MLH1 Lynch Syndrome Colorectal Cancers Are Driven by Heterogeneous Wnt Pathway Gene Mutations

A germline pathogenic variant (PV) in *MLH1* (*path_MLH1/path_MMR*) is 1 of the 2 major instigators of Lynch syndrome (LS), alongside *path_MSH2. Path_MLH1* carriers present with a starkly reduced adenoma, yet equal colorectal carcinoma (CRC) incidence compared with *path_MSH2* carriers.[1] *CTNNB1* mutations are common in CRC from *path_MLH1* carriers, but not in other *path_MMR* CRC,[1–3] and some data suggest enrichment of *CTNNB1* mutations in surveillance-detected *MLH1* CRC.[4] A "2-in-1" mechanism involving a copy number alteration (CNA) of the *MLH1*-mut/*CTNNB1*-mut chromosome harboring the patient's mutant germline *MLH1* allele was recently postulated to explain this tendency.[5] To validate the mechanism, whole-exome sequencing of 51 LS CRC from 42 *path_MLH1* carriers was performed. Normal colonic mucosa was available as reference for 22 cancers.

Formalin-fixed, paraffin-embedded CRC samples were collected through pathology department archives at the Central Finland Hospital Nova in Jyväskylä (Finland) and Helsinki University Hospital (Finland). Tumor genomic DNA was isolated, prepared, and finally sequenced with the NovaSeq System (Illumina). Variant and CN calling were performed using Dragen (Illumina), Mutect2 (GATK), and PureCN (Bioconductor). Only variants annotated as PV were included. *P* values were adjusted for multiple hypotheses using the Benjamini-Hochberg method. The methods are detailed in the Supplementary Material.

Most cancers were stage I (55%) or stage II (31%) with no metastatic tumors. Male patients constituted 62% of the patients and 61% of the tumors were from the right colon. Histologically, 76% were conventional adenocarcinomas and 24% were other adenocarcinomas. The majority (71%) were discovered in surveillance colonoscopies. Cancer clinical data and PVs in select genes are outlined in Figure 1*A* and are provided in Supplementary Table 1.

Analysis of Wnt genes revealed *APC* and *CTNNB1* PVs in 61% and 24% of tumors, respectively (Figure 1*A*). All 12 *CTNNB1* PVs were detected in exon 3; ten were biallelic missense variants in mutation hotspot codons 41 and 45 coding for phosphorylation sites, and 2 were heterozygous p.S45del in-frame deletions, also affecting a phosphorylation site. The p.s45del occurred in APC-mut tumors, meaning that they are likely not genuine driver variants. Of the 10 missense variants, 5 bore little resemblance to an *MLH1*-knockout mutational signature[6] (Supplementary Figure 1*A*). Furthermore, Wnt pathway suppressor genes *RNF43* and *ZNRF3*, the products of which form membrane heterodimers, were examined. It is now known that *RNF43* frequently exhibits a deletion in a 7G microsatellite (c.1976del/p.G659fs, rs755128667, denoted *RNF43_G659fs*), which does not appreciably impair the Wnt signaling pathway suppressor function of *RNF43*, unlike other *RNF43* variants (*RNF43_LoF*).[7–9] The majority (n = 21 of 35 [60%]) of all *RNF43* variants observed occurred in the p.G659fs hotspot; these were not included as Wnt drivers. Mutation rates for *RNF43_LoF* (16%) and *ZNRF3* (18%) were similar, and variants in these 2 genes showed a striking co-occurrence (odds ratio [OR], 107.07; 95% CI, 8.88–6212.18; $P_{adj} < .001$).

Genome-wide CNA analysis then revealed chromosome 3 (chr3) p arm as, by far, the most mutated region (Figure 1*B*). Specifically, in the *MLH1-CTNNB1* locus (chr3: 37,000,000–41,000,000) copy number neutral loss of heterozygosity (CNLOH) was the most common CNA. *MLH1* CNA (n = 32 [63%]) and somatic PV (n = 12 [24%]) exhibited strong mutual exclusivity (OR, 0.026; 95% CI, 0.00–0.22; $P_{adj} < .001$) with only 1 tumor harboring both. Other CRC genes were not affected by CNA above the background rate, whereas chr3 passenger genes were primarily mutated via CNA (Figure 1*C*). *MLH1* PVs tended to co-occur with *APC* PVs (OR, 10.07; 95% CI, 1.24–471.80; $P_{adj} < .05$). Neither CNA nor PV affecting *MLH1* could be identified in 8 tumors (16%).

APC, *MLH1*, *CTNNB1*, *ZNRF3*, and *RNF43_LoF* PV and CNA were examined for association with colonoscopy indication (Supplementary Figure 1*B*). *CTNNB1* mutations were enriched in surveillance-detected (n = 10 of 36) compared with tumors detected at diagnostic (n = 2 of 15) examination (28% vs 13%; $P_{adj} = .47$). *MLH1* CNA events (n = 32) were highly enriched in surveillance-detected tumors as opposed to those detected in diagnostic colonoscopies (75% vs 33%; OR, 5.76; 95% CI, 1.37–27.91; $P_{adj} < .05$).

Clustering was performed based on relevant genetic alterations, including the presence of somatic mutations in Wnt pathway genes (*APC*, *CTNNB1, RNF43_LoF,* and *ZNRF3*) and in *MLH1*. Here, *RNF43_LoF* and *ZNRF3* are proposed to be included alongside *APC* and *CTNNB1*. As is evident from the left-side cluster (Figure 1*D*), the strongest single differentiator of the clusters is the presence of CNLOH of *MLH1.* Further bifurcations occur by means of the presence of *MLH1* and *RNF43_LoF/ZNRF3* variants. The clusters of *CTNNB1*-homozygous–mutated and *RNF43_LoF/ZNRF3*–mutated tumors occurred primarily in surveillance-detected tumors at rates of 9 of 10 (90%) and 7 of 8 (88%), respectively.

Abbreviations used in this paper: **chr3, chromosome 3; CNA, copy number alteration; CNLOH, copy number neutral loss of heterozygosity; CRC, colorectal carcinoma; LS, Lynch syndrome; OR, odds ratio; PV, pathogenic variant.**

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0016-5085
https://doi.org/10.1053/j.gastro.2025.10.012

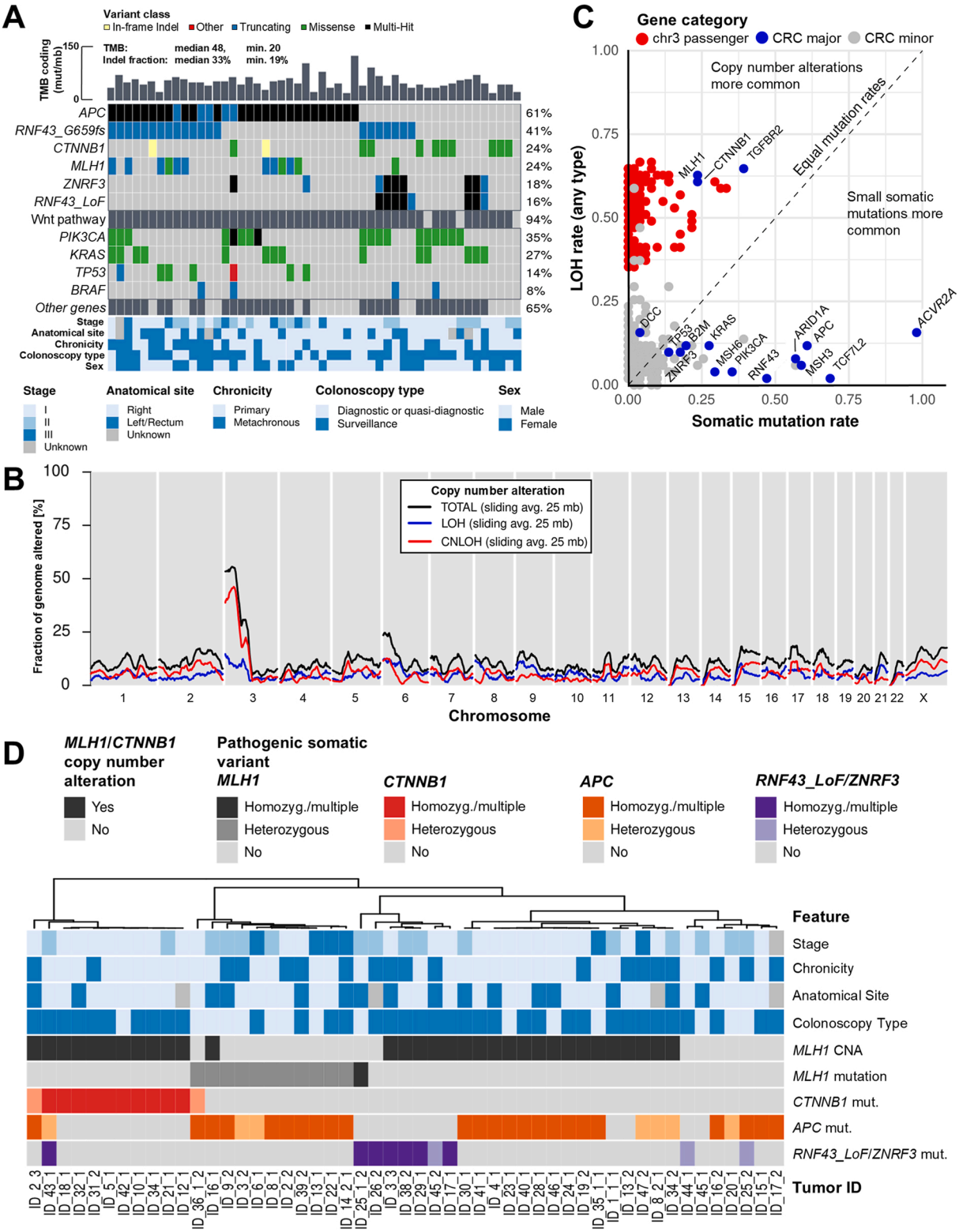

Figure 1. (*A*) *Oncoplot* of pathogenic variants in the select CRC genes. The majority of *APC*-mutated tumors (n = 31) harbored at least 2 pathogenic variants (n = 26 of 31 [84%]). All tumors were microsatellite instable as established by coding tumor mutational burden (TMB) (cutoff 10 mut/mb) and insertion or deletion fraction (cutoff 10%). Furthermore, co-occurring PVs in Wnt suppressor genes *AXIN1* and *AXIN2* were identified in 1 tumor (not shown), bringing the Wnt pathway mutation rate to 96%. (*B*) Genome-wide plot of CNA rate using a sliding average with window size of 25 mb. The figure also includes data for 7 *MSH2* CRC samples (not presented in this research letter), in addition to 51 *MLH1* CRC samples. (*C*) Comparison of somatic mutation and CNA rates for multiple genes among the 51 *MLH1* tumors. In contrast to (*A*), *RNF43* variants have been pooled. (*D*) Clustering of the 51 CRC based on putative carcinogenetic features of *MLH1* and key Wnt genes involved in LS carcinogenesis. The 4 clinical feature annotations (*blue*) were only added after clustering. Refer to Figure 1*A* for clinical legend.

Overall, these observations on *CTNNB1* variants are highly suggestive of the variants already being present in the cell before to chr3 CNLOH because the other viable sequence, namely the independent accumulation of 2 identical *CTNNB1* PVs, is unlikely during tumor progression. Thus, taken together with other reports on *CTNNB1* homozygosity,[10] the results presented here are highly consistent with 2-in-1 hit mechanism.[5] Further evidence is provided by the high rate of CNLOH in not just *CTNNB1*-driven tumors (n = 10 of 10 [100%]), but also in all *CTNNB1* wild-type tumors (n = 22 of 41 [54%]).

The strength of the study lies in the analysis of clinically well-characterized LS CRC defined by a single LS gene, *MLH1*. Comprehensive histologic features, including immunohistochemical staining, are also available for further investigations. A limitation is the relatively low proportion of tumor-normal pairs (43%). However, this is a minor issue because in this research letter the focus was on well-characterized genes and variants; all *CTNNB1* exon 3 hotspots are well-established, and any insertion or deletion mutations in *APC* must be somatic; otherwise, the patient would have familial adenomatous polyposis. Moreover, due to the focus on *MLH1* and *CTNNB1*, the conclusions on carcinogenesis cannot be extrapolated to other *path_MMR* carriers.

In conclusion, this study confirms the 2-in-1 carcinogenetic pathway involving *CTNNB1* in *path_MLH1* carriers. A further *APC*- and *CTNNB1*-independent pathway defined by *MLH1* LOH or somatic variant (or another rarer mechanism of *MLH1* loss) followed by multigene sequential mutations in both *RNF43_LoF* and *ZNRF3*, is observed and hypothesized, although this should be validated in an independent cohort. The latter pathway does not invoke genomic adjacency of any LS and CRC gene pairs; thus, it could be also present in other *path_MMR* carriers.

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Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2025.10.012.

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Received June 12, 2025. Accepted October 9, 2025.

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Acknowledgments
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The authors acknowledge the valuable contribution of the Lynch Syndrome *MLH1* CRC Group authors.

The library preparation and sequencing were performed by the FIMM Genomics Next-Generation Sequencing Unit at University of Helsinki supported by HiLIFE and Biocenter Finland.

The figures in Supplementary Figure 1 include illustrations from third party sources. The following illustrations from National Institutes of Health Bioart (National Institute of Allergy and Infectious Diseases Visual & Medical Arts; https://bioart.niaid.nih.gov/) have been used under public domain allowance: human digestive tract (ID 212, in modified format), gene mutation (ID 170), and diploid chromosome (ID 118, in modified format). For the human digestive tract, the stomach and large parts of the small intestine were made lighter to highlight the colorectum. An icon of colon cancer was overlaid on the ascending colon (see below). For the diploid chromosome, locus marks were added. The following illustration from BioIcons (https://bioicons.com/) has been used in modified format under CC-BY 3.0 Unported license: colon-cancer. Credit is given to Servier. The original image was cropped to only include stage III colon cancer and was then overlaid on human digestive tract.

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Conflicts of interest
These authors disclose the following: Toni T. Seppälä reports consultation fees from Nouscom and Orion Pharma, and being a clinical advisory board member and minor shareholder of Lynsight Ltd. Päivi Peltomäki is a member of clinical advisory board of Lynsight. The remaining authors disclose no conflicts.

Funding
This study was funded by Jane and Aatos Erkko Foundation (21002 to Päivi Peltomäki, Toni T. Seppälä, and Jukka-Pekka Mecklin), Cancer Foundation Finland (63-6409 to Toni T. Seppälä), Sigrid Jusélius Foundation (240194 to Toni T. Seppälä), Research Council of Finland (338657 to Toni T. Seppälä), iCAN Precision Medicine Flagship of the Research Council of Finland (to Toni T. Seppälä), and the State Research Funding through Pirkanmaa Wellbeing Services County (T63354, T66854 and T67954 to Toni T. Seppälä).

Data Availability
Clinical and small variant data of select genes required for the independent replication of key results in this research letter, to the extent allowed by the Finnish law, is provided directly in Supplementary Table 1. Further data are available on reasonable request to the corresponding author.