were produced from mice (Corthell 2014). The first licensed monoclonal antibody, namely, Orthoclone OKT3 (muromonab-CD3), was obtained from mouse (Emmons and Hunsicker 1987). In 1988, Huang et al. reported the first monoclonal antibody, namely pepsinogen, for treating intestinal gastric cancer (Huang et al. 1988). However, as rabbits have better immune responses than mice, recently, novel approaches have been attempted to produce monoclonal antibodies from rabbits (Corthell 2014). Since

1986, ~100 monoclonal antibodies have been designated as a drug, and the approval rate is continuously increasing (Manis and Feldweg 2019). The global value associated with the antibody market is ~$20 billion/year. The global value of the antibody market is approximately $20 billion/year (Maggon 2007).

7.3 Techniques for Monoclonal Antibodies Production

Two most widely methods employed for producing monoclonal antibodies are hybridoma and phage display. In 1975, for the first time, Milstein and Köhler described generating hybridoma as a stable monoclonal antibody production technique (Köhler and Milstein 1975). Hybridoma production involves the removal of activated B lymphocytes from an immunized animal spleen and mingling them along with immortalized myeloma cells that are incapable of producing hypoxanthine-guanine-phosphoribosyltransferase, the key enzyme involved in salvage pathway and is associated with nucleotide production (Chartrain and Chu 2008). For selecting hybridomas, cells pools produced after the fusion (a mixture of non-fused myeloma cells & B lymphocytes and hybridoma cells) are nurtured within a specific medium comprised of aminopterin, which restricts de novo synthesis of nucleotide (Carvalho et al. 2017). Myeloma cells are deprived of the *salvage* pathway that is highly required for nucleotide production. Nevertheless, when these cells are exposed to selective medium comprised of aminopterin, de novo synthesis of nucleotide is also halted, which in turn cause myeloma cells inviable. On the contrary, salvage pathway activated within non-fused B-lymphocytes works perfectly. Thus, in spite of de novo pathway blockage via aminopterin, non-fused B-lymphocytes produce nucleotide continuously. But these cells are not mortal and replicate for limited times and eventually die. Considering this, hybridomas cells were produced that have capability to replicate indefinitely as well as synthesize nucleotides via the *salvage* pathway through selection conditions (Carvalho et al. 2017).

However, the main problem associated with early monoclonal antibodies was to detect availability of suitable myeloma cell line. Hybridomas may also be genetically unstable, and yield is less. Recently several studies have also reported that different expression system for monoclonal antibodies behaves differently. For instance, *E. coli* may be employed for antibody fragments expressions like antigen-binding fragments and single-chain variable fragments. But they are not suitable for the production of full-sized antibodies (Carvalho et al. 2017; Liu 2014). To overcome this problem, another technique, namely, phage display, was developed (Liu 2014). During phage display, at first, B-lymphocytes are isolated from the human blood. Later mRNA is isolated and converted into cDNA employing polymerase chain reaction for amplifying a complete set of the "variable light chain" (VL) as well as "variable heavy chains" (VH) segments. These segments are then cloned with the vector, generally scFv, nearby bacteriophage's PIII protein, and subsequently, *E. coli* is infected for generating a library comprised of 10^{10} cells via inoculating the library with an extra helper phage. Bacteriophage comprised of VL and VH

segments in bacteriophage coat is later secreted via *E. coli*. Distinct VL and VH segments against the antigen are selected and then employed to re-inoculate *E. coli* by bacteriophage. Cells comprising the plasmid can subsequently be isolated and sequenced. The main advantage associated with phage display is that after the generation of a single library, it can be utilized for producing novel antibodies for infinite time. As the complete process is performed under in vitro condition, no immunization is required at any step of processing, and antibodies are produced more in less time in comparison to hybridomas technique. Additionally, the library generated via phage display may also be employed for producing antibodies against toxic antigens (Liu 2014).

7.4 Monoclonal Antibody Therapy in GI Cancer

To date, various monoclonal antibodies have been identified against numerous receptors/genes, namely epidermal growth factor receptor (EGFR), human epidermal growth factor 2 (HER2), HER4, VEGF, CD20, CD30, tumor necrosis factor member11, PD1 and IL4, that play a key role in causing GI cancer at different stages (Table 7.1).

Table 7.1 Monoclonal antibody and its target site

Monoclonal antibody	Mechanism	Target site	Type of cancer	References
Cetuximab	EFGR inhibition	EFGR	Colorectal cancer, gastro-esophageal Cancer	Pinto et al. (2009), Moehler et al. (2011)
Trastuzumab	HER2 inhibition	HER2	Gastroesophageal cancer	Moehler et al. (2011)
Panitumumab	EFGR inhibition	EFGR	Colorectal cancer	
Bevacizumab	VEGFR inhibition	VEGFR	Colorectal cancer	Norguet et al. (2012)
Ramucirumab	VEGFR inhibition	VEGFR	Gastric cancer	Jung et al. (2002)
Afatinib	EFGR inhibition	EFGR	Esophagogastric cancer	Dungo and Keating (2013)
Lapatinib	EFGR and HER2	EFGR	Gastric cancer	Shimoyama (2014)
Docetaxel+ Cetuximab	EFGR inhibition	EFGR	Esophageal carcinoma	Ruhstaller et al. (2011)
Docetaxel+ Oxaliplatin	VEGFR inhibition	VEGFR	Gastroesophageal junction cancer	El-Rayes et al. (2010)
Avelumab	HER2 inhibition	HER2	Gastric cancer	Bang et al. (2010)
Pembrolizumab	Block PD1	PDL1	Gastroesophageal junction cancer	Shitara et al. (2018)

7.4.1 Epidermal Growth Factor Receptor (EGFR)

EGFR is a transmembrane glycoprotein involved in cell proliferation, angiogenesis, and cell transduction pathways (Martinelli et al. 2009; Norguet et al. 2012). When EGFR binds with the ligand, it prevents EGFR dimerization at the extracellular region and enhances the apoptosis of cancerous cells. EGFR on binding with the ligand enhances the changing of ligand confirmation, which in turn activates the tyrosine kinase and phosphorylate tyrosine residue at the intracellular carboxy domain of EGFR (Martinelli et al. 2009). Another EGFR inhibitor, namely cetuximab, is an immunoglobulin G1 antibody that inhibits cell proliferation by interacting with the MAP kinase pathway as well as the PIK3 pathway (Edris et al. 2013). It induces cell cycle arrest at the G1 phase and inhibits cancer cell proliferation. Another phase II trial study reported that 5-fluorouracil in combination with cetuximab can effectively reduce gastric cancer (Norguet et al. 2012; Gold et al. 2010). Cetuximab, along with platinum fluoropyrimidine, has also shown a better effect in metastatic gastric cancer during the phase III trial (Wagner et al. 2006; Lordick et al. 2010). Lorenzen et al. observed that docetaxel, cisplatin, and leucovorin individually provide hematologic based toxicity for gastric cancer (Lorenzen et al. 2007). Afatinib is an irreversible, 4-anilinoquinazoline second-generation tyrosine kinase inhibitor of EGFR. It is widely used for the treatment of various cancers (Tridente 2017; Brody 2018). Panitumumab is another EGFR inhibitor used for the treatment of metastatic colorectal cancer. It is injected along with chemotherapy. In 2013, Waddel et al. reported that panitumumab in combination with epirubicin, oxaliplatin, and capecitabine could be used for treating advanced esophageal gastro adenocarcinoma in phase III trial. For esophago-gastro cancer, panitumumab in combination with epirubicin, oxaliplatin, and capecitabine can be used for treating gastro cancer after the second stage of chemotherapy (Waddell et al. 2013). Nimotuzumab blocks the binding of EGF and transforming growth factor TGFα to EGFR that could inhibit cancer cell inhibition, proliferation, and induce apoptosis (Talavera et al. 2009). Nimotuzumab in combination with irinotecan shows better survival rate in gastric cancer patients. Strumberg et al. reported that nimotuzumab could also be used for treating pancreatic cancer (Strumberg et al. 2012).

7.4.2 Human Epidermal Growth Factor 2 (HER2)

HER2 is a protooncogene and encodes ErbB2, which plays a key role in tumorigenesis. Initially, its overexpression was observed at the primary and secondary stages of stomach cancer. Higher expression of HER2 was reported in ~36% of

gastroesophageal junction tumors, whereas 21% was found in gastric tumors (León-Chong et al. 2007). For the first time, the association between gastric cancer and HER2 was reported in 1986 (Sakai et al. 1986). To date, several inhibitors were used to inhibit or block the expression of receptors and change the conformation of ligand binding to stop cell signaling pathways of cancer cells. The activation of HER2 can be inhibited by trastuzumab that could block the signaling pathway and induce apoptosis by interfering phosphatidyl inositol-3 kinase pathway and mTOR pathway (Bang 2012). RAS protein-mediated signaling pathway can be inhibited by trastuzumab. On binding with HER2 domain, trastuzumab induces antibody-dependent cytotoxicity (Collins et al. 2012). Earlier the trastuzumab antibody, along with cisplatin, is used at phase II and III trials of gastric cancer patients (Cortés-Funes et al. 2007). Barok et al. found that "trastuzumab emtansine," a conjugate for the trastuzumab antibody for HER2 positive cells, may also be used for treating gastric cancer (Barok et al. 2011).

Lapatinib, an inhibitor of tyrosine kinase, inhibits the PI3K and RAS pathway by interfering with the activation of EGFR and HER2 (Chen et al. 2012). In most cases, lapatinib was used to treat the patient from having trastuzumab-resistant cells. Lapatinib interferes with the signaling pathways of HER1 and HER2 by interrupting ATP binding to the ATP binding domain of tyrosine kinase. Previously it was reported that lapatinib could give positive results against gastric cancer. However, the resistance was developed against lapatinib in the patient who has taken it before (Chen et al. 2012). Pertuzumab, another monoclonal antibody, is distinct and complementary to trastuzumab. Pertuzumab binds with the extracellular domain II and dimerization arm of the HER2 receptor, which in turn disrupts HER2-HER3 and HER2-EGFR dimerization (Nahta et al. 2004). Trastuzumab and pertuzumab in combination cause the cell death of cancer cells at the phase II stage. Pertuzumab in combination with trastuzumab, capecitabine, and cisplatin has also used for treating advanced gastric cancer (Matsuoka and Yashiro 2015). Bao et al. reported that the interaction of HER2 with CD44 upregulates the expression of the CXCR4 promoter. The CD44 acts as a mediator to form dimerization between HER2 and HER3 when interacting with nueregulin. The team also reported that on treatment with trastuzumab, the interaction of CD44 with HER2 was inhibited by the disulfide bond present at the 295 position of a cysteine residue in the HER2 positive cancer cells (Bao et al. 2011).

7.4.3 Vascular Endothelial Growth Factor (VEGF) Targeted Pathway

Vascular endothelial growth factor is the tyrosine kinases having five ligands (VEGF-A, B, C, D, H) that directly or indirectly associated with inhibition of tumor cells (Rapisarda and Melillo 2012). The receptor of VEGF is expressed at

the endothelial cells containing the immunoglobulin-like domain and is associated with the regulation of tumor lymphangiogenesis and angiogenesis (Shibuya and Claesson-Welsh 2006). The patients of Western and Asian regions were highly benefited by the VEGF therapy method. It was also known as angiogenic therapy. Bevacizumab, a monoclonal antibody, can be used against vascular endothelial growth factor to treat colorectal cancer in phase III trial of gastric cancer (Norguet et al. 2012). Binding of bevacizumab to the VEGF-A domain inhibits the downstream signaling of VEGF receptor to neutralize tumor cell growth. It was found that bevacizumab, in combination with insulin-like growth factor1, works more effectively on gastric cancer. In metastatic gastroesophageal junction cancer, bevacizumab decreases 65% of cancer cell progression (Shah et al. 2006). Another monoclonal antibody, namely ramucirumab, is reported to bind with the VEGF receptor and increase the survivability of gastric cancer in the phase III study (*NCT number: NCT01170663*). Ramucirumab was used in advance gastric carcinoma after the first stage of chemotherapy. Again ramucirumab along with paclitaxel, also called RAINBOW, was used for treating metastatic gastric cancer. This therapy delayed the growth of cancer cells as compared with the cancer cell treated with the first line of chemotherapy. DC101, another VEGF receptor, induces the endothelial apoptosis by decreasing the tumor vascularity in human gastric cancer patient (Jung et al. 2002). DC101, in combination with C225, reduces gastric tumor growth (Park et al. 2015). Regorafenib is an oral multikinase inhibitor and target angiogenic (VEGFR1, VEGFR2, and TIE2), stromal and oncogenic receptor tyrosine kinases. In a phase II trial, regorafenib significantly increases overall survival and thus may serve as second-line or later-line therapy in advanced gastric cancer (Kiyozumi et al. 2018).

7.4.4 cMET Pathway

MET are the tyrosine kinase receptor present at the extracellular surface that encodes the hepatocyte growth factor (HGF). The activation of MET by HGF enhances cell proliferation, invasion, and tumor formation in cancer cells (Lordick 2014). In gastric cancer, the MET receptor is amplified and overexpressed at a frequent interval. On binding with the HGF ligand, the signaling pathways such as MAPK and AKT also get activated, which in turn develop gastric cancer. In most of the cases, the MET mediated signaling pathway acts directly on HER2 and stimulates the activation of downstream pathways (Chen et al. 2012). Chen and the team also reported that the activated MET provides resistance against the lapatinib of HER2 receptor amplification and decreases the rate of cell proliferation to 70%. Together lapatinib and MET inhibitors show positive results towards the lapatinib resistant gastric cancer cells. Another monoclonal antibody rilotumumab blocks the interaction of MET with HGF. Previously it was found that rilotumumab along with epirubicin, cisplatin, and capecitabine gives a positive effect to gastric esophagus cancer on first-line therapy (Iveson et al. 2014). Onartuzumab also inhibits the

binding of cMET to the HGF receptor (Kiyozumi et al. 2018). Foveau et al. reported that on cMET inactivation, MET did not bind with HGF, which in turn results in impaired dimerization of receptors. Onartuzumab is also reported to be used for the phase III trial of gastric cancer (Foveau et al. 2009).

7.4.5 Cytotoxic T Lymphocyte-Associated Antigen4 (CTL4)

CTL4 is comprised of 149 amino acids, and are mainly expressed in CD4 and CD8 of T lymphocytes. Kordi-Tamandani et al. reported that the methylation at the promoter results in silencing of *CTL4* gene, which in turn increases the risk of gastric cancer cell (Kordi-Tamandani et al. 2014). Anti-CTL4 inhibitors are used to activate the T cells for producing antibodies against colon and gastric cancers. Tremelimumab, a monoclonal antibody, works against anti-CTL4 and can be used for the treatment of gastro cancer, colon cancer, and melanoma. This therapy results in a survival rate of 4.8 months (Ralph et al. 2010; Blank and Enk 2015).

7.4.6 Tyrosine Kinase

Apatinib is a small-molecule tyrosine kinase inhibitor (TKI) that selectively binds to and strongly inhibits VEGFR2. This in turn decreases the VEGF-mediated endothelial cell migration, proliferation, and microvascular tumor density. In a phase III trial, apatinib treatment significantly improved the survival rate of advanced gastric cancer patients. Therefore, apatinib is focused on a novel type of targeted treatment for advanced gastric cancer in several lines of therapy (Li et al. 2016). Regorafenib serves as an inhibitor of angiogenic (VEGFR1, VEGFR2, and TIE2), stromal and oncogenic receptor tyrosine kinases (Pavlakis et al. 2016). Regorafenib significantly increases overall survival of advance gastric cancer patients.

7.4.7 mTOR

The mTOR inhibitor enhances the fluorouracil based apoptosis in gastric cancer cells. The phosphatidylinositol 3-kinase (PI3K) and mTOR get activated in 30–60% of gastric cancer. The PI3K and mTOR pathway dysregulations are associated with chemotherapy resistance (Oki et al. 2005). Although in phase II trial, everolimus was demonstrated to be significantly beneficial, in a phase III trial it failed to improvise the survival after first- or second-line chemotherapy (Doi 2004). The reason for these results was discussed to be partially attributable to the slightly higher percentage of placebo groups, which initiated antineoplastic therapy after a study on drug discontinuation.

7.4.8 Programmed Cell Death Protein 1 (PD1)

Avelumab is an intravenously administered PD-L1 blocking human IgG1 lambda antibody. Avelumab has now been approved by the Food and Drug Administration (FDA) for the treatment of Merkel-cell carcinoma (JAVELIN Gastric 100, NCT2625610). Durvalumab is a human IgG1κ monoclonal antibody that blocks the interaction of PD-L1 with PD-1 and CD80 molecules. This antibody has been approved for the treatment of patients with locally advanced or metastatic urothelial carcinoma. Durvalumab has also been shown to be efficient in gastric cancer. Durvalumab in combination with tremelimumab, which is a human IgG2 fully monoclonal antibody and acts against CTLA-4, is also used for treating metastatic gastric cancer in phase Ib/II trial (Borrie and Maleki Vareki 2018; Guo et al. 2019). Epacadostat is a potent and novel indolemine-2,3-dioxygenase (IDO1) inhibitor (Prendergast et al. 2017). IDO1 is an enzyme responsible for oxidizing tryptophan into kynurenine and is implicated in immune modulation through its ability to limit T cell function and engage mechanisms of immune tolerance (Kiyozumi 2018).

7.5 Side Effects of Monoclonal Antibody Therapy

Though monoclonal antibody therapy is widely employed in the treatment of GI cancer, they too have various side effects (Guan et al. 2015). Anaphylactic hypersensitivity occurs after the injection of the monoclonal antibodies cetuximab and ramucirumab (Guan et al. 2015). A delayed Type III reaction was observed against rituximab, which in turn results in serum sickness in 20% of the patients (Guan et al. 2015). Bevacizumab and ramucirumab directly cause arterial thromboembolic, hypertension, proteinuria, arterial, non-gastrointestinal fistula, and venous thromboembolism in patients (Choueiri et al. 2011; Zuo et al. 2014). Congestive heart failure was observed in patients treated with bevacizumab in the trials on solid tumors (Choueiri et al. 2011). Mucositis was observed for the cetuximab antibody (Dote et al. 2018). Pembrolizumab and pidilizumab causes rashes, diarrhea, fatigue were observed (Linardou and Gogas 2016; So and Board 2018). Trastuzumab causes anemia in the GI tract (Barni et al. 2012). Hypersensitivity occurs due to panitumumab (https://www.gov.uk). Thus, the monoclonal antibody should be employed carefully while treating any diseases, including GI cancer.

7.6 Conclusion

In conclusion, recently, monoclonal antibodies have made a remarkable transformation from scientific apparatuses to powerful human therapeutics. Monoclonal antibodies are single antibodies that are produced by fusing an immortalized cell line with antibody-producing cells, which in turn produce a new form of the cell line, namely, hybridoma. As hybridoma is "immortal," we can generate the exact antibody from them for several years. Additionally, due to high specificity, monoclonal antibodies have fewer side effects. Thus, since 1986, several monoclonal antibodies have been approved for the treatment of a wide range of diseases, including GI cancer. However, nothing is perfect in this world. Few studies have reported that these monoclonal antibodies have side effects. For instance, a delayed Type III reaction was observed against rituximab, which in turn results in serum sickness in 20% of the patients. Thus, the monoclonal antibody should be used carefully under the provision of a medical practitioner. In the near future, the information present in this chapter will be highly useful for treatment in GI cancer.

Conflict of Interest None.

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Chapter 8
Therapeutic Vaccines for Gastrointestinal Malignancies

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Abstract Gastrointestinal (GI) cancers are highly aggressive and display genome instability, gene mutations, immune suppression, immune insensitivity, and desmoplasia. GI cancers represent as one among the most common cancer type with a burden of ~25% worldwide, with each year about 4.5 million global deaths. GI cancers are not preventive, the prognosis of patients with advanced tumors was difficult, and treating the GI cancers is the only option. For many years, the treatment of GI cancer patients involve surgery, radiotherapy, and chemotherapy in combination or alone. The successes oncologists achieved so far was great but not enough, since it is only recently, the very first promising clinical data comes into light in 2015. Hence novel therapeutic ways to treat GI cancer were much required. Presently, it appears that immunotherapy is the answer. Immunotherapy is advancing quickly and outlines, a conventional shift in the treatment of GI cancer through its promising benefits beyond conventional treatments. Currently, researchers are examining a variety of medicines and factors like immune checkpoint inhibitors, ACT, peptide vaccines, cytokines, and antibodies to treat GI cancers. In recent years, the FDA approved the utilization of anti-PD-1, anti-VEGFR2, and anti-CTLA-4, immunotherapy against a few GI cancers including gastric cancer, liver cancer, and colorectal cancers. Among all the GI cancers, biliary tract cancer and pancreatic cancer patients have limited/no immunotherapeutic options at the moment, nonetheless ongoing clinical investigation will provide some assuring therapeutic solutions. It is highly important to overcome the various factors contributing to varied effectiveness of immunotherapy in GI cancers. Researchers are currently investigating the potentiality of cancer stem cells and their specific markers as targets: outcomes from

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such studies may become new waves in immunotherapy treating GI cancers. Let us hope that oncologists will discover the "Magic bullet" to whitewash GI cancers in the near future and we believe it is just the beginning of the new era for immunotherapy and we have a long road ahead to succeed.

Keywords Gastrointestinal cancers · Immunotherapy · Vaccines · PD-1 · CTLA-4 · Clinical trials · FDA

Abbreviations

(Ig)G4	Immunoglobulin G4
5-FU	Fluorouracil
A2AR	Adenosine A2a receptor
AES	Adverse events
AGEJ	Adenocarcinoma of gastric esophageal junction
APC	Antigen-presenting cells
Bcl-2	B-cell lymphoma 2
Bcl2/Bax	B-cell lymphoma 2/CL2 associated X
BRAF	B-Raf proto-oncogene
BTC	Biliary tract cancers
BTK	Bruton's tyrosine kinase
CAIX	Carboxy-anhydrase-IX
CCL2	C-C motif chemokine ligand 2
CCR2	C-C motif chemokine receptor 2
CD152	Cluster of differentiation 152
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD40	Cluster of differentiation 40
CD54	Cluster of differentiation 54
CD8	Cluster of differentiation 8
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CEA	Carcinoembryonic antigen
CHB	Chronic hepatitis B
CMS	Consensus molecular subtypes
CMS1	Consensus molecular subtypes 1
CMS2	Consensus molecular subtypes 2
CMS3	Consensus molecular subtypes 3
CMS4	Consensus molecular subtypes 4
CPS	Combined positive score
CR	Complete response
CRC	Colorectal cancer
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes

DCs	Dendritic cells
DFS	Disease-free survival
dMMR	Poor mismatch repair
DNMT	DNA methyltransferase
EBV-specific CTL	Epstein–Barr virus specific cytotoxic T lymphocytes
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ESCC	Esophageal squamous cell carcinomas
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
GEJ	Esophagogastric junction
GEM	Gemcitabine
GI	Gastrointestinal
GITR	Glucocorticoid-induced TNF receptor
GITRL	Glucocorticoid-induced TNF receptor ligand
GM-CSF	Granulocyte/macrophage-colony stimulating factor
gp100	Glycoprotein 100
GPC3	Glypican-3
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC	Histone deacetylases
HER-2/neu	Human Epidermal Growth Factor Receptor 2/neu
HIV 1	human immunodeficiency virus 1
HLA-24	Human leukocyte antigen alpha 24
HLA-A*2402	Human leukocyte antigen alpha *2402
HLA-A2	Human leukocyte antigen alpha 24
HPV	human papillomavirus
HSP70	Heat shock protein 70
ICC	Intrahepatic cholangiocarcinoma
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-4	Interleukin 4
IL-6	Interleukin 6
ITT	Intent-to-treat
KIF20A	Kinesin family member 20A
KIF20A	Kinesin Family member 20A
KIR's	Killer-cell immunoglobulin-like receptors
KOC1	Kinase of the outer chloroplast membrane 1
KRAS-G12D	Kirsten rat sarcoma G12D
LAG3	Lymphocyte-activation gene 3
LAK	Lymphokine-activated killer
LMP1/2/7	Latent membrane protein 1/2/7
MAGE	Melanoma antigen gene
MART-1	Melanoma-associated antigen recognized by T cells

Mcl-1	Induced myeloid leukemia cell differentiation protein
mCRC	Metastatic colorectal cancer
MDSC	myeloid-derived suppressor cells
MEK inhibitor	Mitogen-activated protein kinase inhibitor
MHC class I	Major histocompatibility complex class I
mPC	Metastatic pancreatic cancer
MSI	Microsatellite instability
MST	Median overall survival time
MUC1-CTL	Mucin 1 cytotoxic T lymphocytes
MUC1-DC	Mucin 1 dendritic cells
NASH	Nonalcoholic steatohepatitis
NF-kB	Nuclear factor NF-κB
NK cells	Natural killer cells
NKT	Natural killer T cells
NLR	Neutrophil/lymphocyte ratio
ORFs	Open reading frame
ORR	Overall response rates
OS	Overall survival
p53	Tumor protein p53
PAC	Pancreatic adenocarcinoma
PBMCs	peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
pDC	Plasmacytoid dendritic cells
PD-L1	Programmed death-ligand 1 (PD-L1)
PFS	Progression-free survival
pMMR	Ultra-mismatch repair
PR	Progesterone receptors
RCC	Renal cell carcinoma
RNF43	Ring Finger Protein 43
SEREX	Serological analysis of recombinant tumor cDNA expression libraries
SOC	Site of care
TAA	Tumor associated antigens
TAP1	Transporter 1, ATP binding cassette subfamily B member
TCB	T-cell bispecific
TGF-β	Transforming growth factor beta
Th1	Type 1 T helper
TIL	Tumor Infiltrating lymphocyte
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR9	Toll-like receptor 9
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
TOMM34	Translocase of outer mitochondrial membrane 34

TRAEs	Treatment-related adverse event
T-reg cells	T regulatory cells
TSA	Tumor specific antigens
VEGF	Vascular endothelial growth factor
VEGFR 1/2	Vascular endothelial growth factor receptor 1/2
WT1	Wilms tumor 1

8.1 Background

The most common kind of cancer that occurs in humans is Gastrointestinal (GI) cancer and a burden of ~25% worldwide with each year about 4.5 million global deaths and around 286,480 people diagnosed with fresh GI cancer, which is a concerning number in which the incidence and mortality are exceeding annually (Bray et al. 2018). GI cancers generally affect the gastrointestinal parts of the human body hence these kinds of cancers are said to be the most life-threatening forms of cancers (MacDonald et al. 2015). Though surgery, radiation, chemotherapy, and targeted therapy were employed as treatment strategies, the overall survival rate of GI cancer subjects continues to exist dull, implicating the necessity of novel therapeutic methods to treat GI cancer.

Researchers began to develop interest in a novel approach such as vaccine that could subdue GI cancers and its relapse. This led to the development of therapeutic vaccines/Immunotherapy for GI cancers on length (Busweiler et al. 2016). In 1891 the first attempt was made for treating cancers with vaccination (Coley 1891). Later in 1909, Smith demonstrated how important it was to activate immune network to repress tumor growth (Smith 1909). In 1970, the recognition of antigens expressed on cancer cells by T cells postulated the existence of immunological surveillance (Ribatti 2017; van der Bruggen et al. 1991). In the last decade, the introduction of immunotherapy for treating GI cancers became a necessity (Bolshinsky et al. 2018). The growth of incidences for GI cancers especially leads to the development of therapeutic vaccines (Shaw et al. 2016).

Ongoing progress in immunotherapy is attributable primarily with the discovery of therapeutic vaccines targeting tumor immunity and its microenvironment (Yaguchi and Kawakami 2016). The therapeutic vaccines specific for GI cancers comprise immune specific as well as non-specific molecular modifiers. Immunotherapy is now attracting more focus than ever as a treatment for GI cancer. The immunotherapy of GI cancers is mainly divided into checkpoint inhibitors (PD1, PD-L1, and CTLA-4), vaccine-based therapy (tumor whole cell lysates, peptide vaccines, dendritic cell vaccines), stromal modulation (FAK inhibition), cytokine-based therapy (CCR2/CCL2 modulation), and adoptive cell transfer (NK cells and T cells). In the recent past immunotherapies were applied successfully against GI cancers including peptide vaccines (*HLA-A*2402-restricted peptides and OCV-C01 from KIF20A*) (Shimizu et al. 2018; Shindo et al. 2014; Miyazawa et al.

2017), dendritic cell vaccines (*DCs transfected with HSP70*) (Maeda et al. 2015), CD8(+) tumor-infiltrating lymphocytes (*TIL*) (Turcotte et al. 2014), lymphokine-activated killer (*LAK*) (Rayner et al. 1985) and non-specific immunopotentiators (*polysaccharide K aka OK-432 and lentinan*) (Rayner et al. 1985; Oba et al. 2016; Oba et al. 2007; Yoshino et al. 2016) and immune checkpoint inhibitors (*blocking PD-L1*, *PD-1*, *or CTLA-4*, *glypican 3,* and *restriction peptides HLA-24*, *HLA-A2*) (Yaguchi and Kawakami 2016; Shimizu et al. 2018). In 2013, anti-CTLA-4 (ipilimumab) and combination of anti–PD-1 with anti–CTLA-4 checkpoint blockers of immune system were highlighted as "Breakthrough of the Year 2013" because of its efficacy against cancer patients (Couzin-Frankel 2013), indicating a promising future to treat GI cancers successfully.

8.2 How Tumors Overcome Host Immune System

The concept of vaccination for cancer began over a century ago by two physicians namely Paul Ehrlich and William Bradley Coley in 1800s. Paul Ehrlich proposed the term "The magic bullet," to kill malignant cells through the use of weakened cancer cells as Immunotherapy (Waldmann 2003). In 1896, Coley treated cancer patients with a blend of heat-inactivated bacteria named "Coley toxin" as immunotherapy against cancer (Vacchelli et al. 2012a). Even though treating cancer patients with vaccines had seen success rate but largely failed to explain the anti-tumor immunity development by cancer cells (Vacchelli et al. 2012a). While understanding the anti-tumor immune response of cancer patients, Frank Macfarlane Burnet theorized "self/non-self" division, briefing that tumors are capable of creating their self-tissue makes them easy to escape immunotherapeutic interventions (Burgio 1990). Polly Matzinger published a paper in 1994, in which he proposed (Matzinger 1994) that, "Immune system does not care about self and non-self, that its primary driving force is the need to detect and protect against danger, and that it does not do the job alone, but receives positive and negative communications from an extended network of other bodily tissues." In the following years, cancer and trauma: the immunologically silent factors now became activators of immune system (Vacchelli et al. 2012b; Bezu et al. 2018). To ameliorate reactions of immune system, cancer cells underwent many diversions to install resistance to immune-therapeutics. Some of the silent features that cancer cells adapted to escape immune system are discussed below.

8.3 Tumor Immunosurveillance

In our body cells are continually watched by vigilant immune system at all times to detect and destroy inceptive cancer cells. However, subsequently, tumor tissue cells accomplished to escape detection or became insensitive to immunological attacks by the immune system, thereby avoiding destruction (Hanahan and Weinberg 2011).

Tumor immunosurveillance theory further confirmed through various experimentations where immunodeficient mice develop tumors more rapidly, particularly the tumor incidences were more significant in mice lacking immune functioning of natural killer (NK) cells or Th1 (CD8+ and CD4+) cells, indicating that both innate and acquired immune responses contributed equally in escaping immune killing and tumor immune surveillance (Vesely and Schreiber 2013; Kim et al. 2007). Various studies from humans show that effective immunosurveillance do exist, and in fact, the prognosis of colon cancer patients became more comfortable due to profoundly infiltrated CTLs and NK, whereas the absence of TILs adds less prognostic significance in ovarian cancer patients (Pages et al. 2010; Leffers et al. 2009). The tumor immunosurveillance theory further confirmed by the existence of particular cancers in immunocompromised people (Vajdic and van Leeuwen 2009) and evoking immune reactions specific to tumor-specific/associated antigens in cancer patients (Wang 1999; Sahin et al. 1995).

8.4 Tumor Immunoediting

Fifty years ago, immune surveillance theory validated that the immune system is sophisticated to detect and eliminate inceptive cancer cells (Burnet 1957); however, the latest evidence on the role of the immune system on eliminating these cancer cells becomes invalidated. Over the past 15 years, a substantial amount of work on immune surveillance, nature of immunity assisted in perfecting and elaborating new concept, Immunoediting—phases of the immune system and tumor interactions took place. Immunoediting composed of three stages, (1) Tumor fixed immune reactions to control growth of tumor cells effectively, thus "Eliminating" cancer cells, (2) The gain of immune insensitivity to cytotoxic functions or loss of immunogenicity to maintain sustainable "Equilibrium." (3) Before-mentioned cells ultimately grow uncurbed leading to a clinically visible tumor, hence the "Escape" (Dunn et al. 2004). Figure 8.1 demonstrates the molecular changes describing how tumor cells evade both (innate and adaptive) immune systems. The occurrence of tumor immunoediting as a core to explain the magnitude of the immune system's synergy with cancer, has, in part, inspired a recent proliferation of the scientific evidence addressing this process as exhibited by dramatically grown citation (Mittal et al. 2014). Immunoediting relates to the transformations that take place unconsciously as the tumor grows in the leadership of an unbroken immune system and the perception of such mechanism provides major suggestion for immunotherapy in human cancers where nullifying immune damage was lately recommended as a prominent trademark of cancer (Hanahan and Weinberg 2011).

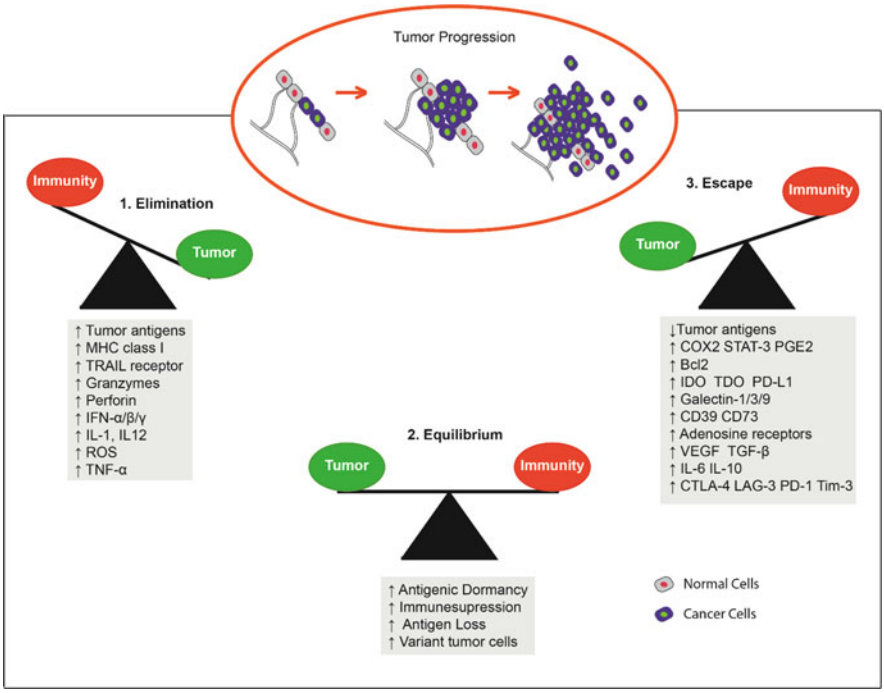

Fig. 8.1 How tumor cells overcome host immune system. Tumor cells follow a series of events in order to prevent destruction by host immune system. The multi-step event occurs at three different stages—(1) Elimination—tumor cells and tumor microenvironment dominates host immunity by expressing tumor antigens, perforin, granzymes, FAS ligand, TNF-related apoptosis-inducing ligand (TRAIL) receptor, major histocompatibility complex (MHC class I), Reactive oxygen species (ROS), Interleukins (IL-1/12), Tumor necrosis factor (TNF-α), Interferons (IFN-α/β/γ). (2) Equilibrium—tumor cells lose their antigens to undergo dormant state where tumor cells can tolerate immune stress. Following functional dormancy, genetic changes occur within tumor cells to produce new tumor cells with defective or low antigen capacity. (3) Escape—Tumor cells grow resistant to immune reactions by secreting angiogenic cytokines including Interleukins (IL-6/10), Transforming growth factor (TGF-**β**), Vascular endothelial growth factor (VEGF) and targets related to immunosuppression such as adenosine receptors, Tryptophan 2,3-dioxygenase (TDO), indoleamine-pyrrole 2,3-dioxygenase (IDO), Galectin-1/3/9, Cluster of Differentiation 39 (CD39), Cluster of Differentiation (CD73) and immune inhibitory receptors like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Programmed cell death protein 1 (PD-1), Lymphocyte-activation gene 3 (LAG-3) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)

8.5 Tumor Immune Response

Despite inhibiting tumor growth, immune responses may promote tumor development through activating chronic inflammation, which in turn provokes the growth, development, and angiogenesis of cancer cells. Hosts counter infections by installing blockades and stimulating various levels of both (innate and adaptive) immune

protection, where the contaminated tissue coordinates the health, condition, and type of the immune response for effective infection elimination and tissue replacement while checking corresponding tissue loss (Matzinger and Kamala 2011). At the site of infection derived inflammation, tumors might originate, and begin to live symbiotically in the host by suppressing extreme inflammation with anti-tumor immune responses. The proinflammatory wound healing microenvironment created by stromal and epithelial cells at the injected site, in turn, promotes tumor development through angiogenic-tissues remodeling expansion that drives tumor cell invasion and progression (Edme et al. 2002; Salcedo et al. 2013)

Malignant cancers stem inside differentiated tissues and have some of the functional, architectural, and immune features of their tissue of origin and mimicking the original tissue (Pierce and Speers 1988). The immune response generated by the neoplasm will be in most examples helpless to destroy it and will install a Darwinian environment picking the genetically adapted cancer cells that unfold into threatening malignant tumors or live momentarily in equilibrium with non-malignant host cells (Schreiber et al. 2011). In addition to tissues of tumor origin, pathogens, carcinogens, the nature of tissue damage, and proinflammatory mediators secreted from tumor or their stroma determine the class of inflammatory/immune response observed in cysts. Establishment of the suppressed immune response through a pathway that promotes tissue growth, T-cells deficient in IFN-**γ**, TNF, granzymes, perforin effector molecule, and high amounts of (CTLA-4, PD-1, LAG3, TIM-3, A2AR and KIR's) inhibitory molecules is inevitable (Goldszmid et al. 2014). The proinflammatory cytokine TNF-α, released from either macrophage or mast cells, is involved in initial melanoma growth during contaminations. TNF-α aids tumor development, continuation following the enrollment of immune effector cells along with active angiogenesis (Dougan and Dranoff 2009). In a colon cancer mouse model, immune cells lacking NF-κβ proved a decline in tumor progression and limited tumor prevalence in intestinal epithelium when NF-κβ was removed (Greten et al. 2004).

8.6 Tumor Immune Escape

The immune evasion mechanisms of tumors become a question to address. The immune surveillance system eradicates a considerable amount of rogue cancer cells, yet for a variety of unknown reasons melanoma cells are still able to progress the suppression by the immune system. Mechanisms of evading immune attention include but not limited to various stages of tumor development. The efficacy of immune mechanisms to discriminate healthy cells from cancerous ones is vital in immunotherapy, which depends to some extent not entirely on cancerous cells storing enough immunogenicity. Tumor displays a mixture having both (mutated and non-mutated) antigens which contain the potentiality toward immune responses that are tumor-specific (Coulie et al. 2014). Though, in bypassing the immune attacks, tumor cell can succumb their immunogenicity. Not having immunogenicity

may come from careful immune assortment of tumor cells which require either defective tumor antigens or attainment of errors or insufficiencies in presenting antigen (Schreiber et al. 2011).

Tumor cells on their own acquire various tactics permitting cancer cell to evade monitoring and removal through immune system. Tolerance stimulation is one among the primary means that comprises different stages. In addition to losing HLA-allele expression, in cases whole MHC class I absence was reported where β2-microglobulin gene mutations are solely responsible for MHC I loss, this loss corroborates with evasion for CTL recognition (Poggi et al. 2005; Upadhyay et al. 2015). In some cases, tumors are sensitive to immune attack though MHC I is lost completely, here NK cells carry out immune reactions; however, certain tumors present themselves with either poor NK cell immunological memory or contain very few NK cells in them (Poggi et al. 2005; Kaufman and Disis 2004). Adding more complexity to the current situation is that absence of TAA in some tumors makes them resistant to immune attack irrespective of MHC I and NK cells, CTL responses (Poggi and Zocchi 2006). Tumor cells acquire defective antigen presentation and processing through down regulation of delivery molecules such as LMP1/2/7 (Hayashi et al. 2011), proteasome components, and TAP1 (Johnsen et al. 1999), and loss of tapasin protein (Shionoya et al. 2017). The downregulation sometimes results in defective molecule synthesis or complete loss (CD80 and CD86) of antigens on cell surfaces (Poggi et al. 2005; Staveley-O'Carroll et al. 1998). The two concepts immunodominance and immunoselection by tumor cells are also linked with immune evasion mechanism. In tumor microenvironment immune responses are always aimed at dominant antigen holding tumor cells, creating a hierarchy within tumor antigens (Cohen et al. 2010). As the tumor growth continues dominant antigens will disappear and a new hierarchy is built within emerging antigens creating immunodominant thus creating an immunoselection process. Defects in molecular pathways like apoptosis (Bcl-2, Mcl-1, p53, and Bcl2/Bax) in tumor host can provoke immune escape (Lopez and Tait 2015; Sayers 2011). Discharge of immune-suppressive cytokines [IL-6/10 (Fisher et al. 2014; Dennis et al. 2013), VEGF (Mulligan et al. 2010), and TGF beta (Massague 2008)] from either tumors or tumor stroma is also considered one of the immune escape plans. Studies recommend that functionally abnormal immune cells residing in tumors play role in developing an immune-suppressive state (Pinheiro et al. 2011). Presence of immature MDSC, non-functional macrophages, and Tregs confers suppressive environment in tumors (Kumar et al. 2016; Noy and Pollard 2014; Chaudhary and Elkord 2016).

8.7 Tumor Immune Checkpoints

A broad array of both stimulatory and inhibitory immune checkpoints molecules is identified and only few of them were discussed in the current section. The neoplastic immune microenvironment comprises a broad array of mixed cooperation between

tumor cell, tumor stroma, and immune cells (APC, T cell, NK cell, B cell). Immune response toward neoplasm is a consequence of competition among stimulatory and signals. Immune checkpoints are primary immune regulators in controlling immune homeostasis and inhibiting autoimmunity. The checkpoints of the immune system consist of both inhibitory and stimulatory agents; these are significant in preserving self-tolerance, monitoring the type, and the extent continuation of the immune response. Under ordinary situations, immune checkpoints support the immune system to react against pathological condition and tumor invasion while defending tissues from any harm that may stem from this action. But, the circulation of immune checkpoint proteins from tumor cells dysregulates the tumor resistance and supports growth and development (Pardoll 2012). The checkpoint protein such as PD-1 always expressed as a cell surface receptor by cells (pro-B or T cells) of immune system binds with one of the PD-L1/2 ligands. The PD-1 signaling prevents T-cell reactions at the former effector step limiting unnecessary activation of T cells. However, the ligands for PD-1 are present on tumor cells to evade anti-cancer immune reactions (Urszula and Krzysztof 2016). The contact of PD-1 receptor with own ligands on tumor cells provokes chronic T-cells inhibition, where T-cells lose their immune potential which leads to a drop in the immune response to cancer cells.

CTLA-4 is another checkpoint molecule expressed by only activated T cells which interacts with APC associated B7-1 and B7-2 ligands with higher affinity (Buchbinder and Desai 2016). CTLA-4 blocks T cell activation and performs an essential part in the initial phases of an immune reactions. Instead, CTLA-4 constitutively expressed on tumor cells to prevent T cell proliferation and effective functioning (Upadhyay et al. 2015; Contardi et al. 2005). GITR is a checkpoint protein highly displayed by T regulatory cells and interacts with GITRL ligand present on DC (Knee et al. 2016). At the inflammatory site, the binding GITR from T-reg cells with GITRL of DC blocks the repressed action of T-reg cells with subsequent enrichment of T-effector cell persistence. GITRL downregulates expression of the immune-stimulatory molecules (CD40 and CD54), influences the expression of TGF β, an immunosuppressive factor released from cancer cell, and blocks the expression of EpCAM (Urszula and Krzysztof 2016). LAG-3 is another immune checkpoint inhibitor protein involved in the immune escape mechanism of tumor cells (Long et al. 2018). LAG-3 is present on immune cells (T/B cells, NK cells, and pDC) and can bind to MHC class II. The LAG-3/MHC-II complex can work as bidirectional preventive mechanism shared by both immune and cancer cells (Hemon et al. 2011). In a similar fashion to CTLA-4 and PD-1 functioning, LAG-3 inhibits immune cell activation, proliferation, and homeostasis. Basic knowledge of the immune evasion procedures employed by checkpoint molecules may direct to better prognostic markers and escort the advancement of targeted medications that are both reliable and more powerful than current standards of care.

8.8 Antigen Identification and Cancer Immunotherapy

Immunotherapy is a procedure that boosts or engineers the immune system as a tumor-killing machine. Controlling the immune system to abolish tumor cells is growing as the most efficient method to treat carcinoma in patients. To earmark and kill tumors our immune system must be proficient in noticing the cancer antigen as "foreign intruder" (Fuchs et al. 2016). Both innate and acquired immune pathways play a central part in surveillance, verification, and removal of cancer tissues (Fuchs et al. 2016). But, when tumor continues to grow, our immune network fails in eradicating malignant cells as the immune network is compromised. Identification and characterization of tumor antigens provide great help in developing vaccines against tumors to stop. When tumor growth continues, immune system fails to recognize most of the antigens present on tumor cells and tumor microenvironment, hence tumor continues to grow exponentially and even spreads to nearby tissues. The antigens corresponding to tumor are divided into two individual groups based on their antigen specificity: TSA (tumor-specific antigens) and TAA (tumor-associated antigens) (Vigneron 2015). Tumor-specific antigens exhibit high tumor specificity and only expressed on tumor cells, including (1) viral antigens, (2) antigens encoded by mutated genes (neoantigen) from tumors, (3) cancer-germline genes (Vigneron 2015). TAAs exhibit low tumor specificity and present on few healthy tissues as differentiated antigens and some derived from overexpression of tumor genes. Figure 8.2 provides some of the familiar tumor antigen types and examples.

In parallel to understand suppressed tumor immunity, significant attempts have been performed in the past 25 years in cancer antigen detection with aims to generate cancer vaccines. Without the knowledge of tumor antigens, it seems dark to develop immunotherapeutics for cancers. Identification of different molecular mechanisms contributing to the formation of various T cell epitopes on tumors aided in discovering novel tumor-reactive T cell epitopes in the past. Among the many mechanisms contributing synthesis of diverse T cell epitopes, few important possibilities were presented here, such as alternative ORFs, somatic mutations, from intronic sequences and protein splicing (Fuchs et al. 2016). The discovery of tumor antigens (T cell specific antigens) involves the use of tumor-responsive T cells from humans with cancer or cancer cell models. Target cells including PBMCs or TILs get transfected with tumor cDNA library. Later by applying peptide recognition and truncation CTL epitopes are defined. This strategy is most direct and mainly used to identify antigens as well as epitopes specific to T cells. Tumor-specific T-cell antigens defined through current immunological procedures include tyrosinase, MART-1, MAGE families, and gp100 (Vigneron 2015). In 1991 for the first time, cDNA library screening with tumor-responsive HLA-res CD8+ T cells led to the discovery of human tumor antigen (van der Bruggen et al. 1991). The overexpressed antigens located in tumors are identified by using gene-expression profiling method since these antigens can be distinguished by CTL to induce an immune reaction. Tumors that are high in the expression of the catalytic subunit of telomerase can

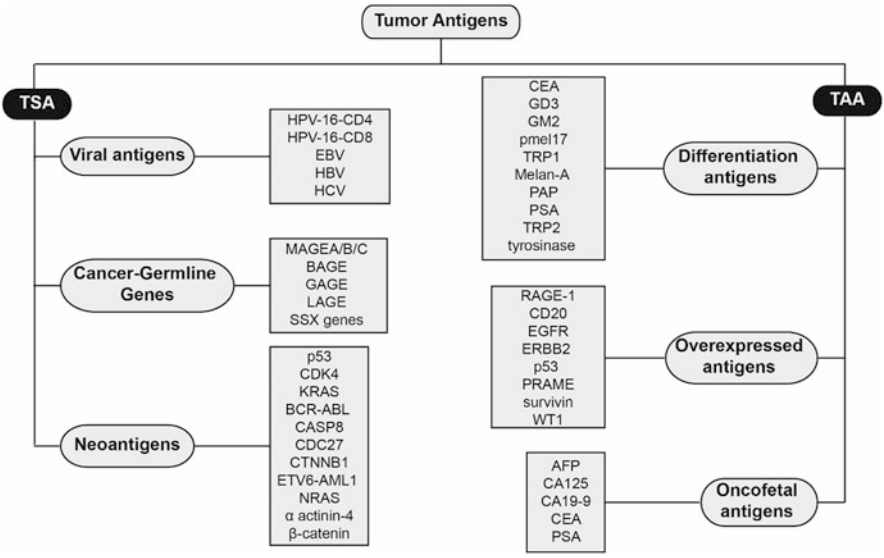

Fig. 8.2 Varieties of tumor antigens expressed by tumor cells. Tumor antigens are classified into two groups called Tumor specific antigens (TSA) and Tumor Associated Antigen (TAA). Viral antigens—Human papillomavirus type 16—Cluster of Differentiation 4 (HPV-16-CD4), Human papillomavirus type 16—Cluster of Differentiation 8 (HPV-16-CD8), Epstein–Barr virus (EBV), hepatitis B virus (HBV) and hepatitis C virus (HCV), Cancer-Germline Genes - Melanoma Antigen Gene (MAGE A/B/C), B melanoma antigen (BAGE), GAGE (G antigen), L Antigen (LAGE) and synovial sarcomas X (SSX) and Neoantigens—Tumor protein (p53), *Cyclin-dependent kinase 4* (CDK4), Kirsten rat sarcoma (KRAS), breakpoint cluster region—Abelson murine leukemia (BCR-ABL), *Caspase 8* (CASP8), Cell division cycle protein 27 (CDC27), Catenin Beta 1 (CTNNB1), ETS Variant 6—Acute Myeloid Leukemia 1 (ETV6-AML1), Neuroblastoma RAS Viral (NRAS), α actinin-4 and β-catenin) belong to tumor specific antigens. Differentiation antigens—Carcinoembryonic antigen (CEA), Ganglioside (*GD3*), Monosialoganglioside (GM2), Premelanosome protein (pmel17), Tyrosinase related protein 1 (*TRP1*), Melan-A, Prostatic acid phosphatase (PAP), Prostate-specific antigen (PSA), Tyrosinase related protein 2 (TRP2) and Tyrosinase and Overexpressed antigens Renal Cell Carcinoma Antigen (RAGE-1), B cell differentiation *antigen* (CD20), Epidermal growth factor receptor (EGFR), Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2), Tumor protein (p53), preferentially expressed *antigen* in melanoma (PRAME), survivin and Wilms Tumor *antigen* WT1), and Oncofetal antigens Alpha-fetoprotein (*AFP*), cancer antigen 125 (CA125), Cancer *antigen* 19-9 (CA19-9), *Carcinoembryonic antigen* (*CEA*) and Prostate-specific antigen (PSA) belong to tumor-associated antigens

mediate cytotoxic T lymphocytes to provoke an immune reaction (Vonderheide et al. 1999). Serological screening of recombinant cDNA expression libraries SEREX is applied to identify T cell targets via B cell responses in patients (Sahin et al. 1995; Fuchs et al. 2016).

8.9 Types of Immunotherapeutic Vaccines

A therapeutic vaccine is generally used by clinicians once the detection of GI cancer. However, therapeutic vaccines have manifested to be potent in case of averting the occurrence of cancers by subduing infections in the human body that could lead to GI cancers. Therapeutic vaccines have been effective in preventing tumor growth in gastrointestinal areas (Spira et al. 2016). There are many types of therapeutic vaccines that could prevent the growth of cancers in gastrointestinal areas. Some major types of therapeutic vaccines are explained.

Autologous vaccines that are used in the treatment of GI cancers are personalized vaccination developed from the cells of an individual. The autologous vaccine is developed by removing few cells from the GI tumor. These cells are then treated in a certain way that would make the cells a target for the human immune system. Engineered cells are then injected into the human body. The immune system then recognizes the injected cells and eventually disables those. In a similar way, the now activated immune cells disable the cancer cells in the body. HLA and blood group antigens are examples of autologous vaccines that were developed using this method.

Allogeneic vaccines are also used in the treatment of GI cancers. The word "allo" is a synonym for "other." Allogeneic vaccines for treating GI cancers are developed from cancer cells that not derived from the body of the patient but are generated in the lab. These kinds of vaccines have already been tested for preventing growth of pancreatic and colorectal tumors. Allogeneic vaccines have high demand because these are cheaper and also the production cost is low. However, these vaccines are not enough effective compared to other therapeutic vaccinations. The vaccine is yet to be licensed. However, at an early stage of GI cancers, this vaccine could be effective to some extent. Human blood group and EGFR antigens is an example of allogenic vaccine.

Peptide vaccines are generally protein parts. These parts could also be smaller components of proteins called peptides. The peptides and protein parts are delivered into the human body as vaccine generally paired with viruses or molecules that could stimulate the immune system. These vaccines are still at trial but some clinics are using these vaccines, for example epitopes.

DNA vaccines are another therapeutic approach to treat GI cancers and designed using tumor antigen DNA via dendrite cells to provoke the immune system against an existing GI tumor. The patient subjected to GI cancer is vaccinated with DNA rings. These rings of DNA are known as plasmids. The plasmids develop antigens for preventing further growth of GI tumors. Cancer vaccines for treating GI are required to be cost-effective so that patients could afford these vaccines. Doctors are required to develop vaccines that could prevent the occurrence of cancers. HPV and HIV 1, BRAF, and stemness are examples of DNA vaccines.

8.10 Molecular Targets of Immunotherapeutic

Oncologists are marching forward to consider immunotherapies against multiple GI cancers pardoning the presently accessible plans. It was notable that treatments like chemotherapy, surgery, anti-angiogenic therapy, and radiation therapies were aiding in the treatment of GI cancer; however, recent reports on immunotherapies and their efficacies against GI cancers attract a lot of interest among oncologists and clinicians. New procedures involving immunotherapies such as checkpoint inhibitors, adoptive cell therapies, protein/peptide, cytokines, whole cell/dendritic cell vaccines, and their efficiencies were well documented through many clinical studies against GI cancers. Figure 8.3 provides a broad understanding on different kinds of molecular targets and means of immunotherapy applied on a range of gastrointestinal cancer.

8.11 Checkpoint Inhibitors

Immunosurveillance crucially engages in tumor growth and development. Similar to cell cycle arrest pathways, cancer cells bear the adaptability to activate different pathways specifically immune checkpoints as a way to suppress the anti-tumor immune function of tumor cells. Therapeutic monoclonal Ab's that target immune checkpoints hold a tremendous potential to treat GI cancer and hold a breakthrough in the realm of immuno-oncology. Immune checkpoint blockers such as PD-1/PD-L1 and CTLA-4 revealed assuring therapeutic upshots in the recent past, and some were recommended for specific tumor treatments, meanwhile other checkpoint protein targets undergoing clinical investigations (Darvin et al. 2018). In 2013, FDA approved the use of ipilimumab (CTLA-4) as an immunotherapeutic agent against metastatic melanoma, colorectal cancer, and renal cell cancers. Following success with ipilimumab approval, a study reported beneficial outcomes in 300 cancer patients with anti-PD1. Later nivolumab, pembrolizumab and atezolizumab approved to treat colon cance, liver cancer, and stomach cancer (Lipson et al. 2013; Topalian et al. 2012). Other potential PD-1/PD-L1 drugs currently employed to treat bladder cancer include pembrolizumab, nivolumab, cemiplimab, atezolizumab, avelumab, and durvalumab.

8.12 Adoptive Cell Therapies (ACT)

In this approach immune cells mostly NK cells or CD8+ T cells from either tumor or guarding lymph node are sequestered, increased in number under ex vivo conditions, and finally infused into the host using fludarabine/cyclophosphamide. Early in 1988, the first successful application of adaptive cell therapy via tumor-infiltrating lymphocytes (TIL's) on solid tumors was conducted by Rosenberg and his colleagues. The use of node-derived autologous CD4+ Th1 cells against colorectal cancer

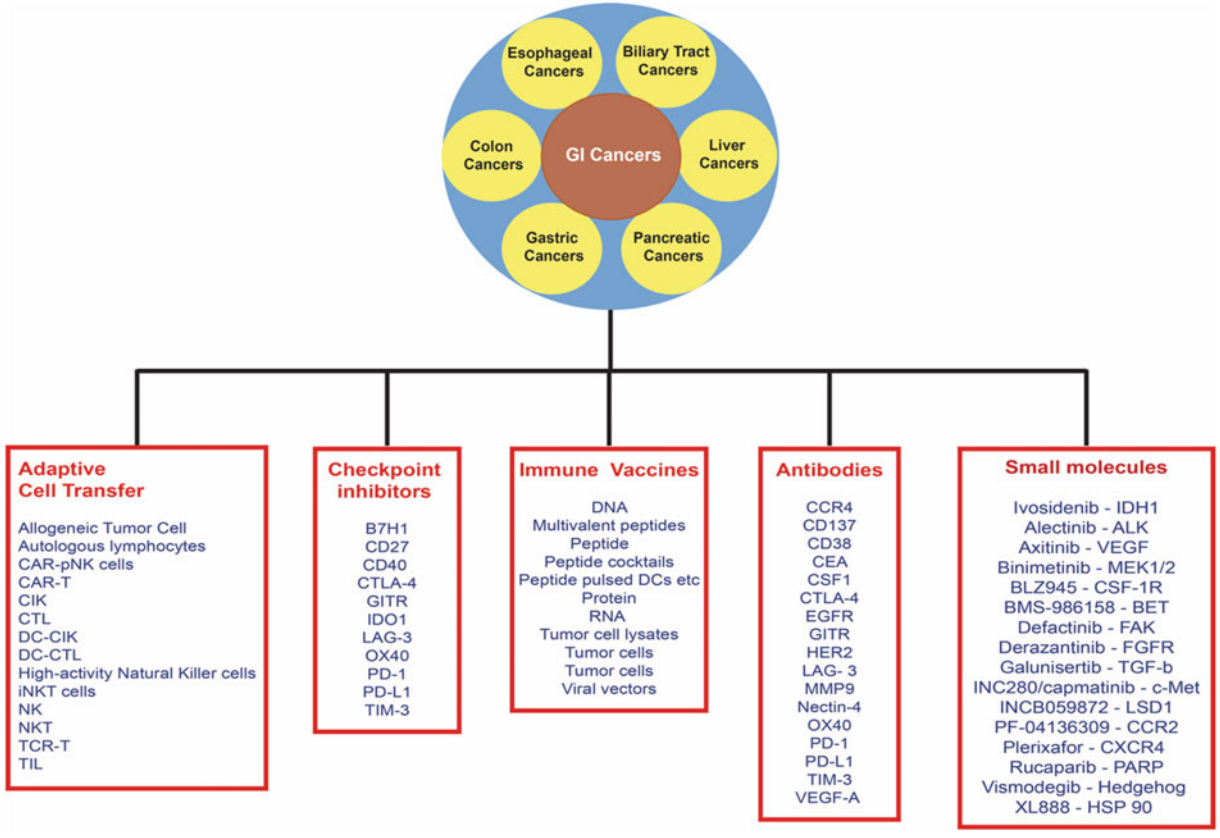

Fig. 8.3 Modes of Immunotherapy on broad range of GI cancers. A schematic representation of classification of general therapeutics against GI cancers. The modern-day immunotherapy in GI cancers including small molecules, monoclonal antibodies, vaccines, checkpoint inhibitors, and adaptive cell transfer

patients resulted in tumor regression and long-term survival (Karlsson et al. 2010). In 2016, a study reported that TILs derived from colon cancer could induce an immune response in tumor-specific driver mutation (KRAS-G12D) bearing tumors implying beneficial outcomes of using adoptive cell therapies involving T cells (Tran et al. 2016). Administrating TILs in humans with metastatic melanoma elicited tumor suppression (Phan and Rosenberg 2013). Activated CTLs such as mucin 1 (MUC1-CTLs) were shown to be effective against pancreatic cancers and liver metastasis in patients who underwent radical pancreatectomy (Matsui et al. 2017). Many independent studies got assuring results in a variety of solid tumors using adaptive cell transfer technology on nasopharyngeal cancer (EBV-specific CTL) (Secondino et al. 2012), hepatocellular carcinoma (Interleukin-2 and anti-CD3) (Takayama et al. 2000), renal cell carcinoma (RCC) anti-carboxy-anhydrase-IX (CAIX) (Lamers et al. 2013) and gastric cancer (T cell specific anti-HER-2/neu peptide) (Kono et al. 2002). So far adoptive cell immunotherapy is proven to be safe, viable in lowering GI cancers. The adoptive cell immunotherapy currently growing as one of the most favorable master plan in cancer treatment, and several clinical studies are happening worldwide.

8.13 Dendritic-Cell Vaccines

The biological nature and active participation in T-cell activation of DC's have been the great importance in developing DC-based vaccines for cancer immunotherapy, hence opening new paradigms in the advancement of practicable clinical protocols (Guo et al. 2013). DCs are functional as APC when challenged with antigens DCs can initiate and sustain immune responses (Banchereau and Steinman 1998). DCs role as antigen presenting cells can trigger the participation of NK and/or NKT cells in both cellular and humoral immune responses (Osada et al. 2006). Both adaptive and innate immune pathways are well attended by DCs thus making a substantiating place in anti-cancer immunotherapy for cancer patients (Sabado and Bhardwaj 2015). DCs can be pulsed with peptides, whole proteins, DNA constructs, tumor lysates, or tumor cells in the process of generating DCs vaccines (Shang et al. 2017). A study treated 16 melanoma patients with DC pulsed with a cocktail of tumor lysates using IL-4/GM-CSF found beneficial results in five patients since then this approach has been applied worldwide as standard procedure (Nestle et al. 1998). A phase I clinical investigation conducted on HCV-based HCC subjects using DCs pulsed with HSP70-mRNA reported that HSP70-DCs based treatment is safe and viable as HCV-based HCC express high levels of HSP70 and linked with loss of HLA-1 and improved tumor differentiation (Maeda et al. 2015). In trail outcomes, at grade III/IV zero side effects were seen; complete response (CR) with an absence of relapse was accomplished in a pair of patients, indicating HSP70-DC adapted therapy efficiency against HCC. Another study employed DC pulsed with mucin1-mRNA/MUC1-CTL complexes in combination with chemotherapeutic drug gemcitabine tested on 42 subjects with cyclical pancreatic cancer (Shindo et al. 2014). The outcomes of the study included 13.9 months of median survival, 51.1%

rate of 1-year survival, and 61.9% disease control with no side effects contributing efficient immunotherapy on pancreatic cancer. Another clinical investigation conducted on progressive pancreatic carcinoma patients treating with DC vaccine plus LAK cells in combination with gemcitabine nearly on 49 subjects reported complete/partial remission, stabilized diseases in 10 with overall survival time increased following no side effects. This study implied that immunotherapy with DC vaccines in combination with chemotherapy provides an effective strategy to treat pancreatic cancer (Kimura et al. 2012). Phase II study in HCC patients with DCs pulsed with tumor lysate demonstrated that DC vaccination is well tolerated, safe, and promising with anti-tumor ability (Palmer et al. 2009). All the above-discussed results indicate the growing importance of DCs as a winning immunotherapeutic strategy to treat GI cancers.

8.14 Peptide Vaccines

Over the decade maneuverings of cancer-vaccination claimed enormous interest and many reports are coming out delineating importance of peptide vaccines in clinical practice to treat cancer. In 1995, the first-rate clinical analysis of the MAGE-1 based vaccine took place. The initial creations of peptide vaccines did constitute of one or more HLA-I based antigen types. The current varieties of peptides based vaccines fall under six different categories such as (1) long multivalent peptides; (2) multiple peptide vaccines (CTL-helper epitope); (3) blend of peptide vaccines; (4) peptide-pulsed DC vaccines; (5) hybrid peptide vaccines; (6) personalized peptide vaccines (Yamada et al. 2013).

The anti-cancer benefits of glypican-3 (GPC3) peptide vaccine against HCC as adjuvant immunotherapy reported through phase II studies. 35 subjects who underwent surgery and vaccination did not show any variations in recurrence rate compared with subjects who underwent surgery alone; however, there was a lower recurrence rate in GPC3 vaccinated subjects who developed GPC3-positive tumors improved for 1-year in a subgroup analysis (Sawada et al. 2016). Clinical treatment with GPC3 derived vaccine on a single patient possessing HCC revealed tumor lysis and durable effects, nonetheless, the subjects died from circulatory failure (Sawada et al. 2013). Another phase I trial study proclaimed safety, anti-tumor efficacy, improved overall survival and clinical responses of GPC-3 peptide vaccination against HCC in humans (Sawada et al. 2012).

In the phase II clinical study, pancreatic cancer patients treated with cocktail peptide vaccines called OCV-C01 derived from kinesin family member 20A (KIF20A), VEGFR 1/2 along with gemcitabine after going through surgery. In this setting, peptide cocktail vaccines combined with chemo drug were endurable with 15.8 months of median disease-free survival (DFS) (Miyazawa et al. 2017). In another phase II study, VEGF receptors and tumor antigens used to derive five HLA-A*2402-limited peptides for treating advanced colorectal cancer subjected in combination with chemotherapy drug oxaliplatin. In this study, the expected endpoints remain the same between treatment and untreated groups, yet a continued response

with <3.0 neutrophil/lymphocyte ratio was observed (Hazama et al. 2014a). Humans with advanced CRC were treated with multiple peptides (HLA-A*2402-restricted peptides, VEGFR1/2, RNF43, oncoantigens, KOC1 and TOMM34) to evaluate the immunological outcomes following endpoint results in alleviating cancer symptoms in phase I clinical study. The treatment was safe, well-tolerated and the presence of peptide-specific CTL reported. The clinical significance included that one subject lived for 10 years without cancer relapse; in six subjects the cancer was stable for 4–7 months and 13.5 months of median overall survival time (MST) recorded (Hazama et al. 2014b).

Currently available immune-based peptide vaccinations for CRC gave a response rate (0.9%) and objective response rate (2.6%) that is regarded as insignificant by National Health Insurance to provide financial support (Nagorsen and Thiel 2006). Although TAA based vaccines proved less efficient on CRC, a clinical trial using monoclonal antibody against EGFR called cetuximab has been approved by FDA to treat CRC (Bou-Assaly and Mukherji 2010). Also, most recently, studies reported that cetuximab combination with chemo treatment improves the infiltration of the immune cell population into metastatic liver sites in CRC patients (Inoue et al. 2017). Two independent clinical investigations revealed the importance of immunotherapy to treat melanoma. Ott et al. showed that the use of the neoantigen vaccines against melanoma patients as a promising therapeutic strategy since the neoantigen vaccination provided safety, feasibility, and immunogenicity (The Cancer Genome Atlas Research Network 2017). Moreover, RNA-based poly-neo-epitope vaccination approach against melanoma invoked T-cell responses, reduced metastasis, and sustained progression-free survival (PFS) in patients (Sahin et al. 2017). Noteworthy to mention that applying abovementioned two strategies can endure a purpose in the future to treat GI cancers.

8.15 Therapeutic Vaccines and GI Cancers

8.15.1 Esophageal and Gastric Cancer Vaccines

Though multiple treatments like radiotherapy, surgery, and chemotherapy have been performed to treat esophageal squamous cell carcinoma, the five-year global survival is quite weak at 30–40%, suggesting an immediate need for improvised treatments. ESCC exhibits high somatic mutation rates making it amenable to treat with therapeutic vaccines (Mimura et al. 2018). Previously over expression of PD-L1/2 has reported in 41 esophageal cancer resection specimens, thus PD-L1/2 becomes a fundamental prediction agent in ESCC patients (Ohigashi et al. 2005; Loos et al. 2011; Zheng et al. 2014). A monoclonal (Ig)G4 antibody commonly referred to as Nivolumab is used against 65 Japanese ESCC patients where the antibody provided safety and promising immune responses involving PD-1 enervation (El-Khoueiry et al. 2017). Limited data support the employment of immunotherapy over ESCC because ESCC now is separated from esophageal adenocarcinoma (The Cancer

Genome Atlas Research Network 2017). A clinical investigation (KEYNOTE-028) enrolled mixed patients with either ESCC (74%) or AGEJ (22%) positive for PD-1 to test pembrolizumab. The study achieved a viable toxicity and grade 3 treatment-linked unfavorable episodes (Doi et al. 2016). Table 8.1 provides clinical investigations undertaken to evaluate different types of immunotherapies on EC.

A clinical study (KEYNOTE-012) with 36 GEJ or advanced stomach cancer patients evaluated and reported the beneficial anti-tumor nature of pembrolizumab (anti-PD-1) for the first time. The ORR was 22%, TRAEs of grade 3 or 4 recorded in 13% (Muro et al. 2016). Currently, phase II and III clinical investigations are ongoing with anti-PD-1 treatments in ESCC. Primary outcomes of multi-cohort study KEYNOTE-059 conducted on advanced gastric cancer subjects (259) reported the use of pembrolizumab and chemotherapy as the first line of treatment. Upon data cut-off in 67% of subjects grade 3/4 TRAEs were recorded, none of which implied to pembrolizumab (Fuchs et al. 2016). A phase II study recently reported the positive outcomes of pembrolizumab on previously treated advanced gastric tumros. Enduring counter was witnessed in subjects with either negative or positive for PD-L1 (Fuchs et al. 2018). Based on the aforementioned data FDA recommended pembrolizumab over PD-L1-positive gastric/GEJ adenocarcinomas in September 2017 (Mehta et al. 2018).

KEYNOTE-180, phase II trial studied the efficacy and safety of pembrolizumab monotherapy on ESCC, advanced/metastatic adenocarcinoma patients and published that pembrolizumab granted long-lasting anti-tumor actions with manageable safety of esophageal cancer patients and awaits data from an ongoing phase III trial (Shah et al. 2018). Another phase III trial, KEYNOTE-061 evaluated chemotherapy vs. pembrolizumab on GEJ adenocarcinomas and metastatic gastric tumors positive with PD-L1 (Ohtsu et al. 2016). The study failed to meet OS, the primary endpoint, but maintained pembrolizumab safety profile (Shitara et al. 2018). To estimate the potency of pembrolizumab as chosen medication in PD-L1 positive/HER-2neu-negative advanced metastatic GEJ adenocarcinomas, KEYNOTE-062 phase III combination studies did not increase OS or PFS when compared with chemotherapy alone. However, in a branch assessment pembrolizumab monotherapy met the primary outcomes of OS in the whole intent-to-treat (ITT) patients whose neoplasms display PD-L1 compared with chemotherapy. Moreover, the safety characterization of the pembrolizumab was uniform with what has been earlier seen in gastric cancer leaving the trial KEYNOTE-062 with ambiguous results (https://clinicaltrials.gov/ct2/show/NCT02494583) (Tabernero et al. 2016). KEYNOTE-181, phase III trial aimed to find out the pembrolizumab ability compared to chemotherapy in adenocarcinoma, ESCC, or GEJ. The study concluded that anti-PD-L1 drug significantly enhanced OS related to chemo suggesting that PD-L1 as second-line therapy for advanced ESCC (Kojima et al. 2019). The ramucirumab (anti-VEGFR2 antibody) as mono or in combination with chemotherapy is shown to increase survival benefit in GEC patients in phase III trial RAINBOW (Wilke et al. 2014). Additionally, preliminary beneficial outcomes of the phase I study (JVDF) using anti-PD-1 and anti-VEGFR2 on advanced GEJ and gastric tumors reported

Table 8.1 Immunotherapies under clinical investigation against esophageal cancers

Trial #	Immunotherapy	Status	Phase		
			I	II	III
NCT02490735	CIK	A-NR			
NCT00004178	Autologous lymphocytes	CT			
NCT01143545	Allogeneic Tumor Cell Vaccine + Celecoxib + Cyclophosphamide	CT			
NCT01003808	IMF-001	CT			
NCT00561275	Peptide cocktail (LY6K, VEGFR1 & VEGFR2)	CT			
NCT00632333	Multiple peptides (URLC10, TTK, KOC1, VEGFR1 & VEGFR2) + Cisplatin + Fluorouraci	A-NR			
NCT00753844	URLC10 peptide	CT			
NCT00020787	G17DT Immunogen + Cisplatin + Fluorouracil	CT			
NCT02743494	Nivolumab	RT			
NCT02564263	Pembrolizumab + Paclitaxel + Docetaxel + Irinotecan	A-NR			
NCT02642809	Pembrolizumab + Radiation	RT			
NCT02569242	Nivolumab + Docetaxel/Paclitaxel	A-NR			
NCT02971956	Pembrolizumab	RT			
NCT02318901	Pembrolizumab + Trastuzumab + Ado-trastuzumab emtansine + Cetuximab	UN			
NCT02735239	Durvalumab + Tremelimumab + Oxaliplatin + Capecitabine + Radiation + Paclitaxel + Carboplatin	RT			
NCT02460224	IMP701 + PDR001	A-NR			
NCT02834013	Ipilimumab + Nivolumab	RT			
NCT00995358	Multiple Peptides (TTK, LY6K & IMP-3)	A-NR			
NCT00682227	Multiple Peptides (TTK, LY6K & IMP-3)	A-NR			
NCT00669292	Multiple Peptides (URLC10-177, TTK-567& CpG-7909)	A-NR			
NCT02520453	Durvalumab	A-NR			
NCT03087864	Atezolizumab + Carboplatin + Paclitaxel + Radiation	RT			
NCT01375842	Atezolizumab	CT			
NCT02830594	Pembrolizumab + Radiation	RT			
NCT02559687	Pembrolizumab	A-NR			
NCT02946671	Mogamulizumab + Nivolumab	RT			
NCT02476123	Mogamulizumab + Nivolumab	A-NR			
NCT03044613	Nivolumab + Relatlimab + Carboplatin + Paclitaxel +Radiation	RT			
NCT02872116	Nivolumab + Ipilimumab+ Oxaliplatin+ Capecitabine+ Leucovorin + Fluorouracil	A-NR			
NCT02544737	Apatinib	A-NR			
NCT02743494	Nivolumab	RT			
NCT02096614	TBI-1201+ Cyclophosphamide + Fludarabine	RT			
NCT02366546	TBI-1301+ Cyclophosphamide + Fludarabine	A-NR			
NCT02457650	Cyclophosphamide + Fludarabine+ Anti-NY ESO-1 TCR-transduced T cells	RT			

RT recruiting, *A-NR* active not recruiting, *UN* unknown, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *CIK* cytokine-induced killer, *LY6K* lymphocyte antigen 6 complex locus K, *VEGFR1* vascular endothelial growth factor receptor 1, *VEGFR2* vascular endothelial growth factor receptor 2, *URLC10* up-regulated lung cancer 10, *TTK* TTK protein kinase, *KOC1* IGF II mRNA binding protein 3, *IMP-3* insulin-like growth factor II mRNA-binding protein 3

Note: the sum of immunotherapy molecules listed under any trial do not represent the combinations designed by the researchers to evaluate potency against mentioned cancer variety

(Chau et al. 2018). Table 8.2 provides clinical investigations undertaken to evaluate different types of immunotherapies on GC.

Another antibody targeting PD-L1 called avelumab, safety, and efficacy on progressive GEJC was evaluated in JAVELIN Solid Tumor trial phase 1b trial (Chung et al. 2016). The study stands out as the first one to report the benefits of avelumab antibody as a switch-maintenance therapeutic agent in advanced GC/GEJC. GC/GEJC cancer subjects who encountered avelumab monotherapy gained help in recovering from cancer symptoms in comparison with chemotherapy (NCT02625623) (Bang et al. 2018). After the satisfactory safety outcomes from avelumab, a phase III study (NCT02625610) on humans with advanced GC/GEJC is currently happening with subject recruitment (Moehler et al. 2018).

Recently in 2017, a phase III trial used nivolumab in subjects with advanced (GEC/gastric) tumors intolerance to chemo drugs was reported. Nivolumab therapy gave a statistically meaningful OS, and ORR was 31% maximum (Kang et al. 2017). In a separate clinical setting where recurrent gastric cancer subjects treated with either nivolumab or a combination of nivolumab+chemotherapy, the nivolumab seems tolerated with grade 3-4 AEs (ATTRACTION-04/ONO-4538-37). A 68.4% ORR with ten patients achieved complete response (CR) (Diaz et al. 2017). Though numerous trials are proceeding with new checkpoint inhibitors (atezolizumab and durvalumab), some studies with nivolumab (anti-CTLA-4) or nivolumab together with ipilimumab (anti-PD-1) presented few assuring effects. Monotherapy with ipilimumab on metastatic GC/GEJ cancers has been validated through phase II trial (NCT01585987) (Moehler et al. 2016). Though this investigation did not obtain its first endpoint, the safety profile of ipilimumab in GC/GEJC installed a framework for possible future use in combination therapy (Bang et al. 2017). Both nivolumab and nivolumab+ipilimumab exhibited clinically essential anti-tumor activity, long-lasting responses, boosting long-term OS, and with moderate side effects in subjects with ECC underwent chemotherapy-refractory in CheckMate-032 Study. Currently, phase III clinical study evaluating both mono nivolumab and ipilimumab +nivolumab as the first-line treatment for ECC is happening (Janjigian et al. 2018). Though EGC continues as a forbidding adversary for patients and surgeons, advancements in resection methods, targeted systemic medicines, and more refined radiation therapy procedures will drive us closer to winning.

8.15.2 Colorectal Cancer

On a global scale, the third most frequently diagnosed cancer type is CRC and the second most leading cause of cancer deaths. This global oppress going to increase by 2.2 million new cases and deaths by 1.1 million in 2030. Notwithstanding the striking progress made at quality therapies, the 5-year survival rate for diagnosed metastatic CRC stays remarkably low with an approximation of 12% (Siegel et al. 2015). The current progress made in terms of therapeutic vaccine development against various cancer types instills hope for the betterment in treating cancer. The

Table 8.2 Immunotherapies under clinical investigation against gastric cancers

Trial #	Immunotherapy	Status	Phase		
			I	II	III
NCT02658214	Oxaliplatin + 5FU + Leucovorin + Durvalumab + Tremelimumab	A-NR			
NCT02678182	Capecitabine + MEDI4736 + Trastuzumab + Rucaparib	RT			
NCT02572687	Ramucirumab + MEDI4736	A-NR			
NCT02734004	Olaparib + MEDI4736 + Bevacizumab	RT			
NCT02554812	Avelumab + Utomilumab + PF-04518600 + PD 0360324	RT			
NCT02625610	Avelumab + Oxaliplatin + 5-Fluorouracil + Leucovorin + Capecitabine	A-NR			
NCT02625623	Avelumab + Irinotecan + Paclitaxel	A-NR			
NCT03281369	5-FU + Leucovorin + Oxaliplatin + Atezolizumab + Cobimetinib+ Ramucirumab + Paclitaxel + PEGPH20 + BL-8040 + Linagliptin	RT			
NCT03126110	INCAGN01876 + Nivolumab + Ipilimumab	RT			
NCT03409848	Nivolumab + Ipilimumab	RT			
NCT02999295	Ramucirumab + Nivolumab	RT			
NCT02935634	Nivolumab + Ipilimumab + Relatlimab + BMS-986205	RT			
NCT02872116	Nivolumab + Ipilimumab + Oxaliplatin + Capecitabine + Leucovorin + Fluorouracil	A-NR			
NCT02746796	ONO-4538 + Oxaliplatin + Tegafur- Gimeracil-Oteracil potassium + Capecitabine	A-NR			
NCT03342417	Nivolumab + Ipilimumab	RT			
NCT02267343	ONO-4538	A-NR			
NCT02370498	Pembroliziumab	A-NR			
NCT02494583	Pembrolizumab + Cisplatin + 5-FU + Capecitabine	A-NR			
NCT03019588	Pembrolizumab	A-NR			
NCT03196232	Epacadostat + Pembrolizumab	RT			
NCT02954536	Pembrolizumab + Trastuzumab + Capecitabine + Cisplatin + Oxaliplatin + 5-FU	RT			
NCT03342937	Oxaliplatin + Capecitabine + Pembrolizumab	RT			
NCT02178722	MK-3475 + INCB024360	A-NR			
NCT03095781	XL888 + Pembrolizumab	RT			
NCT02689284	Margetuximab + Pembrolizumab	A-NR			
NCT02901301	Pembrolizumab + Trastuzumab + Capecitabine + Cisplatin	RT			
NCT02494583	Pembrolizumab+ Cisplatin + 5-FU + Capecitabine	A-NR			
NCT02625610	Avelumab + Oxaliplatin + 5-FU + Leucovori + Capecitabine	A-NR			
NCT02625623	Avelumab + Irinotecan+ Paclitaxel	A-NR			
NCT02689284	Margetuximab + Pembrolizumab	A-NR			
NCT02734004	Olaparib + MEDI4736 + Bevacizumab	RT			
NCT02864381	Andecaliximab + Nivolumab	A-NR			
NCT02872116	Nivolumab + Ipilimumab + Oxaliplatin + Capecitabine+ Leucovorin + Fluorouracil	A-NR			
NCT02935634	Nivolumab + Ipilimumab + Relatlimab + BMS-986205	RT			
NCT03019588	Pembrolizumab+ Paclitaxel	A-NR			
NCT03342417	Nivolumab + Ipilimumab	RT			
NCT03382600	Pembrolizumab + Oxaliplatin + TS-1 + Cisplatin	RT			
NCT03409848	Nivolumab + Ipilimumab	RT			

RT recruiting, *A-NR* active not recruiting, *UN Unknown*, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *5FU* fluorouracil, *PEGPH20* pegvorhyaluronidase alfa, *TS-1* titanium silicate
Note: the sum of immunotherapy molecules listed under any trial do not represent the combinations designed by the researchers to evaluate potency against mentioned cancer variety

novel medications were appreciated quickly because of their potential efficacy and safety in treating cancer patients. In the past, it was evident that gastrointestinal malignancies were becoming insensitive to vaccines, and the insensitivity of colorectal cancers is a big concern since emerging data support the observations that some but not all patients who may profit from these vaccines. Therapeutics with TAS-102 (Mayer et al. 2015), ramucirumab (Goel and Sun 2015), and regorafenib (Grothey et al. 2013) got a green signal from the FDA to treat CRC. Unfortunately, the clinical outcomes involving these drugs remain modest. As a consequence of this, innovative therapeutic strategies involving immunotherapy have come into the evaluation by researchers and clinicians as anti-tumor therapeutics. Molecular genetic instability in chromosomes (CIN) and microsatellite DNA marks the little variation that divided CRC into various subgroups. Following instruction from The Cancer Genome Atlas Project, CRC splits into two: (i) tumors with microsatellite instability (MSI) either ultra-mismatch repair (pMMR) or poor mismatch repair (dMMR), (ii) tumors having high-frequency in DNA copy number mutation are termed microsatellite stable (MSS) and ~84% are non-hypermutated (Muller et al. 2016). Another pedagogy comes from the Consensus Molecular Subtypes (CMS) Consortium investigating CRC pattern in various reports detailed in four groups: (1) CMS1, (2) CMS2, (3) CMS3, and (4) CMS4 (Muller et al. 2016).

In the recent past, many clinical investigations were conducted based on the above stated CRC classification to find suitable therapeutics to these tumor subtypes (Le et al. 2015). An immune checkpoint inhibitor pembrolizumab was tested on 32 subjects having advanced metastatic either positive or negative dMMR (NCT01876511). The study indicated that dMMR positive tumors stand an excellent chance to treat with pembrolizumab (Bang et al. 2015). Predating Le et al., a clinical study with anti-PD1 antibody treatment served only one CRC subject who had dMMR (Brahmer et al. 2010). Next, a study evaluated the potency of anti-PD-1 against patients having advanced dMMR cancers out of 12 distinct tumor types. The reactions were durable and supported that genome instability related to dMMR tumors can be preventable by using immune checkpoint inhibitors (Le et al. 2017). Soon, FDA on 23 May 2017 certified pembrolizumab to treat patients with hard to operate, metastatic, microsatellite instability cancers such as MSI-H and dMMR (Marcus et al. 2019). In another study, nivolumab (anti-PD-1 antibody) has been given to metastatic CRC subjects positive for dMMR/MSI-H in CheckMate-142 (phase II study) and observed long-lasting benefits and disease control (Overman et al. 2017; Overman et al. 2018). Based on the above prospective, nivolumab got approval from the FDA on July 31, 2017 for the treatment for MSI-H/dMMR metastatic CRC. Data from a cohort study on subjects with advanced MSI-H CRC (KEYNOTE-164/NCT02460198) using pembrolizumab offered potential anti-tumor actions with a manageable safety profile (Le et al. 2018). The gathered clinical information recommended a further investigation to evaluate the anti-tumor benefits of pembrolizumab (anti-PD-1) against dMMR/MSI-H mCRC (KEYNOTE-177) (Diaz et al. 2017). A therapeutic approach to treat pMMR mCRC patients using pembrolizumab alone or in combination with either radiotherapy or surgery

following phase II study (NCT02437071) reported ambiguous primary endpoints (Segal et al. 2016).

Moreover, in mismatch repair (MMR) CRC monotherapy with nivolumab (anti-PD-1) further improved when ipilimumab is added to the treatment (Overman et al. 2018) in CheckMate-142 phase II trial. An interim analysis of CheckMate-142 using a combination approach with ipilimumab and nivolumab resulted in 55% ORR among 119 subjects following OS at 85%. The trial CheckMate-142 had multiple arms comparing the mono and combinational treatments majorly with nivolumab implying that combination therapy gave more beneficial reactions compared to monotherapy in CRC patients (Andre et al. 2018). So far combinational therapies proved valid for CRC treatment over single factor treatments, noteworthy to mention that use of atezolizumab (anti-PD-L1) and cobimetinib (MEK inhibitor) on 84 subjects (NCT01988896) confirmed an endurable protection and improved OS compared to pretreated mCRC subjects, inferring advantage of combination therapy (Bendell et al. 2018a). A phase III trial (IMblaze370) has been conducted on chemotherapy-refractory mCRC with atezolizumab in association with cobimetinib vs. regorafenib recently. The outcomes explain that IMblaze370 failed to satisfy its initial endpoint; also the combinational therapies employed fail to demonstrate significant OS benefits, while the safety profile remains consistent with previous studies (Bendell et al. 2018b).

In the past, carcinoembryonic antigen (CEA), which is a TAA found on the majority of CRC tumors, has been tested in clinical tests containing DC pulsed with HLA-A24-restricted peptide of CEA proved worthy in terms of safety and efficiency in producing an anti-tumor response (Fong et al. 2001; Morse et al. 1999; Itoh et al. 2002). These studies remain history because of the absence of futuristic studies since their first outcomes reported. The first line of evidence from phase I a/b (RG7802) using mono agent (CEA CD3 TCB) and in combination with atezolizumab suggested that agent CEA CD3 TCB could induce anti-tumor activity, such activity is further improved in the presence of atezolizumab mCRC patients (Tabernero et al. 2017). Other studies (phase II trial) involving DC pulsed with tumor lysates evoked immune responses but no benefits on OS and PSF (Caballero-Banos et al. 2016). Additionally, DC pulsed with class I and class II WT1 peptides provided beneficial endpoints in mCRC subjects and highlighted the potential nature of DC vaccination for advanced cancer. Though the resistance proceeded for two years providing prolonged survival, the trail failed to represent strong sample size to support immune benefits (Shimodaira et al. 2015; Higuchi et al. 2015). A phase II study (NCT01208194) reported that MGN1703 (TLR9 agonist) maintenance medication has been well accepted and allowed long-lasting prolonged PFS with disease management in a subset of victims bearing mCRC (Riera-Knorrenschild et al. 2015; Schmoll et al. 2014). These outcomes acted as groundwork to invent IMPALA/phase III trial is currently selecting subjects to test TLR9 agonist anti-cancer benefits on CRC subjects. In an open-label, randomized pilot study (NCT02512172), before treating pMMR mCRC patients with pembrolizumab epigenetic priming was suggested to see whether inhibiting HDAC/DNMT influences sensitivity of checkpoint blockers, since in preclinical models the idea was well

established. The results obtained from the NCT02512172 trial manifested that use of pembrolizumab with romidepsin/5-azacitidine was judged safe and tolerable in advanced pMMR CRC. Additional connection of pre- and post-care biopsies is needed in defining presage of reactions (Murphy et al. 2019). In phase II study first-line treatment regimen in CRC such as (bevacizumab + fluoropyrimidine + atezolizumab or bevacizumab + fluoropyrimidine) failed to provide positive outcomes. Trials opting radiation rather than chemotherapy in association with checkpoint inhibitors causing tumor shrinkage effect are ongoing (NCT02291289). Table 8.3 provides clinical investigations undertaken to evaluate different types of immunotherapies on CRC.

8.15.3 Hepatocellular Carcinoma (HCC)

Liver cancer stands as third most reason of cancer-linked deaths globally at nearly 745,000 lives per annum. Certain viral infections like CHB, HCV, NASH, and alcoholic cirrhosis are the most related factor causing liver cancer (Ferlay et al. 2015; Torre et al. 2015). Currently, the treatment options for preventing HCC are short. Surgery and tissue transplantation stand best chances to treat HCC; however, cancer relapse beats the current options for cure (Song and Wai Kit 2004). Expansion of current therapeutics for high-grade HCC has delivered approval of sorafenib freshly (Medavaram and Zhang 2018). In KEYNOTE 224 trail patients with sorafenib, refractory HCC treated with pembrolizumab which executed efficient and sustainable responses confirming pembrolizumab might be a choice to lean on for treating HCC (Zhu et al. 2018). Based on the trial KEYNOTE 224 outcomes another phase III trial is ongoing to evaluate a secondary approach on HCC. KEYNOTE-240 phase III study assessing placebo vs. pembrolizumab effects on sorafenib-refractory HCC humans, the trial failed to report co-primary endpoints of PFS and OS in subjects pretreated with systemic therapy (Merk.com), yet enrollment and continuation of the trial ongoing (NCT02702401) (Finn et al. 2017). In addition, recent clinical investigation proposed effective treatment choices including cabozantinib, lenvatinib, and regorafenib, symbolizing the greatness of immunotherapy in treating humans with liver cancer thus providing a novel paradigm in the era of oncology (Medavaram and Zhang 2018). A phase III (CHECKMATE 459) study currently monitoring the nivolumab vs. sorafenib effects in HCC patients who never received any therapy (NCT02576509) (Sangro et al. 2016).

The TILs of liver cancers display PD-1, and this phenomenon establishes the probability of testing immune-based PD-1 therapeutics (Prieto et al. 2015). In this line of investigation, phase I and II (CheckMate 040/NCT01658878) study investigated the benefits of nivolumab (anti-PD1) in patients with advanced HCC. The study observed that nivolumab was able to provide adequate and tolerable effects in HCC subjects (Melero et al. 2017). The outputs indicated that anti-PD1 provided tractable safety outcomes and positive unbiased responses decoded anti-PD1, potential remedy against advanced HCC (El-Khoueiry et al. 2017; Melero et al. 2017).

Table 8.3 Immunotherapies under clinical investigation against colorectal cancer

Trial #	Immunotherapy	Status	Phase		
			I	II	III
NCT02997228	Atezolizumab + Bevacizumab + Fluorouracil + Leucovorin calcium + Oxaliplatin	RT			
NCT02563002	Bevacizumab + Cetuximab + FOLFIRI + mFOLFOX6 + Pembrolizumab	A-NR			
NCT02227667	MEDI4736	A-NR			
NCT02460198	Pembrolizumab	A-NR			
NCT01885702	DC vaccination	A-NR			
NCT03007407	Durvalumab + Tremelimumab	RT			
NCT02484404	Cediranib + MEDI4736 + Olaparib	RT			
NCT02860546	Nivolumab + TAS-102	CT			
NCT02834052	Pembrolizumab + Poly-ICLC	RT			
NCT03008499	High-activity Natural Killer cells	RT			
NCT02466906	rhGM-CSF	RT			
NCT02688712	Capecitabine + Fluorouracil + LY2157299	RT			
NCT02077868	MGN1703	A-NR			
NCT02715882	CBLB502	NT			
NCT02617134	anti-MUC1 CAR-T cells	RT			
NCT02839954	anti-MUC1 CAR-pNK cells	RT			
NCT01174121	Aldesleukin + Cyclophosphamide + Fludarabine + Pembrolizumab + Young TIL	RT			
NCT03047525	CIK	RT			
NCT02886897	D-CIK & anti-PD-1 antibody	RT			
NCT02280278	Adjuvant chemotherapy + CIK	RT			
NCT02202928	DC-CIK	A-NR			
NCT01741038	AlloStim®Procedure: cryoablation	NRT			
NCT02380443	AlloStimProcedure: cryoablation	A-NR			
NCT02415699	DC-CIK + Fluorouracil + Leucovorin + Oxaliplatin	A-NR			
NCT01929499	CIK	A-NR			
NCT02448173	OncoVAX & Surgery	RT			
NCT02327078	Chemotherapy + Epacadostat + Nivolumab	A-NR			
NCT03026140	Celecoxib + Ipilimumab + Nivolumab	RT			
NCT02060188	anti-LAG-3 + Cobimetinib + Daratumumab + Ipilimumab + Nivolumab	A-NR			
NCT02335918	Nivolumab + Varlilumab	CT			

RT recruiting, *A-NR* active not recruiting, *UN* unknown, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *DC* dendritic cells, *Poly-ICLC* polyinosinic-polycytidylic acid, and poly-L-lysine double-stranded RNA, *rhGM-CSF* recombinant human granulocyte-macrophage colony-stimulating factor, *MUC1* mucin 1, *CAR-T cells* chimeric antigen receptor T cells, *CAR-pNK cells* chimeric antigen receptor NK92 cells, *CIK* cytokine-induced killer, *D-CIK* dendritic-cytokine-induced killer, *anti-PD-1 antibody* anti-programmed cell death protein 1 antibody, *anti-LAG-3* anti-lymphocyte-activation gene 3

Note: the sum of immunotherapy molecules listed under any trial do not represent the combinations designed by the researchers to evaluate potency against mentioned cancer variety

Table 8.3 (continued)

Trial #	Immunotherapy	Status	Phase		
			I	II	III
NCT02992912	Anti-PD-L1 + Atezolizumab	RT			
NCT02713373	Cetuximab + Pembrolizumab	RT			
NCT02437071	Pembrolizumab + Radiotherapy	A-NR			
NCT02260440	Azacitidine + Pembrolizumab	A-NR			
NCT01876511	MK-3475	RT			
NCT02375672	Pembrolizumab + mFOLFOX6	A-NR			
NCT03258398	Avelumab + eFT508	A-NR			
NCT03081494	PDR001 + Regorafenib	RT			
NCT03374254	Binimetinib + Irinotecan + Leucovorin + Oxaliplatin + Pembrolizumab + 5-FU	RT			
NCT02959437	Azacitidine + Epacadostat + INCB057643 + INCB059872 + Pembrolizumab	A-NR			
NCT02512172	CC-486 + Romidepsin + MK-3475	A-NR			
NCT03442569	Ipilimumab + Panitumumab + Nivolumab	RT			
NCT03377361	Ipilimumab + Nivolumab + Trametinib	RT			
NCT03104439	Ipilimumab + Nivolumab+ Radiation	RT			
NCT03271047	Binimetinib nivolumab ipilimumab	A-NR			
NCT02948348	Nivolumab	RT			
NCT02811497	Azacitidine + Durvalumab	RT			
NCT03428126	Durvalumab + Trametinib	RT			
NCT03122509	Durvalumab tremelimumab + Radiation	RT			
NCT02888743	Durvalumab + Radiation + Tremelimumab	A-NR			
NCT02788279	Atezolizumab + Anti-PDL1 + Cobimetinib + Regorafenib	CT			
NCT02873195	Atezolizumab + Bevacizumab + Capecitabine	A-NR			
NCT02876224	Atezolizumab + Bevacizumab + Cobimetinib	A-NR			
NCT03150706	Avelumab	RT			
NCT02912559	Atezolizumab + Fluorouracil + Calcium + Leucovorin + Oxaliplatin	RT			
NCT03202758	Durvalumab + FOLFOX + Tremelimumab	RT			
NCT03414983	Nivolumab + Oxaliplatin + Leucovorin + Fluorouracil + Bevacizumab	RT			
NCT03050814	Avelumab + Ad-CEA vaccine + Bevacizumab + Capecitabine + Leucovorin + Oxaliplatin 5-FU	RT			
NCT01274624	Bevacizumab + Irinotecan + Leucovorin + REOLYSIN® + 5-FU	CT			
NCT03256344	Atezolizumab + Talimogene + Laherparepvec	RT			
NCT02777710	Durvalumab + Pexidartinib	A-NR			
NCT02559024	MEDI6469	A-NR			
NCT02650713	Atezolizumab + RO6958688	A-NR			
NCT02870920	Durvalumab + Tremelimumab	A-NR			

RT recruiting, *A-NR* active not recruiting, *UN* unknown, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *Anti-PD-L1* anti-Programmed cell death protein ligand 1 antibody, *5-FU* fluorouracil
Note: the sum of immunotherapy molecules listed under any trial do not represent the combinations designed by the researchers to evaluate potency against mentioned cancer variety

Another study (NCT01008358) evaluated the potential of tremelimumab in humans bearing HCC having HCV infection, where most of the cancer subjects had altered liver function (Child-Pugh class B). The study reported that tremelimumab is able to

provide an anti-tumor response with safety profile following a reduced burden of viral infection as well (Sangro et al. 2013). Therapy with tremelimumab either in combination with chemo ablation or radiofrequency ablation (NCT01853618) holds the ability to increase CD18 lymphocyte infiltration in sensitive tumors showing median OS of ~1 year (Duffy et al. 2017). Currently, a phase II study (NCT02061761) figuring out the anti-cancer benefits of mono or combination therapy of BMS-986016 and nivolumab in relapsed liver tumor patients (Andrews et al. 2017). A phase II dose-determination trial, Pexa-Vec intramural injection into patients with advanced HCC described tolerable safety and a notable increment in OS in the high-dose group (Breitbach et al. 2015; Heo et al. 2013). Following the previous outcomes, a phase III clinical research aimed at delineating tolerability and efficacy monotherapy with sorafenib or combination of Pexa-Vec + sorafenib is currently on the evaluation on advanced HCC (PHOCUS, NCT02562755) (Abou-Alfa et al. 2016). An oral therapeutic vaccine named Hepko-V5 undergoing a clinical examination to produce beneficial effects on advanced HCC at the moment (NCT02232490- phase III) (Tarakanovskaya et al. 2015). Table 8.4 provides clinical investigations undertaken to evaluate different types of immunotherapies on HCC.

8.15.4 Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) has the potential to grow desmoplasia around the tumor. These PDAC tumors are resistant to immune reactions, cancer medication, and promote melanoma growth rapidly (Neesse et al. 2011). Despite the sophisticated knowledge of the associated molecular pathways, yet no clinically significant change in the 5-year SR (less than five years) for PC subjects. By 2030, the projected pancreatic cancers tend to rise where PC being the second supreme reason for cancer expiries. To avoid such future loss, nearly ~35 therapeutic agents as solo or in grouping undergone evaluation in clinical setups on advanced PAC subjects (Matrisian and Berlin 2016). Indeed, when PC individuals administered with BMS-936559, a negative response was reported (Brahmer et al. 2012). Moreover, desmoplastic stroma appears to be an indispensable block in making new therapeutics (Erkan et al. 2012). Nonetheless, currently, few pre-clinical and clinical investigations ongoing against PC towards finding new immune therapeutic targets. Despite the efforts, currently, there are no successful immune-based therapeutics approved from clinical investigations against PC. Since in other cancer treatments, the immunotherapeutic approach aiming at PC with anti-CTLA-4 or anti-PD-1/PD-L1 seemed insignificant in clinical trials. For instance, a phase II study revealed that ipilimumab as an inefficient therapeutic on PC (Royal et al. 2010). The failure principally ascribed to the immunologically suppressed microenvironment grown out of desmoplasia in PC. To achieve effective treatments for PC, researchers acceded to evaluate immunotherapy in combination with additional therapies—the use of both ipilimumab and gemcitabine in a dose-defining phase Ib study (NCT01473940) gave supporting details like ipilimumab and gemcitabine is

Table 8.4 Immunotherapies under clinical investigation against liver cancers

Trial #	Immunotherapy	Status	Phase			
			I	II	III	IV
NCT03099564	Pembrolizumab + Y90 radioembolization (Device)	RT				
NCT02837029	Nivolumab + Radiation	RT				
NCT03033446	Nivolumab + Y90 radioembolization (Device)	RT				
NCT03143270	Nivolumab + (deb-TACE)	RT				
NCT02325739	FGF401+ PDR001	A-NR				
NCT02474537	INC280	CT				
NCT03095781	XL888 + Pembrolizumab	RT				
NCT02859324	Avadomide + Nivolumab	A-NR				
NCT02423343	Galunisertib + Nivolumab	A-NR				
NCT02988440	PDR001 + Sorafenib	A-NR				
NCT02942329	Apatinib + SHR-1210	RT				
NCT03006926	Lenvatinib + Pembrolizumab	A-NR				
NCT02572687	Ramucirumab + MEDI4736	A-NR				
NCT03071094	Pexa Vec + Nivolumab	RT				
NCT02519348	Durvalumab + Tremelimumab + Bevacizumab	RT				
NCT01658878	Nivolumab + Sorafenib + Ipilimumab + Cabozantinib	A-NR				
NCT02576509	Nivolumab + Sorafenib	A-NR				
NCT02702414	Pembrolizumab	A-NR				
NCT02702401	Pembrolizumab + Placebo	A-NR				
NCT03298451	Durvalumab + Tremelimumab + Sorafenib	RT				
NCT02699515	MSB0011359C	A-NR				
NCT02795429	PDR001+INC280	RT				
NCT03099109	LY3321367 + LY3300054	RT				
NCT03289533	Avelumab + Axitinib	A-NR				
NCT03418922	Lenvatinib + Nivolumab	A-NR				
NCT03170960	Cabozantinib + Atezolizumab	RT				
NCT03434379	Atezolizumab + Bevacizumab + Sorafenib	RT				
NCT01462903	TILs + IL2	UN				
NCT01758679	Licartin + CIK	UN				
NCT01897610	Immuncell-LC	CT				
NCT02008929	MG4101	CT				
NCT01914263	CIK	UN				
NCT02587689	anti-MUC1 CAR T Cells	UN				
NCT02959151	CAR-T cell	UN				

RT recruiting, *A-NR* active not recruiting, *UN* unknown, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *deb-TACE* drug eluting bead transarterial chemoembolization, *FGF401* fibroblast growth factor 401, *TILs* tumor infiltrating lymphocyte, *IL-2* Interleukin-2, *CIK* cytokine-induced killer, *MUC1* mucin 1, *CAR T Cells* chimeric antigen receptor T cells

Note: the sum of immunotherapy molecules listed under any trial do not represent the combination's designed by the researchers to evaluate potency against mentioned cancer variety

Table 8.4 (continued)

Trial #	Immunotherapy	Status	Phase		
			I	II	III
NCT02725996	NK cells + Curative therapy	UN			
NCT02856815	Immuncell-LC	RT			
NCT02715362	TAI-GPC3-CART cells	UN			
NCT02839954	anti-MUC1 CAR-pNK cells	UN			
NCT02854839	MG4101	UN			
NCT03175679	iNKT cells + IL-2 +Tegafur	RT			
NCT03199807	NRT + Radiation	NYT			
NCT03130712	GPC3-CART cells	UN			
NCT03132792	Alpha Fetoprotein ($AFP^{c332}T$)	RT			
NCT02905188	GPC3-CART cells + Cytoxan + Fludarabine	RT			
NCT03441100	IMA202	RT			
NCT02232490	Hepcortespenlisimut-L	RT			
NCT02409524	AlloVax + AlloStim + CRCL	UN			
NCT03203005	IMA970A + CV8102 + Cyclophosphamide	RT			
NCT01853618	Tremelimumab + RFA + TACE + Cryoablation	A-NR			
NCT01821482	DC + CIK	UN			
NCT02562755	Pexa Vec + Sorafenib	RT			
NCT02487017	TACE + DC-CIK	UN			
NCT02432963	p53MVA + Pembrolizumab	UN			
NCT02821754	Durvalumab + Tremelimumab + TACE+ RFA + Cryoablation	RT			
NCT02886897	DC-CIK + anti-PD-1 antibody	RT			
NCT03259867	Nivolumab + Pembrolizumab + TATE	RT			
NCT03380130	Nivolumab	A-NR			
NCT03277352	INCAGN01876 + Epacadostat + Pembrolizumab	A-NR			
NCT03241173	INCAGN01949 + Nivolumab + Ipilimumab	A-NR			
NCT03126110	INCAGN01876 + Nivolumab + Ipilimumab	RT			
NCT03067493	Neo-MASCT	RT			
NCT03482102	Tremelimumab + Durvalumab + Radiation	RT			
NCT03439891	Nivolumab + Sorafenib	RT			
NCT03511222	Vorolanib + Nivolumab + Pembrolizumab	RT			
NCT03412773	BGB-A317+ Sorafenib	RT			
NCT03062358	Pembrolizumab	RT			
NCT03383458	Nivolumab	RT			

RT recruiting, *A-NR* active not recruiting, *UN* unknown, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *NK* natural killer, *TAI* transcatheter arterial infusion, *GPC3-CAR T cells* glypican 3—chimeric antigen receptor cells, *CAR T cells* chimeric antigen receptor T cells, *MUC1* mucin 1, *CAR-pNK cells* chimeric antigen receptor NK92 cells, *iNKT cells* Invariant natural killer T, *IL-2* interleukin-2, *NRT* new antigen reactive immune cells, *CRCL* chaperone rich cell lysate, *RFA* radiofrequency ablation, *TACE* transarterial chemoembolization, *DC* dendritic cells, *CIK* cytokine-induced killer, *p53MVA* p53 modified vaccinia ankara, *anti-PD-1* programmed cell death protein 1, *TATE* trans-arterial tirapazamine embolization, *Neo-MASCT* multiple-antigen specific cell therapy-I

Note: the sum of immunotherapy molecules listed under any trial do not represent the combinations designed by the researchers to evaluate potency against mentioned cancer variety

tolerable and viable therapy vouching for further assessment (Kalyan et al. 2016). The pancreatic tumors do not have intra-tumoral effector T-cells. Activating T-cell mediated immune responses in pancreatic tumor cells via live attenuated and modified Listeria monocytogenes to secrete mesothelin; a tumor-associated antigen (CRS-207) account as a better therapeutic choice for PC (Dalgleish et al. 2016). A phase II study (STELLAR, NCT02243371) is analyzing with or without nivolumab in combination with GVAX/CRS-207 on metastatic PC subjects, those who failed earlier at pre-treatment with any chemotherapy (Dalgleish et al. 2016). Consequently, the blend of GVAX/CRS-207 weighed on pretreated advanced PC patients in phase IIb clinical study (ECLIPSE-NCT02004262). The study flopped at reporting primary conclusions (Le et al. 2017). Table 8.5 provides clinical investigations undertaken to evaluate different types of immunotherapies on PC.

In a different clinical study, a combinational therapy involving nivolumab + DC (monocyte antigens) vaccine in mPC revealed out of seven two PR have been observed (Nesselhut et al. 2016). Tremelimumab and durvalumab immune checkpoint inhibitors provided potency in treating multiple cancer types individually or in a compound. In a randomized phase II trial (ALPS NCT02558894) assessing durvalumab+tremelimumab as secondary choice to treat PC, the disease control rate was low with 9.4%, median PSF was low (1.5 months). The development of this combination in second-line PC canceled at the moment (O'Reilly et al. 2018). A phase II (NCT02362048) analysis tested pembrolizumab with acalabrutinib (Bruton's tyrosine kinase (BTK) inhibitor) together on metastatic PC, at the data cut-off, data suggests that the study had promising prior activity and a manageable side effect profile, patients had stable disease with partial reactions after 3.7 months of treatment (Overman et al. 2016). Stimulating the host immune system to treat pancreatic cancer seems challenging since several clinical investigations failed to achieve significant anti-cancer benefits until today. Adaptive cell therapy involving Algenpantucel-L: made of irradiated malignant cells expressing alpha-1,3-galactosyltransferase, yielded hopeful ends in the NCT01072981 phase II report, where Algenpantucel-L vaccine+radiochemotherapy in an adjuvant background with a median DSF of 1.4 years noted (Hardacre et al. 2013). IMPRESS (NCT01072981) clinical study failed to achieve primary endpoints hence the manufacturing of Algenpantucel-L was terminated (McCormick et al. 2016). Another phase III trial conducted on resectable and non-resectable borderline advanced tumors of the pancreas with algenpantucel-L failed to achieve beneficial outcomes (NCT01836432). Another clinical study (NCT02405585) testing algenpantucel-L and SBRT on borderline resectable PC met the similar end (terminated). Many other clinical investigations such as NCT00358566 and TeloVac, ISRCTN4382138 involving GV1001 (telomerase peptide vaccine) in advanced unresectable PC was annulled shortly due to lack of survival benefits.

Several tumors including PDAC overexpress WT1 gene. In advanced PC patients, application of WT1 peptide along with gemcitabine exhibited a superior PFS in comparison to chemotherapy alone (Nishida et al. 2018; Nishida et al. 2016). IMAGE-1/NCT01303172, a randomized phase II study evaluated treating advancer

Table 8.5 Immunotherapies under clinical investigation against pancreatic cancer

Trial #	Immunotherapy	Status	Phase		
			I	II	III
NCT03153410	Cyclophosphamide + GVAX + IMC-CS4 + Pembrolizumab	RT			
NCT02777710	Durvalumab + Pexidartinib	A- NR			
NCT03277209	Plerixafor	A- NR			
NCT02588443	Gemcitabine + Nab-paclitaxel + RO70097890	CT			
NCT02960594	INO-1400/1401 + INO-9012	CT			
NCT02465983	CART-meso-19 T cells + Cyclophosphamide	CT			
NCT02588443	RO70097890 + Gemcitabine + Nab-paclitaxel	CT			
NCT03214250	APX005M + Gemcitabine + Nab-Paclitaxel + Nivolumab	RT			
NCT02732938	Gemcitabine + Nab-paclitaxel + PF04136309	TER			
NCT02715804	Gemcitabine + Nab-paclitaxel + PEGPH20	A- NR			
NCT03481920	Avelumab + PEGPH20	RT			
NCT02758587	Defactinib + Pembrolizumab	RT			
NCT01876511	MK-3475	RT			
NCT01417000	Cyclophosphamide + CRS-207 + GVAX	CT			
UMIN000004855	DC pulsed with WT1 + Gemcitabine	UN			
ISRCTN4382138	Capecitabine + Gemcitabine + GV1001	CT			
NCT02004262	CRS-207 + Cyclophosphamide + GVAX	CT			
NCT01072981	Gemcitabine + HyperAcute-Pancreas + 5FU	CT			
NCT02546531	Defactinib + Gemcitabine + Pembrolizumab	RT			
NCT03085914	Chemotherapy + Epacadostat + Pembrolizumab	A- NR			
NCT02829723	BLZ945 + PDR001	RT			
NCT02907099	BL-8040 + Pembrolizumab	RT			
NCT02526017	Cabiralizumab + Nivolumab	A- NR			
NCT02826486	BL-8040 + Pembrolizumab	UN			
NCT02880371	ARRY-382 + Pembrolizumab	A- NR			
NCT01896869	FOLFIRINOX + Ipilimumab + Vaccine	A- NR			
NCT02432963	Pembrolizumab + p53MVA vaccine	A- NR			
NCT02451982	Cyclophosphamide + GVAX + Nivolumab + Urelumab	RT			
NCT02517398	MSB0011359C	RT			
NCT01174121	Aldesleukin + Cyclophosphamide + Fludarabine + Pembrolizumab +Young TIL	RT			
NCT02834013	Ipilimumab + Nivolumab	RT			
NCT02451553	Afatinib Dimaleate + Capecitabine	RT			

RT recruiting, *A-NR* active not recruiting, *UN* unknown, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *NK* natural killer, *CART-meso-19* chimeric antigen receptor-modified T cells-*mes-19*, *PEGPH20* pegvorhyaluronidase alfa, *DC* dendritic cells, *WT1* Wilms tumor, *5FU* fluorouracil, *p53MVA vaccine* P53 modified vaccinia ankara, *TIL* tumor infiltrating lymphocyte
Note: the sum of immunotherapy molecules listed under any trial do not represent the combinations designed by the researchers to evaluate potency against mentioned cancer variety

PC patients with IMM-101 (heat-killed Mycobacterium obuense) in combination with chemotherapy that reported IMM-101 + chemotherapy was safe and well-tolerated (Dalgleish et al. 2016). Subsequently, a more extensive phase III study is needed to support the claim of WT1 and IMM-101 as anti-cancer agents. Altogether, it is clear that despite enormous efforts made in evaluating and identifying beneficial effects of chemo-immunotherapies for pancreatic cancer, the journey did not result in satisfactory outcomes so far. In the recent past, the FDA approved only two

treatment regimens like FOLFIRINOX + chemotherapy and irinotecan liposome injection to treat pancreatic cancer.

8.15.5 Biliary Tract Cancer

Extrahepatic cholangiocarcinoma, Intrahepatic cholangiocarcinoma (ICC), and gallbladder carcinoma are groups of Biliary tract cancers (BTC). Chronic infection, inflammation, desmoplasia, and microsatellite instability play vital role in turning cancerous cells into immunosuppressed cells. BTC are highly aggressive tumors linked with immunity to chemotherapy with weak prognostic degrees. Hence, novel therapies are in demand. Immunotherapy signifies ensuring discoveries by using a patient's immune system to fight against cancer. Earlier preclinical and clinical studies insinuate supporting mechanistic results because immunotherapy over BTC presents hope for the growing therapeutic part for this cancer. The two specific antigens namely MUC1 and WT1 are of great interest in treating BTC through immunotherapy (Marks and Yee 2015). In BTC and pancreatic patients MUC1 peptide vaccine was well tolerated though disease succession is observed in seven out of eight subjects (Yamamoto et al. 2005). Clinical trial using vaccines of WT1 or mixture of WT1/gemcitabine on advanced BTC and PC subjects, aimed at studying safety, toxicity and excellent immune responses was carried out (Kaida et al. 2011). Although objective clinical productiveness was not ostensible, the WT1 + GEM combination therapy provided protection. Moreover, peptide cocktail vaccines, personalized multi-peptide vaccine, and 3-peptide vaccine were injected into patients with advanced BTC (Aruga et al. 2013; Aruga et al. 2014; Yoshitomi et al. 2012). The peptide vaccination was able to stimulate peptide-related T cell immune reactions in treated subjects. The outcomes from these trials demand for further evolution of safety and efficacy of peptide cocktail vaccination via Phase II trial in BTC patients.

Dendritic cells pulsed with tumor lysate provided ~3 long-term survival (Higuchi et al. 2006) whereas mutated DNA pulsed DC treatment to metastatic cholangiocarcinoma resulted in tumor deterioration appropriately 30 weeks (Tran et al. 2014). A sum of 36 ICC patients received DC pulsed with tumor lysate and TIL's. The outcomes comprised median PFS up to 18.3 months, and OS with 2.6 years in subjects taking adjuvant immunotherapy (Shimizu et al. 2012). In addition, phase II/III study is required for establishing scientific effectiveness of ACT and DC vaccine blend as adjuvant remedy against ICC. Short term safety and potency terms of KEYNOTE-028 (NCT02054806) study with pembrolizumab evaluated for a small group of patients with BTC, however, failed to achieve primary results for OS and PSF. Eventhough the safety and durability of pembrolizumab were encouraging (Bang et al. 2015). The KEYNOTE-028 trial assuring the safety and efficacy of pembrolizumab in BTC inspired an ongoing KEYNOTE-158/NCT02628067 basket trial in BC with 100 patients. Preliminary outcomes of the KEYNOTE-158 studying the ability and welfare of anti-PD1 in progressive BTC showed that

pembrolizumab is useful in a small subgroup of patients demanding extra studies (Ueno et al. 2018). At present, various clinical investigations considering the influence of checkpoint inhibitors along with other medications as a remedy for advanced BTC could work through acclaiming prospective healing procedures. Effective, substantial, and safe outcomes have been expected from (NCT02834013, NCT02923934, NCT02821754, NCT03111732, NCT03101566, NCT02821754, and NCT03101566) these clinical investigations to strengthen the concept of immunotherapy in BTC. Table 8.6 provides clinical investigations undertaken to evaluate different types of immunotherapies on BTC.

Table 8.6 Immunotherapies under clinical investigation against biliary tract cancer

Trial #	Immunotherapy	Status	Phase		
			I	II	III
NCT01174121	Aldesleukin + Cyclophosphamide + Fludarabine + Pembrolizumab +Young TIL	RT			
NCT02982720	Pembrolizumab + Sylatron	A-NR			
NCT02834013	Ipilimumab + Nivolumab	RT			
NCT02923934	Ipilimumab + Nivolumab	RT			
NCT02703714	Pembrolizumab + Sargramostim	RT			
NCT02628067	Pembrolizumab	RT			
NCT02054806	Pembrolizumab	A-NR			
NCT03111732	Capecitabine/oxaliplatin + Pembrolizumab	RT			
NCT02821754	Cryoablation + Durvalumab + TACE/RFA + Tremelimumab	RT			
NCT03101566	Gemcitabine/cisplatin + Ipilimumab + Nivolumab	RT			
NCT02829918	Nivolumab	A-NR			
NCT03260712	Cisplatin + Gemcitabine + Pembrolizumab	NRT			
NCT02924376	Pemigatinib	RT			
NCT03230318	Derazantinib	RT			
NCT02834780	H3B-6527	RT			
NCT03144661	INCB062079	RT			
NCT02989857	AG-120	A-NR			
NCT02091141	Alectinib + Atezolizumab + Cobimetinib + Erlotinib + Pertuzumab + Trastuzumab + Vemurafenib + Vismodegib	RT			
NCT01953926	Fulvestrant + Neratinib + Paclitaxel + Trastuzumab	RT			
NCT02451553	Afatinib dimaleate + Capecitabine	RT			
NCT02609958	Varlitinib	CT			
NCT02992340	Cisplatin + Gemcitabine + Varlitinib	RT			
NCT03093870	Capecitabine +Varlitinib	A-NR			
NCT02419417	BMS-986158 + Nivolumab	RT			
NCT02091999	Enfortumab vedotin	RT			

RT recruiting, *A-NR* active not recruiting, *UN* unknown, *TER* terminated, *CT* completed, *NYT* not yet recruiting, *TIL* tumor infiltrating lymphocyte, *TACE* transarterial chemoembolization, *RFA* radiofrequency ablation

Note: the sum of immunotherapy molecules listed under any trial do not represent the combinations designed by the researchers to evaluate potency against mentioned cancer variety

8.16 Clinical Significance of Immunotherapy in GI Cancers

Immunotherapies are the new advantageous strategy to fight multiple cancers and researchers are examining a variety of medicines and factors like immune checkpoint inhibitors, ACT, peptide vaccines, cytokines, and antibodies. The immunotherapies must provide significant, reliable, and substantiating outcomes in terms of side effects, safety, disease progression, tolerability, and survival. The therapeutic agent must corroborate the primary endpoints. FDA finally approves the target if it provides at least some or all beneficial effects when administered. Despite the tremendous amount of money, time, and expertise invested in developing successful therapeutic targets to treat GI cancer, the success rate of therapeutic agent crossing FDA approval is limited. Among many therapeutic methods validated on GI cancers, immunotherapy represents promising means to treat these tumors. Since it is evident from the FDA approved immunotherapy list on GI cancers.

Some of the recent FDA approved immunotherapies against GI are discussed here.

(a) On July 10, 2018, FDA granted permission to use a blend of ipilimumab (Yervoy) and nivolumab (Opdivo) in some metastatic CRC patients who previously underwent chemotherapy.
(b) On May 23, 2017, FDA favored the use of pembrolizumab (Keytruda) in MSI-H or dMMR tumors, despite the origin of tumor in the body.
(c) On July 31, 2017, the FDA granted hastened support to the immunotherapy drug nivolumab (Opdivo®) for metastatic CRC with MSI-H and dMMR, whose disease progressed after chemotherapy.
(d) On September 22, 2017, the FDA sanctioned the immunotherapy drug nivolumab-Opdivo® in advanced HCC who previously treated with sorafenib-Nexavar®.
(e) On September 22, 2017, the FDA permitted the use of immunotherapy drug pembrolizumab (Keytruda®) against advanced gastric cancers.
(f) On April 27, 2017, the FDA recommended regorafenib (Stivarga®) in HCC patients. The approval aimed to treat HCC tumors who become insensitive to sorafenib (Nexavar®).
(g) On October 22, 2015, FDA approved the irinotecan liposome-Onivyde AU92® to use on chemotherapy-resistant metastatic PC.
(h) On September 22, 2015, FDA granted a tablet composed of tipiracil hydrochloride and trifluridine (Lonsurf®) to treat mCRC patients.
(i) On April 21, 2014, FDA approved the ramucirumab-Cyramza to treat advanced gastric or GEJ adenocarcinoma.

8.17 Future Prospectus

The future of immune-oncology depends on the choices made in preferring the right vaccine type, selecting correct combination of anti-cancer agents, and also choosing the right person to treat with the selected immunotherapy. GI cancers are aggressive, display genome instability/gene mutations, immune suppression, immune insensitivity, and desmoplasia. All these features make GI cancers invincible to wash with immunotherapy. Whatever oncologists achieved so far was great but not enough, since the very first promising clinical data comes into light just in 2015 and many studies are still figuring out the puzzle, though the first immunotherapy concept came out long ago in 1909. A perfect combination that exerts potential anti-cancer benefits for GI patients must involve precise prognostic factors, knowledge in vaccine development following choice of anti-cancer treatment. Immune based vaccines proved efficient as anti-cancer agents in many GI malignancies; still selection of patients for particular immunotherapy must be done with much more care to achieve probable treatment. The idea lays the foundation for developing personalised immunotheraputics to treat GI cancers in the future. Personalized immunotherapy considers patients' molecular and immune makeup to provide a strong backbone in developing most suitable anti-cancer therapy which can confer safe and prolonged disease control. However, the healthcare cost must be considered while making efficient therapies for GI cancers. In the next 20 years, the future of immunotherapy is going to shift the phase of current treatment choices of GI cancers. The future treatments must be designed in such a way to eliminate unwanted toxicities while shrinking the tumor. Among all the GI cancers, BTC and PC patients have limited/no immunotherapeutic options at the moment, nonetheless ongoing clinical investigation must provide some assuring therapeutic solutions. It is highly important to overcome the various factors contributing to varied effectiveness of immunotherapy in GI cancers. Let us hope that oncologist will discover the "Magic bullet" to whitewash GI cancers in the near future.

Conflicts of Interest None.

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Chapter 9
Immuno-Oncology of Oesophageal Cancer

Bindu Prasuna Aloor and Senthilkumar Rajagopal

Abstract A most worldwide health challenge is oesophageal cancer and is standing in 6th place of cancer deaths all over the world. Oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC) are the two types of oesophageal cancer which are epidemiologically and biologically distinct. More than 90% of the disease is OSCC in East regions of Africa and southern states of America. The disease originates in the epithelial lining. It can metastasize in lungs, liver, stomach, and also other body parts. The disease is most observed in males than females. The major causing agents are tobacco usage and alcohol consumption. The disease is mostly fatal with less survival rate of 5–30%. Patient can be cured if diagnosed in the early stage by surgical removal of the tumour, chemotherapy, radiation. This chapter is mainly focused on possible role of immunotherapy for oesophageal cancer.

Keywords Achalasia · Adenocarcinoma · Barrett's oesophagus · Gastro oesophageal reflux disease · Oesophageal squamous cell carcinoma

Abbreviations

AC	Adenocarcinoma
GERD	Gastro oesophageal reflux disease
HER2	Human epidermal growth factor receptor 2
OAC	Oesophageal adenocarcinoma
OSCC	Oesophageal squamous cell carcinoma
PD-1	Programmed cell death protein I
PET-CT	Positron emission tomography and computed tomography

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SCC Squamous cell carcinoma
VEGF Vascular endothelial growth factor

9.1 Introduction

Oesophagus is the muscular tube that carries food and drink from the mouth to the stomach. Oesophageal cancer refers to malignant tumours of the oesophagus. Oesophageal cancers are named specifically relating to the localization of the tumour as relation "gastro esophageal junction adenocarcinoma" which explains as the adenoma localized where the stomach and oesophagus are jointly related (Montgomery 2014).

9.2 Anatomy

A brief outline described here of the structure and function of the oesophagus will help understand the disease in malignant stage. The oesophagus or food tube is the hollow and muscular tubular structure that connects the oral cavity and the upper end point of the stomach. It conducts swallowed food and liquid to the stomach to be digested. It measures around 25–30 cm in length and has few constrictions present at various points throughout its length. The narrowest portion of oesophagus measures 3/4 inch in diameter. The wall of the oesophagus is four layered. Immediate mucosal lining that surrounds the central lumen. This is made up of flat squamous cell type. Underlying the epithelium is the submucosal layer consisting of blood vessels and nerves.

Next to this is the smooth muscle layer. The contractions of this thick muscular layer help in propelling the food downwards to the stomach for digestion. Beneath the muscular layer is the serosal layer or called adventitia. The upper and lower oesophagus is controlled by the sphincters, cricopharyngeus muscle and gastro oesophageal sphincter, respectively. Oesophagus bears a dense mesh of lymph cells in lamina propria cells and sub mucosa, which runs all along the sub mucosa. Under normal conditions, both sphincters are in closed form. When the lower sphincter is incompetent or fails to relax, it can lead to gastric acid reflux, a condition called achalasia. This condition has the risk of oesophageal cancer increasing slightly. Usually oesophageal cancers occur in the lining epithelium of the oesophagus and spread to the other layers, nearby organs or spread to distant sites called metastasis.

The four main segments of oesophagus are cervical oesophagus of 15–20 cm, upper part of thoracic oesophagus about 20–25 cm, middle part of thoracic oesophagus about 25–30 cm, lower thoracic oesophagus and gastro oesophageal junction about 30–40 cm from the incisors (Fig. 9.1). Tumours caused in the oesophagus are

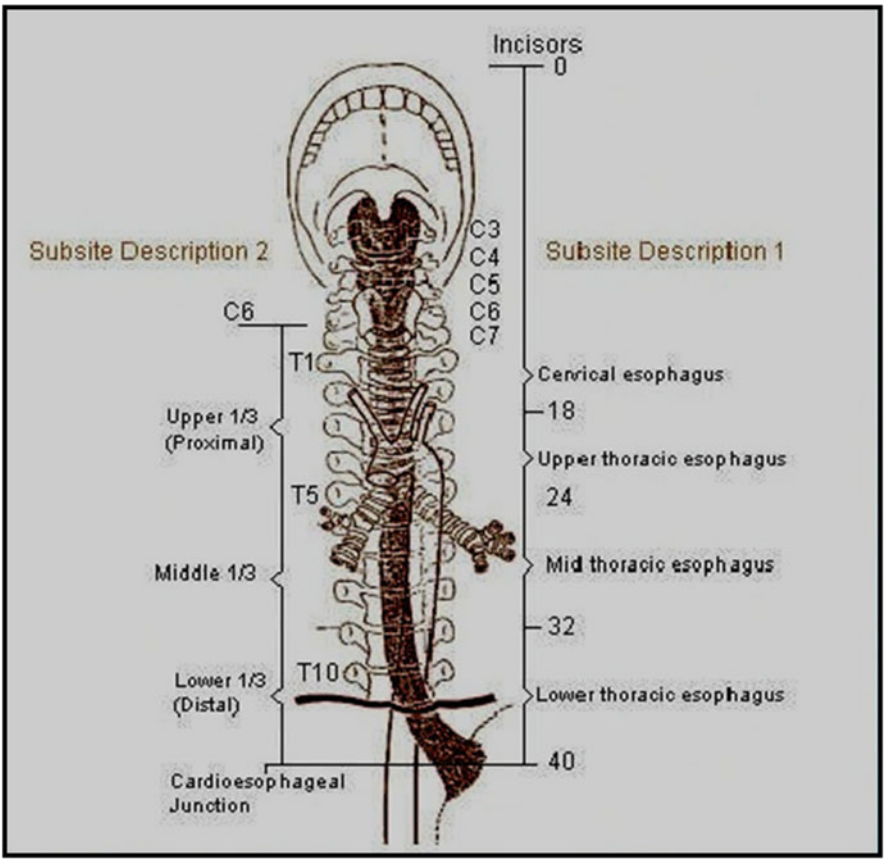

Fig. 9.1 Anatomy of oesophagus (*courtesy:* American Cancer Society: Cancer Facts and Figures (American Cancer Society: Cancer Facts and Figures 2019))

usually measured by the distance of the upper end point of the tumour to the point of incisors location.

9.3 Subtypes

The subtypes are named after their origin in the oesophagus. Squamous cell carcinoma (SCC) begins in the squamous cells that line the oesophageal cells. Mostly originates in the middle portion of the oesophagus. SCC is found in people who have excess usage of cigars and other alcoholic beverages (Prabhu et al. 2014). The disease is also prevalent in people who were diagnosed with head/neck squamous cell carcinoma (Priante et al. 2011; Scherübl et al. 2008).

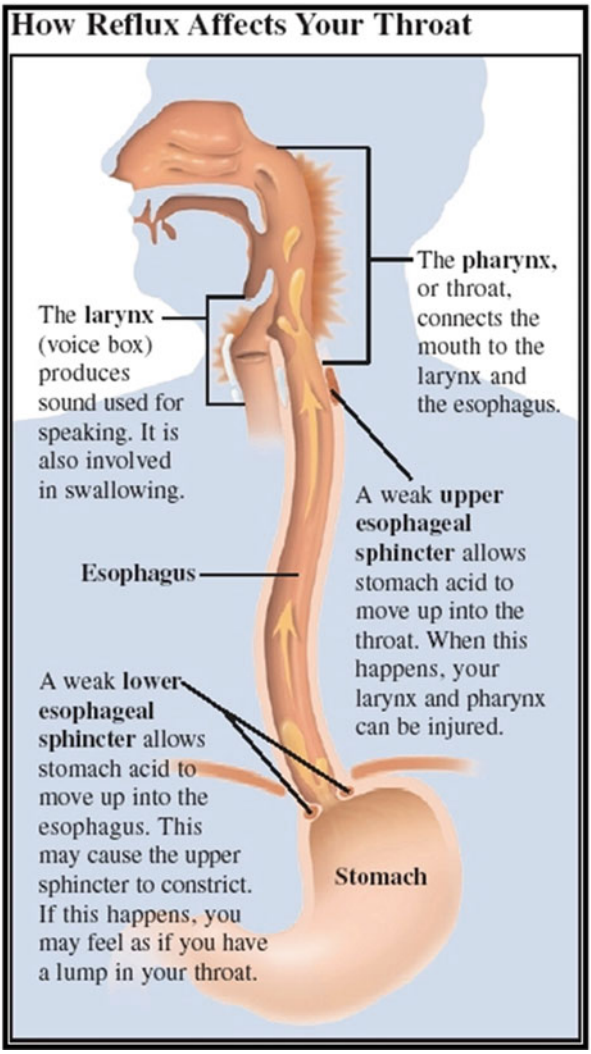

Fig. 9.2 Acid reflux and relation to oesophageal cancer (*courtesy: slide share, GERD*)

Adenocarcinoma (AC) occurs in the distal oesophagus, at the opening to the abdomen. When squamous cells are replaced by glandular cells, and grow abnormally, it is named as adenocarcinoma. It arises from a pathological change occurring in the normal oesophageal lining squamous epithelium (DeJonge et al. 2014). This change is termed as Barrett's oesophagus and is associated with abnormal chronic reflux in gastric juice into the lower oesophagus, chronic symptomatic acid reflux disease (DeJonge et al. 2014). This results in columnar transformation of the normal cells which are lining the oesophagus to that of intestinal and colon lining. The condition is diagnosed as symptom of cancer (Fig. 9.2).

Usually, Barrett's oesophagus is benign and chance of malignancy ranges from 1–5%. ACC incidence is in the last third portion of the oesophagus. Chewing tobacco acts as an enhancer showing less impact in AC and higher in SCC. No report has evidences alcohol as the causing agent (Lagergren and Lagergren 2013). Rarer cases of oesophageal cancers found so far are carcinoma of lymph, chorion, melanoma, and sarcoma.

9.4 Incidence and Mortality

The exact reason behind the disease is still unknown. Researchers say that some factors associated with DNA damage may cause the disease (Pennathur et al. 2013).

This cancer is very rare in United States but most occurred in Asia and parts of Africa. Recorded new cancers and deaths from the disease among US population in 2019 were 17,650 and 16,080, respectively (American Cancer Society: Cancer Facts and Figures 2019). There has been more incidence of oesophageal cancer in recent decades with change in histology and tumour localization. In the USA, SCC is more and the prevalence of adenocarcinoma was also recorded recently in the USA and Western Europe (Brown et al. 2008; Blot and McLaughlin 1999). Disease was recorded more in males (Kubo and Corley 2004). The average age of patients found with oesophageal cancer is 55 years old (Ginsberg 1998). ACs are localized in the distal region of oesophagus. The cause of the disease and demographic distribution are unknown.

Oesophageal carcinoma is the sixth commonly causing cancer related death worldwide and is considered as one of the crucial global health challenges. Even though oesophageal cancer survival rates have improved, the prognosis is poor when compared to other cancers; Survival rate is only 20% for at least 5 years after being diagnosed with oesophageal cancer. These statistics emphasize the need for a new therapy or therapies to prevent and treat oesophageal cancer.

The occurrence of oesophageal carcinoma is very rare in younger population. As age advances, there are higher chances of incidence of the disease with its peak in the 70s and 80s. AC is commonly observed in males, while SCC seems to spread irrespective of sex (Kim et al. 2009). Generally, medical practitioner describes stages of cancer in terms of size, part affected, and organs affected by the spread of cancer cells through blood (Figs. 9.3, 9.4, and 9.5). Staging of the disease helps in proper treatment for the disease. The TNM staging system (Rice et al. 2017) describes cancers by Tumour (T)—primary tumour or slightly extended; Nodes (N)—cancerous cells move to adjacent lymph nodes in the primary tumour located organ; Metastasis (M)—gradual movement of cancerous cells to different organs which are away from the primary tumour.

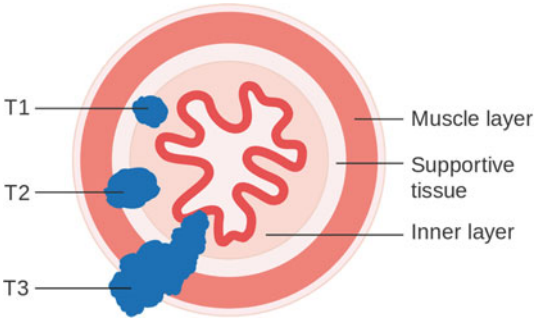

Fig. 9.3 T1, T2, and T3 stages of oesophageal cancer (*courtesy:* American Cancer Society: Cancer Facts and Figures (American Cancer Society: Cancer Facts and Figures 2019))

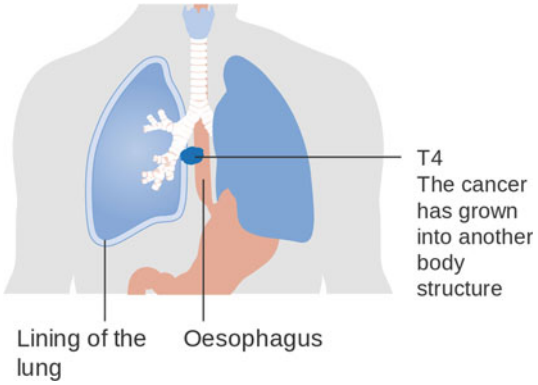

Fig. 9.4 Stage T4 oesophageal cancer (*courtesy:* American Cancer Society: Cancer Facts and Figures (American Cancer Society: Cancer Facts and Figures 2019))

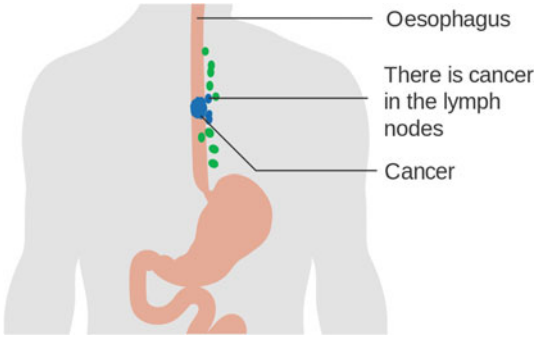

Fig. 9.5 Oesophageal cancer spread to lymph nodes (*courtesy:* American Cancer Society: Cancer Facts and Figures (American Cancer Society: Cancer Facts and Figures 2019))

9.5 Risk Factors

Oesophageal cancer develops when the DNA in the cells is altered or mutated that causes cancer cells to grow and multiply out of control leading to the formation of a tumour mass (Sewram et al. 2014). Rupture or irritation in the mucosal lining of the oesophagus is caused by many factors that include smoking, alcohol consumption, gastro oesophageal reflux disease (GERD) (Falk 2009), bile reflux, Barrett's

oesophagus, drinking hot liquids, obesity, achalasia cardia, radiotherapy given to upper abdomen or chest for lung or breast cancer can increase oesophageal cancer risk.

9.6 Symptoms

Symptoms of oesophageal cancers are Dysphagia—pain or discomfort in swallowing, and sensation of food blockage in the throat or chest. It is very important not to neglect the above symptoms and one has to seek urgent medical attention (Ferri 2013).

Persistent heartburn/indigestion—Tumour present in the lower part of the oesophagus causes dysfunction of the lower oesophageal sphincter and causes acid reflux leading to burning sensation or chest discomfort, soon after eating (Lao-Sirieix et al. 2010). Even though heartburn or indigestion can be harmless, it is important to consult a doctor and get investigated (Cook et al. 2014). Loss of weight—If consuming food becomes less due to swallowing difficulties can lead to marked loss in weight of the human (Yamada 2011).

Regurgitation—Because of difficulty in swallowing, swallowed food starts to come back. This begins initially with solid food and with disease progression, soft mashed food as well as liquids come back (Mayer 2008). Persistent cough or hoarseness of voice—It can be due to pressure on the windpipe. Vomiting blood—This rarely occurs due to an ulcerated growth bleed or there is infiltration of the tumour into a blood vessel (Gerdes and Ferguson 2002). Dark coloured stools. When the blood from the tumour is swallowed, it undergoes digestion by the acid in the stomach and becomes dark coloured and passed out as dark coloured stools.

9.7 Diagnosis

Patients with the above symptoms have to undergo an upper intestinal endoscopy (Stahl et al. 2013). A flexible tube equipped with an illuminated lens at its distal end is passed in to the mouth down the throat into the food pipe. Just before the procedure the throat is numbed with an anaesthetic, gargle and the endoscopic tube is passed into the oesophagus. The doctor can directly view the interior of the oesophagus and identify abnormal areas such as tumour or inflammation (Vazquez-Sequeiros et al. 2001).

Seventy-five percent of ACs occurs in the distal part of oesophagus, while SCCs appear in the region of proximal to middle oesophagus (Montgomery 2014). Biopsies have to be taken from all suspected areas. PET-CT (positron emission tomography and computed tomography) scans and other related tests can visualize cancer cells with rapid metabolism (Bruzzi et al. 2007). Diagnostic tests for different

cancers include mammography, pap smear test, tumour marker test, bone scan, MRI, tissue biopsy (Stahl et al. 2013; Krasna et al. 2001).

Barium Swallow—oesophagus can be used as a marker for imaging metastasized cancer by radiological procedure. Fast for a few hours prior to the procedure is recommended (Cynthia and Barbara 2012). Patient is given some barium containing liquid to swallow and following the swallowing of liquid, x-ray images of the oesophagus are captured. If the results are suggestive of a growth, next set of procedures may be necessary. Blood and urine tests will be conducted to determine the general health status of the patient. Once diagnosis of oesophageal cancer is confirmed, further tests are required to stage the disease. Staging of the carcinoma is necessary to decide medication (Figs. 9.3, 9.4, and 9.5). Tests such as PET-CT and MRI help in staging the disease.

Stage I—tumour growth is confined to the superficial layer of the oesophagus lining. There is no spread to local lymph nodes (Stahl et al. 2013).

Stage II—tumour growth has extended in depth into muscular layers of the oesophagus or into neighbour lymph nodes (Mariette et al. 2014).

Stage III—tumour has extensively involved the wall of the oesophagus, has moved to tissues surrounding or lymph nodes (Montgomery 2014).

Stage IV—tumour has spread all over travelling in blood and metastasized and may be seen in the liver, lungs, brain, or bone (Stahl et al. 2013).

9.8 Prognosis

Favourable prognostic factors are disease recognition in budding stage and complete surgical removal. Acute dysplasia in distal oesophageal mucosal layer may get invasive cancer within the dysplasia region. Prognosis is high in such cases after resection (Reed et al. 2005). In many cases, oesophageal cancer patient's life span can be increased, but is rarely completely curable. The overall 5-year survival time in patients with definitive treatment is from 5% to 30% (Polednak 2003). Regular doctor consultation following therapy is must. Patients' post-surgery of their oesophagus exhibits side effects of narrowing of oesophagus at the site of the surgery. In such complications, need oesophageal dilatations with stents (Tietjen et al. 1994).

9.9 Treatment

In cancer care multidisciplinary doctors collaborate to design a patient's overall medication plan that is in combination with different types of treatments. Cancer care teams are usually associated with physician assistants, oncology nurses, social workers, pharmacists, counsellors, dietitians, and others.

Immunotherapy approaches for oesophageal carcinoma are

9.9.1 Targeted Antibodies

The treatment is by a monoclonal antibody, Ramucirumab that specifically targets the VEGF/VEGFR2 pathway and hinders blood vessel growth in tumours; this is approved for patients in advanced stage of gastro oesophageal cancer.

Trastuzumab is another monoclonal antibody that interferes in the HER2 pathway; and has been approved to medicate advanced, HER2-positive gastro oesophageal cancer as a first-line therapy.

9.9.2 Immunomodulators

Pembrolizumab: A checkpoint inhibitor that has interference in PD-1/PD-L1 pathway; has been used in patients with advanced, PD-L1-positive gastro oesophageal cancer or squamous cell carcinoma of the oesophagus.

Many immunotherapy researches for oesophageal cancer have shown promising results in their early clinical trials (Table 9.1) (Fiorica et al. 2004).

Table 9.1 Standard treatment options for oesophageal cancer (courtesy (Fiorica et al. 2004))

Stage (TNM staging criteria)	Treatment options
Stage 0—oesophageal cancer	Surgery
	Endoscopic resection
Stage I—oesophageal cancer	Chemo radiation therapy followed by surgery
	Surgery alone
Stage II—oesophageal cancer	Chemo radiation followed by surgery
	Surgery alone
	Chemotherapy followed by surgery
	Definitive chemo radiation
Stage III—oesophageal cancer	Chemo radiation followed by surgery
	Preoperative chemotherapy followed by surgery
	Definitive chemo radiation
Stage IV—oesophageal cancer	Chemo radiation followed by surgery (for patients with stage IVA disease)
	Chemotherapy, which has provided partial responses for patients with metastatic distal oesophageal adenocarcinomas
	Nd:YAG endoluminal tumour destruction or electrocoagulation
	Endoscopic-placed stents to provide palliation of dysphagia
	Radiation therapy with or without intraluminal intubation and dilation
	Intraluminal brachytherapy to provide palliation of dysphagia
Recurrent oesophageal cancer	Palliative use of any of the standard therapies, including supportive care

9.10 Conclusion

As there is increased incidence of cancers everywhere, there is the need to create awareness among people. People who are in remote villages have to be educated about the causes of cancer and hygiene should be maintained in relation to the drainages, slums. Any symptom of difficult swallowing, inflammation in throat should not be neglected and ruled out by thorough investigations. Initial stage of oesophageal cancer has prolonged life span. Proper medication has to be followed along with regular doctor checkups. Final stage of oesophageal cancer requires high dosage of chemo and ratio therapies simultaneously. In such cases, patient has to be admitted and given intra venous supplement of proteins to cope with the treatment procedures.

There is the immediate need to enhance the prognosis of final stage oesophageal cancer patients. Treatment should be in such a way that the effect of the drugs should not damage the other organs in the body. A combined administration of supporting drugs that minimize the effect of radiation and chemos has to be recommended to patients. The kind of nutrients that supports patients from treatment has to be developed according to the age of the patients.

Conflicts of Interest None

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Chapter 10
Association Between IL6 Gene Polymorphisms and Gastric Cancer Risk: A Meta-Analysis of Case-Control Studies

Henu Kumar Verma, Neha Merchant, and L. V. K. S. Bhaskar

Abstract Interleukin-6 (IL-6) is a multifunctional cytokine, which plays a vital role in inflammation as well as tumorigenesis. Several studies have demonstrated that the association of IL6 -174 G/C (rs1800795) and -572 G/C (rs1800796) promoter polymorphisms influences transcription and has been found to trigger the risk of gastric tumor advancement with inconsistent and controversial result. The present study aims at collecting eligible articles through extensive search in PubMed, MEDLINE, and Embase databases. Additionally, the analysis also included 15 case–control investigations. MetaGenyo web tool was used to perform the meta-analysis. No substantial association was observed between IL6 polymorphisms and GC. In conclusion, our study signifies that polymorphisms of IL6, -174 G/C, and -572 G/C are not linked with GC risk.

Keywords Gastric cancer · IL-6 gene · -174 G/C · -572 G/C · Meta-analysis

Abbreviations

GC	Gastric cancer
IL-6	Interleukin 6
H. pylori	*Helicobacter pylori*
NLM	National Library of Medicine
SNPs	Single nucleotide polymorphisms
CBLD	Chinese Biomedical Literature Database

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HWE	Hardy–Weinberg equilibrium
P value	Probability value
OR	Odds ratio

10.1 Introduction

Inflammation is an essential innate immune response induced by microbial infection and tissue damage. Numerous studies have provided a wide range of clinical evidence that chronic inflammation is linked with elevated risk of Gastric cancer (GC) (Greten and Grivennikov 2019; Multhoff et al. 2011; Bockerstett and DiPaolo 2017). Cytokines are a wide range of small proteins secreted by immune cells, including nucleated cells and work as an intracellular messenger in the immune system (Lowry 1993). Cytokine is a key mediator of diagnosis and treatment during inflammation in many diseases (Verma et al. 2016). The potential association between multifunctional cytokines and GC has been examined by several investigators. Among those, Interleukin 6 (IL-6) is known to function as both a pro-inflammatory cytokine as well as an anti-inflammatory myokine regulator (Tanaka et al. 2014). IL-6 belongs to a family of pleiotropic cytokine and modulates cell proliferation and differentiation. Previous data has demonstrated that IL-6 level was increased in mucosa, which leads to the inflammatory microenvironment in *Helicobacter pylori* (*H. pylori*) related gastritis (Yamaoka et al. 1996). Further, overexpression of IL6 is strongly associated with an increased risk of GC development and progression (Madej-Michniewicz et al. 2015). Therefore, based on the earlier reports, IL-6 is closely linked to occurrence and development of cancer.

The gene coding for IL6 is located on chromosome 7p21 and comprises 184 amino acids, which fold as a 4 alpha-helix bundle structure (Choy and Rose-John 2017). Understanding the genetic diversity with population genetic structure of IL6 will aid in predicting tumor risk as well as in reducing mortality. To date, several single nucleotide polymorphisms (SNPs) have been identified in the promoter region of IL6 (Terry et al. 2000). Among them, IL6 -174 G/C (rs1800795) and -572 G/C (rs1800796) are the most widely studied polymorphisms in several cancers including GC. However, previous investigations have yielded varying results regarding the relationship between IL6 promoter polymorphisms and gastric cancer (Chakraborty et al. 2017; Markkula et al. 2014). It could be because of the insignificant sample size, variations in genotyping methods, and ethnicity of the populations. In order to assess the precise role of IL6 promoter polymorphism on GC susceptibility, we have conducted this meta-analysis of all existing case–control studies.

10.2 Methods

10.2.1 Study Selection Strategy

To evaluate the relation between IL6 promoter polymorphisms and the risk of GC, all potentially pertinent articles were searched and identified according to the PRISMA guidelines (Liberati et al. 2009). Pubmed, Web of Science, and EMBASE Database were searched using the following keywords: Interleukin-6 and gastric cancer, IL6, IL6 -174 G/C, rs1800795, -572 G/C, and rs1800796. The last search was executed on 26 April 2020.

10.2.2 Literature Inclusion and Exclusion Criteria

Two investigators selected eligible studies independently. Studies that met the following criteria were included in this meta-analysis: (1) case–control study on GC and IL6 promoter polymorphisms; (2) genotypes available for calculating odds ratio (OR) and 95% confidence interval (CI). The exclusion criteria for this meta-analysis were as follows: (1) studies with no specific control group; (2) non-availability of genotype data. The quality evaluation of all eligible studies and data extraction of information was made with consensus and the discrepancy between investigators was resolved by cross-checking the data. From each paper, name of the first author, publication year, country and ethnicity of the participants, genotypes in cases and control subjects were collected and documented in Table 10.1.

10.2.3 Statistical Analysis

The strength of association between IL6 polymorphism (-174 G/C and -572 G/C) and GC was assessed for all studies. The crude ORs and their corresponding 95% confidence interval (CI) limits were calculated. The presence of heterogeneity was evaluated with the Cochran's Q test and inconsistency I2 statistics. Based on the extent of heterogeneity, fixed effects model (FEM) or random effects model (REM) were adopted for pooled analysis. The association between IL6 polymorphisms and GC was analyzed in dominant, recessive, and allelic genetic models. To assess the robustness of the study, sensitivity analysis was performed by overlooking each study one time and estimating the Odd Ratio (OR) for the remaining studies. Publication bias was measured by the use of a funnel plot and Egger's test. MetaGenyo web tool was used to perform the meta-analysis (Martorell-Marugan et al. 2017).

Table 10.1 The characteristics of included studies in present meta-analysis

First author (year)	Country/ ethnicity	Case/ control	Cases			Control			HW *P* value
			CC	GC	GG	CC	GC	GG	
IL6 -174 G/C (rs1800795)									
Dos Santos et al. (2019)	Brazil/ Caucasian	52/87	6	17	29	10	35	42	0.517
Attar et al. (2017)	Iran/ Caucasian	100/ 361	7	30	63	13	187	161	<0.001
Ramis et al. (2017)	Brazil/ Caucasian	0.09/38	0	2	7	2	13	23	0.927
Sampaio et al. (2015)	Portugal/ Caucasian	50/50	8	25	17	6	25	19	0.608
Pohjanen et al. (2013)	Finland/ Caucasian	56/179	8	34	14	56	86	37	0.706
Yong et al. (2010)	China/Asian	142/ 200	0	37	105	0	2	198	0.943
Crusius et al. (2008)	France/ Caucasian	243/ 1138	43	122	78	206	517	415	0.044
Deans et al. (2007)	UK/ Caucasian	197/ 224	43	83	71	44	101	79	0.258
Gatti et al. (2007)	Brazil/ Caucasian	56/112	1	13	42	11	53	48	0.509
Kamangar et al. (2006)	Finland/ Caucasian	102/ 152	27	54	21	43	58	51	0.004
Xing et al. (2006)	China/Asian	65/71	0	3	62	0	4	67	0.807
El-Omar et al. (2003)	USA/ Caucasian	209/ 213	28	98	83	34	91	88	0.205
Hwang et al. (2003)	USA/ Caucasian	30/30	2	9	19	0	8	22	0.399
IL6 -572 G/C (rs1800796)									
Mrtinez-Campos et al. (2019)	Mexico/ Caucasian	122/ 122	18	55	49	15	58	49	0.733
Dos Santos et al. (2019)	Brazil/ Caucasian	52/87	2	10	40	1	22	64	0.555
Kang et al. (2009)	Korea/Asian	332/ 326	21	133	178	17	140	169	0.078
Xing et al. (2006)	China/Asian	65/71	2	4	59	4	11	56	0.005
Hwang et al. (2003)	USA/Asian	30/30	16	13	1	16	13	1	0.394
Hwang et al. (2003)	USA/ Caucasian	30/30	3	16	11	5	7	18	0.020

10.3 Results

10.3.1 Characteristics of Published Studies

Our systematic literature search identified 436 articles. Based on the inclusion and exclusion criteria, unrelated or duplicate studies were excluded by reading titles and abstracts. Ninety-six relevant articles were selected for further assessment and 71 studies were consequently excluded after reading the full text to avoid discrepancy. Finally, 15 case–control studies fulfilled our study criteria (Fig. 10.1). Out of which, IL6 -174 G/C genotypes were extracted from thirteen papers (Dos Santos et al. 2019; Attar et al. 2017; Ramis et al. 2017; Sampaio et al. 2015; Pohjanen et al. 2013; Yong et al. 2010; Crusius et al. 2008; Deans et al. 2007; Gatti et al. 2007; Kamangar et al. 2006; Xing et al. 2006; El-Omar et al. 2003; Hwang et al. 2003). The IL6 -572 G/C genotypes were extracted from six papers (Dos Santos et al. 2019; Xing et al. 2006; Hwang et al. 2003; Martínez-Campos et al. 2019; Kang et al. 2009). Hwang et al. paper has analyzed samples from two ethnicities, hence it is considered as two studies (Hwang et al. 2003). The genotype distributions and main characteristics of studies are presented in Table 10.1. For IL6 -174 G/C, the heterogeneity test indicated significant heterogeneity between studies (CG+CC vs. GG: $P_{\text{heterogeneity}} < 0.001$, $I^2 = 72\%$), but no heterogeneity was observed between studies of IL6 -572 G/C (CG+CC vs. GG: $P_{\text{heterogeneity}} = 0.232$, $I^2 = 27\%$) (Table 10.2).

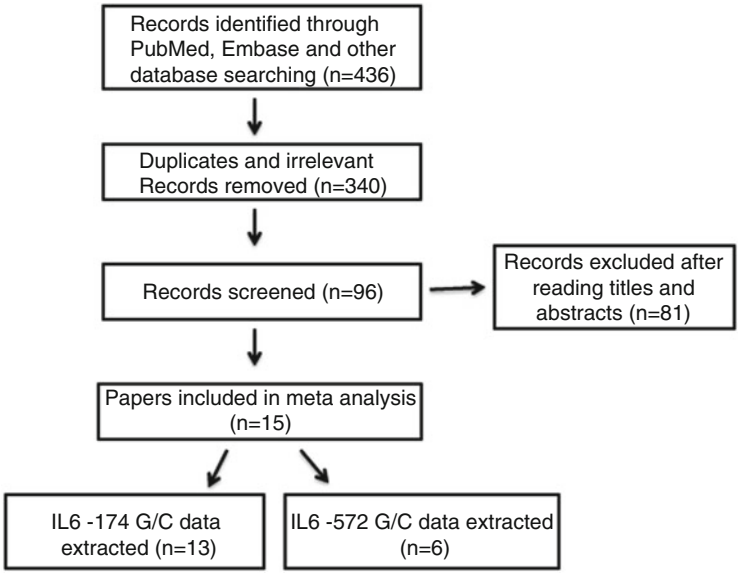

Fig. 10.1 Flowchart of study selection for the current study

Table 10.2 Associations of interleukin 6 gene polymorphisms with the risk of gastric cancer

IL6 -174 G/C (rs1800795)		Allele model (C vs. G)	Recessive model (CC vs. GC+GG)	Dominant model (CG +CC vs. GG)
Number of studies		13	11	13
Test of association	OR	0.96	0.95	1.01
	95% CI	[0.74–1.24]	[0.77–1.16]	[0.69–1.48]
	p value	0.738	0.584	0.960
	Model	REM	FEM	REM
Test of heterogeneity	*p* value	<0.001	0.222	<0.001
	I^2%	76%	23%	79%
Publication bias	Egger's test *p* value	0.903	0.980	0.791
IL6 -572 G/C (rs1800796)		Allele model (C vs. G)	Recessive model (CC vs. GC+GG)	Dominant model (CG +CC vs. GG)
Number of studies		6	6	6
Test of association	OR	0.99	1.11	0.94
	95% CI	[0.82–1.18]	[0.74–1.66]	[0.74; 1.19]
	p value	0.872	0.627	0.611
	Model	FEM	FEM	FEM
Test of heterogeneity	p value	0.440	0.773	0.232
	I^2%	0%	0%	27%
Egger's test *p* value		0.680	0.642	0.968

FEM fixed effect model, *REM* random effect model, *OR* Odds ratio, *95% CI* 95% confidence interval

10.4 Quantitative Data Synthesis

To explore the correlation between IL6 promoter polymorphisms and the risk of GC, 15 studies of IL6 -174 G/C polymorphism (1311 cases/ 2855 control), and six studies of IL6 -572 G/C polymorphism (631 cases /666 controls) were used. Meta-analysis of IL6 -174 G/C polymorphism and GC is documented in Fig. 10.2a, which did not reveal significant association between IL6 -174 G/C polymorphism and gastric cancer in the allelic model (C vs. G; OR = 0.96, 95% CI: 0.74–1.24, P = 0.738), recessive model (CC vs. GC+GG; OR = 0.95, 95% CI: 0.77–1.16, P = 0.584), and dominant models (CG+CC vs. GG; OR = 1.01, 95% CI: 0.69–1.48, P = 0.960). The pooled effect estimates presented in Fig. 10.2b shows that IL6 -572 G/C is not associated with GC in allelic model (C vs. G; OR = 0.99, 95% CI: 0.82–1.18, P = 0.872), recessive model (CC vs. GC+GG; OR = 1.11, 95% CI: 0.74–1.66, P = 0.627), and dominant models (CG+CC vs. GG; OR = 0.94, 95% CI: 0.74–1.19, P = 0.611).

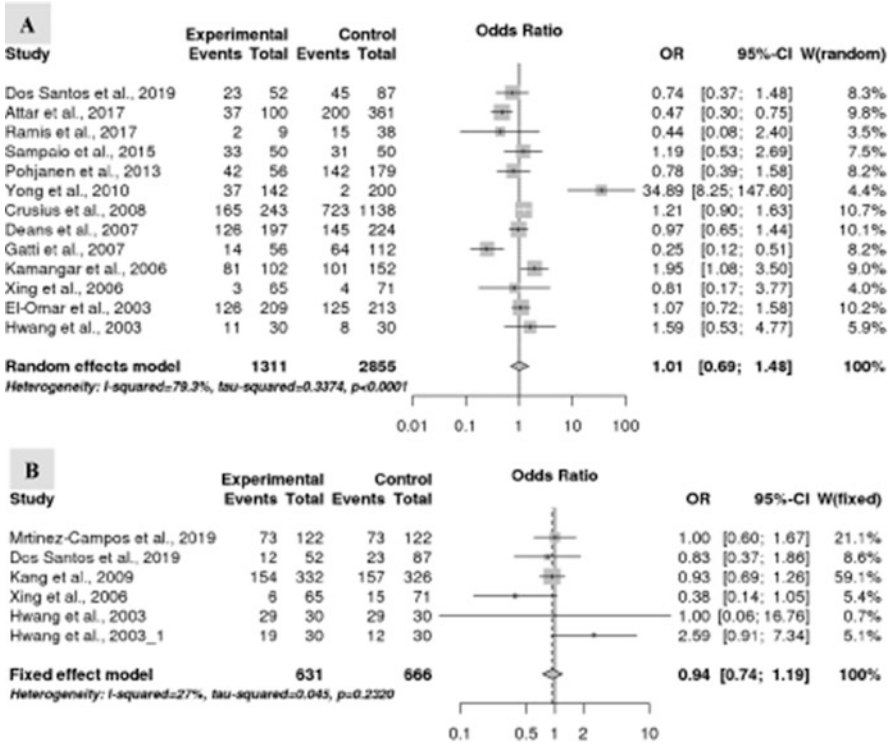

Fig. 10.2 Forest Plot of meta-analysis of the IL-6 polymorphism and gastric cancer risk. (**a**) IL6 -174 G/C; (**b**) IL6 -572 G/C

10.4.1 Sensitivity Analysis and Publication Biases

Sensitivity analysis was carried out with pooled effect estimates by omitting each study one time to evaluate the stability of the outcomes. The outcomes of sensitivity analysis presented in Fig. 10.3 suggest that no single study could influence the pooled ORs of IL6 -174 G/C and IL6 -572 G/C polymorphisms. Visual inspection of Begg's funnel plots did not show asymmetry for both IL6 -174 G/C and IL6 -572 G/C polymorphisms (Fig. 10.4a, b) indicating that there is no publication bias. The same was confirmed by Egger's test p values ($P > 0.05$).

10.5 Discussion

Despite recent progress in clinical practice, GC remains the third most common cancer-related death worldwide. According to current data, in 2017, more than 1.22 million new cases of GC occurred and nearly 8,65,000 patients have died due to GC

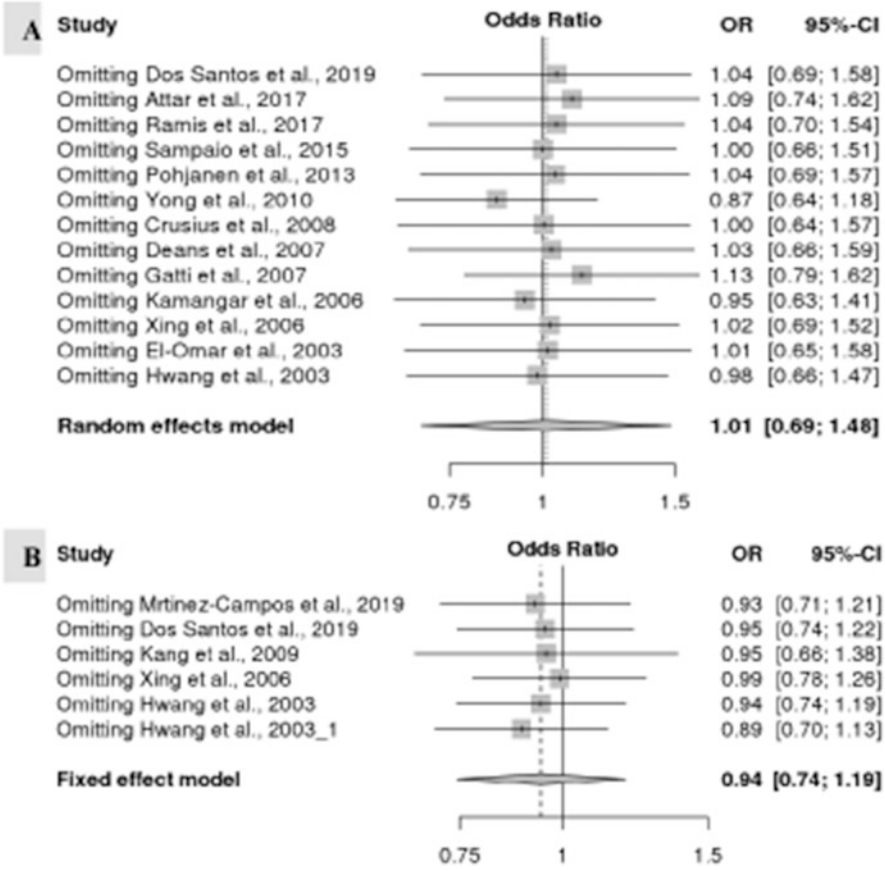

Fig. 10.3 Sensitivity analysis for the association between IL-6 polymorphisms and gastric cancer risk. (**a**) IL6 -174 G/C; (**b**) IL6 -572 G/C

(Russi et al. 2019; Etemadi et al. 2020). To date, the exact causes of GC still remain unknown. Nevertheless, it has been proven that cytokines play a role in inflammation, and can also induce cell transformation in the development of cancer and chemoresistance (Conlon et al. 2019; Verma et al. 2020). Interleukins are low-molecular-weight cytokine expressed by leukocytes and are involved in normal functioning of the immune system. Further, disruptions of interleukins level may lead to immune deficiencies and tumorigenesis (Larsen et al. 2018). Subsequently, it has been reported that some mutations in interleukin genes lead to increased risk of GC development (Wang et al. 2014).

To date, several case–control studies have explored the association between IL6 -174 G/C and IL6 -572 G/C polymorphism on the susceptibility to GC. However, small sample sizes, different genotyping methods, and variation in minor allele frequencies across ethnicities leads to the lack of consistency in results. Therefore, we have performed the present meta-analysis to precisely study the association of

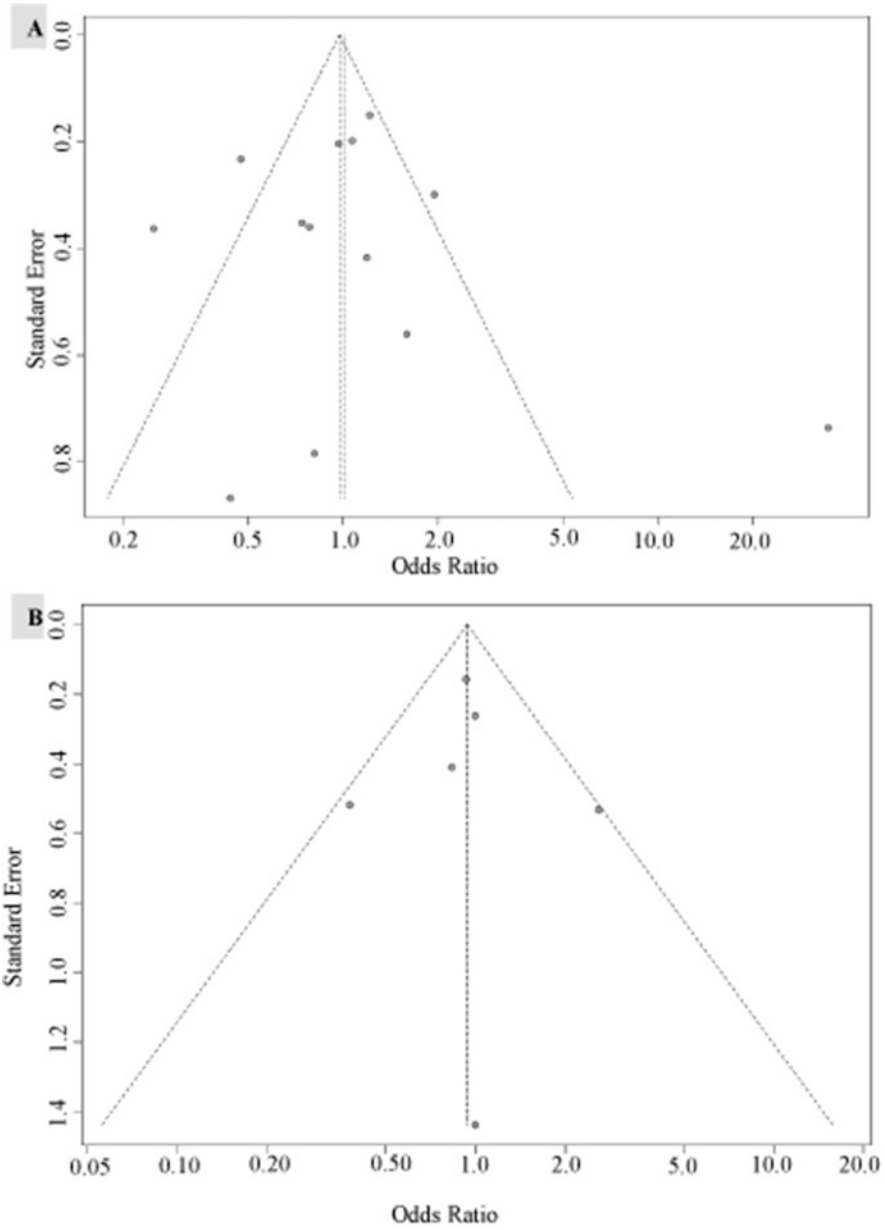

Fig. 10.4 Funnel plot to publication bias in meta-analysis about IL-6 polymorphisms and gastric cancer risk. (**a**) IL6 -174 G/C; (**b**) IL6 -572 G/C

IL6 polymorphism with GC risk. In this comprehensive meta-analysis we have observed that the IL6 -174 G/C and IL6 -572 G/C polymorphisms are not significantly associated with the risk of GC. The results of this meta-analysis are consistent with the results of previous meta-analysis in which no association between GC risk and IL6 -174 G/C (Jafari-Nedooshan et al. 2019; Yunxia Liu et al. 2018; Wang et al. 2018, 2012) or IL6 -572 G/C (Wang et al. 2018, 2012; Peng et al. 2018; Du et al. 2015) was documented. However, some meta-analyses have demonstrated increased GC risk for IL6 -174 G/C (Wang et al. 2018; Tian et al. 2015) or IL6 -572 G/C (Liu et al. 2018) in Asian populations.

In conclusion, our study indicates that the IL6 -174 G/C and IL6 -572 G/C polymorphisms are not correlated with GC risk. Soon, a large population based case–control studies would be potentially needed for validation of Interleukin 6 gene association with GC risk.

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Chapter 11
Immuno-Oncology of Colorectal Cancer

Ramachandra Reddy Pamuru, K. V. Sucharitha, and Ramakrishna Vadde

Abstract The colorectal cancer (CRC) a second leading cancer become most predominant and causing deaths worldwide. Since its spread, more attention has been made to control the CRC. For developing anti-tumor therapies, it is important to know the immune-oncology of CRC. A number of events are identified in the tumor microenvironment of CRC. This chapter gives details of the basics of CRC, immune cells of tumor microenvironment, tumor suppression, and repression. These details of tumor immune-oncology of CRC may help to provide better understanding of CRC and suggest ways to control CRC.

Keywords Colorectal cancer · Microenvironment · Immune cells · Immune response · Immunosuppression

11.1 Introduction

Colon carcinoma (CRC), a third leading cancer reported more than 1.2 million cases worldwide every year and second leading chronic disease in the USA (Rebecca et al. 2019). CRC occupies fourth place in mortality among all cancers in western countries (Globocan, Agency for research on cancer, WHO, 2017) whereas, in the USA 2nd death causing most common carcinoma among other cancers (Tenesa and Dunlop 2009; Jemal et al. 2009). It is very unfortunate that CRCs are silent tumors; they grow slowly and do not show most of the symptoms until they attain large size.

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Due to its heterogeneous nature, CRC does not hold correct prognostic evades and became most common disease. The reasons for getting CRC are not very clear, but found majority of these cases are linked to environmental causes rather than mutations at the gene level (heredity). In the colon and rectum, development of CRC is linked to a variety of risk factors, including microbial environment, food borne mutagens, and inflammation. Inflammation found 2000 years ago by Greek Physician Galenus (Reedy 1975) shares close relation with CRC (angiogenesis, lymphocytes, macrophages, and mast cells). Most commonly people of age 50 and above are more prone to CRC. Though it is not very clear about the CRC risk factors, some of the possible risk factors are mentioned by American Cancer Society (American Cancer Society 2019) in its report. Moreover, the carcinomas can also call 'unhealed wounds' with a characteristic property of inflammation (heal wounds). One of the CRC subtypes difficult to react and has high mortality is colitis associated carcinoma associated with inflammatory bowel disease (Feagins et al. 2009).

The occurrence of CRC is not only restricted to developed and western countries, it is also causing dreadfulness among populations all over the world. There is a shove for control and therapeutic developments for CRC and is one of the thrust areas of research in recent days. In this juncture, lot of information is reviewed on CRC development, histology, screening, and immunotherapy, but still certain aspects of CRC are undercover. One of such part not having lot many reviews is immunology of colorectal cancer, which provides basic information to develop efficient stage specific immune-therapeutics. It gives a lot of importance if we know the different aspects situated in microenvironment of CRC along with basics of risk factors for tumor formation and development. In view of the fact that, the present chapter emphasized on the basics, microenvironment, cells and immuno-oncology of colorectal cancer.

11.2 Risk Factors and Development of CRC

The most common modifiers and prophesied risk factors for development of CRC are lack of physical exercise, diet, chain smoking/chewing tobacco, obesity, low intake of plant based foods and calcium, high intake of processed/red meat, and alcohol drinking (Table 11.1). These are forecasted risk factors for 55% CRC cases in the USA. Family/personal history of CRC, hereditary diseases like inflammatory bowel disease, diabetes, and background of ethnic/race are some of the non-modifiable risk factors of CRC (Reedy 1975). Age (55 years and above) is found to be one of the risk factors for CRC, but an increased % of CRC is found in younger than 55 years and above age group is not limiting the age as a risk factor.

The carcinomas in the colon grow slowly (several years or even a decade) without showing any symptoms at the early and middle stages of cancer. The identified symptom at the last stage is blockage of feces and pain, cramping, bleeding, and rarely tarry stools due to occupation and blockage of polyps/cancer tissue in the colon (Lisanne et al. 2016). Polyps are the external growths occur on the inner lining

Table 11.1 Showing the possible risk factors and symptoms and diagnosis of colorectal cancer

S. No.	Possible conditions/factors	References
Risk factors		
1	A family or personal history of having polyps and CRC	Potter (1999), Chang and Ulrich (2003), Pinczowski et al. (1994)
2	Consumption of high amount of processed and red meat	
3	Having Crohn's disease or ulcerative colitis (inflammatory bowel diseases)	
4	Physical inactivity	
5	Type 2 diabetes	
6	Overweight (obesity)	
7	Chain smoking	
8	African–Americans	
9	High alcohol consumption	
10	Low consumption of folate or vegetable foods	
11	Hormone replacement therapy or use of anti-inflammatory non-steroidal drugs	
Symptoms		
1	Abdominal pain and discomfort	American Cancer Society-Facts and Figures (American Cancer Society 2019)
2	Change in bowel habits	
3	Colon bleeding in the stool	
4	Anemia	
5	Constipation and diarrhea	
6	Sudden weight loss, weakness, and fatigue	
Diagnosis of colorectal polyps and cancer		
1	Medical test and physical examination	American Cancer Society-Facts and Figures (American Cancer Society 2019)
2	Blood tests for complete blood count, liver enzymes, and tumor markers	
3	Proctoscopy: suspected CRC conditions can be identified using proctoscopy by inserting it through anus into colon/rectum to identify the exact location of CRC	

(continued)

Table 11.1 (continued)

S. No.	Possible conditions/factors	References
4	Biopsy: laboratory test can be performed for CRC suspected tissues/polyps by testing genetic mutations, microsatellite instability, and any mismatch repairs in DNA	
5	Imaging test: using sound waves and X-rays under applied magnetic field, images can be obtained to know the area of cancer, spreading of cancer, and CRC response to treatment	
6	Sigmoidoscopy: this is a procedure used to examine the rectum and very last part of the colon. This test can detect polyps, cancer, and other abnormalities in the sigmoid colon and rectum. During this exam, a biopsy (tissue sample) may also be removed and sent for testing	
7	Stool DNA: a stool DNA test looks for changes in genes that are sometimes found in colon cancer cells. This test can find some colon cancers before symptoms develop	
8	Colonoscopy: a colonoscopy examines the entire colon and rectum. During this procedure, polyps can be removed and sent for testing	
9	CT colonography: this is a special X-ray test (also referred to as a virtual colonoscopy) done of the entire colon using a CT (computed tomography) scanner. This test takes less time and is less invasive than other tests. However, if a polyp is detected, a standard colonoscopy needs to be performed	
10	Magnetic resonance imaging (MRI): Using radio waves in magnetic field images of tissue can be obtained. Endorectal MRI can help to find and remove CRCs before and after surgery. Metastasis of CRC can also be detected with MRI	
11	Angiography: used to know the metastasis of CRC	

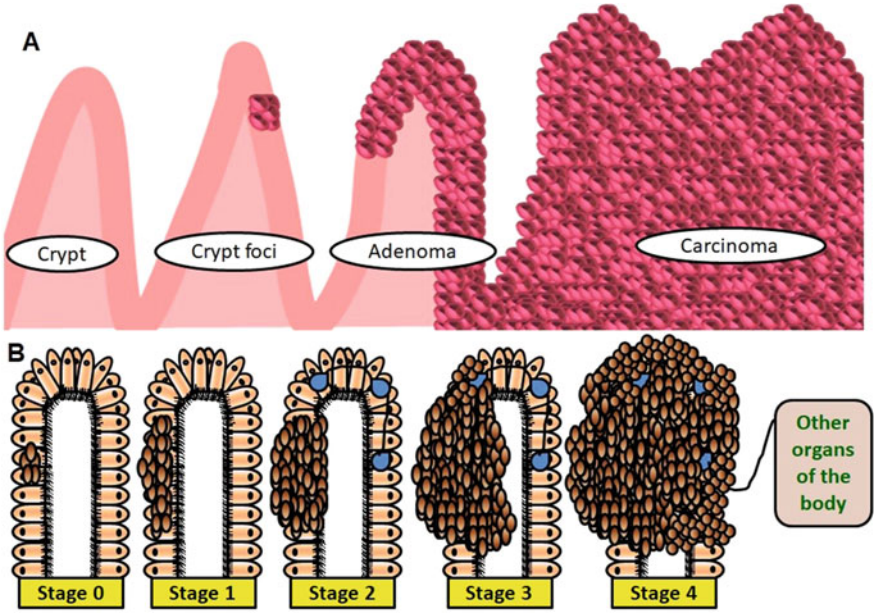

Fig. 11.1 Developmental stages of colorectal carcinoma in colon. (**a**) Transformation of cryptal polyp into colorectal carcinoma. (**b**) Different stages of colorectal cancer and its metastasis to other parts of the body

of the colon and rectum which grows towards intestinal lumen called colorectal tumors (Fig. 11.1a). Two types of polyps are identified in colorectal region. They are adenomas/adenomatous and hyperplasias/inflammatory polyps. Polyps like hyperplastic and inflammatory are generally not pre-cancerous and they do not develop into CRCs, whereas adenomas or adenomatous polyps are pre-cancerous and responsible for CRCs. Enlarge in polyp size along the length of the colon and rectum increases the risk of adenoma to develop CRC (Conteduca et al. 2013). Another pre-cancerous state identified in patients after removal of polyps from the colorectal region is dysplasia, which shows abnormal cells which develop CRC. Dysplasia is common in people suffering with Crohn's disease and/or ulcerative colitis, an inflammatory bowel disease (Fig. 11.2).

The overall risk factors for CRC in general are consumption of high fat diet and low fiber diet, aging, high consumption of alcohol, chain smoking, no physical activity, obesity, CRC history in the family, colon or rectal polyps, irritable bowel disease, and suffering with other cancers. Besides these, consumption of processed foods/meat and having sprouty2 (tumor suppressor) gene are the high risk factors for occurrence of CRC. Men are more prone for CRC than women, even at young stage men can develop the CRC.

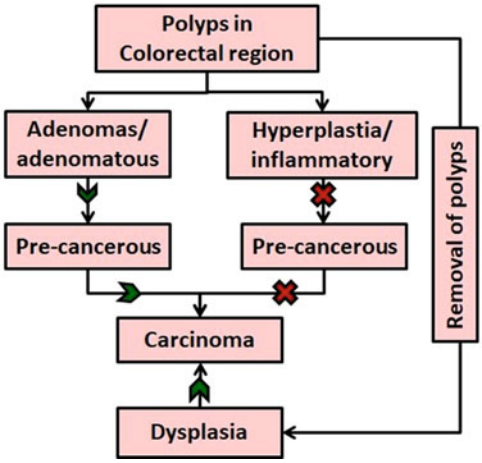

Fig. 11.2 Role of polyps in developing colorectal cancer

11.3 Stages of CRC

The development of CRC is divided into five stages which includes stages 0–4. Each stage of CRC has distinguished with the varied characters of cell mass (Fig. 11.1b). Most of the CRC diagnostic in patients are identified in the stage 4 (metastasis).

11.3.1 "0th" Stage

This stage is named as in situ carcinoma where the tumor cells are developing in the internal layer of rectum/colon and inside the mucosal layer. The cells of this stage are in initial stage of cancer.

11.3.2 "1st" Stage

Cells in this stage come out from the internal layers of the rectum/colon and appear on the outer surface of mucosal layers. Spreading of cancerous cells further than the wall of colon/rectum is not established at this stage.

11.3.3 "2nd" Stage

Cancer cells grow faster and spread towards the lumen of colon/rectum. Cells at this stage are not grown up to nearby lymph nodes.

11.3.4 "3rd" Stage

Nearby lymph nodes are attached to cancer cells and no spreading of cancer cells to other parts of the body is characterized in this stage.

11.3.5 "4th" Stage

Tumor grows much bigger and cancer cells extend to other organs of the body. This stage is also called metastasis. Most affected body parts are lungs, liver, ovaries, and abdominal cavity membrane lining.

11.4 Symptoms of CRC

Symptoms are not specific at the earliest and stage 2 of the CRC. Many symptoms are excelling on the stage 3 and 4 of the CRC. The symptoms are supposed to be observed continuously for not less than 4 weeks to confirm CRC under the doctor's observation. The symptoms are (1) feeling not hungry or heaviness of abdomen even for long time after having food, (2) loss of weight without reason, (3) looks tired or patient feels fatigue, (4) abdominal pain, (5) red/black blood in stools, which comes from rectum, (6) iron deficiency due to continuous loss of blood in stools, (7) constipation/diarrhea, (8) changes in habits of bowel, (9) bowel movement cannot make the bowel empty. The symptoms and diagnosis of CRC is not so easy. However, as per American Cancer Society report (American Cancer Society 2019) some of these are presented in Table 11.1.

11.5 Immunology of CRC Microenvironment

The CRC microenvironment is developed due to oncogenic or inflammatory/infections pathways (Fig. 11.3). A number of cells and events have been taking place in the microenvironment of CRC. The stromal cells are maintained in the CRC microenvironment by transforming gut epithelial cells for their survival, growth, invasion, and metastasis. It is very clear with CRC that these cells are immunogenic and immune response of the host system is a key for survival of patients. The extracellular matrix (ECM), vasculature, tumor-infiltrating cells, and molecules connected to matrix create a microenvironment in colorectal cancer. A variety of cells and agents are identified in the CRC microenvironment which exhibit distinct efficient phenotypes that promote adenocarcinoma. In depth study of cancer

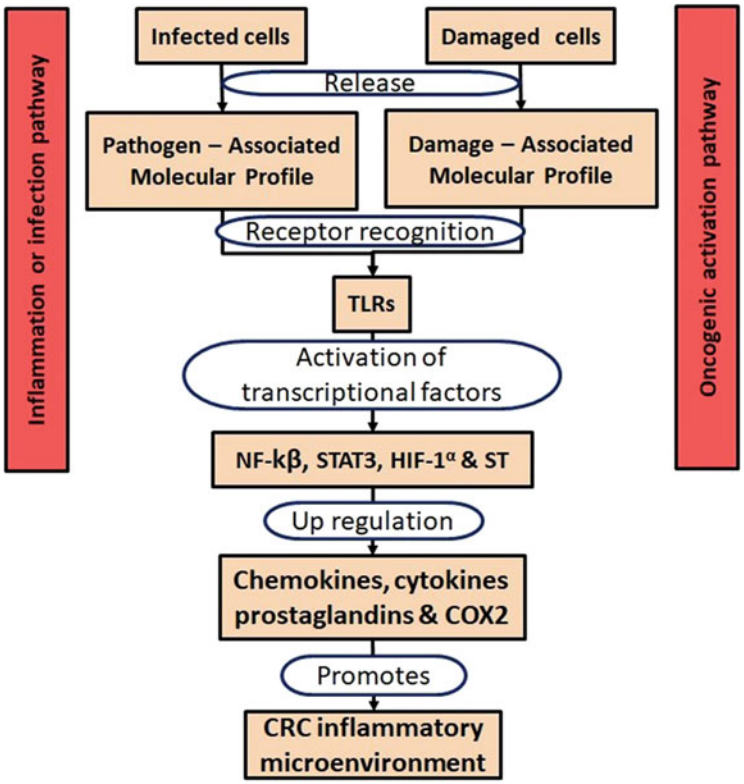

Fig. 11.3 Development of colorectal cancer microenvironment

microenvironment may provide solutions for the development of potential drugs against CRC.

The two main pathways contribute to develop new tumor is extrinsic (inflammation/infection) and intrinsic (oncogenic activation). In this, infected/damaged cells release pathogen/damage-associated molecular profile which recognize TLR receptors for activating the nuclear factor-kβ (NF-kβ), hypoxia-inducible factor-1α (HIF-1α), signal transduce (ST), and its activator of transcription 3 (STAT3). These factors maintain the inflammatory microenvironment by upregulating the gene expression of prostaglandins, chemokines, COX2, and cytokines.

11.5.1 Cells of Immune System

The tumor microenvironment activates the immune system by the involvement of innate immune system, adaptive immune system, and colorectal cancer cells. During CRC the major cells accumulate in the tumor microenvironment are natural killer

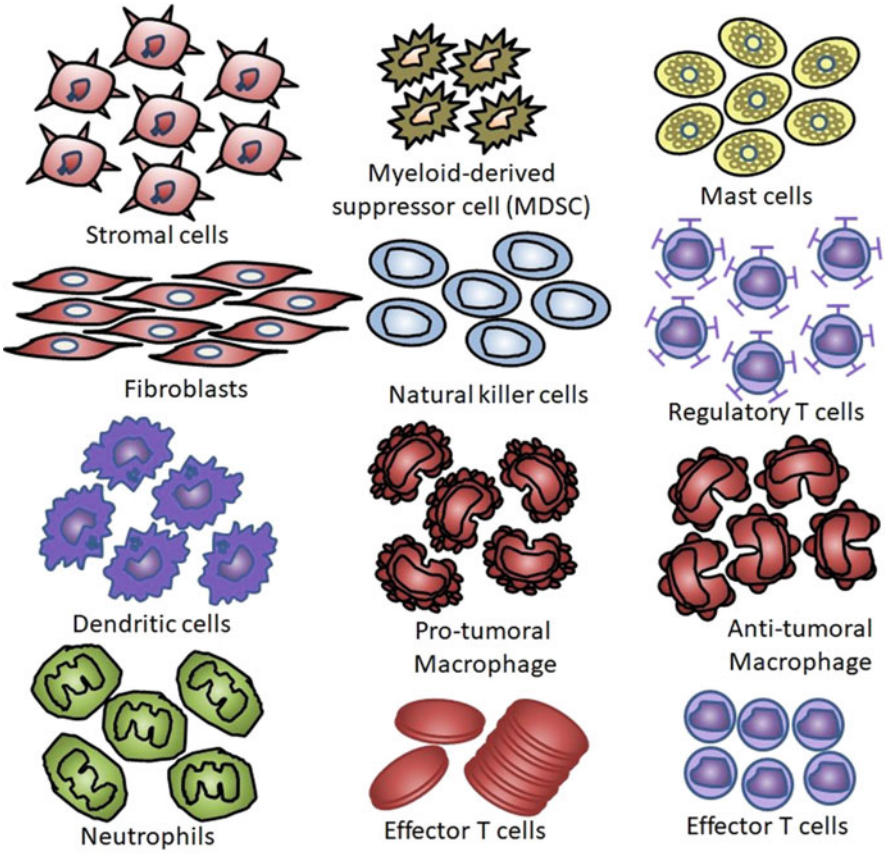

Fig. 11.4 Immune and other cells present in colorectal cancer microenvironment

(NK) cells (Papanikolaou et al. 2004), Macrophages (Algars et al. 2012), Neutrophils (Rao et al. 2012), and CDs (Nagorsen et al. 2007) by the response of innate immune system, whereas adaptive immune system releases T lymphocytes, CD8 cytotoxic, and CD4 helper cells (Koch et al. 2006). These cells can show prometastatic and proangiogenic effects by releasing inflammatory modulators (Coussens and Werb 2002). Vijay et al. (2010) reviewed tumor-infiltrating cells in the CRC microenvironment. The release of a variety of cells in the CRC microenvironment intimately linked to suppression or promotion of the tumor development. Infiltrated cells, such as NK cells, mast cells, myeloid derived suppressor cells (MDSCs), cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), CD4 and CD8 cells, neutrophils, monocytes, dendritic cells (DCs), endothelial progenitor cells (EPCs), endothelial cells, mesenchymal stem cells (MSCs), and platelets are some of them identified in the CRC microenvironment (Fig. 11.4). The detailed account of these cells and its functions facilitates to proceed with immunotherapies.

11.5.1.1 Natural Killer Cells (NKs)

The first line of defense through innate immunity against pathogens is mediated by natural killer cells (NKs). They also called innate lymphocytes holding cytotoxicity, which includes tumor suppression through activated immune function and thereby promots tumor cell apoptosis in CRC (Moriwaki et al. 2009). Besides apoptotic actions NKs are involved in morphogenesis, repair, metabolism, regeneration, and tissue remodeling homeostasis (Paul and Lal 2017). There are different subsets of NKs identified in tissues with diverse homing properties and local maturation (Stabile et al. 2017). NKs are rich in granzyme-containing granules and perforins and show potent in vitro cytotoxicity on cancer cells. This action of NKs is explained with high serum MHC class I molecules which reduce the expression of NKG2D receptor. Doubrovina et al. (2003) demonstrated in vitro and in vivo tumoricidal activity of NK cells bearing NKG2D.

11.5.1.2 Mast Cells

Mast cells can express during cancer, besides allergic and other pathological conditions. Though the higher numbers of mast cells are common in major human cancers, but hypovascularity and better survival of tumor cells are associated with lower numbers of mast cells in CRC condition (Gulubova and Vlaykova 2009; Fisher et al. 1989). In the periphery of developing tumors accretion of mast cells leads to the production of stem cell factor from the cancerous tissue (Huang et al. 2006). Angiogenesis in tumors are triggered by infiltration of mast cell into cancerous tissue during early stage of tumor growth and mast cell independent angiogenesis takes up when tumors grow bigger (Coussens et al. 1999). The release of growth stimulator and proangiogenic factors such as histamine (Dvorak 2005), angiopeptin-1(Nakayama et al. 2004), BFGF (basic fibroblast growth factor; (Lin et al. 2004)), TNF-α (tumor necrosis factor-α; (Kneilling et al. 2009)), heparin (Hallgren et al. 2001), VEGF (vascular endothelial growth factor; (Crivellato et al. 2008), and proteases (Ribatti and Crivellato 2009) in the tumors are mediated by the activated mast cells.

11.5.1.3 Myeloid Derived Suppressor Cells (MDSCs)

The cells that show similar phenotypic characters if granulocytes and macrophages belong to myeloid population with immature features are named as myeloid derived suppressor cells (MDSCs). These cells are identified in the advanced tumor stages of CRC, especially in the peripheral blood and cancer tissues (Zhang et al. 2013). MDSCs produce anti-inflammatory cytokines like prostaglandin E2 and arginase, which are holding strong immune-suppressive functions (Veglia et al. 2018), but are poor prognostics of CRC (Tada et al. 2016).

11.5.1.4 Tumor-Associated T cells and Macrophages (TAT and TAMs)

The tumor-associated T lymphocytes (T cells; Tregs) and macrophages play an important role in the behavior of CRC. The T cells repress cell mediated response of T helper cell type 1 (Th1) and cytotoxic-T cells. The role of T cells in CRC is well explained where these cells accumulate and perform anti-cancer immune response along with Th1 inflammatory response, which in turn resolves the inflammation in colon and rectum (Fridman et al. 2017). In contrast, Tregs immune repression through expressing FOXP3+ CD4+ T cell types promotes inflammation during CRC (Saito et al. 2016).

Based on the phenotype and function, macrophages are divided into two subsets TAM1 and TAM2. The tumor cell death induced molecules like TNF-α, relative oxygen species, and nitric oxide belong to TAM1 type and are raised during pro-inflammatory condition (Mantovani et al. 2004). The immunosuppressive, proangiogenic, and cell growth factors belongs to TAM2 macrophage subtype which releases at chronic inflammatory condition holds pro-tumorigenic function. TAM2 macrophages are majorly called tumor-associated macrophages (Qian and Pollard 2010). However, the elevated phenotypic agility was reported by TAM1 and 2 subtype dichotomization (Aras and Zaidi 2017). The TAMs play a crucial role in tumor growth, immune suppression, angiogenesis, inflammation, tumor invasion/metastasis, epithelial-to-mesenchymal transition (EMT), extracellular matrix, and matrix-associated molecule formation (ECM and MAM) with an association of a number of factors (Fig. 11.5). Nevertheless, more emphasis is needed to use TATs and TAMs as immunotherapeutic agents for CRC treatment.

11.5.1.5 CD4 and CD8 Cells

Immunosuppression, a state of reduced immunogenic cytokines IFN-γ and TNF-α by macrophages or monocytes can initiate the differentiation of $CD4^+$ T lymphocytes into T helper 1 and 2 (Th1 or Th2). Th1 and Th2 are responsible to induce cytokine dependent immune responses. The IFN-γ and TNF-α levels are elevated by Th1 which would initiate cellular immune response through the production and activation of NK cells, cytotoxic $CD8^+$ T lymphocytes, monocytes, and macrophages. IL-10, IL-4, IL-5, and IL-13 another set of cytokines produce by Th2 are involved in the humeral immunity (Rubén and Oscar 2015). Moreover, the tumor-infiltrating lymphocytes (TILs) reduce the response of $CD8^+$ cells and kill the cancer tissue. But the TILs are inhibited by native T cells with a hold of tumor acquiring function (Hsiao et al. 2004). Longer disease free survival time is maintained by T lymphocytes holding a low number of $CD8^+$ where it is correlated positively with neoplastic epithelium (Deschoolmeester et al. 2010). The $Foxp3^+$ $CD8^+$ cells would suppress the secretion of IFN-α and proliferation of T lymphocytes (Chaput et al. 2009).

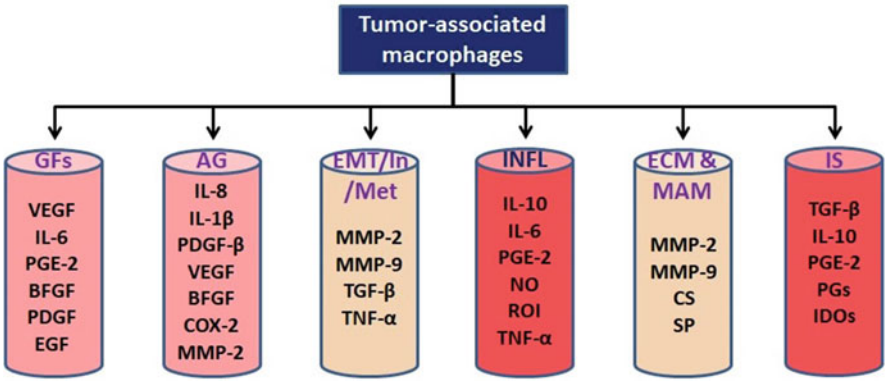

Fig. 11.5 Differential functions of stromal tumor-associated macrophages (TAMs). Various functions of TAMs include (1) cancer progression by releasing growth factors (GFs) such as vascular endothelial growth factor (VEGF), interleukin (IL)-6, prostaglandin (PGE)-2, basic fibroblast growth factor (BFGF), platelet derived growth factor (PDGF), and endothelial growth factor (EGF); (2) angiogenesis (AG) by IL-8, IL-1β, PDGF-β, VEGF, BFGF, cyclooxygenase-2, and matrix metalloprotease (MMP) release; (3) epithelial-to-mesenchymal transition (EMT)/invasion (In)/metastasis (Met) function by MMPs (2 and 9), transforming growth factor (TGF)-β, and tumor necrosis factor (TNF)-α release; (4) release of IL-10, IL-6, PGE-2, nitric oxide (NO), reactive oxygen intermediates (ROI), and TNF-α induces inflammation; (5) formation of extra cellular matrix (ECM) and matrix-associated molecules (MAM) through release of MMPs (2 and 9), cysteine cathepsins (CS), and serine proteases (SP); (6) promotes immunosuppression through TGF-β, IL-10, PGE-2, prostaglandins (PGs), and indoleamine dioxygenase (IDO)

11.5.1.6 Tumor-Associated Neutrophils

The primary innate responsive cells derived from myeloid precursors and belongs to the white blood cell group are predominantly know as neutrophils (Coffelt et al. 2016). The longest life in the CRC microenvironment and the pivotal role of neutrophils in the tumor angiogenesis are becoming popular in recent days (Pillay et al. 2010). The pro-inflammatory factor interferon gamma (INFγ) plays a crucial role in extending longer life span of neutrophil (Akgul et al. 2001) and activated tumor-associated neutrophils function as anti-tumor and pro-tumor (Fridlender et al. 2009). Release of VEGF from tumor cells is stimulated by neutrophils through oncostatin M release (Queen et al. 2005). Moreover neutrophils are identified with inflammatory bowel disease related CRC during oxidative stress associated pathogenesis (Roessner et al. 2008). The anti-cancer action of neutrophils is explained in many studies (Fig. 11.6). Hydrogen peroxide released by the interaction of neutrophils with tumor cells activates $Ca2^{+}$ influx using $Ca2^{+}$ channel (TRPM2) and causes cancer cell death (Gershkovitz et al. 2019). Tumor suppression by nitric oxide mediated by neutrophils through hepatocyte growth factor, Met and its ligands is also reported (Finisguerra et al. 2015). Sun et al. (2018) isolated Fas interaction or Fas ligand mediated cancer suppression of neutrophils from healthy subjects. The collagenase-2, an enzyme expressed during cancer state is released by the action of

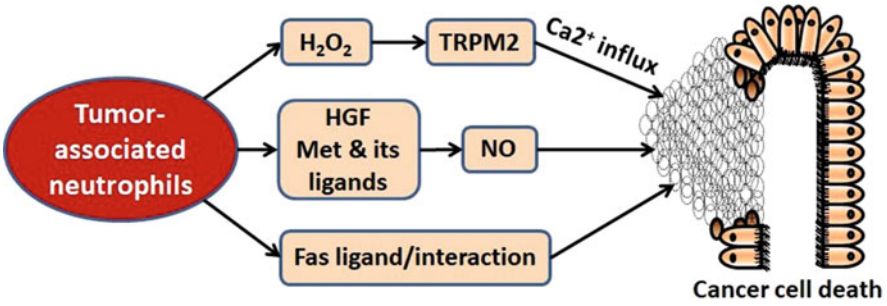

Fig. 11.6 Anti-cancer role of tumor-associated neutrophils

neutrophils. High amount of serum collagenase-2 is an indicator for adverse condition in CRC patients (Bockelman et al. 2018). Furthermore the role of neutrophils is not the same in all types of cancers due to its variation in phenotypic heterogeneity and efficient adaptability.

11.5.1.7 Inflammatory Monocytes

The cells transport from bone marrow to CRC microenvironment shows a typical role in cancer growth, metastasis and resistance to chemotherapy are called monocytes which belongs to myeloid origin. The level of anti-tumor function of inflammatory monocytes always depends on the stage of the tumor and metastasis (Heeren et al. 2015). Initially the counts of peripheral blood monocytes along with differentiation of $CD4^+$ $CD25^+$ regulatory T cells correlated with the immunity level of the CRC patients (Chen et al. 2019). The inflammatory monocytes are shown to have more importance in many cancers, but are still less known in CRC.

11.5.1.8 Dendritic Cells (DCs)

In CRC condition dendritic cells (DCs) show anti-tumor response by induction of immune response and are identified key antigen presenting cells (APCs) (Palucka et al. 2010). During development DCs can exhibit tumerogenic and anti-tumerogenic activities. Steinman et al. (2003) identified the tolerance against T cells in immature CDs where they carry $CD4^+$ and $CD8^+$ self-antigens. Whereas matured and activated CDs are identified with T cell proliferation and its differentiation into the helper and effector lymphocytes (Banchereau et al. 2000). Due to its amphoteric behavior the role of CDs in CRC is contentious and this has been notified in many studies. Low levels of CD83, CD86, CD54, and HLA-DR, and elevated IL-10 besides the suppression of an anti-cancerous IL-12p70 have been reported in the in vitro CRC explants incubation study with DCs (Michielsen et al. 2011). In an another DCs tumor-infiltrating in vitro study, Schwaab et al. (2001) found immature phenotypes

with no tumor-associated antigenic systemic response in correlation with Treg cells. However, the $CD4^+$ and $CD8^+$ polyclonal cell populations are recognized by a range of antigens which includes antigens of cancer, neoantigens and self or viral derived antigens (Gee et al. 2018; Scheper et al. 2019). Taking together the immature CDs can be used as potent immunotherapeutic to improve the survival in the CRC patients.

11.5.1.9 Mesenchymal Stem Cells (MSCs)

The pluripotent nonhemopoietic cells that are produced from bone marrow, umbilical card, muscles, and adipose tissue and differentiate into various types of immune cells are known as mesenchymal stem cells (MSCs) (Yuehua et al. 2002). The human MSCs has receptors of IL-II, TNF-β and γ-interferon. MSCs phenotypes show CD105, CD90, CD29, CD73, CD44, CD34, CD45, CD14, CD31, vWF, leukocyte function-associated antigen-3, and stromal cell antigen-1 (Vijay et al. 2010; Marofi et al. 2017). MSCs are the predominant cells in CRC microenvironment. These cells are responsible for tumor-associated stroma formation in primary tumors. They also possess proangiogenic and immunosuppressive properties, thereby promote tumor growth and metastasis (Kemp et al. 2005). In addition to this, MSCs involved in the production of PDGF, CXCL12 and FGF (proangiogenic factor) from fibroblasts. Cancer growth promoting factors such as endothelial and pericyte-like cells originated from MSCs (Sanz et al. 2008). Moreover, cancer stem cell survival is postulated by MSCs (Ning et al. 2008).

11.5.1.10 Eosinophils

Healthy individuals generally hold eosinophils in the colon mucosa with geographical variations in their number. Eosinophils are identified as anti-CRC immune cells. The amount of eosinophils is low in the early stages and even at invasive carcinomas, whereas highly abundant in adenomas indicates its protective role of eosinophils (Moezzi et al. 2000). But the appearance of these cells in advanced cancer is viewed as a marker than tumor active immune response.

11.5.1.11 Platelets

The platelet components in the blood are important for the restoration and maintenance of endothelial function besides their major function, homeostasis. Early studies of tumor microenvironment suggested that elevated levels of platelets are linked to cancer progression (Verheul and Pinedo 1998). Later studies described the role of platelets in cancer angiogenesis and metastasis (Karpatkin 2003). Activated platelets release α-granules and dense granules (includes proangiogenic factors like CXCL12, VEGF, and PDGF) through thromboxane A2 (Stellos et al. 2009). The

proposed mechanistic action of platelets during tumorigenesis is well explained. At first thrombin production is stimulated by platelets in CRC patients, which activates the development of cancer cells. Platelets can also activate surface molecules of cancer cell membranes either by direct contact or ADP activation (Karpatkin 2003). CRC metastasis spreads by anti-tumor action of the immune system through embryonic cancer cells and circulation clearance of tumor cells is contributed by platelets (Burdick and Konstantopoulos 2004). The studies on CRC in relation to platelets need more focus to establish the clear-cut mechanism, which may help to improve the immunotherapy of CRC.

11.5.1.12 Mesenchymal Stromal Cells (MSCs)

The new set of cells used to identify the crime suspects are mesenchymal stromal cells (MSCs) and are identified in the intestine. In the intestine MSCs are located closely to lymphatic network and blood vessels, nearby to the CRC cells, suggesting the role of these cells in the maintenance of homeostasis and cancer in the intestine. The subsets of MSCs play against pathogens and inflammation by expressing FAP-α^+, ICAM-1^+, α-SMA, $gp38^+$, and $CD90^+$ in the healthy intestine (Owens 2015). MSCs promote carcinogenesis in CRC microenvironment. These cells promote invasion, metastasis, and angiogenesis in the microenvironment of CRC. It is known that the interaction of MSCs plays a key role in function and proliferation of immune cells, such as macrophages, T-lymphocytes, DCs, and natural killer cells which induce tumorigenesis and facilitate tumors to escape from suppression by the immune system. Moreover, the innate and adaptive immunity of cells are influenced by MSCs and its secretary factors (Malley et al. 2016). The interaction of MSCs with cancer cells and immune cells during CRC development and in its microenvironment provides a better understanding to invade the efficacious therapy for CRC.

11.5.2 Endothelial Progenitor Cells

Endothelial cells are specifically lined on the inner side of colon and rectum. Though, these cells are not directly involved in the CRC development but are the mediators for several reactions takes place in the colon/rectum during the development of different CRC stages. Tumor endothelial cells are holding angiopoietin receptors (TIE-2) which act as dominant tumor development factors (Lewis et al. 2007). Colon tumor cells can adhere to the walls of microvascular endothelia in presence of reactive oxygen species promoted by N-nitrosamines of activated human neutrophils (Ten Kate et al. 2007). The CD34, CD31, and vWF are the identified markers of endothelial cells during CRC (Kemp et al. 2005). Endothelial type of MSCs are responsible for cancer progression (Sanz et al. 2008). Moreover, endothelial cells mediate the betaig-h3 extravasation during metastatic transport of tumor cells through Src ($\alpha\gamma\beta5$) signaling pathway (Ma et al. 2008). Platelets mediated

neoangiogenesis in CRC is done through endothelial progenitor cells. MDSCs can promote expression of endothelial markers (CD31 and VEGFR2) during early or in immature cells (Yang et al. 2004) and enter into the cancer tissue endothelia. However, the light on effective role of endothelial cells in CRC progression is still in its infancy and open for researchers to continue in this direction.

11.5.3 Cancer-Associated Fibroblasts (CAFs)

The special cells identified in the CRC microenvironment are cancer-associated fibroblasts (CAFs) crucial molecules for regulation of immunogenicity. The CAF is the main source of immunomodulatory molecules, including TGF-β and initiates TGF-β anti-tumor pathway in innate and adaptive immune cells through a "+"ve feedback mechanism (Hawinkels et al. 2014). Besides this, CAF in association with extracellular matrix proteins constitute a substantial link for direct contact between stroma and CRC cells (Vangangelt et al. 2018). The angiogenic factors secreted by both tumor and the stroma cells interact with respective receptors on endothelial cells, activating tumor-associated angiogenesis (Michele et al. 2017; Yasuhiko 2010).

11.6 Immune Response in CRC

The gut is rich with immune cells due to its continuous exposure to a large variety of antigens and microbial flora, which includes many pathogens and toxicants of different origin. Immunosuppression in tumors is associated with immune response against cancer growth. At first, in cancerous cells immune system responds with elevated neoantigens through the antigen processing pathway, where the produced proteins are converted into peptides by the action of immunoproteasomes (Yewdell et al. 2003). These peptides through transporter associated antigen processing proteins (TAP) enter into the endoplasmic reticulum (ER) and subsequently onto human leukocyte antigen class I (HLA class I; (Neefjes et al. 1993)) holding chaperones such as calreticulin, calnexin, and ER-glycoprotein 57 as associated proteins. The chaperone and TAP dissociate after stabilization of HLA class I—peptide complex which reaches to the cell surface through Golgi complex (Neefjes et al. 2011) and are recognized by $CD8^+$ T cells (Kurts et al. 2010). Furthermore, the attachment of neoantigens and its intermediates to T cell surface is highly determined by its level of affinity towards HLA class I alleles (Garstka et al. 2015).

The immune response against tumor growth of TLRs is notable in the CRC microenvironment. They may show pro- or anti-cancerous effects depends on the conditions in the CRC microenvironment. TGF-β is another molecule shows gut homeostasis in its presence and interrupted signaling or mutation in its gene acts as pro-cancerous.

11.7 Immune Suppression in CRC

Mutations/genetic aberrations of genes responsible to produce antigenic processing pathway would lead to immune suppression in CRC cells. More than 10 mutations/megabase of DNA do not restrict the neoantigens to recognize the T cells in CRC patients. Anyhow the CRCs are strong to evade immune recognition. However, the presence or absence of MMR in CRCs decides the elevation of immune suppression. CRCs with no MMR usually alter the antigen processing pathway thereby complete inhibition of HLA class I expression and immune suppression (Ijsselsteijn et al. 2019). Significant CRC immune suppression is not only restricted to loss of HLA class I gene (B2M) expression, but also other components of antigen processing pathway (Dierssen et al. 2007). The tumor cells execute an extraordinary mechanism to escape from immune action of the cells, even from the self-antigens raised against them. The amount of neoantigens at the time of colorectal tumor removal is low which is an abnormal prediction with no immune selection (Rooney et al. 2015).

11.8 Conclusion

The microenvironment of normal cells is not the same as CRC microenvironment. It is different and a complexed stromal system. CRC microenvironment holds several stromal cells of different phenotypes which promotes both growth and suppression of tumor cell growth. A majority of stromal and its associated cells show anti-tumor activity or tumor suppression function but are not attracted the attention of scientists to develop therapeutics. The cells like TAM2, neutrophils, and CAFs support the survival, growth, and metastasis of CRC. The therapeutics are always raised against the cells involved in the tumor progression. The immuno-oncology of CRC represents by neutrophils, TAMs, COX-2 inhibitors, ECM, matrix-associated molecules, NSAIDS, MDSCs, CAFs, MCs and components of cells like prostaglandins etc., are facilitates the extravagances in the development of therapeutics thereby CRC control worldwide.

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Conflicts of Interest Authors declare no conflict of interest.

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Chapter 12
Immune Targets in Colorectal Cancer

Begum Dariya and Ganji Purnachandra Nagaraju

Abstract Colorectal cancer (CRC) is a multifactorial malignancy, with highest mortality rate amid of the cancer-related deaths in the USA and worldwide. It ranks as the third most recorded cancer irrespective of gender. CRC is that tumor type with high mutation prevalence caused due to mismatch repair (MMR) gene developing MSI and MSS along with high antigenic potentiality. It is impacted by numerous factors like genetic, environment, and inflammation, is always determined to be a dreadful malignancy if detected in its late stages. The other factors that determine the progression and therapy of cancer is the tumor microenvironment (TME). It is critically suggested that the immune system in TME plays a pivotal role in promoting tumor progression. Therefore, understanding the immune cells and their signaling pathways enables the advancement of immune based therapies for better prognosis. For instance, immune checkpoint inhibitors like anti-programmed cell death protein-1 (antiPD-1), anti-programmed cell death ligand protein-1 (anti-PD-L1), and anti-cytotoxic T lymphocyte associated protein 4 (anti-CTLA4) with other growth factor inhibitors or chemodrugs are found effective in treating MSI CRC to inhibit tumor progression. In this article we focused on the immune cells, its pathway, TME of CRC, and immune targeted therapies.

Keywords CRC · TME · MSI · MSS · Immune checkpoint · CTLA4 · PD-1 · PD-L1

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Abbreviations

ACT	Adoptive cell transfer therapy
APC	Antigen presenting cells
B-hCG	Beta-human chorionic gonadotropin
CEA	Carcinoembryonic antigen
CMS	Consensus molecular subtypes
CRC	Colorectal cancer
CTLA-4	Cytotoxic T lymphocyte associated protein 4
DAC	DNMTi 5-aza-2'deoxycytidine
DNMTi	DNA methyltransferase inhibitor
FoxP3	Forkhead box P3
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
LAG3	Lymphocyte activation gene 3
mAbs	Monoclonal antibodies
MDSCs	Myeloid derived suppressor cells
MHC	Major histocompatibility cells
MMP	Matrix metalloproteinases
MMR	Mismatch repair
MMRD	Mismatch repair deficient
MMRP	Mismatch repair proficient
MSI	Microsatellite instability
MSS	Microsatellite stability
NK	Natural killer cells
PD-1	Programmed cell death protein-1
PD-L1	Programmed cell death ligand protein-1
TCR	T cell receptor
Teff	Effector T cells
TGF-β	Transcription growth factor beta
TH	T helper cells
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-alpha
Tregs	Regulator T cells

12.1 Introduction

Colorectal cancer is the heterogenous disease that recorded as third most common cancer diagnosed worldwide in both men and women (Siegel et al. 2020). It is a multifactorial disease with high mortality rate. The risk factors including immune system of the host, microbiota in the gut, and altered risk factors like alcohol consumption and unhealthy lifestyle. This results into a sequence of pathological

conditions that ultimately alters the healthy colon epithelium into an invasive carcinoma (Mármol et al. 2017; Sun and Kato 2016). The immune system is the host defense system on the other side plays crucial role in protecting the body against disease conditions like cancer. Moreover, the tumor immunogenicity is developed due to the elevated release of neoantigens as a result of mutations (Schumacher and Schreiber 2015). As an immune response, the chronic inflammation of the body persuades dysplasia in the epithelial cells of intestine that further initiates CRC progression (Lucas et al. 2017). The somatic mutation induces tumorigenesis causing inactivation of DNA mismatch repair (MMR) develops sporadic and familial microsatellite instability (MSI) in CRC (Galon et al. 2006). This increased the tumor infiltrating lymphocytes in CRC. Tumor necrosis factor (TNF-α) is a pro-inflammatory cytokine that plays crucial role in immune response initiation (Luo and Zhang 2017). Moreover, the tumor microenvironment (TME) that constitutes natural killer cells (NK) detects stress associated molecules and dendritic cells (DC). They activate pre-existing cytotoxic immune cells called T lymphocytes that play crucial part in sensing the tumor associated antigens via their receptors called T cell receptors (TCR) (Jobin et al. 2017). They are involved in tumor regression via attacking CRC cells. The immune response is also supported by other co-receptors like $CD4^+$ and $CD8^+$ (Löfroos et al. 2017). The NK cells together with T cells are found to possess anti-tumor properties via producing enzymes like perforin and granzymes that is further followed by the apoptosis of the cancer cells (Banerjea et al. 2004; Phillips et al. 2004). Moreover, previous research studies showed that lower activity of NK cells results in poor prognosis (Jobin et al. 2017). The T helper cells (Th) support this immune response and promote the production of cytotoxic T lymphocytes. They also help in secreting cytokines like IFN-γ (Sun et al. 2002). This further promotes the production of more NK cells. Similarly, tumor associated macrophages also confer with poor prognosis. However, the heterogenous nature of the tumor prevents them from being recognized by the immune cells due to presence of certain cells like PD-L1. Additionally, the tumor cells also alter the immune cells and to function as immunosuppressive cells. For instance, the tumor associated macrophages are the circulating monocytes initially, later differentiated into macrophages and contribute to angiogenesis and metastasis in CRC under oxidative stress conditions (Grivennikov et al. 2010; Qian et al. 2011).

The immunity strategies developed for cancer focus on restoring the immune system to activate the anti-tumor immunity via generating T cell responses that distinguish and eliminate tumor cells. However, the tumor cells behave trickily with the host immune system by camouflaging themselves as normal cells. Thus, the immune therapy acts to shred away the camouflage to distinguish the tumor cells and kill them. Advancements in understanding about the interaction between tumor and immune system potentiated the therapeutic strategies to boost up the natural defense system against tumorigenesis. The DNA mismatch repair-deficient (MMRD) causing microsatellite instability high (MSI-H) result in positive CRC are found to respond to immunotherapy (Hemminki et al. 1994). This is due to the presence of tumor infiltrating lymphocytes, tumor neoantigens, and immune checkpoints. The therapeutics are further potentiated to improve the efficacy with the revitalizations of

targeted immunotherapy. These therapies include T cell therapy and immune checkpoint blockers that are antibody based. These blockers include anti-PD-1/PD-L1 (programmed cell death 1) and anti-CTLA-4 (cytotoxic T lymphocyte associated protein 4).

12.2 Understanding Immune System

Understanding immune system and its surveillance would potentiate the use of immune cells to inhibit the cancer cell progression. There are advanced therapeutic strategies to activate immune response. The immune system of the host through innate or adaptive is capable of differentiating and eliminating the tumor cells in their early stages of tumorigenesis. The innate immunity is pre-existing and the first line of defense system. It includes immune cells—myeloid derived suppressor cells (MDSCs), neutrophils, macrophages, NK, DC, and mast cells (Hanahan and Weinberg 2011). The adaptive immune cells have memory and can recall before exposed to any stimuli. T and B lymphocytes are the adaptive immune cells (Goldszmid et al. 2014). As determined, both these innate and adaptive immune cells either interact directly with TME or indirectly with the help of signaling cascade of cytokine and chemokine that alters the behavior of tumor cells as per the therapy. The innate immune cells respond to the inflammatory signals generated by the diseased tissue that further activates adaptive immunity via the cascade of inflammations (Goldszmid et al. 2014). This produces the antigen presentation by macrophages and DC on to the T cells. Whereas in case of tumor, the immune cells distinguish the tumor specific antigens present on the cancer cell surface with the healthy cells. Later the NK cells kill the cancer cells that lack MHC-I on their surface that further recruit inflammatory cells via the production of cytokines (Purdy and Campbell 2009). The macrophages and DC phagocyte the tumor cells and present tumor related antigens on the surface of tumor cells (Munn and Cheung 1990). This activates the T cells and directs against tumor cells. As an immune response, the effector T cells divide and infiltrate through the tumor to eliminate it from the body (Van Pel and Boon 1982). However, cancer cells, the cleverest follow few selection mechanisms and have the ability to camouflage the immune system.

12.3 Tumor Immune Microenvironment

The tumor microenvironment (TME) of a CRC patient always affects the progression and metastasis of the tumor. It contains extracellular matrix that constitutes collagen fibers, lymphatic vessels, fibroblast, nerves, and hematopoietic cells (Fridman et al. 2012; Kobayashi et al. 2019). The adaptive and innate immune cells present in the TME interact with the cancer cells directly or through the signaling factors including cytokines and chemokines. They alter the behavior of

tumor cells and retort against the therapy. The immune cells found act variedly as per the host cells and tumor cells factors. The immune cells behave both as anti-tumor and pro-tumor basing on the context. For instance, DC release cytotoxic cytokines like IL-2, TNF-α and IFN-γ and present antigens to T cell during the attack of any pathogen. But, under abnormal conditions it inhibits T cell function and promotes tumor survival and progression. The T cells ($CD8^+$ and $CD4^+$) in general kill the tumor cells and release the cytotoxic cytokines like IFN-γ; however, as a pro-tumorigenic it secretes tumor promoting cytokines like IL-10, IL-13, and IL-4. The Tregs are found to restore homeostasis in order to reduce the chronic inflammation but it inhibits anti-tumor immune response via inducing inflammatory cytokine secretion. The MDSCs are found limited in the microenvironment, yet they are involved in inhibiting T cell activity and recruit immunosuppressive immune cells (Wang et al. 2014). The macrophages and NK cells are cytotoxic to tumor cells, release cytotoxic cytokines, and produce antigen presenting cells (APCs) to T cells. The macrophages however, act abnormally and promote tumor proliferation, angiogenesis, and metastasis. The necrotic cell death other than phagocytosis generates signals for proinflammation in the local tissue for the employment of immune cells (Hanahan and Weinberg 2011). These inflammatory signals comprise high mobility group box-1 and IL-1 that induce angiogenesis and contribute to survival of tumor cells (Grivennikov et al. 2010). Additionally, the activation of cytokines via the immune cells also activates transcription factors like STAT3 and NF-κB that promote growth and survival (Grivennikov et al. 2010). The immune cells in the TME function by interacting tumor cell with the surrounding stroma. This invades the peripheral cells through the activation of macrophages that secretes enzymes like metalloproteinases (MMP) (Coussens et al. 2000) and cysteine cathepsin proteases (Joyce et al. 2004), that later causes metastasis (Grivennikov et al. 2010; Hanahan and Weinberg 2011). The colitis associated CRC and colon cancer produce IL-6 as the inflammatory response was found to activate STAT3 that further promotes tumorigenesis. Further reports suggested that the MMP-9 transcript levels higher in tumor tissues than in the non-tumor tissues in CRC patients. Thus, the presence of MMP-9 in higher levels determines the metastatic nature in CRC (Zeng et al. 1996). The immunocytes affect the progression and evolution of tumor cells (Joyce and Fearon 2015; Spill et al. 2016). The impairments for the success of anti-tumor immunity are due to reduced immunogenicity and potentiating microenvironment with protein factors promote angiogenesis and remodeling of matrix. Additionally, the TME has many immunosuppressive influences. They include increased level of suppressive cytokines, highly expressed Tregs, MDSCs, decreased expression of MHC molecules/ antigens, increased PD-L1 expression by the tumor, and increased levels of checkpoint proteins by the T cells.

Basing on the TME colorectal tumor can be differentiated into different types. For instance, highly infiltrated, medium infiltrated, and low infiltrated by lymphocytes (Dolcetti et al. 1999). The CRC patients with highly infiltration via lymphocytes are with microsatellite instability, low level infiltration is with varied fibroblast, lymphatic and endothelial cells (Spranger et al. 2015; Luke et al. 2019). Whereas, the medium level infiltration is with high density of fibroblast and endothelial cells.

Table 12.1 Molecular classification of CRC

CMS	% of CRC	Mutations	Consequence	TME	T cell inhibited by	Ref
CMS1	14%	Hypermutated MSI, BRAF, and CpG island methylator phenotype	Inhibition of MLHI, MMR gene transcription		PD1, PD-L1, CTLA-4, LAG3	Herman et al. (1998))
CMS2	37%	Canonical, APC	Activation of WNT and MYC	Low number of lymphocytes, endothelial cells, macrophages, and fibroblastic cells		Guinney et al. (2015))
CMS3	13%	Metabolic, KRAS		Decreased levels of immune cell infiltration		Becht et al. (2016b))
CMS4	23%	Mesenchymal	↑ EMT, TGFB, angiogenesis (VEGFA, VEGFB, VEGFC)		PD1, LAG3, CTLA-4, PD-L1	Guinney et al. (2015))

They resulted with high metastatic potentiality and poor prognosis of patient (Becht et al. 2016a). In CRC the tumor infiltration is heavily carried by the macrophages and subsequently by T and B cells (Schumacher and Schreiber 2015).

The deficient MMR or Microsatellite instability-heavy (MSI-H) contributes 15% of CRC cases but encounters for only 4% of mCRC. Whereas for MMRP and MSI-L encounters 85% of CRC cases (Fleisher et al. 2000). The TME for these deficient and MMRP differs that contributes to variation in the immune response and therapy (Mlecnik et al. 2016; Ogino et al. 2009). The high mutation effect on MMRD-MSI-H CRC showed increased neoantigens on MHC-1 molecules expressed on cancer cells thus, promoting T cells to detect them as distant cells. Additionally, the TME of colorectal tumor is classified based on the transcriptome into four consensus molecular subtypes (CMS) (Willett et al. 2012; Roepman et al. 2014; Budinska et al. 2013; Schlicker et al. 2012; Sadanandam et al. 2013; Marisa et al. 2013; Felipe De Sousa et al. 2013; Villamil et al. 2012; Guinney et al. 2015). Among the four CMS, CMS1 and CMS4 are regulated by the immune cells and are found overexpressed in samples that have high proportion of stromal tissue (Alderdice et al. 2018). The classification is illustrated in Table 12.1.

12.4 The T Cell Classification

The mature T cells are the role players, Fig. 12.1 explains the process of T cell maturation. The mature T cells have co-receptors $CD4^+$ and $CD8^+$. They are classified into three different types of T cells such as naïve, effector (Teff), and

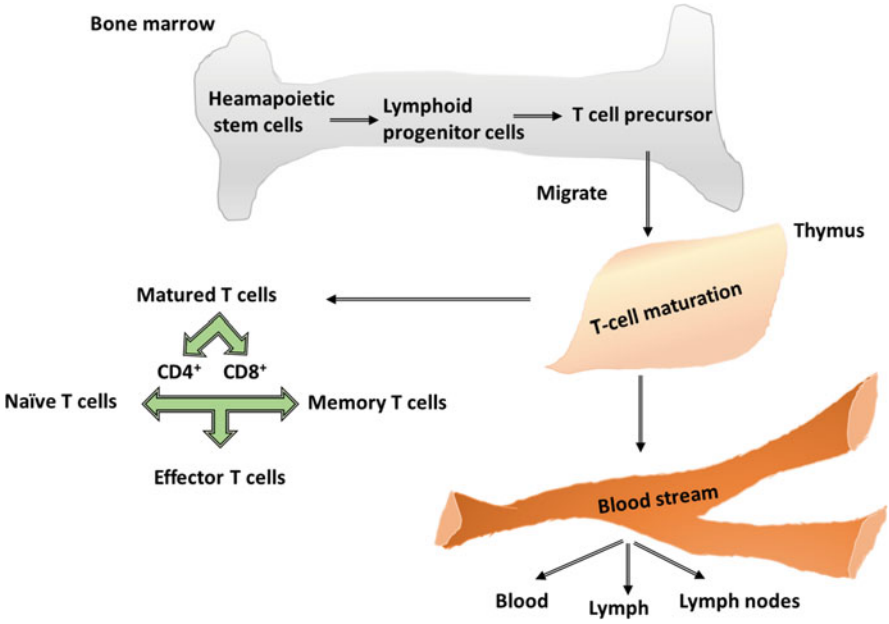

Fig. 12.1 Maturation of T cells and its differentiation. T cell precursors produced from the bone marrow migrate into the thymus where the T cells maturate and are then transferred into the blood streams. The mature T cells are of three types: Naïve T cells, effector T cells, and memory T cells. They have 2 co-receptors: CD4+ and CD8+

memory cells. The naïve cells are the T cells that are not yet encountered by any APCs. The active T cells undergo differentiation and proliferation to develop several T cells called Teff cells. The Teff cells are capable of mediating the immune function. They effectively promote immunotherapies via destroying tumor, mediate its activation, and inhibit the immunosuppressive activity present in the TME. The Teff cells are subdivided into TH cells and CTL. The TH cells assist the activation of other cells in developing immune response. They also regulate antibody production of B cells. These further have subsets including TH1, TH12, TH17, and Tregs. The immune cells Teff (effector T cells) and Tregs (regulatory T cells) act differently toward the progression of tumor cells. The function and classification of T cells are tabulated in Table 12.2. The Treg cells are critical effector cells that maintain homeostasis of immune response and play essential role in averting ailments like autoimmune disease (Brunkow et al. 2001; Bennett et al. 2001). They are categorized as the subgroup of $CD4^+$ T cells that express forkhead box P3 (FoxP3) transcription factor and IL2R α chain (CD25) as the surface molecule along with T cell receptor and CD4 co-receptor. CD25 is otherwise called as IL-2RA and is a receptor for IL-2. IL-2 is the prime cytokine that efficiently potentiates T and B lymphocyte proliferation. FoxP3 is maintained in high level by Tregs. It is a cell lineage marker of Treg and its deletion in the germline would develop improper

Table 12.2 Classification of T cells

T cells	Activation	Types	Subsets	Function
Naïve T cell	Lymphocytes not encountered by any antigens	Naïve $CD4^+$ T cells and Naïve $CD8^+$		Not encountered by antigen
Effector T cells	Lymphocytes capable of mediating immune function/ Short lived	Effector $CD4^+$ T cells→→→ Effector $CD8^+$ T cells→→→	Helper T cells (TH cells)— TH1, TH12, TH17, Tregs Cytotoxic T cells/ CTLs	Respond to APCs and activate T cells differentiation and proliferation
Memory T cells	Long lived antigen specific lymphocytes, responsible for immunological memory			Protects if the same pathogen invades Rapidly generates more T cells and memory cells

Tregs that enhance autoimmune disorders. However, if FoxP3 is overexpressed it gives rise to increased Tregs and promotes expression of IL-10. The identity of Treg is maintained by the signals from TCR, IL2, and TGF-β that promotes the expression of FoxP3. Tregs show different immunoregulatory mechanisms but also have anti-inflammatory function. It interacts with macrophages and prevents pro-inflammatory cytokines secretion including IL1 and IL6, thus prevents the production of $CD4^+$ T cells. Treg also directly competes with $CD4^+$ T cell to bind with IL-2 that is involved in production of T cells and Tregs. However, IL-2 are hardly secreted by Tregs as the FoxP3 alters the transcription factors that are responsible for the secretion of IL-2. But, CD25 that is highly expressed on Tregs competitively binds with IL-2 and inhibits proliferation of T cells. Similarly, Tregs also secrete cytokines like IL-35, TGF-β, and IL-10 that inhibit $CD4^+$ T cells to control down the inflammation. IL10 is an immune suppressive cytokine that downregulates $CD4^+$ cells. Treg often persuades neighboring immune cells including DCs to secrete IL-10 (Whitehead et al. 2012). IL-10 controls the self-activation of DCs and activates CD4+ T cells and CD8+ Teff cells in vitro and in vivo (Ouyang and O'Garra 2019). However, IL-10 activation dependent signaling cascade may not be a proposed Tregs mechanism for immunosuppression. Furthermore, there are certain enzymes secreted by Tregs including granzyme B that causes apoptosis of T cells (Perrella et al. 2014). Additionally, in contact with DCs Tregs also secrete indoleamine 2,3-dioxygenase (IDO) that disrupts T cell function (Munn and Mellor 2013; Jiang et al. 2015). It was found that presence of highly expressed CD25 also constitutes to suppress T cell proliferation.

The high levels of infiltrations of Tregs in various cancer like CRC, head, bladder, and neck cancer are found with better prognosis (Fridman et al. 2012; Saito et al. 2016). However, in certain cancers like gastric, hepatocellular, pancreatic, renal, breast, melanoma, cervical, and non-small cell lung cancer are found with poor prognosis with increased Tregs number (Sasada et al. 2003; Curiel et al. 2004; Bates et al. 2006; Shang et al. 2015). Basing on the functionality, Tregs would be classified

into subtypes effector Tregs (eTregs) and chemokine receptor (CCR4) (Miyara et al. 2009; Sugiyama et al. 2013). The eTregs are highly immunosuppressive and express CD45RA-FoxP3^{++} phenotype. CRC patients showed high tumor infiltration with high subpopulation of CD45RA-FoxP3^{++} and are reported with poor prognosis (Saito et al. 2016). However, in few cases of CRC, low levels of CD45RA-FoxP3^{++} reported with better prognosis. Additionally, these low levels and non-Tregs also secreted pro-inflammatory cytokines such as IL-17 and IFN-γ (Saito et al. 2016; Miyara et al. 2009). Later, high levels of FoxP3^{+}IL-17^{+}CD4^{+} Tregs were detected in microenvironment of colitis with ulcerative colitis related colon cancer. These FoxP3^{+}IL-17^{+} Tregs inhibit T cell proliferation and promote inflammation via inflammatory cytokine stimulation with the release of IL-2 and IFN-γ in the tissue of colitis (Kryczek et al. 2011). The higher levels of Tregs in sporadic colon cancer are also associated with poor prognosis. Thus, Tregs were believed to promote tumorigenesis that more efficiently suppresses the local inflammatory process (Haas et al. 2009).

Tregs also show higher expression of PD-1 and CTLA-4. The blocking of CTLA-4 and PD-1 would deactivate Treg. However, its property of maintaining immune homeostasis, explains the reason for blocking PD-1 and CTLA4 that may develop immune associated inflammation (Francisco et al. 2009; Walker 2013). CTLA4 and PD-1 are the inhibitory checkpoints and known target for immunotherapies in cancer. These immune checkpoints play a crucial role in blocking the activation of T cells, Treg, and other inhibitory cytokines and immunosuppressive cells.

12.5 The Cancer Immunotherapy

12.5.1 Immunosurveillance

Immune system of the body involves in distinguishing the cancer cells (non-self) from the healthy cell (self). This process aims at protecting from tumor development and is called as immunosurveillance. The immunosurveillance is the process where the host immune cells efficiently patrol for the cancerous or abnormal cells, recognize them, and eliminate before they harm the healthy cells (Teng et al. 2008). The immune cells recognize the antigen present on the tumor surface. They can either be oncogenes or tumor suppressor genes or can be viral antigens. These antigens are presented as peptides by the MHC-1 express on the surface of tumor cells. The second phase is the elimination phase. In this phase the cancer cells are incorporated into APCs that are specifically for exposing or presenting tumor antigens as peptides by MHC-II. The APCs further activate TH cells that stimulate B cells for antibody production. Additionally, the TH cells also stimulate the expression of macrophages that engulf the tumor cells and eliminate them. Similarly, the cytotoxic T cells directly bind with the tumor cells and devastate them. Occasionally, the tumor cells escape the immune system as they secrete few mediators to inhibit APCs and T cells. Additionally, they produce mutated or modified tumor antigen on their

surface being non-recognizable by the immune system. This develops into increased proliferation of tumor cells and the immune cells reach the stage called immune tolerance. NK cells are the widely acted immune cells in immunosurveillance that promotes cytotoxicity in tumor cells that have MHC-I on their surface and are highly prone to be attacked by them (Zamai et al. 2007). Similarly, NK cells also develop cytotoxicity in the cancer cells via producing granules that contain granzyme B and perforin (Halama et al. 2011). Additionally, $CD8^+$ T cells also kill cancer cells via promoting cytotoxicity in the tumor cells produced by the activated cytokines like IFN-γ (Pardoll 2002). $CD4^+$ TH1 and TH17 also promote CTL function to produce cytokines including IL-4 and IFN-γ (Munegowda et al. 2011; Gerrard et al. 1981). Thus, these anti-tumor immune cells can be taken as prognostic biomarkers as targets for better outcome in the immunotherapy. Considering the tumor samples of CRC patients with stages ranging from II and IV are found with higher cytotoxic $CD8^+$ ($CD69^+$ and $CD107a^+$) tumor infiltrating lymphocytes (Markman and Shiao 2015). Higher the cytotoxic $CD8^+$ cells, higher will be the tumor antigen-reactive T cells in the bone marrow and blood. Thus, they are inversely corelated, wherein the earlier stage of cancer showed higher proportion of active $CD8^+$ tumor infiltration lymphocytes. This suggest that the initial stages of CRC can be easily detected and endure surveillance by the immune system.

12.5.2 Immunoediting

Immunoediting is a process that selects tumor cells with reduced immunogenicity and maintains the immune response through varied mechanisms in those tumor cells. The communal relationship between the host immune system and TME is differentiated into 3 phases. They include elimination phase, equilibrium, and escape phase. As the initial step, the elimination phase includes cancer immunosurveillance of the host cells that is followed as a two-signal phase which is already discussed earlier. The first signal includes presenting of the tumor antigen on the T cell receptor via MHC and consequently followed by the activation of T lymphocytes. Both the immune response adaptive and innate immunity are activated in the host immune system that efficiently prevents tumor. The equilibrium phase is the extended phase, the immune cells even though active in TME are not capable to destroy tumor cells but maintain the tumor cells in a dormancy state (Dunn et al. 2004). The immune cells, though they are unable to eliminate the tumor cells but prevents metastasis to occur, maintaining tumor cells in a static phase. The tumor cells make use of various biochemical pathways to inhibit the immune response, to reach a state of immune tolerance. Thus, in this stage, the T lymphocytes lose their functionality of suppressing tumor. The dormant stage remains active until the escape phase initiates. In the escape phase the tumor cells are determined to be highly active. The heterogeneity nature of the cancer cells potentiated by various signaling cascade to defend themselves from the activity of immune effector cells. Thus, this phase is highly advantageous for the cancer cells that tolerate the immune response of the host and

suppress it via various physiological pathways. The active tumor cells later bind to the co-inhibitory molecules present on the T cells. For instance, CTLA-4, PD1, T cell immunoglobulin mucin 3, and lymphocyte activation gene 3 (LAG3). Additionally, they also activate the inhibitory co-receptor, PDL-1 that secrete enzymes like IDO which contribute to the secretion of anti-inflammatory IL-10 and TGF-β in TME (Mahoney et al. 2015; Das et al. 2017; Postow et al. 2015). Thus, TGF-β, an immunosuppressive factor secreted by the tumor cells prevents NK cells and CTLs from eliminating them. Secondly, Tregs and MDSCs recruited by the tumor cells camouflage them from the lymphocyte induced apoptosis (Hanahan and Weinberg 2011). Tregs function by inhibiting proliferation, expression of cytokines, and activation of T cells like $CD8^+$ and $CD4^+$ cells. Moreover, intra-tumoral Tregs in the increased number are associated with tumor progression and deprived prognosis (de Leeuw et al. 2012). Additionally, it was detected that CRC patients showed increased percentage of MDSCs in the peripheral blood that promoted metastasis. The in vitro studies also revealed that the MDSCs extracted from the diseased CRC patients were able to inhibit T cell proliferation (Zhang et al. 2013).

Thus, the co-inhibitory molecules otherwise called immune checkpoints play a pivotal role in obstructing immune response of the host. Researchers are now converging to understand the mechanism to restore the immune response via targeting drugs against this altered immune checkpoint to disrupt the immunosuppression signaling against tumor.

12.6 Immunotargets

12.6.1 Immune Checkpoints

The hypermutation in the CRC results in deficient MMR system resulting in the formation of MSI-H that are vigorously expressed on check proteins including CTLA4, PD-1, and PD-L1. Thus, this supports the escape of tumor from being detected by the immune system by acting against MSI-H TME and preventing the exclusion of neoplastic cells. The current immunotherapeutic strategies are aiming to potentiate the activation of effectors of T cells via altering the immune response (Topalian et al. 2016). The targets focused mainly for immunotherapeutic strategies are CTLA4, PD1, and its ligand PD-L1.

12.6.2 CTLA4

CTLA4 is a membrane glycoprotein receptor present on the active T cell surface. It resembles CD28 and thus competes with it, to bind with the common natural ligands of B7 family. The B7 family ligands include CD80 and CD86 that are present on the surface of APCs. As the first step, with the antigenic stimulation at the T cell

receptor, the T cell activates and expresses CTLA4 on its surface that binds with B7 more efficiently than CD28. The interaction of CD28-B7 stimulates the cytotoxic immunity, whereas the interaction of CTLA4 with B7 suppresses T response and promotes immune tolerance (Pardoll 2012). CTLA4 expression is found normal on T cell activation; however, with Tregs, CTLA4 overexpresses due to increased levels of FoxP3 on Tregs that regulate the expression of CTLA4 (Pardoll 2012; Perkins et al. 1996). In case of tumor patients CTLA4 is found highly expressed in both Teff and Tregs (Plitas et al. 2016). The effect of CTLA4 activates the intrinsic signaling pathway of T cells and was found to inhibit production of IL-2 and proliferation of T cell. Furthermore, it cross-talks with other pathways including PI3K, MAPK, and NF-κB to regulate cell survival and proliferation of cells (Intlekofer and Thompson 2013; Chikuma et al. 2005; Schneider et al. 2009; Fraser et al. 1999; Bhandaru and Rotte 2019). The cancer therapy involved in developing anti-CTLA4 antibodies as CTLA4 blockade was tested in murine tumor models (Leach et al. 1996).

12.6.3 PD-1 and PD-L1

The programmed cell death-1/PD-1 (CD279) are the co-receptors expressed on the surface of tumor infiltrating lymphocytes, NK cells, T ($CD8^+$ and $CD4^+$) and B lymphocytes (Postow et al. 2015). PD-1 shows almost 21–33% of similarity with CTLA-4, but PD-1 is a monomer and CTLA4 is a dimeric protein (Rotte 2019). PD-1 has deficiency of extracellular cysteine residue necessary for covalent dimerization and exists as monomer, unlike CTLA4. The presence of PD-1 on the cell surface of T and B cells activates T cell and B cell receptor. PD-1 plays a pivotal role in maintaining the inflammatory response and tumor immunity that alters the functionality of T cells that travel toward TME. PD-L1 (B7-H1) and PD-L2 (B7-DC) are the two ligands for PD-1 receptors. PD-L2 are mostly expressed on DC and macrophages (Francisco et al. 2009; Latchman et al. 2001), whereas PD-L1 are also expressed on organs cells, T, B cells, NK cells, and tumor cells (Topalian et al. 2016; Naboush et al. 2017). The interaction of PD-1 with PD-L1 inhibits T cell proliferation, secretion of cytokines like TNF-α, IFN-γ, and IL-2, and cytotoxic nature of the immune cells. It also avoids the onset of autoimmune diseases by maintaining the immune homeostasis (Kim and Eder 2014). Moreover, during the T cell activation the PD-1 receptor was restricted to bind with PD-L1 and allowed CD80 to bind with PD-L1 (Sugiura et al. 2019). The pathway of PD-1/PD-L1 plays a crucial role in evading tumor cells from the immunosurveillance. PD-1 expressed on the T cells in TME that lost the effector function and PD-L1 expressed on APCs or tumor cells. The interaction of PD-1 and PD-L1 adapts the mechanism of adaptive immune resistance or adaptive suppression and inhibits the infiltration of T cells in TME (Topalian et al. 2016). Thus, PD-L1 is associated with poor prognosis in varied type of cancers. The blockade of this pathway promotes the anti-tumor immune response via recurrence of cytotoxic T cells and is determined to be the successful therapeutic strategy till date.

The current advances in immunotherapies progressed in developing therapeutic strategies including cancer vaccines, adoptive cell transfer therapy (ACT), and antibody-based cancer immunotherapy to treat CRC.

12.6.4 Monoclonal Antibody-Based Cancer Immunotherapy (mAb)

The monoclonal antibodies (mAbs) are found clinically effective since decades (Weiner et al. 2012) (Table 12.3). mAbs like bevacizumab (anti-VEGF mAb) and cetuximab, (anti-EGFR mAb) are approved clinically for CRC therapy in the USA. They focus on targeting vital signaling pathways and promote innate immune effector process. They distinguish Fc portion of Ab through Fc receptor and persuade Ab dependent cytotoxicity via cellular mechanisms (Jiang et al. 2011). mAbs are also called as checkpoint inhibitors, block the CTLA4, PD-1, and PD-L1 that came out with positive result in many cancers. The cancer therapy is thus focusing on developing anti-CTLA4, anti-PD-1, anti-PD-L1 antibodies as to block the activation of CTLA4, PD-1, and PD-L1, respectively.

The current anti-CTLA4 blockade developed are Ipilimumab and tremelimumab against humans. They are used to restore Teff effect to potentiate tumor cytotoxicity. Ipilimumab was approved by FDA for the therapy against metastatic melanoma with no resection history. This is also used as an adjuvant therapy for melanoma with high risk (Rotte et al. 2018; Ascierto et al. 2017; Di Giacomo et al. 2015; Eggermont et al. 2016, 2019; Robert et al. 2011). Ipilimumab showed increased overall survival (OS) rate; however, 20–30% of the patients showed sever autoimmune disease (Topalian et al. 2015). Tremelimumab is a human anti-CTLA4 IgG2 monoclonal antibody (mAb). The phase II clinical trial for tremelimumab is conducted as a single arm multicenter administered intravenously for every 90 days in metastatic CRC patients after the standard chemotherapeutic therapy failure (Chung et al. 2010) (Table 12.3). The median OS was detected to be 19.1 months and median progression-free survival was about 2.3 months. However, this drug was not encouraged for future research against mCRC. Poon et al. (2017) demonstrated the combination activity of MEK inhibitor and anti-CTLA4 in CT26 preclinical tumor model. They used selumetinib as a MEK inhibitor, combining with anti-CTLA4 negatively controlled the upregulation of immunosuppressive mediators including Cox-2 and Arg1 present in TME. This combination decreased the frequency of $CD11^+$ $Ly6G^+$ myeloid cells as well as accumulated monocytes at $Ly6C^+$ MHC^+ tumor state. Anti-CTLA4 increases the T cell proliferation, activation, and improves the infiltration of T cells into the TME that is found more efficient with MEK inhibition. The mutation in Kras gene dysregulated the pathway RAS-RAF-MEK-ERK in various cancers promoting cell proliferation. Thus, targeting MEK with its inhibitors found benefit to the patients when combined with checkpoint blockade like anti-CTLA4. Similarly, there is combination of chemodrug with targeted

Table 12.3 Ongoing clinical trials for immune vaccines and immune checkpoint inhibitors

S. no	Research hypothesis	Condition	Intervention/treatment	Phase	Identifier
1	Combination of chemotherapy with immunotherapy (anti-PD-L1+anti-CTLA4) acting synergistically in CRC patients	mCRC	Durvalumab (Anti-PD-L1), Tremelimumab (Anti-CTLA4) and FOLFOX	Phase I Phase II	NCT03202758
2	Treating CRC patient with immune drugs with liver metastasis that can be removed by surgery.	mCRC in the liver	Durvalumab (Anti-PD-L1), Tremelimumab (Anti-CTLA4). Procedure: Therapeutic conventional surgery	Phase I	NCT02754856
3	The MSS mCRC to liver treated with mAbs	mCRC in liver Gene mutation: MLH1, MSH6, PMS2 CRC:Stage IV, IVA, IVB	Durvalumab (Anti-PD-L1), Tremelimumab (Anti-CTLA4)	Phase I	NCT03005002
4	Multicenter randomized Phase II study to compare the effectiveness and tolerance of avelumab vs standard 2nd line treatment chemotherapy in mCRC with MSI	mCRC, MSI	FOLFOX, FOLFIRI, avelumab (Anti-PD-L1), panitumumab, cetuximab, bevacizumab, aflibercept	Phase 2	NCT03186326
5	Atezolizumab with stereotactic ablative radiotherapy in metastatic tumors (SABR-PD-L1)	CRC, renal cell carcinoma, non-small lung cancer	Atezolizumab (Anti-PD-L1)	Phase II	NCT02992912
	Study of immunodrug against chemotherapy resistant, MSI-like, CRC	MSI, CRC, chemotherapy resistance, APC	Atezolizumab (Anti-PD-L1), bevacizumab	Phase II	NCT02982694
	Immunotherapy in locally advanced rectal cancer (AVANA)	CRC	Avelumab (Anti-PD-L1), capecitabine Radiation: External—Beam irradiation	Phase II	NCT03854799
	Study of cabozantinib in combination with atezolizumab to subjects with locally advanced or metastatic solid tumor	CRC, gastric cancer, hepatocellular carcinoma, ovarian cancer, renal cell carcinoma, lower esophageal cancer	Cabozantinib, atezolizumab (Anti-PD-L1)	Phase I Phase II	NCT03170960

6	Personalized neoantigen cancer vaccine	CRC, non-small cell lung cancer, gastroesophageal adenocarcinoma, urothelial carcinoma	GRT-C901, GRT-R902, nivolumab (Anti-PD-1), ipilimumab (Anti-CTLA4)	Phase I Phase II	NCT03639714
7	Personalized cancer vaccine targeting shared neoantigens	CRC, pancreatic cancer, shared neoantigen-positive solid tumors, non-small cell lung cancer	GRT-C903, GRT-R904, nivolumab (Anti-PD-1), ipilimumab (Anti-CTLA4)	Phase I, Phase II	NCT03953235
8	Study of XmAb®20717 against selected advanced solid tumors	CRC, renal cell carcinoma, melanoma, endometrial cancer, non-small cell lung carcinoma, breast carcinoma, and hepatocellular carcinoma	XmAb®20717	Phase I	NCT03517488
	A study of PI3K inhibition (copanlisib) and anti-PD-1 in refractory solid tumor with expansion in MMR proficient CRC	Unresectable, metastatic MSS solid tumor along with MSS colon cancer	Copanlisib (PI3K inhibitor) Nivolumab (Anti-PD-1)	Phase I Phase II	NCT03711058

Source: www.clinicaltrials.gov

therapy as a standard first line therapy for mCRC. The phase Ib/II study (NCT03202758) was performed for detecting the efficacy of drug combined FOLFOX (5-FU, Leucovorin, and oxaliplatin) with inhibitors of PD-L1 and CTLA4. A research study on a combo drug: durvalumab and tremelimumab is tested for mCRC associated with MSI. Durvalumab is a human mAb found to inhibit binding of PD-L1 with PD-1 and tremelimumab is a CTA4 inhibitor (Fumet et al. 2018). Recently, the phase II study of this combo drug resulted in prolonged overall survival (Chen et al. 2020). Ipilimumab is another drug used in combination with nivolumab against CRC and metastatic renal cell carcinoma with miss match repair and heavy-MSI as CTLA4 and PD-1 blockades (Perez-Ruiz et al. 2019). Thus, these clinically feasible strategies can be used as immune checkpoint blockades, however further clinical studies are still warranted.

The US FDA approved the monoclonal PD-1 antibodies and monoclonal PD-L2 antibodies. The anti-PD1 antibodies include pembrolizumab, cemiplimab, and nivolumab. Similarly, the anti-PD-L1 monoclonal antibodies include atezolizumab, durvalumab, and avelumab (Bhandaru and Rotte 2017; Giaccone et al. 2018; Garon et al. 2015). The gastrointestinal cancer cases experienced pseudoprogression as they are treated with nivolumab and pembrolizumab as anti-PD1 and anti-PDL1 for checkpoint inhibitory therapy (Michalarea et al. 2019). Nivolumab is the PD-1 blocker used against CRC with MSI-H and MMR. Similarly, a multicenter phase Ib, open label study was conducted to compare the overall respond rate for Arm A that includes combination of atezolizumab and bevacizumab with Arm B having combination of bevacizumab and FOLFOX for mCRC patients with MSS to block PD-L1. The Arm B showed better overall response rate that Arm A (Bendell et al. 2015) (Table 12.3). Thus, combination of drugs encourages the clinical activity and also improved survival rate in mCRC patients.

12.6.5 Cancer Vaccine

The cancer vaccine elicits anti-tumor immune response successfully. The main concept for vaccination develops from the immune cells to recognize the reformed self-antigen called as tumor associated antigens present on the tumor cells. Thus, this eventually elicits the immune response against the tumor to eliminate it and continue with the immunosurveillance and avoiding the regrowth. Vaccination agents can be grouped into 4 types: peptide antigens, viral/bacterial vaccination, whole tumor, or dendritic cell. Few are explained here.

12.6.6 Whole Tumor Vaccine

These are the most primitive vaccine as the material for vaccination including the known and unknown tumor associated antigen is readily obtainable. The vaccine

preparation initiates with irradiation of tumor tissue sample, later mixed with immune adjuvant like alum and finally reinjected into CRC patient (Blankenstein et al. 2012). For instance, the autologous whole cancer vaccine is used for several cancers including CRC, renal, and melanoma cancer that induce cytotoxic anti-tumor immune response (Shang et al. 2015; Miyara et al. 2009; Sugiyama et al. 2013). Along with the advantages, it also has limitations as the majority of vaccine having tumor antigens is diluted with normal cells. The Eastern Cooperative Oncology Group performed randomized phase III clinical trial for CRC patients, to compare the disease-free survival in surgically resected patient given autologous whole tumor cell and BCG vaccine with the resection alone. The study however showed no significant result (Hanna Jr et al. 2001). More recently, a neoantigen-based EpiGVAX vaccine was developed against mCRC combined with DNA methyltransferase inhibitor (DNMTi), 5-aza-2'-deoxycytidine (DAC). The DNMTi improved the efficacy of GVAX via inducing antigen specific anti-tumor T cell responses to epigenetically regulated proteins. mCRC have very less neoantigens, therefore DNMTi via the epigenetic therapy induces cancer testis antigen expression and also sensitizes the cancer cells to immunotherapy (Kim et al. 2020). However, further research for using irradiation or chemodrugs would efficiently produce whole tumor vaccine with better anti-tumor immune response.

12.6.7 Peptide Vaccines

The peptide vaccines include fragments or whole protein extracted from the tumor specific protein and is administered together with an adjuvant. The peptides employed for designing vaccine include MHC I recognized by the $CD8^+$ cytotoxic T cells. In CRC, there are varied tumor associated antigens encouraged for vaccine development. They include carcinoembryonic antigen (CEA) (Bilusic et al. 2014), survivin-B/p53 (Idenoue et al. 2005; Speetjens et al. 2009), mucin-1 (Kimura et al. 2013), β-human chorionic gonadotropin (β-hCG) (Moulton et al. 2002), and squamous cell carcinoma antigen recognized by T cells (SART3) (Miyagi et al. 2001). These tumor associated antigens are taken as immunotherapy targets in various cancers like CRC that induce antigen specific immune response. For instance, the β-hCG vaccine induced anti-βhCG antibody production in CRC patients resulted in better overall survival rate (Moulton et al. 2002). Yasuhiro et al. (Shimizu et al. 2019) used heat shock protein 105 (HSP105) as a peptide vaccine for CRC and esophageal cancer as they are overexpressed in CRC patients. HSP105 vaccine was found to induce peptide specific cytotoxic T lymphocytes and cytokine secretion. They also suggested that this vaccine induced immune response as well resulted in better prognosis. More recently, novel oral vaccine was developed with long tumor peptides combined with toll like receptor 2 ligand Pam_2Cys. They are formulated with liposomes with or without emulsions. The novel vaccine increased the activation of T, B cells, and $CD11c^+$ $F4/80^+CD11b^+$ when compared with the control vaccine and is associated with decrease in tumor size (Naciute et al. 2020).

Furthermore, the personalized peptide vaccine have also emerged that could be a promising therapeutic strategy (Parizadeh et al. 2019). However, further clinical trial is necessitated for the benefit of patient.

12.6.8 Adoptive Cell Transfer Therapy (ACT)

Adoptive cell transfer therapy is the novel emerging therapy model for CRC. The process includes collection of cytotoxic T cells from the patient's tumor cell, peripheral blood of lymph. They are then infused into the blood stream of the patient as to distinguish the tumor cell and kill it to attain sustained immune response (Rosenberg and Restifo 2015; Ruella and Kalos 2014). For instance, the NK cells extracted from the umbilical cord of a mouse model resulted positively against BRAF and RAS mutation related malignancy when administered. They also gave positive outcome with cetuximab resistant tumor cells (Veluchamy et al. 2016, 2017). In case of human patients, the administration of IL-2 or IL-15 with incubated NK-cell transplants showed positive results with mCRC and mutated EGFR. The chimeric antigen receptors (CAR) T cell immunotherapy is in preclinical phase that is tested in mCRC mouse model. It includes the engineering of T cells to express more immune stimulator ligands that are designed as lipid nanoparticles encapsulated with IL-2, IL-7, or IL-15 receptor (Yeku and Brentjens 2016; Shum et al. 2018). Thus, they enhance the killing of tumor cells by selectively binding to tumor cells. Similarly, CEA is taken as a biomarker that is targeted by CAR administered in mCRC (Parkhurst et al. 2011; Katz et al. 2015; Zhang et al. 2017). The mCRC patients treated with CAR T cell infusion showed decrease in the size of tumor (Katz et al. 2015). It was suggested as a successful therapy to treat B cell malignancy (Maude et al. 2014; Kochenderfer et al. 2015) as well advantageous for CRC but undetermined (Johnson and June 2017; Newick et al. 2017) (Table 12.3).

12.7 Conclusion

Apart from other therapeutic strategies for CRC, immunotherapy assists as a pioneering step toward novel rational therapeutic option and lays platform for the combinational therapy. For instance, the combination of nivolumab and ipilimumab found efficient with positive results and increased survival in MSI-heavy mCRC patients. Furthermore, discovering novel predictive biomarkers could be the extreme therapeutic option in clinical settings. A thorough understanding about the genetical features and mechanisms related to MMR is very much essential to detect the immune targets. The traditional chemotherapeutic strategies are found effective but with adverse side effects and the patient turn back with recurrence cancer. The immunotherapy is found better with increased survival rate of patient. Other than this the personalized immunotherapy and combinational immunotherapy are found

to be the promising avenues with better survival rate targeting the immune checkpoints. Additionally, combining cytotoxic immune therapies with radiation and chemotherapy are highly advantageous. The clinical findings till now provided are with possible way for CRC therapy. Future preclinical and clinical trials approved by FDA for drugs essential for immune targeted therapies for the benefit of the patient are needed.

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Chapter 13
Applications of Computational Biology in Gastrointestinal Malignancies

Manoj Kumar Gupta and Ramakrishna Vadde

Abstract Gastrointestinal cancers (GICs) are the most common cancers of the digestive tract system in humans. Earlier several techniques have been utilized to understand the molecular mechanism and identification of the key gene or protein–protein interaction that is responsible for causing GICs. Nevertheless, detecting key genes and protein–protein interaction through experimental equipment necessitates huge capital and time. Recently developed computational methods provide a distinct way to address such problems in a short interval of time with less cost. Thus, in the present chapter authors attempted to understand how computational approaches may help us in detecting key genes and protein associated with GICs. Information obtained revealed that several studies have employed computational methods to identify key hub genes, including *COL4A1* and *SERPINH1,* transcription factors (e.g., MYC and MAZ), and miRNAs (e.g., *miRNA-133b* and *miRNA-99a*) that play a key role in the gastric cancer development. Computational studies have also detected key hub genes (e.g., *AMBP* and *APOB*) and miRNAs (e.g., *miRNA-7* and *miRNA-141*) that play a key role in the development of colorectal cancer. However, all these studies performed analysis on the bulk cell level, which in turn provides less information about gene expression at the cellular level, which might be the reason for ineffective treatment and low survival of GICs patients. Thus, there is an urgent requirement to understand gene expression in GICs at the cellular level. In the near future, the information present in the present chapter will be highly valuable for cancer biologists and immunologists toward the treatment of GICs.

Keywords Gastric cancer · Computational approach · Key genes · Drugs

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13.1 Introduction

Cancer is clinically characterized by malignant tumors. The tumor is an abnormal proliferation of cells and may be either "benign" (remains confined to its original location) or "malignant" (invades nearby healthy tissue and spreads throughout the body by lymphatic or circulatory systems ("metastasis")). As tumors can be formed via any cell type, numerous types of cancer differ significantly in their behavior as well as response to treatment (Cooper 2000). Cancer is one of the foremost death cause globally, with 14 million new cases and ~eight million deaths per year worldwide. Though it is well established that residents of developed countries are more prone to cancer, in 2008, more than 70% of cancer death and more than 60% of new cases were reported from developing countries (Shams and Haug 2017). Recently, the incidence of cancer has increased dramatically. In 2019, 606,880 cancer deaths and 1,762,450 new cancer cases were reported to occur in the United States alone (Siegel et al. 2019). In men, bronchus, prostate, colorectal, and lungs cancer account for ~42% of all cancer cases. In women, colorectal, lung, and breast cancer account for ~50% of all cancer cases. However, in women, breast cancer solely constitutes ~30% of all new cases. Additionally, the incident of cancer in men is higher than women, which might be due to differences in endogenous hormones and exposure to numerous biotic and abiotic factors, for instance, cigarette smoking (Siegel et al. 2019). Out of all forms of cancer, gastrointestinal cancers (GICs) are the most common cancers of the digestive tract system in both men as well as women globally. Colorectal cancer, esophageal cancer, gallbladder carcinoma, gastric cancer, hepatocellular carcinoma, and pancreatic cancer are the most common cancer of GICs. Earlier studies have reported that GICs alone count for 30% of the total cancer cases (Ge et al. 2018). Though profound progress has been made toward cancer diagnosis as well as treatment, still the outcome of GICs treatment is unsatisfactory. This might be due to resistance against drugs and lack of information about the complete mechanism associated with pathogenesis, cell differentiation, and origin of the disease (Wu et al. 2012). Therefore, continuous research and effective methods are urgently required for the treatment of GICs patients.

With continuous research suggesting interaction amongst genes, as well as proteins, play a significant role in molecular cancer mechanisms, it highly necessary for introducing computational approaches in cancer research (Gupta et al. 2019a, b; Vemula et al. 2019). Additionally, screening genes and its associated variants responsible for causing various diseases by laboratory approaches take both huge investment and time. However, high screening via a computational methodology saves both money and time (Gupta et al. 2017, 2019c, d; Donde et al. 2019; Gupta and Vadde 2019a; Gouda et al. 2020). Re-analysis of genetic information present in International Consortia like the "International HapMap project," "1000 genomes project," "Simons Genome Diversity Project," and "Exome Aggregation Consortium" (ExAC) utilizing more modern statistical tools will enable us to screen genes along with its variants associated with various diseases or traits. Three-dimension structure for both synthetic drugs and phytochemicals present in the publically

available databases, for instance, TIPdb database (Lin et al. 2013), can be also utilized for identifying novel drug or phytochemicals against gene responsible for causing any disease or trait. These ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) and "Lipinski's rule of 5s" passed natural/synthetic drug molecules will have less or no side effect (Gola et al. 2006; Lagorce et al. 2017) and hence, in future, these phytochemicals/drugs may function as good contestants for the treatment of various human diseases, after further laboratory investigation.

Earlier, Tang and the team employed computational approaches to understand the complex network of known 84 T2D genes based on protein–protein interactions as well as localization. Obtained results revealed that amongst 84 genes, 14 genes (*AKT2, UBC, IRS1, IGF2BP2, HNF4A, IRS2, HNF1A, PPARG, HMGA1, MAPK8IP1, HNF1B, NEUROD1, TCF7L2,* and *GCK*) play a vital role in the T2D complex network (Tang et al. 2016). Recently, Latek and team employed computational approach for understanding glucose homeostasis disturbance and reported that drugs with least binding energy are more capable of stimulating GIPR as well as GLP1R and/or inhibiting GCGR, that in turn enhance insulin secretion and reduce hepatic glucose production, thereby controlling T2D (Latek et al. 2019). Bharti and team confirmed anti-diabetic property of *Withania coagulans* fruit via both in-vivo as well as *in silico* approaches (Bharti et al. 2015). Kaur and the team also identified anti-diabetic property of Theaflavin-3,3′-di-O-gallate and rutin via computational methods (Kaur et al. 2018). Menakha and the team identified the anti-diabetic property of phytochemical quercetin (obtained from *Ipomoea sepiaria*) via computational approaches (Menakha et al. 2018). In 2011, Sawey and the team performed genomic analysis of human cellular carcinoma and reported that an oncogene, namely, *FGF19*, is co-amplified with *CCND1* in human tumors. They also reported that *FGF19* inhibition via RNAi restricts clonal growth as well as tumorigenicity of human HCC cells harboring the "*FGF19/CCND1"* amplicon (Sawey et al. 2011).

Thus, computational investigation of varied data produced from high-throughput sequencing technologies, for instance, RNA sequencing, provides a unique "ontology-based solution for querying distributed databases over service-oriented, model-driven infrastructures by integrating pathology," clinical molecular, and radiology data effectively (González-Beltrán et al. 2012). Besides saving time and money, computational approaches also hasten the process of drug discovery. Considering all this important information, recently, our laboratory has also employed computational approach toward predicting the three-dimensional structure of the "γ-secretase activating protein" (GSAP), an Alzheimer's disease therapeutic target, through comparative modeling approaches and studied its structure as well as function via simulation studies. Docking studies of GSAP with 4153 phytochemicals identified GSAP is having a better binding affinity with "monachosorin B," "(*E*)-1-[2,4-dihydroxy-3-(3-methylbut-2-enyl) phenyl]-3-(2,2-dimethyl-8-hydroxy-2*H*-benzopyran-6-yl)prop-2-en-1-one," and "macaflavanone C" in comparison to "imatinib" (the standard drugs). Subsequently, the molecular dynamics analysis revealed that only two phytochemicals, namely, "macaflavanone C" and "(*E*)-1-[2,4-dihydroxy-3-(3-methylbut-2-enyl)phenyl]-3-(2,2-dimethyl-8-hydroxy-2*H*-benzopyran-6-yl)prop-2-en-1-one)" significantly disrupt the original property of

GSAP; thereby supporting that these two phytochemicals may be utilized in future for curing Alzheimer's disease (Gupta and Vadde 2019b). Thus, in this chapter authors attempted to understand how computational approaches have revolutionized the cancer research, especially GICs. In the near future, the information in the present review will be highly utilized in the GICs treatment.

13.2 Methods for Detecting Cancer

Till date, numerous researcher has employed various computational approaches to detect key gene and associated factors that are responsible for causing GICs. However, in comparison to other GICs, very few computational studies have been performed in the context of gallbladder carcinoma. These factors along with key genes may serve as an important target during drug development against GICs.

13.2.1 Identification of Key Genes and Protein Responsible for Causing GICs

A most important aim in public health research is the identification and development of the best pharmacotherapies for treating disease. The generation and availability of publicly available high-throughput genomic and proteomic datasets provide us with a unique opportunity to scan key candidate gene(s) which can serve as a therapeutic target toward the treatment of any diseases with less cost in short interval of time (Ferguson et al. 2018). To date, numerous approaches have been developed toward in silico drug design and development to examine how drugs interact with key candidate genes and how they modulate the molecular process toward the prevention or treatment of diseases (Table 13.1).

13.2.1.1 Colorectal Cancer

Recently, several independent computational studies have identified six (*Wnt, TGF-β, PI3K, MAPK, RAS,* and *p53*) (Falzone et al. 2018), ten (*ALB, AMBP, F2, APOB, APOH, PLG, APOA1, SERPINC1, AHSG,* and *APOC3*) (Zhang et al. 2019), and six (*COL1A1, TIMP1, CXCL5, SPP1, GNG4,* and *LPAR1*) (Yang et al. 2018) key genes that play vital role in colorectal cancer development. These key genes are mainly involved in significant pathways, namely, extracellular matrix organization, G protein-coupled receptors signaling pathway, and gastrin-CREB signaling pathway through *PKC* and *MAPK* (Yang et al. 2018). Another computational study identified three (*DYNC1H1*, *GRM1*, and *GRIN2A*), four (*IGF1R*, *DSP*, *SPTA1*, and *CPS1*), and three (*GSK3B*, *EIF2B5*, and *GGT1*) key genes that are associated with

Table 13.1 GICs associated genes and miRNAs identified through computational approaches

Cancer		Genes/miRNAs	References
Colorectal cancer	Genes	*AHSG, ALB, AMBP, APOA1, APOB, APOC3, APOH, COL1A1, CPS1, CXCL5, DSP, DYNC1H1, EIF2B5, F2, GGT1, GNG4, GRIN2A, GRM1, GSK3B, IGF1R, LPAR1, MAPK, p53, PI3K, PLG, RAS, SERPINC1, SPP1, SPTA1, TGF-β, TIMP1, Wnt*	Falzone et al. (2018), Zhang et al. (2019), and Yang et al. (2018)
	miRNAs	*miRNA-128, miRNA-4777, miRNA-141, miRNA-143, miRNA-14, miRNA-182, miRNA-183-5p, miRNA-200a, miRNA-21-5p, miRNA-4638, miRNA-497-5p, miRNA-6501, miRNA-6510, miRNA-659, miRNA-675, miRNA-7, miRNA-195-5p, miRNA-200c, miRNA-885, miRNA-200b, miRNA-19b-3p*	Falzone et al. (2018), Zhang et al. (2019), Jiang et al. (2017), Ma et al. (2018), Su et al. (2019), and Chen et al. (2019)
Gastric cancer	Genes	*ACTA2, ADCY7, ADCY9, ADHFE1, AHR, AKR1C1, BGN, BMP2, BRMS1, CALML5, CCNB1, CCNB2, CDKN3, CEP55, COL1A1, COL1A2, COL3A1, COL4A1, COL4A2, COL6A3, CTNNB1, CYP1A1, EGR1,JUN, ERBB2, ERPINH1, FGFR4, FN1, FOS, FOSL1, FYN, GIF, GNAS, GNG7, GPER, GRB2, GSTP1, HSPA4, IGF2, ITCH, ITGA5, JAK3, KDR, MMP2, MMP9, MYH11, ND6, NDC80, NID2, NPY, OLFML2B, PIK3R1, PLCB1, PTGDR, SERPINH1, SRXN1, SST, TGFB1, THBS1, THBS2, TIMP, TIMP1, TMEM59, TOP2A, TPX2, VCAN, WNT7B, XBP1*	Li et al. (2018a), Liu et al. (2019a), Wang et al. (2015), Dai et al. (2018), Zheng et al. (2019), Wu et al. (2019a), Zeng et al. (2018), Liu et al. (2014, 2018a, 2019b), and Saberi Anvar et al. (2018)
	miRNAs	*Let-7i-5p, miRNA-21, miRNA-203, miRNA-212, miRNA-1, miRNA-100, miRNA-368, miRNA-101-3p, miRNA-107, miRNA-10a, miRNA-124a, miRNA-125b, miRNA-129, miRNA-204-5p, miRNA-135b, miRNA-137, miRNA-139, miRNA-145, miRNA-148a, miRNA-150, miRNA-152,*	Su et al. (2019), Ribeiro-dos-Santos et al. (2010), Deng et al. (2013), Pan et al. (2013), Zhang et al. (2015), Baghaei et al. (2017), Gu et al. (2018a), Hwang et al. (2018), Zhang et al. (2018), Yuan et al. (2019), and Hu et al. (2018)

(continued)

Table 13.1 (continued)

Cancer		Genes/miRNAs	References
		miRNA-10b, miRNA-154, miRNA-15b, miRNA-15b-5p, miRNA-181a, miRNA-133a, miRNA-181c, miRNA-18a*, miRNA-18b, miRNA-195, miRNA-143, miRNA-196a, miRNA-196b, miRNA-19b, miRNA-200A-3p, miRNA-200c, miRNA-204, miRNA-215, miRNA-218, miRNA-133b, miRNA-18a, miRNA-369-3p, miRNA-194-5p, miRNA-224, miRNA-26a, miRNA-29a, miRNA-29b, miRNA-29c, miRNA-300, miRNA-302c, miRNA-30e-5p, miRNA-31, miRNA-328, miRNA-148a, miRNA-329, miRNA-34b/c, miRNA-363, miRNA-370, miRNA-375, miRNA-376a, miRNA-381, miRNA-451, miRNA-483, miRNA-497, miRNA-514, miRNA-516a, miRNA-523, miRNA-550, miRNA-551b, miRNA-574-3p, miRNA-586, miRNA-601, miRNA-604, miRNA-611, miRNA-664a, miRNA-767-3p, miRNA-9, miRNA-9*, miRNA-512, miRNA-93-5p, miRNA-96, miRNA-99a, miRNA-125b, miRNA-205-5p*	
Esophageal cancer	Genes	*ACSL1, BCL6, BUB1, BUB1B, CCNA2, CD19, CD226, CD27, CD28, CD37, CD38, CD5, CD74, CD83, CFL1, CHEK1, COL11A1, E2F4, FAM46A, IL1A, IL2, IRF6, JUN, KRT14, KRT5, LAMA3, MME, NDC1, NUP107, NUP155, RAB15, SFN, SLC20A1, SLURP-1, TTK, VEGFA*	He et al. (2017, 2018), Chen et al. (2018), Yue et al. (2017), Dai et al. (2017), and Dong et al. (2018)
	miRNAs	*miRNA-1, miRNA-105-5p, miRNA-1246, miRNA-21-5p, miRNA-1290, miRNA-375, miRNA-206, miRNA-208b-3p, miRNA-21-3p, miRNA-503*	Dai et al. (2017), Xu et al. (2013), Chong et al. (2014), Liu et al. (2015), Lau et al. (2018), and Cai et al. (2018)
Pancreatic cancer	Genes	Albumin, *COL1A1, COL1A2, COL3A1, ECT2,* epidermal	

(continued)

Table 13.1 (continued)

Cancer		Genes/miRNAs	References
		growth factor, fibronectin1, integrin subunitα 2, *ITGA2, MMP2, MMP7, MMP9, NR5A2, NRP2, TGFBI, TIMP1*	Gupta et al. (2019a), Li et al. (2018b), Lv et al. (2019), and Liu et al. (2018b)
	miRNAs	*miRNA-125a, miRNA-126, miRNA-222, miRNA-100, miRNA-454 miRNA-29b, miRNA-21, miRNA-143, miRNA-328, miRNA-148a, miRNA-1301, miRNA-484, miRNA-3613, miRNA-155, miRNA-375, miRNA-193a-3p, miRNA-217, miRNA-221, miRNA-23a, miRNA-31, miRNA-34a, miRNA-376b, miRNA-376c, miRNA-502-3p, miRNA-664a*	Ma et al. (2013), Liang et al. (2018), and Tan et al. (2018)
Hepatocellular carcinoma	Genes	*ABCB1, ACACB, ADH1A, ADH1C, AURKA, CCNB1, CCNB2, CDK1, CDKN3, CENPF, CXCR4, EHHADH, ENO3, ESR1, IGF1, MAD2L1, MAP2K1, NCAPG, NDC80, PLK1, PRC1, PRCC, PRPF4, PSMA7, RACGAP1, RIPK4, RRM2, TLR4, TOP2A, TTK, UBE2C, ZWINT*	Gao et al. (2018), Wu et al. (2019b), Tu et al. (2019), Liu et al. (2019c), and Yan and Liu (2019)
	miRNAs	*miRNA-1296, miRNA-221, miRNA-23c, miRNA-300, miRNA-381-3p, miRNA-494-3p, miRNA-95, miRNA-149, miRNA-15b-5p, miRNA-29c, miRNA-126-3p*	Yan and Liu (2019), Ji et al. (2018), Mei et al. (2018), Lou et al. (2018), and Pan et al. (2019)

stage-II, III, and IV progression in colorectal cancer, respectively (Palaniappan et al. 2016).

13.2.1.2 Gastric Cancer

Recently, Li and team re-analyzed three microarray datasets, namely, GSE27342, GSE33335, GSE29272, present in the GEO database and identified seven novel genes, namely, *COL4A1, THBS2, VCAN, COL1A2, TIMP1, COL6A3*, and *SERPINH1* that are associated with worse overall survival of gastric cancer in human (Li et al. 2018a). Several other studies identified one (*OLFML2B*) (Liu et al. 2019a), two (*CCNB1* and *CCNB2*) (Wang et al. 2015), two (*XBP1* and *GIF*) (Dai et al. 2018), three (*ND6, BRMS1*, and *SRXN1*), three (*NID2, COL4A2*, and

COL4A1), six (*ERPINH1, PTGDR, NPY, ADHFE1, AKR1C1*, and *GPER*) (Zheng et al. 2019), six (*IGF2*, *SST*, *GSTP1*, *TMEM59*, *MYH11*, and *ERBB2*) (Wu et al. 2019a), seven (*FOS*, *AHR*, *EGR1*, *JUN*, *WNT7B, CYP1A1*, and *FOSL1*) (Zeng et al. 2018), nine (*TOP2A, TIMP, TPX2*, *COL3A1, COL1A2, CEP55, NDC80, CDKN3,* and *COL1A1*) *(*Liu et al. 2018a*)*, ten (*GNG7, PLCB1, JAK3, KDR, GNAS, FGFR4, GRB2, ADCY9*, *ADCY7,* and *CALML5*) (Liu et al. 2019b), and fifteen (*CTNNB1, FN1, FYN, MMP9, COL1A1, ITCH, TGFB1, THBS1, MMP2, ACTA2, ITGA5, BMP2, BGN, HSPA4*, and *PIK3R1*) keys genes associated with gastric cancer via bioinformatics approaches. Anvar and the team identified three vital proteins, namely, HNF4A, TAF1, and TP53 that play a crucial role in gastric cancer formation via system biology approaches (Saberi Anvar et al. 2018). In 2014, Liu and the team identified six clusters of proteins responsible for cell-cycle, protein degradation, immunoreaction, and protein trafficking during gastric cancer. Out of all, COPS5 (COP9 Subunit 5) is the critical protein of all the largest cluster (module 1). They also detected two key transcription factors, namely, MAZ (Myc-associated zinc-finger protein) and MYC in module 1 (Liu et al. 2014). These genes may serve as key target molecules during drug discovery against gastric cancer.

13.2.1.3 Esophageal Cancer

In 2017, He and the team identified four essential genes, namely, *CHEK1, CCNA2, COL11A1*, and *MME* that are mainly related to cell-cycle modulation and play a vital role in the development of esophageal cancer (He et al. 2017). In another study, Chen and the team reported that downregulation of *SLURP-1* causes esophageal cancer (Chen et al. 2018). In another study, two sets of genes, *BUB1B, BUB1*, & *TTK* and *NDC1*, *NUP107*, & *NUP155,* were identified to play an essential role in esophageal cancer. While *BUB1B, BUB1*, and *TTK* affect the chemotherapy, *NDC1*, *NUP107*, and *NUP155* modulate the function of the RNA transport pathway during gastric cancer. However, when combined, these six genes do not play an essential role in the development of esophageal cancer (He et al. 2018). Yue and team suggested that dysfunction of *PTK2,* MAPK signaling pathway, PI3K-Akt signaling pathway, p53 signaling pathway, and *MET* plays a vital role in the development of esophageal cancer (Yue et al. 2017). In 2017, Dai and the team suggested that immune-related genes, namely, *CD5*, *CD226*, *CD38*, *CD19*, *CD27*, *CD83*, *BCL6, IL2*, *CD37*, *CD74*, and *CD28*, are highly expressed in subtype I "oesophageal squamous cell carcinoma" (OSCC). Other essential pathways associated with subtype I are drug metabolism, chemokine signaling, and calcium signaling. On the contrary, genes related to epithelium development, for instance, *JUN*, *E2F4*, *VEGFA*, *CFL1*, *SFN*, *KRT14, LAMA3*, *KRT5*, and *IRF6*, are highly expressed in the subtype II OSCC. These genes are mainly associated with numerous biological processes, including focal adhesion, actin cytoskeleton modulation, MAPK pathway, cell-cycle regulation, development of epithelium, glycolysis, programmed cell death, apoptosis (Dai et al. 2017). Another study identified five genes, namely,

FAM46A, *RAB15*, *SLC20A1*, *IL1A*, and *ACSL1* that are associated with the overall survival or relapse-free survival in OSCC (Dong et al. 2018).

13.2.1.4 Pancreatic Cancer

Earlier computational studies have identified two (*MMP7* and *ITGA2*) (Li et al. 2018b), ten (*MMP9, COL1A2, COL1A1, COL3A1, TIMP1, MMP2,* albumin, epidermal growth factor, fibronectin 1, and integrin subunit α 2) (Lv et al. 2019), and two (*ITGA2* and *MMP7*) (Li et al. 2018b) key hub genes that are associated with pancreatic cancer via computational approaches. Both *MMP7* and *ITGA2* are associated with modulating the tumor microenvironment, i.e., tumor proliferation, progression, migration as well as metastasis (Li et al. 2018b). In another study, researchers identified four key genes, namely, *TGFBI, ECT2, NR5A2,* and *NRP2* that are responsible for the poor survival of pancreatic cancer patients (Liu et al. 2018b). For detail information about the usage of computational biology in the pancreatic cancer treatment, the reader can refer to our earlier published review article (Gupta et al. 2019a).

13.2.1.5 Hepatocellular Carcinoma

In 2017, we employed bioinformatics approaches to identify four key genes (*CXCR4, ABCB1, ADH1C*, and *ADH1A*) that play a crucial role in the development of hepatocellular carcinoma (HC). Several other studies have also employed computational approaches to detect eight (*CDK1, CCNB2, CCNB1, ACACB, MAD2L1, TOP2A*, *IGF1,* and *EHHADH*) (Gao et al. 2018), twelve (*TTK, AURKA, NCAPG, ZWINT, CCNB1, PRC1, CDK1, TOP2A, UBE2C, CDKN3, RRM2,* and *RACGAP1*) (Wu et al. 2019b), four (*PLK1*, *PRPF4, PRCC*, and *PSMA7*) (Tu et al. 2019), five (*ACACB, TLR4, IGF1, RIPK4,* and *MAP 2 K1*), and six (*NDC80, ZWINT, ESR1, ENO3, NCAPG,* and *CENPF*) (Liu et al. 2019c) key genes that are responsible for causing HC. These key genes are mainly involved in protein processing in the endoplasmic reticulum and metabolism, the p53 signaling pathway, cell-cycle regulation DNA replication, and oocyte meiosis (Tu et al. 2019; Liu et al. 2019c; Yan and Liu 2019).

13.2.2 Identification of mRNA–Micro RNA (miRNA) Interaction

Earlier several studies have reported that miRNA plays a vital role in the post-transcriptional modulation of genes involved in the development and cellular function, and their dysfunction causes initiation, progression, invasion, and metastasis in

GICs. However, the complete mechanism of how miRNA module GICs remains elusive to date. Thus there is an urgent need to elucidate the biological role of miRNA in gastric cancer (Pereira et al. 2019). To date, several computational approaches have been performed to detect mRNA–miRNA interaction during GICs.

13.2.2.1 Colorectal Cancer

Recently, Falzone and team reported that upregulation of *miRNA-21-5p* and *miRNA-183-5p* and downregulation of *miRNA-195-5p* and *miRNA-497-5p* are directly associated with colorectal cancer development via interaction with the "Mismatch Repair" pathway (Falzone et al. 2018). In another study, Zhang and team reported that *miRNA-885* initiates colorectal cancer via cell migration by partly reducing the expression of vWF and IGFBP5 (Zhang et al. 2019). *miRNA-182*, *miRNA-128*, and *miRNA-143* are reported to play a crucial role in colorectal cancer (Su et al. 2019). *miRNA-19b-3p* is reported to initiate colon cancer proliferation as well as oxaliplatin-based chemoresistance via targeting *SMAD4* (Jiang et al. 2017). In another study, Ma and the team conveyed that five miRNAs (*miRNA-200b, miRNA-200c*, *miRNA-7, miRNA-200a,* and *miRNA-141*) get upregulated during colon cancer (Ma et al. 2018). Another team of researchers reported that *miRNA-4777, miRNA-659, miRNA-6501, miRNA-6510, miRNA-4638,* and *miRNA-675* are associated with better survival of colorectal cancer patients. However, the association between *miRNA-328* and *miRNA-891a* with the overall survival of the patient is relatively lower (Chen et al. 2019). In another study, *miRNA-128, miRNA-143*, and *miRNA-182* play a key role in the initiation and development of colorectal cancer.

13.2.2.2 Gastric Cancer

Gu and team performed a microarray analysis of miRNA expression profiles present in the GEO database and suggested that Hippo and p53 signaling pathways are significantly enriched during gastric cancer and one circular RNA, namely, hsa_circRNA_101504, played a key role in the network associated with gastric cancer (Gu et al. 2018b). Earlier numerous studies have also reported about silencing of several miRNAs, including *miRNA-137, miRNA-1, miRNA-9, miRNA-196b, miRNA-512, miRNA-10b, miRNA-129, miRNA-516a, miRNA-34b/c, miRNA-152, miRNA-18b, miRNA-124a, miRNA-212, miRNA-148a, miRNA-181c*, and *miRNA-203*, during gastric cancer via aberrant DNA methylation of their promoter regions (Pan et al. 2013). Downregulation of six miRNAs, namely, *miRNA-451, miRNA-148a, miRNA-19b, miRNA-29c, miRNA-31,* and *miRNA-29b,* during gastric cancer, was discovered in another study (Ribeiro-dos-Santos et al. 2010).

Baghaei and team identified downregulation of four tumor-suppressive miRNA, namely, *miRNA-194-5p, miRNA-101-3p, miRNA-205-5p,* and *miRNA-200A-3p* during gastric cancer. The downregulation of *miRNA-194-5p* and *miRNA-101-3p* plausibly causes the upregulation of WDR72 in gastric cancer (Baghaei et al. 2017).

Recently, Pereira and the team identified ten miRNAs, namely, *miRNA-9a, miRNA-135b, miRNA-664a, miRNA-21, miRNA-148a, miRNA-204, miRNA-150, miRNA-483*, and *miRNA-215* that are upregulated in gastric cancer (Pereira et al. 2019). Hwang and the team identified 42 aberrantly expressed miRNAs during early gastric cancer. Out of these 42, five miRNAs, namely, *miRNA-375, miRNA-26a, miRNA-574-3p, miRNA-15b,* and *miRNA-145*, experienced reduced expression since adenoma. Six miRNAs, namely, *miRNA-601, miRNA-18a, miRNA-300, miRNA-370, miRNA-107,* and *miRNA-96,* were upregulated, while two miRNAs, namely, *miRNA-29a* and *miRNA-200c*, were downregulated during gastric cancer (Hwang et al. 2018). Recently, Zhang and team reported that *miRNA-329, miRNA-133b, miRNA-129, miRNA-196a, miRNA-376a, miRNA-368, miRNA-204, miRNA-302c, miRNA-145, miRNA-143, miRNA-29c, miRNA-497, miRNA-133a, miRNA-99a, miRNA-381, miRNA-604, miRNA-767-3p, miRNA-148a, miRNA-139, miRNA-218, miRNA-154, miRNA-1, miRNA-363, miRNA-30e-5p, miRNA-125b, miRNA-100, miRNA-195, miRNA-375, miRNA-586, miRNA-328*, *and miRNA-551b* get upregulated, while *miRNA-18a*, miRNA-523, miRNA-611, miRNA-196b, miRNA-9*, miRNA-135b, miRNA-514, miRNA-369-3p, miRNA-550, miRNA-181a**, and *miRNA-224* get downregulated during gastric cancer (Zhang et al. 2018).

Three independent studies reported that *miRNA-15b-5p, let-7i-5p, miRNA-93-5p,* and *miRNA-204-5p* (Yuan et al. 2019), *miRNA-17* (Hu et al. 2018), and *miRNA-125b* (Zhang et al. 2015) play a key role in the development of gastric cancer. In another study, Deng and the team reported that *miRNA-195* is significantly downregulated in gastric cancer (Deng et al. 2013). Thus, identified key genes and miRNAs can serve as a biomarker in the gastric cancer treatment in humans.

13.2.2.3 Esophageal Cancer

In 2017, Dai and the team suggested that upregulation of *miRNA-105-5p, miRNA-21-3p*, and *miRNA-21-5p* and downregulation of *miRNA-206, miRNA-208b-3p*, and *miRNA-375* cause the development of OSCC (Dai et al. 2017). Upregulation of *miRNA-503, miRNA-1290*, *miRNA-21-5p*, and *miRNA-1246* is also found to be associated with OSCC (Lau et al. 2018). Interestingly *miRNA-503* is generally downregulated in most cancer types; for instance, cervical cancer and hepatocellular carcinoma (Xu et al. 2013; Chong et al. 2014; Liu et al. 2015). Another study reported that *miRNA-203* modulates the function of eight upregulated genes, namely, *PXDN, AHR, NRCAM, EIF5A2, FMNL2, GLI3, GREM1*, and *FSL1,* and *miRNA-1* modulates the function of five upregulated genes, namely, *MMD, PTPRG, BICD1, SEMA6D*, and *SDC2* during esophageal cancer (Cai et al. 2018).

13.2.2.4 Pancreatic Cancer

Earlier, Ma and the team identified pancreatic cancer-associated three downregulated (*miRNA-217, miRNA-148a,* and *miRNA-375*) and seven upregulated (*miRNA-31,*

miRNA-143, miRNA-100, miRNA-21, miRNA-155, miRNA-23a, and *miRNA-221*) miRNAs (Ma et al. 2013). Liang and team identified 10 pancreatic cancer-associated novel miRNAs, namely, *miRNA-1301, miRNA-376c, miRNA-328, miRNA-125a, miRNA-454, miRNA-376b, miRNA-29b, miRNA-126*, *miRNA-664a,* and *miRNA-3613* (Liang et al. 2018). Tang and team detected pancreatic cancer related two upregulated (*miRNA-193a-3p* and *miRNA-34a*) and four downregulated (*miRNA-502-3p, miRNA-221, miRNA*-484, and miRNA-*222*) miRNAs (Tan et al. 2018).

13.2.2.5 Hepatocellular Carcinoma

Yan and the team suggested that *miRNA-300* and *miRNA-381-3p* co-regulate the function of *CCNA2*, *UBE2C*, and *AURKA* during liver cancer (Yan and Liu 2019). Earlier other computational studies have reported that four (*miRNA-1296, miRNA-149, miRNA-23c,* and *miRNA-95*) (Mei et al. 2018), two (*miRNA-221* and *miRNA-29c*) (Lou et al. 2018), one (*miRNA-15b-5p*) (Pan et al. 2019), and two (*miRNA-126-3p* and *miRNA-494-3p*) miRNAs also play significant roles in the modulation of transcription, cell proliferation as well as live cancer-associated pathways (Ji et al. 2018). It is pertinent to note that *miRNA-126-3p* and *miRNA-494-3p* were also found to be significantly downregulated and upregulated in HC cell lines, respectively (Lou et al. 2018).

13.2.3 Identification of Drug Toward the Treatment of Gastric Cancer

As stated above, the key hub gene or protein identified through computational approaches may serve as a therapeutic target toward the GICs treatment. For instance, in our laboratory, we have employed computational approaches to scan novel phytochemicals against diabetes (Gupta and Vadde 2019c) and Alzheimer (Gupta and Vadde 2019b). Similarly, several computational studies have been performed to identify the most plausible drug for the GICs treatment. To the best of our knowledge, only a few computational studies have been able to identify drugs against gastric cancer, esophageal cancer, colorectal cancer, gallbladder carcinoma, pancreatic cancer, and hepatocellular carcinoma. Two independent computational studies identified three cycloprotoberberines, and sesame lignans against RAF kinases and β-catenin, important targets for colorectal cancer treatment, respectively (Kaboli et al. 2018; Cavuturu et al. 2019). Earlier two studies utilized both computational as well as experimental approaches and suggested that trifluoperazine (Santofimia-Castaño et al. 2019) and trifluoperazine dihydrochloride (Neira et al. 2017) have a strong affinity toward *NUPR1* (intrinsically disordered proteins that are responsible for causing pancreatic cancer) and inhibit tumor growth. Nevertheless,

trifluoperazine has a side effect on the central nervous system (Santofimia-Castaño et al. 2019).

In 2017, Babu and the team identified anti-*Helicobacter pylori* and urease inhibitory activities of three flavonoids, namely, kaempferol-3-O-b-D-glucopyranoside, 5-hydroxy-7,40-dimethoxy-6,8-di-C-methylflavone, and kaempferol-3-O-a-L-rhamnopyranoside of *Syzygium alternifolium* fruits by employing both experimental and computational approaches (Babu et al. 2017). In another study, Shi and the team identified Nonoxynol-9 and Benzonatate as an inhibitor of HER2 protein, a common gene for ovarian cancer, breast cancer, prostate cancer, and gastric cancer (Shi et al. 2016). In another study, Junaid and the team identified CHEMBL17319 and CHEMBL1183979 as anti-*Helicobacter pylori* molecules (Junaid et al. 2019). Another approach, namely, the drug repositioning approach, is becoming one of the most essential pillars of personalized medicine (Luciano et al. 2019). This approach helps us in identifying drugs whose tolerability and safety have already been examined earlier, which in turn hasten development as well as delivery of novel therapies with less cost. Transcriptomic data associated with drug perturbations, for instance, the "Connectivity Map" (CMap), have been extensively analyzed to scan the most plausible novel indications via matching similar signatures of diseases as well as drugs on the basis of gene expression modification (Chan et al. 2019). These approaches are designed on the presumption that the pattern of gene expression associated with any disease or trait can be modulated via drugs. In this context, earlier researchers examined numerous approaches for restoring physiological markers in dyslipidemia mouse model study. Efficacy of treatments was found to be correlated with their reversal of gene expression abnormalities to normal levels; thereby suggesting that treatment which reverses transcriptomic effects could possibly cure a disease (Chan et al. 2019). Earlier several studies have utilized drug repositioning approach to identify drugs, namely, chloroquine (Sasaki et al. 2010), dehydroepiandrosterone (DHEA) (Osawa et al. 2002), pantoprazole (Zeng et al. 2016), orlistat (Kridel et al. 2004), raloxifene (Luciano et al. 2019), and sodium dichloroacetate (Khan et al. 2016), that may be used for treating gastric cancer.

13.2.4 Mathematical Modeling

Earlier several studies have reported that there is a continuous interaction between the human body and environment for the normal function of the human body. For instance, our body intakes essential nutrient from food present in the digestive tract and imposes organ-specific function. However, irregular and imbalanced nutrient may result in numerous organ or system-specific disorder (Trusov et al. 2016). Thus, there is an urgent requirement to understand the "dose-dependent" effect of any nutrient on the normal function of any organ. Considering this, numerous statistical approaches, as well as mathematical models, have been developed to understand both normal and abnormal function of respiratory, cardiovascular, digestive as well

as other systems (Trusov et al. 2016). These model either work at "macro-level" or "micro-level." "Macro-level" interaction amongst systems and organs is estimated via ordinary differential systems that describe the evolution of damage. Zero designates no functional disorder in any organ, while one designates complete function fails. All these "macro models" consider natural (self-restoration and aging), medical treatment, the impact of non-normative environmental as well as preventive measures. However, all these "macro-model" fails to capture complete mechanisms associated with any body function or human disease at the cellular level. Hence, in the near future, it is highly required to develop "micro-models" which may capture events at the cellular level (Trusov et al. 2016).

Recent developed single-cell RNA sequencing (scRNA-seq) technique provides a unique to capture event at cellular level. For the first time, in 2009, transcriptomic data was estimated at the single-cell level by Tang and the team (Tang et al. 2009). Since then the technique associated with scRNA-seq has experienced an explosive development. In comparison to bulk-based methods, scRNA-seq provides more detailed insights into cellular heterogeneity, which in turn helps us in bringing remarkable new discoveries in biology (Tang et al. 2011; Zeisel et al. 2015). For instance, Deng and the team identified the stochastic expression of monoallelic genes within mammalian cells (Deng et al. 2014). Earlier Xin & team (Xin et al. 2016) and Segerstolpe & team (Segerstolpe et al. 2016) reported expression heterogeneity of human islet cells (for instance, β-cells, α-cells, and δ-cells) using scRNA-seq techniques. They also investigated the modifications in patterns of gene expression and the enriched signaling pathways in T2D in comparison with healthy people. Hence, mathematical models designed based on information obtained from the single-cell RNA-sequencing technology can provide detailed insight about the molecular mechanisms associated with any disease or trait by capturing gene expression at the inter-cell level.

Additionally, several other mathematical models have been proposed to understand cancer progression, giving more emphasis on patient-specific models (Cumsille et al. 2019). However, because of the complex process associated with any cancer, it very hard to predict the absolute model describing a complete mechanism related to all stages of neoplastic growth. Hence, the main objective of most of these mathematical models developed to date is to capture the maximum phenomenon associated with any cancer progression. For developing any mathematical model, parameter estimation is an important step and parameter estimation requires sensible experimental design as well as clinical data collection. Though most of the mathematical models associated with GI-cancer are confined to metastasis to the liver, a mathematical model describing growth as well as therapy failure due to drug resistance is also available. However, these models failed to describe growth as well as therapy failure quantitatively (Cumsille et al. 2019). Additionally, the mathematical model employed for clinical application in GI-cancer does not consider the spatial aspect of tumor growth. Parameter of these models is generally estimated via using statistical methods and may provide tumor diagnosis in the context of other essential elements (Cumsille et al. 2015). Considering this, recently, Cumsille and the team developed patient-specific models that detect the evolution of

tumor metastasis and also describe growth and therapy failure because of drug resistance quantitatively. Clinically, disease progression was mainly detected through CT scans. Later, observation from each CT scan was extracted via hybrid approaches and employed for generating patient-specific mathematical models that describe growth and therapy failure in terms of drug resistance quantitatively (Cumsille et al. 2015). Thus, these mathematical model along with key genes and protein may be utilized in the treatment of GICs.

13.3 Conclusion and Future Perspective

In conclusion GICs are the most common cancers of the digestive tract system in both men as well as women. Though chemotherapy is widely employed for the GICs treatment, to date overall survival rate of GICs is very less. Thus, there is an urgent requirement of the new approaches to identify key genes responsible for causing GICs in humans, which in turn will help us in designing effective treatment and drugs against GICs. Recently developed computational approaches provide us a unique way to identify key genes and drug—using publicly available genomic and proteomic datasets in a short interval of time with less cost. However, earlier developed experimental as well as computational approaches performed analysis on bulk cell, which in turn provide less information about gene expression at the cellular level. Author believes that estimation of gene expression at cellular level by integrating both experimental, for instance, single-cell RNA (scRNA) sequencing technique, and computational approaches together, we will address a timely and urgent need to link genetic and proteomic data to distinguished tumor heterogeneity in GICs at the genomic, transcriptomic, and metabolomic levels that offer, for some, new therapeutic opportunities. In the near future, the information present in the present chapter will be highly valuable for cancer biologists and immunologists toward the GICs treatment.

Conflicts of Interest None

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