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Simultaneous Assessment of Cyclin D1 and Epidermal Growth Factor Receptor Gene Copy Number for Prognostic Factor in Oral Squamous Cell Carcinomas

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Abstract: Cyclin D1 gene (*CCND1*) numerical aberrations are independent prognostic indicators of head and neck squamous cell carcinomas (HNSCCs). High epidermal growth factor receptor gene (*EGFR*) copy number is associated with poor prognosis in lung cancer, but such findings are controversial in oral SCCs (OSCCs). We analyzed copy number status in *CCND1* and *EGFR* in OSCC patients and its association with clinical outcome.

EGFR and *CCND1* statuses were analyzed in 85 OSCC patients by fluorescence *in situ* hybridization (FISH) of specimens obtained by fine-needle aspiration biopsy.

CCND1 numerical aberration was found in 35 of 85 tumors (41%), and aberrant *EGFR* copy number was observed in 36 (42%). Gene amplification (GA) was dominant among *CCND1* copy number changes (14/35:40%). Balanced trisomy (BT) was the most frequently observed *EGFR* aberration (17/36:47%). In a multivariate Cox's proportional hazards analysis, *CCND1* GA was correlated with disease-free survival ($P < 0.001$), whereas *EGFR* BT was significantly correlated with overall survival ($P = 0.001$). Patients with a combination of *CCND1* GA and/or *EGFR* BT had significantly poorer clinical outcome.

CCND1 and *EGFR* copy number changes were frequent in OSCC and had differing aberration patterns. *CCND1* GA and *EGFR* BT statuses by dual-color FISH were the predominant predictors of clinical outcome. Further investigation is needed to determine the implications for EGFR inhibitor therapy in OSCC.

Key words: oral cancer, cyclin D1, EGFR, FISH

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumor worldwide, with an estimated incidence of 500,000 annually[1,2]. Over 50% of HNSCCs arise in the oral cavity. Despite aggressive and often mutilating therapeutic regimens, overall survival in HNSCCs, including oral cancers, has remained

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largely unchanged over the past 20 years[3,4]. To improve the long-term survival rate of patients with this malignant tumor, it is important that we find more accurate prognostic markers that can identify patients with a high risk of recurrence and thus enable us to give more effective treatment.

Chromosomal aberration of the 11q13 region has been detected in HNSCC[5,6]. This region encompasses several putative oncogenes: *CCND1* (cyclin D1) is the major target for study because the amplicon of the 11q13 region always includes *CCND1* and is consistently amplified and overexpressed in tumor cells[7]. Several studies have reported that gene, chromosomal abnormalities and/or overexpression of *CCND1* is significantly correlated with aggressive tumor growth and poor outcome, thus this gene might be useful as a prognostic indicator in HNSCC[8–16]. Since 1998, we have been investigating *CCND1* deregulation by using dual-color fluorescence *in situ* hybridization with fine needle aspiration (FNA-FISH) of primary oral squamous cell carcinomas (OSCCs). We have revealed that: 1) *CCND1* amplification detected by FISH is a more reliable prognostic indicator than cyclin D1 protein overexpression; 2) tumors with aberrant copy numbers of *CCND1* always overexpress cyclin D1 protein; and 3) these *CCND1* numerical aberrations are significantly associated with invasive tumor phenotype and pathologically determined lymph node metastasis[12,13] and are valuable in identifying patients at high risk of late lymph node metastasis in stage I/II OSCC[14].

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of tyrosine kinase receptors, the ligands of which are epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α). EGFR is overexpressed in many kinds of solid tumors, including approximately 80% of HNSCCs[17]. Overexpression of this protein is associated with advanced-stage HNSCC and poor survival[18]. Recently, several clinical trials of EGFR inhibitors in treating patients with HNSCC have demonstrated a clear benefit of these drugs in a small subset of patients. In phase II studies of patients with recurrent or metastatic HNSCC, several EGFR inhibitors have shown encouraging clinical activity[19,20]. Moreover, a large, randomized, phase III trial has shown that a combination of EGFR inhibitors and radiation therapy in locally advanced HNSCC significantly prolongs overall survival[21]. Therefore, a subset of HNSCC tumors respond to EGFR inhibitors, and it is critical that we are able to select those patients who will respond. The identification of predictive markers for treatment response to these drugs is also a task of high priority. In non-small-cell lung cancer (NSCLC), increased *EGFR* copy number, as assessed by FISH, is associated with poor prognosis[22] and is significantly correlated with better clinical outcome in EGFR inhibitor-treated patients[23,24], suggesting that the copy number of this gene may be a useful predictor in patients treated with EGFR inhibitors. However, this association has not yet been clearly demonstrated in HNSCCs, including in oral cancer. Furthermore, recently, Kalish *et al*. suggested that deregulated cyclin D1 overexpression may be associated with resistance of HNSCC to EGFR inhibitors[25]. Thus, from not only a prognostic but also a therapeutic point of view, the simultaneous assessment of *CCND1* and *EGFR* copy number in OSCC by dual-color FISH is essential.

To elucidate the nature of *CCND1* and *EGFR* alterations in OSCC, we used dual-color FISH of FNA biopsy samples to examine *CCND1* and *EGFR* copy numbers and alteration patterns in 85 OSCCs, and we correlated the results with clinical parameters and patient outcome.

Patients and Methods

1. Patients and Tissue Samples

The samples were taken from OSCC specimens resected by primary surgical excision from 85 patients between 1999 and 2005 at the Maxillofacial Surgery, Graduate School, Tokyo Medical and Dental University (Tokyo, Japan) (Table 1). None of the patients had received pre- or postoperative treatment. The study was approved by the institution's review board, and informed consent was obtained from all patients. Clinicopathologic staging and historical grading of tumors followed the

Table 1 Correlation between *CCND1*, *EGFR* positive and clinicopathologic parameters

Clinicopathologic parameters	No. of patients	*P**	
		CCND1 polysomy or gene amplification	EGFR trisomy
Age, y			
＞ 60	38		
＜ 60	47	NS	0.05
Gender			
Male	53		
Female	32	NS	NS
Tumor site			
Tongue	48		
Upper gingiva	4		
Lower gingiva	22		
Floor of mouth	6		
Buccal mucosa	5	NS	NS
Clinical Stage			
I	24		
II	38		
III	7		
IV	16	NS	NS
Cellular differentiation			
Well to Moderate	70		
Poor	15	NS	NS
Mode of Invasion			
1–3	51		
4C to 4D	34	0.027	NS
LN metastasis**	38	0.003	NS
Distant metastasis	12	0.003	NS
Recurrence	37	＜ 0.001	0.012
Cancer death	20	0.001	0.001

NS indicates not significant.
**P* values were determined by the chi-squared test and two-tailed Fisher exact test.
**Histopathologic diagnosis.

current International Union Against Cancer TNM classification[26] and the World Health Organization classification[27] respectively.

2. FNA Biopsy and FISH Analyses

Tumor cell sampling and slide preparation for FISH assay were performed as described previously[12]. A suspension of single cells was obtained by aspirating the tumor with a 21-gauge needle. The cells were soaked in 0.05 M KCl solution for 2 min to disrupt the cell membranes and expose the naked nuclei, and then fixed by addition of an equal volume of methanol/acetic acid (3 : 1) solution (Carnoy). After centrifugation at 3000 revolutions per minute (rpm) for 10 minutes, the upper layer was exchanged for Carnoy solution. Centrifugation and solution exchange were repeated twice and the resulting upper layer was transferred dropwise to glass slides under steam.

To detect changes in *CCND1* and *EGFR* copy number in the oral SCC cells, we used two types of dual color bacterial artificial chromosome clone probes that were specific for those two genes.

FISH assays were performed with the LSI *CCND1* SpectrumOrange/CEP 11 SpectrumGreen and *EGFR* SpectrumOrange/CEP 7 SpectrumGreen

Table 2 Gene and chromosome FISH status

	CCND1		*EGFR*		Criteria		
	Patients		Patients		Average	Average	Average
FISH status	No.	%	No.	%	chromosome/cell	gene/chromosome	gene/cell
Balanced disomy (BD)	50	58.8	49	57.6	< 2.5	0.9–1.2	–
Balanced trisomy (BT)	8	9.4	**17**	**20.0**	2.6–3.0	0.9–1.2	–
Balanced polysomy (BP)	13	15.3	13	15.3	3.0 <	0.9–1.2	–
Gene amplification (GA)	**14**	**16.5**	6	7.1	–	1.2 <	3.0 <

probe (Vysis/Abbott Molecular, Des Plaines, IL). Dual-color FISH was carried out as follows. Briefly, the materials on the slides were aged in 2 × saline-sodium citrate (SSC)/0.1% (v/v) NP-40 at 37℃ for 30 minutes and dehydrated through an ethanol series. The slides were denatured in 70% (v/v) formamide/2 × SSC at 75℃ for 5 minutes and dehydrated through an ethanol series. The probe, denatured at 75℃ for 5 minutes, was placed on denatured slides, covered with Parafilm (American National Can, Greenwich, CT), and incubated in a humid box at 37℃ overnight. After being washed at 45℃ three times in freshly prepared 50% (v/v) formamide/2 × SSC for 10 minutes, SSC for 10 minutes, and 2 × SSC/0.1% (v/v) NP-40 for 5 minutes, the slides were counterstained with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI: 1 μg/ml).

3. Fluorescence Microscopy

An Olympus BX50 epifluoresence microscope equipped with × 60 and × 100 oil-immersion objectives and a triple-pass filter for Spectrum Green/Spectrum Orange and DAPI (Vysis Inc, Downers Grove, IL) was used to count the fluorescent signals. Overlapping and damaged nuclei were ignored and only intact nuclei were evaluated. Hybridization signals were counted in 100 interphase nuclei. The copy numbers of *EGFR* and chromosome 7 centromere signals or *CCND1* and chromosome 11 centromere signals were counted for each nucleus.

4. Evaluation of FISH Analysis

The following criteria were defined for analysis: for each FISH probe tested, the status of the chromosome used as control was classified as disomy, trisomy, or polysomy when, respectively, on average ≤ 2.5, 2.6–3.0 and more copies were scored per nucleus. FISH patterns were considered balanced when the ratio gene/chromosome copy number per cell was 0.9–1.2. When average ratio gene/chromosome copy number was > 1.2 and the gene/cell ratio > 3.0, it was classified as gene amplification (GA)[12–14,28,29]. According to the criteria shown in Table 2, tumors were classified as showing balanced disomy (BD), balanced trisomy (BT), balanced polysomy (BP), or gene amplification (GA).

5. Statistical Analysis

The dual-color FISH results were compared with the clinicopathologic information. Statistical analyses were carried out with SPSS11.0J software (SPSS Japan Inc.). The results of dual-color FISH were compared with clinicopathologic information using the chi-squared test and two-tailed Fisher exact test. The clinicopathologic information included patient age, gender, tumor site, disease stage, histopathologic grading, presence of lymph node metastasis, and the mode of invasion according to the modified Jacobsson criteria.

Multivariate Cox's proportional hazards analysis were used to compare the FISH results across levels of clinicopathologic parameters. Disease-free survival (DFS) and overall survival (OS) rates were also calculated by the Kaplan-Meier method, and statistical significance was determined by the log rank test.

Results

1. Patient Characteristics

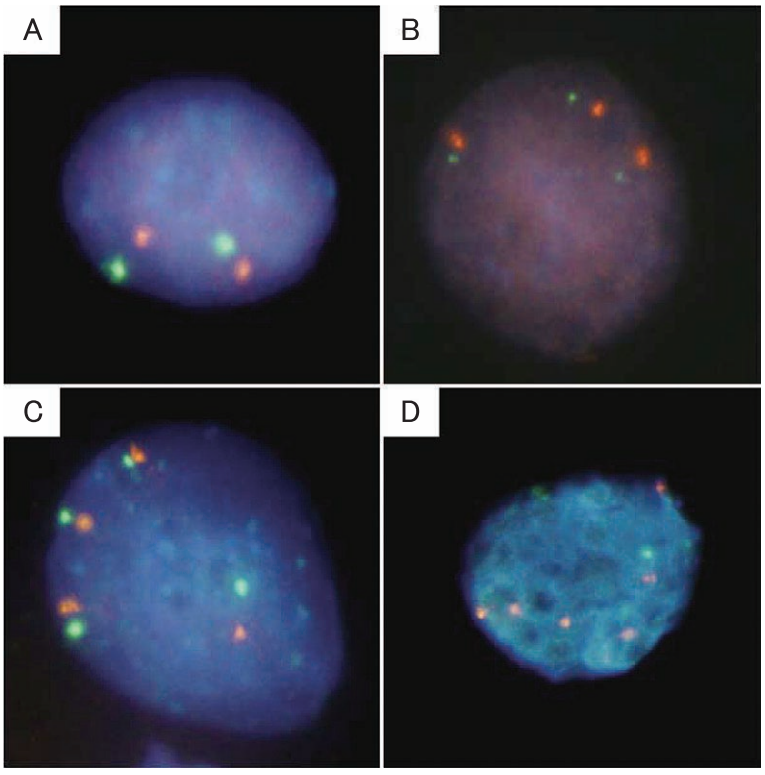

Fig. 1 Evaluation of gene copy number by fluorescent *in situ* hybridization (FISH). Cells are hybridized with probes for the chromosome 11 centromere (green) and *CCND1* (orange). *CCND1* balanced disomy (BD) is normal gene copy number (A), balanced trisomy (BT) of *CCND1* (B), balanced polysomy (BP) of *CCND1* (C), and gene amplification (GA) of *CCND1* (D).

Tumor cells were collected from the surgical specimens of 85 patients with OSCC. Patient characteristics are summarized in Table 1. The median follow-up time after surgery was 1383 days (range 674–2912 days). Postoperative recurrence, including late cervical lymph node metastasis, distant metastasis, and locoregional recurrence, occurred in 37 patients.

2. *CCND1* and *EGFR* Copy Numbers

All 85 OSCC samples were successfully scored for *CCND1* and *EGFR* FISH status. For *CCND1*, there were 50 tumors with BD FISH status (59%), 8 with BT (9%), 13 with BP (15%), and 14 with GA (17%, Fig. 1 and Table 2). Thus, *CCND1* numerical aberration was found in 35 tumors (41%), and GA and BP were the dominant abnormities of this gene. Thirty-six (42%) of the 85 tumors exhibited abnormal *EGFR* copy numbers: 17 with BT (20%), 13 with BP (15%), and 6 with GA (7%, Fig. 1 and Table 2). Therefore, BT was the most frequently observed *EGFR* copy number aberration. Unbalanced gene loss was not observed in the 85 tumors.

3. Association between *CCND1* and *EGFR* Statuses and Survival

The Kaplan-Meier survival curves for DFS and OS (Fig. 2A, 2B) clearly demonstrated the adverse impact of positive *CCND1* BP or GA status on both disease recurrence (BP: log rank $P = 0.0016$; GA: log rank $P < 0.0001$) and OS (BP: log rank $P = 0.0248$; GA: log rank $P = 0.0014$). For *EGFR*, the DFS and OS curves of patients with abnormal copy numbers (BP, BT, or GA) were shorter than those of patients with normal copy number (BD). However, positive *EGFR* BT status was the only abnormality significantly associated with worse DFS (log rank $P = 0.0068$) and OS (log rank $P = 0.002$) (Fig. 2C, 2D). The correlation between *CCND1* polysomy or gene amplification and/or *EGFR* trisomy and clinicopathologic parameters

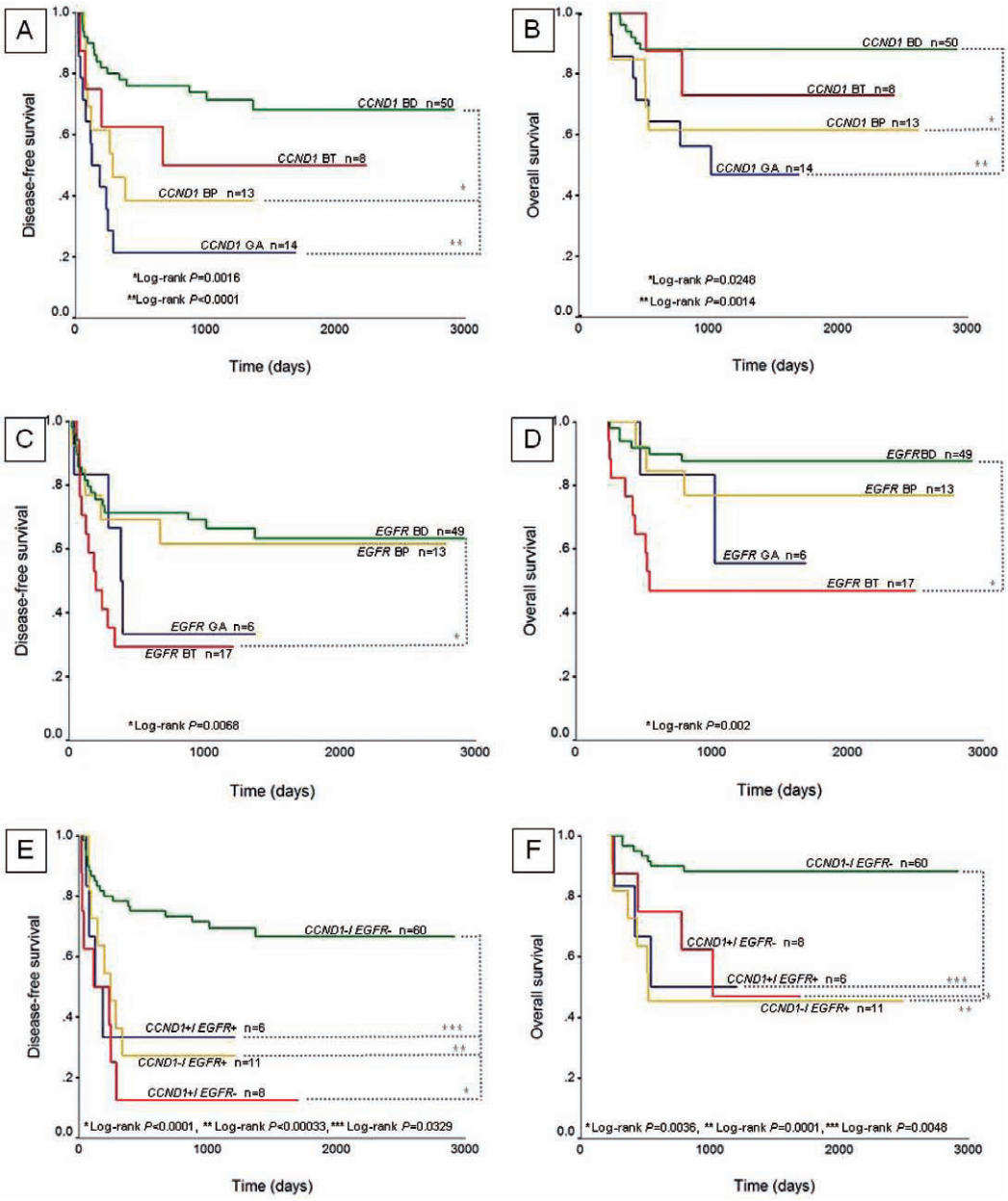

Fig. 2 Kaplan-Meier plots for *CCND1* (A) disease-free survival, (B) overall survival, for *EGFR* (C) disease-free survival, (D) overall survival, and for combined *CCND1* gene amplification and *EGFR* trisomy (E) disease-free survival, (F) overall survival. *CCND1*−; *CCND1* BD, BT or BP, *EGFR*−; *EGFR* not BT, *CCND1*+; *CCND1* GA and *EGFR*+; *EGFR* BT.

in patients with oral SCCs is also summarized in Table 1. The presence of *CCND1* polysomy or gene amplification was not correlated significantly with age, gender, tumor site or tumor clinical stage. Although *CCND1* polysomy or gene amplification occurred more frequently in advanced-stage or poorly differentiated tumors, these differences were not statistically significant. However, a more diffuse invasive pattern (mode of invasion: Grade 4C, 4D) was significantly associated with this genetic abnormality (chi-squared and two-tailed Fisher exact for trend, $P = 0.027$). Moreover, these FISH statuses of *CCND1* were significantly correlated with pathologic lymph node status ($P = 0.003$), distant metastasis ($P = 0.003$) disease recurrence ($P < 0.001$), and survival ($P =$

Table 3 Association between *CCND1* and *EGFR* FISH status

	CCND1 polysomy or gene amplification		
EGFR trisomy	Positive	Negative	Total
Positive	9	8	17
Negative	17	51	68
Total	26	59	85

$P = 0.025$

Table 4 Multivariate Cox proportional hazard analysis

	Disease-free survival		**Overall survival**	
	Risk ratio	95% Cl	Risk ratio	95% Cl
Age	–	–	–	–
Gender (male)	**2.202**	1.016–4.770	**4.000**	1.129–14.169
Tumor site	–	–	–	–
Tumor size	–	–	–	–
Clinical stage I-II vs. III-IV	–	–	**4.224**	1.672–10.675
Cellular differentiation	**2.211**	1.085–4.508	–	–
/CCND1/				
Balanced disomy	–	–	–	–
Balanced trisomy	–	–	–	–
Balanced polysomy	–	–	–	–
Gene amplification	**4.637**	2.086–10.309	–	–
/EGFR/				
Balanced disomy	–	–	–	–
Balanced trisomy	–	–	**6.589**	2.260–19.211
Balanced polysomy	–	–	–	–
Gene amplification	–	–	–	–

0.001).

On the other hand, *EGFR* trisomy was significantly correlated with patient age ($P = 0.05$), and this genetic aberration was significantly associated with disease recurrence and survival ($P = 0.012$ and $P = 0.001$, respectively). However, this abnormality was not significantly correlated with other clinicopathologic factors, and the correlation with pathologic lymph node status and distant metastasis just failed to reach statistical significance. Table 3 shows the correlation between *CCND1* and *EGFR* FISH status. Nine (35%) of the 26 patients with tumors demonstrating *CCND1* polysomy or gene amplification concomitantly exhibited *EGFR* trisomy. Thus, there was significant association between *CCND1* and *EGFR* by FISH analysis ($P = 0.025$).

Multivariate Cox's proportional hazards analysis including clinicopathologic factors and FISH status revealed that male (hazard ratio 2.2, $P = 0.045$), poor cellular differentiation (hazard ratio 2.2, $P = 0.029$), and positive *CCND1* GA status (hazard ratio 4.6, $P < 0.001$) were independent prognostic indicators of poorer DFS. Male (hazard ratio 4.0, $P < 0.032$), clinical stage III/IV (hazard ratio 4.2, $P = 0.005$), and positive *EGFR* BT status (hazard ratio 6.6, $P = 0.001$) were independent prognostic indicators of poorer OS (Table 4).

4. Association of Combined *CCND1* and *EGFR* Status with Disease Outcome in Oral SCC

Given that positive *CCND1* GA status was an independent predictor of DFS and positive *EGFR* BT status was an independent predictor of OS, we attempted to obtain further information by analyzing subgroups on the basis of combined *CCND1*

and *EGFR* FISH statuses. Four subgroups (positive or negative status for *CCND1* GA and *EGFR* BT) were assigned to DFS and OS curves. DFS and OS in patients whose tumors exhibited at least positive *CCND1* GA or *EGFR* BT status (Groups +/+, +/−, and −/+) were significantly lower than in Group −/−. There were no significant differences in DFS and OS rates among these three groups (+/+, +/−, and −/+) (Fig. 2D, 2E).

Discussion

We explored the relationship between *CCND1* and *EGFR* genetic statuses in 85 untreated OSCCs assessed by FISH of FNA biopsies, as well as the association between FISH status and prognosis. We clearly demonstrated that the majority of OSCC tumors exhibited copy number changes in these two genes, and that these genetic aberrations were significantly correlated with aggressive tumor behavior and poorer prognosis. Interestingly, we revealed that the dominant genetic abnormality patterns vary between *CCND1* and *EGFR*.

To the best of our knowledge, this is the first simultaneous dual-color FISH assessment of *CCND1* and *EGFR* copy number in the same OSCC tumor specimens and the first study to categorize FISH status into four subtypes (BD, BT, BP, and GA) and investigate each genetic abnormality as a prognostic indicator for this cancer. *CCND1* numerical aberrations were found in 35 (41%) of all 85 primary OSCCs. GA was seen in 14 (40%) of these 35 patients and was the most frequently identified aberration. In the Kaplan-Meier estimate, the DFS and OS curves of patients with *CCND1* GA or BP were significantly shorter than the curves of patients without *CCND1* numerical aberrations. These findings are in keeping with the evidence that *CCND1* numerical aberrations, as detected by FISH of FNA biopsy samples of primary OSCCs, may be useful predictors of recurrence and survival in patients with these cancers[12–14,29]. Thirty-six (42%) of the patients had tumors with increased numbers of *EGFR* copies per cell, most of them with BT (17 cases: 47%) or BP (13 cases: 36%). GA was found in only 17% of patients whose tumors showed *EGFR* copy number aberrations; it was therefore far less common than in the *CCND1* aberrations. This finding is consistent with the results of previous reports[22,30]. Kaplan-Meier survival curves for DFS and OS clearly demonstrated the adverse impact of *EGFR* BT on both disease recurrence and OS. However, our multivariate Cox's proportional hazards analysis determined that *EGFR* BT was significantly correlated with OS alone and was one of the dominant predictors of OS in OSCC patients.

Many studies have investigated the role of *CCND1* abnormalities in the tumorigenesis of HNSCCs including oral SCCs. Those studies revealed that amplification or overexpression of *CCND1* might be a valuable biologic marker of poor prognosis, tumor aggressiveness, and local and regional recurrence of these malignancies[15,16,31]. Since 1998, we have been investigating *CCND1* deregulation using FISH of FNA biopsies from primary oral SCCs, and have demonstrated clearly that it is possible to investigate chromosomal aberrations, such as amplifications, deletions and chromosomal rearrangements, in these carcinomas using this technique. We have demonstrated that *CCND1* numerical aberration is significantly associated with an invasive phenotype and cervical lymph node metastasis, and that this genetic alteration is a reliable predictor of outcome in oral SCCs[12–14]. In the current study, we observed *CCND1* polysomy or amplification in 27 of 85 primary oral SCCs (31.8%), and these were significantly associated with aggressive tumor behavior and poorer survival (Table 1).

On the other hand, gene amplification and overexpression of EGFR have been reported in various human cancers including oral SCCs. Yamamoto *et al*. has demonstrated that EGFR gene amplification and overexpression is found in many human squamous cell carcinoma cell lines[32]. Kamata *et al*. has also reported the growth inhibitory effect of EGF and the sensitivity to the inhibitory effect of EGF correlated with the level of EGFR in 12 SCC cell lines including oral cavity[33], however the role of those aberrations in the tumorigenesis of human SCCs is unclear. There have been several conflicting reports on the association between

EGFR protein expression level and gene copy number. Many studies have indicated that *EGFR* copy number is significantly correlated with protein expression in many human malignancies, including non-small-cell lung cancer and laryngeal and esophageal cancers[22,28,34]. In contrast, several other studies have found no significant association between EGFR protein expression level and *EGFR* FISH status in HNSCC[35,36]. These discrepancies may result from differences in the site and histology of the tumor, patient numbers, case heterogeneity, and the methods used to assess EGFR. Therefore, this issue remains a matter of controversy, and further investigations will be required to clarify whether EGFR protein expression level is indeed correlated with gene gain of copy number.

We also demonstrated that *EGFR* trisomy was significantly correlated with age of patient in the current study, but none of the other histopathological parameters was statistically significant. A negative correlation between *EGFR* genetic abnormalities or overexpression and clinical features, such as metastasis, or histopathological characters in the HNSCCs including oral SCCs has been reported[36,37]. The question arises as to why EGFR trisomy is the strongest predictor. To address this question, we examined the correlation between *EGFR* and centromere of chromosome 7 FISH status; we found a significant positive correlation between the two genetic factors (Spearman's rho = 0.925, $P < 0.001$). Moreover, we investigated the association between chromosome 7 copy number status and clinical outcome in OSCC, and found that tumors with chromosome 7 trisomy had the worst survival curves of the three chromosome 7 FISH statuses of disomy, trisomy and polysomy (data not shown). These associations are the likely reason why the *EGFR* BT aberration was the most significant predictor of OS. To our knowledge, the association between chromosome 7 copy number and clinical outcome in OSCC has not been reported previously, although Gebhart *et al.* examined 35 OSCCs by comparative genomic hybridization, and those patients whose tumors showed a gain of chromosome 7p, including band p12, had higher rates of relapse and worse survival[38]. Chromosome 7 aneusomy has been found in various carcinomas of the body, such as prostate, lung, larynx, and head and neck[22,30,34,39]. Several groups have demonstrated that aneusomy of chromosome 7 is associated with higher prostate cancer grade, advanced pathological stage, and shorter survival[30,40]. In contrast, in HNSCC, Joris *et al.* used tissue *in situ* hybridization to investigate changes in chromosome constitution with carcinogenesis in laryngeal SCCs and found that 12 (75%) of 16 tumors showed chromosome copy number imbalances and/or polysomy of chromosome 1 and 7; these changes were significantly negatively correlated with cancer-free survival rates[41]. However, they did not clearly demonstrate the association between chromosome 7 aneusomy and prognosis in laryngeal SCC. Therefore, further work is required to clarify whether chromosome 7 copy number changes are in fact correlated with prognosis in HNSCCs including oral cancer.

Of particular interest was the finding that the dominant genetic abnormality patterns vary between *CCND1* and *EGFR*. Different mechanisms may contribute to the development of this discrepancy in genetic status. Recently, Reshmi and Gollin reviewed several sources of chromosomal instability that accounted for the mechanisms by which aneuploidy and gene amplification arise[42]. Our results clearly demonstrated that GA was the most frequently detected aberration of *CCND1*. The chromosomal location of *CCND1* may be intimately associated with this genetic abnormality. *CCND1* is located on 11q13[42], and several fragile sites have been identified in and around this chromosomal locus. These fragile sites are thought to be sites of chromosomal breakage, and breakage-fusion-bridge (BFB) cycles may lead to gene amplification at this site[43,44]. Therefore, it is possible that the amplification of *CCND1* in band 11q13 may be due to its chromosomal location—*i.e.* in a hotspot for chromosomal breakage. For *EGFR*, BT and BP were the dominant genetic abnormalities, suggesting that copy number changes in this gene were significantly associated with chromosome 7 copy number. In general, chromosome aneusomy may be caused by DNA aneuploidy, which may in turn be due to genetic

instability resulting from aberrations in tumor suppressor genes or oncogenes. This may be one of the mechanisms that affect *EGFR* FISH status.

Over the last few years, a substantial amount of structural data has illuminated the role of EGFR in human cancers. EGFR activates a variety of intracellular pathways and proteins that stimulate growth, proliferation, angiogenesis, metastasis, and survival, including the Ras/Raf/MAPK (Mitogen-Activated Protein Kinese), PI3K (phosphatidylinositol-3 kinase)/Akt, phospholipase-Cγ, STATs (signal transducers and activators of transcription), and Src kinase pathways[45]. Signal transducers and activators of transcription 3 (STAT3) represent a point of convergence for several upstream signaling pathways, including EGFR, platelet-derived growth factor receptor, Src, Bcr-Abl, and gp130/IL-6R where activation of STAT3 elicits expression of a variety of target genes, including Bcl-XL, MMP-9, VEGF, MMP-2, and cyclin D1[46–51].

Recently, several *in vitro* studies have suggested that cyclin D1 plays a critical role in EGFR-driven tumorigenesis and that deregulated cyclin D1 overexpression may be significantly associated with resistance of HNSCC to EGFR inhibitors[25]. This suggested that *CCND1* is a pivotal downstream target gene in the EGFR pathway, and that to predict the prognosis of patients with OSCCs and the therapeutic effectiveness of EGFR inhibitors we need to investigate the genetic status of not only *EGFR* but also *CCND1*. We therefore performed a simultaneous assessment of *CCND1* and *EGFR* genetic status, using FISH of FNA biopsy samples to define subgroups of patients at increased risk of disease recurrence and poor prognosis. We clearly demonstrated that DFS and OS of patients with *CCND1* GA and/or *EGFR* BT (Groups +/+, +/−, and −/+) were significantly lower than in patients without these genetic abnormalities. In addition, there were no significant differences among these three groups in terms of DFS and OS, thus we did not determine the synergistic effect of these two genes on prognosis. This finding suggests that *CCND1* and *EGFR* genetic abnormalities equally contribute to clinical impact on prognosis in OSCC patients. However, the mechanism by which these molecules contribute to the initiation and/or progression of this cancer is not clear. Additional studies are required to clarify the relationships between cyclin-dependent kinase pathways, including *CCND1* and *EGFR* pathways, and how these pathways contribute to tumorigenesis in OSCC.

In conclusion, copy number changes in *CCND1* or *EGFR* are frequent events in the carcinogenesis of OSCC, and the aberration patterns vary between these two genes. We clearly demonstrated by dual-color FISH that *CCND1* GA is a prognostic marker of DFS in OSCC and that *EGFR* BT is a marker of OS. Simultaneous assessment of *CCND1* and *EGFR* statuses by means of dual-color FISH is a simple and sensitive method of predicting the prognosis of OSCC patients. However, the question of whether the genetic profiles of these two genes are correlated with response to therapy with EGFR inhibitors in OSCC was not the focus of this study. To address this issue, further investigations are required to determine which kinds of factors, including *CCND1* and *EGFR* FISH statuses, can be used to identify those patients most likely to benefit from *EGFR* inhibitor therapy.

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