Somatic-genetic aberrations, specific protein levels and their prognostic value in colon cancer

Westra, Jantine Lolkje

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CHAPTER 4

Distinct patterns of KRAS mutations in colorectal carcinomas according to germline Mismatch Repair defects and hMLH1 methylation status

Carla Oliveira1, Jantine L Westra2, Diego Arango3, Miina Ollikainen4, Enric Domingo5, Ana Ferreira1, Sérica Velho1, Renee Nijssen1, Kristina Lagerstedt6, Pia Alhopuro7, Paivi Laiho7, Isabel Veiga7, Manuel R Teixeira1, Marjolijn Ligtenberg8, Jan H Kleibeuker9, Rolf H Sijmons2, John T Plukker10, Kohzoh Imai11, Pedro Lage12, Richard Hamelin13, Cristina Albuquerque14, Simo Schwartz Jr.5, Annika Lindblom7, Päivi Peltomaki8, Hiroyuki Yamamoto9, Lauri A Aaltonen3, Raquel Seruca1, Robert MW Hofstra2

1. Institute of Molecular Pathology and Immunology of the University of Porto, IPATIMUP, Portugal
2. Department of Medical Genetics, University of Groningen, The Netherlands
3. Department of Medical Genetics, Haartman Institute, University of Helsinki, Finland
4. Department of Medical Genetics, Biomedicum Helsinki, University of Helsinki, Finland
5. Centre d’Investigacions en Bioquimica i Biologia Molecular (CIBBIM), Hospital Universitari Vall d’Hebron, Barcelona, Spain
6. Department Clinical Genetics, Karolinska University Hospital, Stockholm, Sweden
7. Department of Genetics, Portuguese Institute of Oncology (IPO), Porto, Portugal
8. Department of Human Genetics, UMC Nijmegen, The Netherlands
9. Department of Gastroenterology, University Hospital Groningen, The Netherlands
10. Department of Surgery, University Hospital Groningen, The Netherlands
11. First Department of Internal Medicine, Sapporo Medical University, Japan
12. Serviço de Gastroenterologia, Instituto Portugues de Oncologia Francisco Gentil, Lisbon, Portugal
13. INSERM U434 CEPHI, Paris, France
14. Centro de Investigacao de Patobiologia Molecular-CIPM, Instituto Portugues de Oncologia Francisco Gentil, Lisbon, Portugal

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ABSTRACT

In sporadic colorectal tumours the \textit{BRAF} V600E is associated with microsatellite instability (MSI-H) and inversely associated to \textit{KRAS} mutations. Tumours from HNPCC patients carrying germline mutations in h\textit{MSH2} or h\textit{MLH1}, do not show \textit{BRAF}V600E, however no consistent data exists regarding \textit{KRAS} mutation frequency and spectrum in HNPCC tumours. We investigated \textit{KRAS} in 158 HNPCC tumours from patients with germline h\textit{MLH1}, h\textit{MSH2} or h\textit{MSH6} mutations, 166 MSI-H and 688 MSS sporadic carcinomas. All tumours were characterised for MSI and 81 of 166 sporadic MSI-H CRCs were analysed for h\textit{MLH1} promoter hypermethylation. \textit{KRAS} mutations were observed in 40% of HNPCC tumours, and the mutation frequency varied upon the MMR gene affected: 48\% (29/61) in h\textit{MSH2}, 32\% (29/91) in h\textit{MLH1} and 83\% (5/6) in h\textit{MSH6} (P=0.01). \textit{KRAS} mutation frequency was different between HNPCC, MSS and MSI-H CRCs (P=0.002), and MSI-H with h\textit{MLH1} hypermethylation (P=0.005). Furthermore, HNPCC CRCs had more G13D mutations than MSS (P<0.0001), MSI-H (P=0.02) or MSI-H tumours with h\textit{MLH1} hypermethylation (P=0.03). HNPCC colorectal and sporadic MSI-H tumours without h\textit{MLH1} hypermethylation shared similar \textit{KRAS} mutation frequency, in particular G13D. In conclusion, we show that depending on the genetic/epigenetic mechanism leading to MSI-H, the outcome in terms of oncogenic activation may be different, reinforcing the idea that HNPCC, sporadic MSI-H (depending on the h\textit{MLH1} status) and MSS CRCs, may target distinct kinases within the RAS/RAF/MAPK pathway.

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) accounts for approximately 1-8\% of total colorectal cancer (CRC) burden based on clinical criteria (Aaltonen et al., 1994; Houlston et al., 1992; Lynch and de la Chapelle, 2003; Umar et al., 2004). When considering HNPCC as a syndrome linked to mismatch repair (MMR) mutations, the frequency range from 0.3\% - 3\% of the total colorectal cancer burden (Aaltonen et al., 1998; Salovaara et al., 2000; Cunningham et al., 2001). Germline mutations in a number of MMR genes have been identified in patients with HNPCC. To date, germline mutations in the h\textit{MLH1} and h\textit{MSH2} genes account for about 90\% of all identified MMR gene mutations. h\textit{MSH6} mutations are also found but the number of cases is considerably lower (10\%) when compared to h\textit{MLH1} (~50\%) and h\textit{MSH2} (~40\%) (Umar et al., 2004; Peltomaki et al., 2001). In particular tumours of patients with germline h\textit{MLH1} or h\textit{MSH2} mutations frequently show microsatellite instability (MSI-H) (Aaltonen et al., 1998; Liu et al., 1996; Narayan et al., 2003; Peltomaki et al., 2003). (International Collaborative group on HNPCC Web site, available at http://www.nfdht.nl). The MSI-H phenotype is also found in approximately 15\% of sporadic colorectal carcinoma. In contrast to the CRCs from HNPCC patients, the MMR deficiency seen in these tumours is not caused by (germline) mutations in the MMR genes but is due to h\textit{MLH1} promoter hypermethylation resulting in loss of the MLH1 protein (Umar et al., 2004). In MSI-H colorectal tumours, both sporadic and inherited forms, hundreds of thousands of mutations accumulate within repetitive sequences throughout the genome (Ionov et al., 1993). These mutations occur not
KRAS mutations in hereditary (HNPCC) and sporadic (MSI-H and MSS) CRCs

only in non-coding but also in coding sequences. As a consequence, genes involved in several signalling pathways, such as the WNT/APC/beta-catenin pathway and the TGFbeta pathway, are also subjected to mutation. The genes in these pathways encode proteins, which play a key role in the development of either mismatch-repair deficient and proficient CRCs (microsatellite stable (MSS) CRCs) (Duval et al., 2002).

Although genes with repetitive sequences are clear targets in tumours with a defective mismatch repair system, mutations in non-repetitive sequences are also found in MSI-H tumours. An example of this is the occurrence of the V600E mutation in BRAF (Davies et al., 2002; Rajagopalan et al., 2002). BRAF is a member of the RAS/RAF/MAPK pathway, a crucial pathway in colorectal tumourigenesis (Kinzler and Vogelstein, 1996; Fearon and Vogelstein, 1990). The BRAF V600E has been described in approximately 35% of sporadic MSI-H CRCs (Rajagopalan et al., 2002; Yuen et al., 2002; Oliveira et al., 2003; Koinuma et al., 2004; Wang et al., 2003; Lipton et al., 2003; Fransen et al., 2004) and was found associated with hMLH1 promoter hypermethylation (Koinuma et al., 2004; Wang et al., 2003; Domingo et al., 2004; Deng et al., 2004). BRAFV600E is however, not restricted to MSI-H tumours as it also occurs in approximately 6% of CRCs without MMR deficiency (microsatellite stable (MSS) CRCs) (Rajagopalan et al., 2002; Yuen et al., 2002; Oliveira et al., 2003; Koinuma et al., 2004; Wang et al., 2003; Lipton et al., 2003; Fransen et al., 2004). KRAS, another member of this signalling pathway is mutated in approximately 20% of sporadic MSI-H and 35% of MSS CRCs negative for the BRAF V600E mutation (Rajagopalan et al., 2002; Oliveira et al., 2003; Koinuma et al., 2004; Fransen et al., 2004; Fujiiwara et al., 1998; Konishi et al., 1996). These data demonstrate that point mutations in non-repetitive sequences occur frequently in both mismatch proficient and deficient tumours, but also that this pathway plays a pivotal role in the tumourigenesis of both types of sporadic CRCs. Furthermore, it was shown that mutations in both KRAS and BRAF in the same tumour-sample are rarely found suggesting that the oncogenic capabilities of both genes are mutually exclusive in terms of clonal selection (Rajagopalan et al., 2002; Oliveira et al., 2003; Koinuma et al., 2004; Fransen et al., 2004; Fujiiwara et al., 1998; Konishi et al., 1996; Wynter et al., 2004). Recently, it was found that tumours from HNPCC patients with germline mutations in hMSH2 or hMLH1 do not show BRAFV600E (Wang et al., 2003; Deng et al., 2004; Domingo et al., 2004). Whether the absence of the BRAFV600E mutation in MSI-H CRC tumours from HNPCC patients is due to the presence of a KRAS mutation is not known as the occurrence of KRAS mutations in HNPCC CRCs is not well established.

The frequency of KRAS mutations in HNPCC families, fulfilling the Amsterdam criteria, was first determined by Aaltonen and colleagues (Aaltonen et al., 1993), who found that KRAS mutations occur at similar frequency in familial and sporadic CRCs. The same findings were described latter in two other studies (11/37-30%; 2/8-25%) (Fujiiwara et al., 1998; Tamnergard et al., 1997). However other authors did not confirm this observation (4/23-17% in codon 12 (Lost et al., 1997); 2/20-10% in codon 12 (Young et al., 2001); and 1/20-5% (Konishi et al., 1996)). In most of these studies, however, small numbers of HNPCC carcinomas were included and, in most of them, no demonstration of a hereditary defect in HNPCC patients was made. Moreover, it is not clear whether a specific spectrum of KRAS mutations might characterise tumours according to the presence of a germline
underlying MMR defect and to the presence or absence of hMLH1 hypermethylation in sporadic MSI-H cases. In the present study, we therefore evaluated KRAS mutations in a large series of HNPCC colon carcinomas of patients which were previously characterised for germline mutations in MMR genes and a series of sporadic CRCs characterised for the MSI status and hMLH1 promoter methylation. With this work, we aimed to verify whether a specific spectrum of KRAS mutations might characterise tumours according to the presence of a germline underlying MMR defect and to the presence or absence of hMLH1 promoter hypermethylation in sporadic MSI-H cases. In addition, we compared the frequency of KRAS mutations with the frequency of BRAFV600E in HNPCC, MSI-H and MSS sporadic carcinomas (data from the literature and from our collaborative group (Rajagopalan et al., 2002; Yuen et al., 2002; Oliveira et al., 2003; Koinuma et al., 2004; Wang et al., 2003; Lipton et al., 2003; Fransen et al., 2004, Deng et al., 2004; Fujiwara et al., 1998; Konishi et al., 1996; Wynter et al., 2004; Domingo et al., 2004)) in order to clarify the importance of BRAF and KRAS activation in the development of all sets of colon carcinomas.

MATERIAL AND METHODS

Tumour specimens
Tumour DNA samples were obtained from 1012 colorectal carcinomas, originating from 158 HNPCC patients with pathogenic MMR germline mutations and 854 sporadic cases (MSI-H 166, MSS 688). A family history was obtained in every case and none of the patients reported in this study, as sporadic, had a family history suggestive of hereditary nonpolyposis colorectal cancer. Samples were obtained from: University Hospital, Groningen and University Medical Centre Nijmegen (The Netherlands), Hospital of S. João- Porto, IPO-Porto and IPO-Lisbon (Portugal), Saint-Antoine Hospital, Paris, (France), University of Helsinki, Helsinki (Finland), Sapporo Medical University, Sapporo (Japan), Karolinska University Hospital, Stockholm (Sweden), and from Hospital Universitari Vall d’Hebron, Barcelona (Spain). The study protocol was reviewed and approved by the appropriate Ethics Committees and tumour samples were obtained with informed consent. All HNPCC families selected for this study have been previously characterised for the presence of germline mismatch repair gene mutations and showed a germline mutation in hMSH2, hMSH6 or hMLH1. Only HNPCC patients, for whom the mutations were proven pathogenic, independently of the mutation type, were included. HNPCC families without germline mutations of hMSH2, hMSH6 or hMLH1 were not included in this study. Genomic DNA was isolated from macro-dissected frozen or paraffin-embedded tumour tissue using standard methods.

Microsatellite instability analysis
All colorectal tumours from HNPCC patients and sporadic cases were analysed for microsatellite instability according to the international criteria for the determination of microsatellite instability, using various panels of dinucleotide and mononucleotide repeat
sequences (Umar et al., 2004). Tumours were classified as MSI-H when two of the five standard markers (Brown et al., 1998) or when more than 30% of the total number of markers analysed showed instability, otherwise were classified as MSS (Umar et al., 2004). MSI-H sporadic carcinomas were not investigated for the presence of somatic MMR gene mutations.

**Mutation analysis of KRAS gene**

Mutational analysis of KRAS codons 12, 13 and 61 was performed by SSCP/DGGE and/or Sequencing. The KRAS mutation screening was done according to the methods of analysis in the different collaborative centres.

**Analysis of hMLH1 methylation**

The DNA methylation status of the hMLH1 promoter region was partly determined as described previously (Boland et al., 1998; Herman et al., 1998). Briefly, samples were analysed by methylation-specific PCR (MSP) on bisulfite treated DNA. The primers used for this analysis were; 5’- GAA GAG TGG ATA GTG ATT TTT AAT GT-3’ and 5’-ATC TCT TCA TCC CTC CCT AAA ACA-3’ for unmethylated hMLH1 and 5’-AGC GGA TAG CGA TTT TTA ACG C-3’ and 5’-TCT TCG TCC CTC CCT AAA ACG-3’ for methylated hMLH1 (Boland et al., 1998; Herman et al., 1998).

The other samples, all from IPATIMUP, were analysed according to previously described methods (Esteller et al., 1998). In short, samples were amplified after treatment with bisulfite, using primers flanking the CpG sites in hMLH1: 5’-GTT AGA TAT TTT AGT AGA GGT ATA GT-3’ and 5’-ACC TTC AAC CAA TCA CCT CAA TA-3’. PCR products were digested by BstUI (New England Biolabs). This enzyme cleaves only the CGCG sequence, which is not converted by bisulfite treatment when methylated.

**Statistical analysis**

The statistical analysis was performed using the \( \chi^2 \) test or Fisher statistical test when appropriated. Differences were taken to be significant at P<0.05.

**RESULTS**

**Frequency of KRAS mutations in HNPCC sporadic MSI-H and MSS colorectal cancers**

KRAS mutations in codons 12, 13 and 61 were determined in 1012 primary colorectal carcinomas (CRCs) including 158 tumours from HNPCC patients harbouring pathogenic germline mutations in MMR genes, 166 sporadic MSI-H and 688 sporadic MSS tumours. KRAS mutations were observed in 33% (335/1012) of all colorectal tumours. A higher frequency of KRAS mutations was detected in HNPCC tumours (40%) in comparison to sporadic CRCs (32%), although this difference did not reach statistical significance. However, when sporadic CRCs were divided according to the MSI status, KRAS mutations
were significantly more frequent in HNPCC tumours than in sporadic MSI-H CRCs (22%) (P=0.0006) but similar to MSS (34%) CRCs (P=0.17) (Table 1, Figure 1). Eighty-one of the 166 sporadic MSI-H CRCs screened for KRAS mutations were also analysed for hMLH1 promoter methylation. Fifty (62%) cases showed hMLH1 hypermethylation. hMLH1 hypermethylated tumours showed statistically significantly lower frequency of KRAS mutations when compared to tumours from HNPCC patients (P=0.005), but no differences were found when MSI-H tumours without promoter hypermethylation were compared with HNPCC CRCs (P=0.65) (Table 1, Figure 1).

<table>
<thead>
<tr>
<th>Table 1: KRAS mutations in HNPCC and sporadic MSI-H (MMR deficient) and sporadic MSS (MMR proficient) colorectal tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRCs</strong></td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>All types</td>
</tr>
<tr>
<td>HNPCC</td>
</tr>
<tr>
<td>Sporadic MSI-H</td>
</tr>
<tr>
<td>Sporadic MSS</td>
</tr>
<tr>
<td>MMR deficient</td>
</tr>
<tr>
<td>Sporadic MSI-H *</td>
</tr>
<tr>
<td>hMLH1met -</td>
</tr>
<tr>
<td>hMLH1met+</td>
</tr>
<tr>
<td>Germline mutations</td>
</tr>
<tr>
<td>hMSH2</td>
</tr>
<tr>
<td>hMSH6</td>
</tr>
<tr>
<td>hMLH1</td>
</tr>
</tbody>
</table>

* Total number of sporadic MSI-H CRCs analysed for the methylation status of hMLH1

From the 158 HNPCC patients with germline pathogenic alterations (single nucleotide or deletions) in one of the mismatch repair genes, 61 showed mutations in hMSH2 (39%), 6 in hMSH6 (4%) and 91 in hMLH1 (58%). Within HNPCC CRCs, the frequency of KRAS mutations varied depending on the MMR gene affected (P=0.01). CRCs from patients carrying germline mutations in hMSH2 or hMSH6 harboured a higher frequency of KRAS mutations in comparison to CRCs from patients carrying germline mutations in hMLH1 (P=0.02) (Table 1). No differences were found between the type of germline MMR gene defect and frequency of KRAS mutations in HNPCC tumours. Sixty-three from 158 HNPCC families harboured distinct MMR germline mutations. In these 63 families, no association was found between the type of germline defect and the frequency or type of KRAS mutation (Table 2). All the other families included in this study harboured 21
different germline MMR mutations, every germline mutation was found in at least two families. Within every group of families sharing a specific germline mutation, no association was found between the frequency and type of \( KRAS \) mutation. For instance, in the 27 families with the \( hMLH1 \) founder mutation 1 (genomic deletion of exon 16 and flanking introns) four different types of \( KRAS \) mutations (G12A, G12D, G12V and G13D) were found.

**Table 2: \( KRAS \) mutation frequency and amino acid change of HNPCC colorectal tumours from families carrying distinct MMR germline mutations**

<table>
<thead>
<tr>
<th>MMR gene</th>
<th>Type of germline mutation</th>
<th>( KRAS ) mutation (%)</th>
<th>Amino acid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>( hMSH2 )</td>
<td>Deletion</td>
<td>5/12 (42%)</td>
<td>5 G13D</td>
</tr>
<tr>
<td></td>
<td>Duplication</td>
<td>0/1 (0%)</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Nonsense</td>
<td>3/4 (75%)</td>
<td>1 G12D, 2 G13D</td>
</tr>
<tr>
<td></td>
<td>Splice-site</td>
<td>4/7 (57%)</td>
<td>2 G12D, 1 G12A, 1 G13D</td>
</tr>
<tr>
<td></td>
<td>Frameshift</td>
<td>2/6 (33%)</td>
<td>1 G12D, 1 G12V</td>
</tr>
<tr>
<td></td>
<td>Missense</td>
<td>4/8 (50%)</td>
<td>1 G12D, 2 G12A, 1 G12V</td>
</tr>
<tr>
<td>( hMLH1 )</td>
<td>Deletion</td>
<td>0/3 (0%)</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>Nonsense</td>
<td>1/4 (25%)</td>
<td>G13D</td>
</tr>
<tr>
<td></td>
<td>Splice-site</td>
<td>1/6 (17%)</td>
<td>G13D</td>
</tr>
<tr>
<td></td>
<td>Frameshift</td>
<td>1/6 (17%)</td>
<td>G13D</td>
</tr>
<tr>
<td></td>
<td>Missense</td>
<td>3/4 (75%)</td>
<td>G13D</td>
</tr>
<tr>
<td>( hMSH6 )</td>
<td>Nonsense</td>
<td>1/1 (100%)</td>
<td>G13D</td>
</tr>
<tr>
<td></td>
<td>Missense</td>
<td>1/1 (100%)</td>
<td>G12A</td>
</tr>
</tbody>
</table>

**Frequency of \( KRAS \) mutations in codon 12, 13 and 61 in HNPCC, sporadic MSI-H and MSS colorectal cancers**

From the 335 colorectal tumours, both HNPCC and sporadic tumours, harbouring \( KRAS \) mutations, we found that codon 12 was mutated in 77% (258/335), codon 13 in 21% (69/335), and codon 61 in only 2% (8/335) of the cases. Frequencies of \( KRAS \) mutations in codons 12, 13 and 61 differ significantly between HNPCC, sporadic MSI-H and MSS tumours (\( P=0.0001 \)) (Table 3, Figure 1).

Codon 13 was significantly more mutated in HNPCC tumours when compared to sporadic MSI-H or MSS CRCs (HNPCC versus MSI-H: \( P=0.004 \); HNPCC versus MSS: \( P=0.0001 \)). Within HNPCC CRCs, the frequency of \( KRAS \) mutations in codons 12 or 13 did not vary depending on the MMR gene affected (\( P=0.23 \)). Codon 61 was never mutated in our series.
of HNPCC tumours and rarely in the sporadic colorectal cancers (MSI-H: 5% (N=2) and MSS: 3% (N=6) Table 1, Figure 1). Twenty of the 37 sporadic MSI-H CRCs harbouring KRAS mutations were analysed for hMLH1 promoter methylation. Of the four MSI-H tumours without hMLH1 promoter methylation three proved to have a codon 13 mutation whereas, codon 12 was affected similarly in MSI-H tumours independently of the hMLH1 promoter methylation status (hMLH1met-: 8/16; hMLH1met+: 8/16).

Table 3: Frequency of KRAS mutations in codons 12, 13 and 61 in colorectal carcinomas

<table>
<thead>
<tr>
<th>CRCs</th>
<th>No. mutants</th>
<th>Total (%) codon 12</th>
<th>Total (%) codon 13</th>
<th>Total (%) codon 61</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All types</td>
<td>335</td>
<td>258 (77)</td>
<td>69 (21)</td>
<td>8 (2)</td>
<td></td>
</tr>
<tr>
<td>HNPCC 63</td>
<td>31 (49)</td>
<td>32 (51)</td>
<td>0 (0)</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Sporadic MSI-H-37</td>
<td>27 (73)</td>
<td>8 (22)</td>
<td>2 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporadic MSS 235</td>
<td>200 (85)</td>
<td>29 (12)</td>
<td>6 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sporadic MSI-H* 20</td>
<td>16 (80)</td>
<td>4 (20)</td>
<td>0 (0)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>hMLH1 met -</td>
<td>11</td>
<td>8 (73)</td>
<td>3 (27)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>hMLH1 met +</td>
<td>9</td>
<td>8 (89)</td>
<td>1 (11)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>Germline mutations 63</td>
<td>31 (49)</td>
<td>32 (51)</td>
<td>0 (0)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>hMSH2 29</td>
<td>17 (59)</td>
<td>12 (41)</td>
<td>0 (0)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>hMSH6 5</td>
<td>1 (20)</td>
<td>4 (80)</td>
<td>0 (0)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>hMLH1 29</td>
<td>13 (45)</td>
<td>16 (55)</td>
<td>0 (0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Total number of sporadic MSI-H CRCs analysed for the methylation status of hMLH1.

**KRAS nucleotide substitution in HNPCC, sporadic MSI-H and MSS colorectal cancers**

The frequency of nucleotide substitutions varied significantly between HNPCC, MSI-H and MSS CRCs. The frequency of guanine:thymidine, guanine:cytosine and guanine:adenine nucleotide substitutions was significantly different between HNPCC and MSS sporadic cases (P=0.0001) but not between HNPCC and MSI-H cases (P=0.27) (Figure 1).

**KRAS amino acid change in HNPCC, sporadic MSI-H and MSS colorectal cancers**

The spectrum of amino acid changes in KRAS codons 12, 13 and 61 was distinct in the three groups of CRCs. MSS CRCs showed a wider range of amino acid changes in comparison to MSI-H and HNPCC CRCs. The amino acid changes G12D, G12V, G12A and G13D occurred in all 3 CRC groups. The amino acid changes G12C (9%), G12S (5%), G12R (1%), G13R (0.4%), Q61K (0.4%) and K61L (0.4%) were found only in MSS CRCs. The Q61H amino acid change was present in 2% of MSS and 1% of MSI-H CRCs (Figure 1).
KRAS MUTATIONS IN HEREDITARY (HNPCC) AND SPORADIC (MSI-H AND MSS) CRCs

Figure 1: Frequency and spectrum of KRAS mutations in colorectal carcinomas of three different groups: A, Sporadic microsatellite stable tumours (MSS); B, Sporadic microsatellite unstable tumours (MSI-H), B1, Subgroup of sporadic microsatellite unstable tumours with hMLH1 promoter methylation, B2, Subgroup of sporadic microsatellite unstable tumours without hMLH1 promoter methylation; C, Tumours from hereditary non-polyposis colorectal cancer (HNPCC) patients with mismatch repair gene germline mutations in hMSH2, hMSH6 or hMLH1.
The frequency of \textit{KRAS} amino acid changes varied significantly between HNPCC, MSI-H and MSS CRCs. The frequency of amino acid changes Glycine to Valine (G12V) or Glycine to Alanine (G12A) in codon 12 was significantly different between HNPCC and sporadic MSS cases (G12V: \( P=0.004 \); G12A: \( P=0.0007 \)), but not between HNPCC and sporadic MSI-H cases. The same was true when we compared the frequency of the amino acid change Glycine to Aspartic acid in both codon 12 and 13 between HNPCC and MSS sporadic cases (G12D + G13D: \( P=0.0005 \)), but not between HNPCC and MSI-H cases. However, the frequency of the amino acid change Glycine to Aspartic acid in codon 12 and 13, when analysed separately, was significantly different in HNPCC in comparison to sporadic MSI-H (G12D, HNPCC versus MSI-H: \( P=0.02 \); G13D, HNPCC versus MSI-H: \( P=0.004 \)) (Table 4, Figure 1).

<table>
<thead>
<tr>
<th></th>
<th>HNPCC (%)</th>
<th>MSI-H (%)</th>
<th>MSS (%)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nucleotide substitution*</td>
<td>63</td>
<td>37</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td>Guanine:thymidine</td>
<td>4 (6)</td>
<td>5 (14)</td>
<td>74 (31)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Guanine:cytosine</td>
<td>8 (13)</td>
<td>2 (5)</td>
<td>10 (4)</td>
<td></td>
</tr>
<tr>
<td>Guanine:adenine</td>
<td>51 (81)</td>
<td>28 (76)</td>
<td>146 (62)</td>
<td></td>
</tr>
<tr>
<td>Total amino acid changes*</td>
<td>63</td>
<td>37</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td>Valine (codon 12)</td>
<td>4 (6)</td>
<td>5 (14)</td>
<td>53 (23)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Alanine (codon 12)</td>
<td>8 (13)</td>
<td>2 (5)</td>
<td>6 (3)</td>
<td></td>
</tr>
<tr>
<td>Aspartate (codon 12 and 13)</td>
<td>51 (81)</td>
<td>28 (76)</td>
<td>134 (57)</td>
<td></td>
</tr>
</tbody>
</table>

* Only nucleotide and amino acid changes found in the three CRC groups were considered for statistical analyses.

**DISCUSSION**

**Frequency of \textit{KRAS} mutations in HNPCC, sporadic MSI-H and MSS colorectal cancers**

Activating \textit{KRAS} mutations represent the most common type of abnormality of a dominant oncogene in human cancer, with specificity and type of mutation varying in relation to tumour type (Bos, 1989). It is widely accepted that mutations in \textit{KRAS} are among the critical transforming alterations occurring during colorectal tumourigenesis and occur early in the progression from adenoma to carcinoma (Fearon and Vogelstein, 1990). In this report, \textit{KRAS} mutations were identified in 33% of all colorectal carcinomas. This is consistent with previous studies that, with rare exceptions, have identified \textit{KRAS} mutations in approximately 30 to 40% of colorectal cancer (Kern et al., 1989; Benhatter et al., 1993; Samowitz et al., 2001).

In agreement to previous reports we also found that, tumours from sporadic CRC patients with an MSS phenotype show higher frequency of \textit{KRAS} mutations than MSI-H CRC tumours (Ionov et al., 1993; Fujiwara et al., 1998; Konishi et al., 1996; Losi et al., 1997; Samowitz et al., 2001; Iacopetta et al., 1998; Salahshor et al., 1999). In contrast, other
studies have reported similar frequencies of KRAS mutations in MSI-H and MSS colorectal carcinomas (Aaltonen et al., 1993; Tannergard et al., 1997; Olschwang et al., 1997).

We now report that KRAS is frequently mutated in tumours from HNPCC patients with germline MMR gene mutations. Furthermore, KRAS mutation frequency varies among HNPCC tumours depending on the MMR gene, which carried the germline mutation. The frequency of KRAS mutations is lower in tumours from patients with an hMLH1 germline mutation compared to tumours from patients carrying either hMSH2 or hMSH6 germline mutations. In fact, tumours from patients with germline mutations in hMSH6 harbour the highest frequency of KRAS mutations (it should however be noted that only six tumours from hMSH6 HNPCC patients were included). This finding seems consistent with the role fulfilled by the MSH2:MSH6 complex (hMutSα) in DNA repair (Peltomaki, 2001; Su et al., 1988). As stated earlier, our data show different frequencies of KRAS mutations in tumours of patients with different MMR genes. It remains to be clarified, whether or not this finding is an explanation for the apparently different clinical phenotypes seen in HNPCC families carrying mutations in specific MMR genes (Bandipalliam et al., 2004).

While HNPCC tumours did not differ from MSS sporadic CRCs regarding the frequency of KRAS mutations, they did differ significantly from sporadic MSI-H carcinomas. Our data are in accordance to previously reported findings (Fujiwara et al., 1998) in a series of 24 HNPCC tumours with germline mutations in hMLH1 or hMSH2. These authors found KRAS mutations in 29% (7/24) of HNPCC tumours in comparison to 18% sporadic MSI-H (N=18) (Fujiwara et al., 1998).

Our data and others show that, sporadic MSI-H CRCs are mainly characterised by a relative low frequency mutation in KRAS when compared to HNPCC and MSS colorectal carcinomas (Fujiwara et al., 1998; Konishi et al., 1996; Salahshor et al 1999; Shitoh et al., 2000; Jass et al., 1999). However, when we compare HNPCC with sporadic MSI-H tumours, classified according to the methylation status of the hMLH1 promoter, differences were observed in KRAS mutation frequency. In MSI-H tumours, negative for hMLH1 promoter methylation, the frequency of KRAS mutations was similar to the frequency observed in HNPCC (see Table 1). This subset of sporadic MSI-H may be explained by somatic mutations in MMR genes or gene loss, mimicking the inactivating events occurring in the hereditary counterpart (Kuismanen et al., 2000; Wu et al., 1987). On the other hand, sporadic MSI-H colon carcinomas with hypermethylation of the hMLH1 promoter region showed the lowest frequency of KRAS mutations of all subsets of colon carcinomas. Similarly, preneoplastic lesions of the colon which show hMLH1 promoter hypermethylation or concurrent methylation also harbour low frequency of KRAS mutations (Wynter et al., 2004). The majority of MSI-H colorectal cancers with hMLH1 promoter hypermethylation are frequently mutated on the V600E hotspot of BRAF (71%), one of the downstream targets of KRAS (Koinuma et al., 2004; Wang et al., 2003; Domingo et al., 2004; Deng et al., 2004). This finding and our data suggest that colon tumours progress by targeting distinct genes of the RAS/RAF/MAPkinase pathway, depending on the genetic/epigenetic event underlying MMR deficiency (mutation and loss versus hMLH1 promoter methylation. MSI-H tumours with MMR gene mutations (hereditary and sporadic forms) may preferentially target KRAS whereas MSI-H tumours with hMLH1 methylation may preferentially target the BRAF gene (as depicted in Figure 2). This assumption is in
accordance to the absence of *BRAF*V600E mutations in HNPCC colorectal tumours (Wang et al., 2003; Deng et al., 2004; Domingo et al., 2004).

**Figure 2:** Summary of *BRAF*V600E and *KRAS* mutation frequency and type significantly associated with the distinct groups of CRCs. Grey (*BRAF* data) represents a summary of published data (references are given in the last paragraph of the introduction section) and black (*KRAS* data) summarises the results reported in this manuscript.

**Codon, nucleotide and amino acid changes in *KRAS* in HNPCC, sporadic MSI-H and MSS colorectal cancers**

*KRAS* point mutations cluster at the guanosine triphosphate (GTP)–binding domain (codon 12/13) or at the GTPase domain (codon 61) and determine specific amino acid substitutions that lead to permanent activation of the p21 ras protein (Benhatter et al., 1993; Barbacid, 1987). In our series of 335 CRCs harbouring *KRAS* mutations, 98% carry mutations in codons 12 and 13, confirming that these two codons are preferentially affected in comparison to codon 61, as previously observed (Bos, 1987; Barbacid,1987; Andreyev et al., 2001).

Several observations can be made concerning the frequency and type of *KRAS* mutations in codon 12 and 13 in sporadic MSS, sporadic MSI-H, and HNPCC colorectal cancers. The
frequency of KRAS mutations in codon 12 or 13 significantly differs between sporadic and hereditary settings: KRAS mutations in codon 12 were more common in sporadic cases (overall MSI-H and MSS) whereas mutations in codon 13 were predominant in HNPCC. Moreover, within HNPCC the underlying MMR gene defect did not influence the position or nucleotide substitution of KRAS. Previously, an association was reported between the location of KRAS mutations and the underlying MMR gene involved (Fujiwara et al., 1998). In contrast to our report, these showed that codon 13 was never affected in HNPCC tumours from patients with hMSH2 germline mutations (0/17), while codon 13 KRAS mutations were present in three out of seven cases in HNPCC tumours from patients with hMLH1 germline mutations (Fujiwara et al., 1998). The low number of cases analysed in the aforementioned study may explain these differences (Fujiwara et al., 1998).

Animal models have shown an association between chemical exposure and location and type of RAS activating mutations in resultant tumours (Barbacid, 1987). A good example to illustrate the presence of codon 12 mutations in sporadic carcinomas and its association with environmental factors is the predominance of codon 12 KRAS mutations in environmental associated carcinomas, such as lung and colon cancer associated with tobacco smoking and bladder cancer (Rodenhuis et al., 1992; Slebos et al., 1991; Westra et al., 1993; Diergaard et al., 2003; Przybojewska et al., 2000). Accordingly, to explain the differences found between the location of the mutation in sporadic and HNPCC tumours we hypothesise that sporadic carcinomas may be associated with an increased susceptibility to damage in specific DNA sequences by environmental factors, while hereditary tumours occur due to inherited predisposition.

Further, G:A transitions were the most common alterations found in colorectal carcinomas. In addition, the frequency of G:A transitions was higher in mismatch repair deficient tumours (HNPCC and sporadic MSI-H), than in MSS colon carcinomas. This finding is in agreement with previous studies which showed that G:A alterations are particularly difficult to repair, even within a normal MMR background (Brown et al., 1988). Moreover, our data showed that G:T substitutions are far more frequent in MSS colon carcinomas than in HNPCC or MSI-H tumours. Accordingly, the type of KRAS amino acid substitution varies among the three different groups of colon carcinomas analysed. The frequency of G12V, resulting from a G:T nucleotide substitution, was significantly higher in MSS sporadic than in HNPCC or sporadic MSI-H cases. In a large study of sporadic colon carcinomas, G12V was identified in 24% of all KRAS positive cases (Andreyev et al., 2001). The frequency of G13D, resulting from a G:A nucleotide substitution, was significantly higher in the group of HNPCC in comparison to sporadic carcinomas (MSI-H and MSS) in accordance with a previous report which showed that G13D was the most common type of KRAS mutation (55%) in HNPCC (Fujiwara et al., 1998). The type of KRAS amino acid substitutions was shown to have different impacts on the outcome of colon cancer patients. Clinically, G12V mutations were associated with a 30% increased risk of recurrence or death (Andreyev et al., 2001). Whether or not the better prognosis of HNPCC and sporadic MSI-H patients is related to the low frequency of G12V mutations remains to be clarified.

In conclusion: HNPCC colorectal and sporadic MSI-H tumours without hMLH1 hypermethylation shared similar KRAS mutation frequency, in particular G13D. Although MSI-H colon carcinomas with methylation of hMLH1 show the lowest frequency of KRAS mutations of all subsets of colon carcinomas, they harbour the highest frequency of BRAF.
mutations. Further, we show that depending on the genetic/epigenetic mechanism leading to MSI-H, the outcome in terms of oncogenic activation may be different, reinforcing the idea that HNPCC, sporadic MSI-H (depending on the hMLH1 status) and MSS CRCs, may target distinct kinases within the RAS/RAF/MAPK pathway.

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KRAS MUTATIONS IN HEREDITARY (HNPCC) AND SPORADIC (MSI-H AND MSS) CRCs

REFERENCES


