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Target gene mutational pattern in Lynch syndrome colorectal carcinomas according to tumour location and germline mutation

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Background: We previously reported that the target genes in sporadic mismatch repair (MMR)-deficient colorectal carcinomas (CRCs) in the distal colon differ from those occurring elsewhere in the colon. This study aimed to compare the target gene mutational pattern in microsatellite instability (MSI) CRC from Lynch syndrome patients stratified by tumour location and germline mutation, as well as with that of sporadic disease.

Methods: A series of CRC from Lynch syndrome patients was analysed for MSI in genes predicted to be selective MSI targets and known to be involved in several pathways of colorectal carcinogenesis.

Results: The most frequently mutated genes belong to the TGF-β superfamily pathway, namely *ACVR2A* and *TGFBR2*. A significantly higher frequency of target gene mutations was observed in CRC from patients with germline mutations in *MLH1* or *MSH2* when compared with *MSH6*. Mutations in microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* were significantly more frequent in the distal CRC. Additionally, we observed differences in *MSH3* and *TGFBR2* mutational frequency between Lynch syndrome and sporadic MSI CRC regarding tumour location.

Conclusions: Our results indicate that the pattern of genetic changes differs in CRC depending on tumour location and between Lynch syndrome and sporadic MSI CRC, suggesting that carcinogenesis can occur by different pathways even if driven by generalised MSI.

Lynch syndrome is a highly penetrant, autosomal dominant disease characterised by early-onset colorectal cancer (CRC) and extracolonic tumours of the endometrium, stomach, small bowel, ureter, renal pelvis, ovary and hepatobiliary tract (Lynch and de la Chapelle, 2003). This is the most common hereditary CRC syndrome accounting for up to 4% of all CRC cases

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(Aaltonen *et al*, 1998; Hampel *et al*, 2008) and is commonly caused by a genetic defect affecting one of the four mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* (Lagerstedt Robinson *et al*, 2007). The selection of families for genetic testing is mainly based on personal and family cancer history using the Amsterdam criteria or the Bethesda guidelines (Vasen *et al*, 1999; Umar *et al*, 2004).

More than 95% of the tumours arising in carriers of MMR gene mutations show microsatellite instability (MSI) (Aaltonen *et al*, 1994). Microsatellite instability is characterised by a widespread instability in coding and noncoding short repeat microsatellite sequences, because of MMR deficiency (Perucho, 1996). Through the MSI pathway, CRC progression is accelerated by a rapid mutation accumulation in coding repetitive sequences of target genes with growth-related functions. In Lynch syndrome MSI CRC, somatic mutations have been described in several genes with important cellular roles, such as growth factor receptors (*TGFBR2* and *IGF2R*), genes involved in apoptosis (*BAX*) and DNA repair (*MSH3* and *MSH6*) (Yamaguchi *et al*, 2006), together with many other microsatellite mutations that are not mechanistically responsible for the behaviour of tumour cells.

We have previously reported that, in sporadic MSI CRC, the target genes in MMR-deficient tumours of distal colon and rectum differ from tumours elsewhere in the colon (Pinheiro *et al*, 2010). In this study, we aimed to compare the target gene mutational pattern in MSI CRC from Lynch syndrome patients stratified by tumour location, germline-mutated gene and type of mutation (founder mutations compared with other mutations in the same gene).

MATERIALS AND METHODS

Patients, samples and DNA extraction. This study includes altogether 129 CRC samples from 114 patients belonging to 98 Lynch syndrome families with deleterious (class 5 or 4 according to the InSIGHT variant interpretation committee guidelines) MMR germline mutations (Table 1). The test series includes 78 CRCs, obtained by surgical resection, from 65 patients belonging to 50 Portuguese Lynch syndrome families presenting a germline MMR gene mutation. These mutations were identified by routine genetic diagnosis during the period of 1997–2011 at the Department of Genetics of the Portuguese Oncology Institute, Porto, Portugal, after genetic counselling and informed consent. Thirty-nine families were followed at the Portuguese Oncology Institute and 11 at Centro Hospitalar de S João, Porto, Portugal. Twenty-seven of the families fulfilled the Amsterdam criteria, whereas the remaining presented the Bethesda criteria for genetic testing. Twenty-seven (54%) families carried a pathogenic germline mutation in *MSH2*, 16 (32%) in *MLH1*, 6 (12%) in *MSH6* and 1 (2%) in *PMS2* (Table 1). Furthermore, 9 (18%) and 10 (20%) of these families carried the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del and the *MSH2* c.388_389del Portuguese founder mutations, respectively (Pinheiro *et al*, 2011, 2013). Forty (51%) tumours belonged to patients carrying a germline mutation in *MSH2*, 31 (40%) in *MLH1*, 6 (8%) in *MSH6* and 1 (1%) in *PMS2*. The 65 patients included 32 females and 33 males with a mean CRC diagnosis age of 46 years (range, 15–75 years). Clinical data were drawn from hospital records and tumour staging was performed using the American Joint Committee on Cancer (AJCC) criteria. Summary of the clinicopathological data is presented in Table 1. All large bowel regions up to the splenic flexure were considered as proximal colon and after that as distal colon. All tumour samples were paraffin embedded and were reviewed by a pathologist. Peripheral blood was collected from the same patients. DNA was isolated from paraffin-embedded tumour and from peripheral blood using standard procedures. This study was approved by the Institutional Review Board.

We analysed an additional series that included 51 CRC samples from 49 Swiss patients belonging to 48 Lynch syndrome families referred to and genetically characterised by the Basel research group Human Genomics (Kovac *et al*, 2011). Twenty-six (54%) families carried germline mutations in *MLH1*, 21 (44%) in *MSH2* and 1 (2%) in *MSH6* (Table 1). The 49 patients included 26 females and 23 males, and summary if the clinicopathological data are shown in Table 1. Twenty-eight (55%) tumours belonged to patients carrying a germline mutation in *MLH1*, 22 (43%) in *MSH2* and 1 (2%) in *MSH6*. Thirty-three of these families fulfilled the Amsterdam criteria, whereas the remaining presented the Bethesda criteria for genetic testing (Table 1).

Additionally, the data on Lynch syndrome patients were compared with that of a series of 42 sporadic MSI CRCs (22 and 20 localised in the proximal and distal colons, respectively) (Pinheiro *et al*, 2010).

MMR immunohistochemical and MSI analyses. Assessment of MLH1, MSH2, MSH6 and PMS2 immunoexpression was performed as described previously (Pinheiro *et al*, 2010) in 65 and 51 tumours belonging to the Portuguese and Swiss Lynch syndrome families, respectively. Microsatellite instability evaluation was performed using the Bethesda panel of markers (BAT25, BAT26, D2S123, D5S346 and D17S250) and the 1997 National Cancer Institute guidelines, as described previously (Pinheiro *et al*, 2009).

Target gene analyses. For somatic mutation analysis, we selected 17 genes belonging to pathways involved in CRC carcinogenesis, most of them known to acquire mutations in coding microsatellite sequences in tumours with MSI: *TGFBR2* (A)10, *ACVR2A* (A)8, *BMPR1A* (T)6 (two sequences), *BMPR2* (A)7 and (A)11, *EGFR* (A)13, *E2F4* (CAG)13, *BAX* (G)8, *PRDM2* (A)8 and (A)9, *TCF7L2* (A)9, *APC* (A)6 and (A)4, *AXIN1* (C)6 (two sequences) and (G)6, *AXIN2* (A)6, (G)7, (C)5 (two sequences) and (C)6, *PTEN* (A)6 (two sequences), *MSH6* (C)8, *MSH3* (A)8, *IGF2R* (G)8 and *B2M* (CT)4 (Supplementary Table 1). The selected microsatellite sequences were analysed by PCR and fragment analysis involves using fluorescence-labelled primers (Supplementary Table 1). Fragment length variations were analysed on an ABI Prism 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and allele sizes were determined using the Genemapper software (version 3.7; Applied Biosystems). The results were independently scored by two observers, and an additional round of analyses confirmed the results. Additionally, all cases that presented length variations between tumour and matching blood samples were confirmed by direct sequencing on an ABI 310 DNA sequencer using Big Dye Terminator V1.1 Chemistry (Applied Biosystems), according to the manufacturer's recommendations.

Statistical analysis. Statistical analysis was carried out with SPSS version 22 (IBM, Armonk, NY, USA). Results were expressed in absolute frequencies and percentages. The statistical significance of association between different variables was performed using the Fisher's exact probability test. Mean comparison between two groups was performed using *T*-test. Analysis of variance (ANOVA) was used for mean comparison between more than two groups and the statistical significance was assessed using the Scheffe's multiple comparison test. *P*-values <0.05 were considered statistically significant.

Unsupervised hierarchical cluster analysis and heat maps were performed using the software Multi Experiment Viewer MeV version 4.9 (TM 4 group; Dana Farber Cancer Institute, Boston, MA, USA) using a Pearson's correlation distance metric with average linkage clustering.

Table 1. Clinicopathologic characteristics of Lynch syndrome patients and families

	Portuguese Lynch syndrome families					Swiss Lynch syndrome families			
	Total	*MLH1*	*MSH2*	*MSH6*	*PMS2*	Total	*MLH1*	*MSH2*	*MSH6*
Families	50	16 (32%)	27 (54%)	6 (12%)	1 (2%)	48	26 (54%)	21 (44%)	1 (2%)
Criteria									
Amsterdam	27 (54%)	9 (33%)	17 (63%)	1 (4%)	0 (0%)	33 (69%)	22 (67%)	10 (30%)	1 (3%)
Bethesda	23 (46%)	7 (30%)	10 (43%)	5 (22%)	1 (4%)	15 (31%)	4 (27%)	11 (73%)	0 (0%)
CRC patients	65	25 (38%)	33 (51%)	6 (9%)	1 (2%)	49	27 (55%)	21 (43 %)	1 (2%)
Gender									
Female	32 (49%)	13 (41%)	16 (50%)	2 (6%)	1 (3%)	26 (53%)	14 (54%)	11 (42%)	1 (4%)
Male	33 (51%)	12 (36%)	17 (52%)	4 (12%)	0 (0%)	23 (47%)	13 (57%)	10 (43%)	0 (0%)
Age at diagnosis of CRC (years)									
Mean	46	46	46	44	55	45	45	46	48
Range	15–75	27–68	15–75	21–71		21–79	30–65	21–79	
⩽50	55 (71%)	21 (38%)	29 (53%)	5 (9%)	0 (0%)	35 (69%)	19 (54%)	15 (43%)	1 (3%)
>50	23 (29%)	10 (43%)	11 (48%)	1 (4%)	1 (4%)	16 (31%)	9 (56%)	7 (44%)	0 (0%)
Total CRC	78	31 (40%)	40 (51%)	6 (8%)	1 (1%)	51	28 (55%)	22 (43%)	1 (2%)
CRC localisation									
Proximal	51 (65%)	23 (45%)	24 (47%)	4 (8%)	0 (0%)	35 (69%)	22 (63%)	12 (34%)	1 (3%)
Distal	27 (35%)	8 (30%)	16 (59%)	2 (7%)	1 (4%)	16 (31%)	6 (38%)	10 (63%)	0 (0%)
TNM stage[a]									
I/II	45 (68%)	20 (44%)	23 (51%)	2 (4%)	–	32 (70%)	20 (63%)	12 (38%)	0 (0%)
III/IV	21 (32%)	9 (43%)	9 (43%)	3 (14%)	–	14 (30%)	6 (43%)	7 (50%)	1 (7%)
Differentiation grade[b]									
Well/moderately	53 (90%)	20 (38%)	28 (53%)	5 (9%)	0 (0%)	25 (66%)	15 (60 %)	10 (40 %)	0 (0%)
Poorly	6 (10%)	2 (33%)	3 (50%)	0 (0%)	1 (17%)	13 (34%)	9 (69%)	4 (31%)	0 (0%)

Abbreviations: CRC = colorectal carcinoma; TNM = tumor node metastasis.
[a]Information was not available for 12 and 5 CRC from the Portuguese and Swiss Lynch syndrome families, respectively.
[b]Information was not available for 19 and 13 CRC from the Portuguese and Swiss Lynch syndrome families, respectively.

RESULTS

MMR immunohistochemical analysis. In all tumours from the Porto test series analysed by immunohistochemistry from patients carrying a mutation in the *MLH1* gene, MLH1 and PMS2 protein expression was absent; patients with an *MSH2* mutation did not show MSH2 and MSH6 protein expression; patients with a mutation in *MSH6* gene had no MSH6 protein expression and one case also displayed loss of MSH2 protein expression; and the tumour of the patient with the *PMS2* mutation showed loss of PMS2 protein expression. In the additional Swiss series, all the CRCs analysed by immunohistochemistry from patients carrying a mutation in the *MLH1* gene, MLH1 and PMS2 protein expression was absent; patients with an *MSH2* mutation did not show MSH2 and MSH6 protein expression; and patients with a mutation in *MSH6* gene had no MSH6 protein expression.

Overall MSI. The frequency of MSI in the test series from Porto was 99% (77 out of 78) and the only CRC that did not present MSI belonged to a patient carrying a germline mutation in the *MSH6* gene. In the additional Switzerland series, all 51 Lynch syndrome CRCs presented MSI.

Target gene mutation frequencies. The microsatellite sequence most frequently mutated in the test series from Porto was *ACVR2A* (90.9%), followed by *TGFBR2* (89.6%), *EGFR* (88.3%) and *BMPR2* (A)11 (75%) sequences. Mutations in the *E2F4* (53.3%), *MSH3* (49.4%), *BAX* (44.2%), *TCF7L2* (41.6%) and *BMPR2* (A)7 (35.1%) microsatellite sequences were also frequent. The remaining genes presented a mutational frequency <30% (Table 2). All the MSI CRCs presented MSI in at least one of the genes analysed and the overall mean mutation frequency was 6.7 ± 2.4.

In the additional Switzerland series, we only analysed the target gene sequences showing significant differences between proximal and distal CRCs in the Porto series (see below), namely the *MSH3*, *BMPR2* (A)7 and *PTEN* microsatellite sequences (Table 2). The mutational frequency in this series was 47.1%, 39.2% and

Table 2. Mutational frequency of the target gene microsatellite sequences according to tumour location in the Lynch syndrome MSI-H test (Porto) series

Gene	Total (%)	Proximal colon (%)	Distal colon (%)	*P*-value
ACVR2A	70/77 (90.9)	46/51 (90.2)	24/26 (92.3)	1
TGFBR2	69/77 (89.6)	46/51 (90.2)	23/26 (88.5)	1
EGFR	68/76[a] (88.3)	44/50 (88.0)	24/26 (92.3)	0.708
BMPR2 (A)11	57/76[a] (75.0)	36/50 (72.0)	21/26 (80.8)	0.578
E2F4	40/75[b] (53.3)	30/50 (60.0)	10/25 (40.0)	0.141
MSH3	38/77 (49.4)	20/51 (39.2)	18/26 (69.2)	**0.017**
BAX	34/77 (44.2)	21/51 (41.2)	13/26 (50.0)	0.478
TCF7L2	32/77 (41.6)	19/51 (37.3)	13/26 (50.0)	0.333
BMPR2 (A)7	27/77 (35.1)	13/51 (25.5)	14/26 (53.8)	**0.022**
PRDM2	22/77 (28.6)	15/51 (29.4)	7/26 (26.9)	1
MSH6	19/77 (24.7)	10/51 (19.6)	9/26 (34.6)	0.170
IGF2R	16/77 (20.8)	9/51 (17.6)	7/26 (26.9)	0.382
B2M	7/77 (9.1)	6/51 (11.8)	1/26 (3.8)	0.412
APC	6/77 (7.8)	6/51 (11.8)	0/26 (0.0)	0.091
PTEN	6/77 (7.8)	0/51 (0.0)	6/26 (23.1)	**0.001**
AXIN2	3/77 (3.9)	1/51 (2.0)	2/26 (7.7)	0.262

Abbreviations: CRC = colorectal carcinoma; MSI = microsatellite instability. We did not detect mutations in *BMPR1A* microsatellite sequences. $P<0.05$ are indicated in bold.
[a]One proximal CRC case was not analysed for this gene.
[b]Two cases (one proximal and one distal CRC were not analysed for this gene.

11.8% for *MSH3*, *BMPR2* and *PTEN*, respectively, being 48.4% for *MSH3*, 36.7% for *BMPR2* and 9.4% for *PTEN* in the combined series.

Target gene analysis by germline MMR mutation. The mean frequency of target gene mutations was 7.1 ± 2.1 in the tumours from patients presenting a germline mutation in *MSH2*, 6.8 ± 2.5 in *MLH1* carriers and 3.0 ± 1.6 in *MSH6* carriers, and the single tumour from a *PMS2* carrier had seven mutations. To examine whether there were differences among these groups, one-way ANOVA was conducted and statistically significant differences among the groups were found ($P = 0.001$). *Post hoc* Scheffe's tests revealed statistically significant differences between *MLH1* and *MSH6* ($P = 0.004$) and *MSH2* and *MSH6* ($P = 0.001$), but not between *MLH1* and *MSH2* ($P = 0.865$) (Figure 1).

No association was observed between the target gene mutational frequencies and pattern with the germline-mutated gene or the type of mutation, nor when comparing founder mutations to other mutations in the same gene. Unsupervised hierarchical cluster analysis of target gene mutations and MMR gene is shown in Supplementary Figure 1A.

Target gene mutation analysis by tumour location. The mean frequency of target gene mutations in proximal and distal colon tumours was 6.3 ± 2.4 and 7.5 ± 2.5, respectively ($P = 0.06$). Three microsatellite sequences were preferentially mutated in distal tumours (Table 2). In the CRC test series from Porto, *PTEN*, *BMPR2* (A)7 and *MSH3* mutations were present in 23.1%, 53.8% and 69.2% of the distal MSI carcinomas, whereas in proximal MSI carcinomas mutations were present in 0%, 25.5% and 39.2% of the tumours, respectively ($P = 0.001$ for *PTEN*, $P = 0.022$ for *BMPR2* and $P = 0.017$ for *MSH3*) (Table 2). In the smaller Lynch syndrome CRC series from Switzerland alone, none of these genes were significantly associated with distal MSI carcinomas, but for the combined series mutations in *MSH3* and *BMPR2* (A)7, microsatellite sequences remained significantly associated with distal tumour location ($P = 0.039$ for *MSH3* and $P = 0.012$ for *BMPR2*) (Figure 2 and Table 3). Other genes showed different mutation frequencies according to the large bowel site of origin (Table 2), but their lower mutation frequencies require much larger tumour series to evaluate the statistical significance. For instance, *APC* microsatellite mutations were only detected in the proximal colon, whereas *AXIN1* and *AXIN2* microsatellite mutations only rarely were found in the proximal CRC but were recurrently detected in the distal CRC. No association was observed between target gene mutational pattern or frequency with other clinicopathologic features, namely gender, mean age of CRC diagnosis, tumour staging or differentiation grade. Unsupervised hierarchical cluster analysis of target gene mutations and tumour location is shown in Supplementary Figure 1B.

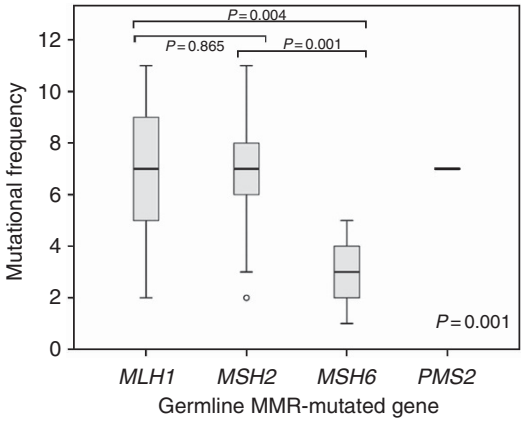

Figure 1. Box-plot analyses of the frequency of target gene mutations (Y axis) in CRC samples from the test series categorised by MMR germline mutation (X axis). The mean comparison was calculated using the one-way ANOVA test. Statistical significance among the samples was assessed using the Scheffe's multiple comparison test.

Target gene mutation comparison between Lynch syndrome and sporadic MSI CRC. In a previous work, we observed that alterations in *MSH3* and *TGFBR2* were less frequent in the distal sporadic MSI CRCs (20.0% and 30.0%, respectively) when compared with the proximal tumours (72.7% and 95.5%, respectively) (Pinheiro *et al*, 2010). Comparing these results with Lynch syndrome MSI CRC, we observed that *MSH3* alterations were significantly more frequent in the proximal sporadic MSI CRCs compared with that in proximal Lynch syndrome tumours (72.7% and 39.2%, respectively, $P = 0.011$), with the opposite occurring in the distal CRCs (20.0% and 69.2%, respectively, $P = 0.001$) (Figure 3). *TGFBR2* alterations were significantly more frequent in distal Lynch syndrome tumours compared with that in the distal sporadic tumours (88.5% and 30.0%, respectively, $P = 0.00006$), with no difference being observed in the proximal CRCs (Table 4 and Figure 3).

DISCUSSION

Microsatellite instability is a hallmark of CRC in Lynch syndrome patients, occurring in >95% of the tumours (Aaltonen *et al*, 1994). The frequency of MSI detected in our study indicates that both series are representative of Lynch syndrome CRC. There is a well-established association between an ineffective MMR system and mutations in genes with key cellular roles in CRC of Lynch syndrome patients. With the purpose of comparing the mutation pattern of target genes in MSI CRC from Lynch syndrome patients stratified by tumour location and germline mutation type, we analysed several genes predicted to be selective target genes of MSI and known to be involved in several pathways of colorectal carcinogenesis. All candidate genes except *BMPR1A* presented frameshift mutations in the microsatellite sequences analysed. Colorectal carcinoma from patients with a germline mutation in *MSH6* presented a significantly lower frequency of target gene mutations when compared with the groups with *MLH1* or *MSH2* germline mutations, whereas no differences were observed regarding target gene mutation frequency or pattern between CRC from carriers of *MLH1* or *MSH2* founder mutations and other mutations in these genes. Wu *et al* (1999) had already suggested that *MSH6* may be involved in a proportion of Lynch syndrome patients presenting MSI-low tumours. More recently, Laghi *et al* (2012) also observed that the mutational rate in specific target genes was significantly lower in *MSH6* compared with that in *MLH1*- and *MSH2*-deficient tumours.

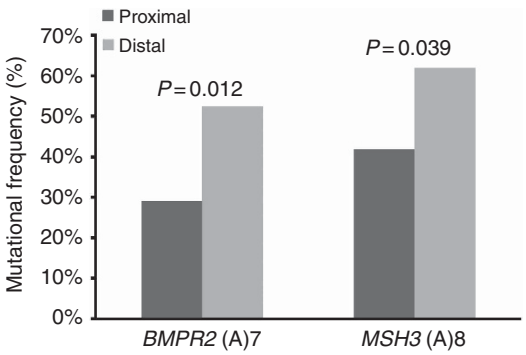

Figure 2. Mutational frequency of the microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* according to tumour location in tumours from both series of Lynch syndrome patients.

Table 3. Mutational frequency of *MSH3*, *BMPR2* (A)7 and *PTEN* microsatellite sequences according to tumour location in the Lynch syndrome MSI-H series of Porto, Switzerland and combined

Cases	Gene	Total (%)	Proximal colon (%)	Distal colon (%)	*P*-value
Porto	*MSH3*	38/77 (49.4)	20/51 (39.2)	18/26 (69.2)	**0.017**
	BMPR2 (A)7	27/77 (35.1)	13/51 (25.5)	14/26 (53.8)	**0.022**
	PTEN	6/77 (7.8)	0/51 (0)	6/26 (23.1)	**0.001**
Switzerland	*MSH3*	24/51 (47.1)	16/35 (45.7)	8/16 (50)	1.000
	BMPR2 (A)7	20/51 (39.2)	12/35 (34.3)	8/16 (50)	0.360
	PTEN	6/51 (11.8)	6/35 (17.1)	0/16 (0)	0.159
Total	*MSH3*	62/128 (48.4)	36/86 (41.9)	26/42 (61.9)	**0.039**
	BMPR2 (A)7	47/128 (36.7)	25/86 (29.1)	22/42 (52.4)	**0.012**
	PTEN	12/128 (9.4)	6/86 (7)	6/42 (14.3)	0.206

Abbreviation: MSI = microsatellite instability. $P<0.05$ are indicated in bold.

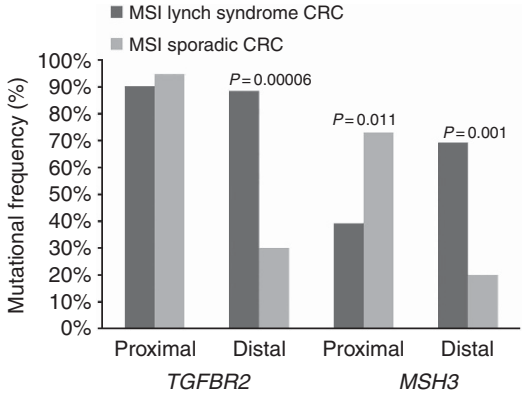

Figure 3. Mutational frequency in *TGFBR2* (A)10 and *MSH3* (A)8 microsatellite sequences categorised by tumour location in sporadic (Pinheiro *et al*, 2010) and Lynch syndrome MSI CRC (present report).

The most frequently mutated genes, *TGFBR2* and *ACVR2A*, encode for proteins belonging to the TGF-β superfamily, supporting the idea that alterations in these genes are important for the development of MSI CRC in the context of Lynch syndrome (Fujiwara *et al*, 1998; Miyaki *et al*, 2001). Additionally, we also observed a high frequency of mutations in the microsatellite sequences of *BMPR2* (75% and 35.1% in (A)11 and (A)7, respectively). Kodach *et al* (2008) also found a high mutational frequency (81.4%) in the (A)11 microsatellite sequence in sporadic MMR-deficient tumours and indicated that loss of BMPR2 expression is associated with MSI in this sequence. Regarding the (A)7 microsatellite sequence, they detected instability in two MSI cell lines and none in the MMR-deficient tumours, whereas none of the MSS cell lines and MMR-proficient tumours analysed presented mutations in either microsatellite sequences (Kodach *et al*, 2008). Furthermore, Park *et al* (2010) detected mutations in *BMPR2* (A)7 sequence in 13.2% of sporadic CRCs associated with loss of BMPR2 expression. Further studies are needed to clarify the role of the (A)7 tract mutations in *BMPR2* function and expression, given that almost all cases presented in concomitance alterations in the more commonly mutated (A)11 tract. Kodach *et al* (2008) also observed that alterations in *BMPR2* occurred in concomitance with *TGFBR2* and *ACVR2A* mutations, suggesting that loss of one of these receptors is insufficient for complete pathway disruption. Some studies indicate that alterations in the WNT pathway may have an important role in the tumorigenesis of MMR deficiency tumours (Miyaki *et al*, 1999; Thorstensen *et al*, 2005). We detected a low frequency of mutations in *APC*, *AXIN1*, *AXIN2* and *PTEN*, but a high frequency in *TCF7L2*. Mutations in *TCF7L2* have been reported with a high frequency (33–58%) in Lynch syndrome CRC (Duval *et al* 1999; Ruckert *et al*, 2002; Yamaguchi *et al*, 2006). Mutations in a polymorphic (A)13 out of 14 repeat within the 3′-UTR of *EGFR* were also described in a high percentage (59–81%) of sporadic MSI colon cancers, but there is no consensus if these are oncogenic mutations (Deqin *et al*, 2012; Sarafan-Vasseur *et al*, 2013). This is the first report showing a high frequency of this type of *EGFR* mutations (88.3%) in Lynch syndrome CRC. The high mutation frequency observed in *BMPR2*, *TCF7L2* and *EGFR* could be an indicator of selective advantage, but some of these mutations may be bystander events that do not have a causal role in carcinogenesis. Additional functional studies are needed to clarify if they are true target genes in the MSI pathway of colorectal carcinogenesis, especially in the context of Lynch syndrome.

A substantial frequency of somatic mutations in *MSH3* and *MSH6* microsatellite sequences have been described in CRC from patients with Lynch syndrome (Akiyama *et al*, 1997; Yamamoto *et al*, 1998). These mutations are considered secondary events resulting from a germline MMR gene deficiency and are defined as 'secondary' mutators in a 'mutator that mutates another mutator' model (Akiyama *et al*, 1997; Yamamoto *et al*, 1998). However, the pathogenetic consequence of these somatic mutations in MMR genes is unclear and may be just a marker of generalised MSI. In fact, in addition to the necessity of biallelic mutations for a biological effect, it is well established that CRC with germline *MSH2* mutations are usually associated with loss of expression of MSH6, thus somatic *MSH6* mutations would not confer an additional selective advantage. Genes involved in cell cycle control and apoptosis were also found to be frequently mutated in Lynch syndrome CRC, namely *E2F4*, *BAX* and *PRDM2*, indicating that these are target genes of genetic instability in Lynch syndrome CRC (Miyaki *et al*, 2001; Moriyama *et al*, 2002; Yamaguchi *et al*, 2006).

Regarding tumour site, we observed that mutations in microsatellite sequences (A)7 of *BMPR2*, (A)8 of *MSH3* and *PTEN* were significantly more frequent in the distal CRC in the Porto series. Although these associations were not clear when only the Switzerland series is considered, which could be because of the lower number of CRC samples in that series or to other factors related to a different genetic background or environmental causes, the analysis of the combined series confirmed those findings for *MSH3* and *BMPR2* (A)7, indicating that these genes are preferentially involved in the development or progression of distal colon cancer in Lynch syndrome patients. It is unclear why the tumour site difference was observed for *BMPR2* (A7) but not *BMPR2* (A11), especially in light of their frequent co-occurrence, being possible that mutations in the (A)11 microsatellite sequence could be just a marker of generalised instability without a functional consequence to the *BMPR2* gene. Interestingly, the results concerning *MSH3* are different from the ones we observed in a previous study of sporadic MSI tumours, where alterations in *MSH3* were less frequent in tumours of the distal colon (Pinheiro *et al*, 2010). Furthermore, the significantly lower frequency of

Table 4. Mutational frequency of *TGFBR2* and *MSH3* microsatellite sequences according to tumour location in Lynch syndrome and sporadic MSI-H carcinomas (Pinheiro *et al*, 2010)

Gene	Cases	Total (%)	Proximal colon (%)	*P*-value	Distal colon (%)	*P*-value
TGFBR2	Lynch	69/77 (89.6)	46/51 (90.2)	0.661	23/26 (88.5)	0.00006
	Sporadic	27/42 (64.3)	21/22 (95.5)		6/20 (30)	
MSH3	Lynch	38/77 (49.4)	20/51 (39.2)	0.011	18/26 (69.2)	0.001
	Sporadic	20/42 (47.6)	16/22 (72.7)		4/20 (20)	

Abbreviation: MSI = microsatellite instability.

TGFBR2 mutations we previously observed in the distal sporadic MSI CRCs (Pinheiro *et al*, 2010) was not seen in the present study with Lynch syndrome MSI CRCs. Qualitative (type of target gene) and quantitative (number and frequency of altered target genes) differences have been observed regarding MSI target genes in different types of cancers. For instance, significant differences have been described between the MSI profiles of endometrial and colorectal cancers from Lynch syndrome patients presenting the same germline mutation. For example, somatic mutations in *TCF7L2* were observed in ~47% of Lynch syndrome CRC, but not in endometrial cancers (Planck *et al*, 2000). Similarly, *TGFBR2* mutational frequency is higher in MSI colon carcinomas (70–90%) compared with that in MSI endometrial carcinoma (17–19%), whereas *PTEN* instability was more frequently observed (~20%) in endometrial carcinomas compared with MSI CRCs (~5%), suggesting that biological features and functional roles of target genes may differ depending on the tissue of origin (Lu *et al*, 1995; Myeroff *et al*, 1995; Kuismanen *et al*, 2002). Our data suggest that target genes differ in colorectal carcinomas depending on large bowel site of origin and between Lynch syndrome and sporadic MSI CRC, suggesting that carcinogenesis can occur by different routes even if driven by generalised MSI (Dierssen *et al*, 2007). Significant differences in the spectrum of molecular alterations between MSI Lynch syndrome and sporadic CRC have been observed previously. *BRAF* mutations, namely p.Val600Glu, have been identified in sporadic MSI CRC associated with *MLH1* promoter hypermethylation but not in Lynch syndrome CRC (Domingo *et al*, 2004). Conversely, *CTNNB1* gene mutations are frequent (43%) in Lynch syndrome tumours but not in sporadic MSI CRCs (Miyaki *et al*, 1999). The differences in the molecular profiles of the two pathways are consistent with the differing carcinogenesis routes of hereditary *vs* sporadic MSI CRCs, specifically the traditional adenoma–carcinoma sequence in the former and a serrated pathway in the latter (Jass, 2007). Furthermore, the different embryological origin of proximal and distal bowel may contribute to the molecular heterogeneity of MSI CRC, similarly to what happens during carcinogenesis of different organs in Lynch syndrome patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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