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KRAS and *BRAF* oncogenic mutations in MSS colorectal carcinoma progression

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In sporadic colorectal cancer (CRC), *KRAS* are alternative to *BRAF* mutations and occur, respectively, in 30 and 10% of cases. Few reports addressed the association between *KRAS–BRAF* mutations and tumour progression specifically in sporadic microsatellite-stable (MSS) CRC. We screened *KRAS* and *BRAF* in 250 MSS primary CRC and 45 lymph node (LN) metastases and analysed the pathological features of the cases to understand the involvement of *KRAS–BRAF* activation in progression and metastasis. Forty-five per cent of primary MSS CRCs carried mutations in at least one of these genes and mutations were associated with wall invasion ($P = 0.02$), presence and number of LN metastases ($P = 0.02$ and $P = 0.03$, respectively), distant metastases ($P = 0.004$) and advanced stage ($P = 0.01$). We demonstrated that *KRAS* and *BRAF* are alternative events in Tis and T1 MSS CRC and, *KRAS* rather than *BRAF* mutations, contributed to the progression of MSS CRC. The frequency of *KRAS* and/or *BRAF* mutations was higher in LN metastases than in primary carcinomas ($P = 0.0002$). Mutated LN metastases displayed *KRAS* associated or not with *BRAF* mutations. *BRAF* mutations were never present as a single event. Concomitant *KRAS* and *BRAF* mutations increased along progression of MSS CRCs, suggesting that activation of both genes is likely to harbour a synergistic effect.

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KRAS and BRAF are members of the MAP kinase (MAPK) pathway, which is hyperactivated in approximately 30% of all cancers (Hoshino *et al*., 1999). The identification of mutationally activated *KRAS* and *BRAF* alleles in several tumour models supports the importance of this signalling pathway in cancer progression (Davies *et al*., 2002; Rajagopalan *et al*., 2002). The RAS/RAF/MAPK pathway regulates cell proliferation, differentiation, senescence and apoptosis. In addition, several reports have shown that MAPK activation, owing to oncogenic *RAS* and *BRAF* mutations, is likely to be involved in promoting cellular invasiveness in different tumour models (Fugimoto *et al*., 2001; Sumimoto *et al*., 2004; Melillo *et al*., 2005). Moreover, it has been shown that G12V RAS mutation has a 50-fold higher transforming and oncogenic activity in NIH3T3 cells than V600E mutation of *BRAF*. By itself, BRAF V600E mutation shows a 138-fold transforming and oncogenic activity over wild-type BRAF (Davies *et al*., 2002).

In sporadic colorectal cancer (CRC), oncogenic mutations affecting *KRAS* and *BRAF* occur in about 30 and 10% of the cases, respectively (Rajagopalan *et al*., 2002; Yuen *et al*., 2002; Brink *et al*., 2003; Wang *et al*., 2003; Oliveira *et al*., 2003, 2004, 2005). *KRAS* mutations have been observed in colorectal tumours independently of their microsatellite instability (MSI) status. In sporadic MSI CRCs, *KRAS* mutations are inversely associated to the oncogenic $BRAF^{V600E}$ mutation, the latter occurring in about 40% of the cases (Rajagopalan *et al*., 2002; Yuen *et al*., 2002; Lipton *et al*., 2003; Oliveira *et al*., 2003; Wang *et al*., 2003; Domingo *et al*., 2004; Fransen *et al*., 2004; Koinuma *et al*., 2004), suggesting that each mutation can induce similar cellular effects and signal through the same pathway. However, the recent report by Solit *et al*. (2006), using MEK (a downstream effector of *KRAS* and *BRAF*) inhibitors showed that *BRAF* mutant cell lines responded differently than *KRAS* mutant ones, raising the possibility that *KRAS* and *BRAF* mutant cancer cells might be differentially dependent on signalling mechanisms that involve MEK.

Although *BRAF* mutations have been observed mainly in sporadic MSI CRC tumours, approximately 5% of microsatellite stable (MSS) CRC cases also show mutations within *BRAF* gene (Rajagopalan *et al*., 2002; Yuen *et al*., 2002; Lipton *et al*., 2003; Oliveira *et al*., 2003, 2005; Wang *et al*., 2003; Fransen *et al*., 2004;

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Koinuma *et al*., 2004). In contrast to sporadic MSI CRC, data on the presence of both *KRAS* and *BRAF* oncogenic mutations in sporadic MSS CRC and their relationship with tumour progression are scarce.

In order to understand the putative involvement of alterations in these two genes in the progression of MSS sporadic colorectal carcinoma, we screened *KRAS* and *BRAF* mutations in a series of 250 MSS CRCs and 45 lymph node (LN) metastases (from 28 distinct cases), and studied the pathological features of these cases.

We found mutations in at least one of the genes (*KRAS–BRAF*) in 45.2% (113/250) primary MSS CRCs, which is in accordance with what has been described previously (*KRAS–BRAF*: 30–50%; *KRAS*: 27–45%; *BRAF*: 2–6%) (Oliveira *et al*., 2003; Deng *et al*., 2004; Fransen *et al*., 2004; Nagasaka *et al*., 2004; Ince *et al*., 2005; Lubomierski *et al*., 2005; Samowitz *et al*., 2005; Velho *et al*., 2005).

We studied the association between pathological parameters of MSS CRCs and the presence of *KRAS–BRAF* oncogenic mutations (Table 1).

We observed an association between the presence of *KRAS–BRAF* mutations and wall invasion ($P = 0.02$). We found that Tis and T1 MSS CRC had either single *KRAS* or *BRAF* mutations and never displayed concomitant mutations (Figure 1a). It was previously shown that *KRAS* activation occurs in the first steps of colorectal carcinoma progression, along the adenoma–carcinoma sequence (Vogelstein *et al*., 1988; Fearon and Vogelstein, 1990). According to the literature, *BRAF* mutations were more frequently found in premalignant colon polyps and early rather than in advanced colorectal carcinomas (Rajagopalan *et al*., 2002; Yuen *et al*., 2002; Ikehara *et al*., 2005). Similar observations have been made in other tumour models, namely activating *BRAF* mutations have been detected in a high proportion of naevi and benign melanocytic skin lesions (Pollock *et al*., 2003; Yazdi *et al*., 2003), although in this specific model, activating mutations of *BRAF* have also been identified in approximately 90% of melanomas (Davies *et al*., 2002; Kumar *et al*., 2003). Our results confirm that *KRAS* and *BRAF* mutations alone are frequent and alternative in CRCs with no extension through the muscularis propria (Tis and T1), as previously demonstrated for MSI CRC (Rajagopalan *et al*., 2002; Oliveira *et al*., 2003). Presumably, in these tumour stages, *BRAF* mutations do not occur concomitantly with *KRAS* mutations because their combined signalling is incompatible with proliferation, as an excess of extracellular signal-regulated protein kinase (ERK) signalling could lead cells to stop cycling and differentiate or to entry senescence (Marshall, 1995; Sewing *et al*., 1997; Woods *et al*., 1997; Kerkhoff and Rapp, 1998).

The frequency of concomitant *KRAS* and *BRAF* mutations increased along with the depth of wall invasion: T2 – 2.8% (1/36), T3 – 3.5% (6/173) and T4 – 9.4% (3/32).

In T2, T3 and T4 CRC, the frequency of *KRAS* mutations increase either owing to the acquisition of *KRAS* mutations in *BRAF*-negative CRC or to the accumulation of *KRAS* and *BRAF* mutations. *KRAS* activation is likely to confer tumour cells a more invasive behaviour. This relationship between the presence of *KRAS* mutations and increased ability of tumour cells to invade and progress through the wall may be explained by the putative capability of mutant *KRAS* to (i) disrupt epithelial cell polarity both by destabilizing adherens junctions and by remodelling cell–matrix interactions through the modulation of

Table 1 Analysis of pathological features of MSS sporadic CRC in stratified groups of tumours with *KRAS* mutations alone, *BRAF* mutations alone and with concomitant mutations in *KRAS* and *BRAF*

Pathological features	*Total* n = *250*	*KRAS mut* n = *93*	*BRAF mut* n = *10*	*KRAS + BRAF mut* n = *10*	*KRAS + BRAF wild-type* n = *137*	P-*value*
Primary tumour (T)	*n* = 250	93	10	10	137	0.02
Tis	2	2 (100%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	—
T1	7	3 (42.9%)	2 (28.6%)	0 (0.0%)	2 (28.6%)	—
T2	36	14 (38.9%)	0 (0.0%)	1 (2.8%)	21 (58.3%)	—
T3	173	58 (33.5%)	7 (4.0%)	6 (3.5%)	102 (59.0%)	—
T4	32	16 (50%)	1 (3.1%)	3 (9.4%)	12 (37.5%)	—
Lymph nodes	*n* = 250	93	10	10	137	0.02
Absent	132	40 (30.3%)	5 (3.8%)	3 (2.3%)	84 (63.6%)	—
Present	118	53 (44.9%)	5 (4.2%)	7 (5.9%)	53 (44.9%)	—
Regional lymph nodes (N)	*n* = 250	93	10	10	137	0.03
N0	132	40 (30.3%)	5 (3.8%)	3 (2.3%)	84 (63.6%)	—
N1	58	21 (36.2%)	3 (5.2%)	3 (5.2%)	31 (53.4%)	—
N2	60	32 (53.3%)	2 (3.3%)	4 (6.7%)	22 (36.7%)	—
Distant metastases (M)	*n* = 192	70	6	1	115	0.004
Absent	152	48 (31.6%)	4 (2.6%)	0 (0%)	100 (65.8%)	—
Present	40	22 (55.0%)	2 (5.0%)	1 (2.5%)	15 (37.5%)	—
TNM staging	*n* = 250	93	10	10	137	0.01
0 + I + II	129	38 (29.5%)	5 (3.9%)	3 (2.3%)	83 (64.3%)	—
III + IV	121	55 (45.5%)	5 (4.1%)	7 (5.8%)	54 (44.6%)	—

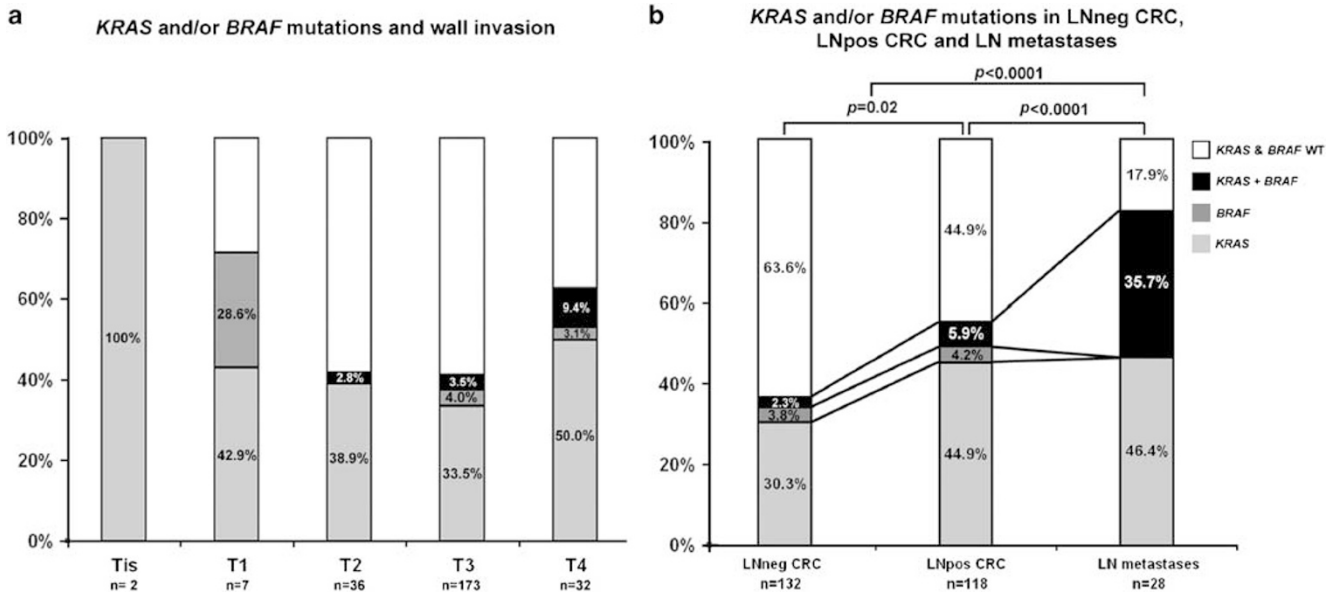

Figure 1 *KRAS–BRAF* mutation frequency in MSS CRCs. Tumours were obtained from the Hospital of S João (Porto, Portugal), the Centre d'Investigacions en Bioquimica i Biologia Molecular Vall d'Hebron (CIBBIM), (Barcelona, Spain) and from the Saint-Antoine Hospital (Paris, France). The criteria used to select the patient were as follows: (1) none of the patients had a positive family history of cancer and (2) all sporadic CRCs and metastases were MSS. Only MSS CRC carcinomas analysed according to Umar *et al*. (2004) were selected for this study. Sample collection was carried out in accordance with previously established ethical protocols. Frozen material or paraffin embedded tissue from 250 primary colorectal carcinomas and 28 LN metastases (one LN metastasis per case) were analysed. In five cases, several LN metastases were analysed (two additional LN metastases in two cases; four additional LN metastases in two cases and five additional LN metastases in another case). Hematoxylin and eosin (HE)-stained sections were used to classify all tumours and allowed their macrodissection. High molecular weight DNA was isolated using standard methods from total sections of the tumours, whenever tumour cells occupied more than 50% of tumour tissue or from macrodissected areas with at least 50% of tumour cells. Analysis of specific hotspot mutations in *KRAS* exon 1 and *BRAF* exon 15 was performed in 295 primary CRC and LN metastases. Pre-screening of *BRAF* and *KRAS* was either performed by PCR-SSCP, PCR-DGGE or direct sequencing depending on the method of analysis in each collaborative centre (Oliveira *et al*., 2004). The statistical analysis was performed using the Student's t test, χ^2 test or Fisher's statistical test when appropriated. Differences were taken to be significant at $P<0.05$. (**a**) Distribution of *KRAS* and/or *BRAF* mutation according to the wall invasion of MSS primary CRCs. The histopathological classification of each specimen was studied by HE-stained tissue sections by board-certified pathologist from each of the Institutions participating in this study. Tumor-node-metastasis system was used for tumour staging: stage 0 represents carcinoma *in situ*, stage I represents tumours that invades the submucosa or the muscularis propria, stage II represents tumours that invade through the muscularis propria into the subserosa or non-peritonealized pericolic or perirectal tissues, or tumours that perforate the visceral peritonenum or directly invade other organs or structures, stage III represents any tumour with regional LN metastases and stage IV represents tumour with confirmed distant metastases. (**b**) *KRAS* and/or *BRAF* mutation frequency in MSS CRC LNneg, MSS CRC LNpos and LN metastasis.

integrin expression, maturation and activity (Hughes *et al*., 1997; Yan *et al*., 1997; Schramm *et al*., 2000), (ii) promote the passage of tumour cells through the epithelial basement membrane by stimulating the expression and/or activation of MMPs and (iii) increase cell motility in stromal tissue through the activation of RHO family of small-GTPases (Yamamoto *et al*., 1995; Thiery, 2002; Liao *et al*., 2003). The number of cases with concomitant *KRAS* and *BRAF* mutations also increases in advanced carcinomas, suggesting that activation of both genes may cooperate in tumour progression. As for *BRAF* alone, as its activation is more prominent in early stages rather than in advanced stages of MSS CRC, we can hypothesize that *BRAF* activation alone is not sufficient to induce cancer progression in a high frequency of MSS CRC. Its activation in few advanced cases suggests that, in these CRCs, *BRAF* activation may contribute to tumour progression by protecting cells from apoptosis as suggested by Hingorani *et al*. (2003) and Ikehara *et al*. (2005).

A significant association was found between the presence of LN metastases and *KRAS–BRAF* mutations ($P=0.02$) (Table 1 and Figure 1b). The frequency of *KRAS* mutations alone was higher in MSS CRC LNpos (44.9% – 53/118) as compared to MSS CRC LNneg (30.3% – 40/132). The number of carcinomas with *BRAF* mutations alone was low and similar in MSS CRC LNneg (3.8% – 5/132) and MSS CRC LNpos (4.2% – 5/118). When comparing the frequency of cases with concomitant *KRAS* and *BRAF* mutations in MSS CRC LNneg (2.3% – 3/132) and MSS CRC LNpos (5.9% – 7/118), we verified that the latter showed a 2.6-fold increase in the frequency of cases with both mutations, suggesting that concomitant activation of *BRAF* and *KRAS* may have a synergistic effect in promoting LN metastasis.

The association between the presence of LN metastases and increased mutation frequency was also verified concerning the number of nodes affected ($P=0.03$). The frequency of concomitant mutations of *KRAS* and *BRAF* was higher in N1 (5.2%) and N2 (6.7%) when compared with N0 (2.3%) carcinomas.

Significant associations were also found between the presence of *KRAS*/*BRAF* mutations and positivity for distant metastases ($P = 0.004$), owing to an increased frequency of *KRAS* mutations in cases with distant metastases. These two observations relate *KRAS* mutations with colorectal tumour invasion and suggest that *KRAS* activation is likely to be crucial to render tumour cells the ability of moving and invading not only LNs but also distant organs (Vogelstein *et al*., 1988; Fearon and Vogelstein, 1990; Pretlow, 1995; Pollock *et al*., 2005). In contrast to what has been observed for *KRAS*, the frequency of *BRAF* mutations alone was not different in primary MSS CRC without and with distant metastases.

The frequency of mutated cases (either *KRAS* or *BRAF* or both *KRAS* and *BRAF* mutations) in LN metastases (82.1% 23/28) was higher in comparison to primary carcinomas ($P = 0.0002$). The detailed analysis of LN metastases showed that all mutated metastases showed *KRAS* mutations. Our results suggest that the majority of MSS carcinomas need *KRAS* activation, through mutation, to be able to metastasize and this activation is crucial for neoplastic cells to acquire invasive potential (Schmidt-Kittler *et al*., 2003; Campbell and Der, 2004; Carter *et al*., 2004). In contrast, none of the mutated LN metastasis had *BRAF* mutations as a single event (Figure 2). These results emphasize the role of *KRAS* activation in metastasis and argues against *BRAF* activation by itself, as a pivotal genetic event in promoting MSS CRC metastasis.

	Tumor		LN metastases	
ID	*KRAS*	*BRAF*	*KRAS*	*BRAF*
1	G12D	V600E	G12D	V600E
2	G12D	V600E	G12D	V600E
3	G12A	V600E	G12A	V600E
4	G12D	K601E	G12D	K601E
5	G12D	K601Q	G12D	K601Q
6	G12D		G12D	
7	G12D		G12D	
8	G12D		G12D	
9	G12D		G12D	
10	G12D		G12D	
11	G12D		G12D	
12	G12D		G12D	
13	G12D		G12D	
14	G12V		G12V	
15	G12S		G12S	
16	G12D		G12D	V600E
17		V600E	G12D	V600E
18		V600E	G12D	V600E
19			G12D	K601Q
20			G12D	D594K
21			G13D	
22			G13D	
23			G12D	
24	G12S			
25	G13D			
26				
27				
28				

Figure 2 Description of *KRAS* and *BRAF* oncogenic mutations in a series of cases with available LN metastasis and matched primary CRC. ■, Multiple metastases analysed.

As 10 of 28 (35.7%) LN metastases harbour concomitant *KRAS* and *BRAF* mutations, we can assume that 'the lethality of this combination' in Tis and T1 carcinomas can be suppressed by dominant survival factors or subsequent oncogenic activations in more advanced cancers.

In 43.4% (10/23) of the mutated LN metastasis mutations of both genes were detected, reinforcing the idea that activation of both genes is likely to play a synergistic role in LN metastasis. This observation is supported by the results of Solit *et al*. (2006) which showed that *KRAS* and *BRAF* may signal through different signalling pathways and whereas *BRAF* mutant cells are preferentially reliant on MEK–ERK signalling, *KRAS* mutant cells have multiple other targets, such as phosphatidylinositol 3′-kinase (PI(3)K) and RalGDS, reducing the requirement for MEK–ERK activation.

In five of 10 LN metastases with concomitant *KRAS* and *BRAF* mutations, a similar picture was observed in respective primary tumours; in the remaining five cases, the mutation pattern in LN metastases was different from primary tumours: one case acquired a *BRAF* mutation (16), two acquired *KRAS* mutations (17,18), and two acquired mutations in both genes (19,20) (Figure 2).

In five LN metastases (cases 24–28), no *KRAS* or *BRAF* mutations were identified, suggesting that alternative pathways are also responsible for colon cancer metastasis (Figure 1b and 2). In cases 24 and 25, *KRAS* mutations were identified in the corresponding primary tumours. Extra LN metastases for these two cases (three for case 24 and four for case 25) were analysed and found to be also wild type for both *KRAS* and *BRAF* in accordance with what was found in the first ones. This observation suggests that populations of carcinoma cells heterogeneous with respect to wild-type and mutant *KRAS* were probably present in the primary carcinoma, but the metastatic clone derived from a *KRAS* negative population, as reported previously (Al-Mulla, 1998).

In cases 6, 13 and 22, we analysed multiple metastases per case. Within each case, the same pattern of mutations was observed in the different LNs analysed. In cases 6 and 13, we demonstrated that all independent metastases, as well as the primary tumours, displayed a G12D *KRAS* mutation demonstrating that a mutation in *KRAS* occurred in the primary tumour and was maintained in all metastases. In case 22, the primary tumour did not display a *KRAS* mutation, but the study of five LN metastases showed that all harboured the same G13D *KRAS* mutation, suggesting that this alteration was acquired before LN invasion and was pivotal for metastasis. Our data is in accordance to Bernards and Weinberg (2002), who suggested that important components of the genotype of metastasis are already implanted early in tumorigenesis, in small primary tumour cells populations that have the ability to dispatch metastatic pioneers to distant sites in the body.

In the present series, we were unable to detect a specific profile of *KRAS* and *BRAF* mutations, namely

codon affected (*KRAS* codons 12 and 13; and *BRAF* codons 600 and 601) nor amino-acid change associated with tumour progression or metastasis, although specific *KRAS* mutations have been previously correlated with more aggressive tumour phenotypes (Finkelstein *et al*., 1993; Moerkerk *et al*., 1994; Span *et al*., 1996; Andreyev *et al*., 2001).

Overall, the results obtained in the present study are supported by the report of Ince *et al*. (2005), which show additional evidence that *KRAS* and *BRAF* mutations are related to disease severity and bad prognosis in CRC patients and demonstrate very elegantly that patients with CRC displaying either *KRAS* (35%) or *BRAF* (5.6%) or both *KRAS* and *BRAF* (0.4%) mutations had worse prognosis, shorter median survival and shorter overall survival than those with wild-type *KRAS* and *BRAF* genotype.

The main conclusions of our work are (1) *BRAF* and *KRAS* mutations are alternative in Tis and T1 MSS CRCs, (2) During MSS CRC progression and metastasis concomitant mutations at *KRAS* and *BRAF* increase, suggesting that activation of both genes is likely to harbour a synergistic effect, (3) None of the metastases harbour *BRAF* mutations alone, suggesting that *KRAS* is the pivotal gene in this process.

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