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SHORT REPORTS

BRAF-V600E is not involved in the colorectal tumorigenesis of HNPCC in patients with functional *MLH1* and *MSH2* genes

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Recently, it was shown that the oncogenic activation of *BRAF*, a member of the RAS/RAF family of kinases, by the V600E mutation is characteristic for sporadic colon tumors with microsatellite instability. Further, it was shown to associate with the silencing of the mismatch repair (MMR) gene *MLH1* by hypermethylation. Moreover, *BRAF* mutations proved to be absent in tumors from hereditary nonpolyposis colorectal cancer syndrome (HNPCC) families with germline mutations in the MMR genes *MLH1* and *MSH2*. These data suggest that the oncogenic activation of *BRAF* is involved only in sporadic colorectal tumorigenesis. In order to further support this hypothesis, we have extended the analysis of the *BRAF* gene to a different subset of HNPCC families without germline mutations in *MLH1* and *MSH2*. *BRAF-V600E* mutations were analysed by automatic sequencing in 38 tumors from HNPCC families with germline mutations in the *MSH6* gene and also in HNPCC (suspected) families that do not have mutations in the MMR genes *MLH1*, *MSH2* and *MSH6*. All patients belong to different families. No mutations were detected in 14 tumors from HNPCC patients with germline mutations in *MSH6*. Further, no mutations of *BRAF* were found in tumors from 23 MMR-negative families, from which 13 fulfilled the Amsterdam criteria (HNPCC) and 10 were suspected for HNPCC as they were positive for the Bethesda criteria. Overall, our data reinforce the concept that *BRAF* is not involved in the colorectal tumorigenesis of HNPCC. The detection of a positive *BRAF-V600E* mutation in a colorectal cancer suggests a sporadic origin of the disease and the absence of germline alterations of *MLH1*, *MSH2* and also of *MSH6*. These findings have a potential impact in the genetic testing for HNPCC diagnostics and suggest a potential use of BRAF as exclusion criteria for HNPCC or as a molecular marker of sporadic cancer.

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Activation of the RAS/RAF pathway is the most common oncogenic event in colorectal tumorigenesis. In addition to mutations in *KRAS*, a V600E hotspot mutation in the exon 15 of *BRAF*, a member of the RAF family of kinases, has been recently reported in colorectal tumors with high microsatellite instability (MSI-H), and was found associated to a defective mismatch repair (MMR) system (Davies *et al*., 2002; Rajagopalan *et al*., 2002; Oliveira *et al*., 2003). Further, it was shown that the *BRAF-V600E* mutation (previously named V599E) is mainly found in proximal colorectal tumors in which the MSI-H phenotype is caused by hypermethylation of the MMR gene *MLH1* (Deng *et al*., 2004; Domingo *et al*., 2004a). Tumors arising in the hereditary nonpolyposis colorectal cancer syndrome (HNPCC) are preferentially found in the proximal colon and show MSI due to germline defects in genes from the MMR system, mainly *MLH1, MSH2* and *MSH6* (Marra and Boland, 1995; Miyaki *et al*., 1997). Therefore, mutations in *BRAF*, as an alternative oncogenic event to *KRAS* activation, were expected to play a role in the tumorigenesis of HNPCC. However, recent data have provided evidence that *BRAF* is not involved in tumors from HNPCC patients with germline mutations in *MLH1* and *MSH2*, suggesting that the oncogenic capability of *BRAF* in MSI colon cancer might be somehow related to the epigenetic mechanisms involved in the inactivation of the *MLH1* gene and not to the germline MMR defect (Wang *et al*., 2003; Deng *et al*., 2004; Domingo *et al*., 2004b). Owing to its absence in the *MLH1* and *MSH2* germline mutation-positive families, a potential use of *BRAF-V600E* in the molecular diagnostics of HNPCC has been suggested (Wang *et al*., 2003; Domingo *et al*., 2004b). Nonetheless,

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Table 1 Features of HNPCC tumors found negative for the *BRAF-V600E* mutation

Patient	*MMR*[a]	*Mutation*	*Location*	*MSI*	*Age*	*Criteria*[b]
Colorectal tumors:						
Y88	*MSH6*	Truncating (3263insT)	Transverse	MSI-H	38	–
Y701	*MSH6*	Truncating (650insT)	Rectum	MSI-H	53	–
Y708[c]	*MSH6*	Truncating (2672delT;2674delT)	Descendent	MSI-H	55	–
Y725	*MSH6*	Truncating (650insT)	Left-sided[d]	MSI-H	83	+
Y1	*MSH6*	Truncating (Glu1258X)	Rectum	MSI-L	55	–
Y37	*MSH6*	Truncating (650insT)	Descendent	MSI-L	59	–
Y241	*MSH6*	Missense (Ala355Val)	Descendent	MSI-H	65	–
Y105	*MSH6*	Missense (Ile725Met)	Transverse	MSI-L	36	–
Y243	*MSH6*	Missense (Gln522Arg)	Rectum	MSI-L	43	–
Y319	*MSH6*	Missense (Pro1087Ser)	Rectum	MSI-L	39	–
Y194	—	—	Rectum	MSI-H	58	+
Y249	—	—	Transverse	MSI-H	48	+
CCH1	—	—	Ascendent	MSI-H	46	+
CCH2	—	—	Cecum	MSI-H	47	+
CCH3	—	—	Descendent	MSI-H	49	+
CCH4	—	—	Transverse	MSI-H	38	+
CCH5	—	—	Cecum	MSI-H	42	+
CCH6	—	—	Descendent	MSI-H	47	+
CCH7	—	—	Ascendent	MSI-H	31	+
CCH8	—	—	Cecum	MSI-H	45	+
CCH9	—	—	Cecum	MSI-H	43	–
CCH11	—	—	Transverse	MSI-H	50	–
CCH12	—	—	Cecum	MSI-H	46	–
CCH14	—	—	Transverse	MSI-H	48	–
CCH15	—	—	Cecum	MSI-H	55	–
CCH16	—	—	Cecum	MSI-H	44	–
CCF265	—	—	Ascendent	MSI-H	82	–
CCF910	—	—	Cecum	MSI-H	81	–
Y74	—	—	Rectosigmoid	MSI-L	48	+
Y168	—	—	Sigmoid	MSI-L	35	+
CCH10	—	—	Ascendent	MSI-L	62	–
CCH13	—	—	Ascendent	MSI-L	44	–
Other tumors:						
Y1	*MSH6*	Truncating (Glu1258X)	Pyelum	MSI-H	63	–
Y37	*MSH6*	Truncating (650insT)	Endometrium	MSI-L	65	–
Y605	*MSH6*	Truncating (650insT)	Endometrium	MSI-L	46	+
Y751	*MSH6*	Truncating (650insT)	Duodenum	MSI-L	51	–
Y77	—	—	Duodenum	MSI-H	54	+

[a]Defective MMR gene. Negative cases do not show mutations in *MLH1*, *MSH2* or *MSH6* genes. Tumor samples were analysed for germline mutations in the *MLH1, MSH2* and *MSH6* genes by several laboratory routines and mutations verified by automatic sequencing. In some cases, large deletions of *MLH1* and *MSH2* were detected by Southern blotting or using alternative strategies (Wahlberg *et al*., 1999; Gille *et al*., 2002; Di Fiore *et al*., 2004). IH for MLH1 (clone G168-728, BD Pharmingen, San Diego, CA, USA), MSH2 (clone FE11, Calbiochem, San Diego, CA, USA), MSH6 (clone 44, Transduction Laboratories, Lexington, KY, USA), PMS1 (sc-615, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and PMS2 (sc-618, Santa Cruz Biotechnology Inc.) were also performed. [b]Positive cases fulfill the Amsterdam criteria and negative cases are from families showing an HNPCC-like pedigree in which at least one first-degree relative is affected by an early-onset colorectal cancer. [c]This patient also showed a synchronous MSI-H tumor in the cecum that was positive for *BRAF-V600E* and showed *MLH1* hypermethylation. [d]This tumor is left-sided, although its exact location is unknown. Tumors were obtained from the University Hospital Groningen (Groningen, The Netherlands), Sapporo Medical University (Sapporo, Japan) and also from several different hospitals in Finland. Sample collection was carried out in accordance with the previously established ethical protocols from each one of the participating institutions, and the respective ethics committees approved the study. Genomic DNA was extracted with phenol-chloroform according to standard procedures. Microsatellite instability was analysed according to the international criteria for the determination of microsatellite instability, using various panels of dinucleotide and mononucleotide repeat sequences as described previously. (Boland *et al*., 1998). Accordingly, tumors were classified as MSI-H or MSI-low (MSI-L) when showing high or low levels of instability, respectively. The analysis of *BRAF* was performed by automatic sequencing. The fragment encompassing exon 15 was amplified by PCR in all carcinoma samples. Primer sequences and PCR conditions were based on those reported previously (Davies *et al*., 2002). Genomic DNA (25–100 ng) was amplified by PCR using the following cycling conditions: 30 s at 94°C, 30 s at 60°C and 45 s at 72°C for 35 cycles. PCR products were purified and sequenced on an ABI Prism 377 Automatic sequencer (Perkin-Elmer, Foster City, CA, USA) using the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). Two-sided Fisher's exact test was used for statistical analysis. We have previously published 40% (82/206) of *BRAF-V600E* mutations in sporadic colorectal MSI tumors (Domingo *et al*., 2004b). According to this, our data suggest that *BRAF* mutations do not associate with HNPCC cases with germline *MSH6* mutations nor with HNPCC cases negative for *MLH1, MSH2* or *MSH6* mutations ($P<0.001$)

approximately 5% of cases show germline mutations in the *MSH6* gene (Miyaki *et al*., 1997; Berends *et al*., 2002) and about half of all families clinically defined as HNPCC (Rodriguez-Bigas *et al*., 1997; Vasen *et al*., 1999) do not have mutations in any of the known MMR genes, and for these cases it is yet not known whether *BRAF-V600E* might play a role in tumorigenesis. Here we report data that support the hypothesis that *BRAF* is not involved in the tumorigenesis of the HNPCC-related tumors considering HNPCC as diagnosed on both

molecular, by the presence of a germline mutation in a MMR gene, or on clinical grounds, fulfilling the revised Amsterdam criteria. Our data suggest a potential use of *BRAF-V600E* as the exclusion criterion for HNPCC or as a molecular marker of sporadic cancer.

We have analysed 38 tumors from two different subsets of HNPCC (suspected) patients: those that harbor an *MSH6* germline defect, and patients from families fulfilling the clinical criteria but in whom no *MLH1, MSH2* or *MSH6* germline mutations could be identified (see Table 1). All patients belong to different families. As shown in Table 1, the analysed cases either fulfilled the Amsterdam criteria or were positive for specific criteria from the Bethesda guidelines (HNPCC-like) (Vasen *et al*., 1991; Rodriguez-Bigas *et al*., 1997; Umar *et al*., 2004). The indexed patients of these HNPCC-like families have early-onset colorectal cancer and at least a first-degree relative with a HNPCC-related tumor (Umar *et al*., 2004).

We have recently suggested the introduction of the *BRAF-V600E* mutation screening in the molecular diagnostic protocols for HNPCC as a low-cost effective strategy that allows simplifying the genetic testing of HNPCC patients (Domingo *et al*., 2004b). The reported absence of V600E mutations in HNPCC cases that harbor germline mutations in the *MLH1* and *MSH2* genes (Wang *et al*., 2003; Deng *et al*., 2004; Domingo *et al*., 2004b) predicted a potential use of *BRAF* as a pre-screening tool in HNPCC as only *BRAF-V600E*-negative cases need to be screened for mutations in the MMR genes *MLH1* and *MSH2*. Further, it suggests that *BRAF* mutations are not involved in HNPCC tumorigenesis, but are restricted to the sporadic cases. However, it is unknown yet whether *BRAF* could be involved in HNPCC tumors without germline mutations in *MLH1* and *MSH2*. In fact, almost half of the HNPCC families show no mutations in the MMR genes (Rodriguez-Bigas *et al*., 1997; Vasen *et al*., 1999) and about 5% of cases are due to germline mutations of the *MSH6* gene (Miyaki *et al*., 1997; Wijnen *et al*., 1999; Berends *et al*., 2002).

To answer these questions, *BRAF-V600E* mutations were screened in 12 colon tumors derived from patients from HNPCC families that fulfilled the Amsterdam criteria but did not show germline mutations in *MLH1, MSH2* or *MSH6*. In none of these tumors, the *BRAF-V600E* mutations were identified. In all, 10 of these tumors were classified as MSI-H and two as MSI-L, according to the international Bethesda criteria (Boland *et al*., 1998). An additional MSI-H tumor from the duodenum of an HNPCC family positive for Amsterdam criteria was also found negative for *BRAF* mutations. Furthermore, no mutations were detected in 10 colorectal tumors, eight MSI-H and two MSI-L, respectively, from suspected HNPCC families not fulfilling the Amsterdam criteria, but being positive for clinical criteria suggestive of familial cancer. Also, we have extended our analysis to colon tumors, five MSI-H and five MSI-L, from 10 HNPCC(like) families showing germline mutations in *MSH6*. Of these families, six proved to have truncating and four missense mutations of this gene (Table 1). Regarding these families, one was positive for Amsterdam criteria and nine were suspected HNPCC, according to the clinical criteria above described. We did not detect *BRAF-V600E* mutations in these cases. Two additional tumors from the endometrium and pyelum from two patients of these families, as well as two extracolonic tumors from the duodenum and endometrium from two additional families showing germline truncating mutations in *MSH6*, were also negative for *BRAF* mutations (Table 1).

Interestingly, in one of the analysed patients with a truncating *MSH6* germline mutation, a synchronous MSI-H colorectal cancer in the cecum was found positive for the *BRAF-V600E* mutation. This tumor, however, showed an absence of MLH1 by immunohistochemistry (IH) and hypermethylation of the *MLH1* promoter. This finding is best explained by the significant reported association of *BRAF-V600E* with the hypermethylation of *MLH1* as seen in a high frequency of sporadic tumors (Deng *et al*., 2004; Domingo *et al*., 2004a), a phenomenon not directly linked to the germline mutation of *MSH6*.

Overall, we did not detect significant *BRAF-V600E* mutations in any of these HNPCC subsets, reinforcing the idea that *BRAF* is not involved in HNPCC tumorigenesis, independently of the MMR gene defect and independent of the presence of high or low MSI phenotypes. These results suggest a potential use of the *BRAF* mutation as a marker of sporadic colorectal cancer.

Therefore, we suggest that detection of a positive *BRAF-V600E* mutation in a colorectal cancer is most likely suggesting a sporadic origin of the disease and the absence of germline alterations of *MLH1, MSH2* and also of *MSH6*. Further, these findings might also have a potential impact in the gene testing for HNPCC diagnostics.

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