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 $\text{BRAF}^{c.1799T\rightarrow A}$ 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 $\text{BRAF}^{c.1799T\rightarrow A}$ 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, 2006; 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;
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 fluorescently primed 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, Buxincasso, 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<sup>1799T→A</sup> 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 POLDI gene.

**Statistical analysis.** Associations between synchronous neoplasia and clinicopathological or molecular features of the index CRC were tested using χ²-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.

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**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 dysplasia, (3) synchrony of colorectal dysplasia, (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.

Even though several studies have demonstrated that S-CRC might be more frequent among cancers of the serrated type, little is known about their association with CIMP phenotype, as opposed to tumours of the conventional adenoma. The discrepancy might reflect the heterogeneity of CIMP-negative 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 type (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).

In the current study, synchronous colorectal neoplasia (i.e., CRC(s) that were detected concurrently with and/or before the primary CRC) was defined as the presence of synchronous CRC (S-CRC) plus synchronous advanced neoplasia, which were defined as synchronous not-advanced adenoma or the presence of more than one invasive CRC, 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.

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**Statistical analysis.** Associations between synchronous neoplasia and clinicopathological or molecular features of the index CRC were tested using χ²-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...
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 \(BRAF^{c.1799T \rightarrow A}\) mutation in determining the association of these two variables with synchronous non-advanced adenoma, whereas no association was detected between the \(BRAF^{c.1799T \rightarrow A}\) mutation and synchronous advanced adenomas and S-CRC. Figure 2 shows that \(BRAF^{c.1799T \rightarrow A}\) 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 \(BRAF^{c.1799T \rightarrow A}\) 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
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 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; Latounerie 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 chromosomally 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 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...
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 \textit{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 \textit{BRAF}^{c.1799T → A} mutations due to the small number of tumours fully characterised for the MS/BRAF status. Finally, our study failed to detect any \textit{MYH} germ-line mutation, not confirming the previously reported association between homozygous or compound heterozygous mutations and S-CRC (Cleary et al, 2009), nor \textit{POLE} or \textit{POLD1} germ-line/somatic mutations, which have been recently associated with multiple CRCs or adenomas (Palles et al, 2013). Cleary et al (2009) found \textit{MYH} mutations in 0.1% 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 \textit{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. | |
| \textit{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** | | | | | | |
| \textit{BRAF}^{WT–sporadic CRC} | 23 | 2 | 1.00 Ref. | | | |
| \textit{BRAF}^{WT–HNPCC} | 30 | 2 | 0.75 (0.11–5.33) | 0.77 | | |
| \textit{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.

*No HNPCC exhibited the \textit{BRAF}^{c.1799T → A} mutation.*
The authors declare no conflict of interest.

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