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A Prospective Cohort Study Shows Unique Epigenetic, Genetic, and Prognostic Features of Synchronous Colorectal Cancers

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Abstract

Background & Aims—Synchronous colorectal neoplasias (2 or more primary carcinomas identified in the same patient) are caused by common genetic and environmental factors and can therefore be used to study the field effect. Synchronous colon cancers have not been compared with control solitary cancers in a prospective study.

Methods—We analyzed data collected from 47 patients with synchronous colorectal cancers and 2021 solitary colorectal cancers (controls) in 2 prospective cohort studies. Tumors samples were analyzed for methylation in LINE-1 and 16 CpG islands (*CACNA1G*, *CDKN2A* [p16], *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, *SOCS1*, *CHFR*, *HIC1*, *IGFBP3*, *MGMT*, *MINT1*, *MINT31*, p14 [ARF], and *WRN*); microsatellite instability (MSI); the CpG island methylator phenotype (CIMP); 18q loss of heterozygosity; *KRAS*, *BRAF* and *PIK3CA* mutations; and expression of β -catenin, p53, p21, p27, cyclin D1, fatty acid synthase, and cyclooxygenase-2.

Results—Compared to patients with solitary colorectal cancer, synchronous colorectal cancer patients had reduced overall survival time (log-rank $p=0.0048$; hazard ratio [HR]=1.71; 95% confidence interval [CI]=1.17–2.50; $p=0.0053$; multivariate HR=1.47; 95% CI=1.00–2.17;

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p=0.049). Compared to solitary tumors, synchronous tumors more frequently contained *BRAF* mutations (p=0.0041), CIMP-high ($\geq 6/8$ methylated CIMP markers; p=0.013), and MSI-high (p=0.037). Methylation levels of LINE-1 (Spearman $r=0.82$; p=0.0072) and levels of CpG island methylation (p<0.0001) correlated between synchronous cancer pairs from the same individuals.

Conclusions—Synchronous colorectal cancers had more frequent mutations in *BRAF*, were more frequently categorized as CIMP- and MSI-high, and a worse prognosis than solitary colorectal cancers. Similar epigenomic and epigenetic events were frequently observed within a synchronous cancer pair, suggesting the presence of a field effect.

Keywords

colon cancer; epigenomics; epigenetics; review; clinical outcome

INTRODUCTION

Synchronous colorectal cancers refer to two or more primary colorectal carcinomas detected in a single individual at the time of the first diagnosis of colorectal cancer. Synchronous neoplasias, which arise in a background of common etiologic (genetic or environmental) factors, can provide a unique model to examine molecular aberrations and field effect.¹ Random molecular aberrations within synchronous tumor pairs may support a stochastic process in carcinogenesis, while nonrandom molecular aberrations may support a specific etiology or cause in carcinogenic process,¹ or the hypothesis of field effect in apparently normal colonic mucosa.^{2–4} Previous studies have analyzed several molecular markers [p53,⁵ microsatellite instability (MSI),^{6–10} *MLH1*,^{6, 7, 9–11} *MSH2*,^{6, 7, 9, 11} *MSH6*,⁷ or CpG island methylation¹] within synchronous colorectal cancer pairs, suggesting both concordant^{1, 8–10} and discordant^{1, 5, 7, 10, 11} alteration patterns. However, no previous study has examined global DNA methylation levels in synchronous colorectal cancer pairs. Global DNA hypomethylation has been linked to genomic instability and carcinogenesis,^{12, 13} and hypomethylation in LINE-1 repetitive sequence has been associated with poor prognosis in colon cancer.¹⁴

Previous studies have examined clinical features of synchronous colorectal cancer patients (Table 1; individual studies are listed in Supplemental Table 1).^{5–11, 15–26} However, the prognostic significance of cancer synchronicity remains inconclusive.^{17–24} While synchronous cancer patients and solitary cancer patients showed similar survival in most studies,^{17, 19–24} one study showed worse survival in synchronous cases.¹⁸ However, in all of these studies,^{17–24} “control” solitary cancers were retrospectively selected, thereby subject to potential selection bias. An optimal control group would be solitary colorectal cancers in a population that has given rise to synchronous colorectal cancers. It is possible to secure such a control group in a prospective cohort setting.

In this study, during follow-up of two well-characterized, prospective cohort studies, we identified 47 cases of synchronous colorectal cancers and 2068 control solitary colorectal cancers that had arisen in the same background population as synchronous cases. We examined patient survival and various molecular changes in synchronous and solitary colorectal cancers in our prospective cohort studies.

MATERIALS AND METHODS

Study population

We utilized two prospective cohort studies; the Nurses’ Health Study (121,701 women followed since 1976),²⁷ and the Health Professionals Follow-up Study (51,529 men

followed since 1986).²⁷ Every 2 years, participants have been sent follow-up questionnaires to identify newly diagnosed cancer and other diseases in themselves and their first degree relatives. For nonresponders, we searched the National Death Index to discover deaths and ascertain any diagnosis of colorectal cancer that contributed to death or was a secondary diagnosis. Study physicians and pathologists reviewed medical and pathology records, and recorded tumor stage, location and synchronicity status.

During prospective follow-up of the cohort participants up to 2002, there were 2068 incident colorectal cancer patients with available pathology reports and follow-up data, which constituted the base of this study (Figure 1). Among them, we identified 47 patients who had synchronous colorectal cancers, which were strictly defined as the presence of two or more colorectal cancers (with at least submucosal invasion, stage pT1) that were grossly, unequivocally separated by normal colorectal mucosa at the first diagnosis of colorectal cancer. In addition, metastasis mimicking synchronous tumors was excluded. Two of the 47 synchronous cases had 3 synchronous cancers, and all of the other cases had 2 synchronous cancers: cancer tissues of the patients with 3 tumors were unavailable. The remaining 2021 patients had solitary colorectal cancers at the first diagnosis, which constituted a control group in this study. These solitary colorectal cancers had arisen in the population that had given rise to synchronous colorectal cancer cases, and thus constituted an optimal comparison (control) group (Table 1). Patients were observed until death or June 2008, whichever came first. We collected paraffin-embedded tissue blocks from hospitals where patients underwent tumor resections,²⁷ and pathologic features were examined by a pathologist (S.O.) unaware of other data. Tumor grade was classified as low or high (<50% or ≥50% solid areas, respectively). Based on tissue specimen availability, we performed pathologic and molecular analysis on a total of 29 cases of synchronous colorectal cancers and 1084 solitary colorectal cancers for comparison. Informed consent was obtained from all 2068 subjects. This study was approved by the Human Subjects Committees at Brigham and Women's Hospital and the Harvard School of Public Health.

Analyses of *KRAS*, *BRAF* and *PIK3CA*, and microsatellites

DNA from paraffin-embedded tissue was extracted, and PCR and Pyrosequencing targeted for *KRAS* codons 12 and 13,²⁸ *BRAF* codon 600,²⁹ and *PIK3CA* exons 9 and 20 were performed.³⁰ MSI status was determined using D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487.³¹ MSI-high was defined as the presence of instability in ≥30% of the markers, MSI-low as instability in 1–29% of the markers, and microsatellite stability (MSS) as the absence of instability. For 18q loss of heterozygosity (LOH) analysis using microsatellite markers (D18S55, D18S56, D18S67, D18S487), LOH at each locus was defined as ≥40% reduction of one of two allele peaks in tumor DNA relative to normal DNA.³² 18q LOH negativity was defined as the presence of ≥2 informative markers and the absence of LOH.

Methylation assays for CpG islands and LINE-1

Bisulfite DNA treatment and real-time PCR (MethyLight) assays were validated and performed.³³ We quantified methylation at 8 CIMP-specific CpG islands [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOC31*].^{34, 35} CIMP-high was defined as ≥6/8 methylated promoters using the 8-marker CIMP panel, CIMP-low as 1/8–5/8 methylated promoters and CIMP-0 as 0/8 methylated promoters, according to the previously established criteria.³⁴ We also quantified methylation at 8 other loci (*CHFR*, *HIC1*, *IGFBP3*, *MGMT*, *MINT1*, *MINT31*, p14, and *WRN*).^{36, 37} The percentage of methylated reference (PMR, i.e., methylation index) at a specific locus was calculated as previously described.³³ Methylation positivity was set as PMR ≥4 as previously validated.³³ LINE-1 methylation level was measured by Pyrosequencing,³⁸

Immunohistochemistry

We constructed tissue microarrays (TMAs).²⁷ Methods of immunohistochemistry were previously described as follows: cyclin D1,³⁹ β -catenin,⁴⁰ p21, p27, p53,⁴¹ fatty acid synthase (FASN) and COX-2.⁴² All immunohistochemically-stained slides for each marker were interpreted by one of the investigators (p53, p21, p27, COX-2 and FASN by S.O.; cyclin D1 and β -catenin by K.N.) unaware of other data. A random sample of 108–402 tumors were reexamined by a second observer (p21, p27 and cyclin D1 by K.S.; p53, FASN by K.N.; β -catenin by S.O.; COX-2 by R. Dehari, Kanagawa Cancer Center) unaware of other data. The concordance rates between the two observers were 0.83 or greater (all $\kappa \geq 0.60$, except for $\kappa = 0.57$ for FASN; all $p < 0.0001$), indicating good to substantial agreement.

Statistical analysis

We used SAS version 9.1 (SAS Institute, Cary, NC) and all p values were two-sided. Rigorous corrections for multiple hypothesis testing were not attempted due to limited statistical power. The χ^2 test (or Fisher's exact test when any expected cell count was < 5) was used to examine an association between categorical variables. The Wilcoxon rank-sum test was performed to compare age and LINE-1 methylation levels between synchronous cancers and solitary controls. Spearman correlation coefficients were used to assess the correlation of LINE-1 methylation levels within synchronous colorectal cancer pairs. The κ coefficient was calculated to assess agreement within synchronous cancer pairs on tumor location (proximal colon vs. distal colorectum) and methylation status (positive vs. negative) at the 16 individual CpG islands.

The Kaplan-Meier method was used to describe the distribution of survival time, and the log-rank test was performed. For analyses of colorectal cancer-specific mortality, death as a result of colorectal cancer was the primary end point and deaths from other causes were censored. Stage-matched (stratified) Cox proportional hazard models were used to calculate mortality by tumor synchronicity status, adjusted for age at diagnosis (continuous), sex, year of diagnosis (continuous), body mass index (BMI; < 30 vs. ≥ 30 kg/m²) prior to diagnosis, family history of colorectal cancer in any first degree relative (present vs. absent), tumor location (proximal vs. distal colon vs. rectum). Tumor stage (I, II, III, IV, unknown) was used as a matching (stratifying) variable using the “strata” option in the SAS “proc phreg” command, to avoid residual confounding and overfitting. For cases with missing data in BMI (14% missing) or tumor location (2.6% missing), we included those cases in the category of BMI < 30 kg/m², or a category of “proximal colon”, respectively, in order to avoid overfitting. Neither BMI nor tumor location was a confounder. We confirmed that excluding cases with missing BMI data (or tumor location data) did not substantially alter results (data not shown).

RESULTS

Clinical features of synchronous colorectal cancer cases

During follow-up of the two prospective cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study), there were 2068 eligible colorectal cancer patients diagnosed up to 2002. Among the 2068 patients, we identified 47 cases of synchronous colorectal cancers (Figure 1). The remaining 2021 solitary colorectal cancers had arisen in the same cohort population that had given rise to the 47 synchronous cases, and thus constituted an optimal control group. We assessed clinical features of the 47 synchronous colorectal cancer cases and the 2021 control solitary cancer controls (Table 2). Compared to the solitary cancer patients, the synchronous cases were significantly older ($p = 0.016$).

Synchronous cancers within pairs tended to co-localize in either proximal colon or distal colorectum (71% concordant; $\kappa=0.42$; $p=0.0057$) (Supplemental Table 2).

Colorectal cancer synchronicity and patient survival

We performed survival analysis on the 2068 patients with incident colorectal cancers. During follow-up, there were 875 deaths, including 579 colorectal cancer-specific deaths. In Kaplan-Meier analysis, synchronous cancer patients experienced worse survival than solitary cancer patients (log-rank $p=0.0048$) (Figure 2). In a univariate Cox model, compared to solitary cancer patients, synchronous cancer patients experienced significantly higher overall mortality [hazard ratio (HR) 1.71; 95% confidence interval (CI), 1.17–2.50; $p=0.0053$] (Table 3). In a multivariate Cox model adjusting for potential confounders, synchronous cases were associated with higher overall mortality (adjusted HR 1.47; 95% CI, 1.00–2.17; $p=0.049$). Similar results were obtained when colorectal cancer-specific mortality was used as the endpoint, though statistical significance was not reached in adjusted models. No major confounder was identified in the Cox regression analyses.

Pathologic and molecular characteristics in synchronous cancers vs. solitary cancers

Among the 2068 colorectal cancer patients, tumor tissues were available for pathologic and molecular analyses in 1113 patients, including 29 synchronous cases for which tissue was obtained from at least one tumor (a tumor with higher stage or larger tumor if synchronous tumors were the same stage). We compared pathologic and molecular characteristics of synchronous cancers with those of solitary cancer controls (Table 2). Compared to the solitary cancers, synchronous cancer cases more frequently showed *BRAF* mutations ($p=0.0041$), CpG island methylator phenotype (CIMP)-high ($p=0.013$) and microsatellite instability (MSI)-high ($p=0.037$). Synchronous cases were associated with hypermethylation at *SOCS1* ($p=0.042$) among the 16 CpG islands (Supplemental Table 3).

The association between synchronicity and *BRAF* mutation might have been due to confounding by tumor location (i.e., proximal colon) or age. Thus, we examined the relation between synchronicity and *BRAF* mutation in the proximal colon. In the proximal colon, *BRAF* mutations were significantly more common in synchronous cancers (58% = 7/12; $p=0.009$) than in solitary cancers (22% = 79/353). We also constructed a logistic regression model including age, *BRAF* mutation, and synchronicity status as an outcome variable. The association between *BRAF* and tumor synchronicity persisted (adjusted odds ratio, 4.02; 95% CI, 1.66–9.72; $p=0.0021$), while age was no longer associated with tumor synchronicity ($p=0.94$).

Since *BRAF* mutation is closely associated with CIMP-high in colorectal cancer,⁴³ we examined the frequencies of combined *BRAF*/CIMP subtypes. Synchronous cancers were more commonly *BRAF*-mutated/CIMP-high (32% = 7/22), compared to solitary controls (8.0% = 63/787; $p=0.0058$) (Table 2).

The relation between tumor synchronicity and high mortality could be attributed to molecular features of synchronous tumors. Thus, we limited our cases to patients with available tumor tissue data and adjusted for tumor stage and molecular variables including *BRAF* mutation,³⁶ 44 CIMP,³⁶ MSI,⁴⁵ and tumor grade. The adjusted HR for overall mortality (synchronous cases vs. solitary controls) was 1.92 (95% CI, 1.10–3.35; $p=0.022$), suggesting that the inferior survival associated with synchronous cancers was independent of *BRAF* mutation, CIMP, MSI and tumor grade.

Molecular analysis in synchronous cancer pairs and matched normal colon mucosa

Among the 29 synchronous cancer cases with available tumor tissue, 10 synchronous cancer cases could provide cancer tissues from both cancers. Among the 10 cases in Table 4, our previous study¹ examined Case Nos. 1–6 for methylation in 6 CpG islands (*CACNA1G*, *CDKN2A*, *CRABP1*, *MLH1*, *NEUROG1* and *MGMT*), MSI, *KRAS* and *BRAF*. In this study, we considerably expanded analyses in terms of the number of markers and sample size, and we examined adjacent normal colon for CIMP, LINE-1 and *BRAF* mutation. Notably, LINE-1 methylation levels in the synchronous cancer pairs were significantly correlated (Spearman $r = 0.82$; $p = 0.0072$) (Figure 3). This correlation appeared to persist even in the tumor pairs of discordant anatomical locations (i.e., one proximal tumor and the other distal tumor). LINE-1 methylation level in adjacent normal colon (from 7 cancer pairs) was consistently high (>71%) even in the cases with low LINE-1 methylation level in both synchronous tumors (Case Nos. 5, 9 and 10; Supplemental Table 4).

With regard to CpG island methylation, Table 5 summarizes pairwise agreements within the 10 synchronous cancer pairs on methylation status at each of the 16 CpG islands. Within all of the 10 synchronous cancer pairs, methylation status at the 16 CpG islands were significantly concordant (78% concordant, $\kappa = 0.53$, $p < 0.0001$). In addition, synchronous cancer pairs of concordant anatomical locations (i.e., proximal colon vs. distal colorectum) showed a significant concordance (81% concordant, $\kappa = 0.41$, $p = 0.010$, for proximal pairs; 94% concordant, $\kappa = 0.80$, $p = 0.0001$, for distal pairs). In contrast, synchronous pairs in discordant locations (i.e., one proximal cancer and the other distal cancer) did not show concordant methylation ($\kappa = 0.15$, $p = 0.18$).

Finally, we analyzed *BRAF* mutation and DNA methylation at the 8 CIMP-specific CpG islands in adjacent normal colon in some synchronous cases, and neither *BRAF* mutation nor substantial CpG island methylation was detected in normal colon (Supplemental Table 4).

DISCUSSION

In this study, we examined genetic and epigenetic features as well as clinical outcome of synchronous colorectal cancer cases. To date, no previous study has examined global DNA methylation levels within synchronous colorectal cancer pairs. In addition, no previous study has compared clinical and molecular features of synchronous colorectal cancers with those of solitary colorectal cancers which had occurred in the same background population as the synchronous cancers in a prospective cohort setting (Table 1). We found that synchronous colorectal cancer patients experienced a significantly higher overall mortality than solitary cancer patients. Compared to solitary cancers, synchronous colorectal cancers more commonly showed *BRAF* mutations, CIMP-high and MSI-high. In our pairwise study, synchronous colorectal cancer pairs exhibited a significant correlation of LINE-1 methylation levels. Synchronous cancer pairs in proximal colon and those in distal colorectum showed a significantly concordant pattern of CpG island methylation, while tumor pairs in discordant locations (one proximal cancer and the other distal cancer) did not show concordant CpG island methylation. Our data indicate that synchronous colorectal cancers give us unique insights into field effect (cancerization) and colorectal carcinogenic process.

The proportion of synchronous cancer cases among colorectal cancer patients vary from 1.1% to 5.3%.^{5, 9, 10, 16–26} The relatively higher reported frequencies (>3.5%) in some studies^{5, 10, 16, 19, 22, 23} might have been due to loose criteria in population-based cancer registry,²³ referral nature of hospital,^{5, 10, 16, 19, 22} or small case numbers with a chance variation^{5, 10, 16, 19} (Supplemental Table 1) Above all, synchronous cancer cases are rare, which has posed a difficulty in conducting a study with an adequate statistical power.

Although one population-based cancer registry reported 596 synchronous colorectal cancer patients, tumoral molecular data were unavailable.²³

Previous studies have examined a number of molecular events within synchronous colorectal cancer pairs.^{5–11} While some studies showed concordant patterns of MSI status within synchronous cancer pairs,^{8–10} others showed discordant patterns of MSI,⁷ *MLH1* methylation,^{10, 11} *MSH2* expression,¹¹ or p53 expression.⁵ Together with previous data,^{9–11} our results (i.e., a concordant CpG island methylation pattern in proximal/proximal and distal/distal pairs and a discordant methylation pattern in proximal/distal pairs) suggest both random and nonrandom components in CpG island methylation.

LINE-1 methylation level has been correlated with global DNA methylation level.^{46, 47} We have previously reported that LINE-1 hypomethylation is associated with poor prognosis among colon cancer patients,¹⁴ and inversely associated with CIMP-high and MSI-high.³⁸ Global DNA methylation level may affect regulation of many proto-oncogenes and anti-oncogenes, and determine clinical, pathologic and molecular characteristics of colorectal cancer.¹⁴ In the current study, we demonstrated a significant correlation of LINE-1 methylation levels within synchronous cancer pairs, which might have been due to field effect. The concept of field effect (field defect or field cancerization) has been proposed to explain the development of multiple (synchronous or metachronous) primary tumors in the same organ.⁴ As a potential mechanism of field effect, epigenetic alterations have been proposed,⁴ and previous studies have reported the field effect due to *MGMT* promoter methylation or other CpG island methylation in normal appearing colonic mucosa.² A study has revealed that LINE-1 methylation level is lower in some colon cancers and adjacent normal colon mucosa than peripheral blood from the same patients or normal colon mucosa in healthy individuals.⁴⁸ However, in the current study, LINE-1 methylation level was consistently high in the adjacent normal mucosa that we tested, even in the cases with LINE-1 hypomethylation in both synchronous tumors. Likewise, neither CpG island methylation nor *BRAF* mutation was detected in the adjacent normal colon from synchronous cases. While these results do not support the field effect hypothesis, these do not exclude it either. Distribution of field effect may be patchy, or alternatively, there may exist an unidentified molecular abnormality in normal mucosa, which drives the development of the described alterations in LINE-1, CpG islands or *BRAF*. Additional studies are necessary to clarify the reasons why synchronous cancer pairs tend to show concordant patterns of some molecular alterations.

In our current study, compared to solitary cancers, synchronous cancers were more likely *BRAF*-mutated, CIMP-high and MSI-high. Accumulating evidence suggests that CIMP-high MSI-high colorectal cancer arise through the serrated pathway to colorectal cancer, which is characterized by a high frequency of *BRAF* mutation.⁴⁹ Thus, compared to solitary cancers, synchronous colorectal cancers more likely arise through the serrated pathway, and possibly from multiple hyperplastic polyps or polyposis.^{3, 49} In addition, synchronous colorectal cancer pairs exhibit a significant correlation of LINE-1 methylation levels and concordant CpG island methylation, suggesting that shared environmental/genetic background may cause concordant patterns of DNA methylation and epigenetic events.

Studying clinical outcome and molecular changes is important in cancer research.^{50, 51} While most studies on clinical outcome of synchronous cancer patients^{17, 19–24} have reported no significant prognostic association with synchronous colorectal cancers, one study¹⁸ reported worse survival associated with synchronous cancers. However, in all of these studies,^{17–22, 24} controls were retrospectively selected; hence selection bias affecting clinical outcome data was a major concern. In contrast, our study represents the first prospective study to examine a survival difference between synchronous and solitary

colorectal cancer patients. Our solitary cancers had arisen in the same background population as the synchronous cancer cases, thus eliminating sources of considerable bias that were inevitably present in any retrospective case-control studies.

Notably, one study²³ utilized the population-based cancer registry, and found no significant survival difference between synchronous cases and solitary cancers. In that study,²³ the proportion of synchronous cases was estimated to be 3.8% (596/15562), which was considerably higher than most previous studies^{5, 9, 17, 18, 20, 21, 24–26} and our current study (2.3%). It may be possible that synchronous cancer cases in the cancer registry study²³ might have included patients with one cancer and a second malignant polyp [intramucosal carcinoma (pTim) or carcinoma in situ (pTis)]. We strictly limited synchronous cancer cases to those with two or more unequivocally separate cancers with at least submucosal invasion (pT1).

Possible reasons for the high mortality associated with synchronous cases remain speculative. There are several hypotheses (which are not necessarily mutually exclusive):

1. It is possible that understaging of cancer may contribute to this phenomenon. Cancer staging is based on limited information. Pathologic staging is based on limited sampling of tissues (primary cancers and lymph nodes) from the entire specimen for pathologic assessment, and tissue sections represent only a two-dimensional profile of tumor or lymph node. Thus, a possibility of not detecting a metastatic or more advanced focus exists, and the presence of two separate cancers in a single individual may increase the likelihood of understaging.
2. The presence of more than one cancer focus increases the likelihood of progressive disease (recurrence or metastasis), due to a larger cancer burden or due to recurrence by field effect.
3. Patients with synchronous cancers may have more chances of postoperative complications than those with solitary cancers.
4. Underlying molecular features of synchronous colorectal cancers may cause poor outcome. In the current study, the inferior survival associated with synchronous cancers was independent of *BRAF* mutation, MSI-high, CIMP-high and tumor grade. Nonetheless, we cannot exclude a possibility of confounding by other unidentified molecular characteristics that contributed to inferior survival of synchronous cancer patients.

In summary, our prospective cohort study suggests that synchronous colorectal cancer cases are associated with poor prognosis. Compared to solitary cancers, synchronous colorectal cancers more commonly show *BRAF* mutations, CIMP-high and MSI-high. In addition, synchronous colorectal cancer pairs exhibit a significant correlation of LINE-1 methylation levels and concordant CpG island methylation. Our study may be an important one step forward to elucidate clinical and molecular characteristics of synchronous colorectal cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and the HUGO Gene Nomenclature Committee-approved official gene symbol

AJCC	American Joint Committee on Cancer
BMI	body mass index
CI	confidence interval
CIMP	CpG island methylator phenotype
FASN	fatty acid synthase
HPFS	Health Professionals Follow-up Study
HR	hazard ratio
LOH	loss of heterozygosity
MSI	microsatellite instability
MSS	microsatellite stable
NHS	Nurses' Health Study
PMR	percentage of methylated reference

References

1. Ogino S, Brahmandam M, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. Epigenetic profiling of synchronous colorectal neoplasias by quantitative DNA methylation analysis. *Mod Pathol* 2006;19:1083–90. [PubMed: 16699497]
2. Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, Houlihan PS, Krouse RS, Prasad AR, Einspahr JG, Buckmeier J, Alberts DS, Hamilton SR, Issa JP. MGMT promoter methylation and field defect in sporadic colorectal cancer. *J Natl Cancer Inst* 2005;97:1330–8. [PubMed: 16174854]
3. Young J, Jass JR. The case for a genetic predisposition to serrated neoplasia in the colorectum: hypothesis and review of the literature. *Cancer Epidemiol Biomarkers Prev* 2006;15:1778–84. [PubMed: 17035382]
4. Giovannucci E, Ogino S. DNA methylation, field effects, and colorectal cancer. *J Natl Cancer Inst* 2005;97:1317–9. [PubMed: 16174847]
5. Yalcinkaya U, Ozturk E, Ozgur T, Yerci O, Yilmazlar T. P53 expression in synchronous colorectal cancer. *Saudi Med J* 2008;29:826–31. [PubMed: 18521459]
6. Velayos FS, Lee SH, Qiu H, Dykes S, Yiu R, Terdiman JP, Garcia-Aguilar J. The mechanism of microsatellite instability is different in synchronous and metachronous colorectal cancer. *J Gastrointest Surg* 2005;9:329–35. [PubMed: 15749592]
7. Lawes DA, Pearson T, Sengupta S, Boulos PB. The role of MLH1, MSH2 and MSH6 in the development of multiple colorectal cancers. *Br J Cancer* 2005;93:472–7. [PubMed: 16106253]
8. Pedroni M, Tamassia MG, Percesepe A, Roncucci L, Benatti P, Lanza G Jr, Gafa R, Di Gregorio C, Fante R, Losi L, Gallinari L, Scorcioni F, Vaccina F, Rossi G, Cesinaro AM, Ponz de Leon M.

- Microsatellite instability in multiple colorectal tumors. *Int J Cancer* 1999;81:1–5. [PubMed: 10077143]
9. Dykes SL, Qui H, Rothenberger DA, Garcia-Aguilar J. Evidence of a preferred molecular pathway in patients with synchronous colorectal cancer. *Cancer* 2003;98:48–54. [PubMed: 12833454]
 10. Norrie MW, Hawkins NJ, Todd AV, Meagher AP, O'Connor TW, Ward RL. The role of hMLH1 methylation in the development of synchronous sporadic colorectal carcinomas. *Dis Colon Rectum* 2002;45:674–80. [PubMed: 12004219]
 11. Aslanian HR, Burgart LJ, Harrington JJ, Mahoney DW, Zinsmeister AR, Thibodeau SN, Ahlquist DA. Altered DNA mismatch repair expression in synchronous and metachronous colorectal cancers. *Clin Gastroenterol Hepatol* 2008;6:1385–8. [PubMed: 18639494]
 12. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R. Induction of tumors in mice by genomic hypomethylation. *Science* 2003;300:489–92. [PubMed: 12702876]
 13. Holm TM, Jackson-Grusby L, Brambrink T, Yamada Y, Rideout WM 3rd, Jaenisch R. Global loss of imprinting leads to widespread tumorigenesis in adult mice. *Cancer Cell* 2005;8:275–85. [PubMed: 16226703]
 14. Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Chan AT, Schernhammer ES, Giovannucci EL, Fuchs CS. A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. *J Natl Cancer Inst* 2008;100:1734–8. [PubMed: 19033568]
 15. Ueno M, Muto T, Oya M, Ota H, Azekura K, Yamaguchi T. Multiple primary cancer: an experience at the Cancer Institute Hospital with special reference to colorectal cancer. *Int J Clin Oncol* 2003;8:162–7. [PubMed: 12851840]
 16. Takeuchi H, Toda T, Nagasaki S, Kawano T, Minamisono Y, Maehara Y, Sugimachi K. Synchronous multiple colorectal adenocarcinomas. *J Surg Oncol* 1997;64:304–7. [PubMed: 9142187]
 17. Chen HS, Sheen-Chen SM. Synchronous and “early” metachronous colorectal adenocarcinoma: analysis of prognosis and current trends. *Dis Colon Rectum* 2000;43:1093–9. [PubMed: 10950007]
 18. Enker WE, Dragacevic S. Multiple carcinomas of the large bowel: a natural experiment in etiology and pathogenesis. *Ann Surg* 1978;187:8–11. [PubMed: 619802]
 19. Oya M, Takahashi S, Okuyama T, Yamaguchi M, Ueda Y. Synchronous colorectal carcinoma: clinico-pathological features and prognosis. *Jpn J Clin Oncol* 2003;33:38–43. [PubMed: 12604723]
 20. Papadopoulos V, Michalopoulos A, Basdanis G, Papapolychroniadis K, Paramythiotis D, Fotiadis P, Berovalis P, Harlaftis N. Synchronous and metachronous colorectal carcinoma. *Tech Coloproctol* 2004;8 (Suppl 1):s97–s100. [PubMed: 15655657]
 21. Passman MA, Pommier RF, Vetto JT. Synchronous colon primaries have the same prognosis as solitary colon cancers. *Dis Colon Rectum* 1996;39:329–34. [PubMed: 8603557]
 22. Adloff M, Arnaud JP, Bergamaschi R, Schloegel M. Synchronous carcinoma of the colon and rectum: prognostic and therapeutic implications. *Am J Surg* 1989;157:299–302. [PubMed: 2537586]
 23. Latournerie M, Jooste V, Cottet V, Lepage C, Faivre J, Bouvier AM. Epidemiology and prognosis of synchronous colorectal cancers. *Br J Surg* 2008;95:1528–33. [PubMed: 18991301]
 24. Nikoloudis N, Saliangas K, Economou A, Andreadis E, Siminou S, Manna I, Georgakis K, Chrissidis T. Synchronous colorectal cancer. *Tech Coloproctol* 2004;8 (Suppl 1):s177–9. [PubMed: 15655614]
 25. Wang HZ, Huang XF, Wang Y, Ji JF, Gu J. Clinical features, diagnosis, treatment and prognosis of multiple primary colorectal carcinoma. *World J Gastroenterol* 2004;10:2136–9. [PubMed: 15237453]
 26. Travieso CR Jr, Knoepp LF Jr, Hanley PH. Multiple adenocarcinomas of the colon and rectum. *Dis Colon Rectum* 1972;15:1–6. [PubMed: 5058410]
 27. Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med* 2007;356:2131–42. [PubMed: 17522398]

28. Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, Mino-Kenudson M, Lauwers GY, Loda M, Fuchs CS. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. *J Mol Diagn* 2005;7:413–21. [PubMed: 16049314]
29. Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. *J Mol Diagn* 2006;8:582–8. [PubMed: 17065427]
30. Nosho K, Kawasaki T, Ohnishi M, Suemoto Y, Kirkner GJ, Zepf D, Yan L, Longtine JA, Fuchs CS, Ogino S. PIK3CA mutation in colorectal cancer: relationship with genetic and epigenetic alterations. *Neoplasia* 2008;10:534–41. [PubMed: 18516290]
31. Ogino S, Cantor M, Kawasaki T, Brahmandam M, Kirkner GJ, Weisenberger DJ, Campan M, Laird PW, Loda M, Fuchs CS. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 2006;55:1000–6. [PubMed: 16407376]
32. Ogino S, Kawasaki T, Kirkner GJ, Ohnishi M, Fuchs CS. 18q loss of heterozygosity in microsatellite stable colorectal cancer is correlated with CpG island methylator phenotype-negative (CIMP-0) and inversely with CIMP-low and CIMP-high. *BMC Cancer* 2007;7:72. [PubMed: 17474983]
33. Ogino S, Kawasaki T, Brahmandam M, Cantor M, Kirkner GJ, Spiegelman D, Makrigiorgos GM, Weisenberger DJ, Laird PW, Loda M, Fuchs CS. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethylLight) for quantitative DNA methylation analysis. *J Mol Diagn* 2006;8:209–17. [PubMed: 16645207]
34. Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. *J Mol Diagn* 2007;9:305–14. [PubMed: 17591929]
35. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006;38:787–93. [PubMed: 16804544]
36. Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL, Fuchs CS. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009;58:90–6. [PubMed: 18832519]
37. Nosho K, Irahara N, Shima K, Kure S, Kirkner GJ, Schernhammer ES, Hazra A, Hunter DJ, Quackenbush J, Spiegelman D, Giovannucci EL, Fuchs CS, Ogino S. Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. *PLoS ONE* 2008;3:e3698. [PubMed: 19002263]
38. Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Int J Cancer* 2008;122:2767–73. [PubMed: 18366060]
39. Nosho K, Kawasaki T, Chan AT, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS, Ogino S. Cyclin D1 is frequently overexpressed in microsatellite unstable colorectal cancer, independent of CpG island methylator phenotype. *Histopathology* 2008;53:588–98. [PubMed: 18983468]
40. Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Dehari R, Meyerhardt JA, Fuchs CS, Ogino S. Correlation of beta-catenin localization with cyclooxygenase-2 expression and CpG island methylator phenotype (CIMP) in colorectal cancer. *Neoplasia* 2007;9:569–77. [PubMed: 17710160]
41. Ogino S, Meyerhardt JA, Cantor M, Brahmandam M, Clark JW, Namgyal C, Kawasaki T, Kinsella K, Micheline AL, Enzinger PC, Kulke MH, Ryan DP, Loda M, Fuchs CS. Molecular alterations in tumors and response to combination chemotherapy with gefitinib for advanced colorectal cancer. *Clin Cancer Res* 2005;11:6650–6. [PubMed: 16166444]
42. Ogino S, Brahmandam M, Cantor M, Namgyal C, Kawasaki T, Kirkner G, Meyerhardt JA, Loda M, Fuchs CS. Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. *Mod Pathol* 2006;19:59–68. [PubMed: 16118624]

43. Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, Murtaugh MA, Wolff RK, Slattery ML. Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. *Gastroenterology* 2005;129:837–45. [PubMed: 16143123]
44. Samowitz WS, Sweeney C, Herrick J, Albertsen H, Levin TR, Murtaugh MA, Wolff RK, Slattery ML. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. *Cancer Res* 2005;65:6063–9. [PubMed: 16024606]
45. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005;23:609–18. [PubMed: 15659508]
46. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 2004;32:e38. [PubMed: 14973332]
47. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW. Analysis of repetitive element DNA methylation by MethyLight. *Nucleic Acids Res* 2005;33:6823–36. [PubMed: 16326863]
48. Suter CM, Martin DI, Ward RL. Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. *Int J Colorectal Dis* 2004;19:95–101. [PubMed: 14534800]
49. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007;50:113–30. [PubMed: 17204026]
50. Hanigan CL, Van Engeland M, De Bruine AP, Wouters KA, Weijenberg MP, Eshleman JR, Herman JG. An inactivating mutation in HDAC2 leads to dysregulation of apoptosis mediated by APAF1. *Gastroenterology* 2008;135:1654–1664. e2. [PubMed: 18834886]
51. Lim U, Flood A, Choi SW, Albanes D, Cross AJ, Schatzkin A, Sinha R, Katki HA, Cash B, Schoenfeld P, Stolzenberg-Solomon R. Genomic methylation of leukocyte DNA in relation to colorectal adenoma among asymptomatic women. *Gastroenterology* 2008;134:47–55. [PubMed: 18166347]

Figure 1. Synchronous colorectal cancer cases and solitary cancer controls in a background of well-defined prospective cohort studies.

Figure 2. Kaplan-Meier curves for colorectal cancer-specific survival (top panel) and overall survival (bottom panel) according to synchronicity status.

Figure 3.

Correlation of LINE-1 methylation levels in synchronous colorectal cancer pairs. Each dot represents a synchronous tumor pair indicated by “location of Tumor 1”/“location of Tumor 2”. Red font indicates tumor pairs in discordant anatomical locations (i.e., proximal colon vs. distal colorectum). * Tumor 1 refers to a tumor at a higher stage or a larger tumor if the two synchronous tumors were at the same stage.

D, distal colon (splenic flexure to sigmoid); P, proximal colon (cecum to transverse colon); R, rectum.

Table 1

Comparison of study designs to examine synchronous and solitary colorectal cancers (A, advantage; D, disadvantage)

	Retrospective case-control study		Prospective cohort study
	Based on a single to several hospitals	Based on population-based cancer registry	
Study example	Refs. ^{5, 6, 9–11, 15–22, 24, 26}	Ref. ²³	Current study
Background populations that have given rise to synchronous and solitary cancers	Largely unknown (D)	Less well known (A or D)	Well known (A)
Source of bias			
1. Selection bias due to a limited number of hospitals	Yes (D)	Small (A)	Small (A)
2. Selection bias due to different background populations that have given rise to synchronous and solitary cancers	Yes (D)	Small (A or D)	No (A)
3. Referral bias	Yes (D)	Small (A or D)	No (A)
4. Recall bias	Yes (D)	Yes (D)	No (A)
Generalizability	Low (D)	Intermediate (A or D)	High (A)
Ease to conduct a study	Very easy (A)	Easy (A or D)	Difficult (D)
Cost to conduct a study	Inexpensive (A)	Inexpensive (A)	Expensive (D)
Number of synchronous cases	Can be large (A or D)	Can be very large (A)	Confined by cohort size (D)
Follow-up for synchronous and solitary cancers to occur	Not necessary (A)	Not necessary (A)	Necessary (D)
Follow-up of cancer patients	Can be thorough (A)	Registry or questionnaire-based (D)	Registry or questionnaire-based (D)
Evaluation and confirmation of cancer by study pathologist	Easy (A)	Possible (D)	Possible (D)
Tumor molecular analyses	Easy (A)	Possible (D)	Possible (D)
Criteria for synchronous cancers	Can be strict (A)	Maybe variable (D)	Maybe variable (D)

A, advantage; D, disadvantage. Individual studies are listed in Supplemental Table 1.

Table 2

Characteristics of colorectal cancer patients according to cancer synchronicity status

Clinical, pathologic or molecular feature	Total No.	Solitary cancer patients (controls)	Synchronous cancer patients (cases)*	P value
Total No. for clinical features	2068	2021	47	
Sex				0.39
Male (HPFS)	629 (30%)	612 (30%)	17 (36%)	
Female (NHS)	1439 (70%)	1409 (70%)	30 (64%)	
Mean age $\pm$ SD	65.6 $\pm$ 8.9	65.5 $\pm$ 8.9	68.9 $\pm$ 8.3	0.016**
Body mass index (BMI, kg/m ²)				0.27
<25	815 (46%)	800 (46%)	15 (37%)	
25–29	654 (37%)	634 (36%)	20 (49%)	
$\geq$ 30	314 (18%)	308 (18%)	6 (15%)	
Family history of colorectal cancer				0.53
Absent	1618 (78%)	1583 (78%)	35 (74%)	
Present	450 (22%)	438 (22%)	12 (26%)	
Year of diagnosis				0.055
Prior to 1990	528 (26%)	523 (26%)	5 (11%)	
1990 to 1999	722 (35%)	701 (35%)	21 (45%)	
2000 or after	818 (40%)	797 (39%)	21 (45%)	
Tumor location				0.30
Proximal colon(cecum to transverse)	868 (43%)	845 (43%)	23 (51%)	
Distal colon (splenic flexure to sigmoid)	696 (35%)	680 (35%)	16 (36%)	
Rectum	453 (22%)	447 (23%)	6 (13%)	
AJCC tumor stage				0.65
I	493 (23%)	486 (24%)	7 (15%)	
II	582 (28%)	568 (28%)	14 (30%)	
III	521 (25%)	507 (25%)	14 (30%)	
IV	268 (13%)	262 (13%)	6 (13%)	
Unknown	204 (9.9%)	198 (9.8%)	6 (13%)	
No. of cases with tissue data	1113	1084	29	
Tumor grade				0.31
Low	1015 (91%)	990 (91%)	25 (86%)	
High	98 (8.8%)	94 (8.7%)	4 (14%)	
Mucinous component				0.24
0%	668 (60%)	650 (60%)	18 (62%)	
1–49%	273 (25%)	269 (25%)	4 (14%)	
$\geq$ 50%	172 (15%)	165 (15%)	7 (24%)	
Signet ring cells				0.74
0%	1013 (91%)	987 (91%)	26 (90%)	
$\geq$ 1%	100 (9.0%)	97 (8.9%)	3 (10%)	
MSI				0.037

Clinical, pathologic or molecular feature	Total No.	Solitary cancer patients (controls)	Synchronous cancer patients (cases)*	P value
MSI-low/MSS	738 (86%)	722 (86%)	16 (70%)	0.013
MSI-high	125 (14%)	118 (14%)	7 (30%)	
CIMP				
CIMP-low/0	723 (85%)	708 (86%)	15 (65%)	
CIMP-high	126 (15%)	118 (14%)	8 (35%)	0.036
MSI & CIMP				
MSI-low/MSS & CIMP-low/0	663 (81%)	650 (81%)	13 (59%)	
MSI-high & CIMP-low/0	36 (4.3%)	34 (4.2%)	2 (9.1%)	
MSI-low/MSS & CIMP-high	39 (4.7%)	37 (4.6%)	2 (9.1%)	0.0041
MSI-high & CIMP-high	85 (10%)	80 (10%)	5 (23%)	
<i>BRAF</i> mutation				
(-)	746 (88%)	731 (88%)	15 (65%)	
(+)	105 (12%)	97 (12%)	8 (35%)	0.0058
<i>BRAF</i> & CIMP				
<i>BRAF</i> (-) & CIMP-low/0	655 (81%)	641 (81%)	14 (64%)	
<i>BRAF</i> (+) & CIMP-low/0	33 (4.1%)	32 (4.1%)	1 (4.6%)	
<i>BRAF</i> (-) & CIMP-high	51 (6.3%)	51 (6.5%)	0	0.92
<i>BRAF</i> (+) & CIMP-high	70 (8.7%)	63 (8.0%)	7 (32%)	
18q loss of heterozygosity (LOH)				
(-)	218 (39%)	212 (39%)	6 (40%)	
(+)	344 (61%)	335 (61%)	9 (60%)	0.91**
Mean LINE-1 methylation (%) $\pm$ SD	61.5 $\pm$ 9.5	61.5 $\pm$ 9.4	61.5 $\pm$ 10.9	
<i>KRAS</i> mutation				
(-)	552 (63%)	537 (63%)	15 (65%)	0.84
(+)	322 (37%)	314 (37%)	8 (35%)	
<i>PIK3CA</i> mutation				
(-)	657 (85%)	635 (84%)	22 (96%)	
(+)	118 (15%)	117 (16%)	1 (4.4%)	0.14
β -catenin ^Δ				
Inactive (score 0–2)	499 (65%)	486 (65%)	13 (62%)	
Active (score 3–5)	270 (35%)	262 (35%)	8 (38%)	
p53 expression				0.55
(-)	515 (57%)	500 (56%)	15 (63%)	
(+)	396 (43%)	387 (44%)	9 (38%)	
p21 (<i>CDKN1A</i>) expression				
(-)	159 (18%)	152 (18%)	7 (30%)	0.13
(+)	707 (82%)	691 (82%)	16 (70%)	
p27 (<i>CDKN1B</i>) expression				
Nuclear expression	163 (19%)	160 (19%)	3 (13%)	
Cytoplasmic expression or loss of expression	692 (81%)	672 (81%)	20 (87%)	0.46
Cyclin D1 expression				
				0.71

Clinical, pathologic or molecular feature	Total No.	Solitary cancer patients (controls)	Synchronous cancer patients (cases)*	P value
(-)	201 (25%)	197 (25%)	4 (21%)	0.95
(+)	614 (75%)	599 (75%)	15 (79%)	
Fatty acid synthase (FASN)				0.49
(-)	609 (86%)	592 (86%)	17 (85%)	
(+)	103 (14%)	100 (14%)	3 (15%)	0.49
Cyclooxygenase-2 (COX-2)				
(-)	145 (16%)	140 (16%)	5 (21%)	0.49
(+)	775 (84%)	756 (84%)	19 (79%)	

(%) indicates the proportion of patients with a specific clinical, pathologic or molecular feature among all patients, solitary cancer patients or synchronous cancer patients.

* For synchronous cases, results were obtained from a tumor at a higher stage or a larger tumor if the two synchronous tumors were at the same stage.

** Wilcoxon rank-sum test.

[^] β -catenin score was calculated as previously described.⁴⁰

AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study; SD, standard deviation.

Synchronous vs. solitary colorectal cancer patients and mortality

Table 3

Total No.		Colorectal cancer-specific mortality				Overall mortality			
		Deaths/ person- years	Univariate HR (95% CI)	Stage- matched HR (95% CI)	Multivariate HR (95% CI)	Deaths/ person- years	Univariate HR (95% CI)	Stage- matched HR (95% CI)	Multivariate HR (95% CI)
Solitary cancer patients (controls)	2021 (98%)	559/16474	1 (referent)	1 (referent)	1 (referent)	847/16474	1 (referent)	1 (referent)	1 (referent)
Synchronous cancer patients* (cases)	47 (2.3%)	20/287	1.63 (1.04–2.55)	1.40 (0.89–2.21)	1.46 (0.93–2.30)	28/287	1.71 (1.17–2.50)	1.53 (1.04–2.24)	1.47 (1.00–2.17)
			p=0.032	p=0.15	p=0.10		p=0.0053	p=0.031	p=0.049

The multivariate, stage-matched (stratified) Cox regression model included sex, age, year of diagnosis, family history of colorectal cancer, body mass index (BMI) and tumor location.

*Tumor stage of a synchronous cancer patient was based on a higher stage tumor if the synchronous tumors were at different stages. CI, confidence interval; HR, hazard ratio.

Table 4

Synchronous colorectal cancer pairs with clinical and molecular data

Case No.	Age ^a	Sex	Family history ^a	Tumor location ^b	TME stage	size (mm)	Differentiation	Mucinous component ^c	CIMP ^d	PMR (degree of methylation) ^e															LINE-1 (methylation %)		18s LOH	KRAS ^g	BRAF	PIK3CA	p53	p21	p27 ^h	CDKN1	FASN		
										CACNA1G ⁱ	CACNA1G ⁱ	CDKN2A (p16) ⁱ	CABP1 ⁱ	SPZ ⁱ	MEP1 ⁱ	NEUROG1 ⁱ	RUND1 ⁱ	SOC1 ⁱ	CHIR	PC1	IGFBP3	MGMT	MNT1	MNT131	p14	WNV										MSI	
1	68F	0	A	T3N0M0	55	W	++	H	504	345	113	447	81	160	133	222	59	0	26	0	27	249	13	208	60	H	ND	WT	+	-	-	+	2	+	-	-	
			A	T3N0M0	28	M	+	H	176	218	86	171	33	46	59	52	36	229	14	88	34	237	5.8	90	73	H	ND	WT	+	-	+	+	1	+	+	+	
2	71F	1	A	TaNoM0	ND	W	-	H	190	47	43	53	20	58	31	0	25	192	0	36	13	203	15	121	72	H	ND	WT	+	-	-	+	+	+	+		
			R	TaNoM0	ND	W	-	0	0	0	0	0	0	0	0	0	0	0	0	0	1.5	0	0	0	68	MSS	ND	WT	-	ND	+	+	2	-	-	+	
3	60F	1	S	T4N1M1	40	P	+	0	0	0	0	0	0	0	0	0	88	0	51	183	0	77	4.1	43	55	MSS	-	12ext	-	+	-	-	-	+	+		
			A	T3N0M1	ND	M	+	L	0	0	32	0	0	0	0	0	73	26	0.9	72	1.9	3.1	0	0	42	MSS	ND	12ext	-	-	-	-	+	+	+		
4	65F	0	A	T3N0M0	50	M	-	H	309	0	43	189	0	17	47	117	30	0	0	5.0	85	25	176	ND	ND	ND	ND	ND	ND	+	-	-	1	ND	ND	-	
			A	T2N0M0	20	W	-	L	0	1.6	39	173	0	0	153	0	0	102	3.2	0	25	42	0	8.0	ND	ND	ND	ND	ND	+	-	-	2	ND	+	+	
5	79M	0	R	T3N0Mx	50	M	++	L	0	93	7.6	0	0	0	0	0	1.3	0	14	36	5.0	7.0	1.3	0	38	MSS	-	12ext	-	ND	-	-	2	+	+	+	
			R	T2N0Mx	20	M	++	L	0	202	3.2	0	0.6	0	0	0	0	0	18	34	4.0	6.2	0	0	42	MSS	-	13ext	-	-	+	+	2	+	+	+	
6	80M	0	A	T3N0M1	45	W	-	0	0	0	0	0	0	0	0	0	0	0	39	0.97	0	0	0	0	72	MSS	+	WT	-	-	-	-	1	-	+	-	
			S	T2N0M1	6	W	+	0	0	0	0	0	0	0	0	0	0	35	0	0	77	2.0	0	0.6	0	57	MSS	ND	12ext	-	-	-	ND	1	-	-	+
7	54F	0	A	T3N2M0	60	M	++	H	982	407	149	747	90	110	275	245	202	975	34	0	41	960	14	361	62	H	ND	WT	+	+	+	+	+	1	+	+	+
			T	T3N2M0	50	P	-	H	459	0	70	208	85	114	28	53	45	668	19	0	34	245	43	225	84	H	ND	WT	+	+	+	+	+	2	+	+	+
8	58M	1	A	T3N1M0	40	W	+	L	0	0	0	0	0	0	0	0	201	0	1.2	0	0	0.6	1.1	0	3.5	72	H	ND	12ext	-	-	-	-	1	ND	-	-
			SF	T2N0M0	70	W	-	L	0	0	0	2.7	0	0	0	0	158	0	15	0	0	107	0	5.4	78	H	ND	12ext	-	-	-	-	ND	ND	+	+	
9	70F	0	A	T3N2M0	50	M	-	L	0	0	0	0	0	0	0	0	110	0	1.0	0	37	9.3	0	1.0	2.8	48	MSS	+	WT	-	-	+	-	1	+	+	+
			S	T3N1M0	30	M	-	L	0	0	0	0	0	0	0	0	132	0	0	0	65	3.7	0	0.9	1.7	95	MSS	+	WT	-	-	+	-	1	-	+	+
10	72M	1	S	T3N0M0	35	W	-	0	0	0	0	0	0	0	0	0	0	11	0	0	1.2	2.6	1.8	0	45	MSS	+	12ext	-	-	-	-	1	-	-	+	
			D	T2N0M0	27	W	-	0	0	0	0	0	0	0	0	0	0	3.7	0	0	1.9	0	0	0	34	MSS	+	12ext	-	-	+	-	2	+	-	-	

a. Family history refers to the number of affective first-degree relatives with colorectal cancer.

b. Proximal colon (cecum to transverse) is shaded as , distal colon (splenic flexure to sigmoid) as .

c. 1-49% of mucinous component is described as +, and ≥50% as ++.

d. CpG island methylator phenotype (CIMP) status; H, CIMP-high; L, CIMP-low; 0, CIMP-0.

e. Grey shading for methylation markers indicates PMR (percentage of methylated reference; methylation index) ≥4.

Table 5

Agreement study of methylation status at each of the 16 CpG islands within 10 synchronous cancer pairs.

		Concordance		κ coefficient	P value
All cases (N=10 pairs × 16 markers)		0.78		0.53	<0.0001
Tumor 1	Tumor 2				
	+	+	-	Total	
	+	45	27	72	
	-	9	79	88	
Total		54	106	160	
Proximal colon [*] /proximal colon [*] (N=3 pairs × 16 markers)		0.81		0.41	0.010
Tumor 1	Tumor 2				
	+	+	-	Total	
	+	34	6	40	
	-	3	5	8	
Total		37	11	48	
Distal colorectum ^{**} /distal colorectum ^{**} (N=2 pairs × 16 markers)		0.94		0.80	0.0001
Tumor 1	Tumor 2				
	+	+	-	Total	
	+	5	2	7	
	-	0	25	25	
Total		5	27	32	
Proximal colon [*] /distal colorectum ^{**} (N= 5 pairs × 16 markers)		0.69		0.15	0.18
Tumor 1	Tumor 2				
	+	+	-	Total	
	+	6	19	25	
	-	6	49	55	
Total		12	68	80	

* Proximal colon refers to cecum to transverse colon.

** Distal colorectum refers to splenic flexure to rectum.