Deletion mapping of chromosome 4q22-35 and identification of four frequently deleted regions in head and neck cancers

E. CETIN[1,2*], B. CENGIZ[1*], E. GUNDUZ[1], M. GUNDUZ[1,3,**], H. NAGATSUKA[1], L. BEKIR BEDER[3], K. FUKUSHIMA[4], D. PEHLIVAN[5], M. OZASLAN[6], N. YAMANAKA[3], K. NISHIZAKI[4], K.SHIMIZU[7], N. NAGAI[1]

Departments of [1]Oral Pathology and Medicine, [4]Otolaryngology, and [7]Molecular Genetics, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Shikatacho 2-5-1, Okayama, 700-8525, Japan, e-mail: mgunduz@md.okayama-u.ac.jp; *Division of [2]Gene Therapy Science, Osaka University Graduate School of Medicine, 2-2, Yamada-oka, Suita-City, Osaka 565-0871, Japan; [3]Department of Otolaryngology, Wakayama Medical University, 811-1, Kimiidera, Wakayama, 641-8509, Japan; [5]Department of Medical Genetics, Istanbul University Istanbul Medical Faculty, Turkey; [6]Department of Biology, Faculty of Science, Gaziantep University, 27310 Gaziantep, Turkey*

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Head and neck squamous cell carcinoma (HNSCC) is a diverse group of cancers that are frequently aggressive in their biologic behavior. Inactivation of tumor suppressor gene (TSG) is one of the most critical steps leading to HNSCC. Loss of heterozygosity analysis is very sensitive method for the detection of frequent allelic loss in a chromosomal locus. This method has been considered as an important evidence for the localization of TSGs. We analyzed loss of heterozygosity (LOH) at chromosome 4q22-35 region by using 14 polymorphic microsatellite markers in 83 matched normal and HNSCC tissues. LOH was detected at least in one location in 71 of 83 (86%) tumor tissues. Frequent deletions were detected at the location of microsatellite markers, D4S2909 (46%), D4S2623 (51%), D4S406 (48%), D4S1644 (45%) and D4S2979 (40%). Four different frequently deleted regions at 4q22, 4q25, 4q31 and 4q34-35 were observed. These regions include several putative TSGs such as Caspase-6, SMARCAD1, SMARCA5, SAP30 and ING2. Further molecular analysis of each gene should be performed to clarify their roles in head and neck squamous cell carcinogenesis.

Key words: Head and neck cancer, Tumor suppressor gene, LOH, 4q

Cancer development is known to be a result of the collection of genetic alterations including multiple genes and chromosomes [1]. Among these changes, inactivation of the tumor suppressor genes (TSG)s is one of the most critical steps. The deletion of targeted chromosomal regions eliminates one of the allele, while inactivating events (mutation, deletion or promoter hypermethylation) affect the other allele of the concerning TSG [2]. Loss of heterozygosity (LOH) analysis is a sensitive genetic method to detect microdeletions on chromosomes. Frequent deletion in a chromosomal region suggests existence of a candidate TSG [2]. Our previous studies and of other authors reported the frequently deleted regions and candidate TSGs on 7q31, 13q34, 18q, 19p13 in head and neck cancers [3–6]. We previously examined genome-wide deletion analysis by using about 191 microsatellite markers spanning all autosomal chromosomes with an average distance of 10 Mbp in head and neck carcinomas. This initial screening study with long interval microsatellite markers revealed highly deleted regions on chromosome 4 such as 4p15.2 and 4q24-25 [7].

Moreover recent microdeletion analysis showed LOH on chromosome 4 alleles in several malignancies such as hepatocellular, colorectal, lung, oral, cervical, bladder, prostate, and head and neck cancers [8–15]. Considering the importance of chromosome 4 alterations in human cancer, we recently reported micro-deletion mapping of this region in oral cancer [16]. Since the human genome sequence project has been completed, we have now more precise and detailed information about location of genes and microsatellite markers. Thus, we examined a commonly deleted region of chromosome 4q22-35 in

[*]EC and BC contributed equally to this work.
[**] Corresponding author

detail by using 14 microsatellite markers and constructed a deletion map of the region and candidate TSGs in head and neck squamous cell carcinomas (HNSCC).

Materials and methods

Tissue samples. Paired normal and tumor samples were obtained from 83 patients with head and neck squamous cell carcinoma at Okayama University Hospital after acquisition of informed consent from each patient. Locations of the tumor samples include oral cavity (n: 42), oropharynx (n: 10), larynx (n: 15), hypopharynx (n: 8), maxilla (n: 6), and ethmoid sinus (n: 2). All tissues were frozen in liquid nitrogen immediately after surgery and stored -80°C until the extraction of DNA. Histopathological examinations were also performed at the Department of Pathology, and all tumors were confirmed as squamous cell carcinoma. Bioethics committee of the institution approved the study.

DNA Extraction. Genomic DNAs were isolated from frozen tissues by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Tumor tissues have been confirmed by H&E staining during initial diagnosis, and tumor cell ratio in the samples was found over 70%.

Microsatellite analysis. Primers for amplification of microsatellite markers D4S2909, D4S1647, D4S1564, D4S2623, D4S406, D4S407, D4S402, D4S2394, D4S175, D4S1644, D4S1625, D4S2979, D4S408 and D4S1652 are available through the human genome database (http://gdbwww.gdb.org/). Polymerase Chain Reaction (PCR) was carried out in 20 µl of reaction mixture with 20 pmol of each primer, 100 ng of genomic DNA, 1X PCR buffer, 200 µM of each deoxynucleotide triphosphate, and 0.5 unit of Taq DNA polymerase (Takara, Kyoto, Japan). Initial denaturation at 94°C for 3 min was followed by 25 cycles of a denaturation step at 94°C for 30s, an annealing step at 52°C (D4S1625), 54°C (D4S1647), 56 °C (D4S1564, D4S2623, D4S2394, D4S175, D4S1644, D4S2979 and D4S1652), 58°C (D4S406, and D4S408) or 60 °C (D4S2909, D4S407 and D4S402) for 30s, and an extension step at 72°C for 1 min. A final extension step at 72°C for 7 min was added. After amplification, 2-4 µl of the reaction mixture were mixed with 8 µl of loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol), heat denatured, chilled on ice, and then electrophoresed through an 8% polyacrylamide gel containing 8 M urea. The DNA bands were visualized by silver staining as described previously [3, 4]. LOH was scored if one of the heterozygous alleles showed at least 50% reduced intensity in tumor DNA as compared with the corresponding normal DNA as described previously [3, 4].

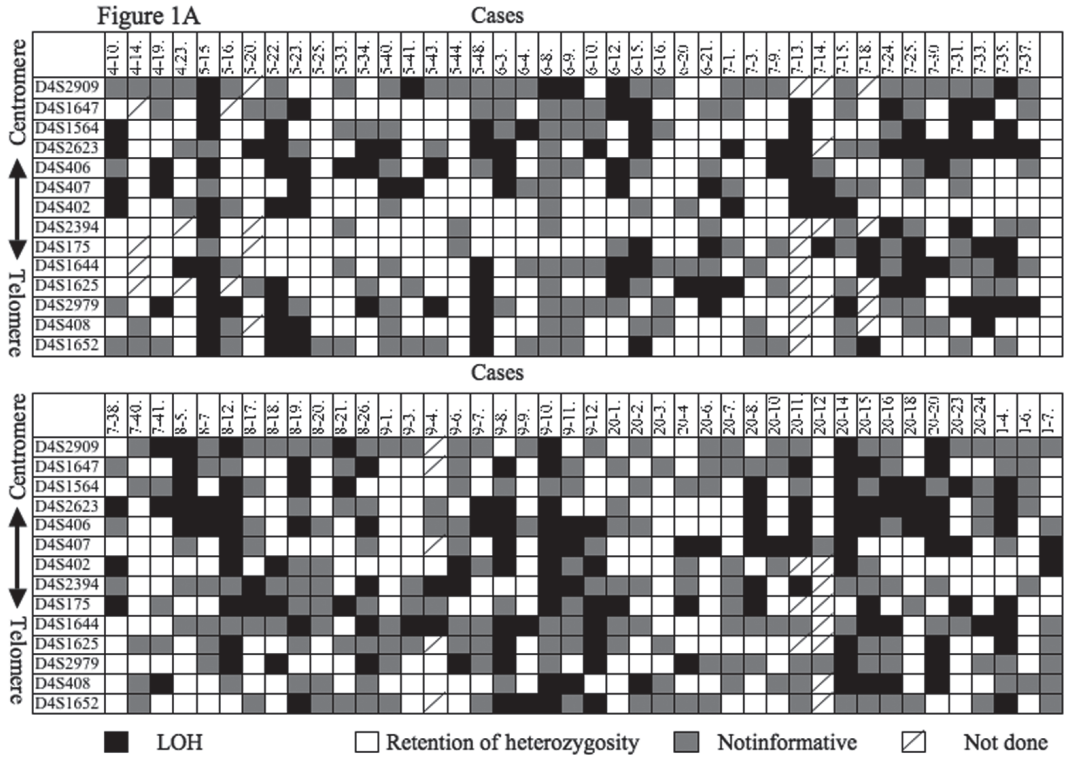
Results

We examined LOH of the chromosome 4q22-35 region (spanning about 100 Mbp) using 14 microsatellite markers in 83 matched normal and head and neck squamous cell carcinoma tissues and identified multiple targeted areas. Overall, LOH was detected at least in one informative location in 71 of 83 (86%) tumor tissues at chromosome 4q22-35 region (Figure 1A). Seven tumor samples (5–15, 7–13, 7–25, 8–12, 9–10, 20–14, and 20–15) showed a large deletion in most polymorphic markers tested. In the other 64 samples, a partial deletion was detected, providing information about the areas of preferential loss. Considering the physical distances between each marker, four different locations showed high frequency of LOH on chromosome 4q22-35 region. These hotspot regions included the chromosomal areas around the marker D4S2979 (4q34-35), D4S1644 (4q31), D4S2623-D4S406 (4q25) and D4S2909 (4q22). These markers D4S2979 (40%), D4S1644 (45%), D4S2623 (51%), D4S406 (48%), and D4S2909 (46%) demonstrated frequent allelic loss than the other markers (Figure 1B).

The highest LOH was detected at the marker D4S2623. Locations of the markers D4S2623, D4S406 and D4S407 are very near to each other. Interestingly, 18 samples (4–19, 5–20, 5–33, 5–41, 5–43, 6–12, 6–21, 7–1, 7–9, 7–30, 7–33, 7–37, 7–38, 8–7, 8–26, 9–7, 20–6 and 20–23) revealed deletion in only one and/or other two locations of this hottest spot with retention of the flanking markers both in the centromere and telomere direction, suggesting distinctive allelic loss of this narrow area and existence of a strong tumor suppressor candidate. Similarly 5 samples (4–19, 5–43, 7–31, 8–18, 20–4) showed LOH only at the marker D4S2979 with retention of the flanking markers, suggesting presence of another candidate TSG in this area. Two other samples (9–9, 20–24) displayed allelic loss only at the marker D4S1644 with keeping the chromosomal locations attached to this location. The marker D4S2909 was located most centromeric side of chromosome 4q and 3 samples (5-41, 7-35, 7-41) demonstrated deletion only at this marker with retention of flanking telomeric locus (Figure 1A).

The distances between the markers D4S2909 and D4S2623, D4S2623 and D4S1644, D4S1644 and D4S2979 are about 20 Mbp, 32 Mbp and 28 Mbp, respectively (contiguous sequence NT_016354 and Human Genome Resources (http://www.ncbi.nlm.nih.gov/genome/guide/human/)). Briefly, high LOH percentage of each hot spot followed by a decreased allelic loss of flanking areas and distinctive deletion of these markers with retention of flanking telomeric and centromeric markers suggested that each of the 4 frequently deleted areas is a separate hot locus and consists of different candidate TSGs. Representative LOH examples are shown in Figure 2.

We redefined map of the chromosomal 4q22-35 region according to the recent genome data for the locations of each markers and genes (www.gdb.org, http://www.ncbi.nlm.nih.gov/genome/guide/human/) (Figure 3). The region spanned about 100 Mbp distance between the markers D4S2909 and D4S1652 and four preferential lost areas were detected.



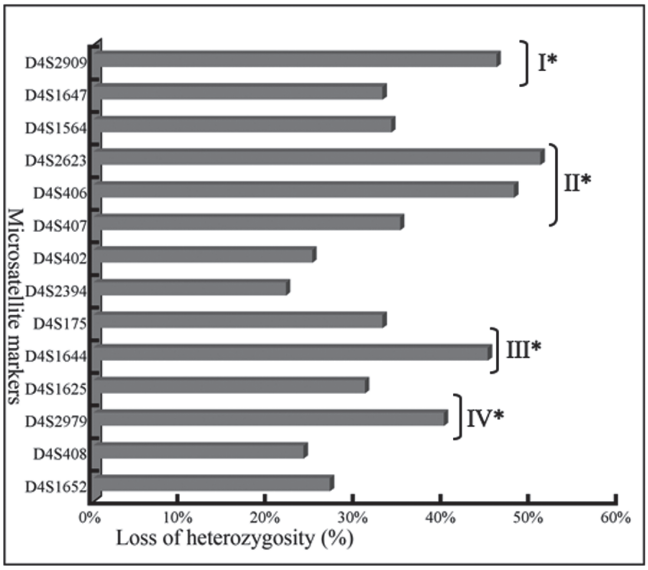


Figure 1. LOH analysis on chromosome 4q22-35 region in HNSCC. (A) Schematic representation of LOH distribution. Microsatellite markers used are shown to the left, cases numbers are on top of the table. Filled box, LOH; open box, retention of heterozygosity; shaded box, not informative (homozygous); box with slash mark, not done. (B) Graphical representation of LOH distribution. *I-IV shows the four preferentially commonly deleted regions.

Discussion

The functional loss of tumor suppressor genes is closely associated with the initiation and/or progression of human cancer [1]. Functional evidence for existence of TSGs on chromosome 4 has been provided by several studies. Introduction of human chromosome 4 in PA-1 teratoma cell line inhibited its tumorigenicity in nude mice, while the PA-1 cell line regained its tumorigenicity when the tagged chromosome 4 was lost under negative selection [17]. In another

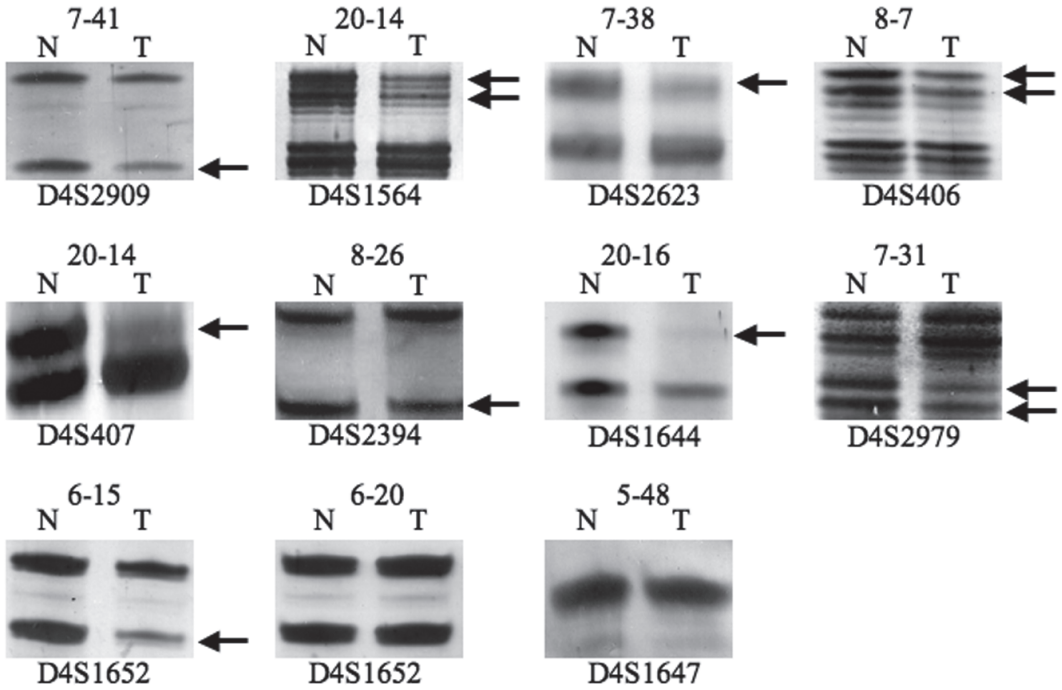


Figure 2. Representative results of microsatellite analysis. DNAs of tumor (T) and corresponding normal (N) tissues are shown with microsatellite markers indicated at the bottom and sample numbers on the top. Lost alleles in samples with LOH are depicted by arrows.

study, hybrid clones of U251 glioma cells containing a transferred neomycin-resistance tagged chromosome 4 showed an inability to form tumors in nude mice and a greatly decreased efficiency of soft agarose colony formation [18]. Later micro-deletion analysis revealed the involvement of LOH on chromosome 4 alleles in various carcinomas including hepatocellular, colorectal, cervical, bladder, lung, and prostate cancers [8–13].

Although karyotype and comparative genomic hybridization studies by using few markers demonstrated the frequent deletion on the long arm of chromosome 4 in head and neck precancerous and cancerous lesions and oral cancer cell lines [19–21], deletion mapping of this region has not been studied in detail. All these and our previous study suggest the existence of TSGs in the long arm of chromosome 4 [7, 16]. Therefore, this time we examined the region of chromosome 4q22-35 in detail based on the recent mapping information of human genome project by using 14 microsatellite markers from this region and constructed a deletion mapping of the region and putative TSGs. Overall a very high deletion ratio was observed in cancer samples. Our data interestingly revealed four independent commonly deleted regions with frequent deletions at the markers D4S2979 (4q34-35), D4S1644 (4q31), D4S2623-D4S406 (4q25) and D4S2909 (4q22). Since the distance between these regions is between 20–32 Mbp and the flanking regions of each marker showed lower percentage of deletion, we believe that these regions represent the location of different putative TSGs.

Current data showed highest frequency of LOH on chromosome 4q25 region in head and neck cancer. Our previous genome-wide LOH analysis on head and neck cancer and deletion mapping of chromosome 4q22-35 region in oral cancer also indicated a hot spot locus at 4q25 region [7, 16]. The candidate TSG in this region was most likely to be around the marker complex D4S2623-D4S406-D4S407 because 18 samples demonstrated LOH only at one of these and/or other with retention of the flanking genomic regions. Heavy gene populations with known and unknown function exist in this region. Several genes reported with an oncogenic function such as PITX2, LEF1, EGF have been mapped to this region decreasing the possibility of their role as a growth suppressor in head and neck carcinogenesis [22]. Some other genes with a low possibility of TSG such as retina specific expressed RRH [23], and PAPSS1 [24], an enzyme, which has a role in sulfate synthesis for the metabolism of endogenous and exogenous larger compounds, also located in this region. The most probable candidate TSG in the region is Caspase-6. Caspase-6 is just localized 280 kbp from the marker D4S2623 and has already been known to be an important molecule in apoptotic pathway [25]. In fact, our previous study in oral cancer narrowed down the allelic loss area 1.5 Mbp around D4S2623, suggesting the strong candidacy of Caspase-6 as a tumor suppressor in a wide range

of HNSCC. Somatic mutations and LOH of several members of caspase family including caspase 3, 7, 9 have recently been reported in various human cancers [26–28]. Considering these reports and our current data, Caspase-6 is a highly possible candidate TSG in the region and further studies including mutation status and mRNA expression of Caspase-6 should be performed.

On the other hand, our results also showed frequent LOH on chromosome 4q22 region. The frequent LOH was detected at the marker D4S2909. Since the distance between D4S2909 and D4S2623 is about 20 Mbp and presence of deletion only at locus of the marker D4S2909 with keeping of the flanking areas in 3 samples, it is likely that another TSG exists in this region. Several candidate TSGs such as SMARCAD1, and GRID2 are localized on chromosome 4q22 region. Anti-oncogenic properties of SMARCAD1 have been shown in hepatocellular carcinoma [29]. Strong expression of BMPR-IB located at this region has been shown to be associated with poor tumor grade, high tumor proliferation, cytogenetic instability, and poor prognosis in breast cancer, suggesting it as an oncogene [30]. The common fragile sites are large regions of genomic instability that are found in all individuals and are hot spots for chromosomal rearrangements and deletions. Two of these common fragile site genes, FHIT and WWOX, have already been demonstrated to function as tumor suppressors [31]. GRID2 was found to be located at the fragile FRA4G and was shown to be common fragile site gene [31] and it is a likely candidate TSG related with allelic loss in HNSCC in our study.

Our third and fourth frequently deletion regions were found at 4q34-35 around the marker D4S2979 and at 4q31 around the D4S1644, respectively. Several genes including SMARCA5, SAP30, and ING2 could be picked up as a candidate TSG from these regions. SMARCA5 and ING2 belong to SWI-SNF chromatin remodeling enzyme, and ING tumor suppressor families, respectively. Previously we demonstrated tumor suppressor character of some members of these gene families [3, 4, 6]. Deregulation of SMARCA5 was recently reported in acute leukemia [32]. A recent report has also demonstrated involvement of ING2 and SAP30 as a possible tumor suppressor in basal cell carcinomas [33]. All these studies suggest that these genes function as TSG candidate in this region.

In conclusion, our data showed that loss of several putative TSGs in the long arm of chromosome 4 are likely to be involved in the carcinogenesis of head and neck cancers. Future functional studies should be planned to clarify the role of these candidate TSGs in head and neck cancers.

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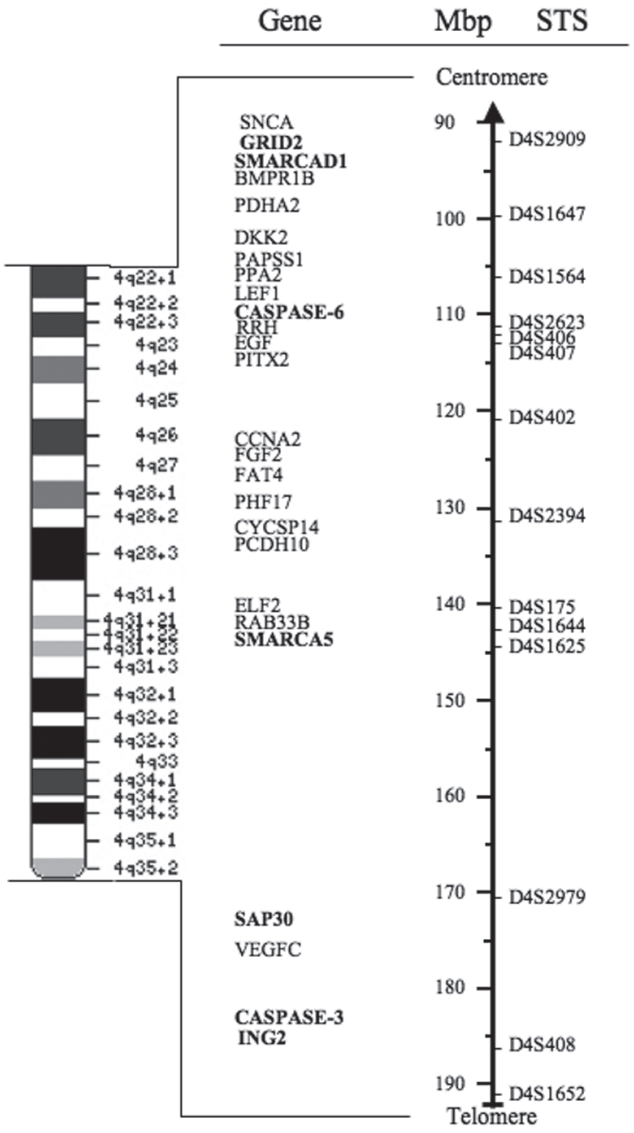

Figure 3. A physical map of the D4S2909-D4S1652 genomic interval. The locations of the markers and genes are based upon the latest mapping information derived from the National Center for Biotechnology Information (NCBI), and the Genome Database (GDB) homepages (http://www.ncbi.nlm.nih.gov/genome/guide/human/, http://www.gdb.org/). Candidate tumor suppressor genes are shown as bold.

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