JOURNAL OF THE
ANUS, RECTUM AND COLON
dx.doi.org/10.23922/jarc.2021-009
http://journal-arc.jp

Review Article

Cancer Genomic Profiling in Colorectal Cancer: Current Challenges in Subtyping Colorectal Cancers Based on Somatic and Germline Variants

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Abstract

Colorectal cancer (CRC) is a heterogeneous disease caused by the accumulation of multistep genetic alterations under the influence of genomic instability. Different backgrounds of genomic instability, such as chromosomal instability, microsatellite instability, hypermutated-single nucleotide variants, and genome stable-induced transformation in the colonic epithelium, can result in adenomas, adenocarcinomas, and metastatic tumors. Characterization of molecular subtypes and establishment of treatment policies based on each subtype will lead to better treatment outcomes and an improved selection of molecularly targeted agents.

In Japan, cancer precision medicine has been introduced in the National Health Insurance program through the addition of the cancer genomic profiling (CGP) examination. It has also become possible to access a large amount of genomic information, including information on pathogenic somatic and germline variants, incomparable to conventional diagnostic tests. This information enables us to apply research data to clinical decision-making, benefiting patients and their healthy family members.

In this article, we discuss the important molecules and signaling pathways presumed to be the driver genes of CRC progression and the signal transduction system in which they are involved.

Molecular subtypes of CRC based on CGP examinations and gene expression profiles have been established in The Cancer Genome Atlas Network with the advent of next-generation sequencing technology. We will also discuss the recommended management of secondary/germline findings, pathogenic germline variants, and presumed germline pathogenic variants obtained from CGP examination and review the current challenges to better understand these data in a new era of cancer genomic medicine.

Keywords

cancer genomic profiling, colorectal cancer, molecular subtype, hereditary colorectal cancer syndrome

J Anus Rectum Colon 2021; 5(3): 213-228

Introduction

Colorectal cancer (CRC) is the major cause of cancer morbidity and mortality. In Japan, the mortality rate owing to CRC has increased significantly over the past 30 years and has become the most prevalent cancer type. The multistep genetic model of colorectal tumorigenesis by Fearon and Vogelstein shed light on the diverse genetic changes that underlie the initiation and progression of adenoma-carcinoma progression[1]. Since then, there has been significant progress in identifying the specific genes and signaling pathways involved in somatic alterations in sporadic CRC and specific gene defects that underlie inherited predisposition to CRC. The adenoma-carcinoma progression model proposes a multistep accumulation of variants in which each histological alteration is the consequence of molecular dys-

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Received: January 31, 2021, Accepted: March 17, 2021

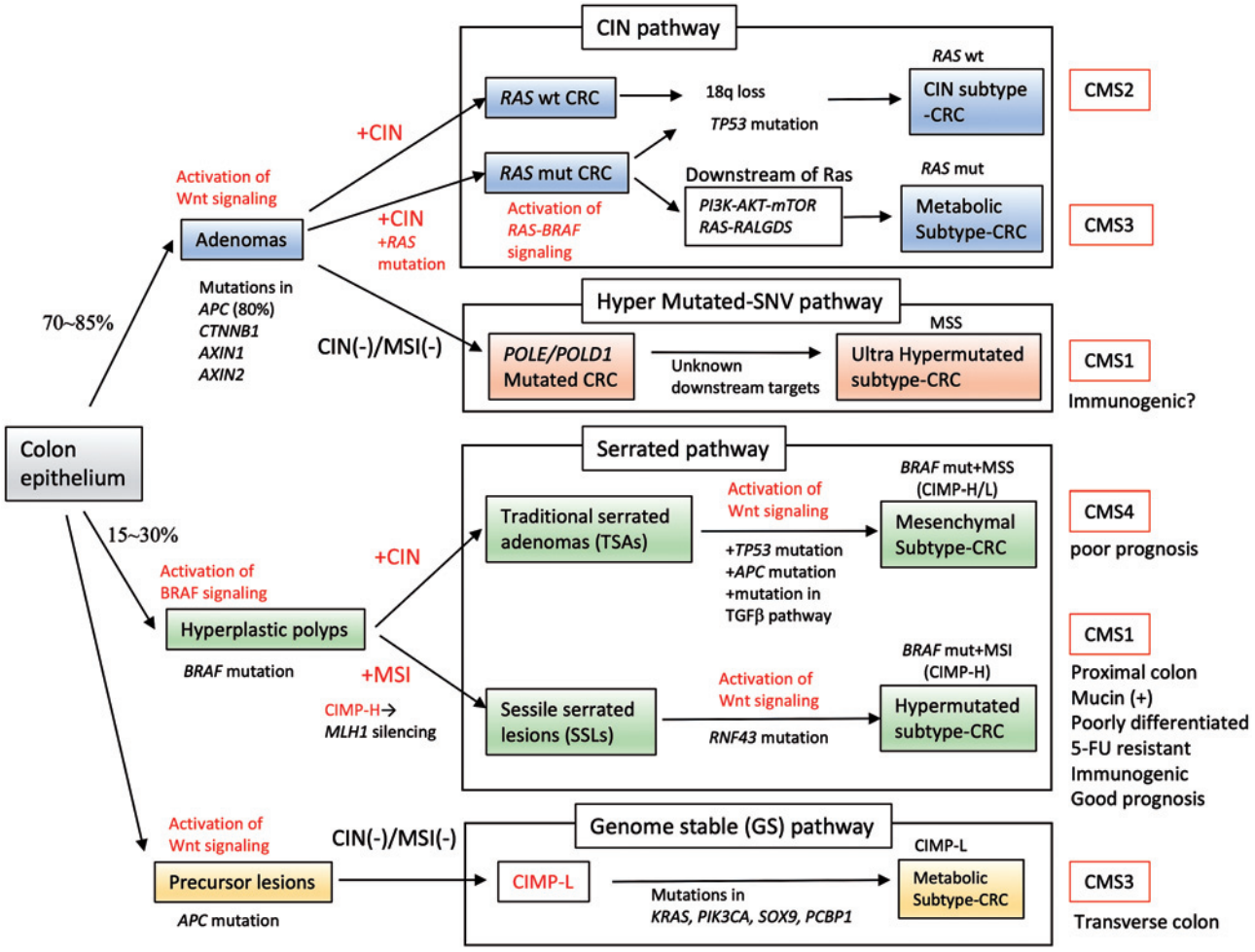

Figure 1. Schematic summary of different pathways of colorectal cancer carcinogenesis with different mechanisms of genetic instabilities and signaling pathway dysregulations.

regulation. In this model, two mechanisms of genomic instability, chromosomal instability (CIN) and microsatellite instability (MSI), have been recognized at the molecular level[2]. Recent advances in endoscopic technology have improved the detection of serrated polyps as precursor lesions to CRC, which are alternative multistep mechanisms of carcinogenesis characterized by epigenetic silencing of *MLH1* in the context of the CpG island methylator phenotype (CIMP) (Figure 1)[3].

Molecular characterization of somatic alterations, including exome sequences of CRC characterized by The Cancer Genome Atlas Network, showed that 16% of CRC were hypermutated (>12 variants per DNA megabase (mut/Mb)): three-quarters of these had MSI-high phenotype and one-quarter were ultra-hypermutated (>100 mut/Mb)[4]. Recent comparative molecular analysis of gastrointestinal adenocarcinomas demonstrated the existence of hypermutated-single nucleotide variants (HM-SNVs) with a polymerase ε (*POLE*) variant, which has been previously categorized as an ultra-hypermutated phenotype[5]. They also revealed a new genome stable (GS) subtype, lacking both aneuploidy/CIN and hypermutation/MSI. Further, a new classification system based on gene expression profiles was established by the CRC subtyping consortium, in which four consensus molecular subtypes (CMS 1-4) correlate clinical outcomes with specific histopathological signatures (Figure 1)[6].

In Japan, the social healthcare system is involved in the development of advanced personalized medicine through the implementation of cancer genomic medicine with the advent of next-generation sequencing (NGS). Therefore, an understanding of the current genetic background of CRC is essential for developing novel targeted therapies.

Thus, we begin this article with an in-depth description of the molecular genetics of CRC and highlight how somatic alterations of genes and signaling pathways play key roles in sporadic cancers. However, 15%-30% of CRCs may have a major hereditary component, given the occurrence of CRC in first- or second-degree relatives, and approximately one-quarter of these familial cases indicate a highly penetrant Mendelian cancer syndrome that predisposes patients to

CRC. Although inherited CRC cases represent a small fraction of the CRC population, studies on the molecular basis of inherited CRC have greatly improved our knowledge of cancer genetics that contribute to sporadic CRC development. I also reviewed studies on inherited tumor syndromes, which might be missed as secondary/germline findings, especially as presumed germline pathogenic variants (PGPVs) in the cancer genomic profiling (CGP) testing of mostly tumor-only panels. This is especially important because the surveillance of patients and family members with potentially pathogenic variants is essential for preventing CRC and its associated cancers.

Adenoma-carcinoma Progression

Most CRCs arise from precancerous lesions that are broadly categorized as either tubular adenomas (70%-85%) or serrated polyps (15%-30%). Variants in the adenomatous polyposis coli (*APC*) tumor suppressor gene[7] or the *BRAF* oncogene[8-10] are initiating events that give rise to traditional adenomas or serrated polyps, respectively. The accumulation of specific gene alterations in a particular predefined order is essential for the progression from adenoma to carcinoma. Additionally, because baseline variant rates are insufficient to account for multiple variants, cancer cells acquire intrinsic genomic instability, a mutator phenotype that increases the rate of new variants. Most cases (~70%) of CRC arise through the CIN pathway characterized by a widespread imbalance of chromosome number (aneuploidy) and loss of heterozygosity (LOH), which induce activation of the Wnt signaling pathway because of variants in the *APC* gene and subsequent somatic alterations[11,12].

CIN Pathway

In the CIN pathway, chromosome changes, including somatic copy number alterations (SCNAs) caused by aneuploidy, amplifications, insertions, deletions, or LOH are observed as a result of defects in chromosomal segregation. The accumulation of a characteristic set of variants in specific tumor suppressor genes (e.g., *APC* and *TP53*) and oncogenes (e.g., *KRAS* and *PIK3CA*) that activate pathways critical for CRC initiation and progression in adenoma-carcinoma progression models are discussed as follows.

Wnt signaling pathway (APC/glycogen synthase kinase 3 beta (GSK-3β)/β-catenin/AXIN)

The earliest genetic event in colorectal tumorigenesis is the activation of the Wnt signaling pathway through the genetic disruption of *APC* on 5q21[13]. Wnt signaling is activated in nearly all CIN tumors, and *APC* variants have been identified in approximately 80% (75.2%, Figure 2) of these tumors[4,6]. Although germline variants responsible for familiar adenomatous polyposis are distributed throughout the gene[2], somatic variants are clustered between codons 1286 and 1513[14]. In the absence of Wnt signaling, *APC*, *AXIN1*, and *GSK-3*β complex phosphorylate β-catenin in the cytosol, marking it for degradation by the ubiquitin-mediated proteasomal pathway[15]. Loss-of-function variant in *APC* results in nuclear translocation of β-catenin and activation of the Wnt signaling pathway, whereas gain-of-function variant in *CTNNB1* gene, encoding β-catenin, which activates the Wnt signaling pathway, has been identified in 50% of colon tumors with intact *APC*[7]. Dysregulated Wnt signaling affects the transcription of *MYC*, the cyclin D1 gene, vascular endothelial growth factor genes, and peroxisome proliferator-activated receptor delta gene, resulting in the disruption of intestinal epithelial cell proliferation and promotion of tumorigenesis[16].

Variants in *AXIN1* genes have been reported, but only in colorectal tumors with MSI[17,18]. *MYC* expression can be upregulated via the activation of the Wnt signaling pathway, and *MYC* amplifications have been found in colorectal and other tumor types, though variants in *MYC* genes are not found in most colorectal tumors. However, a meta-analysis found no clear association between the tumor level of c-MYC protein and overall or disease-specific survival[19,20].

RAS signaling pathway: the EGFR-RAS-RAF-MEK-MAPK (ERK) pathway

The RAS family of small GTPases in three different isoforms (KRAS, NRAS, and HRAS) has been researched since 1982 when its transforming alleles were first identified in human tumors[21]. In a little over 40% (40.8%, Figure 2) of colorectal tumors, activation variants in *KRAS* arise as the second variant after *APC* dysregulation[1]. The RAS protein is activated by numerous extracellular stimuli, thereby switching between the GDP-bound inactive and GTP-bound active forms. The active form of RAS interacts with its effector proteins (RAF, PI3K, and RALGDS) and activates its downstream signaling pathway[21]. *KRAS* and *BRAF* are key oncogenes in the RAS-RAF-MEK-MAPK signaling pathway, the most important oncogenic pathway for the progression to uncontrolled proliferation of cancer cells. Variants in *KRAS, NRAS*, and *BRAF* are usually mutually exclusive. In metastatic tumor treatment, the pharmacological blockade of EGFR with specific monoclonal antibodies is the mainstay of tumor-targeted therapy. However, these are not effective in colorectal tumors with variants in *KRAS, NRAS,* or *BRAF*, which constitutively activate typical EGFR downstream transducers[22,23].

The *BRAF* V600E variant occurs in approximately 10% (11.6%, Figure 2) of patients with metastatic CRC having distinct subtypes with poor prognosis. Although BRAF inhibitors have clinical activity in *BRAF* V600E-mutated melanoma and non-small-cell lung cancer, they alone have lim-

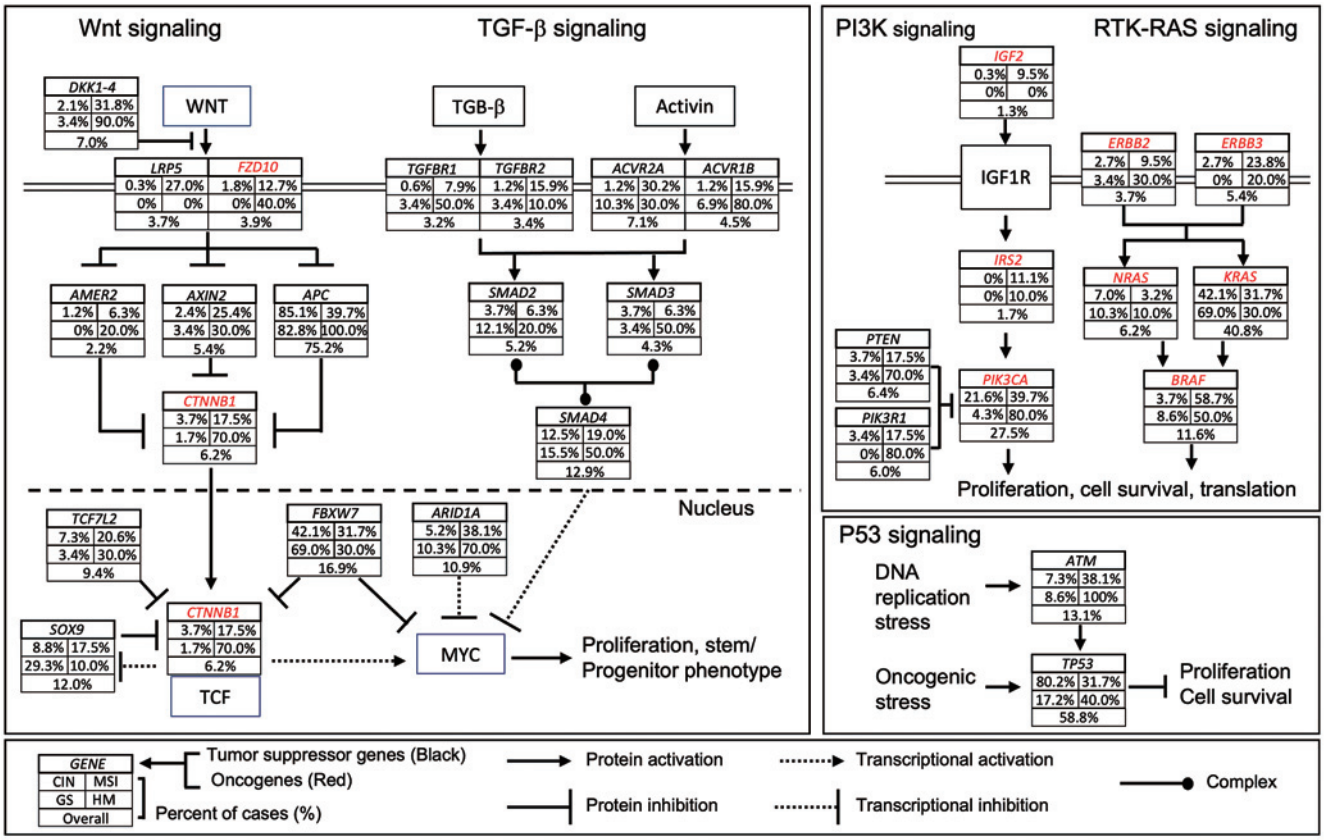

Figure 2. Diversity and frequency of genetic changes leading to deregulation of signaling pathways in CRC. Chromosomal instability (CIN; n = 328), microsatellite instability (MIN; n = 63), genome stable (GS; n = 58), and hypermutated-single nucleotide variants (HM; n = 10) were analyzed separately. Alterations are defined by somatic mutations, homozygous deletions, and high-level focal amplifications. Alteration frequencies are expressed as percentages of all cases. The results shown here are in whole or part based on data generated by the TCGA Research Network: https://www.cancer.gov/tcga."

ited activity against *BRAF* V600E-mutated CRC, whereas triplet regimen of a BRAF inhibitor, an anti-EGFR antibody, and an MEK inhibitor resulted in significantly longer overall survival and higher response rates. This suggests that the combination of agents providing the most effective inhibition of the MAPK pathway was necessary for this subtype[24].

Other RAS signaling pathways (1): phosphatidylinositol 3-kinase (PI3K)-AKT-mTOR signaling

Oncogenic *RAS* variants also activate PI3K, which controls most hallmarks of cancer, including cell cycle, survival, metabolism, mortality, and genomic instability[25,26]. In CRCs, gain-of-function variants in *PIK3CA* (catalytic subunit of *PI3K*) exon 9 or 20 or both arise late in the adenoma-carcinoma sequence and are found in 10%-20% of tumors[27,28]. PIK3CA regulates cell proliferation and survival, inactivating proteins that promote apoptosis. Oncogenic variants in *PIK3CA* activate AKT signaling via mTOR to promote cell growth, proliferation, and survival[29,30]. Since variants in *PIK3CA* exons 9 and 20 trigger different biological effects and concomitant variants in both exons synergistically enhance tumorigenic effects, coexistence of variants in exons 9 and 20, but not a single variant, is associated with the poor prognosis of CRC patients[28].

Other RAS signaling pathways (2): the RALGDS pathway

Previous efforts to inhibit the RAS signaling pathway have produced inhibitors targeting its downstream effectors, including the RAF-MEK-MAPK and PI3K-AKT-mTOR pathways. As the third effector arm, RalGDS/RalGEF, the exchange factor for RAL (RAS-like) GTPases, namely RALA and RALB, has emerged in recent years. However, RALA and RALB play antagonistic roles. This is attributed to their differential usage of effector proteins[31]. RalGDS is a key component of tumor formation in a mouse model of RAS-dependent skin carcinogenesis[32]. In CRC, upregulated *RALA* and *RALB* activation were found in both cell lines and patient samples. Because RALA and RALB play antagonistic roles and RALA is critical for RAS-mediated tumor growth and is activated in human cancer cell lines, anti-RALA selective therapies may provide an effective ap-

proach for *KRAS*-mutated CRCs[33,34].

TP53 pathway

The *TP53* tumor suppressor gene is the most commonly mutated gene in cancer and is located on the short arm of chromosome 17, which encodes a transcription factor and coordinates cellular responses to stress, including DNA damage, oxidative stress, and aberrant proliferative signals. It occurs principally as a late event in the adenoma-carcinoma sequence in five hotspot codons (175, 245, 248, 273, and 282) and in exons 5-8[35,4]. A large cohort analysis revealed that the tumor site, type of variant, and adjuvant treatment are important factors that determine the prognostic significance of these genetic alterations[36]. The risk of CRC increases modestly in patients with Li-Fraumeni syndrome (LFS), a multi-cancer predisposition syndrome with germline variants in *TP53*; therefore, none of the clinical criteria, including both classical LFS and Chompret criteria, define CRC as a component cancer[37].

SMAD pathway

LOH at chromosome 18q is found in approximately 70% of primary CRCs, particularly in advanced stages, suggesting the presence of a tumor suppressor gene locus. *SMAD2* and *SMAD4/DPC4* were identified in the transforming growth factor-beta (TGF-β) pathway on chromosome 18q, although loss-of-function variants in these two genes have been found in <20% and 10% of CRCs, respectively[38,39]. LOH at chromosome 18q is associated with a poor prognosis among patients with stages II and III CRCs[40,41].

MSI Pathway

MSI is observed in approximately 15% and 6% of sporadic colorectal tumors in Western countries and Japan, respectively. This is induced by epigenetic silencing of the *MLH1* gene through promoter hypermethylation as well as induced in patients with Lynch syndrome (LS) caused by germline variants in DNA mismatch repair (MMR) genes (*MLH1, MSH2, MSH6,* and *PMS2*) or *EPCAM* (*EPCAM* deletions cause *MSH2* gene silencing through promoter hypermethylation)[42,43]. TGF-β receptor-2 gene is mutated in more than 90% of MSI-high colorectal tumors[44]. Other target genes for instability, encoding proteins that regulate proliferation (*GRB1, TCF4, WISP3, ACVR2, IGF2R, AXIN*2, and *CDX2*), cell cycle arrest or apoptosis (*CASP5, PRDM2, BCL10, PTEN, PA2G4*, and *FAS*), and DNA repair (*MBD4, BLM, CHK1, MLH3, RAD50, MSH3,* and *MSH6*) have been found[45,46]. In the process of adenoma formation in MSI-high tumors, *APC* variants are found in 35%-50% of cases (39.7%, Figure 2), indicating that genetic instability might be mixed up owing to the MSI and CIN pathways. However, a distinct set of MSI tumors can develop via an initiating *BRAF* variant[8,10] dominated by MSI and serrated pathways as the major clone of the tumor. This is also because tumors develop more rapidly via the MSI pathway than the CIN pathway (hypermutated subtype CRC in Figure 1). Although sporadic MSI-high colorectal tumors have an increased frequency of *BRAF* V600E variants[47], *BRAF* variants are rarely detected in MSI-high tumors in patients with LS[48].

As a predictor of the benefit of adjuvant chemotherapy in stages II and III colon cancer, fluoropyrimidine-based chemotherapy is not effective for patients with MSI-high colorectal tumors[49]. Although the presence of MSI in localized tumors indicates a good prognosis despite poor differentiation, patients with MSI-high metastatic CRC have a shorter overall survival than patients with metastatic CIN CRC. The reason underlying the difference between the prognostic value of MSI status in localized tumors and that in metastatic tumors is unknown. Owing to the accumulation of DNA variants, many mutant forms of proteins allow variant-associated neoantigen recognition, and immunogenicity results in tumor infiltration by immune cells, followed by the expression of checkpoint molecules such as programmed cell death ligand 1. MSI-high tumors are therefore susceptible to treatment with PD-1 inhibitors, resulting in a paradigm shift in CRC treatment.

Serrated Neoplasia Pathway

Serrated benign lesions include hyperplastic polyps (HPs, 60%-75% of serrated polyps), sessile serrated lesions (SSLs, previously called sessile serrated adenoma/polyps, 25%-30% of serrated polyps), and traditional serrated adenomas (TSAs) (Figure 1)[3]. SSLs are characterized by a larger size, location in the proximal colon, and a distinct endoscopic appearance compared with HPs. TSAs are the least common type of serrated polyp and are typically polypoid lesions found in the distal colon and rectum. SSLs and TSAs are considered precursor lesions for CRC. In addition to the conventional adenoma-carcinoma progression model[1,50], clinical, pathological, and molecular data suggest that 15%-30% of CRCs may arise via the serrated pathway, which is a distinct mechanism of carcinogenesis. However, the subset of serrated tumors that acquire high MSI is associated with an accelerated progression similar to MSI-high tumors[51,52]. Activating *BRAF* V600E variants have been detected in most SSLs but not in conventional adenomas. This is the distinguishing characteristic of the serrated pathway, which constitutively activates the downstream MEK-MAPK pathway, resulting in uncontrolled cell division[8,53,54].

After activating *BRAF* variants, serrated tumors develop via two different mechanisms. One mechanism is via the MSI pathway with a variant or hypermethylation of the pro-

moter in MMR genes. Another mechanism is via the microsatellite stable (MSS) pathway with variants in 1) the *TP53* and oncogenic genes in the Wnt pathway, 2) the TGF-β pathway, and 3) the epithelial-to-mesenchymal transition pathway owing to CIN. In the serrated pathway with MSI-high and BRAF variants, the *RNF43* gene encoding E3 ubiquitin ligase, which inhibits the Wnt signaling pathway, is known to undergo a somatic frameshift variant in coding mononucleotide repeats owing to MSI. Furthermore, *RNF43* variant is mutually exclusive to the *APC* variant, suggesting an important role of RNF43 in activating Wnt signaling in this pathway[55,56]. Regarding the discrepancy between the ratio of serrated morphological features in precursor lesions and that in advanced CRC lesions (15%-30% and 10%, respectively)[51], marked loss or reduction of CDX2*, caudal*-related intestine-specific homeobox transcription factors, and *BRAF* variants may play potential cooperating roles in serrated pathways[57]. Compared with patients with CIN tumors with MSS and no *BRAF* variants, patients with MSI-high and mutant *BRAF* tumors have better long-term outcomes, whereas patients with MSS and mutant *BRAF* tumors have worse long-term outcomes[58].

Other Pathways

A recent study based on the comparative molecular analysis of gastrointestinal adenocarcinomas demonstrated that colorectal adenocarcinoma is comprised of four molecular subtypes: CIN, MSI, HM-SNV, and GS[5].

HM-SNV pathway (POLE/POLD1)

Missense variants in polymerase genes *POLE* and *POLD1*, both of which repair errors in DNA replication, have recently been identified as rare causes of multiple colorectal adenomas and carcinomas, a condition termed as polymerase proofreading-associated polyposis (PPAP). PPAP is exhibited in microsatellite-stable and HM-SNV tumors[59]. Among 16% of hypermutated colorectal tumors, three-quarters had MSI with hypermethylation and *MLH1* silencing, and one-quarter had somatic MMR genes and *POLE* variants[4]. Tumors with somatic *POLE* exonuclease domain variants are notable for their extreme genomic instability (their mutation burden is the highest among human cancers), representing nearly 1%-3% of CRC patient populations with distinct mutational signatures, lymphocytic infiltrates, and good prognoses. *APC* variants appear to be the initiating event in this pathway, and pathogenic *POLE* variants are detectable in non-malignant precursors of CRC. However, specific downstream driver variants-developed in the context of hypermutated phenotypes have not been studied yet (Hyper mutated-SNV pathway, Figure 1)[60].

GS pathway

A group of tumors lacking hypermutation and aneuploidy, termed the GS molecular subtype, was identified[5]. The specific mutational spectrum in this phenotype was enriched in DNA hypermethylation and variants in *KRAS* (69.0%), *SOX9*, and *PCBP1*, while the frequency of *TP53* variant was strikingly low (17.2%), consistent with the relative lack of aneuploidy (Figure 2).

The presence of the *SOX9* and *PCBP1* variants may cooperate with the *APC* and *KRAS* variants to facilitate transformation, despite the lack of hypermutation and low levels of aneuploidy[5]. GS CRCs shared features with CIN CRCs and a predilection for the loss of *APC* (GS 82.8% versus CIN 85.1%); GS CRCs are more common in the ascending and transverse colon than are CIN CRCs. Overall, GS CRCs had more frequent pathogenic variants in *RAS/RAF* genes, *PIK3CA,* and the *TGF*-β pathway than did CIN CRCs. This suggests that in the GS molecular subtype, *APC* mutant cells become cancerous by these additional variants without aneuploidy or *TP53* loss.

CDX2 pathway

The intestine-specific homeobox transcription factor CDX 2 plays a key role in intestinal organogenesis and represents a specific marker in gastrointestinal adenocarcinoma differentiation. CDX2 expression is restricted to epithelial cells of the small intestine and colon. Ectopic expression of CDX2 in the esophagus and gastric epithelium induces Barret's mucosa and intestinal metaplasia, respectively, and plays a key role in the gastric cancer intestinal phenotype. In contrast, loss of CDX2 expression is observed in poorly and minimally differentiated colon carcinomas[61-63]. Recent studies have reported the loss of CDX2 as a predictive biomarker for the treatment benefit of chemotherapy in stages II and III CRC[64,65]. In metastatic CRC, *CDX2* expression defines a subgroup of mutated *BRAF* cases with a good prognosis, whereas no *CDX2* expression defines a subgroup of mutated *KRAS* cases with a poor prognosis[66]. To understand the mechanism of interaction between mutated *BRAF* and *CDX2* silencing, mouse serrated CRC models were generated and might be useful to elucidate the mechanism of the prognosis[57].

Molecular Subtypes Based on Somatic Gene Alterations and Gene Expression Profiles

The relationship between molecular subtypes (CIN, MIN, HM-SNV, and GS) based on somatic gene alterations and CMS 1-4 based on gene expression profiles[6] was evaluated as follows (Figure 1):

CMS1 tumors are characterized by hypermethylation of DNA, MSI-high characteristic, and infiltration by immune

cells. A high proportion of CMS1 tumors have the *BRAF* V600E variant and CIMP-high status. These tumors have distinct clinical and histopathological features as they tend to arise in the proximal colon, contain mucin, and be poorly differentiated. CMS2 tumors are characterized by the activation of the Wnt and MYC signaling pathways and by the frequent SCNAs features consistent with the CIN phenotype. CMS3 (also called the metabolic phenotype) tumors are characterized by CIN, but with fewer SCNAs than the CMS 2 subtype. A substantial fraction of GS CRCs was represented in the CMS3, although the CMS system appeared to be largely unable to distinguish between CIN and GS. Gene set enrichment analysis of CMS3 tumors found evidence for the dysregulation of metabolic pathways, including those that involve sugars (such as glucose and fructose), amino acids (such as glutamine), lysophospholipids, and fatty acids. These metabolic aberrations might support tumor growth and are consistent with reports that the activation of *KRAS* affects glucose metabolism and hypoxia.

CMS4 tumors have MSS with CIN, low levels of hypermutation, and high SCNA. These tumors typically develop through the TSA as an intermediate lesion and have CMS4 (mesenchymal subtype) tumor features. CMS4 tumors activate pathways that facilitate an immunosuppressive microenvironment and permit stromal inflammation and tumor invasion, such as the angiogenic pathway. These factors may contribute to the ability of CMS4 tumors to evade immune response, resulting in the lowest survival rates of all CMSs.

CGP tests are performed by extracting genomic DNA from formalin-fixed paraffin-embedded cancer specimens and analyzing genome instability, indels/SNVs, and fusion genes. Therefore, accurate gene expression profiling analysis based on CMSs might be challenging. Even though the translational information from CGP testing to CMS classification can be established, the biological signature of cancer cells may depend on the initiation event (e.g., Wnt signaling or CIMP-*BRAF* variant) and the order of genetic change (CIN or MSI) from precancerous lesions to advanced cancer. Therefore, monitoring cancer genomic profiles using liquid biopsy may provide more precise information.

Hereditary CRC Syndrome and CRC Risk Susceptibility

Approximately 35% of patients with CRC have a family history of the disease. However, only 10% of patients with CRC have been proven to carry pathogenic variants that cause CRC with high (5%) or moderate penetrance (5%), whereas the other 25% have common familial CRC without any cancer syndrome-associated genetic variants. In patients with CRC, MSI testing has occasionally been used as a screening tool for LS, the most common cause of inherited CRC. Recently, the introduction of targeted treatment using immune checkpoint inhibitors for metastatic CRC has increased the use of MSI testing as a companion diagnostic (CDx). Although genetic testing for specific inherited cancer syndromes has been performed based on clinical criteria with family history and/or findings, multigene panel testing appears to be superior to syndrome-specific testing owing to advances in NGS technologies. However, some panels include genes associated with uncertain risks and limited consensus by experts or evidence-based recommendations for management. Patients with these genes require genetic counseling for the interpretation of the results and for individualized recommendations.

In Japan, the social (medical) health-care system is currently in the process of developing precision medicine through the implementation of CGP examination, in which the sequencing of tumor DNA can identify targets for directed therapies and also increase the chance of detecting pathogenic germline variants (PGVs) in matched-pair tests or PGPVs in tumor-only tests for inherited cancer syndrome as secondary/germline findings[67,68]. Current CGP examinations include tumor-only testing, tumor-normal paired testing with germline variant subtraction, and tumor-normal paired testing with an explicit analysis of a group of genes associated with germline cancer predisposition. In tumor-only testing, the FoundationOne® CDx Cancer genomic profiling test (Foundation Medicine, Inc. USA) identified alterations in 324 genes. It is critical to determine which somatic findings may be PGPVs and must be confirmed with follow-up germline testing. The current version of tumor-normal paired testing, OncoGuide™ NCC Oncopanel system (Sysmex Corporation, Kobe, Japan), reports PGVs in 16 genes (*APC, BRCA1, BRCA2, MLH1, MSH2, NF1, PALB2, PTEN, RB1, RET, SMAD4, SMARCB1, STK11/LKB1, TP53, TSC1*, and *VHL*) responsible for hereditary cancers. Thus, the American College of Medical Genetics and Genomics (ACMG) recommends the reporting of secondary/germline findings, though somatic-focused analysis designed to subtract germline variants mask PGVs, except for these 16 genes. Germline findings from CGP testing may be unexpected since many patients do not meet the clinical guidelines for inherited cancer syndromes for genetic testing based on clinicopathological findings. Therefore, management of secondary/germline findings, which are beyond the intended purpose of CGP examinations, is challenging. When tumor-only testing is ordered for CRC patients, clinicians should consider the likelihood of an underlying cancer predisposition syndrome and explain the potential benefit, harm, and limitation of knowing about germline findings by consulting genetic professionals. Moreover, the identification of PGVs in one patient is the entry point for the identification of at-risk family members.

Based on the 59 medically actionable genes on the ACMG secondary findings v2.0 list[69], the larger panel or

whole exome/genome sequencing may reveal PGVs, including cardiovascular (27 genes) disease and other diseases (7 genes) other than hereditary tumor syndromes (25 genes). In Japan, policy statements regarding their clinical management have been proposed by Kosugi et al. as a project of the Japan Agency for Medical Research and Development (https://www.amed.go.jp/news/seika/kenkyu/20200121.html). Inherited forms of CRC with/without polyposis syndromes, high/moderate penetrance genes, and tumor phenotypes are listed on the basis of the genes (Table 1)[70-74].

LS

LS is found in approximately 3% of CRCs and is the most common inherited CRC syndrome. LS is associated with elevated risks of colonic and extracolonic malignancies caused by germline variants in one of the MMR genes (*MLH1, MSH2, MSH6, PMS2*, or *EPCAM)*. Pathogenic variants in the *MLH1* and *MSH2* genes were originally thought to be the most common (〜90%) among patients with LS owing to the high penetrance of *MLH1* and/or *MSH2* carriers detected using clinicopathological findings. The prevalence of pathogenic variants in the *MSH6* and *PMS2* genes has increased since universal screening was introduced[75,76]. Among the population carrying any MMR PGVs (1 in 279), the number of *PMS2* (1 in 714) and MSH6 (1 in 758) variant carriers was higher than that of *MLH1* (1 in 1946) and *MSH2* (1 in 2841)[77], suggesting a difference in penetrance according to the specific gene. In tumor-only CGP examinations, the distribution of variant allele frequency for true germline variants of *MLH1, MSH2, MSH6*, and *PMS2* was 30%, 45%, 50%, and 90%, respectively[78].

High penetrance genes (BRCA1/2, PALB2, CDH1, CDKN2A, and TP53)

Variants in high penetrance genes were identified in a notable number of CRC probands, most of which lacked the phenotypic features of these syndromes. *BRCA1, BRCA2,* and *PALB2,* categorized as high penetrance genes in breast ovary syndromes, are associated with DNA repair, such as double-strand break repair by homologous recombination. However, surveillance for CRC tumors is not recommended on the basis of existing evidence. Other genes, such as *CHEK2, ATM, NBN,* and *BRIP1,* are categorized as moderate penetrance genes.

Other high penetrance genes in non-polyposis CRC lists are *CDH1, TP53*, and *CDKN2A*. Germline *CDH1* variants confer a high lifetime risk of developing diffuse gastric cancer and lobular breast cancer. Although there is no evidence to suggest that the risk of CRC in patients with *CDH1* variants is significantly elevated and there are insufficient data to provide recommendations for surveillance, cases of colorectal and appendiceal signet ring cell carcinoma have been reported[79,80].

LFS with a pathogenic *TP53* germline variant should be suspected in individuals who meet the Chompret criteria, which is characterized by a variety of early onset cancers and family history of young onset cancer or multiple primaries at any age. However, the penetrance of LFS may have been overestimated as more individuals who were recently diagnosed with a germline *TP53* pathogenic variant do not meet this criterion because of a less significant family and personal history of cancer[81]. *CDKN2A* is the major high penetrance susceptibility gene with germline variants identified in 20%-40% of melanoma families. Studies have documented the association between *CDKN2A* variants and pancreatic cancer; however, no significance in any specific organ cancer was found other than a 5-fold increase in the risk of cancer at all anatomic sites.

Familial Colorectal Cancer Type X (FCCTX) is a type of hereditary nonpolyposis CRC in accordance with the Amsterdam criteria I for LS, with no related mutation in the MMR gene. Previous studies describe the correlation between FCCTX and genes such as *FAN1* and *RPS20*[82,83].

Hereditary CRCs with polyposis phenotype

Hereditary CRCs with adenomatous polyposis, such as familiar adenomatous polyposis, Gardner syndrome, and Turcot syndrome, have been well studied. AXIN2, initially cloned as Axil (Axin-like)[84] binds to GSK-3β, APC, and β-catenin as a complex and regulates the degradation of β-catenin in the Wnt pathway. Germline variants in *AXIN2* have been associated with colorectal adenomatous polyposis similar to AFAP and tooth agenesis (oligodontia)[85].

In the serrated pathway, RNF43 inhibits Wnt signaling, and the variant of *RNF43* is mutually exclusive with APC variants. This suggests that RNF43 has an important role in activating Wnt signaling in this pathway[55,56]. Although the prevalence of serrated polyposis syndrome (SPS) is ~1% in the general population on colonoscopy screening, SPS is characterized by multiple SSLs with a CRC risk approaching 50% by 63 years of age. Pathogenic variants of the *RNF 43* gene were detected in 15%-25% of SPS cases.

Missense variants in polymerase genes *POLE* and *POLD1* were identified as the cause of the HM-SNV phenotype. Some studies indicate that patients with variants in *POLE* have a 28% risk and patients with *POLD1* variants have an 82%-90% risk of CRC by 70 years of age[86].

Patients with biallelic mismatch repair deficiency (BMMRD) syndrome, also called constitutional mismatch repair deficiency (CMMRD) syndrome, are born with a biallelic inactivation of any one of the MMR genes that have no DNA MMR activity in any tissue. The most frequent underlying gene defects were *PMS2* variants, which were reported in approximately 60% of cases. The most common cancers observed in BMMRD/CMMRD patients are hematological

Table 1A. Genetics of Inherited Cancer Tumor Syndromes: Inherited Cancer Tumor Syndromes Associated with Non-Polyposis CRC.

Categories of polyposis or nonpolyposis syndromes	Syndrome	Gene defect(s)	Mode of inheritance	Penetrance	Associated cancer spectrum	Pathway	Frequency	CRC risk	*Disclosure recommendation level	**Necessity of Germline testing in T-only panel	NCC Oncopanel somatic	NCC Oncopanel germline	FoundationOne CDx	References
Non-polyposis CRC	Lynch syndrome	*MLH1, MSH2, EPCAM*	AD	High	Lynch-associated cancers, CRC	MMR-deficient	1/280	52%~82%	AAA	◎	*MLH1, MSH2*	*MLH1, MSH2*	*MLH1, MSH2,*	[70-72, 74-76]
		MSH6, PMS2	AD	Moderate	Lynch-associated cancers, CRC	MMR-deficient	1/280	10%~22%	AAA	◎	-	-	*MSH6, PMS2*	[70-72, 74-76]
	Hereditary breast and ovarian cancer syndrome	*BRCA1, BRCA2*	AD	High	breast/ovarian, pancreas, and prostate cancers	DNA repair, cell cycle	1/400-800	increased	AAA	◎	*BRCA1, BRCA2*	*BRCA1, BRCA2*	*BRCA1, BRCA2*	[70-74]
	Hereditary breast cancer syndrome	*PALB2*	AD	High	breast, pancreatic, and ovarian cancers	DNA repair, cell cycle	RARE	unknown	AA	◎	*PALB2*	*PALB2*	*PALB2*	[70-74]
	Hereditary breast cancer syndrome	*CHEK2*	AD	Moderate	breast, CRC	DNA repair, cell cycle	~1%	unknown	A	○	*CHEK2*	-	*CHEK2*	[70-74]
	Hereditary breast cancer	*ATM*	AD	Moderate	breast, pancreatic cancers	DNA repair, cell cycle	~1%	2.5-3.0 fold increase	A	◎	*ATM*	-	*ATM*	[70-74]
	Breast ovarian cancer	*NBN*	AD	Moderate	breast, possibly prostate cancers	DNA repair, cell cycle	RARE	unknown	A	□	-	-	*NBN*	[71, 72]
	Breast ovarian cancer	*BRIP1*	AD	Moderate	ovarian, possibly breast cancers	DNA repair	RARE	unknown	A	◎	-	-	*BRIP1*	[71, 73]
	Breast ovarian cancer	*BARD1*	AD	Moderate	breast cancers	DNA repair	RARE	unknown	not listed	not listed	*BARD1*	-	*BARD1*	[71, 73]
	Hereditary diffuse gastric cancer syndrome	*CDH1*	AD	High	gastric (diffuse type) and breast (lobular) cancers	Adheion molecle	1~3% of gastric cancer	CRC: ~5% (gastric ca: 30%~40%, breast ca: 55%)	AA	○	-	-	*CDH1*	[72, 73, 79, 80]
	Li-Fraumeni syndrome	*TP53*	AD	High	various cancer (breast, sarcoma, brain, adenocortical, leukemia, CRC, gastric, and others)	cell cycle regulation and apoptosis	1/5000	8%~16%	AA	△	*TP53*	*TP53*	*TP53*	[36, 71-74, 81]

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dx.doi.org/10.23922/jarc.2021-009

Table 1A. Continued.

Categories of polyposis or nonpolyposis syndromes	Syndrome	Gene defect(s)	Mode of inheritance	Penetrance	Associated cancer spectrum	Pathway	Frequency	CRC risk	*Disclosure recommendation level	**Necessity of Germline testing in T-only panel	NCC Oncopanel somatic	NCC Oncopanel germline	FoundationOne CDx	References
Non-polyposis CRC	Familial atypical multiple mole-melanoma syndrome (FAMMM)	*CDKN2A*	AD	High	melanoma and pancreatic cancers	Cell cycle	2%~5% of melanoma	unknown (5-fold increase in risk of all cancers)	A	○	*CDKN2A*	-	*CDKN2A*	[70-74]
	Familiar colorectal cancer type X (FCCTX)	*RPS20*	AD	unknown	CRC (Amsterdam I)	rebosomal RNA formation	RARE	unknown	not listed	not listed	-	-	-	[72, 83]
	Familiar colorectal cancer type X (FCCTX)	*FAN1*	AD	High	CRC (Amsterdam I)	DNA repair (MMR-proficient, DNA inter-strand cross-link repair)	2.8% of Amsterdam-positive, MMR proficient families	unknown	not listed	not listed	-	-	-	[72, 82]

*Disclosure recommendation level based on the minimum list of actionable genes to be reported as secondary/germline findings by Kosugi et al. (https://www.amed.go.jp/news/seika/kenkyu/20200121.html): (1) AAA: there exist Japanese domestic guidelines for medical treatment policies for the pathogenic variants carrier. (2) AA: the gene is in the list of ACMG 59 genes (ACMF SF v2) or in the list of NCCN guideline, which is unanimously recommended for reporting in the main articles. (3) A: the gene is listed in the NCCN guideline, although recommendation policies are not concordant among major articles. (4) B: the gene is in the recommendation list for reporting in one article.

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Is germline testing recommended for secondary findings in tumor-only testing?

(1) ◎ Germline testing must be performed with suspicion since it is unconditionally recommended in any of major articles. (2) ○ Germline testing should be performed as much as possible since it is conditionally recommended in any of the major articles. (3) □ Germline testing may be performed if possible. (4) △ Germline testing can be performed only when it is strongly suspected in special cases. (Rather, it should not be performed proactively).

Table 1B. Genetics of Inherited Cancer Tumor Syndromes: Inherited Cancer Tumor Syndromes Associated with Polyposis-Related CRC.

Categories of polyposis or nonpolyposis syndromes	Syndrome	Gene defect (s)	Mode of inheritance	Penetrance	Associated cancer spectrum	Pathway	Frequency	CRC risk	*Disclosure recommendation level	**Necessity of Germline testing in T-only panel	NCC Oncopanel somatic	NCC Oncopanel germline	FoundationOne CDx	References
Adenomatous polyposis (>100 polyps)	FAP/Gardner syndrome/Turcot syndrome type II	*APC*	AD	High	FAP: CRC, duodenal polyps and carcinomas; fundic gland polyps in the stomach, thyroid (cribriform-molula variant), Gardner: desmoid, mandibular ostoma, Turcot: brain tumor (glioblastoma multiforme)	WNT signaling	1/10,000, ~0.5% of CRC	50% risk by 40 years. 100% risk by 60 years	AAA	△	*APC*	*APC*	*APC*	[7, 13, 14, 70-72, 74]
Adenomatous polyps and CRC	Familiar CRC	*APC* (I1307K)	AD	Moderate/ Low	CRC-associated	WNT signaling	6% of Ashkenazi Jews carry this mutation	2-fold increased risk (20% lifetime risk) for CRC	(AAA)	△	*APC*	*APC*	*APC*	[70-72, 74]
Adenomatous polyposis (10~100 polyps)	AFAP	*APC* (predominantly 5' mutations)	AD	High	CRC-associated	WNT signaling	unknown	70% risk by age 80 years	AAA	△	*APC*	*APC*	*APC*	[70-72, 74]
	AFAP With oligodontia	*AXIN2*	AD	High	CRC and oligodontia	WNT signaling	RARE	unknown	not listed	not listed	-	-	-	[18, 84, 85, 72-74]
	NTHL1 associated polyposis	*NTHL1*	AR	High	CRC-associated/extracolonic cancers	Base excision repair	1/115,000	unknown	not listed	not listed	-	-	-	[72, 74, 92]
	MSH3 associated polyposis	*MSH3*	AR	High	CRC-associated	MMR	RARE	unknown	not listed	not listed	-	-	*MSH3*	[73, 74, 92]
	MUTYH-associated polyposis (MAP)	*MUTYH* (biallelic)	AR	High	CRC-associated	Base excision repair	~0.5% of CRC	43%~63% (60 years)	AA (biallelic)	◎	-	-	*MUTYH*	[70-72, 74, 89, 90]
CRC	CRC	*MUTYH* (monoallelic)	AD	Moderate	CRC-associated	Base excision repair	1/~50 population	unknown	not listed	not listed	-	-	*MUTYH*	[70-72, 74, 89, 90]
Adenomatous polyps and CRC	polymerase proofreading-associated polyposis (PPAP)	*POLE*	AD	High	CRC, duodenal, and brain cancers	polymerase proofreading	RARE	40% in male, 32% in female risk by 70 years	B	□	*POLE*	-	*POLE*	[59, 60, 72-74]

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dx.doi.org/10.23922/jarc.2021-009

Table 1B. Continued.

Categories of polyposis or nonpolyposis syndromes	Syndrome	Gene defect (s)	Mode of inheritance	Penetrance	Associated cancer spectrum	Pathway	Frequency	CRC risk	*Disclosure recommendation level	**Necessity of Germline testing in T-only panel	NCC Oncopanel somatic	NCC Oncopanel germline	FoundationOne CDx	References
Adenomatous polyps and CRC	polymerase proofreading-associated polyposis (PPAP)	*POLD1*	AD	High	CRC, endometrial, and breast cancers	polymerase proofreading	RARE	63% in male, 52% in female risk by 70 years	B	○	*POLD1*	-	*POLD1*	[59, 72-74]
	CMMRD	*PMS2* (60%), *MLH1, MSH2, MSH6* (40%)	AR	High	brain tumor (Glioblastoma), CRC, and hematogical (NHL and other lymphoma)	MMR	RARE	100% in childhood	AAA	◎	*MLH1, MSH2*	*MLH1, MSH2*	*MLH1, MSH2, MSH6, PMS2,*	[87, 88]
Hamartomatous polyposis	Peutz-Jeghers syndrome	*STK11/LKB1*	AD	High	Peutz-Jeghers polyps (may have adenomatous features) in the stomach, small bowel, CRC, and sex cord tumors of ovary and testes	Cell polarity	1/200,000	39% lifetime risk	AA	△	*STK11/ LKB1*	*STK11/ LKB1*	*STK11/ LKB1*	[72, 74]
	Cowden disease	*PTEN*	AD	High	colon, breast, endmetrial, thyroid (papillary, follicular), and kidney cancers	PI3K signaling	1/200,000	9%~16% lifetime risk	AA	△	*PTEN*	*PTEN*	*PTEN*	[72, 74]
	Juvenile polyposis syndrome	*SMAD4/ DPC4, BMPR1A*	AD	High	Multiple hamartomas/ juvenile polyps in the colon and stomach	TGF-β signaling	1/100,000	increased risk of CRC and stomach cancer	AA	○ (*SMAD4*)	*SMAD4*	*SMAD4*	*SMAD4*	[70-72, 74]
Mixed polyp types	*GRE1*-associated mixed polyposis	*GREM1*	AD	High	CRC-associated	TGF-β signaling	RARE	9%~16% lifetime risk	not listed	not listed	-	-	-	[72, 74, 94-96]
Serrated polyposis syndrome (SPS)	*RNF43*-related serrated polyposis	*RNF43*	AD	High	CRC, breast, endometrial, urothelial, and brain cancers	WNT signaling	~2% of unexplained polyposis	50% risk by the age of 60 years	not listed	not listed	-	-	*RNF43*	[55, 56, 73, 74]

*Disclosure recommendation level based on the minimum list of actionable genes to be reported as secondary/germline findings by Kosugi et al. (https://www.amed.go.jp/news/seika/kenkyu/20200121.html): (1) AAA: there exist Japanese domestic guidelines for medical treatment policies for the pathogenic variants carrier. (2) AA: the gene is in the list of ACMG 59 genes (ACMF SF v2) or in the list of NCCN guideline, which is unanimously recommended for reporting in the main articles. (3) A: the gene is listed in the NCCN guideline, although recommendation policies are not concordant among major articles. (4) B: the gene is in the recommendation list for reporting in one article.

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malignancies (NHL and other lymphomas), brain tumors (glioblastoma), and LS-associated tumors (CRCs)[87,88].

In approximately 0.5% of CRC cases, *MUTYH*-associated polyposis (MAP) is caused by biallelic germline variants in the *MUTYH* gene associated with base-excision repair. MAP is diagnosed in 8%-13% of FAP-like clinicopathological backgrounds without *APC* germline variants and is associated with the risk of CRC in 43%-63% at the age of 60 years, and the median age of onset is 48 years[89]. It is recognized that monoallelic *MUTYH* variants are detected in 1%-2% of the general population. However, the presence of these variants increases the risk of CRC by approximately 2-fold in individuals with a family history of CRC[90]. This is presumably owing to the interaction between other driver gene variants and monoallelic *MUTYH* mutations.

Homozygous nonsense germline variants in the *NTHL1* gene were detected as relevant variants through their association with base-excision repair[91]. Although studies that screened polyposis patients detected the prevalence of *NTHL 1* biallelic variants in approximately 2% of cases[92], the lifetime risks of CRC and extracolonic cancers were 64% and 86% in men and 47% and 100% in women, respectively, suggesting that constitutional *NTHL1* deficiency underlies high-risk hereditary multi-tumor syndrome[93].

Hamartomatous polyposis syndromes are rare (occurring in 1 in 100,000-200,000 persons) but well defined clinicopathologically and genetically, which include Peutz-Jeghers syndrome, juvenile polyposis syndrome, and PTEN hamartoma tumor syndrome. Individuals with these syndromes develop hamartomatous polyps in the gastrointestinal tract and have an increased risk of cancer, which warrants endoscopic surveillance and, occasionally, surgical intervention.

Hereditary mixed polyposis syndrome is a rare colon cancer predisposition syndrome caused by a duplication of a noncoding sequence near *GREM1* originally described in Ashkenazi Jews[94]. This variant is associated with an increased allele-specific *GREM1* expression, and excess GREM1 proteins suppress the bone morphogenetic protein pathway, a mechanism that also underlies tumorigenesis in juvenile polyposis of the large bowel[95,96].

Future Directions

Owing to the enormous progress in defining genetic alterations and gene expression profiles in CRC in the past three decades as well as the recent introduction of CGP examinations in the clinical setting, we are in the process of obtaining a huge amount of complex data, although identification of critical gene alterations and characterization of their contribution to cancer will be important yet challenging future tasks; therefore, much work remains to be done for the comprehensive understanding of the pathogenesis of the biologically and clinically distinct subsets of CRC. In addition, efforts to define and characterize changes in DNA methylation and chromatin modification, changes in the mRNA and noncoding RNA expression patterns, and protein expression and posttranslational modification in CRC are only in the early stages. Moreover, there is little understanding of the complex interactions among dietary and environmental agents, gut microbiome, and inflammation that are associated with an increased risk of CRC.

Advancement in care for patients with CRC will depend on the establishment in the classification of molecular subtypes on the basis of genetic alterations, gene profiling, and/or proteome, and on the development of target therapy based on the specific mechanism of tumorigenesis in each subtype. Furthermore, information from secondary/germline findings should be handled with care and used to formulate recommendations for patients and their family members. Hopefully, continued and cooperative efforts of researchers and clinicians will not only yield in-depth and comprehensive insights into the molecular changes that underlie CRC but will also result in advances in preventing and treating this disease.

Acknowledgements

The author would like to thank Hiroaki Niitsu, M.D., Ph.D. (Vanderbilt University Medical Center, USA) and Naoya Sakamoto, M.D., Ph.D. (National Cancer Center Hospital East, Japan) for editing and reviewing the figures. The author would like to thank Editage (www.editage.com) for the English language editing.

Conflicts of Interest

There are no conflicts of interest.

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