Deletion Mapping in Squamous Cell Carcinomas of the Esophagus Defines a Region Containing a Tumor Suppressor Gene within a 4-Centimorgan Interval of the Distal Long Arm of Chromosome 9

Koh Miura, Kohei Okita, Yoichi Furukawa, Seiki Matsuno, and Yusuke Nakamura

Abstract

Recent studies in our laboratory indicated that inactivation of a putative tumor suppressor gene on chromosome 9q is likely to be associated with an early step of esophageal carcinogenesis. To further define a region containing the putative tumor suppressor gene, we have examined loss of heterozygosity in 37 esophageal squamous cell carcinomas using 14 microsatellite markers mapped to 9q31-q34.1. Loss of heterozygosity was observed in 30 (81%) of 37 tumors at one or more of the loci examined, and partial or interstitial deletions at 9q31-q34.1 were detected in 13 of these tumors. On the basis of these results, we constructed a detailed deletion map and defined a commonly deleted region between the D9S154 loci at 9q31-q32. The genetic distance between these two loci is estimated to be approximately 4 cM.

Introduction

Recent studies based on molecular biology have clarified some basic mechanisms of carcinogenesis. Like cancers in other organs, ESCs are thought to result from accumulation of certain genetic alterations that include amplifications of the c-myc, epidermal growth factor receptor (1), and cyclin D genes (2). The involvement of one or more tumor suppressor genes on chromosome 9q, as well as the p53 and RB1 genes, in ESC has also been suggested (3, 4). In allelotyping studies of ESCs, we and others have observed high frequencies of LOH on chromosomal arms 3p, 9q, 9q, 17p, and 17q with RFLP markers (5, 6), implying the presence of tumor suppressor genes on these chromosomal arms. Furthermore, during LOH analysis of these candidate loci in ESCs at various clinicopathological stages, we detected a high frequency of LOH for polymorphic loci on 9q31 in tumors at an early stage of esophageal carcinogenesis, even in low-grade dysplastic lesions (7).

Human bladder tumors, originating mainly from stratified epithelia, and squamous cell carcinomas of the lung, head, and neck have also shown frequent LOH on 9q in recent studies (8). The combined evidence suggests that a tumor suppressor gene at 9q31 is likely to be associated with development of squamous cell carcinomas in various tissues. To further define this candidate locus, we screened 37 primary ESCs using 14 microsatellite markers and constructed a detailed deletion map of the distal long arm of chromosome 9.

Tumor Samples. We selected 37 tumor samples from 93 ESCs which had been previously analyzed for LOH by RFLP markers (5). These 37 tumor specimens were considered to be less contaminated with normal cells than the others, because the loss of one allele was unequivocal in each of them. Of the 37 patients from whom the tissues were derived, 20 were Chinese who lived in an area with a high incidence of esophageal cancer (9); they had undergone surgery at the Fourth Affiliated Hospital of Hebei Medical College (Hebei, China). The other 17 were Japanese patients who had undergone surgery at the Cancer Institute Hospital (Tokyo, Japan) or at Tohoku University Hospital (Miyagi, Japan). All 37 tumors were histopathologically diagnosed as squamous cell carcinomas. Paired DNA samples from tumors and adjacent normal tissues were frozen in liquid nitrogen immediately after resection. Extraction of DNA from tissue samples was carried out later according to methods described previously (10).

LOH Analysis. All of the marker names used for detection of LOH in this study are listed in Table 1 (11). Each PCR was performed in a 12.5-μl mixture containing 40 ng of genomic DNA; 20 pmol each of primer (one of them was end labeled with [γ-32P]ATP); 500 μM each of dATP, dGTP, dCTP, and dTTP; and 0.125 unit Taq DNA polymerase (Boeringer Mannheim). The mixture was subjected to 40 PCR cycles, each consisting of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final 3-min extension time at 72°C. For some primers, the annealing temperature was increased to 64°C on the basis of preliminary PCR results, to eliminate nonallelic bands. Loading buffer was added to each product before it was heat-denatured and electrophoresed in 6% denaturing polyacrylamide gel. Gels were dried and exposed to Fujix-XR film overnight.

Allelic loss was scored if the intensity of one allele was at least 50% reduced in the tumor DNA as compared with the corresponding normal DNA. In almost all cases that either were not informative or were ambiguous for LOH with the microsatellite markers, PCR was repeated once or more until the results were ascertained to be reliable.

FISH. We determined the order and chromosomal localization of four loci (D9S155, D9S177, D9S170, and D9S154) by the multicolor FISH method (12). In brief, metaphase chromosomes, prepared by the thymidine synchronization/bromodeoxyuridine release technique (13), were used for hybridization with biotin- and digoxigenin-labeled YAC probes. Fluorescent signals were enhanced by avidin-FITC or antidigoxigenin rhodamine.

Results

Allelic losses involving at least 1 of the 14 microsatellite loci on 9q31–34.1 were detected in 30 (81%) of the 37 esophageal tumors. Locus symbols, markers, and frequencies of LOH are summarized in Table 1. High frequencies of LOH were observed at D9S155 (71%), D9S177 (68%), and D9S282 (68%), but frequencies of LOH were relatively low at D9S266 (33%) and D9S260 (31%), both of which are located in the distal portion of 9q32. Among the 30 tumors which lost an allele for at least one locus, 13 revealed a partial or interstitial deletion of 9q31–34.1. Fig. 1 shows some examples of autoradiographs revealing interstitial deletions. In tumor 113, both D9S262 and D9S154 showed LOH, but retention of both alleles at each of three other loci (D9S172, D9S275, and D9S282) lying outside the interval.

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3 The abbreviations used are: ESC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; FISH, fluorescence in situ hybridization; YAC, yeast artificial chromosome; cM, centimorgan; MSSE, multiple self-healing squamous epithelioma.
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defined by D9S262 and D9S154 indicated an interstitial deletion between D9S172 and D9S275. Similarly, LOH analysis revealed an interstitial deletion between D9S172 and D9S154 in tumor 211.

The order and orientation of D9S155, D9S177, D9S170, and D9S154 were critical for defining the commonly deleted region. For prior evidence that YAC867e8 contains D9S155 and D9S177, that YAC774b10 contains D9S177 and D9S170, and that YAC954d2 contains D9S170 and D9S154 (Genome Interactive Databases, Vol.1, 1994), we were able to conclude that the order of the four clones is D9S155-D9S177-D9S170-D9S154, assuming that the human inserts in each YAC are complete and not partially deleted. Then, to determine the orientation of these loci, we undertook FISH analysis using two YAC clones (867e8 and 954d2) as probes; YAC867e8 hybridized proximally to YAC954d2 (data not shown). Hence, the order and direction of the four loci were determined as centromere-D9S155-D9S177-D9S170-D9S154-telomere.

The results of LOH analyses in the 13 tumors showing partial or interstitial deletions are summarized schematically in Fig. 2. For example, tumor 3 showed LOH at the D9S282 locus, but not at the D9S262; a partial chromosomal loss between the D9S172 and D9S260 loci was confirmed in this case. On the basis of these mapping data, the commonly deleted region was defined between microsatellite marker loci D9S262 and D9S154 at 9q31-32; the interval of them represented a genetic distance of approximately 4 cM (11).

![Fig. 2. Schematic representation of LOH distribution on chromosomal arm 9q in esophageal squamous cell carcinomas. Case numbers are shown above, and locus symbols on the left. (L) LOH; (R) retention of both alleles; no symbol, noninformative. Genetic distance (cM) under the assumption of sex-averaged recombination estimates were obtained from Ref. 11. * order of the four loci was determined based on Genome Interactive Data bases and FISH methods. The commonly deleted region is indicated by a dotted rectangle.](image)

**Table 1 Allelic loss at loci on chromosome arm 9q31-34.1 in esophageal carcinoma**

<table>
<thead>
<tr>
<th>Locus</th>
<th>Marker</th>
<th>Locus symbol</th>
<th>Chromosome location</th>
<th>Allelic losses/informative cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AMF263f1</td>
<td>D9S271</td>
<td>9q31</td>
<td>15/23 (65)</td>
</tr>
<tr>
<td>2</td>
<td>AMF199zr10</td>
<td>D9S172</td>
<td>9q32</td>
<td>10/19 (53)</td>
</tr>
<tr>
<td>3</td>
<td>AMF297w1b1</td>
<td>D9S279</td>
<td>9q32</td>
<td>12/20 (60)</td>
</tr>
<tr>
<td>4</td>
<td>AMF211w9c9</td>
<td>D9S262</td>
<td>9q32</td>
<td>18/28 (64)</td>
</tr>
<tr>
<td>5</td>
<td>AMF402z3a4</td>
<td>D9S155</td>
<td>9q32</td>
<td>15/21 (71)</td>
</tr>
<tr>
<td>6</td>
<td>AMF234z5c</td>
<td>D9S177</td>
<td>9q32</td>
<td>17/25 (66)</td>
</tr>
<tr>
<td>7</td>
<td>AMF164ya11</td>
<td>D9S170</td>
<td>9q32</td>
<td>7/15 (47)</td>
</tr>
<tr>
<td>8</td>
<td>AMF029y5</td>
<td>D9S154</td>
<td>9q32</td>
<td>16/24 (67)</td>
</tr>
<tr>
<td>9</td>
<td>AMF185x3</td>
<td>D9S258</td>
<td>9q34.1</td>
<td>12/18 (67)</td>
</tr>
<tr>
<td>10</td>
<td>AMF266yc5</td>
<td>D9S275</td>
<td>9q34.1</td>
<td>12/23 (58)</td>
</tr>
<tr>
<td>11</td>
<td>AMF308vb1</td>
<td>D9S282</td>
<td>9q34.1</td>
<td>15/22 (68)</td>
</tr>
<tr>
<td>12</td>
<td>AMF225y62</td>
<td>D9S266</td>
<td>9q34.1</td>
<td>2/6 (33)</td>
</tr>
<tr>
<td>13</td>
<td>AMF296xa9</td>
<td>D9S260</td>
<td>9q34.1</td>
<td>4/13 (31)</td>
</tr>
<tr>
<td>14</td>
<td>AMF077xa9</td>
<td>D9S159</td>
<td>9q34.1</td>
<td>15/26 (58)</td>
</tr>
</tbody>
</table>

* All of the markers are as described by Gyapay et al. (11), and this reference also provides information about marker position relative to selected markers from the Centre d’Etudes du Polymorphisme Humain data base.

* Chromosome location of each marker is referred from Ref. 11.

Fig. 1. Analysis of ESCs for LOH at microsatellite loci on 9q31-34. a, case 113; b, case 211. Left lanes, DNA from corresponding normal tissues (N); right lanes, DNA from tumor tissues (T). LOH (L) or retention of both alleles (R), above each pair of lanes; locus symbols, below the lanes; arrows, polymorphic bands. Both a and b show interstitial deletions.

**Discussion**

In our previous studies of ESCs using polymorphic DNA markers, we found frequent allelic losses on several chromosomal arms; the most frequent LOH was observed at the D9S17 locus on chromosomal band 9q34 (76%) (5). LOH at this locus seemed to occur at a very early stage of esophageal carcinogenesis, even in low-grade dysplasia (7). In the present study, we investigated this chromosomal arm in detail to define the region containing the putative tumor suppressor gene. On the basis of deletion mapping, we defined a critical region between the D9S262 and D9S154, loci that are approximately 4 cM apart on 9q31-32 (11).

Frequent allelic losses on 9q have also been found in early-stage bladder carcinomas (14, 15), and detailed deletion mapping has assigned a candidate locus to chromosomal band 9q34.1-9q34.2 (8). Furthermore, genetic analysis of squamous cell carcinomas of the lung...
revealed frequent LOH on chromosomal arm 9q (67%, 12 of 18 informative cases; Ref. 16). These results suggested to us that inactivation of a tumor suppressor gene on 9q might play a significant role in the development of squamous cell carcinomas of the esophagus as well as cancers of the bladder and lung.

Moreover, genes responsible for two cancer-predisposition syndromes were recently localized to the distal long arm of chromosome 9 by linkage analysis of families carrying these diseases. Nevoid basal cell carcinoma syndrome (or Gorlin syndrome; Ref. 17), which is characterized by multiple basal cell carcinomas and dental malformations, bifid ribs, cleft lips, kidney malformations and/or other developmental defects, was localized to an approximately 2-cM region between D9S196 and D9S180 on 9q22.3-q31 (18). The gene responsible for MSSE, an autosomal dominant disease characterized by locally invasive multiple skin tumors (19), was also mapped to chromosome 9q22-q31 (20). It is unclear whether the commonly deleted region we have detected at 9q31–32 in ESCs detected in this study is the same as the region containing genes responsible for nevoid basal cell carcinoma syndrome or MSSE. However, since the tumors characteristic of MSSE, like ESCs, originate from squamous epithelial cells, it is conceivable that the gene in this region may be associated with the development of squamous cell carcinomas in various tissues.

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References

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