Clinical, Pathology, Genetic, and Molecular Features of Colorectal Tumors in Adolescents and Adults 25 Years or Younger

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BACKGROUND & AIMS: **Colorectal cancers (CRCs) are rare in adolescents and adults ages 25 years or younger. We analyzed clinical, pathology, and molecular features of colorectal tumors from adolescents and young adults in an effort to improve genetic counseling, surveillance, and, ultimately, treatment and outcomes.**

METHODS: **We analyzed clinical data and molecular and genetic features of colorectal tumor tissues from 139 adolescents or young adults (age, ≤25 y; median age, 23 y; 58% male), collected from 2000 through 2017; tumor tissues and clinical data were obtained from the nationwide network and registry of histopathology and cytopathology and The Netherlands Cancer Registry, respectively. DNA samples from tumors were analyzed for microsatellite instability, mutations in 56 genes, and genome-wide somatic copy number aberrations.**

RESULTS: **Mucinous and/or signet ring cell components were observed in 33% of tumor samples. A genetic tumor risk syndrome was confirmed for 39% of cases. Factors associated with shorter survival time included younger age at diagnosis, signet ring cell carcinoma, the absence of a genetic tumor risk syndrome, and diagnosis at an advanced stage of disease. Compared with colorectal tumors from patients ages 60 years or older in the Cancer Genome Atlas, higher proportions of tumors from adolescents or young adults were microsatellite stable with nearly diploid genomes, or contained somatic mutations in *TP53* and *POLE*, whereas lower proportions contained mutations in *APC*.**

CONCLUSIONS: **We found clinical, molecular, and genetic features of CRCs in adolescents or young adults to differ from those of patients older than age 60 years. In 39% of patients a genetic tumor risk syndrome was identified. These findings provide insight into the pathogenesis of CRC in young patients and suggest new strategies for clinical management. Performing genetic and molecular analyses for every individual diagnosed with CRC at age 25 years or younger would aid in this optimization.**

Keywords: SCNA; Colon Cancer; MSI; MSS.

[a]Authors share co-first authorship.

Abbreviations used in this paper: AYA-CRC, children, adolescents, and young adults with colorectal cancer; CDX2, Caudal Type Homeobox 2; CK20, keratin 20; CMMRD, constitutional mismatch repair deficiency syndrome; CMS, consensus molecular subtypes; CRC, colorectal cancer; IBD, inflammatory bowel disease; IHC, immunohistochemistry; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; NCR, The Netherlands Cancer Registry; NOS, not otherwise specified; PALGA, Nationwide network and registry of histo- and cytopathology; *POLE*-exo, *POLE* exonuclease domain; SCNA, somatic copy number alteration; smMIP, single-molecule molecular inversion probe; SRCC, signet ring cell carcinoma; TCGA, The Cancer Genome Atlas.

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Colorectal cancer (CRC) is the third most common cancer in both males and females in The Netherlands.[1] However, CRC in children, adolescents, and young adults (AYA-CRC; in this study defined as age 25 years or younger) is very rare and represents fewer than 0.1% of CRC cases.[1] In contrast to adult CRC, the clinical and molecular background of AYA-CRC is scarcely studied.[2] Studies have shown that the incidence of CRC in young individuals is increasing,[3] highlighting the importance of further research in this group.[2]

CRC at a young age has a worse prognosis than in older adults.[4–6] The reason for this discrepancy is not completely understood, but AYA-CRC more often presents with an advanced disease stage and unfavorable histology.[4–8] In individuals who developed CRC up to age 50 a genetic predisposition often is suspected, with estimates as high as approximately 40%.[9–11] Next to genetic predispositions, it has been suggested that molecular pathways underlying CRC at younger than age 50 years differ from those in in CRC at an older age (ie, age, >60 y). For example, somatic *POLE* exonuclease domain (*POLE*-exo) mutations are associated with younger age,[12,13] and *BRAF* V600E, associated with *MLH1* hypermethylation, is identified more often in CRCs at an older age.[14] However, despite the clinical recognition that AYA-CRC may differ from CRC at an older age, case series on AYA-CRC in general are small, and most studies focused on 1 or 2 aspects of CRC. Furthermore, most studies published on AYA-CRC hardly include individuals age 25 years and younger.[4–14]

Here, we retrospectively collected clinical information and (tumor) tissues from individuals who developed CRC at 25 years of age or younger between 2000 and 2017 in The Netherlands. We aimed to make a comprehensive analysis of CRC at 25 years of age or younger on both a clinical and molecular level. A better understanding of these early onset CRCs can lead to directions for personalized therapies and improvements in genetic counseling and surveillance.

What You Need to Know

Background

Colorectal cancers (CRCs) in adolescents and adults age 25 years or younger require analysis of clinical, genetic, and molecular features to improve genetic counseling, surveillance, and treatment.

Findings

Clinical, molecular, and genetic features of CRCs in adolescents or young adults differ from those of patients older than age 60. These findings might provide insight into the pathogenesis of CRC in young patients and new strategies for treatment.

Implications for patient care

Clinical, molecular, and genetic information should be collected from all patients with CRC ages 25 years or younger to optimize their management.

Methods

Study Cohort

We retrospectively collected clinical information and matched tumor and normal tissues from individuals who developed CRC and/or appendiceal cancer at 25 years of age or younger, between 2000 and 2017 in The Netherlands, via the nationwide network and registry of histopathology and cytopathology (PALGA) and The Netherlands Cancer Registry (NCR). The data were collected from both sources independently and linked to each other by PALGA. The study cohort and details on the statistical analysis are described in detail in the Supplementary Methods (Figure 1, Supplementary Table 1).

Review of Histopathology

Tumor tissue blocks from 140 CRCs (139 individuals) were requested through the Dutch National Tissue Portal. Slides of 88 CRCs (87 individuals) were available and reviewed for histologic subtype, growth pattern, immune cell reaction, and presence of mucin and signet ring cells by 2 gastroenterology-dedicated pathologists (R.S.v.d.P. and I.D.N.). Tumors were categorized into serrated carcinoma, medullary carcinoma, signet ring cell carcinoma (SRCC), mucinous carcinoma and adenocarcinoma not otherwise specified (NOS).

Immunohistochemistry. To identify consensus molecular subtypes (CMS) in CRC, next to microsatellite instability (MSI) analysis (see later), immunohistochemistry (IHC) was used (Supplementary Table 2).[15,16] In addition, multiple (neuroendocrine) differentiation markers were used. Details of IHC are listed in the Supplementary Methods and Supplementary Table 3.

Somatic Mutation Analysis

Microsatellite instability analysis. MSI analysis using 5 markers (BAT25, BAT26, NR21, NR24, and NR27) was performed as described previously.[17]

Single-molecule molecular inversion probe–targeted enrichment and sequencing. To identify germline and somatic variants we designed a single-molecule molecular inversion probe (smMIP) panel. We included genes reported to be mutated significantly in CRC, genes with a known (germline) role in CRC development, and genes with a therapeutic potential (Supplementary Table 4).[14,18] Target regions preferentially were covered by 2 independent smMIPs. On average, 99% of the target regions were covered by at least 1 smMIP probe (range, 91%–100%) (Supplementary Table 5).

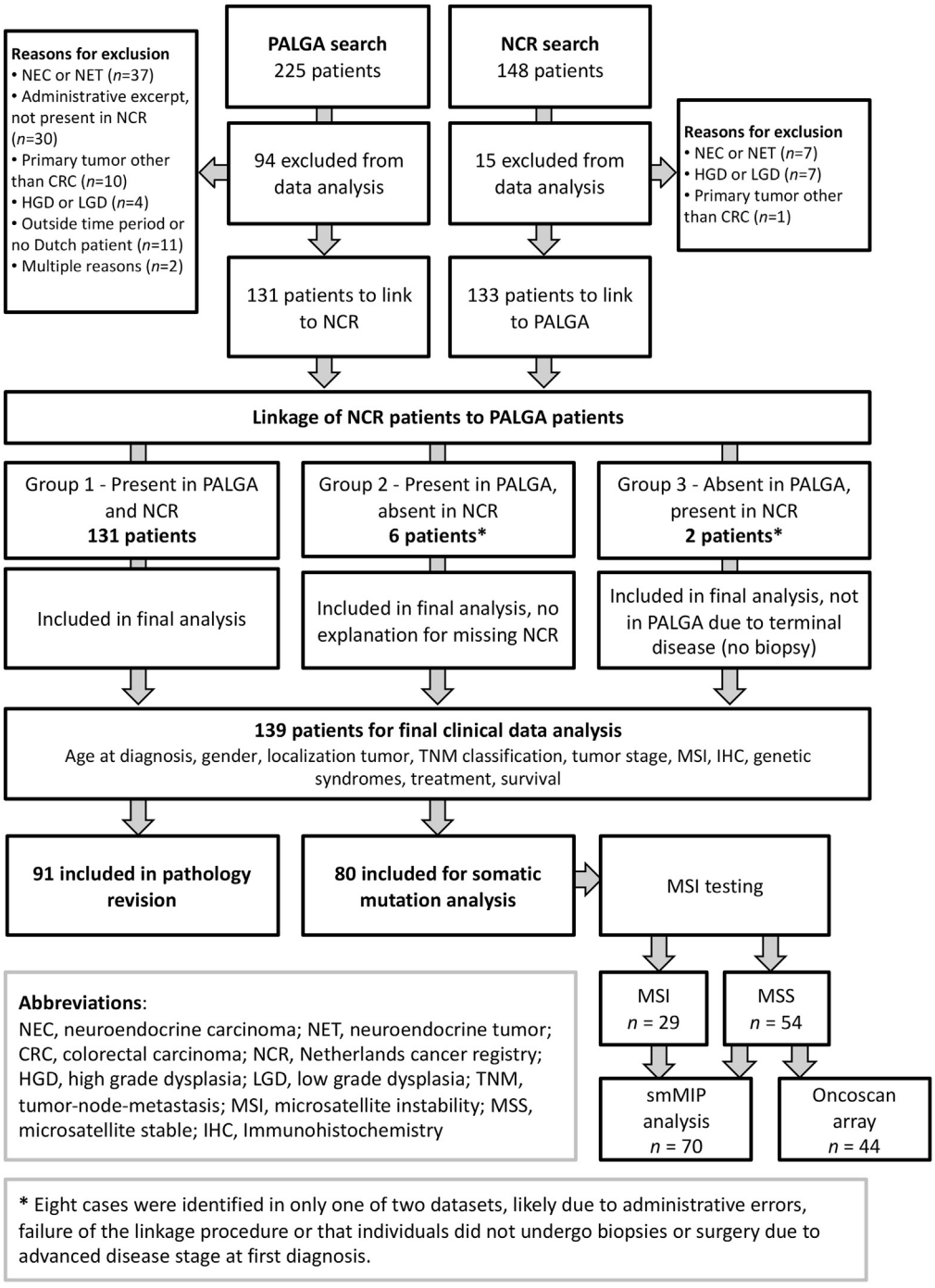

Figure 1. Flowchart of study.

Details of the smMIP-based sequencing, and the identification and selection of germline and somatic variants, are described in the Supplementary Methods section. All somatic variants are reported in Supplementary Table 6.

Multiplex ligation-dependent probe amplification. MSI-high samples without an explanatory pathogenic germline variant were subjected to (methylation-specific) multiplex ligation-dependent probe amplification analysis of *MLH1*, *PMS2*, *MSH2*, and *MSH6* according to the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands).

OncoScan formalin-fixed, paraffin-embedded arrays. To identify regional copy number alterations and loss of heterozygosity, each microsatellite stable (MSS) sample was subjected to an OncoScan formalin-fixed, paraffin-embedded array (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol (see the Supplementary Methods section).

Late-onset (sporadic) colorectal cancer from The Cancer Genome Atlas. To compare our findings in the AYA-CRC group regarding histology and mutations in the target regions, we extracted clinical and molecular data from The Cancer Genome Atlas (TCGA)[15] for 184 CRCs diagnosed in patients at 25 years of age or younger (67.4% of the published cohort) (Supplementary Table 7).

Results

Clinical Manifestation of Colorectal Cancer in Children, Adolescents, and Young Adults

Between 2000 and 2017, 139 AYA-CRC cases were identified using PALGA and NCR (Figure 1), which were diagnosed with CRC ($n = 135$) or an appendiceal adenocarcinoma ($n = 4$) at age 25 years or younger (Table 1, Supplementary Table 1). On average, 8 individuals were diagnosed annually with CRC at age 25 years or younger (range, 5–13 y), which translates to an incidence rate of 1.60 per 1 million person-years. The mean age at diagnosis was 22 years (range, 12–25 y). The distribution of cancers was 31% in the proximal colon, 34% in the distal colon, 31% in the rectum, and 4% in other sites (Supplementary Table 1).

Most tumors were diagnosed at stage III (40.3%), followed by stage IV (25.2%), stage II (18.0%), and stage I disease (16.5%). Follow-up data on survival ($n = 134$ individuals) showed that 52 individuals were deceased. No differences in overall survival were seen by sex, tumor location, or presence of MSI (Supplementary Figure 1*A* and *B*). Disease stage was a significant predictor of survival ($P = .000$) (Supplementary Figure 1*C*). The 5-year survival for individuals with stage IV, III, II, and I disease was 19%, 51%, 78%, and 87%, respectively. Individuals with a diagnosis between ages 10 and 15 had a significantly worse prognosis compared with individuals diagnosed at 16 years of age and older ($P = .015$) (Supplementary Figure 1*D*).

Thirty-one individuals (22%) were diagnosed with benign tumors next to their primary CRC. A second primary malignancy was diagnosed in 13% of the cohort ($n = 18$). Ten individuals with a second primary malignancy also were diagnosed with gastrointestinal adenomatous polyps (Supplementary Table 1).

Nine individuals (6.5%) were diagnosed with inflammatory bowel disease (IBD) (Supplementary Table 1). Six of these individuals had been diagnosed with IBD before the diagnosis of CRC and were followed up and treated for IBD for a duration of 6 to 9 years (mean, 8.3 y).

Table 1. Clinical Characteristics of Individuals With Colorectal Cancer ≤25 Years of Age

	Right-sided colon[a]	Left-sided colon[a]	Rectum[a]	Total (%)
Sex				
Male	27	32	21	80 (57.6)
Female	17	21	21	59 (42.4)
Age at diagnosis, *y*				
10–15	3	4	2	9 (6.5)
16–20	9	8	9	26 (18.7)
21–25	32	41	31	104 (74.8)
Histology				
Adenocarcinoma, NOS	13	19	12	44 (31.7)
Serrated carcinoma	6	10	9	25 (18.0)
Medullary carcinoma	4	2	1	7 (5.0)
Signet ring cell carcinoma	8	2	0	10 (7.2)
Mucinous carcinoma	2	2	0	4 (2.9)
Not revised	11	18	20	49 (35.3)
Disease stage				
Stage I	5	9	9	23 (16.5)
Stage II	8	14	3	25 (18.0)
Stage III	17	18	21	56 (40.3)
Stage IV	14	12	9	35 (25.2)
Microsatellite instability[b]				
Yes	13	13	11	37 (26.6)
No	25	31	19	75 (54.0)
Unknown	6	9	12	27 (19.4)
Vital status at end of follow-up period				
Deceased	18	18	16	52 (37.4)
Alive	23	34	24	81 (58.3)
Unknown	3	1	2	6 (4.3)
Genetic diagnosis[c]				
Lynch syndrome	8	7	7	22 (29.3)
Familial adenomatous polyposis	1	1[d]	4	5 (6.7)[d]
CMMRD	0	1	1	2 (2.7)
No genetic diagnosis	20	16	9	45 (60.0)

CMMRD, constitutional mismatch repair deficiency syndrome; NOS, not otherwise specified.

[a]Right-sided tumors are classified as originating in the cecum, ascending colon, hepatic flexure, or proximal two thirds of the transverse colon. Left-sided tumors are classified as originating in the distal one third of the transverse colon, splenic flexure, descending colon, or sigmoid colon.

[b]Microsatellite instability (MSI) status was based on our analyses in 71 individuals. From the remaining cases, information on MSI was obtained from PALGA reports, if available (Supplementary Table 1).

[c]These genetic diagnoses include only cases that were confirmed by sequence analysis.

[d]One patient had a variant of uncertain significance in the *APC* gene, which was not included in the total number of patients with familial adenomatous polyposis.

Histopathologic and Molecular Characterization of Colorectal Cancers in Children, Adolescents, and Young Adults

A review of the histopathology was possible for 91 tumors (90 individuals), which is representative of the complete cohort (Supplementary Figure 2*A*). The majority of tumors were adenocarcinoma NOS (48%), other histologic subtypes were serrated carcinoma (27%), SRCC (11%), medullary carcinoma (8%), and mucinous carcinoma (6%) (Figure 2*A* and *B*). Next to the 15 CRCs classified as SRCC or mucinous carcinoma, another 14 CRCs had a mucinous component (Supplementary Table 1). In total, 33% of the reviewed cohort ($n = 29$) showed a mucinous or signet ring cell component. Individuals with a SRCC have a significantly worse prognosis than individuals with an adenocarcinoma NOS ($P <$ .001) (Figure 2*C*).

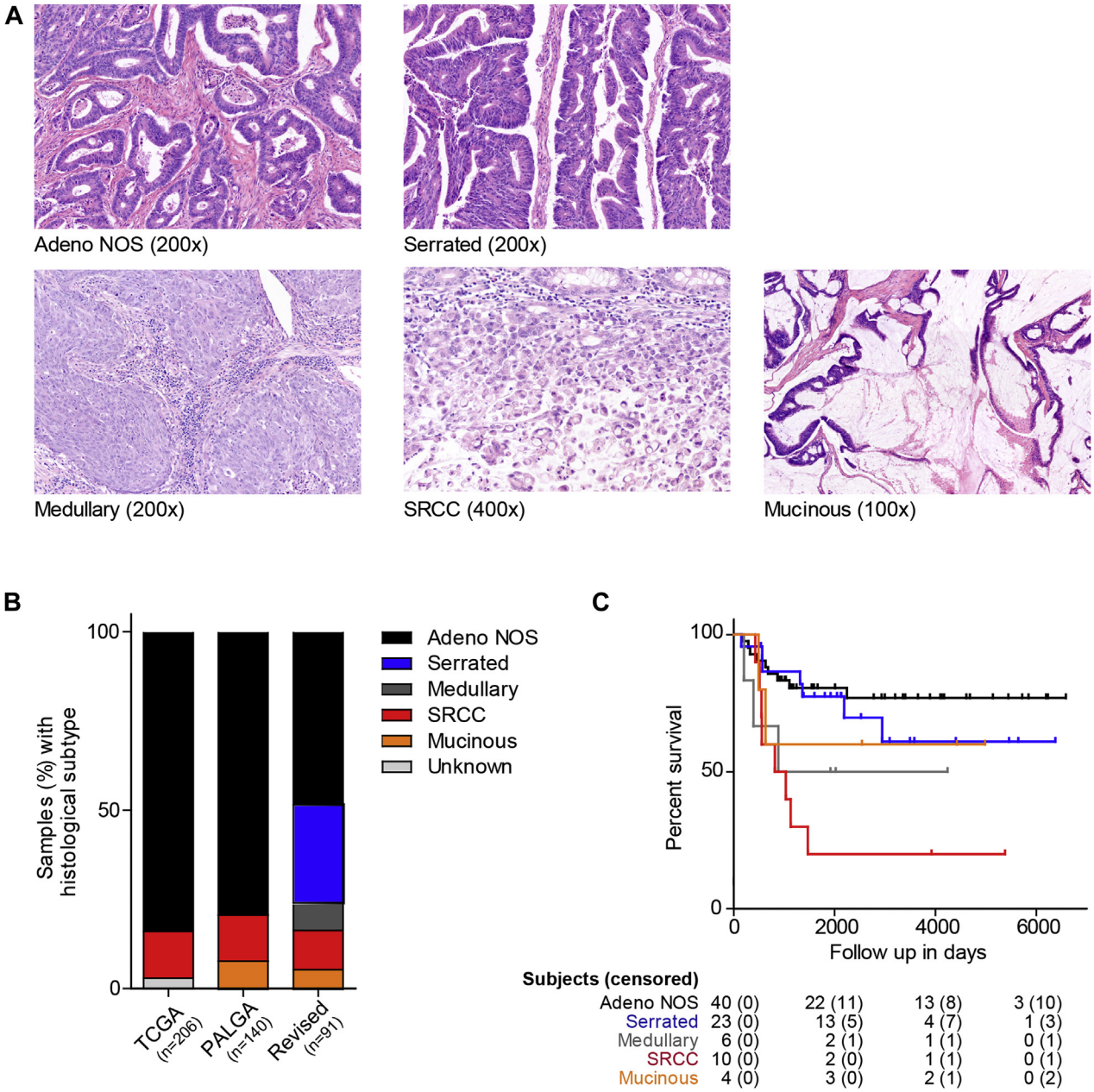

Figure 2. Histologic subtypes and survival analysis of colorectal cancer in patients age 25 years or younger. (*A*) Representative images of the 5 histologic subtypes observed. (*B*) Percentage of samples with a histologic subtype in each analyzed group. The Cancer Genome Atlas: colorectal cancers (CRCs) developed in patients age 60 years and older.[14] PALGA: CRCs developed in patients age 25 years and younger as reported in the Dutch Pathology Registry (n = 140). Revised: CRCs developed in patients age 25 years and younger that were revised by 2 expert pathologists (n = 91). (*C*) Survival analysis of adolescents and young adults (AYAs) with CRC based on the revised histologic subtypes. NOS, not otherwise specified; SRCC, signet ring cell carcinoma.

Most cases showed an intestinal IHC pattern with strong expression of Caudal Type Homeobox 2 (CDX2) (94%), moderate to strong expression of Keratin 20 (CK20) (97%), and, in a minority, CK7 expression (19%). Neuroendocrine IHC expression was observed in 16 of 74 available tumors (22%) (Supplementary Tables 1 and 8). Survival related to neuroendocrine differentiation showed no difference in prognosis between positive and negative tumors (Supplementary Figure 1*E*).

CMS of CRC was possible for 80 tumors (79 individuals). In 32 tumors MSI was reported and/or detected (see later), and these were classified as CMS1 (Supplementary Tables 1, 2, and 9). Twenty-seven tumors were classified as CMS2/3. In 8 of these tumors a *KRAS* mutation was identified (see later), which may suggest a CMS3 subtype. The other 21 cases had moderate-to-strong expression of 2 or 3 mesenchymal markers with or without CDX2 expression and were classified as CMS4 (Supplementary Tables 1, 2, and 9). AYA-CRC with a CMS4 classification showed a poorer prognosis than the CMS1/2/3 subtypes (Supplementary Figure 1*F*).

DNA was isolated from 83 tumors (82 individuals) and from 81 matched normal tissues (80 individuals). Twenty-nine tumors (35%) were MSI-high and 54 tumors were MSS (65%), which is representative of the complete cohort (Supplementary Figure 2*B*). The germline and somatic mutation status of each tumor was investigated by targeted sequencing. For 73 tumors (72 individuals) sufficient coverage was achieved (median unique coverage of consensus reads per smMIP, 146; range, 12–1308). For 75 individuals a normal sample was available (median unique coverage per smMIP, 160; range, 14–1174).

For 75 individuals pathogenic germline variants in genes associated with gastrointestinal cancer were assessed (Supplementary Table 4). In 22 of 27 MSI-high cases a monoallelic germline pathogenic variant or deletion in *MLH1* ($n = 10$), *EPCAM* ($n = 2$), *MSH2* ($n = 9$), or *MSH6* ($n = 1$) was observed (Supplementary Table 1). In addition, in 2 MSI-high cases a homozygous pathogenic germline variant was detected in *MSH2* or *MSH6*, indicating constitutional mismatch repair deficiency syndrome (CMMRD). In 3 MSI-high tumors we did not identify a germline pathogenic variant: 2 tumors harbored 1 somatic mismatch repair (MMR) mutation and 1 tumor showed somatic *MLH1* promoter methylation. Five MSS CRCs harbored a pathogenic germline variant in *APC*. Taken together, a germline genetic tumor risk syndrome was identified in 29 of 75 cases (39%). Survival analyses suggest that the 5-year survival for cases with a genetic tumor risk syndrome is slightly better (73%) compared with cases without a genetic tumor risk syndrome (63%), albeit this was not significant (Supplementary Figure 1*G*).

To analyze the somatic mutation spectrum we extracted all somatic nonsynonymous and canonical splice-site mutations. Two samples were excluded owing to low neoplastic cell counts (<30% neoplastic cells). Four genes were mutated in more than 50% of MSI-high tumors: *ACVR2A* (91%), *APC* (82%), *KRAS* (68%), and *ARID1A* (55%) (Figure 3*A*). In contrast, in MSS tumors only *TP53* was mutated in more than 50% of tumors (73%), which is a significant enrichment compared with MSI-high tumors (73% vs 36%; $P < .01$). *APC* and *KRAS* were mutated in 28% ($n = 16$) and 21% ($n = 15$) of MSS samples, respectively, which is significantly less in MSI-high tumors ($P < .001$ and $P < .01$, respectively) (Figure 3*B*). Three MSS samples without mutations in *APC* harbored mutations in *CTNNB1*. Five MSS AYA-CRCs harbored a pathogenic *POLE*-exo mutation and the somatic mutation profile of these tumors highly resembled Catalogue of Somatic Mutations in Cancer signatures SBS10a and SBS10b (Supplementary Figure 3). Furthermore, we detected 1 *BRAF* V600E mutation, which was present in a MSS tumor (PT029).

In total, 44 MSS AYA-CRCs were analyzed for somatic copy number alterations (SCNAs). We classified these tumors into 3 groups: (1) tumors with no or only 1 focal SCNA smaller than 15 Mb in size ($n = 18$); (2) tumors with 2 or more SCNAs more than 15 Mb in size ($n = 20$); and (3) tumors with more than half of the autosomes affected by a SCNA ($n = 6$) (Figure 3*D*, Supplementary Figure 4). In group II we observed recurrent gains (≥4 tumors) of 7p and q, 8q, 13q, 16q, and 20p and q, and losses (≥4 tumors) of 1p, 4p and q, 8p, 9p, 14q, 15q, 17p, 18p and q, 21q, and 22q. In group III ($n = 6$), 5 tumors showed an almost-complete genome duplication and 1 tumor was near-haploid.

We performed an integrated analysis using the available clinical, histopathologic, copy number, and/or mutation data. MSI-high tumors were located mostly in the left side of the colorectum and in stages I and II, albeit this is not significantly different from MSS tumors (72% vs 67% and 45% vs 33%, respectively). MSI-high tumors were enriched significantly for tumors with serrated histology compared with MSS tumors (45% vs 20%; $P = .04298$) (Figure 3*C*). Next, we compared the histopathologic findings in each of the SCNA groups. SCNA group I tumors were located more frequently in the proximal colon than tumors in group II ($P < .001$). SCNA group II tumors were of lower tumor stage (36% stage I; $P = .05$). Furthermore, SCNA group I tumors were enriched significantly for mucinous histology (33%; $P = .01$) and enriched for neuroendocrine differentiation (43%; $P = .05$) compared with groups II and III. Next, we analyzed the top most frequently mutated genes in each group and observed that the 5 *POLE*-exo mutated samples were enriched significantly in SCNA group I (29%; $P = .02$), although this was not significant after correction for multiple testing ($P = .057$). All tumors with pathogenic germline *APC* variants clustered into SCNA group II (Figure 3*D*).

When comparing the percentage of the genome affected by a SCNA and specific SCNAs between AYA-CRC and in patients age 60 years and older from the TCGA,[15] we observed that MMR-proficient AYA-CRCs were more frequently chromosomal stable (32% vs 10%; $P < .001$). Furthermore, MMR-proficient AYA-CRC less frequently harbored deletions of chromosome 17p (28% vs 56%; $P < .001$) and 18q (26% vs 66%; $P < .001$), regions that carry the *TP53* and *SMAD4* genes, compared with TCGA-CRCs in patients age 60 years and older, respectively. When comparing SCNA groups, SCNA group II was most similar to sporadic late-onset CRC based on gains of chromosome arms 7p and q, 8q, 13p and q, and 20p, and losses of chromosome arms 8p, 14q, 15q, 17p, and 18p and q.

Next, we compared the mutation frequency of the most frequently mutated genes in AYA-CRC with that of the TCGA-CRCs in patients age 60 years and older. MSI-high AYA-CRCs more frequently harbored mutations in *APC* (62% vs 35%; $P = .001$) and *KRAS* (68% vs 23%; $P = .003$) compared with the MSI-high TCGA tumors (Supplementary Figure 5*A*). Somatic mutations in *APC* occurred less frequently (34% vs 82%; $P < .001$), and mutations in *TP53* occurred more frequently in MSS

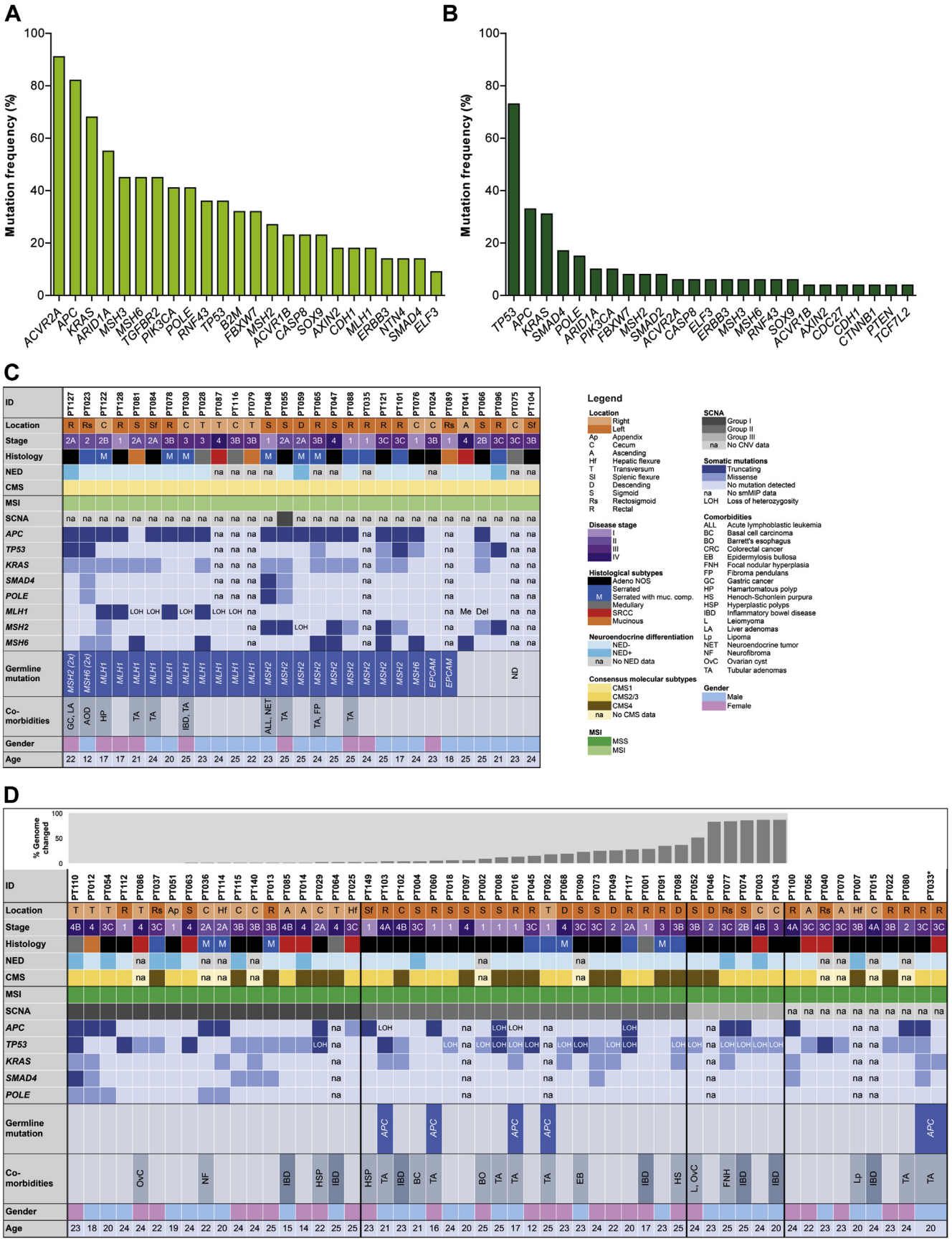

Figure 3. Mutation frequencies in microsatellite instability (MSI)-high and mismatch repair–proficient colorectal cancers (CRCs) at adolescent and young adult (AYA) age. Recurrently mutated genes in (*A*) MSI-high and (*B*) mismatch repair–proficient CRCs at AYA age. Integrative analysis of (*C*) genomic changes in MSI-high and in (*D*) mismatch repair-proficient CRC at AYA age.

AYA-CRCs ($n = 47$) than in the MSS TCGA tumors (74% vs 56%; $P = .02783$) (Supplementary Figure 5*B*). Furthermore, *POLE*-exo mutations occurred significantly more frequently in MSS AYA-CRCs than in the MSS TCGA tumors (11% vs 3%; $P = .03$). Collectively, these data suggest that AYA-CRCs develop through different molecular pathways than those diagnosed in patients older than age 60 years.

Discussion

We investigated the clinical and molecular features of CRC developed at an extremely young age (age, ≤25 y), representing the 0.1% youngest individuals who develop CRC. Important hallmarks of AYA-CRCs are advanced disease stage at presentation, a high incidence of second primary malignancies (14%), genetic tumor risk syndromes (39%), and IBD (8.4%). A substantial part of AYA-CRC shows mucinous or signet ring cell tumor components (33%). Furthermore, we identified an overrepresentation of MSS and chromosomal-stable CRCs, and tumors with a *POLE*-exo mutation in the AYA population compared with CRC diagnosed in patients age 60 years and older. These data indicate that AYA-CRC may develop through different pathways than CRC at an older age, which may open up new opportunities for treatment of CRC at this extremely young age.

Negative predictors of outcome in our study were a younger age at diagnosis (age, <16 y), SRCC histology, and advanced disease stage at diagnosis. In our cohort, 66% of individuals presented with advanced stage of disease (stages III or IV), compared with approximately 46% of adult individuals in The Netherlands.[1] This difference may be explained by a doctors delay in the recognition of CRC in young individuals because it is a rare phenomenon. In addition, AYA-CRC might have more aggressive behavior, leading to rapid development of metastases. An increasing incidence of CRC in individuals younger than age 50 has been reported,[3] but we did not observe this in our AYA cohort. This discrepancy might be explained by the fact that CRC in patients age 25 years or younger is associated frequently with genetic predisposition, for which the incidence likely will not increase over time.

Nine individuals in our cohort had CRC and a concomitant diagnosis of IBD, reflecting an annual incidence of IBD-related CRC of 8.4%, which is a 10- to 15-time increase compared with IBD-related CRC in The Netherlands (0.6%).[19] The literature suggests that the risk of CRC in individuals with IBD is increased, with a cumulative incidence of 7.5% to 18% after being affected by colonic inflammation for 30 years.[20,21] Approximately 8 to 10 years after diagnosis of IBD the risk increases significantly above that of the general population. Six individuals in our cohort had relatively long-standing inflammatory disease (6–9 y), and therefore inflammation may have increased their CRC risk significantly. Young age at IBD presentation (age, <20 y) has been described as a risk factor for developing CRC at a young age,[21] but generally CRC does not develop before age 25 years. Because it is known that IBD is associated with a genetic predisposition, certain genetic predispositions possibly are associated with IBD and also may play a role in developing AYA-CRC.

A genetic tumor risk syndrome was identified in 39% of individuals with AYA-CRCs, which is in line with previous findings.[10] However, this percentage may be higher because we have not tested all CRC-predisposing genes, such as *MUTYH* and *NTHL1*. Nevertheless, the percentage of individuals with a genetic predisposition is much higher than in adults with CRC,[22] highlighting the importance of referral of these young individuals for germline genetic testing. The majority of individuals tested positive for Lynch syndrome (29%) and 5 individuals had CMMRD. Individuals with CMMRD developed CRC at younger ages compared with the total cohort (median age, 15 vs 23 y; $P = .027$). In 13 of 19 cases (68%) with a second primary malignancy, a genetic predisposition was identified, confirming that in individuals with 2 malignancies at a young age, a genetic tumor risk syndrome is very likely.[23]

Our analysis showed notable differences for the somatic mutation spectrum in *APC*, *TP53*, and *POLE* between MSS AYA-CRC and CRC at later onset. Lieu et al[24] recently reported that *APC* also was mutated less frequently in MSS CRC patients younger than age 40 years compared with CRC in patients older than age 50 years, although a higher mutation frequency was observed for *APC* by Salem et al[25] in a similar group of patients. Interestingly, CRCs without an *APC* mutation were described to have a worse prognosis.[26] Indeed, MSS AYA-CRCs lacking *APC* mutations had an overall worse survival in our cohort, albeit this was not significant, likely owing to the small cohort size (Supplementary Figure 1*H*). In contrast, MSS AYA-CRCs more frequently harbor *TP53* mutations compared with late-onset CRCs, which also was seen by Lieu et al,[24] again suggesting a different etiology in early onset compared with late-onset CRC.

We observed an increased incidence of *POLE*-exo mutations in the in MSS AYA-CRCs compared with adult-onset CRC. Somatic *POLE*-exo mutations have been associated previously with CRC at a younger age,[12] but thus far have not been described in detail in individuals who developed CRC at age 25 years and younger. This finding may have clinical implications because *POLE*-mutated samples are associated with hypermutated genomes and an increased number of mutation-associated neoantigens.[14] Such tumors can be sensitive for new therapeutic modalities such as immune-checkpoint inhibitors.[27,28] This treatment also is relevant for stage III or IV MSI-high AYA-CRC, which is a substantial percentage of MSI-high CRCs at this age (45%). Collectively, based on MSI-high status and *POLE*-mutations, immune-checkpoint inhibitors may be relevant for more than 30% of individuals who develop AYA-CRC. Furthermore,

because both MSI-high and *POLE* mutated tumors are (near-)diploid (TCGA[14] and this study), we hypothesize that SCNA group I tumors (which are near-diploid), may unravel additional hypermutated samples that may benefit from these types of therapy.

In conclusion, AYA-CRC is different from later-onset CRC, both at a clinical and molecular level. A substantial proportion of AYAs with CRC have a genetic tumor risk syndrome, which may have a big impact on the clinical management, in terms of treatment and screening, for the patient and his relatives. Furthermore, a significant number of AYA-CRCs harbor somatic mutations that may have important therapeutic consequences. Therefore, each individual diagnosed with CRC at age 25 years or younger should at least undergo germline and somatic sequencing for an extensive panel of tumor-predisposing and actionable genes.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2020.06.034.

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The authors disclose no conflicts.

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Supplementary Methods

Study Cohort

Cases with CRC and/or appendiceal cancer age 25 years and younger between 2000 and 2017 in The Netherlands were collected via the nationwide network and registry of histopathology and cytopathology (PALGA) and NCR. The linked data were processed and sent to us deidentified. From each case, information about age at diagnosis, sex, location of the primary tumor, pathologic TNM (American Joint Committee on Cancer, 8th ed), tumor stage, MSI, IHC, presence of a genetic tumor risk syndrome, treatment, and survival was collected. Follow-up data on the vital status of the individuals was collected until February 2019. All data on previous or later medical conditions that were confirmed by a pathologist were obtained.

Exclusion criteria were as follows: diagnosis of a nonepithelial tumor, cases with adenomas with only low-grade or high-grade dysplasia (including carcinoma-in-situ) and cases from abroad (that had a consultation in The Netherlands).

Statistical Analysis

Demographics, clinical data, and pathologic characteristics were analyzed. A chi-square test was performed to determine the association between variables. The incidence of CRC in patients age 25 years and younger was calculated by dividing the number of cases by the total Dutch population of patients age 25 years and younger for each of the years between 2000 and 2017 (available at statline.cbs.nl). Overall survival was defined as the time in days between cancer diagnosis and death resulting from any cause. Survival was displayed using Kaplan–Meier analysis for various variables and probability distributions were compared using the exact log-rank test using SPSS software (IBM SPSS Statistics, version 25, New York). Differences between groups were determined using the Fisher exact test using R (RStudio, version 1.1.456, Vienna, Austria).

Immunohistochemistry

IHC was performed on 4-μm, paraffin-embedded, whole-slide sections of cases in which sufficient material was available ($n = 74$). Whole slides were stained for CDX2 (differentiation marker with high expression in epithelial-like tumors), FRMD6 (marker for goblet cells expressed in mesenchymal-like CRCs), HTR2B (high expression in mesenchymal CRCs), and ZEB1 (marker for epithelial to mesenchymal transition). IHC for CK7, CK20, CDX2, chromogranin, synaptophysin, and CD56 was performed using the Immunologic Autostainer 480 (Immunologic, Duiven, The Netherlands). Staining for ZEB1, HTR2B, and FRMD6 was performed manually on the MSS cases for which tissue was available ($n = 48$). Sections were deparaffinized in xylene and rehydrated through an ethanol series. Heat-induced epitope retrieval was performed in either EDTA buffer, pH 9.0, or 10 mmol/L sodium citrate (pH 6.0) buffer, depending on the antibody. After cooling to room temperature, endogenous peroxidase was blocked for either 10 or 20 minutes in 3% H_2O_2, depending on the antibody. Slides were incubated with primary antibodies as indicated in Supplementary Table 3, followed by incubation with a 1:1 dilution of poly-horseradish peroxidase–anti-mouse/rabbit IgG secondary antibody (Brightvision; Immunologic, Duiven, The Netherland) for 30 minutes at room temperature. Visualization was performed with a 1:25 dilution of diaminobenzidine (Immunologic) for 7 minutes at room temperature. Between every incubation, slides were washed in phosphate-buffered saline, and after incubation with DAB, slides were washed in tap water. Slides were counterstained with Mayer's hematoxylin.

Single-Molecule Molecular Inversion Probe Targeted Enrichment and Sequencing

We designed a smMIP panel using the procedure described elsewhere.[1] The genes included in the analyses were divided into 2 smMIP pools: 1 pool with genes that recently were described in the literature to play a role in CRC development (pool CRC1) (Supplementary Table 4), and a second pool for genes associated with germline predisposition, classic drivers of CRC, and genes with a therapeutic potential (pool CRC2) (Supplementary Table 4). In brief, a total of 100 ng of each normal and tumor genomic DNA was used per capture reaction with a molecular ratio of 1:800 between genomic DNA and smMIPs for every individual smMIP. After denaturation, the mixes were incubated for probe hybridization, extension, ligation, and exonuclease treatment. Per sample, 4 individual polymerase chain reactions were performed, these subsequently were pooled and purified, followed by semiautomated library preparation and sequencing on a NextSeq500 (Illumina, San Diego, CA) according to the manufacturer's protocol (300 cycles High Output sequencing kit), resulting in 2 $\times$ 150 bp paired-end reads. Sequencing reads were aligned to the reference genome (human genome 19) and variants were called using Sequence Pilot (JSI Medical Systems, Ettenheim, Germany) as described previously.[1] A consensus read was built for each single molecule and the following settings were used for variant calling: minimal absolute coverage of 20 combined reads, minimal absolute coverage of 5 variant reads, and minimal 5% variant reads per direction. Each unique smMIP provides a read depth of 2 based on both a forward and a reverse read using this software package. Variant call format files were extracted and annotated using an in-house developed annotation pipeline.[2] After mapping and variant calling, each sample was assessed for

coverage of the target regions. A sample was excluded if more than 25% of the smMIP probes in each pool had a coverage of less than 10 unique (consensus) reads (Supplementary Table 5). We obtained sufficient sequencing reads for 72 AYA tumor samples (22 MSI and 50 MSS tumors). The sequencing data were analyzed in a 2-step approach: an analysis of pathogenic variants in genes associated with CRC predisposition in both the normal and tumor tissues, and an analysis of the somatic mutations in the genes associated with CRC tumorigenesis and treatment.

Identification and Selection of Germline Variants in Colorectal Cancer–Predisposing Genes

All potential pathogenic germline variants in noncancerous colorectal tissues in *APC*, *CDH1*, *MLH1*, *MSH2*, *MSH6*, *POLD1* (only exonuclease domain), *POLE*, *SMAD4*, *PTEN*, and *TP53* were selected by the following steps: (1) all variants covered by less than 10× unique reads were removed (on average, 95.4% of targets; $n = 1053$ probes to the aforementioned genes were covered by ≥10× reads) (Supplementary Table 5); (2) all variants covered by less than 30% variant unique reads were removed; (3) all variants with an allele frequency greater than 0.01% in our in-house variant database and greater than 0.01% in The Exome Aggregation Consortium were removed; and (4) all synonymous variants or variants located outside of the coding sequence and canonical splice sites (outside intronic positions −2 till +2) were removed. Next, each variant was classified based on existing guidelines for variant classification that were determined by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.[3] Furthermore, presence of the germline variant was assessed in the matched tumor sample. Samples that tested positive for MSI but remained without a germline pathogenic variant in 1 of the MMR genes were resequenced using smMIPs to exclude missed pathogenic variants in MMR genes *MLH1*, *MSH2*, *MSH6*, and *PMS2*.[1]

Identification and Selection of Somatic Variants in Genes Associated With Colorectal Cancer Tumorigenesis and Treatment

All somatic variants in each smMIP pool were selected using the following steps: all synonymous variants or variants located outside of the coding sequence and canonical splice sites (outside intronic positions −2 till +2) were removed, and all variants called in the matched normal genomic DNA sample were removed. Subsequently, we selected all variants that were called in at least 2 independent smMIP regions, or variants that were supported by at least 10 unique variant reads and at least 10% unique variant read percentage covered by a single smMIP. All selected variants were checked manually by comparing normal and tumor sequencing data in Sequence Pilot and variants that still represented spontaneous deaminated cytosine mutations, germline variants, or variants present only directly at the start or end of reads and/or absent in the overlapping MIP were removed.

Somatic variants in samples for which a normal tissue sample was missing or in which insufficient sequencing data were generated ($n = 6$) were selected as follows: all synonymous variants or variants located outside of the coding sequence and canonical splice sites (outside intronic positions −2 till +2) were removed, and all variants with a frequency greater than 0.001% in ExAC were removed. Subsequently, we selected all variants that were called in at least 2 independent smMIP probe regions or variants that were supported by at least 10 variant reads and at least 10% variant read percentage. Furthermore, variants that were removed in the matched normal and tumor analysis (described earlier) were removed manually to prevent inclusion of false-positive somatic mutations. All variants are listed in Supplementary Table 6.

Mutational Signature Analysis

For cases with more than 10 nonsynonymous single-nucleotide variants we extracted all synonymous and nonsynonymous single-nucleotide variants and inferred the contribution of the 49 mutational signatures (V3) available at the Catalogue of Somatic Mutations in Cancer using the R package DeconstructSigs.[4]

OncoScan Formalin-Fixed, Paraffin-Embedded Arrays

SCNA profiles and B-allele frequency plots were generated and analyzed using Nexus Copy Number 9.0 software (BioDiscovery, El Segundo, CA) using the SNP-FASST2 segmentation algorithm, specifically designed for OncoScan formalin-fixed, paraffin-embedded arrays. For each sample the baseline was set manually using the copy number status and B-allele frequencies and individual SCNAs were called with at least 10 consecutive probes per segment. All called SCNAs were checked manually and curated if needed. We estimated the percentage of genome altered based on the length of each SCNA on the autosomal regions as determined by Nexus Copy Number.

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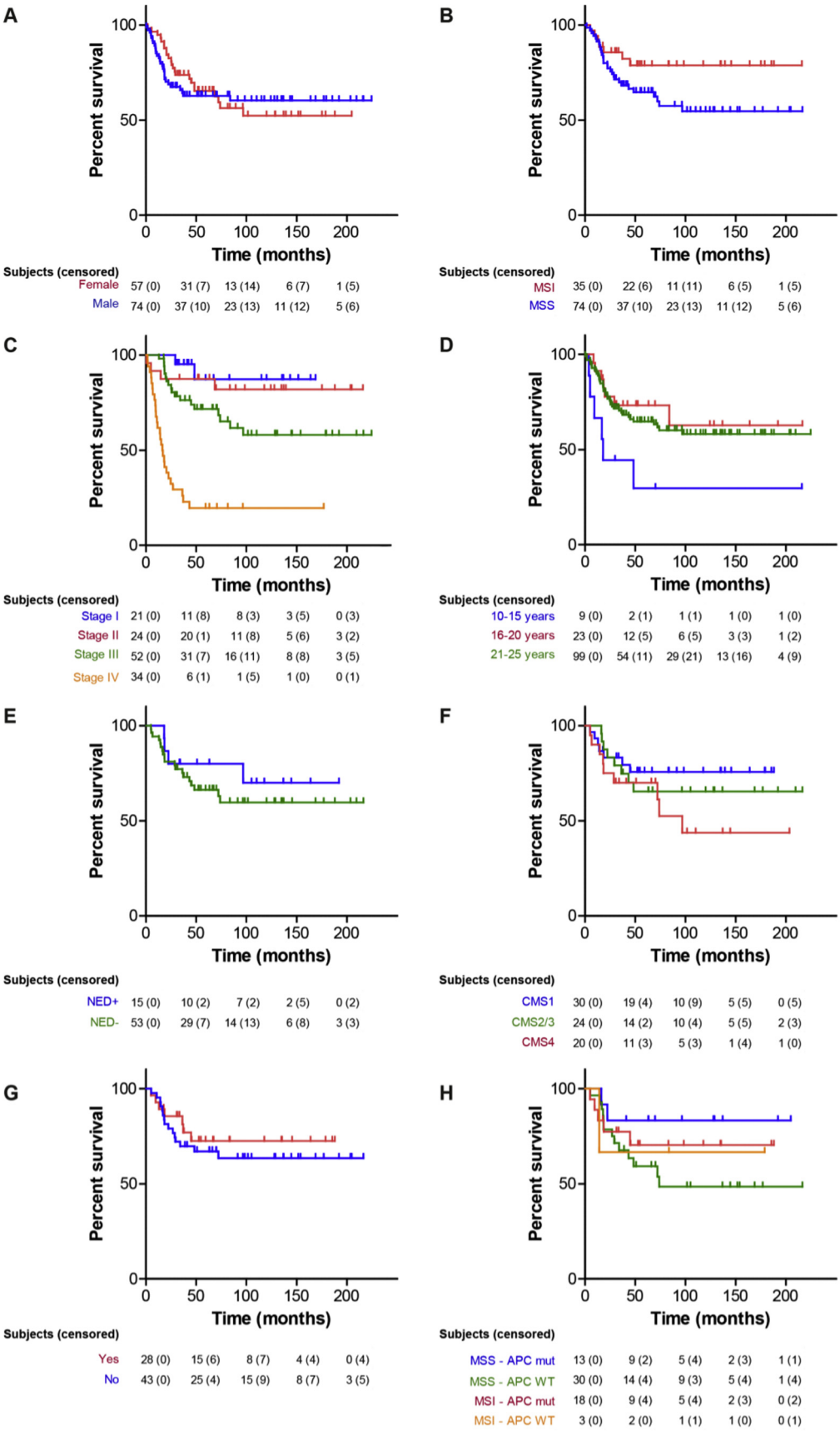
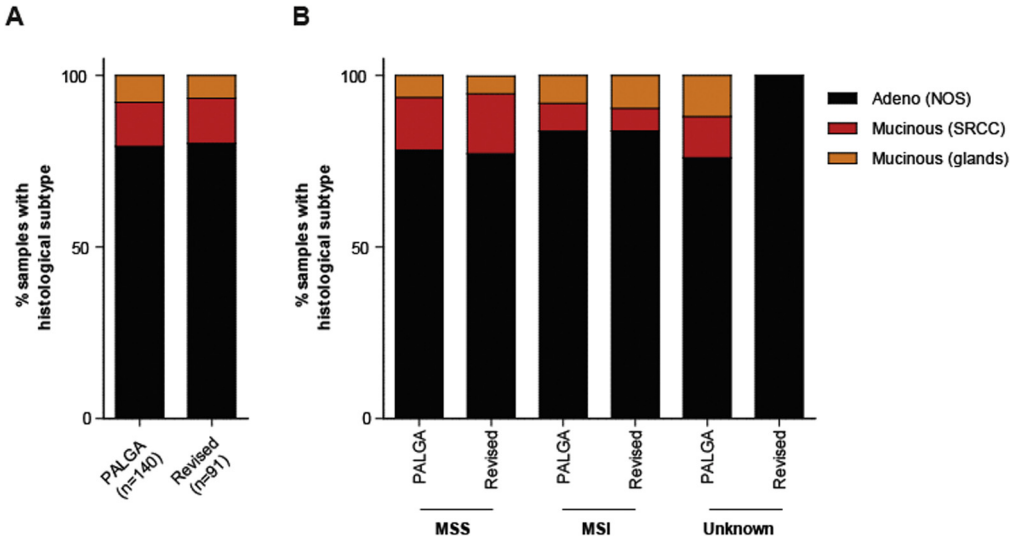
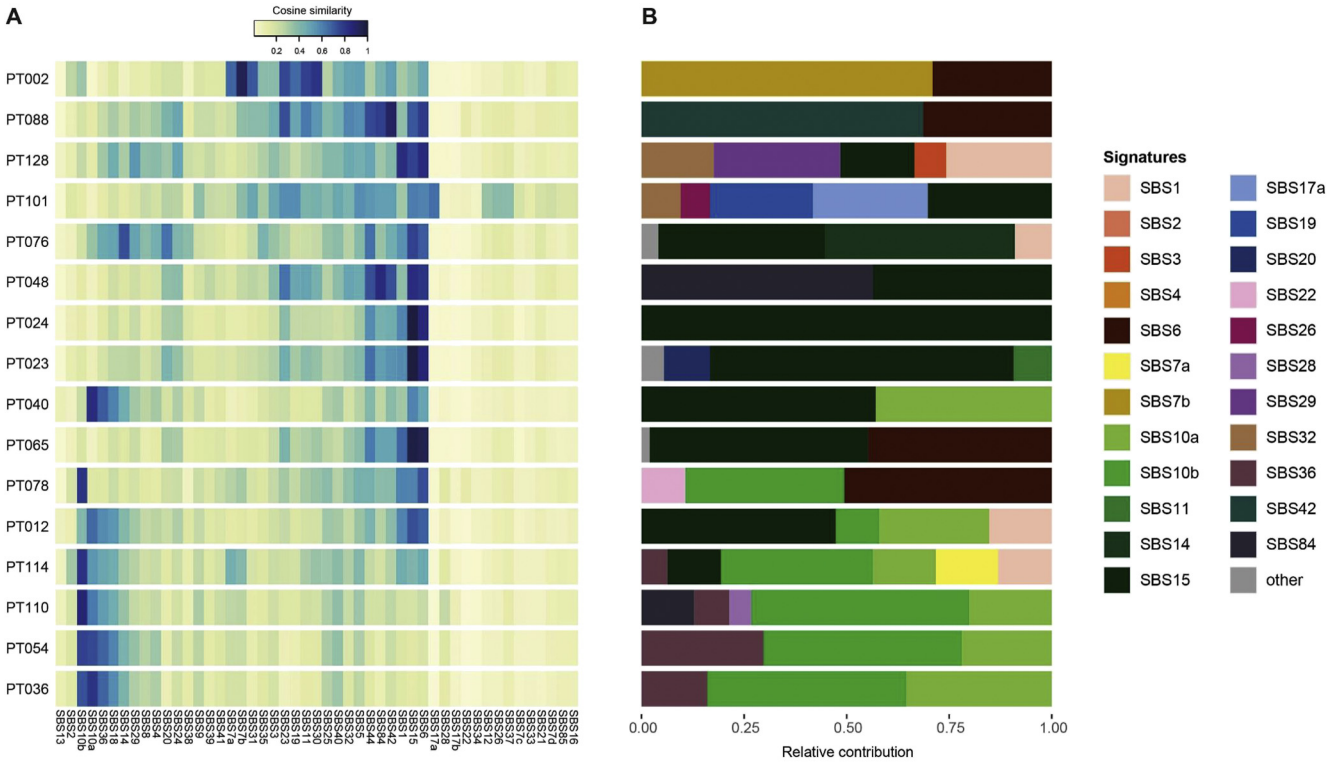


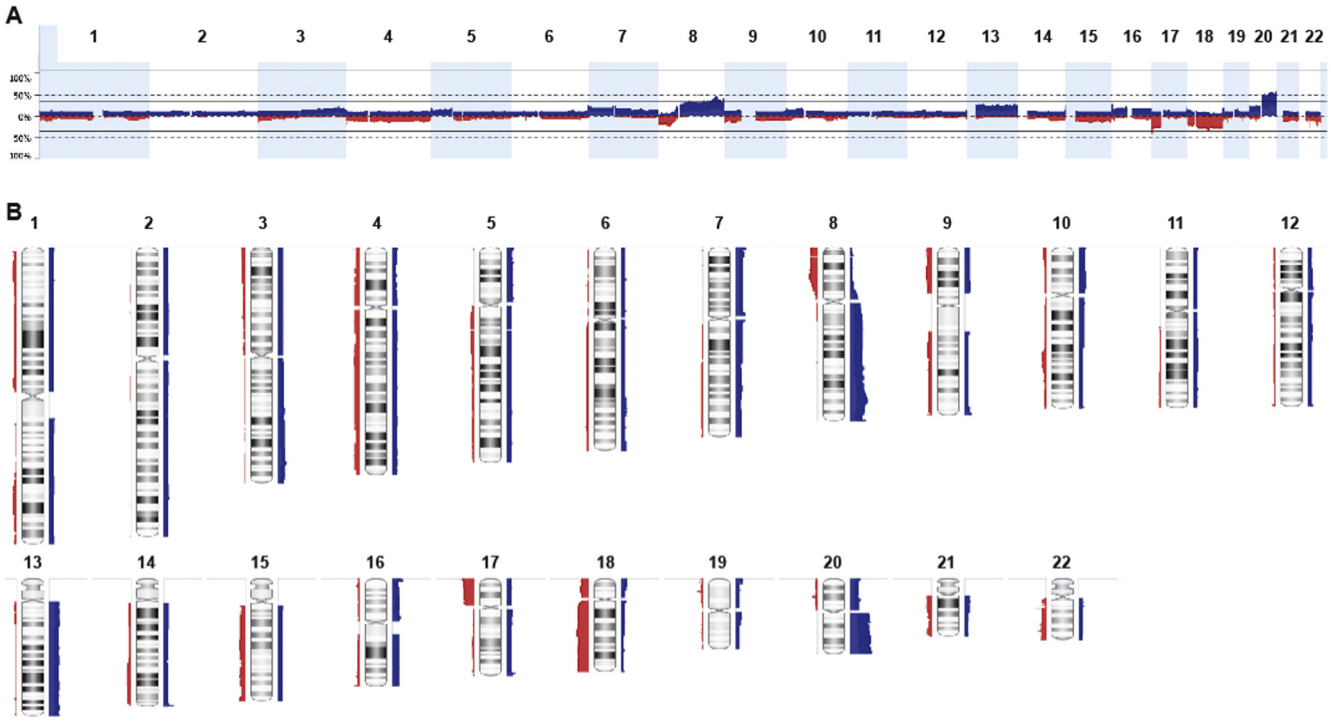
Supplementary Figure 1. Survival of children adolescents, and young adults (AYAs) with colorectal cancer (CRC) based on clinical and molecular features. Survival was based on (*A*) sex, (*B*) microsatellite instability (MSI) status, (*C*) disease stage, (*D*) age, (*E*) neuroendocrine differentiation (NED), (*F*) consensus molecular subtypes (CMS), (*G*) presence of a genetic tumor risk syndrome, and (*H*) somatic *APC* mutation status in MSI and microsatellite stable (MSS) CRCs.



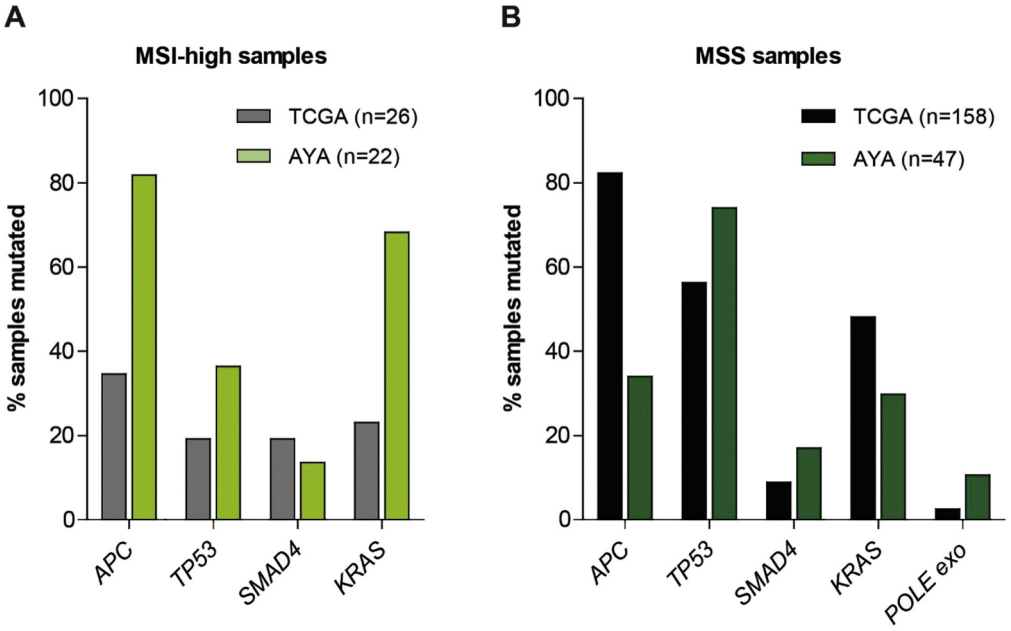
Supplementary Figure 2. Comparison histology as reported in PALGA for complete and revised cohorts. (*A*) Complete Nationwide network and registry of histo- and cytopathology and revised cohort. (*B*) Histologic subtypes were divided among those who have microsatellite stable (MSS) and microsatellite instability (MSI) CRCs. NOS, not otherwise specified; SRCC, signet ring cell carcinoma.



Supplementary Figure 3. Mutational signature analysis of *POLE* mutated colorectal cancers (CRCs). (*A*) Heatmap showing the cosine similarity scores for the mutation spectrum of each indicated *POLE*-mutated CRC and the 49 (V3) Catalogue of Somatic Mutations in Cancer (COSMIC) mutational signatures. (*B*) The estimated relative contribution of each COSMIC V3 signature to the mutation spectrum of each indicated CRC after refitting to the 49 V3 COSMIC signatures is shown.



Supplementary Figure 4. Somatic copy number alteration (SCNA) analysis in colorectal cancer (CRC) in children adolescents, and young adults (AYAs). (*A*) Aggregate view of SCNAs identified in 44 microsatellite stable (MSS) CRCs in AYAs. (*B*) Aggregate per chromosome of SCNAs identified in 44 MSS CRCs in AYAs. Red, SCNA losses; blue, SCNA gains.



Supplementary Figure 5. Mutation frequency of the most frequently mutated genes in colorectal cancer (CRC). The percentage of samples with driver gene mutations is shown for CRCs developed in adolescents and young adults (AYA) who have microsatellite instability (MSI-high) (*A*) or are mismatch-repair proficient (microsatellite stable [MSS]), (*B*) compared with the set of tumor samples from The Cancer Genome Atlas (TCGA) cohort that were developed at 60 years of age and older.

Supplementary Table 2. Colorectal Consensus Molecular Subtypes

CMS subtype	CMS1 (MSI immune)	CMS2 (canonical)/CMS3 (metabolic)	CMS4 (mesenchymal)
Type	MSI, mucinous, inflammatory subtype	Epithelial-like, chromosomal instable	Mesenchymal-like, EMT, MSS, stemness
Distribution/histology	Left-sided, serrated subtype	Right-sided, traditional subtype	Evenly distributed, enriched with poorly differentiated cancers
Immunohistochemical profile	MSI	CDX2+, ZEB1- (low), HTR2B+, FRMD6+/-	CDX2-, ZEB1+, HTR2B++ (higher intensity), FRMD6++ (higher expression)

CDX2, Caudal Type Homeobox 2; CMS, consensus molecular subtype; EMT, epithelial to mesenchymal transition; FRMD6, Ferm domain-containing protein 6; HTR2B, Hydroxytryptamine Receptor 2B; MSI, microsatellite instability; MSS, microsatellite stable; ZEB1, Zinc finger E-box-binding homeobox 1.
Data are from Trinh et al[5] and Roseweir et al.[6]

Supplementary Table 3. Details of Immunohistochemical Antibodies

Antigen	Antibody	Manufacturer	Cat number	Retrieval, *min*	Peroxidase block, *min*	Dilution	Incubation time and temperature	Cellular location
CK7	Mouse (M)	Cell Marque (Rocklin, CA)	ILM 54411 C1	10 EDTA	10	1:800	1 h, room temperature	Membrane
CK20	Rabbit (M)	Immunologic	ILM2133-C1	10 sodium citrate	10	1:400	1 h, room temperature	Membrane
CDX2	Mouse (M)	Cell Marque	235R-16	10 EDTA	10	1:50	1 h, room temperature	Nucleus
Chromogranin	Mouse (M)	ThermoFisher Scientific (Waltham, MA)	MS-324-P1	10 EDTA	10	1:8000	1 h, room temperature	Cytoplasm
Synaptophysin	Rabbit (M)	Cell Marque	336R-96	10 EDTA	10	1:100	1 h, room temperature	Membrane
CD56	Rabbit (M)	Cell Marque	156R-96	10 EDTA	10	1:500	1 h, room temperature	Membrane
ZEB1	Rabbit (P)	Sigma-Aldrich (St. Louis, MO)	HPA027524	20 sodium citrate	20	1:400	o/n, 4°C	Nucleus and cytoplasm
HTR2B	Rabbit (P)	Sigma-Aldrich	HPA012867	20 sodium citrate	20	1:400	1 h, room temperature	Membrane
FRMD6	Rabbit (P)	Sigma-Aldrich	HPA001297	20 sodium citrate	20	1:800	1 h, room temperature	Cytoplasm

CDX2, Caudal Type Homeobox 2; CK20, Keratin 20; FRMD6, Ferm domain-containing protein 6; HTR2B, Hydroxytryptamine Receptor 2B; M, monoclonal antibody; o/n, overnight; P, polyclonal antibody; ZEB1, Zinc finger E-box-binding homeobox 1.

Supplementary Table 8. Immunohistochemical Staining Results ($n = 74$)

Antibody	No expression, <1%	Slight–moderate expression	Strong expression	Total number of samples
Cytokeratin 7	60	11	3	74
Cytokeratin 20	2	23	49	74
CDX2	2	2	70	74
CD56	70	2	2	74
Chromogranin	60	14	0	74
Synaptophysin	69	3	2	74

CDX-2, Caudal Type Homeobox 2.

Supplementary Table 9. Colorectal MSS Consensus Molecular Subtypes ($n = 48$)

Antibody	No expression (<1%)	Slight expression	Moderate expression	Strong expression
FRMD6	5	29	7	7
HTR2B	6	25	9	8
ZEB1 (membranous/cytoplasm)	9	31	0	8
ZEB1 (nuclear)	41	7	0	0

NOTE. Thirty cases showed microsatellite instability and were categorized as CMS1; of 48 stained cases, 27 cases were classified as epithelial (CMS2/3) and 21 were classified as mesenchymal (CMS4) subtype.
FRMD6, Ferm domain-containing protein 6; HTR2B, 5-Hydroxytryptamine Receptor 2B; MSS, microsatellite stable; ZEB1, Zinc finger E-box-binding homeobox 1.