Microsatellite Instability profiling of Lynch syndrome-associated cancers
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CHAPTER 3

Do Microsatellite Instability Profiles Really Differ Between Colorectal and Endometrial Tumours?

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ABSTRACT

Microsatellite instability (MSI) occurs in more than 90% of the tumours of Lynch syndrome patients, and in 15-25% of sporadic colorectal (CRC) and endometrial carcinomas (EC). Previous studies comparing EC and CRC using BAT markers showed that the frequency of unstable markers is lower in EC, and that the size of the mutations is smaller in EC. In the present study we analyzed the type (insertions/deletions), size and frequency of mutations occurring at three BAT and three dinucleotide markers in CRC and EC, in order to elucidate whether it is possible to establish different MSI profiles in carcinomas of different tissue origin. We show that mononucleotide markers nearly always become shorter whereas dinucleotide markers can become shorter or longer, in both CRC and EC. We therefore conclude that the type of mutation is a marker-dependent feature rather than tissue-dependent. However, we observed that the size of the deletions/insertions differs between CRC and EC, with EC having shorter alterations. The frequency of mono- and dinucleotide instability found in both tissues is comparable, with mononucleotide and dinucleotide markers being affected at similar rates. We conclude that it is not possible to define clearly different MSI profiles that could distinguish MSI-H in CRC and EC. We propose that the differences observed might indicate different durations of tumour development and/or differences in tissue turnover between colorectal and endometrial epithelium, rather than reflecting truly different MSI profiles. We therefore suggest that the same MSI tests can be used for both tumour types.
INTRODUCTION

Microsatellite instability (MSI) is a form of genetic instability caused by defects in the DNA mismatch repair (MMR) pathway. MSI was first associated with Lynch syndrome patients in 1993 (Aaltonen et al., 1993; Ionov et al., 1993; Peltomäki et al., 1993; Thibodeau et al., 1993) and occurs in over 90% of the tumours of these patients. It is also found in a large proportion (15-25%) of sporadic colorectal (CRC) and endometrial (EC) carcinomas (Boland et al., 1998).

The MMR pathway corrects mispaired nucleotides as well as small insertions and deletions, this last group resulting from slippage of the polymerases during replication of short DNA repeat sequences (microsatellites). When inactivating mutations occur within the MMR genes, such as MLH1, MSH2, and MSH6, the MMR pathway becomes deficient and an accumulation of insertions or deletions is observed in microsatellite sequences (Ionov et al., 1993). This phenomenon is referred to as microsatellite instability. MSI is therefore easily recognized by increased or decreased microsatellite lengths. An international consensus panel of five microsatellite markers was established to facilitate the detection and analysis of MSI and this panel is widely used (Boland et al., 1998). Samples that are unstable for two or more of these markers are considered MSI-high (MSI-H); samples unstable for one marker are called MSI-low (MSI-L); samples stable for all markers are called microsatellite stable (MSS). When a different number of markers are used, a sample is MSI-H when it shows instability in more than 30% of the markers used.

Microsatellite mutations occur both at coding and non-coding repeats. Genes frequently found mutated in MSI-H tumours (also called target genes) play important roles in tumour development pathways and show mutations in their coding microsatellite sequences. The profile of target genes is thought to be different in CRC and EC, both in quantitative and qualitative ways (Duval et al., 2002). Previous studies also compared patterns of MSI in sporadic and Lynch syndrome-associated CRC and EC at the non-coding level, in particular by analyzing the mononucleotide BAT markers. It was shown that in both sporadic and Lynch syndrome-associated tumours, the proportion of unstable markers is
lower in EC than in CRC, and that the size of the allelic variations is smaller in EC than in CRC (Furlan et al., 2002; Kuismanen et al., 2002).

These studies thus show quantitative differences in non-coding mononucleotide instability between the two types of tumours. Data on MSI of different types of markers and on qualitative differences are, however, scarce. Do CRC and EC show different ratios of insertions/deletions? Do EC display smaller insertions/deletions than CRC also in dinucleotide markers? Do CRC and EC have different “preferences” for specific types of MSI markers, as they have for target genes? In this study we addressed these questions in order to elucidate whether it is possible to define different profiles of MSI for tumours with different tissue origin, such as colorectal and endometrial cancers.

MATERIALS AND METHODS

Samples
The patients participating in this study were all suspected of having Lynch syndrome and were selected from other research studies being conducted by our group (Berends et al., 2002; Niessen et al., 2006). All patients gave their informed consent for the study. The patients had either been diagnosed with CRC or EC under the age of 50 years, or had two or more Lynch syndrome-related cancers, including at least one CRC, irrespective of age and family history. The cases had been analyzed for germline mutations in the MLH1, MSH2, and MSH6 genes (Berends et al., 2001; Berends et al., 2003; Niessen et al., 2006). In total we included paraffin-embedded tumour tissue sections and normal tissue/blood samples from 194 colon carcinomas (CRC) and 68 endometrial carcinomas (EC). Thirteen out of the 194 CRC and 7 out of the 68 EC were from patients who carried a pathogenic germline mutation in one of the three MMR genes (MLH1, MSH2, and MSH6). These patients are referred to as mutation carriers. Average age of tumour onset for the different groups of patients is presented in table 2.
**MSI Analysis**

MSI analysis was performed by fragment analysis using a panel of three mono-nucleotide markers (BAT25, BAT26, BAT40) and three dinucleotide markers (D2S123, D5S346, D17S250) as described previously (Berends *et al.*, 2002). DNA was extracted from formalin-fixed, paraffin-embedded tumour sections and compared with DNA isolated from normal tissue from paraffin-embedded sections (when available) or peripheral blood lymphocytes from the same patient, as described previously (Berends *et al.*, 2002). The samples were classified as MSI-H if more than 30% of the markers analyzed were unstable. Only samples with informative results for four or more of the six MSI markers (independent of the type of marker) were included in this study. For the MSI-H tumours, type of microsatellite mutation (deletion/insertion) and the size of these mutations were analyzed for every marker, and frequencies of instability were calculated. The size of mutations was measured as the difference between the highest peak in the normal tumour and the farthest peak in the unstable tumour.

**Statistical Analysis**

For the differences in type of mutations and frequencies of instability, the $\chi^2$ test or Fisher’s Exact test were used. P values <0.05 were considered to be significant. Two-way factorial ANOVA was used for the differences in mutation size between markers and tumour tissues.

**RESULTS**

**Frequency of microsatellite instability is highest in mutation carriers**

One-hundred and five tumors (40%) were classified as MSI-H and selected for further analysis. The frequencies of instability were overall as we expected from the literature, with mutation carriers showing significantly higher frequencies of MSI-H than non-carriers, both in colorectal tumors and in endometrial tumors (table 1). MSI-L was found in mutation carriers in the CRC group only, in two cases, both harboring an *MSH6* mutation. In the EC mutation carriers, two MSS cases were detected; one carried an *MSH6* mutation and the other an *MSH2* mutation.
**Table 1.** Distribution of the samples by MSI status, presence or absence of germline mismatch repair mutations, and tumour tissue type

<table>
<thead>
<tr>
<th></th>
<th>Non-carriers</th>
<th>Mutation carriers</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRC</td>
<td>EC</td>
<td>CRC</td>
</tr>
<tr>
<td>N</td>
<td>181</td>
<td>61</td>
<td>13</td>
</tr>
<tr>
<td>MSI-H</td>
<td>36%</td>
<td>40%</td>
<td>85%</td>
</tr>
<tr>
<td>MSI-L</td>
<td>28%</td>
<td>29%</td>
<td>15%</td>
</tr>
<tr>
<td>MSS</td>
<td>36%</td>
<td>31%</td>
<td>0%</td>
</tr>
</tbody>
</table>

CRC colorectal carcinomas; EC endometrial carcinomas; MSI-H microsatellite instability high; MSI-L microsatellite instability low; MSS microsatellite stable; N, absolute number.

**Table 2.** Average age of tumor onset of the different groups of patients

<table>
<thead>
<tr>
<th></th>
<th>Non-carriers</th>
<th>Mutation carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CRC</td>
<td>EC</td>
</tr>
<tr>
<td>MSI-H</td>
<td>48.4</td>
<td>48.3</td>
</tr>
<tr>
<td>MSI-L+MSS</td>
<td>46.8</td>
<td>46.9</td>
</tr>
</tbody>
</table>

CRC colorectal carcinomas; EC endometrial carcinomas; MSI-H microsatellite instability high; MSI-L microsatellite instability low; MSS microsatellite stable.

**Type of microsatellite mutations (insertions/deletions) depends on type of repeat**

Frequencies of deletions and/or insertions occurring at each microsatellite marker were calculated for the MSI-H CRC and EC samples. No association between the prevalence of deletions or insertions and tumour type was found. A strong correlation between type of markers (mononucleotide vs. dinucleotide markers) and type of microsatellite mutation (insertion/deletion) was, however, observed. Mononucleotide markers were almost exclusively targets of deletions (98% in CRC and 100% in EC), whereas dinucleotide loci showed both deletions and insertions (Fig. 1). Simultaneous insertions and deletions were also detected in all dinucleotide markers (Fig. 1).
Figure 1. Frequencies of deletions, insertions, and simultaneous deletions and insertions in mononucleotide (BAT25, BAT26, BAT40) and dinucleotide (D2S123, D5S346, D17S250) MSI markers in MSI-H colorectal (upper panel) and endometrial (lower panel) carcinomas.

Size of mutations in EC is smaller than in CRC
The size of insertions/deletions was analyzed for each unstable locus of MSI-H tumours. The difference in size as defined in this study corresponded to the allele with the maximum length difference from the normal allele (observed in normal tissue/blood from the same patient). The mutation sizes were determined for both tumour types (Fig. 2). The mutations were significantly smaller in EC (average 6.02±0.45bp) than in CRC (average 7.67±0.34bp) (ANOVA: $F_{(1,203)}=11.25$, $p<0.001$).
However, these differences are not statistically significant when analyzing each marker separately, except for D2S123.

Different markers showed different mutation sizes (ANOVA: $F_{(5,203)}=14.67$, $P<0.001$), but the relative differences between them remained similar in both tissues, as there is no interaction between the two variables (ANOVA: $F_{(5,203)}=1.3$, $P>0.1$).

**Figure 2.** Size of the insertions/deletions (in bp) for each MSI marker, in MSI-H colorectal (CRC) and endometrial (EC) carcinomas. * Statistically significant differences between the CRC/EC pair for each marker.

**Distribution of microsatellite instability**

Frequencies of instability were calculated for each MSI marker in both types of tumours. Neither of the two tissues showed a statistically significant preference for a specific type of marker. Both mononucleotide and dinucleotide markers are equally affected in the two tumour types and none of the markers was differently affected when we compared colorectal and endometrial tumours (Fig. 3).
Figure 3. Frequencies of instability observed for the mononucleotide (BAT25, BAT26, BAT40) and dinucleotide (D2S123, D5S346, D17S250) microsatellite markers, in MSI-H colorectal and endometrial tumours. The upper panel shows the frequencies for each marker; the lower panel shows the total of mononucleotide and dinucleotide instability. CRC, colorectal carcinomas; EC, endometrial carcinomas.

DISCUSSION

We report an analysis of mononucleotide and dinucleotide MSI markers, with regard to type, size and frequency of the mutations in MSI-H tumours with different tissue origins, namely colorectal and endometrial carcinomas. Looking at these features we found no significant differences between the EC and CRC MSI profiles, or at least not great enough to justify applying different MSI tests for the two tumour types.

No statistically significant differences between mutation carriers and non-carriers were found. For this reason we were able to group all MSI-H cases
together for the analyses of the different MSI features. Nevertheless, we should keep in mind that the number of mutation carriers used in this study is much smaller than the number of non-carriers. Considering the age of the patients, mutation carriers developed CRC earlier than non-mutation carriers for MSI-H cases (table 2). Overall the average age of onset did not differ significantly among the mutation carriers and the non-mutation carriers.

Our data show that the ratio of insertions and deletions is a marker-dependent feature rather than a tissue-dependent one, as all the mononucleotide markers we studied showed almost exclusively deletions, while dinucleotide markers showed deletions, insertions, and simultaneous deletions and insertions, both in CRC and EC.

The occurrence of mainly deletions in the mononucleotide markers was what we expected from the literature, since mutations in these markers are commonly referred to as shortenings. In the first reports on the involvement of microsatellite mutations in colon carcinogenesis mediated by a mutation in the MMR system (“mutator mutation”) (Ionov et al., 1993), a striking imbalance of deletions over insertions in Poly (A) sequences in CRC cell lines, with various degrees of microsatellite instability, was described. It was also known that, in *Saccharomyces cerevisiae*, frameshifts on single base pair tracts tend to be deletions (Kunkel et al., 1989; Henderson and Petes, 1992). With respect to dinucleotide instability, if the three dinucleotide markers are taken as a whole, a tendency for only insertions over only deletions was observed in both CRC (44% vs. 37%) and EC (49% vs. 32%). Simultaneous deletions and insertions were found in 19% of dinucleotide loci in CRC and in 20% in EC. These results are in agreement with previous studies suggesting that insertions are more common than deletions among dinucleotide repeats (Twerdi et al., 1999; Ellegren, 2000; Yamada et al., 2002).

This close association of the occurrence of insertions or deletions with the type of MSI marker suggests that characteristics of the repeats, such as repeat length, have more influence on the type of mutation occurring at a microsatellite repeat than the tissue origin of the tumour in which those mutations arise. Repeat length, together with base composition and number of repeat units per tract, are some of the features known to influence the mechanism of “slipped-strand mispairing” (Boyer et al, 2002), the main mechanism generating insertions or

While analyzing the size of the deletions/insertions, we observed significant differences between CRC and EC, with EC showing smaller mutations than CRC for all markers, as previously described in the literature for the BAT markers. The differences are, however, not statistically significant in our study when comparing each marker alone. For instance Kuismanen et al. (2002) reported a mean deviation (bp) of 6.7 in CRC and 4.1 in EC for BAT25; and a mean deviation (bp) of 13.5 in CRC, and 8.5 in EC for BAT26. For the same markers we observed the same tendency to larger mutations in CRC: a mean deviation (bp) of 6.08 ± 0.41 in CRC, and 5.25 ± 0.62 in EC for BAT25; and 7.82 ± 0.65 in CRC and 6.00 ± 0.89 in EC for BAT26. The apparent differences found between our data and the mentioned study (Kuismanen et al., 2002) might be explained by differences in the classification of MSI by different observers. The number of samples included might also play a role in these differences, namely the inclusion of tumours with MMR mutations, typically more unstable than those not carrying MMR mutations. The stage of the tumour – more specifically the rounds of replication that a given tumour has undergone – might, in our opinion, also influence the size of microsatellite mutations.

Furthermore, although the different markers showed different mutation sizes, the relative differences between them remained similar in both tissue types, leading to comparable patterns of instability, as observed for the type of mutation.

Analyzing each marker alone, the frequencies of instability were not significantly different between CRC and EC for any of the markers. If we consider two groups, one of the three mononucleotide markers and one of the three dinucleotide markers, the pattern was again similar, with mono- and dinucleotide markers being affected in equal amounts and both of them similarly affected in CRC and EC.

In conclusion, our results suggest that it is not possible to define specific profiles of MSI marker instability to distinguish tumours of different tissue origins. The features analyzed in our study – type, size and frequency of instability of MSI markers – seem to be representative of common patterns of MSI in CRC and EC.
A possible explanation for the quantitative differences described between CRC and EC, with EC having usually less unstable markers and smaller deletions/insertions, might be that they indicate different durations of tumour development, rather than reflecting real differences in profiles of the two tumour types. Tissue specificities, such as differences in tissue turnover between colorectal and endometrial epithelium might lead to different timings of tumour development and, in practice, result in different levels of instability. This would be in agreement with the tumour clock model of Shibata et al. (1996), who proposed that microsatellite alterations could be seen as a proxy for the number of cell divisions. Mutations accumulate with the number of replications, serving as a molecular clock to define the time of tumorigenesis and tracing the history of the tumour.

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