Allele loss occurs frequently at *hMLH1*, but rarely at *hMSH2*, in sporadic colorectal cancers with microsatellite instability

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Summary Mutations at the *hMSH2* and *hMLH1* mismatch repair loci have been implicated in the pathogenesis of colorectal cancer. Tumours with two allelic mutations at a mismatch repair locus develop replication errors (RERs). In the hereditary non-polyposis colorectal cancer (HNPPC) syndrome, one mutation is inherited and the other acquired somatically; in RER⁺ sporadic colorectal cancers, both mutations are somatic. RER⁺ tumours tend to have a low frequency of allele loss, presumably because they acquire most mutations through RERs. However, before a second mismatch repair mutation has occurred somatically, there is no reason to suppose that allele loss occurs less frequently in tumours that are to become RER−. Indeed, this second mutation might itself occur by allele loss. We have searched for allele loss at the *hMSH2* and *hMLH1* loci in RER⁺ and RER− sporadic colorectal cancers. Loss occurred at the *hMLH1* locus in 7/17 (41%) RER⁺ tumours, compared with 6/40 (15%) RER− cancers ($\chi^2=3.82$, $P<0.05$). At *hMSH2*, 2/22 RER⁺ sporadic cancers (9%) had lost an allele, compared with 2/40 (5%) RER− cancers ($\chi^2=0.03$, $P>0.5$). Taken together with previous studies which focused on colorectal cancers from HNPPC families, the data suggest that allele loss at *hMLH1*, but not at *hMSH2*, contributes to defective mismatch repair in inherited and sporadic colorectal cancer.

Keywords: allele loss; colon cancer; *hMSH2; hMLH1*

Germ-line mutations at the DNA mismatch repair loci *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* cause the hereditary non-polyposis colorectal cancer (HNPPC) syndrome (Peltomäki, 1995). Patients develop carcinomas of the colon, stomach, ovary, uterus and other specific sites (Jass et al., 1994). The cancers of HNPPC patients show replication errors (RERs), a manifestation of which is instability at microsatellite loci (Fishel and Kolodner, 1995). Some sporadic cases of colorectal cancer (CRC) also have microsatellite instability, presumably because of somatically acquired defects in mismatch repair (Aaltonen et al., 1993). Mutations of mismatch repair genes have been found in sporadic colorectal cancers (Liu et al., 1995).

It has been suggested that RERs comprise an alternative to allele loss [loss of heterozygosity (LOH), allelic imbalance] in the genetic pathways of colorectal tumorigenesis (Aaltonen et al., 1993). Hence, whereas RER⁺ tumours acquire many mutations by LOH, RER⁺ tumours tend to acquire mutations through errors of replication. This divergence between genetic pathways can only occur, however, after the cell becomes deficient in mismatch repair. If this deficiency is recessive, as seems probable (Radman and Wagner, 1993), two allelic mutations must first occur at the *hMSH2*, *hMLH1*, *hPMS1* or *hPMS2* loci (or some related locus). It follows that in RER⁺ tumours, LOH may occur relatively frequently at mismatch repair loci, thereby inactivating one mismatch repair allele, in a way similar to allele loss at tumour-suppressor loci.

This study determines whether or not allele loss at the *hMSH2* and *hMLH1* mismatch repair loci is important in the pathogenesis of RER⁺ sporadic colorectal cancers. We have characterised a set of sporadic colorectal cancers as RER⁺ and RER−. Allele loss at *hMSH2* and *hMLH1* (the mismatch repair loci most commonly involved in colorectal tumorigenesis) has been analysed in these cases. A set of colorectal cancer cell lines has also been analysed with respect to RER status and studied for LOH at *hMSH2* and *hMLH1*.

Materials and methods

Selection of sporadic CRC cases and CRC cell lines

Samples of sporadic CRC and matched normal tissue or blood were obtained from the collection of St Mark’s Hospital, Harrow, UK. These cases were chosen at random and were unselected with respect to family history. DNA was extracted from tumours and normal tissue/blood using standard methods. All visible normal tissue was removed from tumours before extraction of DNA, but tumours were not microdissected. A set of 24 sporadic CRCs was identified as RER⁺, using the methods below, and 48 RER− CRCs were chosen at random for comparison.

Thirty CRC cell lines were available from public sources, from Imperial Cancer Research Fund sources or from individual laboratories (Table 1). The colorectal adenoma cell line, PC/AA, was included in this group of samples. DNA was extracted from these cell lines using standard methods.

RER analysis in sporadic CRCs

Microsatellite instability was assessed at seven dinucleotide repeat markers. PCR reactions were performed on each tumour/normal pair using 50–250 ng DNA template in a final volume of 50 µl. The reaction mixture contained 1× standard PCR buffer (Promega), 1.5 mM Mg²⁺, 0.5 mM dNTPs, 0.4 mM of each specific oligonucleotide primer and 1 U Taq polymerase. The thermal cycling protocols were as follows: for D6S434, D11S968, D11S901 and D11S1313, 94°C (1 min)×1, 94°C (1 min)/55°C (1 min)/72°C (1 min)×35, 72°C (5 min)×1 (Gyapay et al., 1994); for D11S29, 94°C (1 min)×1, 94°C (1 min)/55°C (1 min)/72°C (1 min)×35, 72°C (10 min)×1 (Warnich et al., 1992); for NCAM94, 94°C (1 min)×1, 94°C (1 min)/50°C (1 min)/72°C (1 min)×30, 72°C (5 min)×1 (Telatar et al., 1994); and for DRD2, 94°C (1 min)×1, 94°C (30 s)/58°C (30 s)/72°C (30 s)×30, 74°C (5 min)×1 (Hauge et al., 1991). After heating to 90°C for 5 min the PCR products underwent electrophoresis under denaturing conditions on a 6% acrylamide sequencing gel (Sequagel) for 2–4 h. DNA was blotted onto Hybond N⁺ membranes (Amersham, UK) and
Microsatellite instability and hMSH2/hMLH1 status of CRC cell lines

Microsatellite instability is difficult to assess in the CRC cell lines, because in nearly all cases, no constitutional DNA is available for comparison with the tumour samples. However, the fact that these tumour samples are not contaminated by constitutional DNA (as may occur with frozen tumour samples) means that such an assessment is possible. The same loci and methods for determining microsatellite instability were used for the cell lines as had been used for the sporadic CRCs. In addition, published or publicly-available information on the RER status and mismatch repair genotypes of these cell lines was used (Table I).

LOH at mismatch repair loci in sporadic CRCs

Allele loss was assessed at the D2S123 and D2S391 microsatellite loci (2p15–p16) in order to determine whether or not LOH had occurred at hMSH2 (Figure 1). Allele loss at hMLH1 (Figure 1) was assessed using the D3S1611 and D3S1612 microsatellite loci (3p21.3). PCR reactions were performed as for locus D6S434 (see above and Gyapay et al., 1994). PCR products were electrophoresed for 2–4 h on an 8% non-denaturing polyacrylamide gel and were detected by silver-staining using standard methods. Allele loss was scored by eye in informative (heterozygous) cases if the intensity of one allelic band in the tumour DNA was diminished in comparison with the other allele, allowing for the relative intensity of the alleles in the constitutional DNA. If microsatellite instability occurred at any locus, the results at that locus were excluded from the LOH analysis and deemed non-informative. In practice, no case showed instability at both D2S123 and D2S391, or at both D3S1611 and D3S1612.

LOH at mismatch repair loci in sporadic colon cancer

Owing to the absence of constitutional DNA in most samples, allele loss could only be assessed indirectly in the CRC cell lines using the following method. Given that about 20% of individuals are homozygous at any one of the microsatellite markers used here, it is most unlikely (P=0.2^6 i.e. P~0.002) that an individual is homozygous in the germ line at four of the markers. In fact, linkage disequilibrium between closely linked markers and some degree of consanguinity in marriages mean that the probability of homozygosity is slightly greater than the value given. Nevertheless, any tumour homozygous at four adjacent markers has very probably lost an allele and is unlikely to be a constitutional homozygote. Tumour cell lines lend themselves well to this means of detecting LOH, because the absence of containing normal DNA in the samples means that tumour DNA can be analysed for homozygosity without difficulty. One problem with the method is that very small regions of LOH cannot be detected. For this reason, microsatellite loci as close together as possible were chosen from the Genethon map (Gyapay et al., 1994) to study LOH at hMSH2 (D2S123, D2S391, D2S288 and D2S119) and at hMLH1 (D3S1611, D3S1612, D3S1277 and D3S1561) (Figure 1). PCR reactions were carried out as for D6S434 above and products were detected using the same method as was used for study of LOH in the sporadic CRCs. As above, any cases of microsatellite instability at the eight loci used to assess LOH were excluded from the analysis and the tumour deemed non-informative at that microsatellite locus.

Clinical data

From the sporadic cases, the following data were obtained: patient age at presentation; Dukes’ tumour stage; Jass group; tumour grade (degree of differentiation); mucinous differentiation; and tumour site in the colon (left- or right-sided).

### Table I

| Name            | RER status | Mutation | 2p LOH? | 3p LOH? |
|-----------------|------------|----------|---------|---------|
| HCT116          | +          | MLH      | N       | N       |
| COL202DM        | -          | N        | N       | N       |
| SW620           | -          | N        | N       | N       |
| SW480           | -          | N        | N       | N       |
| SW837           | -          | N        | N       | N       |
| SW1222          | -          | N        | N       | N       |
| SW48            | +          | MLH      | N       | N       |
| T84             | -          | N        | N       | N       |
| SW1417          | -          | N        | N       | N       |
| HT29            | -          | N        | N       | N       |
| WIDR            | -          | N        | N       | N       |
| HCT1S/DLD1      | +          | ?        | N       | N       |
| COLO201         | -          | N        | N       | N       |
| COLO206         | -          | N        | N       | N       |
| HCA46           | -          | N        | N       | N       |
| HCA7            | +          | ?        | N       | N       |
| JW              | -          | N        | N       | N       |
| VAC05           | +          | MLH      | N       | N       |
| VAC04S          | -          | N        | N       | N       |
| CACO2           | -          | N        | N       | N       |
| VACO10OMS       | -          | N        | N       | N       |
| SW838           | -          | N        | N       | N       |
| LS174T          | +          | ?        | N       | N       |
| LS411           | +          | ?        | N       | N       |
| LS1034          | -          | N        | N       | N       |
| LIM1863         | -          | N        | N       | N       |
| SW403           | -          | N        | N       | N       |
| LOVO            | +          | MSH      | N       | N       |
| PC/AA           | -          | N        | N       | N       |
| SKCO-1          | -          | N        | N       | N       |

Blank entries, no mutation detected.

### Figure 1

Genetic maps of the microsatellite markers used to assess allele loss at hMSH2 (D2S123, D2S391, D2S288 and D2S119) and at hMLH1 (D3S1611, D3S1612, D3S1277 and D3S1561).

PCR products were detected by the enhanced chemiluminescence technique (Amersham, UK), using a randomly elongated oligonucleotide primer as a specific probe for each locus. Membranes were exposed to Hyperfilm (Amersham, UK) for up to 60 min to allow visualisation of PCR products. Microsatellite instability was scored if extra bands differing by 2 bp (one repeat unit) from the constitutional DNA were present in the tumour DNA at two or more of the microsatellite loci. If instability was present at just one of the seven microsatellite loci, only cases with more than one extra band were scored as RER+ (owing to the possibility of false positives arising from random PCR errors or from slippage of microsatellite repeat units unrelated to mismatch repair defects).
Results

RER analysis

The set of 24 RER+ and 48 RER- sporadic CRC cases was determined as described above. A negative association had been found between the frequency of LOH and RER+ status in these tumours (Tomlinson and Bodmer, 1996). In the CRC cell lines, microsatellite instability was confirmed in those eight lines already reported as being RER+. All other CRC lines were found to be RER- (Table I).

LOH at hMSH2 and hMLH1 in sporadic CRCs

In the sporadic CRCs, LOH occurred at the hMLH1 locus (Figure 2) in 7/17 (41%) RER+ tumours and in 6/40 (15%) RER- CRCs (Table II). This difference is of borderline significance at the 5% level (Table II). At hMSH2, a much lower frequency of LOH was found (Figure 2). Of 22 informative RER+ tumours, two (9%) had lost an allele at hMSH2; a similar frequency of allele loss (2 of 40 cases, 5%) occurred at hMSH2 in the RER- CRCs (Table II).

None of the sporadic CRCs had lost an allele at one of the two microsatellite loci used to study hMLH1, while retaining alleles at the other locus. At hMSH2, however, one case had lost an allele at D2S391, but not at D2S123; the significance of this result is uncertain. Of the 51 sporadic CRCs, which were informative at both hMSH2 and hMLH1, 15 (29%) lost an allele at either hMSH2 or hMLH1; eight of 17 (47%) informative RER+ cancers lost an allele at either hMSH2 or hMLH1. No tumour had lost an allele at both hMSH2 and hMLH1.

Figure 2 Examples of allele loss at hMLH1 and hMSH2 in sporadic colorectal cancers. Arrows denote 'lost' alleles. Case 226 is non-informative (constitutionally homozygous) at D2S391.

Associations between clinical data and LOH in sporadic CRCs

There was no significant difference between the clinicopathological features of the RER+ and RER- tumours studied here (details of statistical analyses not shown). There were trends in the data towards the RER+ tumours having a higher Dukes' stage and to being right-sided, in agreement with other authors. By chance, however, there was a predominance of left-sided colon cancers in our sample (63/ 72, 88.5%) and this probably prevented a significant association from being found between RER+ status and right-sided tumours. We found no association between RER status and patient age, the degree of tumour differentiation or Jass score. Too few tumours showed mucinous differentiation for a meaningful comparison to be made between the RER+ and RER- groups.

Further analysis showed no association between LOH at hMLH1 or hMSH2 and any of the clinical data (age, stage, grade or site). This was true when all of the 72 sporadic CRCs were included in the analysis, or when only the 24 RER+ CRCs were included.

LOH at hMSH2 and hMLH1 in CRC cell lines

No cell line showed apparent homozygosity at all four microsatellite loci analysed, whether close to hMSH2 or hMLH1. Indeed, every line was heterozygous at two or more of the four microsatellite marker loci used to study hMSH2 or hMLH1.

Discussion

We have detected a relatively high frequency of LOH (41%) at hMLH1 in sporadic RER+ colorectal cancers (that is, tumours with microsatellite instability). This frequency may slightly underestimate the true frequency of LOH, because tumours were not microdissected from normal tissue. A lower frequency of LOH (16%) occurred in RER- sporadic CRCs. This frequency may reflect random events or truly RER+ cancers in which microsatellite instability has not been detected. At hMSH2, allele loss was uncommon in both RER+ and RER- sporadic CRCs.

In 30 CRC cell lines studied, microsatellite instability was confirmed in eight cases and not detected in the other 22 lines. We found no cell line that had lost an allele at hMLH1 or hMSH2 according to the criteria used in this study (see above), although small regions of loss cannot be excluded at hMLH1 in particular, because some of the markers used on chromosome 2 were several cM apart (Figure 1). Some CRC lines were apparently homozygous at the D2S391 and D3S1611 loci, which are the closest markers known to hMSH2 and hMLH1 respectively, and it is possible that these tumours had lost alleles. This fact, and the small number of RER+ CRC cell lines identified, explain why the frequency of LOH at hMLH1 is apparently lower in the cell lines than in the sporadic CRCs.

Table II Numbers of sporadic colorectal cancers subdivided according to RER status and LOH at hMSH2 or hMLH1

|       | hMLH1A |       | hMLH1B |
|-------|--------|-------|--------|
| RER-  | 2      | 2     | 4      |
| RER+  | 38     | 22    | 62     |
| Total | 40     | 22    | 62     |

aχ² = 0.03, P > 0.5 (Yates' correction). bχ² = 3.82, P = 0.05 (Yates' correction). Informative cases only are shown (that is, cases that are heterozygous at either of the two microsatellite loci used to study hMSH2 and hMLH1). For both hMSH2 and hMLH1, LOH denotes allele loss at either of the two microsatellite markers studied.
The frequencies of LOH at hMSH2 and hMLH1 reported here are in agreement with the results of Hemminki et al. (1994). This previous study found a frequency of allele loss of 56% at D3S1611 in a sample of nine informative cancers from HNPCC patients and sporadic RER+ cancers. Combined, the data suggest that, at hMLH1, mismatch repair is dominant at the cellular level (that is, repair is normal before a second 'hit' occurs). The frequency of allele loss at hMLH1 in the RER+ cancers (41%) is similar to that found at many putative tumour-suppressor loci in CRCs (Devilee et al., 1991).

Given the results at hMLH1 and the apparently similar effects of mutations at hMLH1 and hMSH2, it is difficult to explain the low frequency of allele loss at hMSH2 that was found in this study and had also been found by Aaltonen et al. (1993). One explanation is that LOH at hMSH2 and hMLH1 affects unusually small regions of the chromosome: the markers used at hMLH1 lie within or close to the gene, whereas the markers used for hMSH2 are up to 2cM away, perhaps too far to detect some cases of LOH at hMSH2. There are, however, other explanations for the difference between allele loss at hMSH2 and hMLH1. The relative frequencies of hMLH1 and hMSH2 mutations in sporadic CRCs are not fully known and it is possible that hMLH1 mutations are more common, the low frequency of LOH at hMSH2 merely reflecting this fact (despite the finding that germ-line hMSH2 mutations are actually more frequent than hMLH1 mutations in HNPCC). Alternatively, we can speculate that LOH at hMSH2 is disadvantageous, presumably because of effects on the dosage of contiguous genes with important cellular functions; the same reasoning may not apply to hMLH1. A further possibility is that hMSH2 mutations are at least partially dominant and that some defect in mismatch repair in the \( \mu_{hMSH2} + hMSH2 \) heterozygote biases the second 'hit' towards mismatch repair mutations away from LOH. Finally, we cannot discount the possibility that LOH at hMLH1 is not directly related to mismatch repair, but primarily targets a nearby gene such as \( \beta \)-catenin.

We conclude that allele loss at hMLH1 probably contributes to the RER+ status of a significant proportion of sporadic colorectal cancers. Loss of heterozygosity at hMSH2 appears to be rare. Allele loss at both these mismatch repair loci occurs less frequently in RER- CRCs.

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