Loss of 3p26.3 is an independent prognostic factor in patients with oral squamous cell carcinoma

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Abstract. Oral squamous cell carcinoma (OSCC) is a common malignancy worldwide and the prognosis for patients with advanced-stage OSCC is particularly poor. To identify DNA copy number aberrations and candidate genes associated with a poor or favorable outcome, we analyzed the genome profiles of OSCC tumors by array-based comparative genomic hybridization (A-CGH). This technique uses DNA microarray technology to detect genomic copy number variations at a higher resolution level than chromosome-based CGH. Fifty patients with primary OSCCs were included in the study. Of these 50 patients, 37 were treated surgically and 13 were treated without surgery and had received irradiation and/or chemotherapy. All samples were analyzed by A-CGH. Gains were detected frequently (>50%) at chromosomal regions 5p15.33, 7p22.3, 8q21.1-24.3, 9q34.3, 11q13, 16p13.3 and 20q13.3. Losses were frequently detected at 3p22, 3p14 and 4q35.2. High-level gains were recurrently (>10%) detected at each of 5p15, 7p22, 7p11, 8q24, 11q13, 11q22 and 22q11. Gains of 2p25.1, 11p15, 16p13.3, 16q24.3 and 20q13.3 were inversely correlated with nodal metastasis. In 37 of the 50 OSCC patients treated with surgery, gains of 8q12.1-24.22 and losses of 3p26.2-3 were associated with disease-specific survival ($p<0.01$). Loss of a 0.2 Mb chromosomal region in 3p26.3 was associated with a poor prognostic outcome in the Kaplan-Meier analysis ($p<0.01$ by the log-rank test). Multivariate analysis revealed that loss of 3p26.3 is an independent prognostic factor ($p<0.01$) of OSCC. Loss of a 0.2 Mb chromosomal region in 3p26.3 including the CHL1 (cell adhesion molecule with homology to L1CAM1) gene was identified as a novel potential marker for predicting the prognosis of patients with OSCC.

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Introduction

There are approximately 270,000 cases of oral cancer worldwide per year, with deaths exceeding 128,000 (1,2). Most cases of oral cancer involve oral squamous cell carcinoma (OSCC). Diagnostic and therapeutic modalities for OSCC have markedly improved, but the prognosis has not changed significantly for more than two decades and is particularly poor for patients with advanced-stage OSCC (3,4). One of the important prognostic factors of OSCC is nodal metastasis. The biological characteristics of tumors are primarily affected by genetic and epigenetic changes in tumor cells, and identification of genomic alterations underlying malignant behavior of tumors may lead to an improvement of prognosis prediction and anticancer strategies for OSCC patients.

The accumulation of genomic aberrations, especially in proto-oncogenes and tumor suppressor genes, plays an important role in carcinogenesis and tumor progression. Comparative genomic hybridization (CGH) is a powerful tool that allows detection of genomic aberrations in a tumor sample in a single experiment (5). We have analyzed OSCCs by conventional (chromosome-based) CGH and identified frequent DNA copy number aberrations (DCNAs) as gains of 3q, 5p, 8q, 12p, 20 and X; and losses of 3p, 4, 8p, 13q, 17p, and 18q (6-10). Loss of heterozygosity (LOH) at 9p, 3p, and 17p as the early event and LOH at 4q, 6p, 8,11q, 13q, and 14q as the late event have been reported in OSCC development (11,12). However, few genes implicated in oral tumorigenesis have been identified in these studies. For example, allelic imbalances of p16 (CDKN2A) and FHIT loci have been associated with these chromosomal alterations and have been implicated in OSCC development (13,14).

Array-based CGH (A-CGH), in which DNA microarray technology is used for CGH, has recently been developed and provides high-resolution information at the gene level in genome-wide screenings of DCNAs (15-18). A-CGH makes it possible to detect sufficiently narrow chromosomal regions to allow identification of genes involved in each tumor. DCNAs of clinical OSCC specimens have been investigated in several studies using A-CGH, but a significant association between DCNAs and the prognosis of OSCC patients has been scarcely shown (19-21). In the present study, we applied A-CGH to

samples from 50 patients with primary OSCC to identify DCNAs and candidate genes associated with nodal metastasis or outcome.

Materials and methods

Patients and tissue samples. Fifty patients with primary OSCC who were admitted to the Yamaguchi University Hospital between 2003 and 2007 were enrolled in the study. The clinicopathological features of the patients are summarized in Table I. Their age ranged from 38 to 91 years old (mean, 69.2 years old). Of these 50 patients, 37 were treated surgically (including excisional biopsy). Thirteen of 50 patients were inoperative cases and were treated with radiation and/or chemotherapy. TNM stage and tumor grade were classified according to the UICC TNM staging system (22). Frozen tissue specimens were obtained from biopsy samples collected for diagnostic or therapeutic purposes. The specimens were stored at -80˚C until use. Of the 37 patients who underwent surgery, 10 died due to recurrence of OSCC and two died from non-cancer related causes. The study was approved by the ethics review committee of gene analysis research at the Yamaguchi University School of Medicine (submission no. 98) and informed consent was obtained from all patients.

DNA extraction. Microdissection was performed as previously described to reduce contamination with normal DNA (8). Cancerous regions were collected in which >80% of the cells were cancer cells. Test DNAs derived from microdissected tissue fragments and reference DNA derived from peripheral lymphocytes of healthy volunteers were isolated with a DNA extraction kit (DNeasy Tissue Kit®; Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Array-based comparative genomic hybridization (A-CGH). A commercial array (MAC array Karyo 4K®, Macrogen Inc., Seoul, Korea) was used in the study. This array consists of 4030 BAC clones spotted in duplicate at an average spacing of about 1 Mb over the whole genome. Data for each BAC clone are available at http://www.macrogen.co.kr. The BAC clones in this platform were established by Macrogen Inc. The unique name of each BAC clone is defined as a plate number by Macrogen Inc. and for consistency was used in our results. The position of the BAC clones in the human genome was established according to the NCBI Map Viewer 36.3 archived at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). A-CGH was performed as previously described (23).

Analysis of A-CGH data. Data analysis was performed according to previous reports using the MAC array Karyo 4K (23,24). Spots with fluorescent intensity that was too low or too high to be analyzed were excluded from the analysis. The average $\log_2$Cy5/Cy3 signal ratios of duplicate BAC clones were calculated for each sample and the ±0.25 $\log_2$Cy5/Cy3 signal ratios were used as the threshold for defining a copy number increase (gain) or decrease (loss). A BAC clone with an average $\log_2$Cy5/Cy3 signal ratio >0.75 and an average $\log_2$Cy5/Cy3 signal ratio of an adjacent BAC clone >0.5 was defined as a high-level gain.

Table I. Clinicopathological features of 50 patients with primary OSCCs.

Patient age (years)	
Mean	69.2
Range	38-91
Gender	
Male	26
Female	24
Tumor site	
Mouth floor	12
Buccal mucosa	2
Maxillary gingiva	7
Mandibular gingiva	8
Tongue	21
OSCC differentiation	
Well	21
Moderate	24
Poor	5
TNM classification	
T1	8
T2	25
T3	9
T4	8
N0	25
N1	12
N2-3	13
M0	50
Stage I	8
Stage II	14
Stage III	11
Stage IV	17
Habitual alcohol consumption[a]	
Yes	20
No	30
Habitual smoking[b]	
Yes	18
No	32

[a]Past history of habitual alcohol consumption on the first examination in our hospital. [b]Past history of habitual smoking on the first examination in our hospital.

Statistical analysis. Statistical analysis was performed with the JMP 6.0.2® software (SAS Institute, Cary, NC). A χ^2 test was used to determine the association between DCNAs and nodal metastasis or disease-specific survival. The probability of disease-specific survival was calculated by the Kaplan-Meier method, and statistical differences were evaluated by the log-rank test. A Cox proportional hazard model was applied for multivariate analysis. The 37 OSCC patients who underwent surgery were categorized as survivors or patients who died from a non-cancer-related cause (group A) and those who died from OSCC (group B). The 13 OSCC patients who

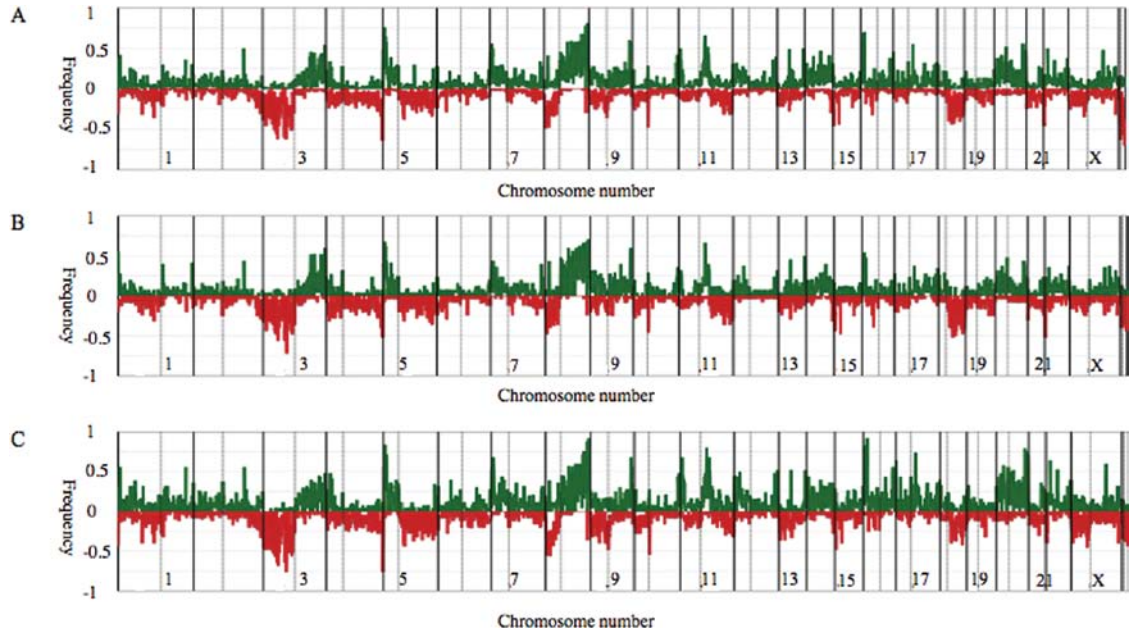

Figure 1. Genome-wide frequencies of DNA copy number aberrations (gains, above 0; losses, below 0) detected by A-CGH in 4030 BAC clones. (A) Fifty primary OSCCs. (B) Twenty-five cases with nodal metastasis. (C) Twenty-five cases without nodal metastasis.

Table II. Summary of high-level gains detected recurrently in 50 OSCCs.

Chromosomal site	BAC start	BAC end	Plate no.[a]	Located genes	Frequency
5p15.33	473145	562560	5262	AHRR, LOC442126, SEC6L1, SLC9A3	5/50
7p22.3	1650312	1929418	2601	MAD1L1, LOC402663, LOC442696, LOC442609	5/50
7p11.2	54808715	55102035	839	LOC442683, EGFR	5/50
8q24.3	145647088	145761879	5242	CYHR1, KIFC2, FOXH1, PPP1R16A	10/50
8q24.3	145648976	145759358	607	CYHR1, KIFC2, FOXH1, PPP1R16A	11/48
11q13.1	65068927	65159129	2897	LTBP3, SSSCA1, MTVR1, EHBP1L1, KCNK7, MAP3K11, PCNXL3	7/50
11q13.1	65111809	65159161	2623	EHBP1L1, KCNK7, MAP3K11, PCNXL3	8/50
11q13.3	68979903	69057580	124		13/50
11q13.3	69118585	69195569	677	CCND1, FLJ42258, ORAOV1	11/50
11q13.3	69236712	69325605	2817	FGF4	9/49
11q13.3	69266851	69383149	2272	FGF4, FGF3	10/50
11q13.3	69385428	69499328	2288		11/50
11q13.3-11q13.4	69888330	70001148	243	PPFIA1, CTTN, SHANK2	5/50
11q22.1	99915046	100015315	1174		5/50
11q22.2	101640046	101725137	2296	LOC120318, BIRC3, LOC440064, BIRC2	5/42
22q11.21	18059640	18139199	239	SEPT5, GP1BB, LOC441972, TBX1	5/50

[a]Plate no. of the BAC clone: the name of each BAC clone is defined as a plate number by Macrogen Inc. on the chip.

were treated non-surgically were excluded from survival analysis, since they included inoperable cases of advanced OSCC and patients who refused surgery. BAC clones with DCNAs detected in <20% of the 37 OSCCs were omitted from survival analysis. A χ^2 test was performed to assess relation-ships between DCNAs and disease-specific survival. To ensure statistical robustness, $p<0.01$ was considered to be significant for all statistical tests.

Results

DCNAs detected by A-CGH. The genome-wide frequencies of DCNAs detected by A-CGH are summarized in Fig. 1A. Gains were detected in 50% or more of the 50 OSCCs in a 1 Mb chromosomal region of 5p15.33, 1.1 Mb of 7p22.3, 71.3 Mb of 8q21.1-8q24.3, 0.6 Mb of 9q34.3, 0.3 Mb of 11q13.1, 1.6 Mb of 16p13.3, and 1.2 Mb of 20q13.33. Losses

Table III. Chromosomal regions in which copy number increases were inversely associated with metastasis to cervical lymph node in 50 OSCCs.

Chromosomal region	BAC start	BAC end	Plate no.[a]	Located genes
2p25.1	10213910	10559495	1347, 249	HPCAL1, ODC1
11p15	2390128	2973479	2932, 202, 5308, 789	TRPM5, KCNQ1, KCNQ1DN, CDKN1C, SLC22A18AS, SLC22A18
16p13.3	360924	2157921	2980, 456, 5885, 2228, 1560, 788	TMEM8, RPL23AP5, DECR2, SOLH, LMF1, SOX8, TSC2, PKD1, RAB26
16q24.3	87473666	88664779	2078, 5717, 1553, 5816	CBFA2T3, SPG7, RPL13, CPNE7, DPEP1, AFG3L1, DBNDD1, GAS8
20q13.33	61482280	62375822	5188, 5523, 3025, 5709, 5561	KCNQ2, EEF1A2, PRPF6, PRR17, SOX18, MYT1, PCMTD2

[a]Plate number of the BAC clone: the name of each BAC clone is defined as a plate number by Macrogen Inc. on the chip.

Table IV. Associations between DNA copy number aberrations of BAC clones and disease-specific survival.

						Frequency of gains	
Chromosomal site	BAC start	BAC end	Plate no.[a]	Located genes	p-value	Group A[b]	Group B[c]
Gains							
3q23	143731339	143817430	2411	ATR	0.0009	3/27	6/10
3q25.2-3q25.31	156280154	156393342	556	MME	0.0075	4/26	5/8
5p15.33	2593881	2676370	1258		0.0018	4/27	6/10
8q12.1	58530498	58626304	1293		0.0087	13/27	0/10
8q22.1	98299966	98380663	4950	TSPYL5	0.0019	15/24	0/9
8q22.3	102513356	102619292	505		0.0047	14/27	0/10
8q24.21	128110171	128193833	939		0.0014	21/27	2/9
8q24.22	133489743	133608097	5269	KCNQ3	0.0010	19/27	1/10
8q24.22	134015801	134112080	318	TG	0.0016	23/27	4/10
8q24.22	134344743	134440638	2598	NDRG1, LOC392271	0.0052	19/26	2/9
14q31.1	80562798	80644440	776	TSHR, NMNATP	0.0070	5/23	6/8
Losses							
3p26.3	142175	224030	2246	LOC642891, CHL1	0.0041	5/27	7/10
3p26.3	180950	339983	5677	LOC642891, CHL1	0.0073	6/27	7/10
3p26.2	4637828	4736173	4541	ITPR1	0.0073	8/27	8/10
4q35.1	187265799	187236711	1263	LOC442122, TLR3	0.0007	3/27	6/10
8p23.3	1289201	1436212	923	LOC442376	0.0042	10/27	9/10

[a]Plate number of the BAC clone: the name of each BAC clone is defined as a plate number by Macrogen Inc. on the chip. [b]Group A, survivors and patients who died from non-cancer related cause. [c]Group B, patients who died from OSCC.

were detected in 50% or more of the 50 OSCCs in a 10.1 Mb region of 3p22, 5.2 Mb of 3p14, and 0.2 Mb of 4q35.2. High-level gains were found for BAC clones at 5p15, 7p22, 7p11, 8q24, 11q13, 11q22, and 22q11 in >4 of the 50 OSCCs (Table II).

Correlations between DCNAs and metastasis to cervical lymph nodes. Associations between copy number gain, loss, and high-level gains of each of the 4030 BAC clones and metastatic status of cervical lymph nodes were analyzed by χ^2 test. The genome-wide frequencies of DCNAs in cases with and without nodal metastasis are shown in Fig. 1B and C, respectively. In the statistical analysis using each BAC clone, gains in 52 BAC clones and losses in 9 BAC clones were significantly correlated with nodal metastasis. Gains of a 0.2 Mb region of 2p25.1, a 0.6 Mb region of 11p15, a 1.8 Mb

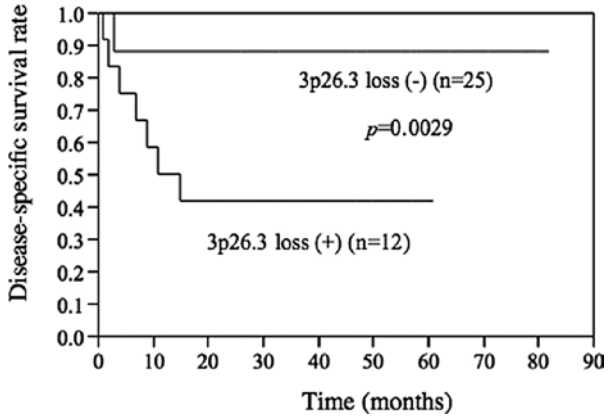

Figure 2. Kaplan-Meier curves showing a significant difference in survival between patients with and without loss of 3p26.3 (p=0.0041 by log-rank test).

Table V. Multivariate analysis of risk factors for disease-specific survival in patients with OSCC.

Factor	Risk ratio	95% CI	p-value
Nodal metastasis (0, 1, 2 or 3)	3.54	1.34-12.2	0.009
Age (≤70 vs. >70)	0.70	0.21-2.02	0.519
Histological grade (1, 2, or 3)	0.75	0.18-2.97	0.680
3p26.3 loss (plate no. 2246)[a] (Positive vs. negative)	0.22	0.07-0.59	0.002
Habitual alcohol consumption[b] (Positive vs. negative)	2.11	0.82-5.66	0.12
Habitual smoking[c] (Positive vs. negative)	0.99	0.33-3.17	0.985

[a]The name of each BAC clone is defined as a plate number by Macrogen Inc. on the chip. [b]Past history of habitual alcohol consumption on the first examination in our hospital. [c]Past history of habitual smoking on the first examination in our hospital.

region of 16p13.3, a 1.2 Mb region of 16q24.3, and a 0.9 Mb region of 20q13.3 were inversely correlated with nodal metastasis (Table III). In these 5 chromosomal regions, the interval between the BAC clones was narrower than 1 Mb. There was no correlation between BAC clones with high-level gains and nodal metastasis.

Correlations between DCNAs and disease-specific survival after surgery. Gain of 3p23, 3q25.2-25.31, 5p15.33, 8q12, 8q22.1, 8q22.3, 8q24.1, 8q24.21, 8q24.22 (including 3 different BAC clone regions) and 14q31.1, and losses of 3p26.2, 3p26.3 (including 2 different BAC clone regions) 4q35.1, and 8p23.3 were correlated with disease-specific survival in univariate analyses (p<0.01, Table IV). Two sets of BAC clones (plate nos. 5269, 318 and 2598 located at 8q24.22; and plate nos. 2246 and 5677 located at 3q26.3) were mapped to <1 Mb of the chromosomal region. The first set at 8q24.3 was inversely correlated with disease-specific survival, but three other BAC clones located between plate nos. 318 and 2598 did not show a significant association. The second set was located contiguously at 3p26.3. Univariate Kaplan-Meier analysis showed that disease-specific survival of patients with OSCC was negatively affected by loss of 3p26.3 (Fig. 2) and multivariate Cox proportional hazard model analysis showed that copy number loss of 3p26.3 was an independent factor predicting poor disease-free survival after surgery (Table V). Three early-stage OSCC patients with poor prognosis had a copy number loss at 3p26.3 and metastasis occurred in the cervical lymph node after resection of the primary tumor.

Discussion

In this study, DCNAs of 50 primary OSCCs were investigated by A-CGH. As far as we are aware, this is the largest study of the correlation between genomic aberrations detected by A-CGH and prognosis in OSCC. DNA copy number gains were detected frequently (50% or more) at chromosomal regions 5p15.33, 7p22.3, 8q21.1-24.3, 9q34.3, 11q13, 16p13.3, and 20q13.3, and losses were detected frequently at chromosomal regions 3p22, 3p14, and 4q35.2. We have identified similar changes previously, but the changes in the current study are more frequent and narrower than those in the earlier study (6-10).

Recurrent high-level gains (10% or more) were detected at 8q24.3, 11q13, and 11q22 (Table II). In these high-level gains, two different BAC clone regions in 8q24.3 were located 17 Mb away in the direction of q-ter from c-MYC. This area includes CYHR1, KIFC2, FOXH1, and PPP1R16A, but an association of these genes with OSCC has not been reported. The aberration in the 11q13 region includes CCND1 and FGF4, which have been associated with OSCC (25-27). Numerical aberrations of CCND1 have been linked with nodal metastasis and poor prognosis in OSCC and FGF4 overexpression detected immunohistochemically has been correlated with a worse prognosis in primary OSCC (21,28). BIRC2 (baculoviral IAP repeat-containing protein 2) is a candidate gene included in the 11q22 high-level gain, and overexpression of BIRC2 related to the amplification of 11q22 has previously been found in OSCC (20). BIRC2 amplification has also been linked to inhibition of apoptosis in lung cancer (29).

Copy number increases of 16p13.3, 16q24.3, and 20q13.33 have been detected recurrently in OSCC by A-CGH; however, the prognostic value of the aberrations was unclear (20,21). In the present study metastasis to cervical lymph nodes was less common in OSCC patients with a copy number increase of 16p13.3, 16q24.3, or 20q13.33. These findings suggest that gains of these chromosomal regions constitute markers for estimating nodal metastasis in OSCCs. Moreover, a gene associated with prevention of OSCC metastasis to lymph nodes might be included in 16p13.3, 16q24.3, and 20q13.33. Overexpression of TSC2 (tuberous sclerosis 2) located at 16p13.3 has been reported as a negative regulator of cell growth in OSCC cell lines; TSC2 encodes tuberin and seems to regulate the cell cycle by induction of the $p27^{kip1}$ protein (30-32). Deletions of 16q24.3 have been frequently detected in

breast and prostate cancers and in neuroblastoma (33-35). Gains of 16q24.3 are commonly detected in colon cancers, and DPEP1 and CDK10 have been reported as candidate genes in these gains of 16q24.3 (36). Candidate genes associated with over-representation of 20q13.33 have not been reported in OSCCs, but gain of 20q13.33 and overexpression of EEF1A2 (eukaryotic translation elongation factor 1 α2), which maps to this chromosomal region, have been found in hepatocellular carcinoma (37).

DCNAs are often associated with aberrant expression of oncogenes and tumor-suppressor genes, and many reports have shown that particular DCNAs are linked with poor prognosis of patients in many kinds of cancers (38,39). The association between DCNAs and outcome after surgery was analyzed by the χ^2 test, and DCNAs of 16 BAC clone regions, including gain of 11 and loss of 5 BAC clone regions, were identified as candidate prognostic markers for OSCC ($p<0.01$, Table IV). To ensure reliability of the prognostic markers, we looked for sets of DCNAs where the gain or loss in two adjacent areas was present within 1 Mb. This analysis showed that loss of BAC clone regions in 3p26.3 had the most reliable prognostic value among the 16 DCNAs. OSCC patients with 3p26.3 loss showed a significantly poorer outcome in Kaplan-Meier analysis ($p<0.01$ by log-rank test). Furthermore, the 3p26.3 loss was an independent prognostic marker in multivariate analysis using a Cox proportional hazard model. Loss of 3p has been associated with nodal metastasis and poor outcome in the previous study of OSCC using conventional CGH (40). This indicates that 3p26.3 loss is a novel prognostic marker for OSCC, and it is of note that three cases of early-stage OSCCs with poor outcome had loss of 3p26.3 and metastasis occurred after surgical treatment. Therefore, this marker might be useful for prognostic prediction in patients with early-stage OSCC. The only protein-coding gene that maps to this chromosomal region is CHLI (cell adhesion molecule with homology to L1CAM1). CHL1 is a member of the family of L1 neural cell adhesion molecules and has been reported to have regulatory functions that are pivotal to nervous system development (41). Additionally, a low-level transcript of CHL1 associated with loss of 3p26.3 has been reported in esophageal squamous cell carcinoma (42). These observations in combination with our results suggest that CHL1 is a candidate tumor suppressor gene associated with aggressiveness of OSCC.

In conclusion, we found many significant relationships between DCNAs and clinicopathological features using A-CGH on biopsy specimens from 50 cases of OSCC. Among these DCNAs, a copy number decrease of a 0.2 Mb chromosomal region at 3p26.3 was identified as a new potential marker for predicting the prognosis of OSCC. We have designed a prospective study to examine the association of prognosis with the DCNA at 3p26.3, and a further study will also be necessary to investigate the role of CHL1 protein in progression of OSCC.

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