Detailed Deletion Mapping in Squamous Cell Carcinomas of the Esophagus Narrows a Region Containing a Putative Tumor Suppressor Gene to about 200 Kilobases on Distal Chromosome 9q

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ABSTRACT

We previously reported definition of a region containing a putative tumor suppressor gene for esophageal squamous cell carcinoma within an ~4-cM genomic segment at 9q31-q32. We have investigated this region further using six new microsatellite markers isolated from yeast artificial chromosome clones covering the deleted region and have narrowly defined the commonly deleted region to a segment between two loci, KM9.1 and D9S177. On the basis of the contig map of cosmid and yeast artificial chromosome clones, we estimate the physical size of the region of interest to be about 200 kb. Because the distal 9q region also has been implicated as the site of a tumor suppressor gene(s) related to squamous cell carcinomas of other tissues, our map provides useful information for attempts to identify a common gene for carcinomas of this cell type.

INTRODUCTION

ESCs1 are the sixth most common type of cancer among the male population in Japan, and the number of people who die of ESC is increasing significantly (1). Because its clinicopathology is severe, a better understanding of the genetic mechanism of esophageal carcinogenesis is critical for development of new approaches to chemoprevention or treatment of ESCs. Like other cancers, ESCs result from accumulation of deleterious alterations in growth-regulating genes; these changes often include amplifications of the c-myc, epidermal growth factor receptor (2), and cyclin D genes (3). The involvement of one or more tumor suppressor genes on chromosome 9q as well as the p53 and RBl genes has also been implicated in ESCs (4, 5). To further clarify the carcinogenesis of ESC, we have been investigating these genes as well as other genetic alterations in this type of tumor. With RFLP markers, we documented frequent allelic losses on chromosomal arms 3p, 5q, 9p, 9q, and 17p (6), the most frequent LOH being observed at 9q34, even in low-grade dysplasias (7).

Allelic losses on 9q have also been found in many early stage bladder carcinomas (8, 9), and detailed deletion mapping in those tumors has assigned a candidate locus to chromosomal band 9q34.1–9q34.2 (10). Furthermore, genetic analyses of SCCs of the lung and of the head and neck have disclosed frequent LOH on the long arm of chromosome 9q (11–14). These results suggested to us that inactivation of a tumor suppressor gene on 9q is likely to play a significant role in development of SCCs of the esophagus as well as of the other tissues.

Toward positional cloning of the putative tumor suppressor gene on 9q, we constructed a deletion map on chromosome 9q in ESCs and defined a commonly deleted region within an interval of ~4 cm at 9q31–9q32 (15). In the study reported here, we investigated the commonly deleted region in more detail by constructing a YAC contig covering the region and analyzing LOH using microsatellite markers isolated from the contig. We have been able to narrow the region of interest to a 200-kb interval.

MATERIALS AND METHODS

Preparation of YAC DNAs and Construction of Cosmid Libraries. On the basis of information available in Genome Interactive Databases, Vol. 1, 1994, we selected four Centre d’Etude du Polymorphisme Humain YAC clones that were likely to span the commonly deleted region on 9q31–9q32. High-molecular-weight DNAs were isolated from yeast spheroplasts by sucrose-density gradient centrifugation (16). Cosmid libraries were constructed from these YAC DNAs according to methods described elsewhere (17). A total of 200–250 cosmid clones (about five-genome equivalents), each of which contained ~35–42 kb human DNA, were selected from each YAC by hybridization with [a-32P]dCTP-labeled total human DNA. The cosmid clones were inoculated into 96-well microtiter plates, and their DNAs were extracted by means of an automatic plasmid system (PI-100; Kurabo).

Construction of a YAC Contig Spanning the Commonly Deleted Region. Cosmid clones that contained DNAs corresponding to the ends of the human insert in YACs (cosmid end clones) were selected by hybridization with [y-32P]dATP-end-labeled oligonucleotides L0522 (5’-CGGTAGCCAAAGTTGGTTTAAGG-3’) or R0521 (5’-AGTCGAACGCCCGATCTCAAG-3’); each of these probes corresponds to part of the TRP or URA region of the YAC vector pYAC4. To develop a YAC contig spanning the deleted region on 9q31–9q32, the end clones or several randomly selected clones from each YAC were used as probes for hybridization with EcoRI-digested YAC DNAs. If a cosmid clone hybridized with two or more YACs, those YACs were ascertained to overlap (Fig. 1a).

Isolation of Microsatellite Markers and LOH Analysis. To isolate cosmids that contained (CA)n, repetitive sequences, cosmid clones isolated from the YACs were hybridized with [y-32P]dATP-end-labeled (CA)n probes. DNAs of (CA)n-positive cosmid clones were extracted, and their nucleotide sequences were determined by the dideoxynucleotide termination method (18). As described previously (19), PCR primers on either side of each (CA)n repetitive sequence were designed (Table 1). PCR analyses for LOH were performed (15) for 13 ESC samples known to have partial deletions of chromosome 9q and for 5 other ESC samples in which both alleles had been retained at all the loci examined in our previous study (15). LOH was scored if the intensity of one allele was at least 50% reduced in the tumor DNA as compared with the corresponding normal DNA.

To examine precise locations of the newly isolated microsatellite marker loci on the YAC contig map, each cosmid DNA from which the respective microsatellite was isolated was used as the probe for hybridization with EcoRI-digested YAC DNAs (Fig. 1b). To further determine the order of the isolated markers, we constructed a cosmid contig map by a method reported previously (20). In brief, sets of Southern blots of EcoRI-digested cosmid DNAs from YAC B67e8 were prepared. A cosmid clone corresponding to one of the markers was selected as a probe for the first hybridization. Cosmids that shared one or more bands with the probe were considered contiguous (Fig. 1c). Then, one of the contiguous clones was used as the probe for the next Southern

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1 The abbreviations used are: ESC, esophageal squamous cell carcinoma; LOH, loss of heterozygosity; YAC, yeast artificial chromosome; FISH, fluorescence in situ hybridization; SCC, squamous cell carcinoma.

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Table 1 Characteristics of newly isolated microsatellite markers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer sequences (CA/CT strand)</th>
<th>Size range (bp)</th>
<th>No. of alleles</th>
<th>Allelic losses/informative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>KM9.1</td>
<td>GAGATGCAGGCCAAGCTAAAAAG</td>
<td>106–118</td>
<td>6</td>
<td>9/14</td>
</tr>
<tr>
<td>KM9.2</td>
<td>AAACAAACAAAGGATAAATCATC</td>
<td>93–113</td>
<td>8</td>
<td>7/11</td>
</tr>
<tr>
<td>KM9.3</td>
<td>GTCTCCACTCCTGGAGAAG</td>
<td>225–235</td>
<td>6</td>
<td>9/12</td>
</tr>
<tr>
<td>KM9.4</td>
<td>TCAAGGCTTCTAAATGATTGGAAG</td>
<td>82–94</td>
<td>3</td>
<td>7/10</td>
</tr>
<tr>
<td>KM9.5</td>
<td>AGTAGACCTTTCTGATGAGCA</td>
<td>79–91</td>
<td>6</td>
<td>7/12</td>
</tr>
<tr>
<td>KM9.6</td>
<td>CAATGGATACGGCCTCATCG</td>
<td>198–208</td>
<td>4</td>
<td>5/10</td>
</tr>
</tbody>
</table>

* These data were determined by observation of samples in this study.

Fig. 2. Schema of the YAC contig spanning the deleted region on 9q31–32 and the localization of isolated microsatellite markers. Genetic distances (cM) under the assumption of sex-averaged recombination estimates were obtained from a study by Gyapay et al. (33). Isolated end clones are indicated. Locations of the six newly isolated markers are indicated below the contig.

**FISH Analysis.** A multi-color FISH method (21) was applied for physical ordering of adjacent cosmid clones on 9q. To resolve DNA loci that were close together, interphase chromosomes were used for hybridization with biotin- and digoxigenin-labeled cosmid probes. Fluorescent signals were enhanced by avidin-FITC or antidigoxigenin rhodamine.

**RESULTS**

To construct a YAC contig map spanning the target region on 9q31–32, we selected four YAC clones, each of which contained multiple loci corresponding to microsatellite markers known to lie in the commonly deleted region. PCR analyses using each of the four YAC DNAs as a template confirmed that Centre d’Etude du Polymorphisme Humain clone YAC867e8 contains the D9S155 and D9S177 loci; YAC774bl0 and YAC798d3 both contain the D9S177 and D9S170 loci; and YAC954d2 contains the D9S170 and D9S154 loci (Fig. 2).

From each of four cosmid libraries constructed from the YAC-containing yeast DNAs, 200–250 cosmid clones were selected (about five-genome equivalents) by hybridization with labeled total human DNA. From these clones, we attempted to isolate the TRP- and URA-end clones by hybridization with oligonucleotide probes L0522 and R0521. Although end clones containing URA were identified for all four YACs, no TRP-end clones of YACs 954d2 and 774b10 were obtained. All of the end clones of 867e8, 798d3, and 954d2 were mapped on 9q31–32; however, the URA-end clone and five randomly selected clones from YAC774b10 were localized to 9q32, 9q32, or 10q15.3, implying that 774b10 is chimeric. The order and direction of YAC clones 867e8, 798d3, and 954d2 were determined by Southern hybridizations with the end clones or with several randomly selected clones from each YAC (Fig. 1a) and also by the FISH method (data not shown).

To further investigate LOH in the region covered by the YAC contig, we isolated five new microsatellite markers from 867e8 and one from 954d2 (Table 1). To determine precise locations of these hybridization. After repeated experiments, a contig map emerged, and the order of the markers was determined.

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markers, each cosmide clone from which a microsatellite marker was identified was used as a probe for hybridization with the four YAC DNAs (Fig. 1b). For example, the marker KM9.4 hybridized with YACs 774a10, 798d3, and 867e8, indicating its location within the overlapping portions of these three YACs (Fig. 2). To determine the linear orders of markers KM9.1 and KM9.2 and KM9.3, KM9.4, and KM9.5, we constructed a cosmid-contig map. On the basis of this map, the order of the markers was determined to be cen-KM9.1-KM9.2-D9S177-KM9.3-KM9.4-KM9.5-tel.

To better define the deleted region in ESCs, PCR analysis for LOH was performed using DNA from 18 tumors; in 13 of which we had previously detected interstitial or partial deletions; in the other five, our previous study had detected no LOH with markers on 9q (15). Fig. 3 shows some examples of autoradiographs revealing interstitial deletions. In tumor 211, the loci D9S155 and KM9.1 showed LOH, but retention of both alleles at D9S172 and KM9.5 indicated an interstitial deletion between those two loci (Fig. 3a). Of the five tumors in which both alleles had been retained in the earlier study, three also retained heterozygosity with all six newly isolated markers. However, in the other two tumors (cases 4 and 12), the analysis using the new markers disclosed a small interstitial deletion. For example, in tumor 4, the locus KM9.2 showed LOH; retention of both alleles at other loci (KM9.1, D9S177, and KM9.3) indicated an interstitial deletion between KM9.1 and D9S177 (Fig. 3b). Similarly, LOH analysis revealed an interstitial deletion between D9S262 and marker KM9.4 in tumor 12 (Fig. 3c).

The results of LOH analyses in the 15 tumors showing partial or interstitial deletions are summarized schematically in Fig. 4. On the basis of these mapping data, the commonly deleted region was defined between microsatellite loci KM9.1 and D9S177; the interval between them represented a physical distance of approximately 200 kb, on the basis of information inferred from the cosmid-contig map.

**DISCUSSION**

When we and others investigated LOH in ESCs using polymorphic DNA markers, frequent observations of allelic losses on chromosomal arms 3p, 5q, 9p, 9q, 13q, and 17p (6, 7, 22) suggested that tumor suppressor genes associated with development or progression of ESCs are present on these chromosomal arms. Furthermore, during LOH analysis of these candidate loci in various stages of esophageal tumors, 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 dysplasia (7).

A high frequency of LOH on 9q has also been reported in cancers of organs other than the esophagus. In bladder cancers, LOH on 9q is frequently observed; 45% at 9q34.1-q34.3 (10) and 60% at D9S7 (9q34) (9). Frequent allelic losses on 9q have also been found in early stage bladder carcinomas (8, 9). Other studies have identified several candidate loci of tumor suppressor genes for bladder tumors by deletion mapping of tumors at 9q (23, 24); one of those candidate loci is assigned to the telomeric band of 9q (4-2, 24). Because human bladder cancer originates mainly from the transitional epithelium, which is classified into stratified tissues such as the squamous epithelium, carcinogenic mechanisms may be similar in the bladder and esophagus. In SCCs of the lung, the frequent loss of 9q is also reported (11-13); among lung cancers, LOH on 9q is reported more frequently in SCCs than in adenocarcinomas (12, 13); therefore, LOH on 9q is considered important in the development of SCC. Furthermore, Sato et al. (12) observed frequent allelic losses distal to 9q21.1 in SCCs of the lung and concluded that putative tumor suppressor genes associated with SCC of the lung should be located there (12). Considering that the bronchus and esophagus are both derived from the foregut, allelic loss on 9q may play an important role in the tumorigenesis of these developmentally related tissues. In head and neck cancers, which originate mainly from squamous epithelia, as do ESCs, a high incidence of LOH is reported on 9q22.1-34 (35%), 3p (44%), 5q (43%), 11q (45%), and 17q (31%) (14). Esophageal cancers share not only histological features with head and neck cancers, but also epidemiological and clinical characteristics (1, 25).

Genes responsible for two cancer predisposition syndromes were recently localized to the distal long arm of chromosome 9 by linkage analysis. Nevoid basal cell carcinoma syndrome (or Gorlin syndrome; Ref. 26), which is characterized by multiple basal cell carcinomas and diverse developmental defects, was localized to a ~2-cM region between D9S196 and D9S180 on 9q22.3-q31 (27) and by others between D9S196 and D9S109 in the 9q22.3-q31 region (28) or in a 2.6-cM interval on 9q22.3 (29). The gene responsible for multiple self-healing squamous epitheliomata (ESS1), an autosomal dominant disease characterized by locally invasive but spontaneously self-healing multiple skin tumors (30), was also mapped to 9q22-q31 (31). In the present study, to investigate in more detail the region of
9q31−32 that is commonly deleted in ESCs, we isolated six new microsatellite markers in the region, constructed a more precise deletion map, and narrowed the commonly deleted region to an estimated physical distance of 200 kb between the marker KM9.1 and the D9S177 locus. According to the linkage map presented at the Second International Workshop in 1993 (32, 33), the region containing genes responsible for Gorlin syndrome and multiple self-healing squamous epitheliomata was considered proximal to our candidate locus on 9q32. Nevertheless, because allelic loss of the distal region of 9q is so frequently observed in tumors of several developmentally related tissues, including esophagus, urinary bladder, and lung, our contig map will provide useful information for attempts to identify a tumor suppressor common to SCCs. Isolation of the gene should yield important clues for resolving the mechanism of carcinogenesis in organs affected by this type of tumor.

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