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EGFR gene copy number alteration is a better prognostic indicator than protein overexpression in oral tongue squamous cell carcinomas

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ABSTRACT

Although Epidermal growth factor receptor (EGFR) is particularly important in the pathogenesis of head and neck squamous cell carcinomas (HNSCCs), conflicting data have been reported on the correlation between *EGFR* copy number and survival and the association between *EGFR* copy number and protein expression. Anatomical site of the tumour in HNSCCs may likely contribute to the discordance of the above points as EGFR expression may differ between the sub-sites of HNSCCs. Thus, in this study, we focused on oral tongue squamous cell carcinomas (OTSCCs). To investigate the association between *EGFR* copy number alteration and overexpression and to determine which is the more reliable prognostic indicator, Fluorescence in situ hybridisation (FISH) and immunohistochemical staining (IHC) were performed at a single institution on samples from 89 patients with OTSCCs undergoing surgery as the primary treatment modality. Thirty-two (36%) of 89 cases demonstrated an *EGFR* copy number alteration. EGFR protein expression was found in all 89 cases, of which 82.0% showed overexpression. No significant correlation was found between gene copy number and protein overexpression. Gene copy number alteration was significantly associated with reduced disease-free survival ($P = 0.048$) and overall survival ($P = 0.001$). Multivariate Cox proportional hazards analysis demonstrated that *EGFR* copy number increase was significantly correlated with overall survival ($P = 0.001$). *EGFR* copy number status is a more reliable indicator than protein overexpression of the survival rate in OTSCCs. FISH analysis of the *EGFR* status is useful in predicting poor prognosis in OTSCCs.

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1. Introduction

The epidermal growth factor receptor (*EGFR*) located at the region of p12 on chromosome 7 is a member of the ErbB family of tyrosine kinase receptors that regulates cell growth. Binding of its specific ligands, such as epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), promotes homo- or hetero-dimerisation with other family members (ERBB2, ERBB3 and ERBB4) and subsequent autophosphorylation and initiates a number of signalling pathways. Upregulated EGFR signalling in tumours has been correlated with cell proliferation, invasion, angiogenesis, metastasis, migration and inhibition of apoptosis.[1] EGFR is overexpressed in many epithelial malignancies, including approximately 80% of head and neck squamous cell carcinomas (HNSCCs),[2] and overexpression occurs early during the course of malignant development.[3] Thus, EGFR is important in the pathogenesis of HNSCCs, and is an interesting target for therapy.

Recently, several clinical trials of EGFR inhibitors in HNSCC treatment have demonstrated a clear benefit of these drugs in a small subset of patients. Phase II studies of patients with recurrent or metastatic HNSCCs found encouraging clinical activity of several EGFR inhibitors.[4,5] Moreover, a large randomised phase III trial showed that a combination of EGFR inhibitors and radiation therapy in locally advanced HNSCCs significantly prolongs overall survival.[6] Therefore, subsets of HNSCCs appear to respond to EGFR inhibitors, and it is critical that we are able to select those patients who will best respond to such treatment. The identification of predictive markers for treatment response is also a task of high priority. In non-small cell lung cancer (NSCLC), increased *EGFR* copy number, as assessed by fluorescence in situ hybridisation (FISH), is significantly correlated with improved clinical outcome in EGFR inhibitor-treated patients,[7,8] suggesting that gene copy number may be a useful predictor. However, this association has not yet been clearly demonstrated for HNSCCs.

In addition, there have been several conflicting reports on the association between EGFR protein expression by immunohistochemistry (IHC) and survival in HNSCCs. Many studies have indicated that EGFR protein expression is significantly correlated with survival in several human malignancies, including NSCLC[9] and HNSCC,[10,11] whilst others found no association between EGFR protein expression and HNSCC prognosis.[12,13] Furthermore, conflicting data have been reported on the correlation between *EGFR* copy number and HNSCC survival and the association between *EGFR* copy number and protein expression.[14–18] These discrepancies may result from differences in tumour site and histology, patient numbers, case heterogeneity and the methods used to assess copy number. In particular, variations in case heterogeneity and treatment strategies may contribute to conflicting evaluations of EGFR as a prognostic indictor for HNSCCs.

HNSCC is itself a heterogeneous disease, and there might be differences in signalling depending on the pathobiology of the tumour. EGFR expression may also differ between the site of HNSCC; for example, it was reported to be low in laryngeal tumours compared with those from the pharynx and oral cavity.[19] Additionally, the presence of human papilloma virus (HPV), more common in the base of the tongue than in the oral cavity, is associated with favourable prognosis in oropharyngeal cancer.[20–22] Therefore, to evaluate the prognostic significance of EGFR protein expression and copy number status, it is essential to evaluate at separate sub-sites.

Thus, in the present study, we focused on oral tongue SCCs (OTSCCs) in 89 patients with OTSCCs undergoing surgery as the primary treatment modality at a single institution. We examined the association between *EGFR* copy number alteration and protein expression to determine which is more reliable as a prognostic indicator in this malignancy. Using this approach, we could correctly determine prognostic significance of this gene alteration without affection of other factors such as site-specific factors and treatment modalities.

2. Materials and methods

2.1. Patients

Tissue samples were obtained from 89 patients with OTSCC who had undergone primary surgical excision with curative intent at the Department of Maxillofacial Surgery, Graduate School, Tokyo Medical and Dental University (Tokyo, Japan) between 1999 and 2009. None of these patients received preoperative treatment. All protocols of this study were reviewed and approved by the Research Ethics Committee of Tokyo Medical and Dental University. Informed consent was obtained from all patients in accordance with our Institutional guidelines. Clinical staging was defined according to the International Union against Cancer TNM classification system.[23] Tumours were classified histopathologically as well, moderately or poorly differentiated according to their cellular differentiation as defined by the World Health Organisation criteria.[24] The mode of tumour invasion at the tumour–host borderline was classified according to the modified Jacobsson criteria.[25,26] Disease-free survival (DFS) was calculated from the time of initial examination to the time of local, regional or distant recurrence of the disease or the time of last follow-up. Overall survival (OS) was calculated from the time of initial examination to the time of death or last follow-up.

2.2. FISH analysis

Samples were taken from 89 tumours by fine-needle aspiration (FNA) technique and FISH analysis were performed as described previously using the EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Vysus/Abbott Molecular, Des Plaines, IL).[27] *EGFR* FISH patterns were classified as follows: balanced disomy, chromosome/nucleus ratio (C/N) $\leqslant$2.5; balanced trisomy, C/N 2.6–3.0; balanced polysomy, C/N >3.0 (where balanced patterns had an average ratio gene/chromosome copy number per nucleus (G/C) of 0.9–2.0); and amplification, G/C >2.0 and gene/nucleus ratio >3.0.[18,27–30] Tumours showing disomy were regarded as unchanged in gene number, with all other tumours being considered to have gene numerical alterations.

2.3. *Immunohistochemistry (IHC)*

IHC staining was performed using the streptavidin–biotin immunoperoxidase technique with a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Formalin-fixed paraffin-embedded tissue sections (4-μm) were stained with the mouse monoclonal EGFR antibody 31G7 (Invitrogen, Carlsbad, CA) at a 1:50 dilution according to the manufacturer's instructions. Staining with PBS buffer instead of EGFR antibody solution was carried out as a negative control. Positive staining was defined as membranous staining above background level but staining intensity was not assessed. For each tissue section, squamous cell carcinoma cells were counted in three different regions at the deepest level of the invasion site. The number of EGFR-positive squamous tumour cells was also counted and the average positive rate for three different regions was calculated. Expression was scored as grade I (<50% tumour cells stained), grade II (50–90%) and grade III (>90%). Grade II and III was defined as protein overexpression. Grades were assigned by an oral pathologist and two oral surgeons blinded to the clinical parameters.

2.4. *Statistical analysis*

FISH and IHC results were compared with the clinicopathologic information using the two-tailed Fisher's exact test. The clinicopathologic information included patient age, gender, smoking habit, alcohol consumption, disease stage, histopathological grading, mode of invasion, nodal status, disease recurrence, and survival. DFS and OS were calculated by the Kaplan–Meier method and statistical significance was determined by the log-rank test. Multivariate survival analysis was performed using Cox's proportional hazard model. The level of significance was set at $P < 0.05$. All statistical analyses were performed using SPSS 15.0 J software (SPSS Inc., Chicago, IL).

3. Results

3.1. *FISH*

Thirty-two (36.0%) of the 89 cases exhibited *EGFR* numerical alteration: 16 with trisomy (18.1%), 14 with polysomy (15.7%) and two with amplification (2.2%). Fifty-seven cases showed disomy (64.0%) and none showed gene deletion (Fig. 1).

3.2. *IHC*

EGFR protein expression was observed in all samples including normal epithelium and tumour tissue. Amongst the 89 OTSCC cases analysed, EGFR protein overexpression was found in 73 cases (82.0%), of which 31 (34.8%) scored grade III, 42 (47.2%) scored grade II, and 16 scored grade I (18.0%) (Fig. 2).

3.3. *Statistical analysis*

The two-tailed Fisher's exact test found no correlation between *EGFR* copy number status and protein expression ($P = 0.778$) (Table 1). The correlation between EGFR numerical

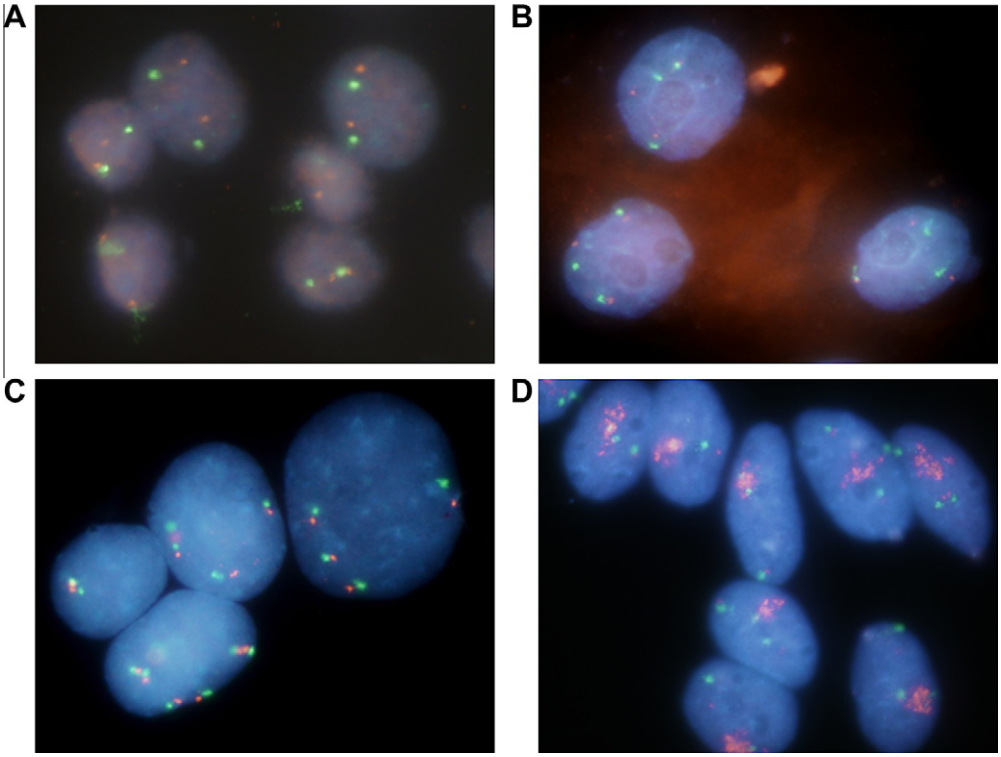

Fig. 1 – Evaluation of gene copy number by fluorescence in situ hybridisation (FISH). Cells were hybridised with probes for the chromosome 7 (green) and *EGFR* (orange). (A) Balanced disomy, (B) balanced trisomy, (C) balanced polysomy, (D) gene amplification.

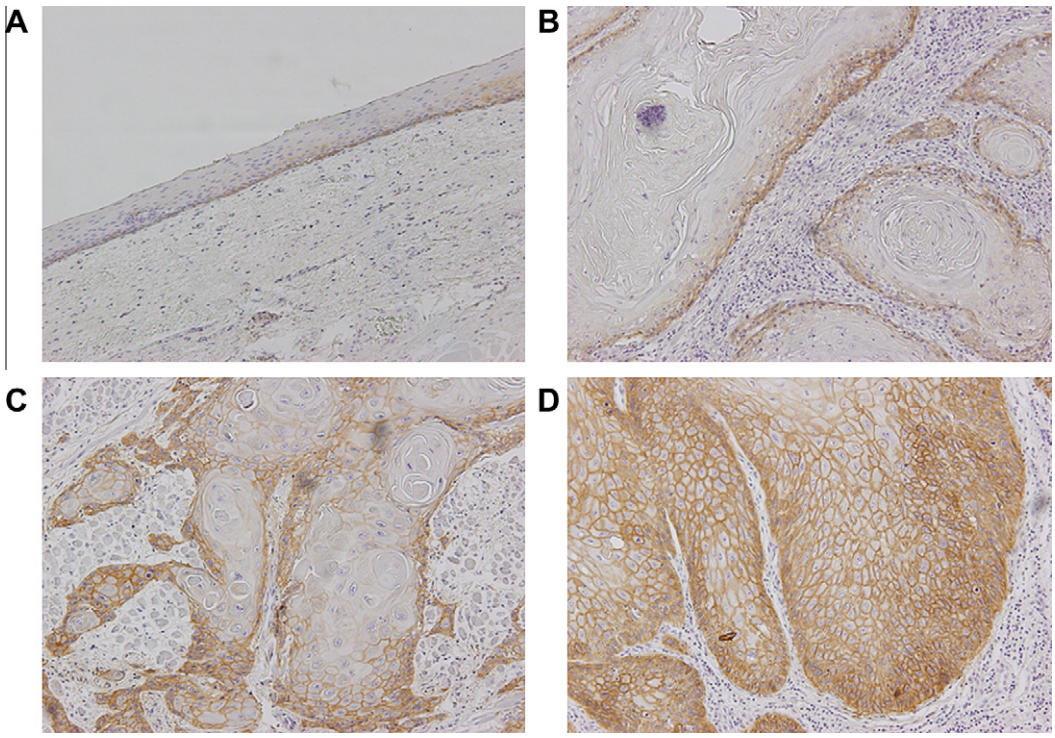

Fig. 2 – Immunohistochemical staining of EGFR protein expression. (A) Normal epithelium, (B) grade I, (C) grade II, (D) grade III.

Table 1 – Relation between EGFR FISH and IHC status.

	FISH status		
	Negative	Positive	Total
IHC status			
Overexpression (–)	11	5	16
Overexpression (+)	46	27	73
Total	57	32	89

FISH: fluorescence in situ hybridisation.
IHC: immunohistochemical staining.
Negative: disomy.
Positive: trisomy, polysomy and amplification.

alteration/protein overexpression and clinicopathological features of the 89 primary OTSCCs are summarised in Table 2. *EGFR* copy number increase was significantly correlated with a more diffuse tumour invasion pattern ($P = 0.006$) and survival ($P = 0.002$). By contrast, there was no correlation between EGFR overexpression and clinicopathological features.

Sixteen of 32 patients (50.0%) with tumours that had *EGFR* numerical alteration and 39 of 57 patients (68.4%) with tumours that lacked such a genetic abnormality had no disease recurrence. Twenty of 32 patients (62.5%) with *EGFR* numerical alteration and 52 of 57 patients (91.2%) without such alterations remained alive. Kaplan–Meier survival curves clearly demonstrated the adverse impact of *EGFR* copy number alteration on both disease recurrence ($P = 0.048$) and OS ($P = 0.001$) (Fig. 3A, B).

Conversely, 44 of 73 patients (60.3%) whose tumours overexpressed EGFR and 11 of 16 patients (68.8%) without overexpression did not have recurrence. Forty-seven of 73 patients (78.1%) with EGFR overexpression and 15 of 16 patients (93.8%) without overexpression survived. The DFS and OS curves of the patients with EGFR overexpression were lower than that of patients without overexpression, although this just failed to reach statistical significance ($P = 0.506$, $P = 0.181$) (Fig. 3C, D).

Multivariate analysis showed that diffuse invasion pattern (RR = 2.117, 95% CI = 1.077–4.162, $P = 0.030$) was retained as an independent prognostic indicator for DFS. Clinical stage III/IV (RR = 3.266, 95% CI = 1.239–8.607, $P = 0.017$) and *EGFR* copy number increase (RR = 5.557, 95% CI = 1.933–15.975, $P = 0.001$) were independent prognostic factors of poor OS. However, EGFR overexpression was not an independent prognostic indicator in these models (Table 3).

To confirm that *EGFR* copy number status is a more reliable indicator than protein overexpression in OTSCCs, patients with tumours demonstrating EGFR protein overexpression were divided into two groups based on their *EGFR* FISH status and assigned Kaplan–Meier survival curves. Twenty-seven patients whose tumours exhibited a simultaneous *EGFR* copy

Table 2 – Correlation between *EGFR* FISH-positive/overexpression with clinicopathological parameters.

	Total No. of patients	*EGFR* FISH-positive	P[a]	EGFR overexpression	P
Age (yrs)					
<60	45	16		38	
⩾60	44	16	NS	35	NS
Gender					
Male	62	26		51	
Female	27	6	NS	22	NS
Smoking					
Yes	40	18		32	
No	49	14	NS	41	NS
Alcohol consumption					
Yes	29	10		25	
No	60	22	NS	48	NS
Disease stage					
I, II	61	22		50	
III, IV	28	10	NS	23	NS
Cellular differentiation					
Well to moderate	74	27		60	
Poor	12	4	NS	10	NS
Mode of invasion					
1 to 3	54	14		45	
4C to 4D	32	18	0.006	25	NS
Nodal status[b]					
No metastasis	66	24		53	
Metastasis	23	8	NS	20	NS
Recurrence					
Negative	55	16		44	
Positive	34	16	NS	29	NS
Survival					
Alive	72	20		57	
Dead	17	12	0.002	16	NS

NS: not significant.
[a] By two-tailed Fisher exact test.
[b] Histopathlogic diagnosis.

number increase and protein overexpression (Group +/+) had significantly worse survival curves than 46 patients with tumours negative for FISH status and positive for protein overexpression (Group −/+) in both DFS ($P = 0.033$) and OS ($P = 0.002$) curves (Fig. 4).

4. Discussion

EGFR is particularly important in the pathogenesis of HNSCC, and is a promising biomarker candidate for this malignancy. In addition, it is anticipated to be a good drug target for HNSCC treatment as it is overexpressed in most cases of this cancer. However, there are three unsolved issues: (1) the association between protein expression and prognosis, (2) the correlation between gene copy number status and prognosis, and (3) the relationship between protein expression level and gene copy number. Despite of several studies in these areas, the data are conflicting and the issues remain controversial. These discrepancies may result from differences in methodologies used to assess EGFR and also from case heterogeneity in HNSCCs.[19]

In particular, anatomical site of the tumour in HNSCCs may likely contribute to the discordance of the above points as EGFR expression may differ between the sub-sites of HNSCCs. Thus, in this study, we focused on OTSCCs, which were treated with surgery as the primary treatment modality, and tried to examine the above unsolved issue using FISH and IHC. Kaplan–Meier analysis showed that the OS period in patients whose tumours exhibited an *EGFR* copy number increase was significantly shorter than in patients without such genetic abnormalities. Although EGFR overexpression occurred more frequently in patients with an unfavorable outcome, these differences failed to reach statistical significance. Furthermore, multivariate analysis using the Cox proportional hazard model revealed that *EGFR* copy number alteration, but not EGFR protein overexpression, was an independent prognostic indicator for OS. These results suggest that *EGFR* copy number status is a more reliable prognostic indicator than EGFR overexpression in OTSCCs.

Of particular interest was the finding that the DFS and OS rates for Group +/+ patients (FISH-positive and protein overexpression-positive) were significantly lower than those of Group −/+ patients (FISH-negative and protein

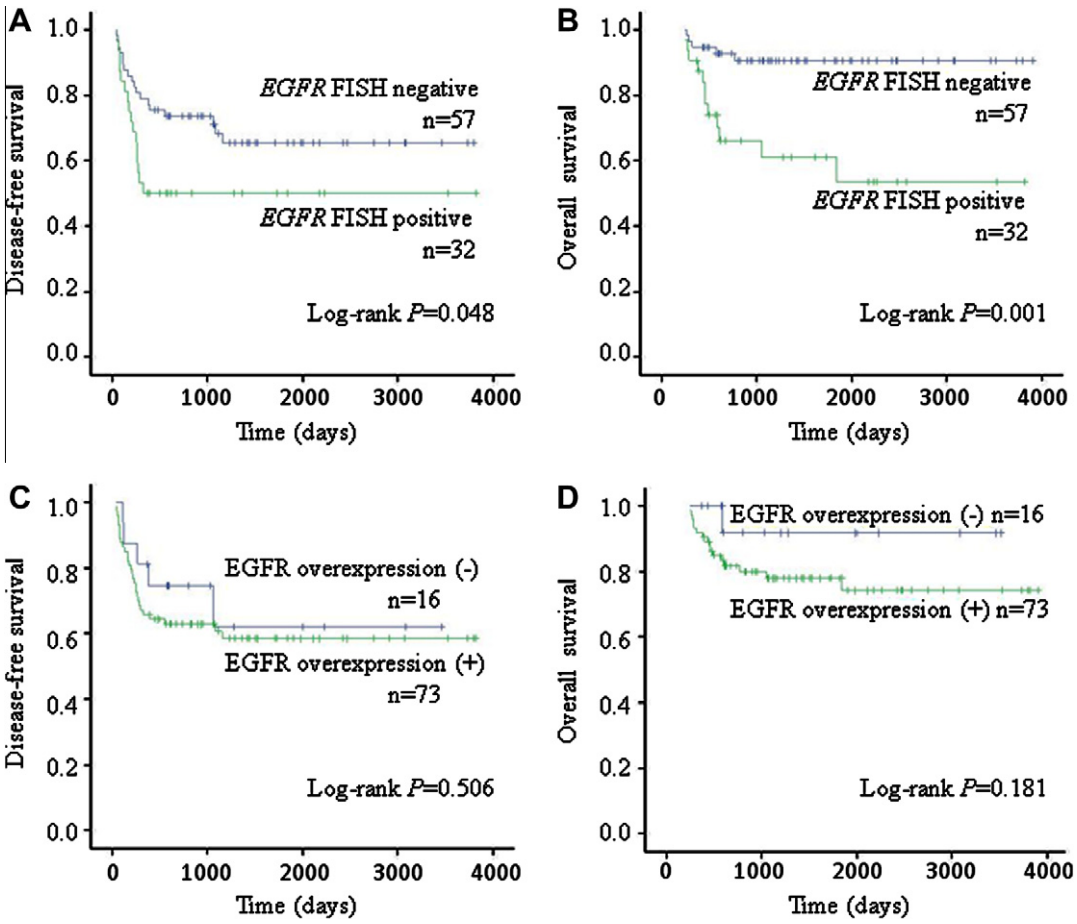

Fig. 3 – Kaplan–Meier plots for (A) disease-free survival according to *EGFR* gene status, (B) overall survival according to *EGFR* gene status, (C) disease-free survival according to EGFR protein status, and (D) overall survival according to EGFR protein status.

Table 3 – Multivariate survival analysis with Cox proportional hazards model.

Clinicopathological parameters	Disease-free survival			Overall survival		
	p Value	Risk ratio	95%CI	*p* Value	Risk ratio	95%CI
Age	NS	–	–	NS	–	–
Gender	NS	–	–	NS	–	–
Smoking	NS	–	–	NS	–	–
Alcohol consumption	NS	–	–	NS	–	–
Disease stage	NS	–	–	0.017	3.266	1.239–8.607
Cellular differentiation	NS	–	–	NS	–	–
Mode of invasion	0.030	2.117	1.077–4.162	NS	–	–
EGFR overexpression	NS	–	–	NS	–	–
EGFR FISH-positive	NS	–	–	0.001	5.557	1.933–15.975

CI: confidence interval.
NS: not significant.

overexpression-positive). These observations indicate that *EGFR* copy number alteration may not be related to EGFR protein expression alone. To determine why gene copy number increases rather than protein overexpression should be the strongest prognostic indicator in OTSCCs, we examined the correlation between copy number and centromere of chromosome 7, and found a significant positive correlation between the two genetic factors. This finding indicates that *EGFR* copy number changes occur frequently in association with chromosome 7 aneusomy. And we demonstrated that OTSCCs with chromosome 7 aneusomy had worse survival curves than those without (data not shown). Therefore, one possibility is that additional genes on chromosome 7 that are coamplified with *EGFR* are involved in mediating these aggressive behaviours. In support of this theory, Gebhart et al. examined 35 OSCCs by comparative genomic hybridisation, and found that patients whose tumours exhibited a gain of short arm on chromosome 7 had higher rates of relapse and worse

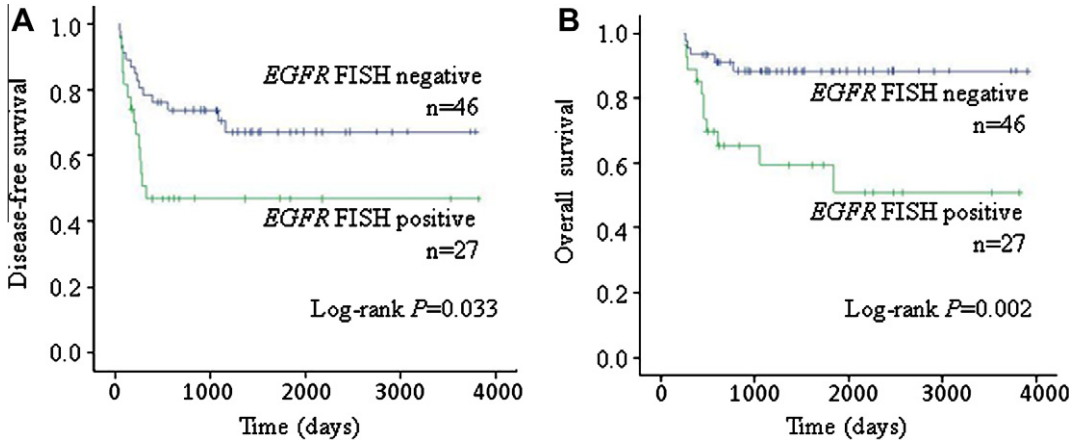

Fig. 4 – Kaplan–Meier plots of 73 patients with tumours that exhibited EGFR protein overexpression according to *EGFR* gene status. (A) disease-free survival, (B) overall survival.

survival.[31] Alternatively, *EGFR* copy number alteration may be a surrogate marker of chromosomal instability, which confers an adverse prognosis in many tumours. Recently, we examined the chromosomal instability of OSCCs using FISH with chromosome 7, 9 and, 11 centromere probes and clearly demonstrated that high-grade chromosomal instability was associated with reduced DFS and OS.[32] However, the detailed mechanism by which *EGFR* FISH status contributes to the oncogenic effect in cells with EGFR protein overexpression remains unclear.

To our knowledge, ours is only the second study to exclusively investigate *EGFR* gene copy status and protein expression levels in OTSCCs. The first was reported by Ryott et al. in 2009, and examined *EGFR* gene copy number and protein expression in 78 pretreatment OTSCC paraffin samples. They demonstrated a significant correlation between copy number and protein expression, but not between *EGFR* FISH and IHC status and survival.[17] Although their analytic methods (FISH and IHC) and the anatomical site (OTSCCs) were similar to ours, their findings conflict with ours. These discrepancies could be a result of the evaluation method used to assess *EGFR* FISH status. In the present study, we performed dual-colour FISH using *EGFR*- and centromere-specific chromosome 7 probes, a procedure typically used in many previous studies and evaluated copy number status of both the *EGFR* gene and chromosome 7 centromere. On the other hand, Ryott et al. used a centromere-specific chromosome 4 probe as a control for sectioning artifacts, but this was not used to interpret the *EGFR* FISH status. There were additional differences in sampling methods used (FNA biopsy from fresh tumours versus paraffin-embedded tissue) and in the primary treatment modality (surgery versus preoperative radiotherapy followed by surgery). Moreover, with regard to the association between *EGFR* FISH and protein status, no positive association has been consistently observed in NSCLC[33] and colon cancer.[34] Discrepancies may also result from differences in the site and histology of the tumour, patient number, case heterogeneity and the methods used to assess EGFR. However, most importantly, *EGFR* copy status, but not protein expression level, is associated with the response to EGFR-targeted therapies in NSCLC[35] and colorectal cancers,[36–38] indicating that gene copy number status may be a predictive marker for the response to EGFR-targeted therapies in HNSCCs. Thus, when further investigating *EGFR* copy number status, especially for correlation with response to EGFR-targeted agents, it will be necessary to investigate HNSCC sub-sites and to develop a consensus evaluation of *EGFR* copy number status.

In conclusion, we clearly demonstrate that *EGFR* copy number status is a more reliable indicator than EGFR protein overexpression in OTSCCs and that the copy number increase is not correlated with EGFR protein levels alone. Although copy number changes of this gene may indicate the existence of additional genes that are coamplified with EGFR, or chromosomal instability in the tumour, the mechanism by which the FISH-positive status contributes to the malignant phenotype of this cancer is not clear. Thus, further studies are required to clarify this point. It will be of interest to determine whether *EGFR* FISH status will prove useful in selecting patients who would benefit from EGFR inhibitor therapy for OTSCCs.

Conflict of interest statement

None declared.

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