Genome-wide Loss of Heterozygosity Analysis in Head and Neck Squamous Cell Carcinomas

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Abstract: Identifying the tumor suppressor gene (TSG) loci by genomic studies is an important step to uncover the molecular mechanisms involved in HNSCC pathogenesis. We therefore performed comprehensive analyses on loss of heterozygosity (LOH) using a genome-wide panel of 191 microsatellite markers in 22 HNSCC samples. We found 53 markers with significantly high LOH (>30%) on 21 chromosomal arms, the highest values of those were observed on 3p, 9p, 13q, 15q, and 17p, corresponding to D3S2432 (67%), D9S921-D9S925 (67%) and GATA62F03 (86%), D13S1493 (60%), D15S211 (62%) and D17S1353 (88%), respectively. Fifteen hot spots of LOH were defined in 13 chromosomal arms reported previously in HNSCCs. Furthermore, we identified 5 novel hot spots of LOH on 3 chromosomal arms in HNSCC at 2q33 (D2S1384), 2q37 (D2S125), 8q12-13 (D8S1136), 8q24 (D8S1128) and 15q21 (D15S211). In conclusion, our comprehensive allelotype analyses have unveiled and confirmed a total of 20 possible TSG loci that could be involved in the development of HNSCC. These results provide useful clues for identification of putative TSGs involved in HNSCC by fine mapping of the suspected regions.

Key words. LOH, head and neck cancer, tumor suppressor gene.

Introduction

In carcinogenesis, inactivation of the tumor suppressor genes (TSGs) is one of the most critical steps. In this process, deletion of targeted chromosomal regions eliminates one allele while inactivating events (mutation, promoter hypermethylation) affect the other allele of concerning TSG[1)]. Detection of frequent loss of heterozygosity (LOH) in a locus is considered to be critical evidence for localization of a TSG. Large-scale genomic studies identified chromosomal location of several different human TSGs including *p53* in HNSCCs, however some others should remain to be unveiled.

Previously three different genome-wide allelotype analyses [2-4)] of HNSCC by using 50, 58 and 145 microsatellite markers, respectively, revealed frequent LOH at several chromosomal regions. However, our study is the most comprehensive genomic study of HNSCCs to date, covering all of the non-acrocentric chromosome arms with 191 microsatellite markers most of which are different from those used previously. In the present study, we aimed to determine frequently deleted chromosomal locations.

Materials and Methods

Tumor samples with localization of oral cavity (9 cases), hypopharynx (4), larynx (4), oropharynx (3) and maxillary sinus (2) were obtained at the Department of Otolaryngology, Okayama University Hospital. Squamous cell carcinoma was confirmed by histological studies.

DNA Extraction & Microsatellite Analysis

Genomic DNAs were isolated from frozen tissues by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. For each marker, PCR was carried out in 20 µl of reaction mixture with 10 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 ºC for 3 min was followed by 25 cycles of a denaturation step at 94 ºC for 30s, an annealing step between 50 and 60 ºC depending on each primer for 30s, and an extension step at 72 ºC for 1 min. A final extension step at 72 ºC for 7 min was added. After amplification, 2 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. 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.

Results

LOH Frequency of Each Chromosomal Arm

DNA from matched pairs of tumor and normal tissues of 22 patients were analyzed for LOH by using 191 microsatellite markers (Fig. 1). LOH frequency (number of cases with LOH / number of informative cases) ranged from 0 to 88% (23 ± 17%; mean ± SD). LOH frequencies for each chromosomal arm, varied between 0% (16p) and 73% (9p) (21 ± 15%; mean ± SD) (Fig. 2). Chromosomal arms 3p, 9p, 13q, 15q, and 17p showed LOH frequency of higher than 40%. Furthermore 3p and 15q seemed to have total deletion of each chromosomal arm in 4 and 5 tumor samples respectively. We could not find frequent LOH on chromosomes 1, 7, 16, 21 and 22 at microsatellite markers used in this study.

Loss of Heterozygosity at Specific Loci& Hot Spots of LOH

Among 191 primers, we found 53 markers with significantly

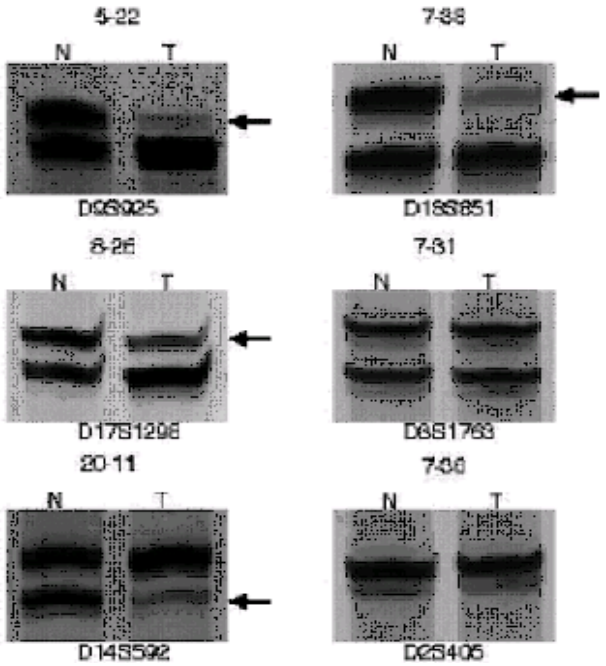

Fig. 1. Representative results of microsatellite analysis for LOH by using highly polymorphic microsatellite markers at several chromosomal regions in the samples of HNSCCs. DNA of tumor (T) and corresponding normal tissues (N) are shown with microsatellite markers indicated at the bottom and sample numbers on the top. Lost alleles in samples with LOH are depicted by arrows. Samples 7-31 (D3S1763) and 7-38 (D2S405) represent cases for retention of heterozygosity and not-informative, respectively.

high LOH (>30%) on 21 chromosomal arms, the highest values being seen with D13S1493 (60%), D15S211, (62%), D3S2432 (67%), D9S921 (66%), D9S925 (66%), GATA62F03 (86%), D17S1298 (75%) and D17S1353 (88%). All the markers located on 3p, 9p, 13q and 17p arms showed LOH frequency higher than 40%.

We found 20 hot regions (markers with frequent LOH but retaining heterozygosity for the flanking microsatellites) on 13 chromosomal arms; 2q33 (D2S1384), 2q22-23 (D2S1334-D2S1399), 2q37 (D2S125), 4p15.2 (D4S2639), 4q24-25 (D4S2623), 5q31.3 (D5S1480), 8p23.2 (D8S264), 8q13.1 (D8S1136), 8q24 (D8S1128), 9p23-24 (D9S925, GATA62F03), 9q31.3 (D9S930), 9q34.2 (D9S158), 10q21 (D10S1221), 11q21-22.3 (D11S2000, D11S2002), 14q11-13 (D14S608-D14S306), 14q22.3 (D14S592), 15q21(D15S211), 17p13 (D17S1353), 18q11 (D18S877), and 19q12 (D19S433). Among these areas, 2q22-23, 2q37, 8q13, 8q24 and 15q21 were identified as new hot loci in HNSCCs. In respect to the 15q arm, we found a significantly high frequency of LOH at D15S211 (62%), though the entire 15q arm seemed to be lost in around 25% of HNSCC specimens.

Discussion

We found frequent allelic losses in 53 different loci by comprehensive microsatellite allelotype analysis in HNSCCs. Detection of hot spot is critical for defining the possible TSGs. In this regard, five of 20 hot spot loci we found appeared to be new candidate TSG regions responsible for HNSCC development.

Chromosome 3p and 15q arms were likely to be completely deleted in 4 out of 20 tumor samples. Although it does not specify the location regarding to putative TSG, it implies the existence of new TSGs in these chromosomal regions remaining to be clarified by further analysis. Allelic loss of 3p is predicted as an early event in HNSCC development [5].

On chromosome 2q, three different hot spots were defined by D2S1384, D2S125 and D2S1334-D2S1399. In HNSCC, only study using LOH analysis regarding chromosome 2 found 32% loss in 2q32[6]. For 2q33, *Caspase 8* was shown to be related with apoptosis in HNSCC cell line. This study combined with our results suggested *caspase 8* gene as a candidate TSG in HNSCCs. The regions at 2q22-23 and 2q37 are new hot loci for HNSCCs, although frequent LOH in other cancers were shown.

Deletions on chromosome 4 were suggested to appear in the late stages of HNSCC[7]. Our study defined two different loci with significant LOH: 4q24-25 overlaps with some previous reports in HNSCCs[8]. The other locus, 4p15, is involved in a more extensive band (4p14-pter) in HNSCC [5]. Similar results were also found in studies of other cancers, while none of the genes located in these regions was shown to fulfill the requirements for a TSG.

On chromosome 5, we found frequent deletions in 5q31.3 and 5q13-14. Both loci were defined as hot spot in various tumors including nasopharyngeal carcinoma, oral carcinoma, esophageal cancers. Our data in the light of previous studies suggest that, some candidate TSGs in these regions are involved in HNSCC carcinogenesis.

Concerning LOH analysis on chromosome 8, the present study is the first report for the 8q13 and 8q24 loci with high LOH frequency in HNSCCs. In other solid tumors, 8q24 loci in hepatocellular carcinoma, lung cancer and 8q12-13 region in prostate cancer were found to be commonly deleted. In 8p, p23 band was a frequently deleted region in HNSCCs. Our findings confirmed those studies.

Chromosome 9p23-24 region showed the second highest frequency of deletion in this study. Field *et al*.[3] found the highest LOH frequency on 9p of HNSCC in accord with our results, although they focused on 9p21 locus in which at least 4 TSGs, *p15, p16, p19* and *MTAP* are located. We infer that an unknown TSG closely related to HNSCC resides on 9p24 rather than 9p21. In 9q arm, frequent LOH at 9q31.3 and 9q34.2 in our study are consistent with the previous studies in HNSCC[3,4].

In the current study, frequent LOH was detected in 10q21 region. Comparative genomic hybridization analysis revealed 10q21 as a

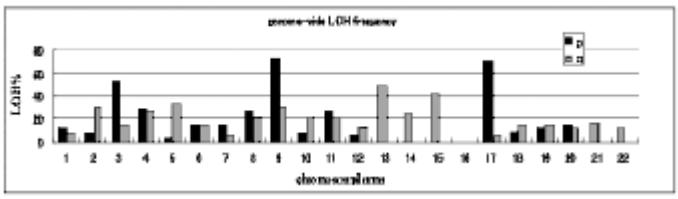

Fig. 2. Frequency of LOH at each chromosome arm in 22 cases of HNSCC. LOH frequencies were calculated by total number of markers with LOH/total number of informative markers on the same arm level.

hot spot locus in HNSCC. Recently the *ANX7* gene in 10q21 locus was proposed as TSG in prostate cancer.

Regarding chromosome 11, our study revealed 11q21 and 11q22.3 with 41% and 32% of LOH, respectively. Similar results with different microsatellite markers were shown in HNSCC[5].

Multiple minimal deleted regions including 13q14 and 13q33-34 were determined for 13q in HNSCC. Previously we demonstrated the *ING1* gene as a TSG in 13q33-34 region[9]. In the current study, all markers on 13q showed LOH frequency more than 40%.

In previous studies, 14q13-21 and 14q31-32 loci were appeared to be frequently deleted in HNSCC[4,7]. In addition to these regions, significant loss of 14q11 was also found in our study. However, no TSG was defined on chromosome 14 so far.

Our results confirmed loss of chromosome 17p13 region, which harbors *p53*, as the hottest spot though no other significant deletion was found in other areas of chromosome 17. Our LOH analysis on 19q revealed 19q12 locus as a hot spot, which appeared to be selectively deleted in HNSCC [3].

In conclusion, the present comprehensive LOH analysis of HNSCC revealed 5 novel loci at which candidate TSGs may reside, in addition to previously reported 15 loci. We also showed lots of new microsatellite markers with frequent LOH, which can be useful to narrow down the minimally deleted regions by compromising with previously reported data.

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