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Comparison of DNA Copy Numbers in Original Oral Squamous Cell Carcinomas and Corresponding Cell Lines by Comparative Genomic Hybridization

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We analyzed regional DNA copy numbers in 4 oral squamous cell carcinomas (SCCs) by using comparative genomic hybridization, and compared them with those in cell lines derived from the SCCs. In the original tumors, DNA copy number increases were observed on chromosomes 5p (4/4 cases), 8q (4/4), 20p (3/4), 3q (2/4), 5q (2/4), 7p (2/4), 7q (2/4), 11p (2/4), 11q (2/4) and 13q (2/4). Although most of these changes have been described previously for SCC tumors in the head and neck, the incidence of increases in 8q and 20p was much higher in the present study; this may be important in relation to cell line establishment, since 8q contains c-*myc*, which is involved in immortalization. No common chromosomal region with DNA copy number decreases was observed, except for 18q (2/4). When the original tumors and the cell lines were compared, their profiles were essentially similar with one exception. Further, there was no region that commonly changed in the cell lines, but not in the original tumors, suggesting that the DNA copy number changes observed in the cell lines mostly represent those of the original tumors.

Key words: Comparative genomic hybridization — Squamous cell carcinoma — Oral cancer — Chromosome — DNA copy number

Cancer cells are considered to be formed by a multi-hit process of malignant transformation, as the culmination of a number of aberrant genetic events. In squamous cell carcinomas (SCCs) of the head and neck, abnormalities of both oncogenes and tumor-suppressor genes have been described, but the changes of these genes during the course of the disease remain obscure.[1)] Further, the involvement of as-yet unidentified genes is not excluded. There are at least 2 critical processes in the *in vitro* model of carcinogenesis: escape from crisis (immortalization), and development of the ability of activated oncogenes to cooperate in tumorigenic transformation.[2,3)] The process of immortalization is distinct from cell transformation, probably requiring different changes in specific genes related to cell line establishment.[4)]

To examine these problems, comparative genomic hybridization (CGH),[5)] a recently developed molecular cytogenetic method, was applied to 4 original tumors and 4 derived cell lines to detect regions with DNA copy number abnormalities. It is possible simultaneously to assay for physical genetic gains and losses in tumor DNAs. CGH is very useful, because DNA copy number changes can be identified rapidly and tumors can be analyzed directly without culturing or karyotyping.

In this study, we identified the regions with DNA copy number abnormalities in the original SCCs of the oral cavity and in cell lines derived therefrom, in order (1) to look for regions with differences in DNA copy number abnormalities between the original tumors and the derived cell lines and (2) to look for regions that may be important for the establishment of cell lines.

MATERIALS AND METHODS

Clinical data Clinical data for the 4 patients with the original oral SCCs from which the 4 cell lines were derived are summarized in Table I.

DNA samples High-molecular-weight DNAs were extracted by proteinase K digestion and phenol-chloroform extraction as previously described.[6)] DNA probes were labeled either with biotin-14-dATP (tumor DNAs) or digoxigenin-11-dUTP (normal DNAs from peripheral blood lymphocytes of a normal male) using the BioNick Labeling System (Gibco BRL, Gaithersburg, MD). The DNase I concentration was adjusted so that the distribution of fragment sizes was 300–2300 bp.

CGH The target metaphase slides made from peripheral blood lymphocytes of a normal male (46,XY) were provided by SRL (Tokyo). Two hundred nanograms of biotin-labeled tumor DNA, 200 ng of digoxigenin-labeled reference DNA and 10 μg of unlabeled Cot-1 DNA were mixed and precipitated with ethanol. The DNA probes were dried and dissolved in 10 μl of hybridization solution (50% formamide, 2× SSC and 10% dextran sul-

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Table I. Clinical Data for the 4 SCC Patients

Case	Clinical stage[a)]	Sex	Age (yr)	Tumor site	Derived cell line
1	T4N1M0	Female	51	Mouth floor	HOC313
2	T3N3M0	Male	67	Tongue	HOC815
3	T3N0M0	Male	40	Tongue	HOC927
4	T4N1M0	Female	38	Mandible	HOC605

a) TNM classification of malignant tumors according to the International Union Against Cancer, 1987.

fate). Metaphase slides were denatured at 72.5°C in 70% formamide and 2× SSC for 2.5 min, and dehydrated in 70%, 85% and 100% ethanol. The DNAs were denatured at 72.5°C for 5 min, immediately applied to the denatured metaphase slides, and hybridized for 2–3 days at 37°C in a humidified chamber.

After hybridization, the slides were washed, and the biotinylated DNA was detected by using avidin-fluorescein isothiocyanate (Vector, Burlingame, CA) (to visualize bound biotinylated probes with green fluorescence) at 5 μg/ml and digoxigenin-labeled DNA with 1 μg/ml anti-digoxigenin rhodamine (Boehringer Mannheim, Mannheim, Germany) (to visualize bound digoxigenin-labeled probes with red fluorescence) in 5% skim milk, 0.1% Triton X-100 and 4× SSC. Samples were counterstained with 0.2 μg/ml DAPI in anti-fade solution. Coverslips were applied and sealed with nail polish.

Digital image analysis Three gray level images were taken separately for each fluorochrome using an epifluorescence microscope (XFEDII, Nikon, Tokyo) equipped with a cooled charge-coupled device camera and filter system consisting of a triple band-pass beam splitter and emission filters. Chromosomes were identified by using the fluorescence banding pattern obtained after DAPI staining. The fluorescence signals were quantitatively analyzed and ratio profiles of fluorescence intensity along the chromosomes were generated by a digital analysis system (CytoVision CGH, Applied Imaging, Santa Clara, CA). The average green-to-red ratio of these images was calculated from at least 4 metaphase spreads. The criteria used to define increased DNA-sequence copy number and decreased DNA-sequence copy number in tumors were based on comparisons of normal DNAs labeled and stained with two different colors: green-to-red ratios >1.2 (increase) and <0.8 (decrease). Control CGH experiments using normal-normal DNAs from oral mucosa and peripheral blood lymphocytes showed a range of green-to-red fluorescence ratios between 0.8 and 1.2 (data not shown).

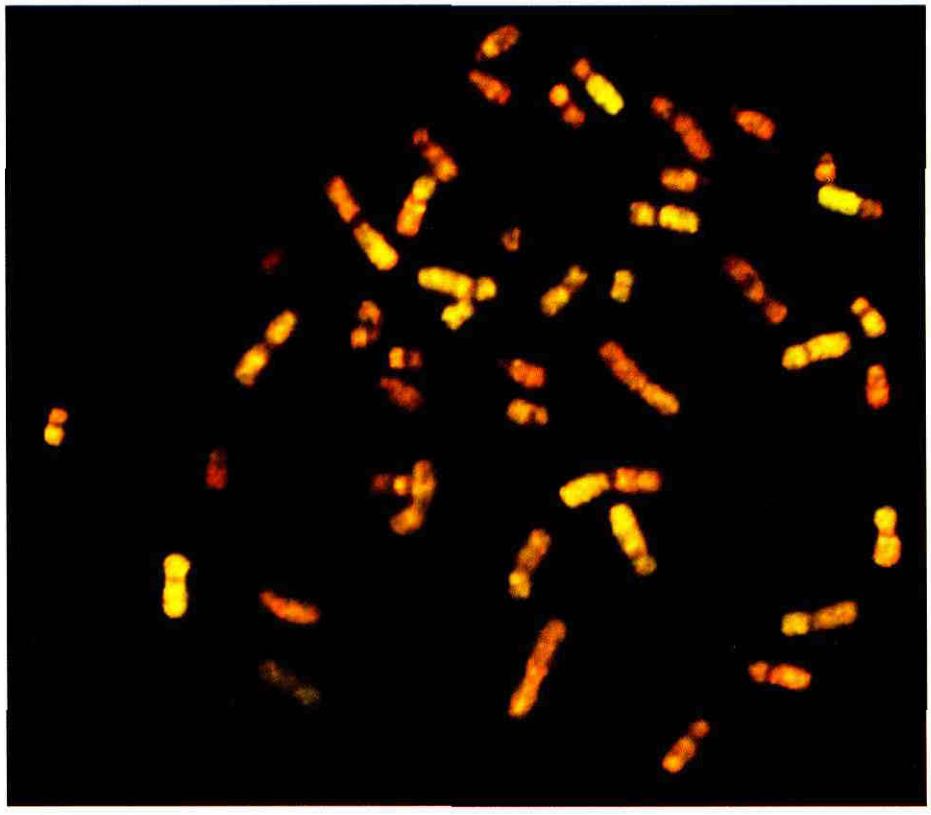
Southern blot hybridization Ten microgram aliquots of cellular DNAs (case 3, HOC927, case 4 and HOC605) were digested with *Eco*R I under the conditions recommended by the supplier, electrophoresed in agarose gels (1% w/v) and blotted onto nylon filters (Gene Screen Plus, Dupont, Boston, MA) according to Southern.[7] The filter was hybridized at 65°C for 12–16 h in a hybridization solution (5× SSC, 1.5% SDS, 0.1 mg/ml of salmon testis DNA and DNA probes labeled with [α-^{32}P]dCTP). Probes used were the 0.8 kb *Eco*R I fragment of *hst-1* genomic DNA[8] and the 0.86 kb *Pst* I-*Sma* I of a c-*mos* cDNA fragment[9] (c-*mos* cDNA fragment was used as a control), labeled with a random primer DNA labeling kit (Takara, Kyoto) using [α-^{32}P]dCTP (ICN, Costa Mesa, CA). After hybridization, the filters were briefly washed twice with 2× SSC/1% SDS at room temperature, once with this wash solution at 60°C for 30 min, and twice with 0.2× SSC/1% SDS at 60°C for 30 min, followed by exposure to an imaging plate for a few hours. Signals were quantitatively analyzed by an image-analysis device (BAS 2000, Fuji Film, Tokyo). The copy numbers of *hst-1* DNA were calculated from band intensities relative to those of control DNA as a standard.

RESULTS

CGH was applied to identify the regions with DNA copy number abnormalities in the original squamous cell carcinoma tissues and the cell lines derived from them, and to compare DNA copy number abnormalities among them. Fig. 1 shows the result of CGH analysis of an oral SCC tissue (case 1). The two-color image is shown in Fig. 1A when tumor (female) and normal (male) DNAs were simultaneously hybridized to metaphase cells of lymphocytes from a normal human male and detected by means of differentially labeled ligands. The biotinylated tumor DNAs were detected with a green fluorochrome, so DNA regions that are over-represented in the tumor appear green. In contrast, the digoxigenin-labeled normal DNAs were detected with a red fluorochrome, so regions that are under-represented in the tumor appear red. In this study, analysis of sex chromosomes was not done, since we used DNAs of original tumors, cell lines and normal lymphocytes from different sexes. The fluorescence banding pattern (based on the Q-banding pattern) of normal lymphocyte metaphase spreads after DAPI staining was used for chromosome identification (Fig. 1B). The image in Fig. 1A was captured digitally and used to generate a profile of the green-to-red fluorescence intensity ratio along the length of each chromosome. A compilation of fluorescence ratio profiles was made from at least 4 different metaphases, as shown in Fig. 1C. The fluorescence ratio profiles for HOC313, which was established from the tumor of case 1, are shown in Fig. 1D. The other 3 sets of tumors and cell lines were analyzed analogously (data not shown).

The regions with DNA copy number changes in the original tumors and the derived cell lines are schemati-

A



C

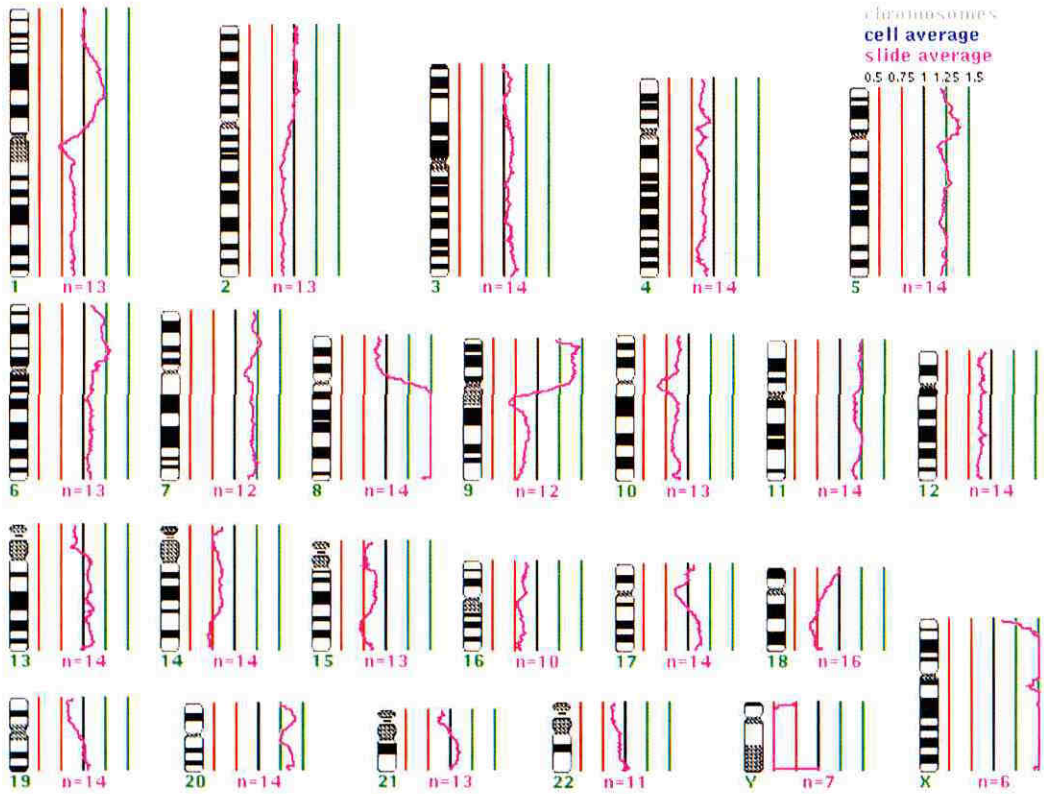

chromosomes
cell average
slide average
0.5 0.75 1 1.25 1.5
1 n=13
2 n=13
3 n=14
4 n=14
5 n=14
6 n=13
7 n=12
8 n=14
9 n=12
10 n=13
11 n=14
12 n=14
13 n=14
14 n=14
15 n=13
16 n=10
17 n=14
18 n=16
19 n=14
20 n=14
21 n=13
22 n=11
Y n=7
X n=6

Fig. 1. A, CGH image of case 1. Metaphase chromosomes from normal human male lymphocytes were hybridized to DNA of case 1 (green) and normal reference DNA (red). B, Paired chromosomes identified by DAPI banding pattern (right in each chromosome) and CGH image (left in each chromosome). The same metaphase chromosomes shown in A were sorted. C, Ratio profiles of CGH analysis of case 1. Each profile represents the relative amounts of labeled tumor DNA that hybridized to a normal metaphase chromosome. The central black line represents a green-to-red fluorescence ratio of 1.0. The green lines on the right represent fluorescence ratios of 1.25 and 1.5, respectively. Likewise, the red lines on the left represent fluorescence ratios of 0.75 and 0.5. The chromosomal number is indicated in the lower part of each ideogram. N means number of chromosomes analyzed. D, Ratio profiles of CGH analysis of the cell line (HOC313) derived from case 1.

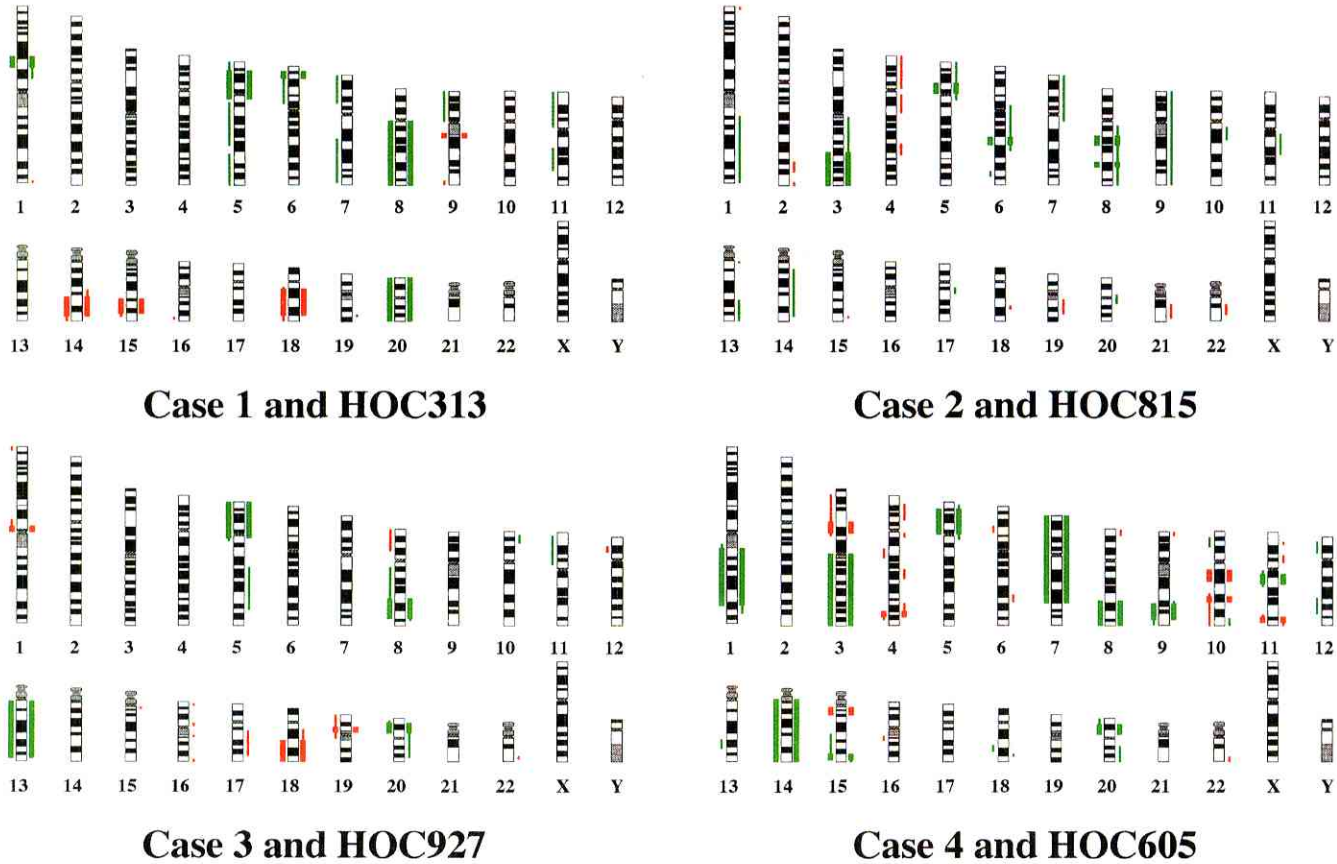


Fig. 2. Summary of all chromosomal regions with DNA copy number changes in the 4 original tumors and 4 derived cell lines. CGH data for these tumors and cell lines are plotted in ideogram form. DNA copy changes of the original tumors are indicated on the left side of the chromosome and changes for the cell lines are indicated on the right. Green and red lines indicate regions of increased (>1.2) and decreased (<0.8) DNA copy numbers, respectively. Bold lines represent the common regions between the original tumor and the corresponding cell line.

cally displayed in Fig. 2, where DNA copy number changes in the original tumors are indicated on the left side of each chromosome and those in the cell lines on the right. Green and red lines indicate regions with increased and decreased DNA copy numbers, respectively. Regions with abnormalities in DNA copy numbers are summarized in Table II, where DNA copy number changes of 13 cell lines reported by Matsumura are included for comparison.[10]

In the original tumor DNAs, DNA copy number increases were observed on chromosomes 5p and 8q in all cases. Three out of four had increased DNA copy numbers on chromosome 20p, and, in one or two cases, on chromosomes 1p, 1q, 3q, 5q, 6p, 6q, 7p, 7q, 9p, 9q, 10p, 11p, 11q, 12p, 12q, 13q, 14q, 15q, 18q and 20q. As for copy number decreases, there was no common region, except that two cases had a decreased copy number on chromosome 18q. Copy number decreases observed in one case were on chromosomes 1p, 3p, 4q, 6p, 8p, 9p, 9q, 10q, 11q, 14q, 15q, 16q, 19p and 19q.

When a comparison was made between the original tumors and the corresponding cell lines, the DNA copy number changes observed in the cell line mostly corresponded well with those of the original tumor, and the number of regions with abnormalities was not greatly changed. Although there were minor differences, the profiles were very similar. However, in case 2 and the cell line, HOC815, most regions with DNA copy number changes observed in the cell line were not represented in the original tumor tissue: many regions with DNA copy number changes were observed in HOC815, but far fewer regions were observed in the original tumor.

To confirm the results obtained by CGH, Southern blot hybridization with *hst-1* probe which maps on chromosome 11q13 was performed. DNA amplifications at this locus have often been reported in other SCCs of the

Table II. Summary of Regions with Abnormalities in DNA Copy Numbers

	Regions with increased DNA copy number	Regions with decreased DNA copy number
Original tumor (case 1)	**1p31-p22, 5pter-q11.2,** 5q12-q23, 5q31-qter, **6p24-p12,** 7pter-p13, 7q21-q36, **8q,** 9p, 11p, 11q14-q23, **20**	**9q13,** 9q34, **14q24-qter, 15q22-q26,** 16q24, **18q11.2-qter**
Cell line (HOC313)	**1p31-p21, 5p15.1-q11.2, 6p24-p23, 8q,** 19q13.3, **20**	1q44, **9q13, 14q22-q32, 15q23-q26, 18q12-q22**
Original tumor (case 2)	**3q24-qter, 5p13-cen, 6q21,** 6q25, **8q13-q21.2, 8q23**	
Cell line (HOC815)	1q22-qter, **3q11.2-qter, 5pter-q11.2, 6p12-q22,** 7pter-q11.2, **8q11.2-qter,** 9, 10q11.2-q21, 11q12-q14, 13q12, 13q31-qter, 14q12-q32, 17q12-q21, 20p11.2-q11.2	1q36.3, 2q34-q36, 2q37, 4pter-p12, 4q12-q21, 4q28-q31.1, 15q26, 18q21-q22, 19q12-q13.3, 21q21-q22, 22 q12-q13
Original tumor (Case 3)	**5pter-q11.2, 8q12-q24.2,** 11p15-p11.2, **13q12-q34, 20p13-p11.2**	1p36.3, **1p13-cen,** 8pter-p12, 12p12, **18q21-qter, 19pter-q12**
Cell line (HOC927)	**5pter-q11.2,** 5q15-q33, **8q22-q24.2,** 10p15-p13, **13q12-q34, 20p13-q13.2**	**1p13-cen,** 15q11.2, 16p13.3, 16p11.2, 16q12.1, 16q24, 17q21-25, **18q12-qter, 19p13.1-p12,** 22q13
Original tumor (Case 4)	**1q12-q41, 3q, 5p15.2-cen, 7pter-q31, 8q22-qter, 9q31-qter,** 10p14-p12, **11cen-q13,** 12p13-p12, 12q21-q24.1, 13q22-q32, **14q, 15q22-q26,** 18q21-q22, **20pter-p11.2**	**3p25-p14,** 4q21-q22, **4q32-q34,** 6p22-p21.3, **10q21, 10 q22-qter,** 11q23-q25, **15q11.2-q14,** 16q12.1-q13
Cell line (HOC605)	**1q12-q42, 3q, 5p15.3-q11.2, 7pter-q31, 8q22-qter, 9q31-q34,** 10q26, **11q12-q13, 14q, 15q26,** 18q22, **20p12-p11.2,** 20q12-qter	**3p21-p14,** 4p16-p14, 4q12, 4q25-q26, **4q31.3-q34,** 6q22-q23, 8p23, 9p24, **10q21, 10q23,** 11p15, 11p13-p11.2, **11q24-qter, 15q11.2-q14,** 22q13
Cell lines[a)]	20q (11/13), 11q (8/13), 13q (8/13), 3q (7/13), 7p (7/13), 8q (7/13), 14q (7/13), 20p (7/13), 15q (6/13), 1q (5/13), 9q (5/13), 10q (5/13), 22q (5/13), 1p (4/13), 2p (4/13), 12p (4/13), 16q (4/13), 17p (4/13), 5p (3/13), 7q (3/13), 11p (3/13), 12q (3/13), 3p (2/13), 6p (2/13), 8p (2/13), 9p (2/13), 18p (2/13), 19p (2/13), 19q (2/13), 4p (1/13), 5q (1/13), 6q (1/13), 10p (1/13), 16p (1/13), 17q (1/13)	3p (6/13), 8p (6/13), 18q (6/13), 5q (5/13), 7q (5/13), 10p (4/13), 19p (4/13), 19q (4/13), 2p (3/13), 12q (3/13), 16p (3/13), 1p (2/13), 1q (2/13), 2q (2/13), 4q (2/13), 6p (2/13), 6q (2/13), 9p (2/13), 11q (2/13), 18p (2/13), 3q (1/13), 4p (1/13), 7p (1/13), 8q (1/13), 10q (1/13), 11p (1/13), 14q (1/13), 15q (1/13), 16q (1/13), 17p (1/13), 21q (1/13), 22q (1/13)

a) Data from 13 cell lines previously reported (Matsumura[10]).

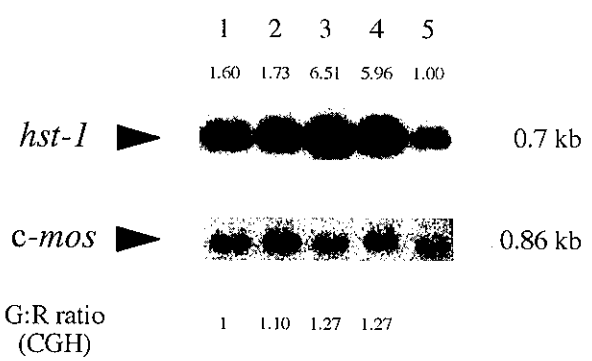


Fig. 3. Southern blot analysis of 2 original tumors and 2 derived cell lines. Lane 1, case 3; lane 2, HOC927; lane 3, case 4; lane 4, HOC605; lane 5, normal control (normal lymphocyte DNA). The number below the lane numbers represents the extent of amplification of *hst-1* after adjusting for the amount of DNA hybridization with the c-*mos* probe. The G/R ratio at 11q13 was calculated from the profiles of the original tumors and cell lines (data not shown).

head and neck region.[11] Further amplification of *hst-1* within this region has previously been found in 30–54% of human head and neck and esophageal primary SCCs, although this gene does not appear to be transcribed in appreciable amounts.[11,12] Fig. 3 shows the results of Southern blot hybridization of *Eco*R I-digested cellular DNAs probed with *hst-1*. There was more than five-fold amplification in case 4 and HOC605, but no significant amplification in case 3 and HOC927. This result is thus consistent with that obtained by CGH.

DISCUSSION

In this study, we examined regional DNA copy number changes in oral squamous cell carcinomas by using CGH and compared the changes with those of cell lines derived from them.

In the original tumors, DNA copy number increases occurred with high frequency on chromosomes 5p (4/4 cases, 100%), 8q (4/4, 100%), 20p (3/4, 75%), 3q (2/4, 50%), 5q (2/4, 50%), 7p (2/4, 50%), 7q (2/4, 50%),

11p (2/4, 50%), 11q (2/4, 50%) and 13q (2/4, 50%). In contrast, there was no frequently altered region with DNA copy number decreases, except for chromosome 18q (2/4, 50%). When the present result was compared with those obtained by CGH in two recent reports,[13,14] there was a significant difference; the incidence of DNA copy number increases in the previous reports was higher for 3q (50–77%), and 5p (20–62%), but not as high (15–30%) for 8q and 20p. Although the number of cases examined in this study was only 4, our preliminary data on 17 oral SCC cell lines established in our faculty also showed a high incidence of copy number increases for 8q (67%) and 20p (56%) (Matsumura and Tsuchida, unpublished data). Since cell lines were established from all of the 4 present cases, while only tumor samples were examined in the other reports, at least one of the regions detected in this study may contain amplified gene(s) that are important for cell line establishment (immortalization). In fact, it has been reported that chromosome 8q24 harbors c-*myc*, which may be associated with immortalization.[2,15] DNA copy number increases of 7p and 11q may correspond to the amplification of regions containing *EGFR* on 7p12 and *hst-1* on 11q13, respectively, in head and neck cancers.[11,16,17] With respect to the copy number increases on 3q, 5p, 5q, 7q, 11p, 13q and 20p, no oncogene has yet been reported on these chromosomes, so these arms may contain genes important for cancer progression, at least of oral SCCs.

Comparisons between the original tumors and the cell lines showed that DNA copy number changes observed in a cell line represented mostly those of the original tumor, except for case 2 and HOC815. Similar results have been reported previously, although the cell lines used in that study were not directly derived from the corresponding original tumors.[18] We looked for a region that was commonly changed in cell lines, but not in the original tumors. However, we could not detect such a common region, indicating that not many changes occurred in the cells during the course of establishment and further propagation. This result further suggests that changes necessary for establishment, such as an increase on 8q, had occurred before the cell line establishment, which may explain the reason why we observed a DNA increase in this region in all 4 cases, and also frequently in established cell lines, as described above. Alternatively, the commonly changed region(s) could have been too small to be detected by CGH. The ability of CGH to detect amplification depends on the level of amplification and the size of the region affected.[19] The detection limit has been calculated based on $L(N\text{-}2)$, where L is the size (Mb) and N is the copy number, and should be greater than about 2 Mbase.[19]

One possible explanation of the discrepancy between case 2 and HOC815 is contamination with normal cells in the original tumor, although the same *p53* mutation was detected by polymerase chain reaction-single strand conformation polymorphism in both the original and cell line DNAs used in this study.[20] (Komiyama and Tsuchida, unpublished data) To avoid normal cell contamination, the use of tumor DNA from a formalin-fixed and paraffin-embedded sample is required.[21,22] Furthermore, a retrospective CGH analysis can be done by using such DNA preparations. CGH provides a global analysis of chromosomal copy number changes that may be etiologically relevant, as well as having diagnostic and prognostic application in cancer.[23]

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