Ubiquitous Somatic Alterations at Microsatellite Alleles Occur Infrequently in Barrett’s-associated Esophageal Adenocarcinoma

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Abstract

Microsatellite alterations have been documented in a subset of sporadic tumors, including those of the colon, lung, bladder, stomach, and esophagus. This study documented the frequency of microsatellite alterations at 139 loci, comprising predominantly dinucleotide and tetranucleotide repeat units, in 17 cases of primary esophageal adenocarcinoma arising against a background of Barrett’s metaplasia. Each tumor demonstrated alterations in at least one locus studied. Widespread microsatellite alterations, occurring at 45.3% (58 of 128) of loci tested, were detected in a single case. The remaining 16 tumors exhibited low levels of microsatellite instability, ranging from 0.8% (1 of 128) to 8.1% (10 of 123) of loci tested. The single case with ubiquitous somatic alterations showed no significant difference in the incidence of novel alleles at di- and tetranucleotide repeat loci. The 16 cases showing a low level of microsatellite alterations demonstrated a 3.3-fold higher incidence of novel alleles at tetranucleotide repeat loci compared to dinucleotide repeat loci. These data suggest that ubiquitous somatic alterations at microsatellite loci, considered a phenotypic expression of defective mismatch repair, occur infrequently in Barrett’s-associated adenocarcinoma. However, the majority of these tumors demonstrate a low level of microsatellite alterations, perhaps reflecting the inherent instability of these markers.

Introduction

The development of cancer is a multistep process involving the activation of oncogenes and the loss, or inactivation, of tumor suppressor genes. In recent years, a second distinct mechanism of tumorigenesis, which results in widespread RERs (RER+ phenotype) throughout the cancer cell genome, has been documented (1). Microsatellites have been shown to be useful markers of this genetic instability in tumor cells. Microsatellites are highly polymorphic, short, tandem repeat DNA sequences. They are abundantly and evenly distributed throughout the genome and are easily typed by PCR (2). Genetic instability is evident as alterations in the length of microsatellite alleles in tumor DNA as compared to matched normal DNA (3).

Widespread microsatellite instability is a characteristic feature of tumors from HNPCC kindreds (3, 4). These families are associated with an increased incidence of colorectal cancer and also extracolonic tumors, including those of endometrial, gastric, and ovarian origins (5). The microsatellite instability detected in these tumors is a phenotypic manifestation of an underlying defect in DNA mismatch repair (6). Four genes involved in DNA mismatch repair have been found to be mutated in the germ line of HNPCC families (7–11). The RER+ phenotype is also associated with a subset of sporadic tumors typical of HNPCC, i.e., colorectal, endometrial, and gastric tumors (12–19). A number of studies report that microsatellite instability occurs at varying frequencies in a number of other sporadic tumors, including those of the bladder, breast, lung, and esophagus (20–24). This study presents an extensive analysis of microsatellite instability in 17 cases of sporadic primary esophageal adenocarcinoma arising against a background of Barrett’s metaplasia and discusses the significance of low-level microsatellite alterations in sporadic carcinoma in general.

Materials and Methods

Tissue Collection. Matched tumor and control tissue samples were obtained intraoperatively from 17 patients with esophageal adenocarcinoma arising against a background of Barrett’s metaplasia. The Barrett’s esophagus study was approved in 1993 by The Research Ethical Committee (The Queen’s University of Belfast). The tissue was snap-frozen in liquid nitrogen and stored at −70°C until utilized for DNA extraction.

Tumor and control tissue were cryostat sectioned prior to DNA extraction. Frozen sections were evaluated to determine the percentage of tumor and normal tissue present. Where possible, microdissection of the specimen was performed, and normal tissue was excised to maximize the percentage of tumor in each specimen. Only samples demonstrating greater than 40% tumor were selected for analysis. A number of serial sections were obtained for DNA extraction, and a final section was examined to confirm the percentage tumor present. In addition, the frozen sections were reviewed by a consultant pathologist (J. M. S.) and histologically classified according to the procedure of Lauren (25). Penetration of the esophageal wall as well as the presence of lymph node metastases was recorded in every case, using the American Joint Committee on Cancer (AJCC) and the International Union against Cancer (UICC) criteria for pathological staging.

DNA Extraction. DNA was extracted from matched normal and tumor tissue samples by standard methods (26). The tissue was placed in 1 ml lysis buffer [100 mM Tris-HCl (pH 8.5), 0.2% SDS, and 200 mM NaCl containing 100 μg/ml proteinase K], digested overnight at 55°C with agitation, and precipitated by addition of an equal volume of isopropyl alcohol. The DNA was washed in 70% ethanol and resuspended in 50–200 μl TE (10 mM Tris-HCl and 0.1 mM EDTA (pH 7.5)).

Microsatellite Analysis. A total of 139 microsatellite loci containing di-, tri-, and tetranucleotide repeat sequences representing all autosomal arms, excluding acrocentric arms, was analyzed. An average of 130 markers (range, 117–136) was analyzed per tumor. Prior to amplification, 0.75 μM forward primer from each pair was end-labeled with 22 μCi [32P]ATP (NEN DuPont; 10 μCi/μl) and 6.7 units of T4 polymerase kinase in a total volume of 9.9 μl. Following incubation at 37°C for 30 min, 23.1 μl distilled H2O was added. PCR reactions were carried out in a total volume of 5 μl PCR buffer [10 mM Tris, 1.5 mM MgCl2, and 50 mM KCl] containing 50 ng genomic DNA, 0.01 μM labeled primer, 0.15 μM of each unlabeled primer, 0.2 mM dNTPs, 0.15 units of Taq polymerase (Boehringer Mannheim). PCR amplifications were performed for 27 cycles consisting of 30 s at 94°C, 75 s at 55°C, and 15 s at 72°C, plus a final extension step of 6 min at 72°C. Two volumes of gel loading solution [0.3% xylene cyanol, 0.3% bromophenol blue, 10 mM EDTA (pH 8.0) and 90% (v/v) formamide] were added to the PCR product. Samples were denatured at 95°C for 10 min, and 1 μl...
resolved on a 6.5% polyacrylamide DNA sequencing gel. The gel was exposed to film for 4–72 h. Autoradiographs were independently scored by C. M. G and S. E. H. R. A sample was scored positive for microsatellite instability when additional new alleles were observed in tumor DNA, as compared with control DNA from the same individual.

Results

This study examined the incidence of microsatellite alterations at 139 loci in 17 cases of primary esophageal adenocarcinoma arising against a background of Barrett’s metaplasia. Of the 139 markers analyzed, 37% (52 of 139) were dinucleotide repeat sequences, 4% (5 of 139) were triplet repeat sequences, and the remaining 59% (82 of 139) were tetranucleotide repeat sequences. The clinicopathological features of the 17 cases are listed in Table 1. The patient group demonstrated a male:female ratio of 14:3, and the median age at diagnosis was 65 years (range, 31–75 years). The tumors were classified according to the procedure of Lauren (25) and included 16 intestinal-type adenocarcinomas (well to moderately differentiated) and one poorly differentiated adenocarcinoma.

Representative examples of microsatellite alterations are shown in Fig. 1. The most frequently observed alterations included an additional larger allele, representing the gain of a single repeat unit, and an additional smaller allele, representing the loss of a single repeat unit (Fig. 1A, Lanes 1–7). Occasionally, two additional bands were detected in the tumor DNA (Fig. 1B, Lanes 8 and 9). Larger alterations involving the gain or loss of a number of repeat units were detected infrequently (Fig. 1B, Lanes 10 and 11). A laddering pattern of larger alterations was demonstrated in tumor DNA (Fig. 1B, Lanes 12–14). Alterations in microsatellite alleles were detected in all tumors analyzed. A single tumor (no. 123) exhibited ubiquitous somatic mutations throughout the genome (>45% of loci tested). A varying level of microsatellite alterations, ranging from 0.8% (1 of 128) to 8.1% (10 of 123) of loci tested, was detected in the remaining 94% (16 of 17) of cases. The specific microsatellite alterations detected in each of the 17 tumors are listed in Table 1. The variable expression of the instability phenotype in these tumors is indicated in Fig. 2.

A similar incidence of novel alleles was detected at both dinucleotide and tetranucleotide repeat loci in sample no. 123 (Table 2). The remaining 16 tumors demonstrated a 3.3-fold higher incidence of novel alleles at tetranucleotide repeat loci than at dinucleotide repeat loci. For these 16 tumors, the average incidence of a novel allele was 1.6% (12 of 753 genotypes) at dinucleotide repeat loci and was 5.2% (65 of 1253 genotypes) at tetranucleotide repeat loci. This difference in mutation rate was highly significant ($\chi^2$, $P < 0.001$; Table 2).

Discussion

DNA mismatch repair is a highly conserved cellular process that results in the recognition, excision, and repair of mismatched bases (27). In recent years, defects in this process have been identified as a novel mechanism of tumorigenesis. This phenomenon was initially described in sporadic colorectal cancer, and defective mismatch repair has also been identified as the underlying genetic defect in HNPCC kindreds (1, 4, 28). Analyses of colorectal tumors from these patients demonstrated widespread genomic instability (3, 4, 6). This instability is readily identified by the detection of ubiquitous somatic alterations in microsatellite alleles (RER+ phenotype).

To date, most studies of microsatellite instability in colorectal carcinoma have analyzed an average of 4–7 microsatellite markers (3, 4, 13, 28). These studies have used predominantly dinucleotide repeat

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**Table 1  Clinical features and microsatellite alterations in 17 cases of Barrett’s-associated adenocarcinoma**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Grade</th>
<th>T, N, M</th>
<th>Loci demonstrating microsatellite rearrangement in tumor DNA</th>
</tr>
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<tbody>
<tr>
<td>43</td>
<td>59</td>
<td>M</td>
<td>PD</td>
<td>3,1,0</td>
<td>D15S547</td>
</tr>
<tr>
<td>90</td>
<td>66</td>
<td>M</td>
<td>WD</td>
<td>3,0,0</td>
<td>D12S292</td>
</tr>
<tr>
<td>54</td>
<td>69</td>
<td>M</td>
<td>MD</td>
<td>3,1,0</td>
<td>D7S817,D9S302</td>
</tr>
<tr>
<td>88</td>
<td>75</td>
<td>F</td>
<td>WM/MD</td>
<td>3,0,0</td>
<td>D6S305,D10S1237</td>
</tr>
<tr>
<td>76</td>
<td>70</td>
<td>M</td>
<td>MD</td>
<td>2,1,0</td>
<td>D15S1985,D14S306,D19S394</td>
</tr>
<tr>
<td>29</td>
<td>65</td>
<td>M</td>
<td>MD</td>
<td>3,1,0</td>
<td>D25S434,D25S406,D21S1090</td>
</tr>
<tr>
<td>108</td>
<td>50</td>
<td>M</td>
<td>MD</td>
<td>1,0,0</td>
<td>D25S405,D25S406,D7S820,D19S246</td>
</tr>
<tr>
<td>50</td>
<td>53</td>
<td>M</td>
<td>MD</td>
<td>3,1,0</td>
<td>D25S1334,D3S1293,D3S1763,D12S192,D20S115,</td>
</tr>
<tr>
<td>120</td>
<td>74</td>
<td>M</td>
<td>PD</td>
<td>2,0,0</td>
<td>D25S1766,D7S1830,D8S1110,D12S1757,D20S480</td>
</tr>
<tr>
<td>112</td>
<td>33</td>
<td>M</td>
<td>MD</td>
<td>2,0,0</td>
<td>D25S1764,D6S262,D7S513,D7S1850,D13S156</td>
</tr>
<tr>
<td>99</td>
<td>58</td>
<td>M</td>
<td>MD</td>
<td>3,1,0</td>
<td>D25S406,D25S1764,D5S474,D11S1392,D16S541</td>
</tr>
<tr>
<td>104</td>
<td>69</td>
<td>M</td>
<td>W/MD</td>
<td>1,0,0</td>
<td>D25S405,D5S819,D5S199,D9S301,D12S375,D4S306</td>
</tr>
<tr>
<td>110</td>
<td>68</td>
<td>M</td>
<td>MD</td>
<td>3,0,0</td>
<td>D25S134,D5S434,D5S815,D7S1830,D7S1805,D12S391,D12S395</td>
</tr>
<tr>
<td>124</td>
<td>31</td>
<td>M</td>
<td>MD</td>
<td>3,1,0</td>
<td>D25S166,D6S262,D7S1805,D9S319,D9S299,D11S192,D12S374, D18S535</td>
</tr>
<tr>
<td>74</td>
<td>59</td>
<td>F</td>
<td>PD</td>
<td>3,1,0</td>
<td>D25S406,D25S134,D3S1764,D4S1652,D5S408,D5S133,D3S303, D10S1237,D12S374,D20S470</td>
</tr>
<tr>
<td>125</td>
<td>69</td>
<td>M</td>
<td>MD</td>
<td>3,0,0</td>
<td>D25S406,D25S134,D3S1764,D4S1652,D5S408,D5S133,D3S303, D10S1237,D12S374,D20S470</td>
</tr>
<tr>
<td>123</td>
<td>54</td>
<td>F</td>
<td>PD</td>
<td>2,0,0</td>
<td>D25S406,D25S134,D3S1764,D4S1652,D5S408,D5S133,D3S303, D10S1237,D12S374,D20S470</td>
</tr>
</tbody>
</table>

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*All patients had primary esophageal adenocarcinoma arising against a background of Barrett’s metaplasia. The tumors are listed in order of increasing frequency of microsatellite alterations.

**Age** at diagnosis.

**M**, male; **F**, female.

The tumors were classified according to the procedure of Lauren (25). Case no. 43 was a diffuse-type adenocarcinoma; the remaining 16 tumors were intestinal type. WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

**TNM classification** system agreed by the AJCC and the UICC. T, depth of penetration of the primary tumor, N, the status of regional lymph nodes with regard to malignant involvement; M, the presence or absence of distant metastases.
Fig. 1. Representative examples of microsatellite alterations observed at different loci in esophageal adenocarcinoma. The alterations in microsatellite alleles are indicated by arrows. N, control DNA; T, tumor DNA. Lane 1, case no. 99, D2S406; Lane 2, case no. 112, D7S1830; Lane 3, case no. 120, D8S1110; Lane 4, case no. 123, D8S1110; Lane 5, case no. 123, D5S814; Lane 6, case no. 123, D4S404; Lane 7, case no. 123, D2S406; Lane 8, case no. 123, D7S821; Lane 9, case no. 124, D3S1766; Lane 10, case no. 123, D7S1830; Lane 11, case no. 123, D3S1766; Lane 12, case no. 123, D13S175; Lane 13, case no. 123, D17S579; Lane 14, case no. 123, D7S513.

markers, with the exception of a single trinucleotide repeat marker (3). In these studies, the RER\(^+\) phenotype has frequently been defined as two or more loci exhibiting alterations, i.e., at least 29% (2 of 7) of loci tested exhibited alterations (3). Using these criteria, Thibodeau et al. (28) found 17% (15 of 90) of an unselected series of colorectal carcinomas exhibited an RER\(^+\) phenotype, whereas Lothe et al. (13) reported that 10% (19 of 207) of sporadic colorectal carcinomas exhibited microsatellite alterations at two or more loci. Aaltonen et al. (4) found that 16% (8 of 49) of sporadic colorectal carcinomas demonstrated an RER\(^+\) phenotype, with each tumor exhibiting alter-

Fig. 2. Frequency of microsatellite alterations observed in individual cases of esophageal adenocarcinoma. Individual case numbers are listed on the X-axis. The Y-axis indicates the percentage of total markers analyzed which were rearranged in each case.
alterations at dinucleotide repeat loci than HNPCC tumors (3). These exhibited microsatellite instability at >45% (58 of 128) of loci analyzed. Evidence suggests that specific molecular defects and sequence homology has identified it as a member of the MSH defective GTBP (32, 33). Thus, GTBP represents the most recently defined criteria for identifying microsatellite instability in sporadic tumors.

In addition to those of the colon, a subset of other sporadic tumors also demonstrate microsatellite alterations, leading to the suggestion that defective mismatch repair may represent a general mechanism in tumorigenesis (12—24). However, many studies have not rigorously defined criteria for identifying microsatellite instability in sporadic carcinoma, and frequently tumors showing single or at least two alterations have been classified as RER⁺. In the present study, we investigated the occurrence of microsatellite alterations in 17 cases of sporadic esophageal adenocarcinoma and assessed the significance of low-level microsatellite alterations in sporadic carcinoma.

Each of the 17 tumors analyzed demonstrated microsatellite alterations in at least one marker analyzed. A single tumor (no. 123) exhibited microsatellite instability at >45% (58 of 128) of loci analyzed. In this case, the patient history showed no evidence of cancers in previous or subsequent generations. This widespread instability is comparable to that described in HNPCC-associated tumors and may reflect a similar defect in mismatch repair. Four genes involved in mismatch repair; hMSH2, hMLH1, hPMS1 and hPMS2 are mutated in the germ line of kindreds with HNPCC, and mutations in these genes occur somatically in sporadic carcinoma (7—11, 29).

The remaining 94% (16 of 17) of cases exhibited a relatively low level of microsatellite alterations, ranging from 0.8% (1 of 128) to 8.1% (10 of 123) of markers analyzed. The detection of a low level of microsatellite alterations at di- and tetranucleotide repeat sequences may reflect inactivation of a mismatch repair gene with a less severe phenotype than that exhibited in HNPCC. This has been observed in yeast, where inactivation of MSH2 leads to a less severe phenotype than MSH2 (30). More recently, a number of groups have demonstrated variability in the expression of a mutator phenotype in extracts of tumor cell lines. Evidence suggests that specific molecular defects underly this variability (31, 32). Extracts from cell lines deficient in hMSH2, hMLH1, and hPMS2 exhibited widespread alterations in both mononucleotide and dinucleotide repeat sequences, whereas extracts from cell lines deficient in GTBP demonstrated alterations primarily in mononucleotide repeat sequences, with few mutations detected in dinucleotide repeat loci (32). A heterodimer of hMSH2 and GTBP was shown to restore mismatch repair capability to hMSH2-deficient LoVo cells and also to MT1 and HCT 15 cells, both of which contain defective GTBP (32, 33). Thus, GTBP represents the most recently identified human gene involved in DNA mismatch binding and repair, and sequence homology has identified it as a member of the MSH family (33). Numerous studies have indicated that many sporadic tumors exhibit a quantitatively less severe phenotype of microsatellite alterations at dinucleotide repeat loci than HNPCC tumors (3). These include bladder, breast, lung (small cell lung cancer and non-small cell lung cancer), stomach, and esophagus (Refs. 15—24; Table 1). It remains to be established whether these tumors are defective in GTBP or a similar gene, resulting in destabilization of mononucleotide repeat sequences and DNA sequences other than dinucleotide and tetranucleotide repeat loci.

Alternatively, the detection of low-level microsatellite alterations in human tumors may simply reflect the inherent instability of the highly polymorphic markers analyzed. Studies on neoplasms unrelated to the Lynch syndrome have indicated that when analyzed with a range of 12—22 markers, approximately 8% of all cancers exhibited instability at a single microsatellite locus. It was estimated that the incidence of a novel allele was 0.5% for a dinucleotide marker and 0.5%—2.5% for tri- and tetranucleotide markers (22, 34). Tetranucleotide repeat sequences comprised 59% (82 of 139) of the markers analyzed in the present study, whereas dinucleotide and dinucleotide repeat sequences accounted for 4% (5 of 139) and 37% (52 of 139) of markers, respectively. The mutation rate of short tandem repeat sequences varies at different loci. It has been reported that the baseline mutation rate at tetranucleotide repeat sequences is 4-fold higher than that at dinucleotide repeat sequences (35). Furthermore, a number of tetranucleotide repeat sequences demonstrate very high mutation rates (35, 36). Excluding the data for tumor no. 123, which is most likely to have a specific biochemical defect in DNA mismatch repair, the average incidence of a novel allele was 1.6% (12 of 753 genotypes) at dinucleotide repeat loci and 5.2% (65 of 1253 genotypes) at tetranucleotide repeat loci. Therefore, in these 16 tumors, tetranucleotide repeat loci demonstrated a 3.3-fold higher incidence of novel alleles than dinucleotide repeat loci. Statistical analysis indicated that this difference in mutation rates was highly significant (χ², P < 0.001). This higher incidence of somatically occurring novel alleles at tetranucleotide repeat loci compares directly with the reported higher incidence of germ line mutations at these loci. Therefore, the low level of microsatellite alterations (0.8% (1 of 128) to 8.1% (10 of 123)) detected in the majority of tumors analyzed in the present study may be due to the inherent instability of these markers, reflecting both the large number of markers studied and the fact that these comprised a majority of tetranucleotide repeat loci. These infrequent alterations may not result from a specific biochemical defect; rather, they may serve as an indicator of relaxed genomic stability and of an increased susceptibility to mutation throughout the cancer cell genome. In the case of tumor no. 123, which exhibited ubiquitous somatic alterations at microsatellite alleles, there was no significant difference in the incidence of novel alleles at di- and tetranucleotide repeat loci (48% (21 of 44) and 43% (34 of 79), respectively). One potential interpretation of these findings is that microsatellite alterations resulting from a biochemical defect in mismatch repair do not exhibit preferential mutability at tetranucleotide repeat loci, unlike those resulting from the inherent instability of these markers. These observations, if confirmed by other studies, may have implications for the discernment of an RER⁺ phenotype in sporadic carcinoma.

A previous report indicated that microsatellite instability occurred in 22% (8 of 36) of Barrett’s-associated adenocarcinomas. These tumors were analyzed for instability at five loci. Of the eight tumors demonstrating instability, 62.5% (5 of 8) demonstrated microsatellite instability at a single locus. A single case exhibited instability at 2 of 5 loci, whereas the remaining two tumors exhibited instability at 4 of 5 and 5 of 5 loci, respectively (24). These data support the present observation that microsatellite instability exhibits variable phenotypic expression in esophageal adenocarcinoma.

In summary, this study has demonstrated that the RER⁺ phenotype, defined as ubiquitous somatic alterations in microsatellite alleles, occurs in a relatively small subgroup of esophageal adenocarcinomas. However, the majority of tumors demonstrate a low level of micro-

Table 2  Incidence of novel alleles at short tandem repeat sequences in esophageal adenocarcinoma (n = 17)

<table>
<thead>
<tr>
<th>Size of repeat unit</th>
<th>Sample no. 123a</th>
<th>Remaining 16 samplesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinucleotide</td>
<td>21/44 (48)</td>
<td>12/753 (1.6)</td>
</tr>
<tr>
<td>Trinucleotide</td>
<td>3/5</td>
<td>0/75</td>
</tr>
<tr>
<td>Tetranucleotide</td>
<td>34/79 (43)</td>
<td>65/1253 (5.2)</td>
</tr>
<tr>
<td>Combined</td>
<td>58/128 (45.3)</td>
<td>77/2081 (3.7)</td>
</tr>
</tbody>
</table>

a No significant difference in the incidence of novel alleles at di- and tetranucleotide repeat loci; χ², P > 0.05.

b Significant difference in the incidence of novel alleles at di- and tetranucleotide repeat loci; χ², P < 0.001.

MICROSATELLITE INSTABILITY IN BARRETT’S ADENOCARCINOMA

Data downloaded from cancerres.aacrjournals.org on April 15, 2017. © 1996 American Association for Cancer Research.
satellite alterations at dinucleotide and tetranculotide repeat loci. The specific biochemical effects underlying the heterogeneous expression of the instability phenotype in these tumors remain to be elucidated. The relative frequency of novel alleles at di- and tetranculotide repeat loci in the single case with ubiquitous somatic alterations differed from that in cases exhibiting a low level of microsatellite alterations. It remains to be established if this observation will aid the accurate identification of an RER+ phenotype in sporadic carcinoma.

References
Ubiquitous Somatic Alterations at Microsatellite Alleles Occur Infrequently in Barrett’s-associated Esophageal Adenocarcinoma

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Cancer Res 1996;56:259-263.

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