Review Article (Current Status of Colorectal Cancer Surgery)

Genomics and metagenomics of colorectal cancer

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Contributions: (I) Conception and design: SH Wong, J Yun; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: C Ng, H Li; (V) Data analysis and interpretation: None; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Colorectal cancer (CRC) is a common cancer globally. It is a complex disease influenced by genetic and environmental factors. Early studies on familial cases have identified major genes involved in CRC, such as proto-oncogenes *KRAS*, *PIK3CA* and *BRAF*, and tumour-suppressor genes *APC* and *TP53*. These genes have provided valuable insight into the molecular pathogenesis of CRC, and some have made ways to clinical utility to help diagnose cancer syndromes, prognosticate oncological outcomes and predict treatment responses. While these genetic factors are important, recent studies have suggested contribution of microorganisms to colorectal carcinogenesis. Observational studies, animal experiments and translational works have identified several microorganisms as potential carcinogenic bacteria, such as *Fusobacterium nucleatum* and *Peptostreptococcus anaerobius*. With the advent of sequencing technology and bioinformatics, more genomic and metagenomic factors are being uncovered as important players in CRC carcinogenesis. This article aims to review recent genomic and metagenomic discoveries relating to CRC.

Keywords: Colorectal cancer (CRC); genomics; metagenomics; microbiota; cancer syndrome

Submitted Apr 16, 2019. Accepted for publication Jun 11, 2019.
doi: 10.21037/jgo.2019.06.04
View this article at: http://dx.doi.org/10.21037/jgo.2019.06.04

Introduction

Colorectal cancer (CRC) is among the top three commonest cancers globally (1). It is a complex disease influenced by genetic and environmental factors (2,3). Currently, the United States has the highest incidence in the world. Nevertheless, over the past two decades, many Asian countries have witnessed rapidly soaring CRC incidence rates (1). This upward trend, especially in developed Asian countries like Japan, Korea and Singapore, is attributable to shifts towards a sedentary lifestyle and adoption of a Western lifestyle (4,5).

The predisposition to develop CRC can be resolved down to ethnicity, as evident in several Asian countries. For example, in multi-racial countries like Singapore and Malaysia, CRC incidence rates in Malays are lower than their Chinese counterparts (6,7). On a broader regional scale, CRC rates in Chinese, Japanese, and Koreans are higher than that of Indians, Malays and Indonesians (8,9). These differences likely result from a combination of genetic and environmental factors, which are both important for cancer development in the colorectum. There is growing evidence that in addition to host genetic factors, the gut microbiota is associated with CRC and its major risk factors, and may be casually linked to colorectal carcinogenesis. This article aims to review genomic and metagenomic factors relating to CRC.

Early genetic studies in CRC

Early studies estimated that genetic factors contribute to 35% of the inter-individual variability in CRC risk (10). People with one first-degree relative with CRC experience a 2-fold higher risk, while those with two or more relatives with CRC experience a 4-fold increased risk (11). Less than 10% of CRC patients are associated with germline mutations that constitute hereditary CRC syndromes (12). These includes diseases with adenomatous polyposis such as *APC* mutations in familial adenomatous polyposis (FAP), hamartomatous polyposis like *STK11* mutations in Peutz-Jeghers syndrome (PJS), and non-polyposis CRC from DNA mismatch repair (MMR) gene mutations in Lynch syndrome (LS) (12). These mutations are highly penetrant and are associated with high risk of developing CRC. Although these familial syndromes are uncommon and only account for a small proportion of all CRC, mutations at these genes allowed our first glimpse into the molecular basis of CRC.

The early genetic model of colorectal carcinogenesis was described by Fearon and Vogelstein, who proposed CRC as a multistep process in which a clonal population acquires successive mutations to outnumber neighboring epithelial cells (13). This manifests phenotypically as the adenoma-carcinoma sequence, in which small adenomas increase in size and become dysplastic in their development into invasive carcinomas. Classically, this involves mutations causing inactivation of *APC* at early adenomatous stage, followed by those at *KRAS* and *SMAD4*, and eventually *TP53* as a late event in the carcinogenesis process.

Apart from the adenoma-carcinoma sequence, serrated lesions are thought to undergo an alternative pathway in carcinogenesis. Serrated lesions include hyperplastic polyps, sessile serrated adenoma/polyps (SSA/P), and traditional serrated adenoma (TSA). Harboring malignant potentials, SSA/P and TSA are usually located in the right-sided colon and are now recognized precancerous lesions that account for up to 30% of all CRC (14). This serrated pathway of CRC is associated with distinct molecular features, including CpG island methylator phenotype (CIMP) and *BRAF* mutation (V600E) (15,16).

Somatic mutations and molecular changes in CRC

Massive sequencing efforts have been spent to identify somatic mutations important in colorectal carcinogenesis. A genome-scale analysis of 276 CRC samples by the Cancer Genome Atlas Network has provided a comprehensive genomic landscape of CRC (17). In this study, up to 16% of all CRC samples were hypermutated, among which three-quarters harbored microsatellite instability (MSI) with hypermethylation and *MLH1* silencing, and one-quarter had somatic MMR gene and polymerase ε (*POLE*) mutations. As for the non-hypermutated CRC samples, recurrent mutations have been identified in *APC*, *TP53*, *SMAD4*, *PIK3CA* and *KRAS*, and other novel genes including *ARID1A*, *SOX9* and *FAM123B*. A subsequent target sequencing study involving 1,134 CRC samples confirmed recurrent mutations in *APC* and *CTNNB1* genes, implicating the oncogenic WNT pathway in up to 96% of cancers (18). *APC* is a major component of the β-catenin degradation complex. Mutant *APC* failed to be exported from the nucleus to the cytoplasm, where it normally induces β-catenin degradation and thereby it accumulates to activate oncogenic targets such as c-Myc, cyclin D1 and c-Jun (19-22). Given the essential role of the WNT pathway in intestinal homeostasis, aberrant activation of this pathway would promote cell proliferation and impose stemness to the epithelium (23). It represents a final common pathway in colorectal carcinogenesis as multiple signaling abnormalities converge on it for the final step.

Furthermore, these large-scale studies have revealed distinct molecular features between left-sided versus right-sided tumors. Left-sided tumors were found to have less mutational burden with predominant genetic alternations in receptor tyrosine kinase genes (e.g., *EGFR*), whereas right-sided tumors were observed to have greater mutation burden and more aberration activation of mitogenic oncogenes (e.g., *KRAS*, *BRAF*). These distinct molecular features are thought to account for the disparate biology, clinical courses and treatment responses of tumors (18).

Apart from genomic alternations, some studies have focused on non-coding regions for cancer-driving mutations. Using high-throughput chromosome conformation capture (Hi-C) techniques, the regulatory landscape of CRC was studied with 19,023 promoter fragments (24). The study identified a recurrently mutated cis-regulatory element interacting with the *ETV1* promoter to affect its gene expression and subsequently cell viability. Moreover, an international consortium has worked to integrate transcriptomic data into four consensus molecule subtypes (CMSs) with distinguishing features (25): CMS1 (microsatellite instability immune) with hypermutation, microsatellite instability and strong immune activation,

CMS2 (canonical) with marked WNT and MYC signaling activation, CMS3 (metabolic) with metabolic dysregulation, and CMS4 (mesenchymal) with prominent TGF-β activation, stromal invasion and angiogenesis. This represents a robust molecular classification system for CRC, with a strong biological basis that may affect tumor behavior, clinical progression and treatment outcomes.

Germline alternations and susceptibility to CRC

While highly penetrant and specific germline mutations are responsible for inherited CRC syndromes, other sequencing or genome-wide association studies (GWAS) have identified genetic variants that confer susceptibility to the cancer. Based on the common-disease-common variant hypothesis (26,27), these genetic alternations are expectedly more common (defined as polymorphism if variant frequency >1%) but have smaller effect sizes to the relevant phenotype (odds ratios commonly <1.5). This approach often involves collection of unrelated samples in a population, DNA extraction, genotyping or sequencing, followed by comparing the frequencies of genetic variants (or their markers) between cases and controls. Here we review findings from these studies.

Over the past decade, numerous GWAS have identified over 90 genetic loci that were associated with CRC at genome-wide level of significance. The first wave of results came from studies in the United Kingdom and Canada (28,29), which reported several genetic associations including the chromosome 8q24 locus. This region is a major locus associated with CRC, and harbors risk variants that alter long-range interaction with the proto-oncogene *MYC* (30,31). Subsequent GWAS have also identified other CRC loci such as *SMAD7*, *BMP4*, *CDH1* and others.

Sequencing studies and meta-analyses of major GWAS have been performed to achieve greater statistical power in detecting genetic variants. In a recent study that performed whole-genome sequencing of 1,439 CRC cases (and 720 controls) and combined meta-analysis of 125,478 individuals, the authors identified 40 new genetic variants and bought the total number of signals beyond genome-wide significance to ~100 (32). Apart from cancer signaling pathways, this study also highlighted importance of low-frequency variants and long non-coding RNAs in CRC. The study was first to identify a rare variant protective signal for sporadic CRC, occurring at 0.3% frequency near genes *CDH1* and *RGMB*. Heritability analyses suggest that CRC risk is highly polygenic, and that larger studies on rare variants will likely influence personalized treatment of CRC.

Metagenomic studies in CRC

Having more than 10^{13} microorganisms (33), the gut microbiota colonizes the human gastrointestinal tract with a role to maintain gut integrity, metabolism, immunity, and protect against pathogens (34,35). The gut microbiota consists of commensal organisms that include bacteria, archaea, eukarya, and viruses (36). Containing one of the most populous bacterial communities in the body, there is good evidence that the gut microbiota is associated with CRC and can modulate colorectal tumorigenesis. This proposition is supported by multiple studies using metagenomic shotgun (37-40) or 16S ribosomal RNA (rRNA) sequencing (41-43).

The current working hypothesis is that microbial carcinogenesis of CRC hinges on the interplay between (I) keystone pathogens and (II) the bacterial driver-passenger model (44,45). Keystone pathogens are present at low abundance within a community, but are capable of instigating inflammation and supporting a dysbiotic microbiota which to manifest a disease phenotype (44). In CRC, enterotoxigenic *Bacteroides fragilis* (ETBF) is considered a keystone pathogen that secretes a metalloprotease toxin (encoded by the *bft* gene) to result in genotoxicity, epithelial damage, and colorectal neoplasia (46,47). Evidence of its pro-inflammatory and carcinogenic effects were demonstrated in murine models orally colonized with ETBF (47). ETBF disrupts the balance of the colonic commensal microflora, outcompeting other beneficial Gram positive bacteria including *Lactobacilli* and *Bifidobacteria* (48). Within the context of the bacterial driver-passenger model, ETBF acts as the 'bacterial driver' initiating niche alterations that support the proliferation of 'bacterial passengers', which are opportunistic pathogens that outcompete the bacterial drivers during CRC progression (45).

Apart from ETBF, *Fusobacterium nucleatum* is another bacterium that has been extensively studied in CRC. Studies showed that *F. nucleatum* could promote intestinal tumorigenesis, through adhering to cancer cells (49) and modulating immune cells (50) via its Fap2 protein. The bacterium could also modify the tumor microenvironment (42), activate β-catenin caner pathway (51), and induce microRNA-21 expression (52). Increased intra-tumor levels of *Fusobacterium* have been associated with lower T-cell infiltration (53), proximal tumor location (54), advanced

disease stage and poorer patient survival (55,56).

Non-bacterial microbiome

Intestinal bacteriophages make up 90% of the gut virome (57). A recent network analysis of bacteriophage communities in ~60 healthy individuals revealed a core group (58). Bacteriophages can be either beneficial or detrimental to their host, and are important players in maintaining microbiota homeostasis in the human body (59). In microbial ecosystems, lytic bacteriophages adopt a "kill-the-winner" model where dominant bacterial species are preferentially infected and killed, reducing populations of susceptible hosts and affecting the overall bacterial composition (60). In contrast, lysogenic phages infect their hosts and integrate into bacterial chromosomes (61). This coexistence imparts fitness to the bacterial host to adapt to changes and undergo lysis to release progeny phage triggered by environmental cues (61).

Through examining the viral component of CRC-associated microbiomes, fecal virome profiles was shown to be able to predict CRC status and segregate individuals at early and late stages of CRC (39). From a metagenomics cohort analysis of 74 CRC patients and 92 healthy controls from Hong Kong, 22 key viral markers discerned CRC patients from controls. The top three differentiating taxa were *Orthobunyavirus*, *Inovirus* and *Tunalikevirus* (27). The latter two are known to infect Gram-negatives such as ETBF, *F. nucleatum*, and pks-producing *E. coli* which are implicated in CRC tumorigenesis. Furthermore, it was established that correlations between 14 pairs of phage and gut-oral bacterial species remained through the early and late stages of CRC progression. *Streptococcus*-, *Vibrio*- and *Enterobacteriaceae*-specific phages increased in the early-stage (I and II) of CRC while *Parabacteroides*-, *Nocardia*-, *Enterococcus*- and *Lactobacillus*-specific phages dominated in the late stages (III and IV). These phages may have a mechanistic and distinct role in shaping the bacterial microbiome which affects tumor growth or metastasis. In a study by Hannigan *et al.* [2018] of North American subjects which included healthy controls (n=30), and individuals with adenomas (n=30) and carcinomas (n=30), bacteriophage signatures belonging to families *Siphoviridae*, *Myoviridae*, and other unclassified taxa were associated with individuals in a cancerous state (62). They concluded that the cancer-associated virome primarily consisted of lysogenic bacteriophages which have an indirect role on CRC progression through modulation of bacterial host communities. These seminal studies of viromes in CRC cohorts have brought attention to the influence of viruses over the control of bacterial community dynamics within microbiomes.

Apart from the virome, metagenome of the fungal microbiota has also been studied in the context of CRC. Fungal dysbiosis has been reported in CRC with increased Basidiomycota-Ascomycota ratio in patients when compared with healthy subjects (63,64). The fungal class *Malasseziomycetes* was enriched in CRC, while classes *Saccharomycetes* and *Pneumocystidomycetes* were depleted. Ecological analysis revealed a higher number of co-occurring fungal intra-kingdom correlations, and more co-exclusive correlations between fungi and bacteria in CRC compared with healthy controls. This indicates that synergistic intra-fungal and antagonistic bacterial-fungal associations may play role in colorectal carcinogenesis.

Potential applications in medicine

One major difference between the host genome and the microbial metagenome is the scalability and maneuverability. While the host genetic factors cannot be modified, the gut microbiota can rapidly change in response to both external environmental factors and internal physiological conditions. This provides a valuable window for manipulation for clinical purposes. In the context of CRC, this offers an opportunity for cancer prevention and treatment.

Customizing cocktails of probiotics as microbiome therapies is an area of research that is worth exploring. As for CRC, some *Bifidobacterium* and *Lactobacillus* species have shown anti-cancer properties. In a small study of CRC patients, probiotic intervention was used to manipulate CRC-associated microbiota to one that was enriched in butyrate-producing bacteria (65). Butyrate has been shown to ameliorate the effects of CRC by reducing pro-inflammatory cytokines, inhibiting cell proliferation and promoting apoptosis and expression of tumor suppressor genes (66). Such studies reinforce the potential of tapping into gut ecosystem to deliver positive health outcomes for patients suffering from microbiome mediated disorders.

The efficacy and response of patients to cancer treatment can also be affected by the resident gut microbiota. In the case of chemotherapy and immunotherapy, the microbiome is capable of either facilitating drug efficacy, abrogating and compromising anticancer effects or mediating toxicity. One example is irinotecan, a chemotherapeutic drug used to treat advanced stages of colon cancer by killing

rapidly proliferating tumor cells. Upon eliminating tumor cells, the excreted drug is reactivated by β-glucuronidase-producing bacteria causing a side effect of diarrhea and server intestinal toxicity which is detrimental for patient recovery (67). Furthermore, gut microbiota has been shown to affect cancer response to checkpoint inhibitors including those that aim at the programmed cell death protein 1 (PD-1)-PD-1 ligand 1 (PD-L1) axis. In a meta-analysis that combined data from three studies on PD-1 antibody response, enrichment of *Akkermansia muciniphila* and *Ruminococcus champanellensis* were observed in immunotherapy responders. This raised the possibility of modifying these bacteria for improving immunotherapy response.

Conclusions

With massive sequencing and bioinformatics technologies, there are rapidly accumulating data on the genomic and metagenomic landscapes of CRC. These valuable data have expanded our understanding on the oncogenic mechanisms, meanwhile, they offer new opportunities to develop new applications for CRC diagnosis and treatment. Just like the way genomics have contributed to personalized medicine, metagenomics is likely to join as an important component of future medical care with these exciting developments.

Acknowledgments

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Ng C, Li H, Wu WKK, Wong SH, Yu J. Genomics and metagenomics of colorectal cancer. J Gastrointest Oncol 2019;10(6):1164-1170. doi: 10.21037/jgo.2019.06.04

