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Genome-Wide Analyses on Loss of Heterozygosity in Head and Neck Squamous Cell Carcinomas

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SUMMARY: Head and neck squamous cell carcinoma (HNSCC) is a frequent malignancy with a poor survival rate. 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: 2q22-23, 4p15.2, 4q24-25, 5q31, 8p23, 9p23-24, 9q31.3, 9q34.2, 10q21, 11q21-22.3, 14q11-13, 14q22.3, 17p13, 18q11, and 19q12 as loci reported previously in HNSCCs. Furthermore, we identified five novel hot spots of LOH on three 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 and subsequent analysis for functional genes. (*Lab Invest 2003, 83:99–105*).

Head and neck squamous cell carcinomas (HNSCCs) are a diverse group of cancers and are frequently aggressive in their biologic behavior. They account for 1% to 2% of all cancer deaths in both the United States and Japan. Most patients with this malignancy have advanced disease at presentation, with regional disease in 43% and distant metastases in 10% (Pfister et al, 1997). The overall survival rate for this disease group remains poor, largely because of the high incidence of recurrent disease at the primary site or in the regional lymph nodes. Patients presenting with HNSCC also demonstrate a high incidence of second primary tumors in the upper aerodigestive tract, which may be synchronous or metachronous.

In neoplastic progression, most of the sporadic solid tumors result from a multistep process of accumulated genetic and epigenetic alterations (Renan, 1993). Among these changes, inactivation of the tumor suppressor genes (TSGs) is one of the most critical steps. In this process, the deletion of targeted chromosomal regions eliminates the one allele, while inactivating events (mutation, deletion, or promoter hypermethylation) affect the other allele of the concerning TSG (Knudson, 1971). The detection of frequent loss of heterozygosity (LOH) in a chromosomal locus is considered to be critical evidence for the localization of a TSG. Large-scale genomic studies identified the chromosomal locations of several different human TSGs. A substantial number of TSGs involved in the genesis of several cancer types, including HNSCCs, have already been discovered. On the basis of such studies, the *p53*, *p16*, *ING1*, and *ING3* genes have been reported as TSGs involved in HNSCCs (Gunduz et al, 2000, 2002; Reed et al, 1996; Somers et al, 1992). However, a variety of large scale genome analysis techniques suggested that a number of such genes remain to be unveiled.

Previously three different genome-wide allelotype analyses (Ah-See et al, 1994; Field et al, 1995; Nawroz et al, 1994) of HNSCC using 50, 58, and 145 microsatellite markers, respectively, revealed frequent LOH at several chromosomal regions including 3p, 4q, 5q, 6p, 8p, 8q, 9q, 11q, 13q, 14q, 17p, 18q, and 19q. However, our study is the most comprehensive genomic study of HNSCCs to date, covering all of the nonacrocentric chromosome arms with 191 microsatellite markers, most of which are different from those used previously. In the present study, we aimed to determine frequently

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deleted chromosomal locations and ultimately to reveal new putative TSGs in HNSCCs.

Results

LOH Frequency of Each Chromosomal Arm

DNA from matched pairs of tumor and normal tissues of 22 patients were analyzed comprehensively for LOH by using 223 highly polymorphic microsatellite markers. PCR was successfully performed for 191 (85%) of 223 markers with about 70% of informative allelotypes. By using these markers, the frequency of LOH (number of cases with LOH/number of informative cases) ranged from 0% to 88% (23 ± 17%; mean ± SD). Representative profiles of microsatellite analysis with LOH are shown in Figure 1. LOH frequencies for each chromosomal arm, calculated by dividing the total number of cases with LOH by the total number of informative cases on the same arm level, varied between 0% (16p) and 73% (9p) (21 ± 15%; mean ± SD). Chromosomal arms 3p, 9p, 13q, 15q, and 17p showed LOH frequencies of higher than 40%, whereas the LOH frequencies of 2q, 3q, 4p, 4q, 5q, 8p, 8q, 9q, 10q, 11p, 11q and 14q were more than 20%. Furthermore, 3p and 15q seemed to have total deletion of each chromosomal arm in four and five tumor samples, respectively, which made it difficult to focus on the target area carrying putative TSG. On the other hand, LOH was not detected on 16p arm (Fig. 2). All the markers explored on sex chromosomes showed almost no informative results; consequently, those could not be evaluated.

LOH at Specific Loci

When evaluating the significance of the markers, LOH frequency of more than 30% of the informative cases (which represents losses presumably associated with the cancer-specific phenotype; Girard et al, 2000) was considered to be significant for the presence of putative TSGs. Among 191 primers, we found 53 markers with significantly high LOH on 21 chromosomal arms. The highest values were seen with D13S1493 (60%), D15S211 (62%), D3S2432 (67%), D9S921 (66%), D9S925 (66%), GATA62F03 (86%), D17S1298 (75%), and D17S1353 (88%). All the markers located on the 3p, 9p, 13q, and 17p arms showed LOH frequency higher than 40%. On the other hand, we could not found any significant marker on several chromosomal arms including 1p, 1q, 2p, 3q, 5p, 6p, 7p, 7q, 10p, 12p, 12q, 16p, 16q, 18p, 19p, 20p, 20q, 21q, and 22q.

Hot Spots of LOH

We defined the hot spot loci for markers with frequent LOH but that retained heterozygosity for the flanking microsatellites. According to this definition, 19 hot regions were found 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), 17p13 (D17S1353), 18q11 (D18S877), and 19q12 (D19S433). Results of LOH analysis on these markers are shown in Figure 3. Among these areas, 2q22-23, 2q37, 8q13, and 8q24 were identified as new hot loci in HNSCCs. With respect to the 15q arm, we found a significantly high frequency of LOH at D15S211 (62%), although the entire 15q arm seemed to be lost in approximately 25% of HNSCC specimens.

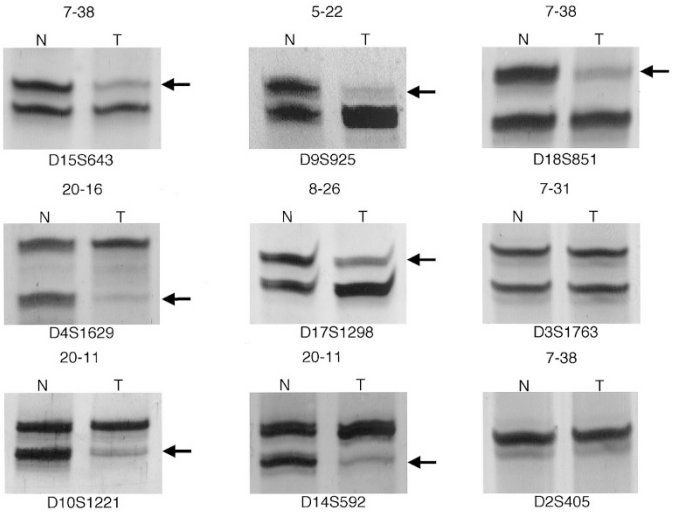

Figure 1.

Representative results of microsatellite analysis for loss of heterozygosity (LOH) by using highly polymorphic microsatellite markers at several chromosomal regions in the samples of head and neck squamous cell carcinomas (HNSCCs). DNA 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*. Samples 7-31 (D3S1763) and 7-38 (D2S405) represent cases for retention of heterozygosity and noninformative, respectively.

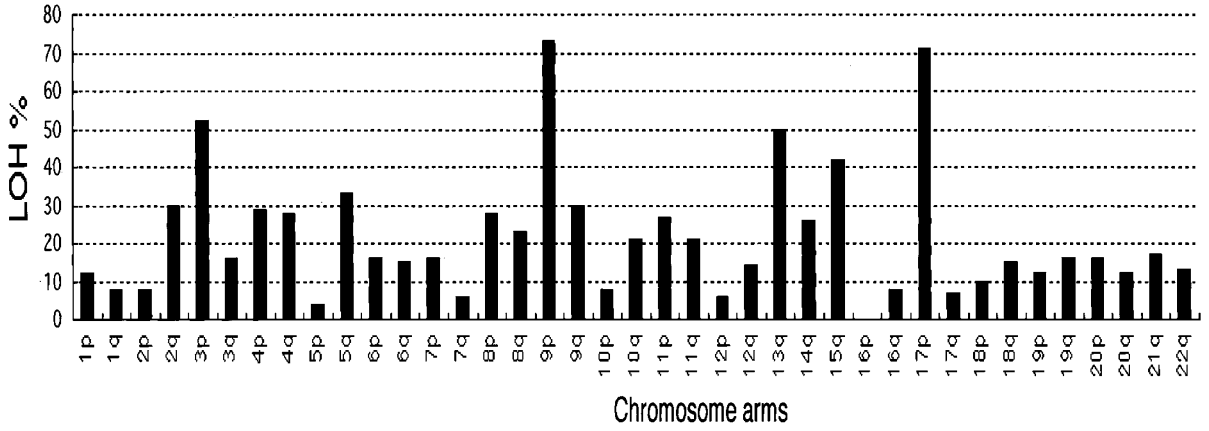

Figure 2.

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

Discussion

We found frequent allelic losses (more than 30%) in 53 different loci on 21 chromosomal arms by comprehensive microsatellite allelotype analysis in HNSCCs. Detection of hot spots is critical for defining the possible TSGs. In this regard, 4 to 5 of the 20 hot spot loci we found seemed to be new candidate TSG regions responsible for HNSCC development.

Chromosome 3p and 15q arms were likely to be completely deleted in 5 and 4 of the 20 tumor samples, respectively. Although this does not specify the location regarding the putative TSG, it implies the existence of new TSGs in these chromosomal regions, which remain to be clarified by further analysis.

On chromosome 2q, three different hot spots were defined: D2S1384 (2q33.1), D2S125 (2q37), and

Figure 3.

Complete allelotyping results of 22 HNSCC samples. Microsatellite markers are shown on the left and sample numbers on the top of the tables. The percentages of LOH at each location are displayed on the right of each panel. Hot spots are depicted by cytogenetic map position on the far right of each panel. Among these areas, 2q22-23, 2q37, 8q13, 8q24, and 15q21 were identified as novel hot loci for LOH in HNSCCs. *Filled box*, LOH; *open box*, retention of heterozygosity; *gray box*, noninformative; *hatched box*, not done; *Ch*, chromosome. *Cytogenetic location of hot spots.

D2S1334–D2S1399 (2q22.1-2q23.1), locations with LOH frequencies of 50%, 42%, and 31%, respectively. In HNSCC, only a few studies were reported in the literature regarding significantly deleted regions on chromosome 2. The only study using microsatellite analysis found a 32% loss in 2q32 (Ransom et al, 1998). Other studies using comparative genomic hybridization denoted losses in 2q35-37 (43%) (Bockmuhl et al, 1996) and 2q33 (29%) (Weber et al, 1998) in oral malignancies. On the 2q33 band, frequent LOH was also revealed in various human tumors, including neuroblastoma and lung cancer. In this locus, the *caspase 8* gene for neuroblastoma (Takita et al, 2001), *TPEF* for various tumor cell lines, and the *PLC-L* gene for small cell lung carcinoma (Kohno et al, 1995) were suggested as TSGs. Inhibition of caspase 8 in a HNSCC cell line, KB, resulted in the reduction of the apoptotic cell fraction and changes in expression of Fas-related genes after irradiation (Uno et al, 2002). This study and our data that showed frequent deletion in the 2q33 region suggest the *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 breast carcinoma at the 2q22 (41%) region and in lung and esophageal carcinomas at the 2q37 locus were shown (Hu et al, 2000; Otsuka et al, 1996).

Fractional or complete allelic loss of 3p is predicted as an early and basic event in HNSCC development (Bockmuhl et al, 1996). We found frequent LOH at the 3p region, with an average deletion ratio of 52%. At least five tumors (25%) revealed loss at all informative markers, suggesting complete deletion of 3p. Our findings on 3p are consistent with those of previous studies. Based on discrete regions of deletion on the 3p arm in a variety of human cancers, several candidate TSGs were suggested, including *VHL*, *FHIT*, *RASSF1*, and *H37* (Dammann et al, 2000; Kisielewski et al, 1998; Latif et al, 1993; Oh et al, 2002).

Deletions on chromosome 4 were suggested to have appeared in the late stages of HNSCC (Califano et al, 1996). Our study defined two different loci with significant LOH. The more prominent locus, 4q24-25 (D4S2623), overlaps with some previous reports on HNSCCs (Pershouse et al, 1997; Wang et al, 1999). The other discrete locus, 4p15, is involved in a more extensive band (4p14-pter) in HNSCC (Bockmuhl et al, 1996). On the other hand, the same region was found to be significantly deleted in breast carcinoma, small cell lung carcinoma, and colorectal carcinoma (Girard et al, 2000; Shivapurkar et al, 1999, 2001). Despite these regions showing a high incidence of deletion, none of the genes located in these regions was shown to fulfill the requirements for a TSG.

Several studies on chromosome 5 revealed deletion of the 5q21-22 region, the locus of the *APC/MCC* gene, involved in HNSCCs (Ah-See et al, 1994; Field et al, 1995; Mao et al, 1998). Nevertheless, mutation of the *APC* gene could not be displayed in SCC of the oral cavity or esophagus (Cooper et al, 1996; Shibagaki et al, 1994). In our study, the same locus showed a relatively high LOH frequency of 18% to 27%. On the other hand, we found much more frequent deletions in 5q31.3 (D5S1480, 54%) and 5q13-14 (D5S1501, 45%; D5S1462, 41%). Both loci were defined as hot spots in various tumors, including nasopharyngeal carcinoma (Shao et al, 2001), oral carcinoma (Wolff et al, 1998), esophageal cancers (Ogasawara et al, 1996), and myeloid neoplasms (Horrigan et al, 2000). Our data, together with previous studies, suggest that some candidate TSGs in these regions are involved in the carcinogenesis of various cancers, including HNSCC.

Regarding the short arm of chromosome 8, the 8p23 band was the most frequently deleted region in several solid tumors (Li et al, 2001; Pineau et al, 1999), including HNSCCs (Sunwoo et al, 1999; Wu et al, 1997). Our findings showed similar results, with a high incidence of LOH at the 8p23 location. Concerning LOH analysis on chromosome 8q, the present study is the first report of the 8q13 and 8q24 loci with high LOH frequency in HNSCCs. In other solid tumors, the 8q24 loci in hepatocellular carcinoma and lung cancer (Girard et al, 2000; Li et al, 2001) and the 8q12-13 region in prostate cancer (Perinchery et al, 1999) were found to be commonly deleted. Conversely, the chromosomal region of 8q24 was also found to be amplified in various malignancies, including hepatocellular carcinoma (Wang et al, 2001), prostate cancer (El Gedaily et al, 2001), and endometrial carcinoma (Suehiro et al, 2000). Most of the amplified chromosomal regions were detected by the comparative genomic hybridization technique, which shows loss or gain of DNA copy in an area rather than a specific locus. However, microsatellite analysis determines deletion in a specific locus. Thus, the comparison of these data obtained by two different methods should be done with caution.

The chromosome 9p23-24 region showed the second highest frequency of deletion in this study. Comparative genomic hybridization analysis on chromosome 9 also defined 9p23 and 9p24 loci as the most highly deleted regions for 73 different types of tumor entities (Knuutila et al, 1999). Field et al (1995) found the highest LOH frequency on 9p of HNSCC in accord with our results, although they focused on the 9p21 locus, in which at least four TSGs (*p15*, *p16*, *p19*, and *MTAP*) are located. We infer that an unknown TSG closely related to HNSCC resides on 9p24 rather than on 9p21. It is notable that at least 5 (25%) of 20 tumors exhibited total loss of the short arm of chromosome 9 in our results (Fig. 3). In the 9q arm, frequent LOH at the D9S930 (9q31.3) and D9S158 (9q34.2) markers in our study are consistent with previous studies in HNSCC (Ah-See et al, 1994; Field et al, 1995).

In the current study, frequent LOH was detected at a microsatellite marker D10S1221 in the 10q21 region. Comparative genomic hybridization analysis on chromosome 10 revealed 10q21 as a hot spot locus with a high lymph node metastasis in HNSCC (Bockmuhl et al, 2000). Recently the *ANX7* gene in the 10q21 locus was proposed as a TSG in prostate cancer (Srivastava et al, 2001).

Regarding LOH analysis of chromosome 11, nasopharyngeal carcinomas of the Chinese population were reported to display frequent deletion of the 11q21-24 region (Harn et al, 2002; Hui et al, 1996). Our study revealed the 11q21 (D11S2000) and 11q22.3 (D11S2002) bands with 41% and 32% of LOH, respectively. A microsatellite marker, ATA34E08 at the 11p14 location, showed high LOH. Similar results were shown in HNSCC, although the microsatellite markers used were different from ours (Bockmuhl et al, 1996).

Multiple minimal deleted regions including 13q14 and 13q33-34 were determined for 13q in HNSCC (Gupta et al, 1999; Maestro et al, 1996). Previously we demonstrated the *ING1* gene as a TSG at a hot spot of LOH in the 13q33-34 region (Gunduz et al, 2000). Concerning the 13q14 region, at least two candidate TSGs other than *RB*, which have been proposed as oncosuppressors in diverse tumor types, were suggested in HNSCC (Maestro et al, 1996; Ogawara et al, 1998). In the current study, all markers on 13q showed an LOH frequency more than 40%. Moreover, 4 of 20 tumors exhibited loss of all informative markers on 13q. All but one marker on chromosome 15 displayed a similar level of LOH frequency, whereas four samples demonstrated allelic loss of all markers, which made it difficult to define a minimal deleted region.

LOH analysis of 14q in HNSCC, except nasopharyngeal carcinoma, was poorly defined, and the 14q13-21 and 14q31-32 loci seemed to be frequently deleted (Califano et al, 1996; Nawroz et al, 1994). In our study, in addition to these regions, significant loss was found at the 14q11 region. However, no TSG has been defined on chromosome 14 so far.

Deletion of the chromosome 17p13 region, which harbors a well-known TSG, *p53*, was shown in the transition from the preinvasive to the invasive lesion in various tumor types, including HNSCC (Boyle et al, 1993). Our results also confirmed the region proximal to the *p53* gene (D17S1353) at 17p13 as the hottest spot, although no other significant deletion was found in other areas of chromosome 17.

Our microsatellite analysis on 19q revealed the 19q12 locus as a hot spot, which seemed to be selectively deleted in HNSCC (Field et al, 1995). We could not find frequent LOH on chromosomes 1, 7, 16, 21, and 22 at microsatellite markers used in this study; however, we have previously demonstrated that a specific narrow region at 7q31, including the *ING3* gene, exhibited a significantly high frequency of LOH in HNSCC (Gunduz et al, 2002). In addition, sex chromosomes could not be evaluated, because most markers examined were not informative.

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

Materials and Methods

Tissue Samples

Paired normal and tumor samples were obtained from 22 patients with primary HNSCCs at the Department of Otolaryngology, Okayama University Hospital, after acquisition of written informed consent from each patient. Localizations of tumors included oral cavity (nine cases), hypopharynx (four), larynx (four), oropharynx (three), and maxillary sinus (two). All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80° C until the extraction of DNA and RNA. Histologic studies were also performed at the Department of Pathology, and all tumors were confirmed as squamous cell carcinoma.

DNA Extraction

Genomic DNAs were isolated from frozen tissues by SDS/proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation.

Microsatellite Analysis

A genome-wide screening was performed with 191 markers spaced an average of 10 cm apart (The Map Pairs Human Screening Set, version 8.0; ResGen, Invitrogen Japan K.K., Tokyo, Japan). PCR was performed in 20 μl of reaction mixture with 10 pmol of each primer, 100 ng of genomic DNA, 1$\times$ PCR buffer, 200 μM of each deoxynucleotide triphosphate, and 0.5 U of *Taq* DNA polymerase (Takara, Kyoto, Japan). Initial denaturation at 94° C for 3 minutes was followed by 25 cycles of a denaturation step at 94° C for 30 seconds, an annealing step between 50° C and 60° C depending on each primer for 30 seconds, and an extension step at 72° C for 1 minute. A final extension step at 72° C for 7 minutes was added. After amplification, 2 μl of the reaction mixture was 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 (Bassam et al, 1991). 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. In some cases, PCR with fluorescent dye–labeled primers were used to analyze LOH with ABI-3100 capillary sequencer and the GeneScan software (Applied BioSystems/Hitachi, Tokyo, Japan).

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