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Colorectal Carcinomas With CpG Island Methylator Phenotype 1 Frequently Contain Mutations in Chromatin Regulators

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BACKGROUND & AIMS: Subgroups of colorectal carcinomas (CRCs) characterized by DNA methylation anomalies are termed CpG island methylator phenotype (CIMP)1, CIMP2, or CIMP-negative. The pathogenesis of CIMP1 colorectal carcinomas, and their effects on patients' prognoses and responses to treatment, differ from those of other CRCs. We sought to identify genetic somatic alterations associated with CIMP1 CRCs. **METHODS:** We examined genomic DNA samples from 100 primary CRCs, 10 adenomas, and adjacent normal-appearing mucosae from patients undergoing surgery or colonoscopy at 3 tertiary medical centers. We performed exome sequencing of 16 colorectal tumors and their adjacent normal tissues. Extensive comparison with known somatic alterations in CRCs allowed segregation of CIMP1-exclusive alterations. The prevalence of mutations in selected genes was determined from an independent cohort. **RESULTS:** We found that genes that regulate chromatin were mutated in CIMP1 CRCs; the highest rates of mutation were observed in *CHD7* and *CHD8*, which encode members of the chromodomain helicase/adenosine triphosphate–dependent chromatin remodeling family. Somatic mutations in these 2 genes were detected in 5 of 9 CIMP1 CRCs. A prevalence screen showed that nonsilencing mutations in *CHD7* and *CHD8* occurred significantly more frequently in CIMP1 tumors (18 of 42 [43%]) than in CIMP2 (3 of 34 [9%]; $P < .01$) or CIMP-negative tumors (2 of 34 [6%]; $P < .001$). CIMP1 markers had increased binding by CHD7, compared with all genes. Genes altered in patients with CHARGE syndrome (congenital malformations involving the central nervous system, eye, ear, nose, and mediastinal organs) who had *CHD7* mutations were also altered in CRCs with mutations in *CHD7*. **CONCLUSIONS:** Aberrations in chromatin remodeling could contribute to the development of CIMP1 CRCs. A better understanding of the biological determinants of CRCs can be achieved when these tumors are categorized according to their epigenetic status.

Keywords: Colon Cancer; Hypermethylation; Microsatellite Instability; Gene Silencing.

Approximately 75% of all colorectal cancers (CRCs) are sporadic and characterized by genetic lesions, most commonly mutations of the *TP53*, *KRAS,* or *APC* gene.[1,2] In addition, epigenetic alterations in CRCs are also widely reported, mainly gene promoter DNA methylation. Classification of CRCs according to DNA methylation status has identified a subset of tumors with extensive epigenetic instability, characterized by concordant promoter hypermethylation.[3] The existence of a CpG island methylator phenotype (CIMP) and its correlation with clinicopathologic features have been confirmed extensively by use of high-throughput techniques.[4,5] Typical high-level CIMP (CIMP-high, CIMP1) CRCs are associated with microsatellite instability through epigenetic silencing of mismatch repair gene *MLH1*, often have *BRAF* mutation, and occur predominantly in the proximal colon, and low-level CIMP (CIMP-low, CIMP2) has been characterized by DNA methylation of a limited group of genes and mutation of *KRAS*.[6] Recent pathologic studies have shown that sessile serrated adenomas, mainly observed in the proximal colon, are associated with frequent *BRAF* mutation and CIMP,[7] suggesting that CIMP-positive CRCs arise from a different precursor than CIMP-negative tumors. Importantly, CIMP-positive CRCs are usually associated with better prognosis,[8] although patients with CIMP-positive CRC do not benefit from 5-fluorouracil–based adjuvant chemotherapy regimens.[9]

The events that lead to different clinicopathologic manifestations of CIMP1 CRCs are not well described. Although the increased frequency of DNA methylation can determine the behavior of these tumors, it is also possible that somatic mutation of a gene or a group of genes other than *BRAF* that co-occur with CIMP1 modulates the genesis and progression

†Deceased.

Abbreviations used in this paper: **CIMP, CpG island methylator phenotype; CRC, colorectal carcinoma; SNP, single-nucleotide polymorphism; TCGA, The Cancer Genome Atlas.**

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of these tumors. To test this hypothesis, we used next-generation sequencing technology to analyze the exome of 16 colorectal tumors. We found that CIMP1 CRCs have frequent mutations in genes encoding proteins that function in chromatin organization, most frequently *CHD7* and *CHD8,* members of the chromodomain helicase/adenosine triphosphate–dependent (CHD) chromatin remodeling family. These results suggest a prevalent role for aberrant chromatin remodeling in CIMP1 CRCs.

Materials and Methods

Preparation of Clinical Samples

We examined genomic DNA samples from 100 primary CRCs, 10 adenomas, and adjacent normal-appearing mucosae from patients undergoing surgery or colonoscopy at the Johns Hopkins Hospital, Sapporo Medical University, or Akita Red Cross Hospital. Specimens were gathered in accordance with institutional policies and all patients provided written informed consent. All DNA were obtained from frozen specimens, and none of the CRCs had been treated with chemotherapy or radiation. Tumors were selected solely on the basis of availability. Both CRCs and adenomas used in this study were characterized previously for CIMP; microsatellite instability; and *BRAF*, *KRAS*, and *TP53* mutation status.[6,10] For CIMP classification, DNA methylation of 7 classical markers (*p16*, *MLH1*, MINT1, MINT2, MINT12, MINT17, and MINT31) was evaluated by bisulfite polymerase chain reaction followed by combined bisulfite restriction analysis (COBRA) or pyrosequencing analysis. Specimens were classified as CIMP1 when *MLH1* and at least 4 of the 6 remaining markers where hypermethylated. CIMP-negative cases presented methylation of none or 1 of the markers, and CIMP2 cases were defined as those with hypermethylation of at least 2 markers but no *MLH1* hypermethylation. Adenomas were classified into CIMP groups according to the methylation profiling of their corresponding carcinoma.

Exome Sequencing

Genomic DNA specimens from 16 colorectal tumors and their adjacent normal tissues were submitted to Otogenetics Corporation (Norcross, GA) for exome capture and sequencing. Briefly, genomic DNA was subjected to agarose gel and optical-density ratio tests to confirm the purity and concentration before fragmentation. Fragmented genomic DNAs were tested for size distribution and concentration using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Illumina libraries were made from qualified fragmented genomic DNA using Next reagents (New England Biolabs, Ipswich, MA), and the resulting libraries were subjected to exome enrichment using NimbleGen SeqCap EZ Human Exome Library v2.0 (Roche NimbleGen, Inc, Madison, WI) according to manufacturer's instructions. Libraries were tested for enrichment by quantitative polymerase chain reaction and for size distribution and concentration by an Agilent Bioanalyzer 2100. The samples were then sequenced on an Illumina HiSeq2000 (Illumina, Inc, San Diego, CA), which generated paired-end reads of 90 or 100 nucleotides. Reads from both replicates were combined in the final analysis. Data were analyzed for quality, exome coverage, and exome-wide single-nucleotide polymorphism (SNP)/InDel using the platform provided by DNAnexus (Mountain View, CA).

A sequence variation in tumor DNA was considered a potential somatic mutation when it was present in 3 or more distinct tags of at least 10 total tags. We excluded all variants with a PHRED-encoded probability score <35, those that were present in the DNA of the corresponding normal samples (excluding germline events), and those that were not in coding regions, as well as silent changes and known SNPs (except for clinically associated SNPs). The ratio of variant tag count/reference tag count was also calculated, and all variants with a ratio >0.5 were removed. DNAnexus Genome Browser was used for visual validation of all potential somatic mutations to ensure that they were present in forward and reverse strands.

Pyrosequencing and Sanger Sequencing

Mutations in *CHD7* and *CHD8*, and selected additional mutations in 4 genes detected by exome sequencing (*ITGA10*, *CLSTN2, TTN,* and *KCNMA1*), were validated by pyrosequencing or Sanger sequencing. The list of primers is provided in Supplementary Table 4. Pyrosequencing was carried out on a PSQ96 system with a Pyro-Gold reagent Kit (Qiagen, Valencia, CA), and the results were analyzed by PyroMark Q96 ID software version 1.0 (Qiagen). For evaluation of *CHD7* and *CHD8* genes, the coding regions from 94 additional colorectal tumors and matched normal colonic tissues were sequenced using the primers listed in Supplementary Table 11. The sequence chromatograms were visually inspected with DNA Dynamo Sequence Analysis Software (Blue Tractor Software, Llanfairfachan, Wales, UK). All mutations were confirmed by independent sequencing reactions from both forward and reverse strands. Known database polymorphisms were excluded.

Immunohistochemistry Analysis

Expression of CHD7 (anti-CHD7 antibody, ab31824; Abcam, Cambridge, MA) and CHD8 (anti-CHD8 antibody, ab84527; Abcam) was studied using the DAKO Envision system (DAKO, Carpinteria, CA), as described previously.[11]

Gene Function Analysis

Functional enrichment of mutated genes was determined by gene ontology analysis using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). *P* values were corrected for multiple hypothesis testing using the Benjamini method. Comparison of the spectrum of mutations in our cohort to known mutations in cancer was done using the Catalogue of Somatic Mutations in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Gene expression data downloaded from The Cancer Genome Atlas (TCGA) data portal (https://tcga-data.nci.nih.gov/tcga/) and published by Lalani et al were subjected to gene set enrichment analysis.[12]

Statistics

The statistical significance of the differential frequency of *CHD7* and *CHD8* mutations in CIMP groups was determined using Fisher's exact test. Two-tailed *P* values were calculated using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA).