Left-Sided Early-Onset vs Late-Onset Colorectal Carcinoma

Histologic, Clinical, and Molecular Differences

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ABSTRACT

Objectives: Carcinomas of the left colon represent a neoplasm of older patients (late onset), but epidemiologic evidence has been showing an increasing incidence in patients 50 years or younger (early onset). In this study, we investigate pathologic and molecular features of early- and late-onset carcinoma of the left colon.

Methods: We selected 22 patients 50 years or younger and 21 patients 70 years or older with left-sided colorectal carcinoma (CRC). All samples were evaluated for pathologic features, microsatellite instability, and KRAS and BRAF mutations. Moreover, both groups were analyzed to identify CpG island methylator phenotype features and assessed with restriction landmark genome scanning technique (RLGS) to unveil differential DNA methylation patterns.

Results: Early-onset patients had advanced pathologic stages compared with late-onset patients (P = .0482). All cases showed a microsatellite stable profile and BRAF wild-type sequence. Early-onset patients (43%) more frequently had mutations at KRAS codon 12 compared with late-onset patients (14%) (P = .0413). RLGS showed that patients younger than 50 years who had CRC had a significantly lower percentage of methylated loci than did patients 70 years or older (P = .04124), and differential methylation of several genomic loci was observed in the two groups.

Conclusions: Our results suggest that left-sided CRCs may present differential patterns of aberrant DNA methylation when they are separated by age.

Colorectal carcinomas (CRCs) represent, in most cases, a neoplasm of older patients (median age 70 years for late-onset CRCs in developed countries).1 Early-onset CRC (aged ≤50 years) is regarded as a marker of a hereditary syndrome when it shows proximal location. Early-onset CRCs, with proximal location, very often harbor microsatellite instability (MSI+) due to germline defects in mismatch repair genes and cause the syndrome known as hereditary nonpolyposis colorectal carcinoma (HNPCC).2 However, epidemiologic evidence has been showing an increasing incidence in patients 50 years or younger, especially in the left colon.3,4 In past years, single-institution studies have been performed on early-onset CRCs to investigate clinical, pathologic, or molecular peculiarities. Most works have shown that early-onset left-sided CRCs are more likely to present at a higher stage compared with late-onset cases and, despite early age of presentation, do not develop in the context of hereditary...
syndrome or cancer risk factors. However, most of these studies included CRCs selected only for age, regardless of the tumor site (right vs left colon), implying the inclusion of Lynch syndrome–associated carcinoma, which has a different specific mechanism of tumorigenesis and prognostic implication. The aim of our study was to investigate pathologic and molecular features of early-onset vs late-onset carcinoma of the left colon (descending colon, sigmoid colon, and rectum). For this reason, we selected 22 patients 50 years or younger with left-sided CRC and, as a control group, 21 patients 70 years or older with left-sided CRC. To identify specific features, both groups were evaluated for the following pathologic and molecular features: grading, staging, pattern of growth, inflammatory infiltrate, presence of MSI, mutation in KRAS codons 12 and 13, and mutation in BRAF codon 15 (V600E). Moreover, we extended our observations to epigenetic features. For instance, DNA methylation alterations play an important role in human colon tumor progression. DNA hypermethylation of the hMLH1 mismatch repair gene leads to silencing of hMLH1 gene expression, which results in a specific phenotype (MSI+) of sporadic CRC.

A specific DNA hypermethylation pattern, known as CpG island methylator phenotype (CIMP), has been identified in a subgroup of CRCs and represents a major mechanism of genomic or epigenetic instability. Therefore, early-onset and late-onset left-sided colorectal tumors were analyzed by methylation-sensitive polymerase chain reaction (MSP) to identify CIMP features and by restriction landmark genome scanning (RLGS) analysis to unveil differential DNA methylation patterns. We used RLGS as a method to identify genomic regions that were frequently methylated in CRCs. RLGS identified a number of genomic loci that were frequent targets of CpG island methylation in human left-sided CRCs and showed that the two age-related groups might present specific patterns of DNA hypermethylation.

Materials and Methods

Patients and Clinicopathologic Features

Twenty-two patients 50 years or younger affected by adenocarcinomas of the left-sided colon and, as a control group, 21 patients 70 years or older with left-sided colon adenocarcinoma were included in the study. Patients were selected from archive files of our institution from 2003 through 2010 according to tumor location (descending, sigmoid colon, and rectum), age (<50 years and ≥70 years), and availability of frozen tissue from resection specimens (tumor and paired normal mucosa) to be used for the RLGS technique (see below). No patient included in the study received neoadjuvant chemoradiotherapy. Patients did not have any history of chronic inflammatory bowel disease or colonic polyposis. For all cases, the following histologic features were scored: grade of differentiation: well (G1), moderate (G2), or poor (G3); mucinous adenocarcinoma: mucinous differentiation of more than 50% of the tumor; mucinous aspects: presence of mucinous differentiation of less than 50% of the tumor; pattern of growth: infiltrative (diffuse/insidious growth at the infiltration frontline) or compressive (pushing growth at the infiltration frontline); Crohn-like inflammatory infiltration (discrete lymphoid aggregates outside the muscularis propria as described by Graham and Appelman14); presence or absence; and tumor-infiltrating lymphocytes as described by van den Bos et al15: presence or absence (none to few). Presence of polyloid lesions in the resection specimen was recorded. The study was carried out according to ethical guidelines of Sant’Andrea Hospital of Rome.

Immunohistochemistry

The specimens were formalin fixed and routinely processed. Immunohistochemistry was performed on 4-μm-thick sections using the following antibodies for mismatch repair protein—hMLH1 (clone G168-15; BD Pharman-gen, Franklin Lakes, NJ), hMSH2 (clone G219-1129; BD Pharmaningen), hMSH6 (clone G4; Diagnostic BioSystems, Pleasanton, CA), and PMS2 (clone A16-4; Biocare Medical, Concord, CA)—and visualized by Envision-Flex (DAKO, Glostrup, Denmark) in a DAKO Autostainer instrument. Nuclear protein expression in neoplastic cells was assessed as retained (normal) or lost. Inflammatory cells in the samples were used as a positive internal control.

DNA Extraction

A fragment of cancer tissue and normal mucosa were collected from each patient. For each sample of paraffin-embedded tissue, three 7-μm sections were cut and put on slides, dewaxed, rehydrated, and stained with hematoxylin. A pathologist (E.P.) performed microdissection of the normal mucosa and neoplastic tissue under a microscope by using a needle. DNA extraction was carried out with 50 μL of lysis buffer containing 10 mmol/L of proteinase K, with overnight digestion at 37°C followed by incubation at 95°C for 10 minutes. After centrifugation, supernatant was stored at −20°C.

KRAS and BRAF Mutation Analysis and Microsatellite Analysis

KRAS (codons 12 and 13) and BRAF (exon 15) mutational analysis was performed using direct sequencing of polymerase chain reaction (PCR)–amplified products. The primer sequences were KRAS-FW, 5′-CTG-GGTGAGTATTTGATAGTGTATT-3′; KRAS-REV, 5′-GTGGAGTATTTGATAGTGTATT-3′; BRAF-FW, 5′-CTG-GGTGAGTATTTGATAGTGTATT-3′; BRAF-REV, 5′-GTGGAGTATTTGATAGTGTATT-3′.
5'-ATCTGTATCAAAGAATGGTCTCGT-3'; **BRAF-FW,** 5'-TCATATGGCTGCTGATAGGA-3'; and **BRAF-REV,** 5'-GGCCAAAATTTTTAACTCAGTGGA-3'. All PCR products were sequenced bidirectionally using Big Dye Terminator chemistry and an automated 3100 DNA analyzer (Life Technology, Carlsbad, CA). For evaluation of high MSI, PCR amplification was carried out using a panel of two mononucleotide (BAT25, BAT26) targets (primer sequence: BAT25-FW, 5'-TCGCCCTCAAAGAATGTAAG-3'; BAT25-REV, 5'-TCTGCAATTTAACTATGGGTC-3'; BAT26-FW, 5'-TGACTACTTTTGACTTCAGCC-3'; BAT26-REV, 5'-AACCATTCAACATTTTTAACC-3') by using fluorescently labeled primers. Microsatellite analysis was performed with a genetic analyzer ABI 3100 and by using the GeneMapper (Life Technology) software to analyze data. MSI was referred to as the presence in both loci of extra alleles in neoplastic tissue compared with matched normal tissue.

**Bisulfite Treatment**

In total, 3 μg of genomic DNA from normal colorectal mucosa and colorectal tumor samples was bisulfite modified as previously described.16

**MSP Followed by Restriction Enzymes Digestion (Combined Bisulfite Restriction Assay)**

Methylation status (CIMP) of **MLH1-3′,** **MINT31,** **p16**<sup>ink4a</sup>, **p14**<sup>ARF</sup>, **MGMT,** and **MINT2** was determined in early-onset and late-onset left-sided colorectal tumors, in 20 tumor-related normal mucosa samples (10 for each group), and in one CRC sample showing MSI+ and **BRAF** mutation by methylation-specific PCR of bisulfite-modified DNA followed by restriction digestion of the PCR product (combined bisulfite restriction assay). The digested DNA was electrophoresed on 3% agarose gel and visualized by ethidium bromide staining. The quantitative methylation levels (ratios of methylated to unmethylated DNA) were determined from the relative intensities of cleaved and noncleaved PCR products. A marker was considered methylated if it showed 5% or more methylation density and methylation negative if it had 5% or less, in accordance with canonical CIMP criteria.17 Primers sequences, restriction enzymes, and PCR conditions used were previously described by Nagasaka et al.18

**RLGS**

Briefly, RLGS was performed according to published protocols using the enzyme combination *NotI*-EcoRV-*Hinfl*.19-24 Nonspecific sheared ends of 1 to 3 μg genomic DNA were blocked in a 10-μL reaction by the addition of nucleotide analogues (αS-dGTP, αS-dCTP, ddATP, and ddTTP) with 2 U DNA polymerase 1 (37°C, 20 minutes), followed by enzyme inactivation (65°C, 30 minutes). We adjusted the buffer and digested DNA (37°C, 2 hours) with 20 U *NotI* (Promega, Madison, WI), which is sensitive to methylation. We then used Sequenase (version 2.0; U.S. B. Cleveland, OH) to fill in the *NotI* ends with [α-32P]dGTP and [α-32P]dCTP (Amersham GE Healthcare Europe, Freiburg, Germany) for 30 minutes at 37°C. We digested the labeled DNA (37°C, 1 hour) with 20 U EcoRV (Promega) and separated a portion by electrophoresis through a 60-cm–long, 0.8% agarose tube gel (first-dimension separation). The agarose gel was next equilibrated in *Hinfl* digestion buffer, and the DNA was digested in the gel with 700 U *Hinfl* (Promega) at 37°C for 2 hours. We then placed the gel horizontally (rotated 90° relative to the first direction of electrophoresis) across the top of a nondenaturing 5% polyacrylamide gel, connected the two gels with molten agarose, and separated the DNA by electrophoresis in the second dimension. Gels were dried and exposed to X-ray film for 2 to 14 days. We carried out RLGS at least twice for each tumor. RLGS analysis was performed by visual inspection of spot intensity and comparing RLGS profiles of colorectal tumor DNA with a “master” RLGS profile that was derived from the analysis of six normal colorectal mucosa DNAs (three normal mucosa from patients aged ≤50 years and three from patients aged ≥70 years) and one peripheral blood lymphocyte (PBL) DNA. The “master” RLGS profile considered only those genomic loci consistently unmethylated in all colorectal normal mucosa and PBL DNAs. This diminished the contribution of restriction site polymorphism to apparent spot loss. To avoid erroneous interpretation of spot intensity, we considered genomic loci methylated when the spot was almost visually undetectable on autoradiography; we did not consider spots with diminished intensity, diffuse spots, or spots with a high local background. RLGS profiles from tumor DNA were compared with control profiles by two independent observers. RLGS spot intensity and identity was determined as described22 and confirmed by Human February 2009 GRCh37/hg19 draft assembly of the human genome by the University of California at Santa Cruz.

**Results**

**Clinicopathologic Features**

Clinicopathologic features of patients investigated are summarized in Table 1. Mean age of the group 50 years or younger was 43 years (range, 27-50 years). Seven (32%) cases occurred in nonperitonealized rectum, while 15 were located in the sigma or descending colon, and a similar distribution was observed in the group 70 years or older. Fourteen (64%) patients 50 years or younger had
advanced stage (III or IV) disease at diagnosis, while 14 (67%) patients 70 years or older had early-stage disease (ie, not metastatic disease: stages I and II) \((P = .0482)\). In both groups, most tumors showed G1 or G2 features; G3 and a mucinous aspect were present only in three of 22 patients 50 years or younger. A pattern of infiltrative growth was observed in most (64%) cases in the group 50 years or younger, while in the group 70 years or older, a compressive growth pattern was more frequent (67%). Two of 22 patients 50 years or younger had a small (<1 cm) synchronous adenoma in the resection specimen. In the group 70 years or older, four patients had synchronous adenoma (0.7-1.2 cm) in the specimen.

**MSI and Expression of Mismatch Repair Proteins**

In both groups, no case showed MSI at any mononucleotide target (BAT26 and BAT25) tested. Immunohistochemistry for mismatch repair proteins (MLH1, MSH2, MSH6, and PMS2) was performed in 20 of 22 patients 50 years or younger and 21 of 21 patients 70 years or older in which sections were available. No loss of nuclear expression was observed in any case **Table 2**.

**KRAS and BRAF Mutations**

Nine (43%) of 21 and four (19%) of 21 patients 50 years or younger and 70 years or older, respectively, showed **K**RAS exon 2 mutation. Specifically, all nine patients 50 years or younger showed mutations exclusively in codon 12, while codon 12 was mutated in three (14%) of 21 patients 70 years or older (43% vs 14%; \(P = .0413\)). No mutations in codon 13 were observed in patients 50 years or younger. In one patient, **K**RAS exon 2 sequencing failed. In patients 70 years or older, one (5%) of 21 patients showed mutations in **K**RAS codon 13. **B**R**A**F showed a wild-type sequence in all cases in both groups (Table 2).

**DNA Methylation Analyses**

To unveil any difference at the DNA methylation level, we analyzed CIMP\(^9\)-\(^13\) in our age-related cohorts (≤50 years and ≥70 years), restraining the analyses to the classic CIMP markers set: **M**LH1-3, **M**INT31, **p**16\(^{ink4a}\), **p**14\(^{ARF}\), **MG**MT, and **M**INT2.\(^{10}\) Methylation status of **MLH1-3**, **MINT31**, **p**16\(^{ink4a}\), **p**14\(^{ARF}\), and **MGMT** was analyzed in 18 colorectal tumors, whereas **MINT2** was assessed in eight samples from patients 50 years or younger. Methylation experiments results showed that colorectal tumor samples (MSI–) in patients 70 years or older. Methylation experiments results showed that colorectal tumor samples (MSI–) in patients 70 years or older. Methylation experiments results showed that colorectal tumor samples (MSI–) in patients 70 years or older. Methylation experiments results showed that colorectal tumor samples (MSI–) in patients 70 years or older. Methylation experiments results showed that colorectal tumor samples (MSI–) in patients 70 years or older.
Image II CpG island methylator phenotype (CIMP) assessment in age-related colorectal carcinoma (CRC) cohorts. Representative figure of methylation status of classic CIMP markers5-13 (MLH1-3', MINT31, p16ink4a, p14ARF, MGMT, and MINT2) in our MSI– age-related colorectal tumor cohorts—CRC in patients 70 years or older (A) and CRC in patients 50 years or younger (B).
differences were observed in the methylation status of the CIMP markers between the two groups. Importantly, MLH1-3′ was unmethylated in all samples. As expected, the colorectal tumor sample identified as MSI+ and BRAF mutated showed a classic CIMP phenotype consisting of MLH1-3′, p16, p14, MGMT, and MINT2 promoter methylation.[18,25] Based on the DNA quality, some of the samples chosen between the two age-related groups were analyzed by RLGS.[22-24] RLGS was performed using NotI as a methylation-sensitive enzyme and restriction landmark. Since a methylated NotI site is not digested and is not labeled, the absence of a spot indicates DNA methylation. Although spot loss could be due to DNA deletion or rearrangement, previous results indicate that most spot loss is due to DNA methylation.[21]

By RLGS, we analyzed three normal mucosa samples and five colon cancer samples from patients 50 years or younger and three normal mucosa samples and seven colon cancer samples from patients 70 years or older. The RLGS analyses included 1,116 genomic NotI sites,[23] for 672 NotI sites, the chromosomal location and corresponding gene were known based on an arrayed NotI-EcoRV genomic library[26,27] and on an in silico restriction digestion method previously described.[22] The comparisons showed that 450 of 672 NotI sites were methylated in at least one of the tumor samples independently of the age-related group they belonged to and that patients 50 years or younger had a significantly lower percentage of methylated sites compared with patients 70 years or older (8.4% ± 2.4% vs 11.6% ± 2.4%, P = .04124). Furthermore, our analyses showed gene-specific methylation among the two age-related groups. In patients 70 years or older, 34 of 250 methylated loci were specifically hypermethylated compared with patients 50 years or younger (Figure 2A). In contrast, in patients 50 years or younger, site-specific hypermethylation was limited to very few loci and in a small percentage of tumor samples (Figure 2A). In addition, a certain number of genomic loci were more hypermethylated in patients 70 years or older than in patients 50 years or younger (Figure 2B). The same tendency was also observed when unknown genomic loci were analyzed (data not shown).

**Discussion**

The definition of early-onset colorectal carcinoma refers to cases diagnosed in early decades, usually before 50 years. However, a real limit has never been established, even though Bethesda criteria fixed a limit of 50 years for screening of HNPCC/Lynch syndrome. Most recent colon cancer screening guidelines recommend starting screening...
We investigated MSI using immunohistochemistry for mismatch repair proteins (hMLH1, hMSH2, hMSH6, and PMS2) and a panel of two mononucleotide markers that have been shown as the most sensitive and specific to identify colorectal tumors harboring real mismatch repair system deficiency in a large population series.

Our data are in agreement with most previous reports, in which left-sided early-onset CRC has been shown to be microsatellite stable (MSS). However, it has been recently observed by Goel et al. that in a large series of 75 patients 50 years or younger with nonfamilial CRC, 12 cases of distal colon carcinoma showed MSI. A possible explanation for this discrepancy is the large number of cases they investigated (54 cases of distal colon carcinoma) and a higher proportion of patients aged 31 to 40 years (75%) compared with our series (22%). Moreover, Goel et al. found loss of hMSH6 protein in two patients with distal colon carcinoma without a molecular phenotype of instability.

Since it has been observed that hMSH6-associated CRC may show an MSS phenotype, to not miss hMSH6-deficient tumors, we investigated the expression of hMLH6 in patients 50 years or younger, but we did not find loss of the protein in any (data not shown). Mutation of KRAS is an early event in the development of CRC and has been observed in 30% to 40% of unselected cases. A prognostic role for KRAS in CRC remains debated. Previous studies on early-onset CRC have arbitrarily fixed the limit in their cohorts to patients 50 years or younger or those younger than 45 or 40 years. In our study, we selected the cutoff of 50 years so that we could include as many cases in the “early-onset group” as possible for which frozen material was available. The selection of patients had two main peculiarities compared with previous studies on early-onset CRC: (1) to investigate and compare clinical and biological aspects specifically of left-sided CRC in the early-onset (≤50 years) group of patients since previous reports on early-onset carcinoma took into consideration colonic neoplasm, regardless of patient’s age and site of involvement, and (2) to separate as much as possible the early-onset group from the group of patients significantly older (≥70 years) who we defined as “late onset.”

Our data, in agreement with previous studies, show that early-onset CRC presents at advanced pathologic stages compared with late-onset CRC (63% vs 33%, \( P = .0482 \)). The early-onset group morphologically displayed more adverse histologic features as an infiltrative pattern of growth compared with the late-onset group. In our cohorts, early-onset CRC did not underlie Lynch syndrome. Indeed, no case showed MSI-H, a molecular signature of Lynch syndrome.

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meta-analyses (RASCAL I and II) showed that mutations in KRAS were associated with worst outcome, while a recent larger series showed that in univariate and multivariate analysis, KRAS mutation was not a prognostic factor.\textsuperscript{35} KRAS mutation has been associated with more advanced disease in CRC.\textsuperscript{36} In our study, the average value of KRAS mutation, considering both groups together, is 31%, which is in accordance with the incidence of KRAS mutations reported in unselected series.\textsuperscript{37,38} However, in our study, the early-onset group showed KRAS mutation in 43% of cases; in contrast, in the late-onset group, KRAS mutations were observed in 18% of patients. This difference can be explained with the highest incidence of advanced stage in our series of the early-onset group. Significantly, KRAS-mutated CRCs in patients 50 years or younger showed mutations exclusively on codon 12, whereas in the late-onset group, KRAS codon 12 was mutated only in three (14%) of 21 cases (Table 2) (43% vs 14%; \( P = .0413 \)). Data on KRAS mutations in early-onset CRC are heterogeneous.

Gunal et al\textsuperscript{36} showed a very high incidence of KRAS mutations in patients younger than 40 years compared with those older than 40 years (66.7% and 36%, respectively). This discrepancy could be related to the method of KRAS mutation investigation (single strand confirmation polymorphism in the study by Gunal et al\textsuperscript{36} vs direct sequencing in our study) and to the selection of patients. Indeed, in their analyses, Gunal et al\textsuperscript{36} also took into consideration cases from the right colon, which have been shown to be statistically associated with KRAS mutation. On the contrary, Chang et al\textsuperscript{36} are the only authors reporting a much lower incidence of KRAS mutation in early-onset patients and in the control group (4% and 15%, respectively). The reason for this discrepancy is not clear. It could relate to high frequency in their series of signet ring histology (13%), while no case in our series showed that morphology. BRAF exon 15 mutation (mainly V600E) has been observed in 7% to 8% of an unselected series of CRC. It is well known that in CRC, the BRAF mutation is related to the MSI pathway of carcinogenesis, mostly involved in right-sided colon carcinoma. BRAF is frequently mutated in sporadic MSI-H colon carcinoma and associated with high levels of CpG island methylation. To our knowledge, CIMP has not been specifically investigated in early-onset CRC. Our samples series showed neither CIMP nor BRAF mutation.

Our data were substantially in agreement with the observations by Nagasaka et al\textsuperscript{18} relative to the MSI-colorectal tumor subgroup. Exceptions were represented by MINT2 methylation status (41% vs 20%). Thus, our data are in agreement with previous reports\textsuperscript{6,33} that have shown no implication of the BRAF gene in early-onset colon carcinoma and indicate that the CIMP pathway is not involved in the neoplastic progression of left-sided early-onset CRCs. By using RLGSS, a genome-wide approach allowing the analyses of DNA methylation status of a limited number of gene promoters, we observed that patients 50 years or younger had a significantly lower percentage of methylated loci compared with patients 70 years or older (8.4% ± 2.4% vs 11.6% ± 2.4, \( P = .04124 \); Figure 1). It could be argued that this is likely due to age-related DNA hypermethylation,\textsuperscript{39} but normal colorectal mucosa from the two age-related groups did not show significant differences in the global levels of DNA hypermethylation (Figure 1). Our RLGSS data are in agreement with a previous study investigating the methylation profile in CRC by RLGSS from a series of 33 colon cancer samples.\textsuperscript{40} Most methylated loci identified in our study were also observed methylated by Smith et al.\textsuperscript{40} However, Smith et al\textsuperscript{40} performed their analysis on an unselected series of colon cancer samples irrespective of cancer site location or patient age. Notably, our study shows that, taking into consideration cancer site location and especially patient age, it is possible to distinguish preferential genomic loci hypermethylation in one age-related group with respect to the other (Figure 2). Although our data are based on a small group of cases, the two groups showed differences in...
Figure 2i) Histograms showing the percentage of colorectal carcinoma (CRC) samples from each age-related group (full box, CRC in patients 70 years or older; empty box, CRC in patients 50 years or younger) presenting hypermethylation of specific gene promoter regions and identified by restriction landmark genome scanning. A, Percentage of CRC samples showing hypermethylation of exclusive genes in patients 70 years or older or patients 50 years or younger. B, Percentage of CRC samples, from the two age-related groups, showing differential hypermethylation occurrence for a specific subset of genes.
the methylation of specific gene promoters. Taken together, these data suggest that the DNA methylation profile in the two groups might be different and may imply different pathways in neoplastic progression.

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References


