Colorectal cancer (CRC) with CpG island methylator phenotype (CIMP) is recognized as a subgroup of CRC that shows association with particular genetic defects and patient outcomes. We analyzed CIMP status of 229 individuals with CRC using an eight-marker panel (CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3 and SOCS1); CIMP- (+) tumors were defined as having ≥ 5 methylated markers. Patients were divided into individuals who developed a “unique” CRC, which were subclassified into early-onset CRC (EOCRC) and late-onset CRC (LOCRC), and patients with multiple primary CRCs subclassified into synchronous CRC (SCRC) and metachronous CRC (MCRC). We found 9 (15.2%) CIMP- (+) EOCRC patients related with the proximal colon (p = 0.008), and 19 (26.8%) CIMP- (+) LOCRC patients associated with tumor differentiation (p = 0.045), MSI status (p = 0.021) and BRAF mutation (p = 0.001). Thirty-five (64.8%) SCRC patients had at least one CIMP- (+) tumor and 20 (44.4%) MCRC patients presented their first tumor as CIMP- (+). Thirty-nine (72.2%) SCRC patients showed concordant CIMP status in their simultaneous tumors. The differences in CIMP- (+) frequency between groups may reflect the importance of taking into account several criteria for the development of multiple primary neoplasms. Additionally, the concordance between synchronous tumors suggests CIMP status is generally maintained in SCRC patients.
pathways are widely accepted to be involved in the etiology of CRC: Chromosomal Instability (CIN), Microsatellite Instability (MSI) and CpG Island Methylator Phenotype (CIMP)5–7. Structural rearrangements as well as gains and losses of chromosome fragments are characteristic features of CIN tumors, possibly associated with higher mutation rates; the majority of sporadic cases are in this group9. MSI is associated with changes in short microsatellite repeats, caused by deficient mismatch repair (MMR) genes, and is related to hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch Syndrome (LS), and some sporadic cases in the elderly population8. Lastly, tumor suppressor and DNA repair genes are frequently transcriptionally silenced in CIMP cases. Furthermore, several studies have shown that CIMP positivity is associated with proximal colon location, presence of mucinous features, poor tumor differentiation, MSI, female gender and high BRAF mutation rates10,11.

Genetic, biological and clinical differences have been identified depending on the age of onset of CRC in many studies, thus it has been suggested that CRC should be subclassified attending this major criterion12–15. There are other features that should also be taken into account for subclassification, such as the development of two or more different tumors because these cases provide a good model to examine “field effect”. This effect is associated with the tendency of healthy colorectal mucosa to suffer early molecular alterations that trigger malignant transformation of the tissue16,17. Synchronous CRC (SCRC) is defined by the presence of more than one tumor simultaneously, while metachronous CRC (MCRC) is characterized by the development of a second lesion after surgery and/or diagnosis of the primary tumor18–20.

The given above, when focusing on CIMP status, it is important to take the type of CRC, into consideration, since CIMP status affects the response to therapy12,22 and it may have a relation to the “field effect” linked to CRC23,24. For this reason, we examined CIMP status in different CRC subtypes: patients with a single tumor (“unique” CRC), divided into early-onset CRC (EOCRC; age at diagnosis ≤45 years old) and patients with late-onset CRC (LOCRC; age at diagnosis >70 years old); and individuals diagnosed with multiple primary CRC, i.e. patients diagnosed with SCRC or MCRC.

Methods

Patients. A total of 229 CRC patients were included in this study at 12 de Octubre Hospital (Madrid, Spain). Among these patients (all of them Caucasian), 59 had EOCRC, 71 had LOCRC, 54 were diagnosed with SCRC and 45 were diagnosed with MCRC. SCRC was diagnosed when two or more histologically different lesions were developed simultaneously or within a time lapse shorter than six months after the detection of the first tumor19. When a secondary neoplasm was detected outside the anastomosis area after more than 6 months of the initial tumor diagnosis, it was considered as MCRC25. Samples with the highest content of tumor tissue were selected for molecular analysis. A multi-site database was used to collect clinicopathological, therapeutic and pre- and post-operative information. Informed consent was signed by all patients or by a first degree relative when the patient had died. The protocol of this study was approved by the Ethics Committee of this Institution.

DNA isolation. Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were selected by a pathologist. To be suitable for molecular analysis, one sample should contain a minimum percentage of 70% tumor cells. Briefly, DNA was extracted from FFPE samples using mineral oil to dissolve the paraffin, followed by proteinase K digestion and ethanol precipitation. DNA was further purified and eluted using CLART® HPV2 kit (Genomica S.A.U., Madrid, Spain).

Microsatellite analysis and analysis of BRAF mutation, hypermethylation of MLH1 and germline mutations in MMR genes. Microsatellite instability status of each tumor was defined using the Bethesda five-marker microsatellite panel (NR-21, BAT-26, BAT-25, NR-24 and MONO-27)26. Fluorescence labeled primers were included for amplification of markers, then the PCR products were separated using capillary electrophoresis and analyzed. When 2 or more markers showed instability, the sample was defined as microsatellite instable (Fig. 1). Moreover, MSI tumors were analyzed for the BRAFV600E mutation and hypermethylation of the MLH1 gene promoter in order to verify their sporadic nature, using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA; ME011-B3, ME0042-CIMP, MRC-Holland, Amsterdam, The Netherlands). MSI cases also were screened for Lynch Syndrome by evaluating germline mutations in the MMR genes (MLH1, MSH2 and MSH6) by high-resolution melting analysis using a LightCycler® 480 real-time PCR system (Roche, Mannheim, Germany), as previously reported27.

KRAS mutations. Mutations in KRAS (codons 12, 13 and 61) were determined previously in patients younger than 45 and older than 70 years. Briefly, DNA was extracted from the neoplastic material, and Sanger sequencing was carried out in both orientations on a 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA)28. The genetic alterations of KRAS in individuals diagnosed with SCRC and MCRC were evaluated by next generation sequencing using a gene panel related to cancer (Ion PGM System, ThermoFisher, Waltham, MA, USA) (Fig. 2).

CpG island methylator phenotype analysis. For the evaluation of CIMP, we examined the methylation status of the promoter regions of CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3 and SOCS1 using the SALSA MS-MLPA Probemix (ME0042-CIMP, MRC-Holland, Amsterdam, The Netherlands). Each patient was classified as CIMP (+) or CIMP (−) depending on whether tumors showed ≥5/8 or <5/8 methylated promoters, respectively (Fig. 3A,B)11. Patients diagnosed with multiple CRC and distinct CIMP statuses in their tumors were categorized as CIMP-MM (mismatching).

Statistical analysis. Clinical characteristics were compared between different groups according to CIMP status, including age, sex, stage, tumor location, presence of mucinous features, BRAF and KRAS mutations, and MSI status. Categorical variables were expressed as number of cases and their percentage, and continuous variables were expressed as mean values plus/minus standard deviation (SD). Comparison of categorical variables was done using Pearson's Chi Square (X²) test. For comparisons between two groups Student's t test was performed,
and for comparisons between more than two groups analysis of variance (ANOVA) (for normal distributions) or the Kruskal-Wallis test (for nonparametric distributions) were used. Statistical analysis was carried out using IBM SPSS 17.0 (SPSS Inc., Chicago, IL, USA). P value of < 0.05 was considered statistically significant differences.

Ethics approval and consent to participate. Study approval was obtained from the Ethics Committee of the 12 de Octubre University Hospital in Madrid, Spain. All procedures were performed in accordance with the ethical standards of the institutional research committee and the Declaration of Helsinki.

Results

Global features of “unique” colorectal cancer. We collected clinical characteristics of 130 individuals with “unique” CRC, of which 59 were EOCRC cases and the remaining 71 patients were LOCRC cases. The average age at diagnosis was 40 ± 5 and 78 ± 6 years old for EOCRC and LOCRC, respectively. Male to female ratio was >1 in the EOCRC group, whereas in the LOCRC set it was < 1. When we assessed the anatomical location of the tumors, the EOCRC group showed most tumors (47.5%) at the distal colon, while LOCRC patients presented more rectum location (42.3%). Among the 59 patients with EOCRC, 8 (13.6%) patients showed MSI status, of
which 3 (37.5%) were sporadic cases: 1 (12.5%) presented a BRAF mutation and 2 (25.0%) showed hypermethylation of the MLH1 gene promoter; the remaining 5 (62.5%) individuals were diagnosed with LS. Moreover, 22 (40.7%) EOCRC patients had a KRAS mutation. In the LOCRC group, 6 (8.5%) patients showed MSI status, all of them sporadic cases, 6 (8.5%) patients had a BRAF mutation and 1 (16.7%) case presented a KRAS mutation (Tables 1 and 2).

Global features of primary multiple colorectal cancer. Among 99 cases with multiple CRC, 54 individuals were diagnosed with SCRC and 45 patients with MCRC. In both groups, the mean age at onset was around 70 years old, with a male to female ratio > 1. Moreover, the most common tumor location for both groups was the entire colon, defined as the location of the synchronous or metachronous tumors at different sides of the colon. Only one patient in the SCRC group (1.8%) had MSI in both tumors whereas discordant MSI status was found between synchronous tumors in 3 cases (3.6%), of which 1 (25.0%) case was diagnosed with LS. Five

Figure 3. (A) Sample with CpG Island Methylator Phenotype negative (CIMP –). (B) Sample with CpG Island Methylator Phenotype positive (CIMP +).
(10.4%) and 31 (64.6%) patients presented BRAF mutations and KRAS mutations in at least one tumor, respectively. In the MCRC group, several remarkable features were the diagnosis of metachronous neoplasm at an early stage (82.2%), the total concordance of MSI status between paired tumors, and only 1 (2.2%) patient who was identified as a LS case, showed MSI status. Regarding a BRAF mutation, MCRC patients showed concordance between both tumors, of which 1 (2.2%) patient presented a BRAF mutation. Eighteen (40.0%) MCRC patients showed a KRAS mutation in at least one tumor and 11 (24.4%) in paired-tumors (Tables 3 and 4).

### CIMP analysis

We analyzed CIMP status in the four subtypes of CRCs patients. The number of methylated genes in each tumor of the patients of the different subgroups is shown in Fig. 4. In the cohort of 59 patients with EOCRC, 50 (84.7%) tumors were CIMP− (−) and 9 (15.2%) were CIMP+(+) . In the 71 patients with LOCRC, 52 (73.2%) tumors were CIMP− (−) and 19 (26.8%) tumors were CIMP+(+) (Tables 1 and 2). Interestingly, the subset of 54 diagnosed individuals with SCRC showed 20 (37.0%) patients with CIMP+(+) tumors and 19 (35.2%) with CIMP− (−) tumors, where both tumors presented same CIMP status, and 15 (27.8%) patients with CIMP-MM tumors. In the cohort of 45 patients with MCRC, 11 (24.4%) were CIMP+(+) for both tumors, 16 (35.6%) were CIMP− (−) for paired tumors, and 18 (40.0%) showed CIMP-MM: 9 (20.0%) were first tumor CIMP+(+) and

| Table 1. Clinical variables of interest and CIMP status in the EOCRC. *Statistical comparison was performed using Pearson’s Chi Square test (χ²). Statistical comparison was performed using Student’s t-test. Parenthesis refer to percentage numbers. Brackets are used to identify standard deviation. Percentages come from different initial number of patients because some cases were excluded: when only one biopsy was available or in cases of carcinoma in situ with severe dysplasia in which other features could not be studied. CIMP: CpG island methylator phenotype. No. Number. HGD: High-grade dysplasia. MSI: Microsatellite instability. MSS: Microsatellite stability. HNPCC: Hereditary non-polyposis colorectal cancer. NS: Not significant. |
| No. of patients & 59 (100.0) & 50 (84.7) & 9 (15.2) & — | p-value* |
| Average age of onset & 40 [5] & 40 [5] & 39 [5] & NS* |
| Sex & Female & 23 (39.0) & 19 (62.0) & 4 (44.4) & NS |
| & Male & 36 (61.0) & 31 (38.0) & 5 (55.6) & |
| Location & Proximal colon & 11 (18.6) & 6 (12.0) & 5 (55.6) & 0.008 |
| & Distal colon & 28 (47.5) & 26 (52.0) & 2 (22.2) & NS |
| & Rectum & 20 (33.9) & 18 (36.0) & 2 (22.2) & |
| Tumor histology & Adenocarcinoma & 47 (79.7) & 40 (80.0) & 7 (77.8) & NS |
| & Adenoma with HGD & 12 (20.3) & 10 (20.0) & 2 (22.2) & |
| Tumor stage & I & 16 (27.2) & 13 (26.0) & 3 (33.3) & NS |
| & II & 23 (39.0) & 19 (38.0) & 4 (44.4) & |
| & III & 10 (16.9) & 9 (18.0) & 1 (11.1) & |
| & IV & 10 (16.9) & 9 (18.0) & 1 (11.1) & |
| Tumor differentiation & Well differentiated & 18 (38.3) & 17 (41.5) & 1 (16.7) & NS |
| & Moderately differentiated & 24 (51.1) & 21 (51.2) & 3 (50.0) & |
| & Poorly differentiated & 5 (10.6) & 3 (7.3) & 2 (33.3) & |
| & Mucin production Tumor & 15 (31.9) & 12 (29.3) & 3 (50.0) & NS |
| & “Signet ring” cells Tumor & 3 (7.3) & 3 (7.3) & — & NS |
| Microsatellite status & MSS & 51 (86.4) & 43 (86.0) & 8 (88.9) & NS |
| & MSI & 8 (13.6) & 7 (14.0) & 1 (11.1) & |
| BRAF & Wild type & 58 (98.3) & 49 (98.0) & 9 (100.0) & NS |
| & Mutated & 1 (1.7) & 1 (2.0) & — & |
| KRAS & Wild type & 32 (59.3) & 27 (58.7) & 5 (62.5) & NS |
| & Mutated & 22 (40.7) & 19 (41.3) & 3 (37.5) & |
| Familial cancer history & Sporadic & 31 (52.5) & 26 (52.0) & 5 (55.6) & NS |
| & Familial aggregation & 23 (39.0) & 20 (40.0) & 3 (33.3) & |
| & HNPCC & 5 (8.5) & 4 (8.0) & 1 (11.1) & |
second tumor CIMP-(−), and 9 (20.0%) were first tumor CIMP-(−) and second tumor CIMP- (+) (Tables 3 and 4). Furthermore, the concordance of CIMP status between paired tumors in the SCRC and MCRC groups is summarized in Tables 5 and 6. Thirty-nine (72.2%) SCRC patients showed the same CIMP status in both tumors. In the cohort of MCRC, 27 (60%) patients presented concordant CIMP status in both tumors.

Correlation between CIMP phenotype and clinical features. Regarding clinical correlation with CIMP status, CIMP-(+) tumors in the EOCRC group were mainly located in proximal colon (p = 0.008), with a very low rate of well-differentiated tumors (Table 1). The CIMP-(+) tumors of patients with LOCRC presented moderate tumor differentiation (p = 0.045), MSI status (p = 0.021) and a higher BRAF mutation rate (p = 0.001) (Table 2). Other interesting features were the higher proportion of females as well as the rectal tumor location (both 63%). In the SCRC group (Table 3), cases with both tumors CIMP-(+), 16 (80.0%) patients were male and 11 (55.0%) tumors were distal-sided both paired tumors. Most patients diagnosed with MCRC and CIMP- (+) for both tumors showed entire colon location (81.8%), mucin production in the first tumor (25%) and early stage at diagnosis of the second tumor (72.7%) (Table 4). Differences in diagnostic tumor stage, sex, age and other clinicopathological features analyzed between different and concordant CIMP status in the SCRC and MCRC groups were not statistically significant (Tables 3 and 4).

| Total | Tumor CIMP- (−) | Tumor CIMP- (+) | p-value |
|---|---|---|---|
| No. of patients | 71 (100.0) | 52 (73.2) | 19 (26.8) | NS |
| Average age of onset | 78 [6] | 78 [6] | 79 [5] | NS |

**Sex**

- Female: 38 (53.5) vs. 26 (50.0) vs. 12 (63.2) | NS |
- Male: 33 (46.5) vs. 26 (50.0) vs. 7 (36.8) |

**Location**

- Proximal colon: 28 (39.4) vs. 22 (42.3) vs. 6 (31.6) | NS |
- Distal colon: 13 (18.3) vs. 12 (23.1) vs. 1 (5.3) |
- Rectum: 30 (42.3) vs. 18 (34.6) vs. 12 (63.2) |

**Tumor histology**

- Adenocarcinoma: 71 (100.0) vs. 52 (100.0) vs. 19 (100.0) | NS |
- Adenoma with HGD: — vs. — vs. — |

**Tumor stage**

- Stage I: 3 (4.3) vs. 3 (6.0) | NS |
- Stage II: 34 (49.3) vs. 24 (48.0) vs. 10 (52.6) |
- Stage III: 18 (26.1) vs. 15 (30.0) vs. 3 (15.8) |
- Stage IV: 14 (20.3) vs. 8 (16.0) vs. 6 (31.6) |

**Tumor differentiation**

- Well differentiated: 16 (24.2) vs. 15 (30.6) vs. 1 (5.9) | 0.045 |
- Moderately differentiated: 47 (71.2) vs. 33 (67.4) vs. 14 (82.4) |
- Poorly differentiated: 3 (4.6) vs. 1 (2.0) vs. 2 (11.8) |

**Mucin production**

- Tumor: 11 (16.7) vs. 9 (18.4) vs. 2 (11.8) | NS |

**Microsatellite status**

- MSS: 65 (91.5) vs. 50 (96.2) vs. 15 (78.9) | 0.021 |
- MSI: 6 (8.5) vs. 2 (3.8) vs. 4 (21.1) |

**BRAF**

- Wild type: 65 (91.5) vs. 51 (98.1) vs. 14 (73.7) | 0.001 |
- Mutated: 6 (8.5) vs. 1 (1.9) vs. 5 (26.3) |

**KRAS**

- Wild type: 5 (83.3) vs. 4 (80.0) vs. 1 (100.0) | NS |
- Mutated: 1 (16.7) vs. 1 (20.0) vs. — |

**Familial cancer history**

- Sporadic: 62 (91.2) vs. 45 (90.0) vs. 17 (94.4) | NS |
- Familial aggregation: 6 (8.8) vs. 5 (10.0) vs. 1 (5.6) |
- HNPCC: 3 (4.4) vs. 2 (4) vs. 1 (5.6) |

**Table 2.** Clinical variables of interest and CIMP status in the LOCRC subgroup. *Statistical comparison was performed using Pearson’s Chi Square test (χ2). Statistical comparison was performed using Student’s t-test. Parenthesis refer to percentage numbers. Brackets are used to identify standard deviation. Percentages come from different initial number of patients because some cases were excluded: when only one biopsy was available or in cases of carcinoma in situ with severe dysplasia in which other features could not be studied. CIMP: CpG island methylator phenotype. No.: Number. HGD: High-grade dysplasia. MSI: Microsatellite instability. MSS: Microsatellite stability. HNPCC: Hereditary non-polyposis colorectal cancer. NS: Not significant.
CpG-island methylation testing has been proposed as a tool for cancer detection and prognosis, and indicates that methylation status is of clinical relevance, despite the timing of its occurrence and its interaction with other genetic defects are not fully understood. In this study, we assessed CIMP status in different subsets of CRCs according to age of onset and the number of primary neoplasms: EOCRC and LOCRC with “unique” CRCs, and SCRC and MCRC.

In the assessment of CIMP status, we found that only 15.2% of EOCRC patients and 26.8% of LOCRC patients showed CIMP-(+) tumors. However, 37.0% of SCRC patients were CIMP-(+) for both tumors and total.

| Tumor | CIMP-(-) | CIMP-(+) | Total |
|-------|---------|---------|-------|
| Tumor A | 19 (35.2) | 20 (37.0) | 39 (73.5) |
| Tumor B | 20 (37.0) | 15 (27.8) | 35 (65.5) |
| p-value* | — | — | NS |

Table 3. Clinical variables of interest and CIMP status in the SCRC subgroup. *Statistical comparison was performed using Pearson's Chi Square test ($\chi^2$). Statistical comparison was performed using analysis of variance (ANOVA). Parenthesis refer to percentage numbers. Brackets are used to identify standard deviation.

Discussion

CpG-island methylation testing has been proposed as a tool for cancer detection and prognosis, and indicates that methylation status is of clinical relevance, despite the timing of its occurrence and its interaction with other genetic defects are not fully understood. In this study, we assessed CIMP status in different subsets of CRCs according to age of onset and the number of primary neoplasms: EOCRC and LOCRC with “unique” CRCs, and SCRC and MCRC.

In the assessment of CIMP status, we found that only 15.2% of EOCRC patients and 26.8% of LOCRC patients showed CIMP-(+) tumors. However, 37.0% of SCRC patients were CIMP-(+) for both tumors and
Table 4. Clinical variables of interest and CIMP status in the MCRC subgroup. *Statistical comparison was performed using Pearson’s Chi Square test ($\chi^2$). †Statistical comparison was performed using analysis of variance (ANOVA). Parentheses refer to percentage numbers. Brackets are used to identify standard deviation. Percentages come from different initial number of patients because some cases were excluded: when only one biopsy was available or in cases of carcinoma in situ with severe dysplasia in which other features could not be studied. CIMP: CpG island methylator phenotype. No.: Number. HGD: High-grade dysplasia. MSI: Microsatellite instability. MSS: Microsatellite stability. HNPCC: Hereditary non-polyposis colorectal cancer. NS: Not significant.
27.8% of the tumor pairs showed at least one CIMP- (+) tumor, suggesting that the serrated pathway of carcinogenesis could be the main mechanism for SCRC development\textsuperscript{34,35}. In the MCRC group, 24.4% were CIMP- (+) for both tumors and 40.0% were CIMP- (+) for at least one tumor. The reported differences in CIMP- (+) frequency between different groups could reflect the relative involvement of this particular molecular phenotype throughout the development of multiple neoplasms. In summary, our findings show a higher CIMP- (+) frequency in patients who develop multiple tumors than in patients who develop a single tumor. These different epigenetic patterns may be due to a “field effect” and possibly a highly-susceptible tissue microenvironment could be generated by the interplay of different etiologic factors including lifestyle, eating habits or environmental issues leading to the appearance and development of malignant tumors\textsuperscript{20,36}.

Moreover, in this study we observed that most pairs of tumors had concordant CIMP status in patients with SCRC (72.2%) which suggests that CIMP is maintained throughout neoplasm development; this has been taken as a evidence for the high probability that synchronous tumors could be developed through the same genetic pathway in each particular patient\textsuperscript{15}. On the other hand, 40% of the patients diagnosed with MCRC did not present concordance concerning CIMP status. The clinical implication of this finding is that the analysis of the CIMP status in any of the synchronous tumors could provide a reasonably reliable prediction of CIMP status in the other tumors even when it is not directly assayed. However, this prediction would be less reliable in MCRC. Comparison of these epigenetic patterns in synchronous and metachronous lesions may suggest that there is a tendency to have clonal features or a stronger “field effect” in patients diagnosed with SCRC, while the tumor heterogeneity in patients with MCRC may be caused by the contribution of different carcinogenic pathways in tumors developed at different time points.

About the results from the correlation between CIMP status and the clinical features within each group of tumors, recent systematic reviews have confirmed the association between CIMP phenotype and older ages, female gender, proximal tumor location, mucinous histology, poor differentiation and MSI\textsuperscript{36,37}. According to the “unique” colorectal cancers, EOCR\textsubscript{C} subset showed the proximal location, mucinous and poorly-differentiated
phenotype, while LOCRC linked with a higher proportion within this subset, and gender and MSI phenotype. Maybe the age-of-onset criterion gives rise to this stratification in the characteristics related to the CIMP in this type of CRC. Nevertheless, this characteristics didn’t correlate with Multiple Primary CRCs.

Our results seem to confirm the fact that there are distinct groups of CRC patients. This study, focused on the current knowledge of epigenetic alterations in CRC, could represent a substantial contribution to this research line, since it may have implications in terms of prevention, diagnosis and therapy.

Conclusion
Our results underscore the importance of taking into account several criteria for the development of multiple primary tumors when analyzing CRC. There is higher CIMP-(+) frequency in patients diagnosed with multiple CRC than in patients with “unique” CRC. Additionally, we conclude that there is a concordance of CIMP status of synchronous tumors in SCRC. Therefore, it could be suggested that CIMP status in one of the simultaneous tumors could predict CIMP status of other tumors.

Data Availability
The data that support the findings of this study are available from the corresponding author on reasonable request.

References
1. Siegel, R. L. et al. Colorectal Cancer Statistics, 2017. CA Cancer J Clin. 67, 177–193 (2017).
2. Network, T. C. G. A. et al. Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330–337 (2012).
3. Tsai, M.-H. et al. DNA Hypermethylation of SHISA3 in Colorectal Cancer: An Independent Predictor of Poor Prognosis. Ann. Surg. Oncol. 22, 1481–1489 (2015).
4. Duffy, M. J., O’Donovan, N. & Crown, J. Use of molecular markers for predicting therapy response in cancer patients. Cancer Treat. Rev. 37, 151–159 (2011).
5. Dienstmann, R. et al. Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. Nat. Rev 17, 79 (2017).
6. Ogino, S., Nosho, K. & Kikkeri, G. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut 58, 90–96 (2009).
7. Pino, M. S. & Chung, D. C. The Chromosomal Instability Pathway in Colon Cancer. Gastroenterology 138, 2059–2072 (2010).
8. Simons, C. C. J. M. et al. A novel classification of colorectal tumors based on microsatellite instability, the CpG island methylator phenotype and chromosomal instability: Implications for prognosis. Ann. Oncol. 24, 2048–2056 (2013).
9. Nojadeh, J. N., Shafie, S. B. & Sahinia, E. Review article: Microsatellite instability in colorectal cancer. EXCLI J. 17, 159–168 (2018).
10. Weisenberger, D. J., Liang, G. & Lenz, H. REVIEW DNA methylation aberrancies delineate clinically distinct subsets of colorectal cancer and provide novel targets for epigenetic therapies. Nat. Publ. Gr. 37, 566–577 (2017).
11. Weisenberger, D. J. et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat. Genet. 38, 787–793 (2006).
12. Arriba, M. et al. DNA copy number profiling reveals different patterns of chromosomal instability within colorectal cancer according to the age of onset. Mol. Carcinog. 55, 705–716 (2016).
13. Magnani, G. et al. Molecular Features and Methylation Status in Early Onset (<40 Years) Colorectal Cancer: A Population Based, Case–Control Study. Case. 2015 (2015).
14. Yeo, H. et al. Early-onset Colorectal Cancer Is Distinct From Traditional Colorectal Cancer. Clin. Colorectal Cancer 16, 293–299.e6 (2017).
15. Kirzin, S. et al. Sporadic early-onset colorectal cancer is a specific sub-type of cancer: A morphological, molecular and genetics study. PLoS One 9 (2014).
16. Hawthorn, L., Lan, L. & Mojica, W. Genomics Evidence for field effect cancerization in colorectal cancer. Genomics 103, 211–221 (2014).
17. Sugih, T. et al. Analysis of the DNA methylation level of cancer-related genes in colorectal cancer and the surrounding normal mucosa. Clin. Epigenetics 9, 55 (2017).
18. Wang, X. et al. The molecular landscape of synchronous colorectal cancer reveals genetic heterogeneity. Carcinogenesis 39, 708–718 (2018).
19. Lam, A. K. et al. Synchronous colorectal cancer: Clinical, pathological and molecular implications. 20, 6815–6820 (2014).
20. Arriba, M. et al. Towards a Molecular Classification of Synchronous Colorectal Cancer: Clinical and Molecular Characterization. Clin. Colorectal Cancer (2016).
21. Min, B.-H. et al. The CpG island methylator phenotype may confer a survival benefit in patients with stage II or III colorectal carcinomas receiving fluoropyrimidine-based adjuvant chemotherapy. BMC Cancer 11, 344 (2011).
22. Shiwitz, S. et al. CpG island methylator phenotype is associated with response to adjuvant irinotecan-based therapy for stage III colon cancer. Gastroenterology 147, 637–645 (2014).
23. Baba, Y. et al. Epigenetic Field Cancerization in Gastrointestinal Cancers Running Title: Epigenetic field defect in GI Cancers. Cancer Lett. 396, 101601 (2016).
24. Park, S. et al. Field Cancerization in Sporadic Colon Cancer. 10, 773–780 (2016).
25. Perea, J. et al. Defining synchronous colorectal cancers based on tumor clonality. Int. J. Cancer 144, 1596–1608 (2019).
26. Bacher, J. W. et al. Development of a fluorescent multiplex assay for detection of MSI–High tumors. Dis. Markers 20, 237–50 (2004).
27. Perea, J. et al. Early-onset colorectal cancer is an easy and effective tool to identify retrospectively Lynch syndrome. Ann. Surg. Oncol. 18, 3285–3291 (2011).
28. Perea, J. et al. Frequency and Impact of KRAS mutation in early onset colorectal cancer. Hum. Pathol. 61, 221–222 (2017).
29. Sanchez, J. A. et al. Genetic and epigenetic classifications define clinical phenotypes and determine patient outcomes in colorectal cancer. Br. J. Surg. 96, 1196–1204 (2009).

| CIMP status | 2nd Tumor (+) | 2nd Tumor (−) |
|------------|--------------|---------------|
| 1st Tumor (+) | 11 (24.4%) | 9 (20.0%) |
| 1st Tumor (−) | 9 (20.0%) | 16 (35.6%) |

Table 6. Concordance of CIMP status in paired-tumors from patients diagnosed with MCRC using eight-marker panel. CIMP: CpG island methylator phenotype. MCRCs: Metachronous colorectal cancers.
30. Murcia, O. et al. Serrated colorectal cancer: Molecular classification, prognosis, and response to chemotherapy. World J. Gastroenterol. 22, 3516–3530 (2016).
31. Bettington, M. et al. The serrated pathway to colorectal carcinoma: Current concepts and challenges. Histopathology 62, 367–386 (2013).
32. Szylberg, A., Janiczek, M., Popiel, A. & Marszałek, A. Serrated polyps and their alternative pathway to the colorectal cancer: A systematic review. Gastroenterol. Res. Pract. 2015, 1–7 (2015).
33. Ogino, S. et al. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J. Mol. Diagn. 9, 305–14 (2007).
34. Gao, Q. et al. Serrated polyps and the risk of synchronous colorectal advanced neoplasia: A systematic review and meta-analysis. Am. J. Gastroenterol. 110, 501–509 (2015).
35. Nosho, K. et al. A Prospective Cohort Study Shows Unique Epigenetic, Genetic, and Prognostic Features of Synchronous Colorectal Cancers. NIH Public Access 137, 1609–20 (2009).
36. Lochhead, P. et al. Etiologic Field Effect: Reappraisal of the Field Effect Concept in Cancer Predisposition and Progression. NIH Public Access 28, 14–29 (2015).
37. Dykes, S. L., Qui, H., Rothenberger, D. A. & Garcia-Aguilar, J. Evidence of a preferred molecular pathway in patients with synchronous colorectal cancer. Cancer 98, 48–54 (2003).

Acknowledgements
We thank the Tumor Registry of the Pathology Department of the 12 de Octubre University Hospital for providing the paraffin-embedded tissues, and Ron Hartong for his help with the English revision of this manuscript. This work was funded by Projects PI10/00683 and PI16/01650 to J.P, and PI16/01920 to R.G.S., from the Spanish Ministry of Health and Consumer Affairs and FEDER, and by Project 2012-0036 from the Mutua Madrileña Foundation. This work was supported by R01 (CA72851, CA181572, CA184792, CA202797) and U01 (CA187956, CA214254) grants from the National Cancer Institute, NIH; R0140784 from the Cancer Prevention Research Institute of Texas; grants from the Baylor Foundation and Baylor Scott & White Research Institute, Dallas, TX, USA to AG.

Author Contributions
Conceptualization: Sandra Tapial, José Perea, Mariano García-Arranz, Damián García-Olmo, Miguel Urioste, Rogelio González-Sarmiento, Ajay Goel. Methodology Validation: Sandra Tapial, Susana Olmedillas-López, Daniel Rueda, María Arriba. Resources: Alfredo Vivas, Yolanda Rodriguez. Formal analysis and investigation: Sandra Tapial, Susana Olmedillas-López, Daniel Rueda, Juan L. García, María Arriba, Daniel Rueda, Jessica Pérez, Laura Pena, Rocío Olivera. Data Curation and visualization: Juan L. García, Jessica Pérez, Laura Pena, Rocío Olivera. Writing-Original Draft Preparation: Sandra Tapial, José Perea, Daniã³n García-Olmo, Miguel Urioste, Rogelio González-Sarmiento, Ajaý Goel. Supervision: José Perea, Mariano García-Arranz, Daniã³n García-Olmo, Miguel Urioste, Rogelio González-Sarmiento, Ajay Goel. Writing-Review and Editing: All authors. Project Administration: José Perea. Funding Acquisition: Rogelio González-Sarmiento, José Perea, Ajay Goel.

Additional Information
Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019