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Molecular heterogeneity and prognostic implications of synchronous advanced colorectal neoplasia

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Background: It is uncertain whether synchronous colorectal cancers (S-CRCs) preferentially develop through widespread DNA methylation and whether they have a prognosis worse than solitary CRC. As tumours with microsatellite instability (MSI) may confound the effect of S-CRC methylation on outcome, we addressed this issue in a series of CRC characterised by *BRAF* and MS status.

Methods: Demographics, clinicopathological records and disease-specific survival (DSS) were assessed in 881 consecutively resected CRC undergoing complete colonoscopy. All tumours were typed for $BRAF^{c.1799T>A}$ mutation and MS status, followed by search of germ-line mutation in patients with MSI CRC.

Results: Synchronous colorectal cancers (50/881, 5.7%) were associated with stage IV microsatellite-stable (MSS) CRC (19/205, 9.3%, $P=0.001$) and with HNPCC (9/32, 28%, $P<0.001$). *BRAF* mutation (60/881, 6.8%) was associated with sporadic MSI CRC (37/62, 60%, $P<0.001$) but not with S-CRC (3/50, 6.0%, $P=0.96$). Synchronous colorectal cancer (HR 1.82; 95% CI 1.15–2.87; $P=0.01$), synchronous advanced adenoma (HR 1.81; 95% CI 1.27–2.58; $P=0.001$), and $BRAF^{c.1799T>A}$ mutation (HR 2.16; 95% CI 1.25–3.73; $P=0.01$) were stage-independent predictors of death from MSS CRC. Disease-specific survival of MSI CRC patients was not affected by S-CRC (HR 0.74; 95% CI 0.09–5.75; $P=0.77$).

Conclusion: Microsatellite-stable CRCs have a worse prognosis if S-CRC or synchronous advanced adenoma are diagnosed. The occurrence and the enhanced aggressiveness of synchronous MSS advanced neoplasia are not associated with *BRAF* mutation.

Two or more primary colorectal carcinomas are detected in 2–5% of all patients newly diagnosed with colorectal cancer (CRC) (Adloff *et al*, 1989; Passman *et al*, 1996; Chen and Sheen-Chen, 2000; Oya *et al*, 2003; Papadopoulos *et al*, 2004; Latournerie *et al*, 2008; Nosho *et al*, 2009; Mulder *et al*, 2011). The recognition of synchronous colorectal cancer (S-CRC) as a clinical entity has enhanced the awareness that an accurate perioperative exploration of the entire colon is mandatory in patients undergoing CRC resection. However, it remains uncertain whether S-CRC has prognostic and molecular features distinct from those of solitary CRC.

Case–control studies examining characteristics and outcome of patients with S-CRC have provided discrepant results. Male gender (Oya *et al*, 2003; Latournerie *et al*, 2008; Mulder *et al*, 2011), older age (Passman *et al*, 1996; Papadopoulos *et al*, 2004; Wang *et al*, 2004;

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Mulder *et al*, 2011), coexisting adenomas (Chen and Sheen-Chen, 2000; Latournerie *et al*, 2008), and worse survival (Oya *et al*, 2003; Nosho *et al*, 2009; Mulder *et al*, 2011) were associated with S-CRC in some series but not in others. These inconsistencies likely reflect a bias in the selection of index cases as well as in the recruitment of controls with solitary CRC. In particular, different series may have variable prevalences of cancers with microsatellite instability (MSI), which are associated with multiple lesions (Pedroni *et al*, 1999; Dykes *et al*, 2003; Nosho *et al*, 2009; Bae *et al*, 2012) but also with a better prognosis (Gryfe *et al*, 2000; Malesci *et al*, 2007).

It is also uncertain whether S-CRC is the result of a stochastic oncogenic event or, alternatively, of an increased susceptibility of the colonic mucosa to neoplastic transformation, as supported by the established association of S-CRC with metachronous CRC (Mulder *et al*, 2011). Beside the controversial association of S-CRC with conventional adenomas, evidence also exists that S-CRC might be more frequent among cancers of the serrated neoplastic pathway, which is characterised by a CpG island methylation (CIMP) phenotype, as opposed to tumours of the chromosomal instability pathway. Two studies found a higher degree of gene promoter methylation in tumour samples from multiple lesions than from solitary CRC, thus suggesting an epigenetic field effect as the basis for S-CRC (Konishi *et al*, 2009; Gonzalo *et al*, 2010; Moon *et al*, 2010). More recently, Nosho *et al* (2009) found that S-CRCs more frequently exhibited the $BRAF^{c.1799T>A}$ mutation, an established marker of CIMP phenotype (Kambara *et al*, 2004; Samowitz *et al*, 2005a; Spring *et al*, 2006), and that they had a worse outcome. Conversely, Bae *et al* (2012) failed to detect any association of *BRAF* mutation with S-CRC. The discrepancy might reflect the heterogeneity of CIMP-positive cancers, a subclass encompassing tumours that do not undergo MLH1-methylation, remain microsatellite-stable (MSS), and have a poor prognosis but also MLH1-deficient MSI CRC, which have a much more favourable prognosis.

Given the complex interactions between outcome and distinct molecular profiles, it is obvious that the prognostic significance of S-CRC can be safely assessed only through the analysis of single molecular subgroups of CRC. In addition, the analysis should be extended to synchronous advanced adenomas that are also associated with metachronous CRC (Moon *et al*, 2010). We retrospectively searched for synchronous adenomas or S-CRC the records of perioperative colonoscopies performed in 881 consecutive patients, whose resected CRC had been originally classified in molecular subclasses according to microsatellite and *BRAF* status. Clinicopathological features and outcome of patients with synchronous advanced neoplasia were then assessed.

MATERIALS AND METHODS

Study population and CRC subgrouping by synchronous neoplasia. The study intended to include 1000 consecutive patients who had undergone resective surgery for CRC at the Humanitas Clinical and Research Center between 2 February 1998 and 6 April 2006. Exclusion criteria were limited to the following: (1) absence of submucosal invasion at pathology; (2) anastomotic recurrence of a previously resected colorectal tumour; (3) diagnosis of familial adenomatous polyposis; (4) CRC associated to inflammatory bowel disease. The protocol was approved by the Ethical Committee of the Institution and the informed consent of patients regarding the treatment of their personal data was obtained by the referring physician or by other clinicians involved in the study. At preliminary analysis of records, 119 patients (none of which with S-CRC) were excluded because of incomplete or poor-quality perioperative colonoscopy, leading to a final study population of 881 subjects.

Demographic and clinicopathological records were obtained for each patient from the hospital's intranet system. Synchronous colorectal cancers were defined as the simultaneous detection of two or more invasive (at least pT1) tumours, separated by at least 5 cm of normal colorectal mucosa, at the time of diagnosis or within 6 months for obstructing tumours. The most invading lesion (greatest pT) was taken as the reference lesion for pathological and molecular classification of S-CRC. By combining macroscopic and histological findings, the following CRC subsets were defined: (I) no synchronous neoplasia ($n=548$); (II) synchronous not-advanced adenoma ($n=177$); (III) synchronous advanced adenoma (tubular adenoma 10 mm or greater in diameter, and/or $>25\%$ villous component, and/or high-grade dysplasia), ($n=106$); (IV) S-CRC ($n=50$). To define stage IV disease, pathological reports were combined with surgical findings and with perioperative imaging. The disease-specific survival (DSS) was calculated from diagnosis until death, or until data were censored, as of 30 September 2011. On this date, each patient was confirmed to be alive by direct phone call or by formal enquiry at the local registry of vital statistics.

Tumour molecular subtyping. Tumour samples from all patients, and all cancers from patients with S-CRC, were screened for MSI. *BAT-26* mononucleotide, and *BAT-25* mononucleotide in patients fulfilling the Amsterdam Criteria II and/or the Bethesda Criteria ($n=279$), were used as molecular markers of MSI (Hatch *et al*, 2005; Laghi *et al*, 2008). DNA was obtained from paraffin-embedded sections of tumours containing at least 50% tumour cells or from tumour micro-dissections. *BAT*-25 and *BAT-26* loci were amplified by fluoresceinated primers and analysed by capillary gel electrophoresis (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Monza, Italy) (Aaltonen *et al*, 1998; Malesci *et al*, 2007).

In all MSI CRC, a defect in mismatch repair (MMR) protein was assessed by the lack of nuclear expression of hMLH1 (clone G-168–15, BD Bio sciences, Buccinasco, Milan, Italy), hMSH2 (clone FE11, Calbiochem, Merk Millipore, Darmstadt, Germany), or hMSH6 (clone 44, BD Bio sciences) at immunohistochemistry (Truninger *et al*, 2005). Mismatch repair protein expression was also checked in MSS tumours from patients fulfilling the Amsterdam Criteria ($n=10$). Patients with MSI CRCs underwent germ-line genetic testing for *MLH1*, *MSH2* and *MSH6* mutations by sequencing according to the MMR defect in the primary cancer (Wahlberg *et al*, 2002). Multiple ligation probe amplification analysis (SALSA MLPA P003 MLH1-MSH2 probemix, P248 MLH1-MSH2 probemix, P072 MSH6 probemix, Medical Research Council-Holland, Amsterdam, The Netherland) was performed in mutation-negative patients.

All CRC samples were scrutinised for $BRAF^{c.1799T>A}$ mutation by Real Time–PCR using a TaqMan SNP Genotyping Assay (Applied Biosystem). TaqMan MGB probes were designed using the Custom TaqMan Assay Design Tool (Applied Biosystem). The chosen reporter fluorophores were VIC for detecting the wild-type allele and FAM for the mutant allele.

Sequencing of all exons of the *MYH* gene was performed to assess germ-line mutations in all patients with MSS S-CRC. We also investigated all tissues from MSS S-CRC for germ-line or somatic mutations in the exon 13 of the *POLE* gene and in the exon 11 of the *POLD1* gene.

Statistical analysis. Associations between synchronous neoplasia and clinicopathological or molecular features of the index CRC were tested using χ^2-test or, if appropriate, Fisher's Exact test for categorical variables and by Student's *t*-test for continuous variables. Pathological and molecular factors significantly associated with S-CRCs at univariate analysis were entered into a multivariate logistic regression analysis. Survival curves were drawn according to the Kaplan–Meier method to comparatively evaluate the DSS of patients with synchronous colorectal neoplasia.

To better assess the prognostic role of S-CRCs, as well as of synchronous adenomas, and of tumour MS/*BRAF* status, Cox proportional-hazard models were also used. For all statistical tests, $P<0.05$ was considered statistically significant.

RESULTS

Out of 1000 patients undergoing resective surgery for newly diagnosed CRC, 50 (5%) were found to have S-CRC (two cancers in 47 patients and three cancers in three patients). At full colonoscopy, 17 of 50 (34.0%) patients with S-CRC and 283 of 831 (34.1%, $P>0.5$) patients with solitary cancer had at least one distinct concomitant adenoma.

Table 1 reports the clinicopathological and molecular features of the index CRC stratified by the absence or the presence of a synchronous colorectal neoplasia. As compared with patients with no synchronous neoplasia, subjects with synchronous adenoma or cancer were older (66.4 ± 9.9 *vs* 63.9 ± 11.8 years; $P=0.001$), were more frequently men (66.4% *vs* 53.3%; $P<0.001$), and more frequently had

Table 1. Demographics, pathology and molecular features of CRC with synchronous colorectal neoplasia

	Synchronous colorectal neoplasia						
	None[a]	Not-advanced adenoma[b]		Advanced adenoma[c]		Invasive cancer[d]	
	n **= 548, ref.**	***n*** **= 177**	***P****	***n*** **= 106**	***P****	***n*** **= 50**	***P****
Age							
Years, mean ± s.d.	63.9 ± 11.8	66.8 ± 9.7	0.003	66.6 ± 10.0	0.02	64.6 ± 10.8	0.70
Gender							
Male	292 (53.3)	115 (65.0)	77 (72.6)	29 (53.4)			
Female	256 (46.7)	62 (35.0)	0.006	29 (27.4)	<0.001	21 (46.6)	0.52
Site							
Distal	374 (68.2)	101 (57.1)	64 (60.4)	30 (61.2)			
Proximal	174 (31.8)	76 (42.9)	0.006	42 (39.6)	0.11	19 (38.8)	0.31
Stage							
I–III	419 (76.5)	139 (78.5)	80 (75.5)	30 (60.0)			
IV	129 (23.5)	38 (21.5)	0.57	26 (24.5)	0.83	20 (40.0)	0.01[e]
Histotype							
Adenoca	506 (92.3)	162 (91.5)	100 (94.3)	48 (96.0)			
Variant	42 (7.7)	15 (8.5)	0.73	6 (5.7)	0.47	2 (4.0)	0.34
Grade[f]							
G1–G2	406 (79.3)	143 (83.6)	78 (77.2)	38 (77.6)			
G3	106 (20.7)	28 (16.4)	0.22	23 (22.8)	0.64	11 (22.4)	0.77
Vein invasion							
No	423 (77.2)	140 (79.1)			82 (77.4)	36 (72.0)	
Yes	125 (22.8)	37 (20.9)	0.60	24 (22.6)	0.97	14 (28.0)	0.40
MS status							
MSS	495 (90.3)	153 (86.4)	100 (94.3)	39 (78.0)			
MSI-sporadic	36 (6.6)	19 (10.7)	0.04[g]	5 (4.7)	0.44	2 (4.0)	0.64
HNPCC	17 (3.1)	5 (2.8)	0.92	1 (0.9)	0.20	9 (18.0)	<0.001[e]
BRAF							
BRAF WT	516 (94.2)	158 (89.3)	100 (94.3)	47 (94.0)			
BRAF $^{c.1799T>A}$	32 (5.8)	19 (10.7)	0.02[g]	6 (5.7)	0.94	3 (6.0)	0.96

Abbreviations: CRC = colorectal cancer; MSI = microsatellite instability; MSS = microsatellite-stable. At Fisher's exact test.

[a]'No adenoma'.

[b]'Low-grade dysplasia <10 mm tubular adenoma, no serrated adenoma.

[c]Forty-five patients with low-grade dysplasia adenoma ⩾10 mm or with villous component >25%, 59 with high-grade dysplasia adenoma, 2 with ⩾10 mm low-grade dysplasia serrated adenoma.

[d]Pathological and molecular characteristics of the most advanced cancer ('index' lesion, by pT) were to be inserted. Of 23 pairs with identical pT (no 'index' lesion assessable), 22 had fully concordant pathological and molecular features, whereas one pair with discordant tumour site was excluded from the analysis of this variable.

[e]Interactions at multivariate analysis (logistic regression): 'Stages I–II *vs* III–IV' * 'MS status', $P=0.03$.

[f]Not assessed in 48 cases (34, 6, 7 and 1 in the four subclasses, respectively).

[g]'*BRAF* status' * 'MS status' (excluding HNPCC), $P=0.04$.

a right-sided CRC (41.3% *vs* 31.8%; $P=0.003$). Synchronous colorectal cancer was strongly associated with stage IV disease and with MSI hereditary cancer (HNPCC); however, a statistically significant ($P=0.03$) interaction of the two variables was detected at multivariate analysis. Accordingly, Figure 1 details how only MSS S-CRCs were associated with stage IV ($P=0.001$), whereas MSI CRC presented a low prevalence of metastatic disease even in the presence of synchronous invasive cancer ($P=0.88$). A full concordance in the MS status was observed in all pairs and triplets of S-CRC, except in one patient carrying an MSI-sporadic index CRC and a second MSS cancer. An interaction was also observed between the MSI status and $BRAF^{c.1799T>A}$ mutation in determining the association of these two variables with synchronous non-advanced adenoma, whereas no association was detected between the *BRAF* status and synchronous advanced adenomas and S-CRC. Figure 2 shows that $BRAF^{c.1799T>A}$ mutation was strongly associated with MSI-sporadic CRC (37/62, 59.7% *vs* 23/787, 2.9% in MSS CRC; $P<0.001$), and that the prevalence of the mutation was higher in MSI-sporadic CRC with synchronous lesions than in those with no concurrent neoplasia (21/26, 80.8% *vs* 16/36, 44.4%; $P=0.005$). Conversely, in MSS CRC the presence of synchronous colorectal adenomas or cancer was not associated with $BRAF^{c.1799T>A}$ mutation. No *MYH* germ-line mutations and no *POLE* or *POLD1* germ-line and/or somatic mutations were detected in patients with S-CRC.

Over a mean post-surgical follow-up of 4.3 ± 2.4 years, a total of 231 CRC-related deaths were registered, 220 (28.0%) among the

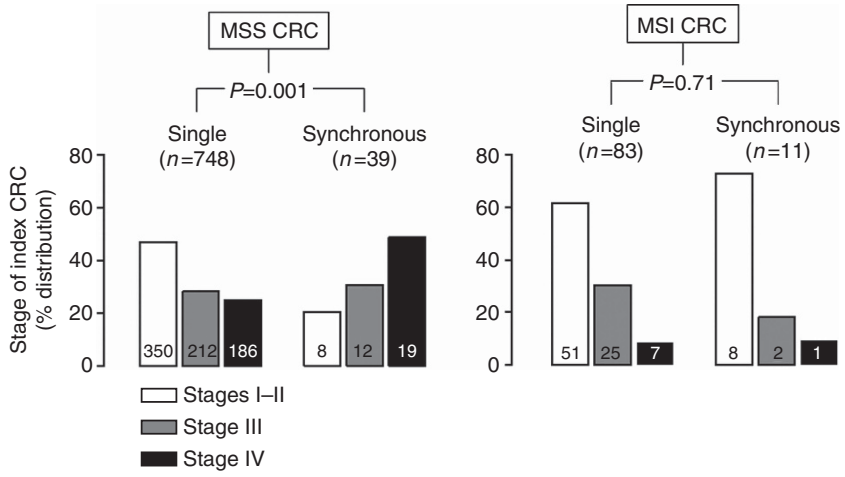

Figure 1. Interaction of CRC microsatellite status and of synchronous colorectal malignancy in determining the association of these two variables with TNM stage. Stage distribution of synchronous CRC is compared with that of single CRC, stratifying by microsatellite status. Stage IV was significantly associated with synchronous CRC in patients with MSS cancer (19/39, 48.7% *vs* 186/748, 24.9%; $P=0.001$). The frequency of stage IV disease was not different in MSS CRC patients with no concomitant adenoma (124/495, 25.1%, ref.), with synchronous not-advanced adenoma (36/153, 23.5%; $P=0.70$), and with synchronous advanced adenoma (26/100; 26.0%; $P=0.84$). The frequency of stage IV disease, in patients with MSI CRC, was not associated with S-CRC (7/83, 8.4% *vs* 1/11, 9.1%; $P=0.88$). *P-values at χ^2 or Fisher's exact test, as appropriate (stage IV *vs* others).

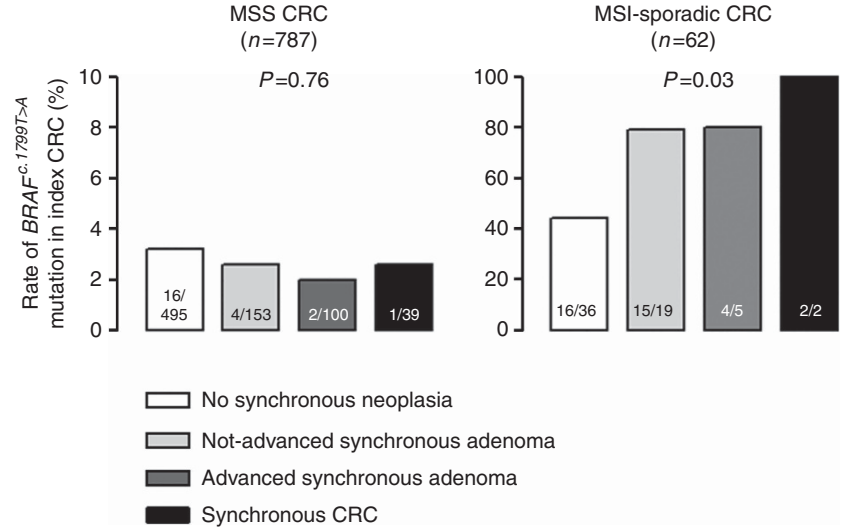

Figure 2. $BRAF^{c.1799T>A}$ mutation was significantly ($P<0.001$) more frequent in MSI-sporadic (37/62, 59.7%) than in MSS CRC (23/787, 2.9%). In MSS CRC, no association was found between the occurrence of *BRAF* mutation in the index CRC and the presence of synchronous neoplasia. Conversely, the mutation in MSI-sporadic CRC was less frequent in the absence of synchronous neoplasia (16/36, 44.4%) than in (a) any synchronous neoplasia (21/26, 80.8%, $P=0.005$), (b) synchronous not-advanced adenoma (15/19, 78.9%, $P=0.01$), (c) synchronous advanced adenoma or CRC (6/7, 85.7%, $P=0.05$). HNPCC, which invariably carry no *BRAF* mutation, was excluded from analysis. *P*-values are from χ^2 or Fisher's exact test, as appropriate.

787 patients with MSS CRC and only 11 (11.7%) among the 94 patients with MSI cancer ($P<0.001$). At Kaplan–Meier curves, the presence of S-CRC significantly affected the DSS of patients with MSS CRC ($P<0.001$) but not that of patients with MSI cancer ($P=0.83$) (Figure 3). MSS CRC had a poorer prognosis also in the presence of a synchronous advanced adenoma ($P=0.02$) but not in the presence of a not-advanced adenoma ($P=0.29$) (Figure 4). The negative prognostic effect of S-CRC or synchronous advanced adenoma was limited to MSS cancers with no $BRAF^{c.1799T>A}$ mutation ($P<0.001$), whereas *BRAF*-mutated MSS CRC had a much poorer outcome independent of the presence of a synchronous advanced neoplasia ($P=0.98$) (Supplemental Material 1). At Cox proportional-hazard models (Table 2), the presence of synchronous advanced neoplasia was confirmed to be associated with a worse outcome of MSS CRC (S-CRC: HR 2.66; 95% CI, 1.69–4.19; $P<0.001$; synchronous advanced adenoma: HR 1.59; 95% CI, 1.11–2.26; $P=0.01$). Given the higher prevalence of stage IV disease in patients with MSS S-CRC but not in those with synchronous advanced adenoma (see Figure 1 and its legend), the incremental risk of death associated with synchronous advanced adenoma (HR 1.81; 95% CI, 1.27–2.58; $P=0.001$) and that conferred by the presence of synchronous invasive cancer (HR 1.82; 95% CI, 1.15–2.87; $P=0.01$) were almost identical at stage-adjusted multivariate analysis. The occurrence of $BRAF^{c.1799T>A}$ mutation in the index cancer also predicted a higher risk of death from MSS CRC, independently of the presence of a synchronous advanced neoplasia and of TNM stage (HR 2.16; 95% CI 1.25–3.73; $P=0.01$). Synchronous advanced neoplasia (HR 1.75, C.I. 95% 1.30–2.37, $P<0.001$) and $BRAF^{c.1799T>A}$ mutation (HR 1.74, C.I. 95% 1.00–3.03, $P=0.05$) remained stage-independent predictors of death when adjuvant 5-fluorouracil-based chemotherapy was entered into the multivariate analysis (Supplemental Material 2). On the contrary, neither the presence of synchronous advanced neoplasia nor the *BRAF* status of the tumour significantly affected the DSS of patients with MSI CRC.

DISCUSSION

In this large, hospital-based study, patients with MSS CRC had a significantly poorer outcome if originally diagnosed with a S-CRC or even with a synchronous advanced adenoma. The finding is important in that it contributes to a highly controversial issue generated by the fact that most studies failed to recognise any association between S-CRC and poor prognosis (Passman *et al*, 1996; Chen and Sheen-Chen, 2000; Papadopoulos *et al*, 2004; Latournerie *et al*, 2008), whereas the only prospective study reported a higher mortality in patients with multiple primary cancers (Nosho *et al*, 2009). Notably, our study is unique in having investigated the prognostic role of S-CRC in molecularly defined subgroups of CRC, so as to avoid the confounding effect of MSI cancers that more likely occur as synchronous malignancies but also have an overall better prognosis. In addition, $BRAF^{c.1799T>A}$ mutation, an established marker of CIMP and of poor prognosis (Samowitz *et al*, 2005b; Weisenberger *et al*, 2006), was not associated with MSS S-CRC, indicating that neither the occurrence nor the outcome of chromosomal-unstable synchronous cancers are likely due to an epigenetic field effect.

Several studies have documented the association between MSI and S-CRC (Pedroni *et al*, 1999; Dykes *et al*, 2003; Nosho *et al*, 2009; Bae *et al*, 2012). The strong concordance in the MSI status among synchronous cancers also led to the concept that, for genetic and/or environmental reasons, some individuals may be prone to develop multiple cancers through the pathway of MSI secondary to widespread CIMP and to silencing of the mismatch repair gene MMR *MLH1* (Leggett and Worthley, 2009). This concept was mainly based on the assumption that the majority of MSI S-CRCs were sporadic tumours, as suggested by the typically old age of patients with synchronous colorectal malignancies and by the established association between MSI-sporadic tumours and older age (Leggett and Worthley, 2009; Nosho *et al*, 2009). As a matter of fact, no previous study addressing the issue of synchronous cancers systematically screened patients with MSI S-CRC for germ-line mutations in the *MMR* genes. Therefore, it was a novel, and somehow unexpected finding of our series to see that HNPCC largely accounted for MSI S-CRC (9 of 11, 82%) so that about one out of five of all S-CRC were diagnosed in patients with hereditary cancer. Consistently, $BRAF^{c.1799T>A}$ mutation was associated only with sporadic MSI CRC, whereas no mutation was detected in any HNPCC. Overall, data cannot exclude the existence of an epigenetic field effect favouring the development of multiple neoplasia in patients with sporadic MSI CRC, but certainly contradict the idea that this mechanism may account for most synchronous MSI cancers. Rather, our results confirm the appropriateness of the Bethesda criteria, which recommend MSI testing of CRC in the presence of multiple primary tumours (Umar *et al*, 2004).

The interaction between the MS status and advanced stage in their association with S-CRC indicated the need to analyse

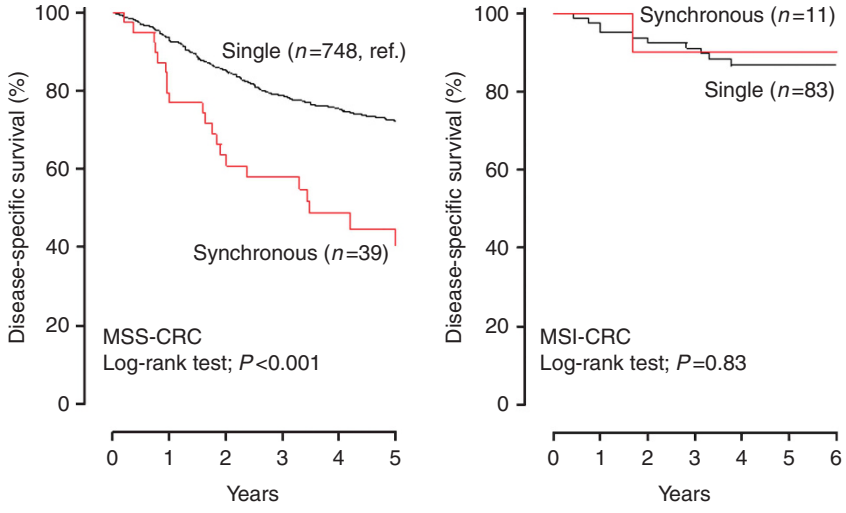

Figure 3. Disease-specific survival of patients with CRC by MS status and by synchronous invasive cancer. Synchronous CRC significantly affected disease-specific survival of patients with MSS cancer but not of those with MSI tumour (Kaplan–Meier curves, log-rank test).

separately the prognosis of MSI and MSS S-CRC. The analysis revealed that the prognosis of MSI cancers was not affected by any concurrent neoplasia, whereas MSS CRC had a significantly poorer outcome if S-CRC, or even a synchronous advanced adenoma, had been diagnosed. Interestingly, at stage-adjusted analysis, the negative prognostic effect of S-CRC equalled that of synchronous advanced adenoma, indicating that the worsened prognosis of synchronous advanced neoplasia likely reflects a more aggressive biological behaviour rather than a larger cancer burden. This concept is consistent with the well-recognized value of S-CRC, as well as of synchronous advanced adenomas, in predicting the future development of metachronous colorectal neoplasia (Balleste *et al*, 2007; Moon *et al*, 2010). Of note, both the presence of synchronous advanced neoplasia and the rare *BRAF*$^{c.1799T>A}$ mutation were unrelated and stage-independent predictors of poor prognosis for MSS CRC. These findings also contradict the hypothesis that an epigenetic field defect may predispose to MSS synchronous neoplasia, which account for the vast majority of multiple primary colorectal malignancies (Leggett and Worthley, 2009). In this respect, the study by Nosho *et al* (2009) may have failed to recognise the existing interactions of S-CRC with MSI and *BRAF*$^{c.1799T>A}$ mutations due to the small number of tumours fully characterised for the MS/BRAF status.

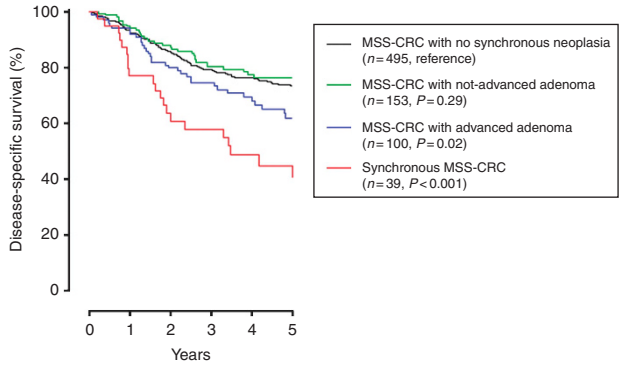

Figure 4. Disease-specific survival of patients with MSS CRC stratified by synchronous colorectal neoplasia. The presence of synchronous advanced neoplasia, but not that of synchronous not-advanced adenoma, negatively affected the survival of CRC patients (Kaplan–Meier curves, log-rank test).

Finally, our study failed to detect any *MYH* germ-line mutation, not confirming the previously reported association between homozygous or compound heterozygous mutations and S-CRC (Cleary *et al*, 2009), nor *POLE* or *POLD1* germ-line/somatic/ mutations, which have been recently associated with multiple CRCs or adenomas (Palles *et al*, 2013). Cleary *et al* (2009) found *MYH* mutations in <1% of the general population with CRC and in about 6% of patients with S-CRC, so that discrepancies might still reflect a type II statistical error. Alternatively, we might have been more selective in excluding mild polyposis syndromes from our colonoscopy-based clinical series.

Our study has the intrinsic limitation of being a case–control, retrospective analysis. This may have altered the relative contribution of different molecular and clinical subsets of CRC, but a bias in the selection of controls to S-CRC is unlikely, given the consecutive series and the use of complete colonoscopy are the only criteria for inclusion of patients with solitary CRC. Then, the correlations found between synchronous neoplasia and

Table 2. Synchronous advanced colorectal neoplasia and *BRAF*$^{c.1799T>A}$ mutation as predictors of death from CRC (Cox proportional-hazard models)

	Death		Univariate		Stage-adjusted multivariate	
	No	Yes	HR (95% CI)	*P*	HR (95% CI)	*P*
MSS CRC						
Synchronous advanced neoplasia						
None	487	161	1.00 Ref.		1.00 Ref.	
Advanced adenoma	62	38	1.59 (1.11–2.26)	0.01	1.81 (1.27–2.58)	0.001
Invasive cancer	18	21	2.66 (1.69–4.19)	<0.001	1.82 (1.15–2.87)	0.01
***BRAF* status**						
WT	558	206	1.00 Ref.		1.00 Ref.	
BRAF $^{c.1799T>A}$	9	14	3.29 (1.91–5.70)	<0.001	2.16 (1.25–3.73)	0.01
MSI CRC						
Synchronous advanced neoplasia						
None	67	10	1.00 Ref.			
Advanced adenoma	6	0	NA	0.45		
Invasive cancer	10	1	0.74 (0.09–5.75)	0.77		
***BRAF* status by sporadic/HNPCC**[a]						
BRAF WT–sporadic CRC	23	2	1.00 Ref.			
BRAF WT–HNPCC	30	2	0.75 (0.11–5.33)	0.77		
BRAF $^{c.1799T>A}$–sporadic CRC	30	7	2.68 (0.55–12.9)	0.22		

Abbreviations: HR = hazard ratios <1 represent a decreased risk of death, whereas HR >1 represent an increased risk of death; MSI = microsatellite instability; MSS CRC = microsatellite-stable colorectal cancer; NA = not applicable.
[a]No HNPCC exhibited the *BRAF* $^{c.1799T>A}$ mutation.

prognosis in single molecular subsets can hardly be interpreted as the result of selection artifacts. The analysis was also limited by the use of $BRAF^{c.1799T>A}$ mutation as the only marker of DNA methylation. Although the *BRAF* status is validated as a reproducible and very specific marker of cancers with methylator phenotype (Weisenberger *et al*, 2006), we might have missed a few methylated tumours potentially identifiable at analysis of multiple markers of CIMP. However, we believe that the lack of association between MSS S-CRC and tumour methylation status in our series cannot be disputed on the basis of this limitation. Finally, synchronous serrated adenomas, which may coexist with an advanced colorectal neoplasia (Álvarez *et al*, 2013), were surely underestimated in a series collected when there was little or no awareness for endoscopic removal of 'hyperplastic' lesions.

In conclusion, our study demonstrates that MSS cancers presenting with the phenotype of multiple advanced lesions account for the vast majority of S-CRC, are not associated with BRAF mutation, and have a worse prognosis than corresponding solitary tumours. The association between non-hypermutated multiple advanced neoplasia and poor prognosis might have important implications for post-surgical surveillance and for adjuvant therapeutic strategies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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