

Mutational and LOH Analyses of the Chromosome 4q Region in Esophageal Adenocarcinoma

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Key Words

Cancer · CARF · Chromosome 4q · Esophagus ·
hCDC4 · LOH · Mutation

Abstract

Objective: Mortality due to esophageal adenocarcinoma has risen markedly, but the molecular mechanisms underlying this carcinogenesis are still incompletely understood. Findings from loss of heterozygosity (LOH) studies have suggested that the long arm of chromosome 4 might harbor tumor suppressor genes relevant to esophageal adenocarcinoma. **Methods:** We performed LOH analysis of 4q in esophageal adenocarcinomas. Regions of LOH were further evaluated by studying two candidate tumor suppressor genes, *hCDC4* and *CARF*, located within them. **Results:** 54% of the adenocarcinomas examined showed allelic deletion. LOH was observed in 53, 40, 32, 38, and 27% of tumors at positions D4S1554 (the locus of *CARF*), D4S1572, D4S1548, D4S2934, and D4S3021, respectively. An area of allelic deletion (spanning 3 mil-

lion bases) was identified at 4q31.1–3 in 37% of tumors. This region harbors a candidate tumor suppressor gene: *hCDC4*. However, sequencing of the coding regions of *CARF* and *hCDC4* at 4q35 and 4q31, respectively, did not identify mutations. **Conclusions:** Our findings demonstrate frequent LOH in esophageal adenocarcinoma at several loci including a novel area of allelic deletion at 4q31.1–3. The results imply that mutational or other alterations at these loci may be involved in the pathogenesis of esophageal adenocarcinoma. Candidate tumor suppressor genes located within these regions merit further study.

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Introduction

The incidence of esophageal adenocarcinoma has risen markedly over the past two decades, now being more prevalent than squamous cell cancer in Western Europe and in the United States. Despite advances in diagnostic and surgical techniques, and improved pre- and postoperative care, the mortality from esophageal adenocarcinoma is increasing, and the prognosis remains poor. Gas-

A.S. and T.K. contributed equally to this work.

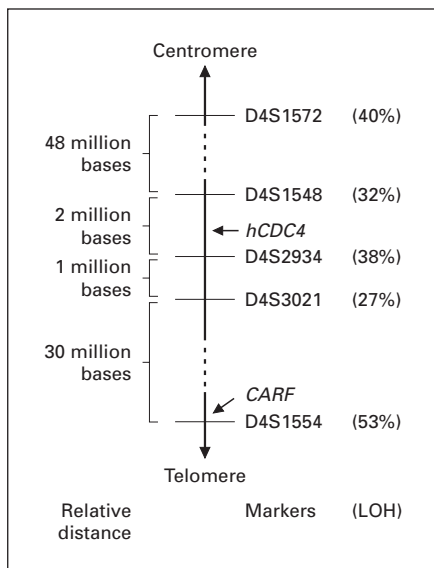


Fig. 1. Map of chromosome 4q with the relative positions of microsatellite markers. Five microsatellite markers were used on chromosome 4q. Arrows indicate the positions of *CARF* and *hCDC4* genes relative to the markers. Percentage of LOH at each locus in esophageal adenocarcinoma is indicated in the parentheses next to the markers.

oesophageal reflux disease and its sequella, Barrett's esophagus, are the major recognized risk factors for esophageal adenocarcinoma.

The molecular mechanisms underlying the carcinogenesis in this tumor type are still incompletely understood. Previous studies using comparative genomic hybridization found that genetic losses on chromosome 4 are a common occurrence [1, 2]. Allelotyping indicated loss of heterozygosity (LOH) on chromosome 4q in numerous malignancies including colorectal, hepatocellular, transitional bladder, and lung carcinomas [3–6]. Introduction of human chromosome 4 into various immortalized cell lines resulted in loss of proliferation and reversal of the immortal phenotype [7–9]. These findings suggested that the long arm of chromosome 4 may harbor one or more tumor suppressor genes.

Therefore, we investigated the frequency of LOH at several loci on the long arm of chromosome 4 in esophageal adenocarcinoma and evaluated two candidate genes, *hCDC4* and *CARF*, as targets of allelic loss by testing them for point mutations. *hCDC4* (also known as *Fbxw7* or *Ago*) is a putative tumor suppressor gene that encodes an F-box protein and has been found to be mutated in a number of primary cancers and cancer-derived cell lines

[10–16]. *CARF* (collaborates/cooperates with *ARF*), a serine-rich nuclear protein, was identified as a potential interacting partner of *ARF* in the *ARF-MDM2-p53* pathway [17]. New evidence shows that *CARF* may have *ARF*-independent tumor suppressor function by directly interacting with the p53 protein [18].

Materials and Methods

Tissue Samples

27 esophageal cancer and matching normal tissue specimens were obtained by endoscopic or surgical resection performed at the University of Maryland Medical Center, Baltimore, Md., USA. All patients gave informed consent. The specimens were immediately frozen in liquid nitrogen and stored at -80°C . Genomic DNAs from normal and tumor samples were extracted using standard protocols.

Loss of Heterozygosity Analysis

Allelic losses on chromosome 4q were evaluated using 5 polymorphic markers: D4S1572, D4S1548, D4S2934, D4S3021, and D4S1554. Information about these loci and primer sequences were obtained from the Genome Database (www.gdb.org). Three markers (D4S1548, D4S2934, and D4S3021) closely flank the *hCDC4* locus (4q31.2). D4S1554 is present at the *CARF* locus (4q35.1, fig. 1). PCR was carried out as described previously [19]. PCR products were separated on a LongRanger polyacrylamide gel for the ABI PRISM 377 (PE Applied Biosystems, Foster City, Calif., USA) with a labeled marker (TAMRA 500) as an internal size standard. GeneScan 3.1 and Genotyper 2.1 software (PE Applied Biosystems) were used for LOH scanning and analysis. In constitutional heterozygotes, when the peak area for one allele in tumor DNA decreased to less than 50% of the peak area for the corresponding allele in matching normal DNA, the tumor was read as LOH positive. Specimens that presented LOH at the *hCDC4* or *CARF* locus were further investigated for mutations.

Mutational Study

hCDC4- and *CARF*-coding exons were amplified from genomic DNA by PCR using exon-specific primers selected in the flanking intron sequences. The sequences of primers for exons 2–10 of the *hCDC4* gene had been published in a recent paper [16]. This gene presents alternative splicing at its 5'-end, generating three variants (NM_033632, NM_018315, and NM_001013415) with a different sequence of the first exon. The newly designed primers are listed in table 1. Exon 3 of *CARF* and exon 1 (variant NM_033632) of *hCDC4* required amplification in two or more overlapping fragments due to their extended size.

PCR reactions were carried out in a final volume of 25 μl with 40 ng of genomic DNA as template. The buffer contained 5 pmol of forward and reverse primers, and 12.5 μl of Accuprime Supermix I (Invitrogen, Carlsbad, Calif., USA). Cycling parameters were as follows: activation for 30 s at 96°C ; PCR throughout 35 cycles, at 94°C for 45 s, at 55°C for 45 s and at 72°C for 60 s. The final extension was carried out at 72°C for 5 min. PCR products were purified and then sequenced by DYEEnamic ET terminator chemistry (Amersham Biosciences, Little Chalfont, UK) using the manufacturer's

Table 1. Primers used in the mutation studies of *CARF* and *hCDC4* genes

Exon	Forward primer (5'–3')	Reverse primer (5'–3')
<i>CARF</i>		
Exon 1	GTTTGGTCTTTAGGCCTGCG	ATTCCC CGCTTCACA ACT
Exon 2	CACTTTCTTGATTGCAGCCA	GGACTTCAA AATCTAAAGCAGTC
Exon 3-1	GAAATGTTTTTCACTTTGTGCCTTTA	TTTAGGTGATCCACTTTGTGTCTAG
Exon 3-2	CCAGTGTGAATAGTCACATGACCC	AACAGCTACCAACTTCCATGCC
Exon 3-3	GTACTTCACAGTCAAGTGAGAGTTCTG	AATTTGTGAAAGCCTGTGTTTTATACTA
<i>hCDC4</i>		
Exon 1 (NM_018315)	CTGCATTGCTGAATCCTGGA	AATTAGAGGATACTGCAGCCATCTCT
Exon 1-1 (NM_033632)	AATGCCTTGGTGGCATCAATAC	TCCTCTTGTTCCTTCTTGGTTTCCT
Exon 1-2 (NM_033632)	GGAGTAGAACCTAGACCTGGAGGC	CCATTTGTACTCAGATTGTCCCAT
Exon 1 (NM_001013415)	TGAAAGACAAAAGCAGCAGGC	GGTGA ACTGGGTAACCC TATCTTC
Exon 11	CCTAAAATACTGAGGACATGGGTTTC	AAGCCAACATCCTGCACCA

protocol. Electrophoresis was performed on a MegaBace 1000 automated sequence detector (Amersham Biosciences). Samples were analyzed by Sequence Analyzer, v. 3.0 (Amersham Biosciences), and Mutation Surveyor, ver.2.41 (SoftGenetics, State College, Pa., USA). Whenever a presumptive mutation was identified, it was compared to the sequence of the reverse strand and of matching normal specimens in order to confirm any sequence alterations.

Results

Twenty-seven pairs of matched esophageal adenocarcinomas and normal esophageal epithelia were examined for LOH at five microsatellite markers on the long arm of chromosome 4. Figure 2 shows a summary of the results. Fifteen (54%) of 27 informative tumors showed LOH at one or more loci. Rates of LOH at markers D4S1572, D4S1548, D4S2934, and D4S3021 markers were 40, 32, 38, and 27%, respectively. The most frequent loss was encountered at marker D4S1554 (10/19 informative cases, or 53%), which is located in the vicinity of the *CARF* locus. Allelic deletion involving one of the three markers between D4S1548 and D4S3021 and spanning approximately 3 million bases, was found at 4q31.1–3 in 11 (40%) of 27 informative tumors.

We further sequenced the coding region of *hCDC4* in the samples presenting LOH at 4q31 to test whether *hCDC4* was a target of this LOH. The coding region of *CARF* was also sequenced in 10 samples showing LOH at 4q35. No mutations were identified in the entire coding regions of either *hCDC4* or *CARF* in these LOH-positive samples. Reproducibility of results was confirmed by sequencing the specimens at least twice on both sense and antisense strands.

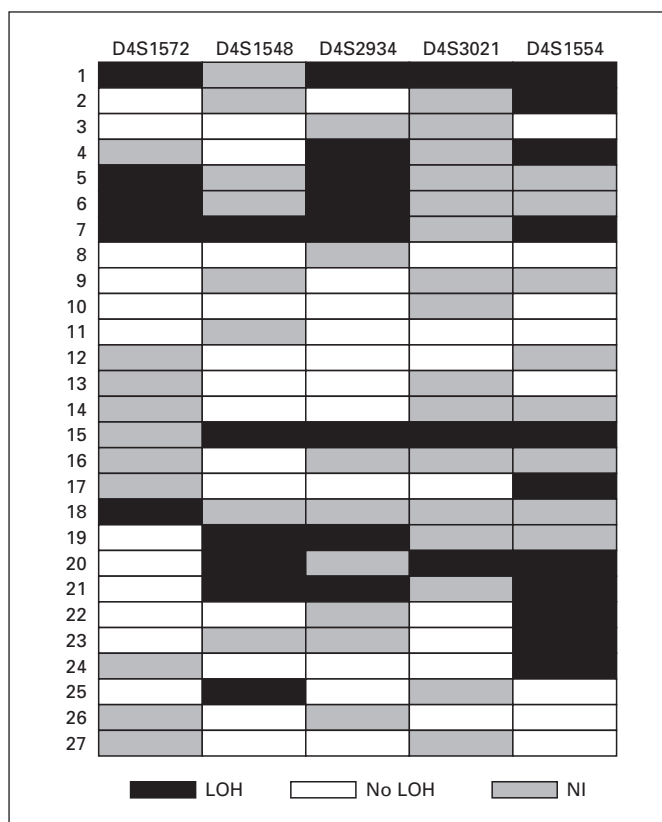


Fig. 2. Allelotyping results of chromosome 4q. Deletion mapping of chromosome 4q was performed using five different polymorphic markers (D4S1572, D4S1548, D4S2934, D4S3021, and D4S1554) in DNA samples from 27 patients with esophageal adenocarcinoma. Designations of the DNA samples run from 225T to 1446T. The LOH status of each tumor at each marker location was tested and is presented. Filled box designates the presence of LOH at a given site (LOH), and an empty box the absence of LOH (no LOH). Shaded box symbolizes results that were not informative regarding the LOH status (NI) of the sample.

Discussion

Several lines of evidence have suggested the presence of tumor suppressor genes on the long arm of chromosome 4. Introduction of human chromosome 4 into the PA-1 human teratocarcinoma cell line suppressed the PA-1 tumorigenicity in nude mice [9]. Chromosomal transfer containing chromosome 4 restored the senescent phenotype of certain cancer cell lines [7]. Immortal human squamous cell carcinoma keratinocytes showed high rates of LOH between the D4S1554 and D4S171 loci (4q33–35) of chromosome 4 [20]. Accordingly, several studies have attempted to find deletions associated with the long arm of chromosome 4 in many different human solid tumors. Tumors tested have included hepatocellular, colorectal, bladder, cervical, basal cell, oral, and small cell lung carcinomas, esophageal squamous cell carcinoma, and Hodgkin's lymphoma [4–6, 20–26]. A minimal common region of deletion was identified at 4q35 [4–6, 23, 27, 28].

The existence of a tumor suppressor locus on the long arm of chromosome 4 was also suggested by previously published systematic screenings. A comprehensive allelotyping study identified two chromosomal regions, 17p and 4q, which presented high rates of allelic loss, especially in the area of 4q33–35 (deleted in 65% of cases) [3]. Rumpel et al. [29] reported three non-overlapping loci of consensus deletion: at 4q21.1–22, 4q32–33, and 4q35. In our study, the highest frequency of deletion (53%) was found at marker D4S1554 (4q35), which points to 4p35 as the most likely tumor suppressor locus for this tumor type.

A potential tumor suppressor gene, *CARF*, is located in the 4q35 region [17, 30]. Previous studies identified *CARF* as a potential tumor suppressor based on its interaction with *ARF*, which carries out its growth-suppressive function by stabilizing p53 as part of the *ARF-MDM2-p53* pathway [31–38]. Studies by Eymin et al. [39] showed, however, that *ARF* may interact with partners other than *MDM2*. On the other hand, *CARF* was shown to have *ARF*-independent interacting partners. *CARF* can interact with p53 protein directly, while it stabilizes and activates p53 [18]. *CARF* also caused some reduction (up to 20–30%) in colony-forming efficiency of cells in the absence of *ARF* [30]. These findings strongly support a tumor-suppressive function for *CARF*.

We observed frequent allelic loss at the *CARF* locus in esophageal adenocarcinomas. Accordingly, we performed a mutational analysis to identify the presence of biallelic inactivation of *CARF*. The coding region, however, did not contain any mutations, suggesting a different mechanism

of inactivation. This alternative mechanism could have been epigenetic silencing, homozygous deletion, or other non-coding region alterations affecting transcription and/or protein stability. Although it is possible that *CARF* lacks involvement or plays a relatively minor role in esophageal adenocarcinogenesis, further studies are needed to surely rule out its role in this cancer type.

A potential tumor suppressor gene, *hCDC4*, is located in the 4q31 region. The 4q31 region has been reported to be deleted in several cancers, such as glioblastoma multiforme [28], nasopharyngeal carcinoma [40], and small-cell carcinoma of the breast [41]. Our study identified a novel allelic deletion at 4q31.1–3 spanning approximately 3 million bases. This region harbors a putative tumor suppressor gene, *hCDC4*, the human ortholog of the *Ago* gene in *Drosophila*. *hCDC4* is a component of the ubiquitin protein ligase complex known as SCF (SKP1-Cullin-F box protein), and it may be involved in the regulation of the G1-S cell cycle checkpoint by targeting cyclin E for ubiquitin-mediated degradation. On the other hand, findings showed that genetic inactivation of *hCDC4* resulted in chromosomal instability [16]. Reports indicated that *hCDC4* is mutated in breast and ovarian cell lines, as well as in pancreatic, endometrial and colorectal carcinomas, and colorectal adenomas [10, 11, 13–16]. Accordingly, we conducted a mutational analysis of *hCDC4* in esophageal adenocarcinoma specimens that presented LOH at 4q31. Although no mutations were discovered at this locus in the current study, it is possible that the *hCDC4* gene is inactivated by alternate mechanisms.

We performed a deletion and mutational analysis of the chromosome 4q region that has been implicated in esophageal adenocarcinoma. Frequent LOH was found at the D4S1554 locus and at a new region on 4q. Two putative tumor suppressor genes in close proximity to these regions, *CARF* and *hCDC4*, did not contain coding region point mutations in tumors showing LOH. These findings imply that either these genes are not involved in tumorigenesis or that inactivation of these genes is due to alternative mechanisms. Based on our findings, further study of these putative tumor suppressor genes and testing of other candidate tumor suppressor genes in this region are merited.

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