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Title	Expression of p53, p16, cyclin D1, epidermal growth factor receptor and Notch1 in patients with temporal bone squamous cell carcinoma
Author(s)	Morita, Shinya; Nakamaru, Yuji; Homma, Akihiro et al.
Citation	International Journal of Clinical Oncology, 22(1), 181-189 https://doi.org/10.1007/s10147-016-1026-5
Issue Date	2017-02
Doc URL	https://hdl.handle.net/2115/68250
Rights	The final publication is available at Springer via http://dx.doi.org/10.1007/s10147-016-1026-5
Type	journal article
File Information	IntJClinOncol22_181.pdf



**Expression of p53, p16, cyclin D1, EGFR and Notch1 in patients with the
temporal bone squamous cell carcinoma**

Shinya Morita, M.D.¹, Yuji Nakamaru, M.D., Ph.D.¹, Akihiro Homma,
M.D., Ph.D.¹, Shinichiro Yasukawa, M.D.², Hiromitsu Hatakeyama, M.
D., Ph.D.¹, Tomohiro Sakashita, M.D., Ph.D.¹, Satoshi Kano, M.D., Ph.
D.¹, Atsushi Fukuda, M.D.¹, Satoshi Fukuda, M.D., Ph.D.¹

Affiliations:

1 Department of Otolaryngology - Head and Neck Surgery,

Hokkaido University Graduate School of Medicine, Sapporo, Japan

2 Department of Translational Pathology, Hokkaido University Graduate
School of Medicine, Sapporo, Japan

Corresponding author: Akihiro Homma

Department of Otolaryngology - Head and Neck Surgery

Hokkaido University Graduate School of Medicine.

Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan

Tel: +81-11-706-5958; Fax: +81-11-717-7566

E-mail address: ak-homma@med.hokudai.ac.jp

Abstract

Background: The aim of this study was to investigate the expression of p53, p16, cyclin D1, EGFR and Notch1 in temporal bone squamous cell carcinoma (TBSCC) tissue samples by immunohistochemistry (IHC), and to evaluate the association between these biomarkers and clinicopathological features.

Methods: We performed a retrospective, single-institution review of 30 patients with TBSCC treated with curative intent between April 2006 and March 2015. All tissue samples were obtained from pre-treatment biopsy specimens or surgical specimens and IHC staining performed.

Results: Ten patients were categorized as T1, 7 patients as T2, 5 patients as T3 and 8 patients as T4. Nine patients had clinically positive lymph node metastasis. The positive expression of p53 and EGFR was significantly associated with T classification ($P=0.042$ and $P=0.0039$). EGFR expression was significantly more frequent in patients with positive lymph node metastasis compared with patients without node involvement ($P=0.017$). In the analysis of the association between protein expression by IHC staining and prognosis, the positive expression of EGFR and Notch1 was significantly correlated with poor survival outcomes in TBSCC ($P=0.015$ and $P=0.025$).

Conclusion: The overexpression of p53 and EGFR may be valuable biomarkers for identifying individuals at high risk of developing tumors in TBSCC. Furthermore, the positive expression of EGFR was significantly associated with poor survival outcome. Anti-EGFR therapy has potential for use as the treatment modality of choice for advanced-stage TBSCC as well as other head and neck squamous cell carcinomas.

Keywords: temporal bone squamous cell carcinoma; clinicopathological features; EGFR; protein expression; immunohistochemistry; prognostic factor.

Introduction

Temporal bone cancer is rare form of malignant tumor representing less than 0.2% of all tumors of the head and neck, with squamous cell carcinoma being the most common histology [1, 2]. Surgical resection is widely performed as the mainstay of treatment for this aggressive tumor with a poor prognosis, and the surgical margins have been reported to be important prognostic factor [1-3]. However, pathological margin status alone has been insufficient to assess the clinical prognosis and determine the treatment strategy for patients with temporal bone squamous cell carcinoma (TBSCC).

Alterations in expression level, molecular weight, subcellular localization, and post-translational modification of proteins have been implicated in the tumorigenesis and development processes of this carcinoma on the basis of studies on several factors controlling the cell cycle, cell proliferation and apoptotic pathways [4-6]. Head and neck squamous cell carcinomas (HNSCCs) are thought to be initiated and to progress through a series of genetic alterations, such as those involving TP53 and CDK2NA [7]. HNSCCs also exhibit many chromosomal abnormalities, including amplifications of region 11q13, which contains the cyclin D1 gene, and region 7p11, which encodes epidermal growth factor receptor (EGFR) [8]. Recently, aberrant Notch signaling in a variety of human tumor cells has been suggested to play an essential role in cancer promotion [9, 10]. The expression of proteins involved in genetic and epigenetic alterations has been clinically investigated using immunohistochemical staining, and these expression levels are expected to have a significant correlation with an unfavorable

prognosis in patients with various cancers. Meanwhile, the clinical relevance of immunohistochemistry (IHC) expression in TBSCC remains undefined due to the rarity of disease. Thus, the identification of biological markers for tumor aggressiveness is required to aid in predicting prognosis and to define individual treatment modalities for patients with TBSCC.

The present study aimed to investigate the expression levels of the above five proteins (p53, p16, cyclin D1, EGFR and Notch1) in TBSCC tissue samples by IHC, and to evaluate the association between these biomarkers and clinicopathological features.

Material and methods

Patients and tissue samples

We performed a retrospective, single-institution review of patients with previously-untreated TBSCC treated with curative intent in the Department of Otolaryngology, Head and Neck Surgery, Hokkaido University Hospital between April 2006 and March 2015. Staging was performed using the University of Pittsburgh modified TNM staging system [2]. All patients were initially evaluated by a multidisciplinary team consisting of otolaryngologists, radiation oncologists and medical oncologists. The selection of treatment was based on the extent of the disease, performance status, comorbidities, patient wishes and/or the attending physician's preference in each case. All tissue samples were obtained from pre-treatment biopsy specimens or surgical specimens. Patients for whom the specimens afforded insufficient tissue volume to perform IHC were excluded from this analysis. Pairs of paraffin-embedded tissue samples, consisting of TBSCC tissue and adjacent normal tissues as controls, were examined. The relationship between the expression levels of five proteins (p53, p16, cyclin D1, EGFR and Notch1) and clinicopathological parameters as well as survival outcome was analyzed.

All patients had to be instructed on the potential risks and benefits of treatment, and written-informed consent for the use of their tissue specimens and clinical data was obtained after a full explanation. This research adhered to the tenets of the Declaration of Helsinki and was approved by our Institutional Review Board.

Analysis of candidate proteins expression by immunohistochemistry

The specimens were embedded in paraffin and cut into 4- μ m-thick sections. They were then deparaffinized in xylene, dehydrated through graded alcohols, and placed in 0.1% hydrogen peroxide to quench any endogenous peroxidase activity. Antigen retrieval was performed using a 750 W microwave oven for 15 minutes in 10 mM sodium citrate buffer (10 mmol/L, pH 6.0). The sections were blocked with a 10% normal goat serum for 30 minutes at room temperature to prevent the non-specific binding of antibodies. The slides were then incubated with a p53 mouse monoclonal antibody (DO-7; Roche Diagnostics Ltd.; Tokyo, Japan), an anti-p16^{INK4a} mouse monoclonal antibody (E6H4; Roche Diagnostics Ltd.; Tokyo, Japan), an anti-human cyclin D1 monoclonal rabbit antibody (EP12; Dako; Glostrup, Denmark), an anti-mouse EGFR monoclonal antibody (M7298; Dako; Glostrup, Denmark) and an anti-Notch1 monoclonal antibody (#3608; Cell Signaling Technology Inc.; Danvers, Massachusetts, U.S.A.) in a humid chamber at 4 °C overnight. The sections were then incubated with a biotin-labeled goat anti-rabbit secondary antibody (Histofine SAB-PO (M) kit; Nichirei; Tokyo, Japan) for 30 minutes at 37 °C, followed by reaction with a streptavidin-biotin horseradish peroxidase complex. The reaction products were observed by immersing the slides in a freshly prepared diaminobenzidine solution for 10 minutes and counterstaining them with hematoxylin before dehydration and mounting.

Immunohistochemical assessment

All slides were evaluated by light microscopy scanning the entire tissue specimen under low magnification ($\times 40$) and then confirmed under high magnification ($\times 200$ and $\times 400$), and were examined by 2 experienced pathologists who were blinded to clinical information.

Positive immunostaining of proteins was determined from the staining intensity as well as the percentage of immunoreactive cells in the most highly stained area of each slide based on a previously reported method with some modifications [11-14]. The intensity score was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage score was graded as 0 ($<1\%$ positive tumor cells), 1 (1-10%), 2 (10-50%), 3 (50-90%), and 4 ($>90\%$). For p53 and p16, the sum of the staining intensity and percentage of positive cells was scored and simply graded as positive (sum of scores exceeded 3) or negative (sum of scores were 2 or less; i.e., no or weak staining in less than 10% of cells) in line with previous reports [11, 13]. For cyclin D1, an overall score (0-12) was calculated by multiplying the intensity score by the percentage score. Samples with an overall score of more than 3 were considered positive [12].

EGFR results were divided into four categories, as follows: 0 (no or weak staining in $<10\%$ of tumor cells), 1+ (weak staining in part of the membrane in $>10\%$ of the tumor cells), 2+ (complete staining of the membrane with weak or moderate intensity in $>10\%$ of the neoplastic cells), and 3+ (strong staining in $>10\%$ of the neoplastic cells). The samples were also categorized into two groups: 0 and 1+ were considered negative, while 2+ and 3+ were

considered positive [12].

For Notch1, an overall score (0-12) was calculated by multiplying the intensity score by the percentage score, as described previously [14]. The intensity score was rated as 0 (colorless), 1 (pallideflavens), 2 (yellow), and 3 (brown). The percentage score was graded as 0 (<5% positive tumor cells), 1 (5-25%), 2 (25-50%), 3 (50-75%), and 4 (>75%). The overall score was stratified as absent (0 score), weak (1-4 score), moderate (5-8 score), and strong (9-12 score). Tumors with moderate or strong immunostaining were classified as having positive expression, whereas tumors with absent or weak immunostaining were classified as having negative expression.

Specimens were rescored if the difference in overall scores from 2 experienced pathologists was more than 3. The concordance of scoring between the 2 observers indicated substantial agreement.

Statistical analysis

Statistical analyses were performed using JMP software (version 10.0; SAS Institute Inc.; Cary, NC, U.S.A.). Statistical differences were analyzed using the Mann-Whitney U-test to assess the correlation between protein expression levels and clinicopathologic parameters. A Kaplan-Meier time-to-event method was used to calculate the overall survival (OS) rates. For the calculation of survival rates, death was counted as an event, whereas the patient being alive at the latest contact, regardless of disease status, was counted as censored. The time of interest included the beginning of treatment, the last follow-up date and death. Survival status was updated in

January 2016. Stepwise regression analysis was performed to build an appropriate model through the addition and removal of predictor variables. Multivariable analysis was performed using the Cox proportional hazard model. Statistical differences were analyzed using the log rank test. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological features

Patient profiles are summarized in Table 1. Thirty patients with TBSCC were enrolled according to inclusion criteria used in this analysis. The study population consisted of 15 males and 15 females, ranging in age from 39 to 86, with a median age of 67.5 years. The follow-up period ranged from 9 to 112 months, with a median of 36 months. Regarding T classification, 10 patients were categorized as T1, 7 patients as T2, 5 patients as T3 and 8 patients as T4. Nine patients had clinically positive lymph node metastasis. The histopathological diagnoses consisted of well-differentiated SCC in 21 patients (70.0%), moderately-differentiated SCC in 8 patients (26.7%) and poorly-differentiated SCC in 1 patient (3.3%). Surgical resection was performed in 10 patients with T1, 7 with T2, 5 with T3 and 4 with T4 disease. Of the seven T2 patients, 2 with pathologically positive surgical margins received postoperative RT. All T3-4 patients receiving surgery were followed by postoperative RT. Four patients with T4 underwent definitive CRT using platinum-based regimens.

Immunohistochemical analysis of p53, p16, cyclin D1, EGFR and Notch1 expression in TBSCC and adjacent normal tissues

The positive expression of p53, p16, cyclin D1, EGFR and Notch1 was detected in 21 (70.0%), 8 (26.7%), 18 (60.0%), 16 (53.3%) and 4 (13.3%) of the TBSCC samples, respectively (Table 2 and Fig. 1a-e). In contrast, none of the adjacent normal external auditory canal skin specimens were positive for

p53, p16, cyclin D1, EGFR or Notch1.

Relationships between clinicopathologic features and immunohistochemistry expression of p53, p16, cyclin D1, EGFR and Notch1

There was a significant association between the positive expression of both p53 and EGFR and T classification ($P=0.042$ and $P=0.0039$). EGFR expression was more frequent in patients with positive lymph node metastasis compared with patients without node involvement ($P=0.017$). However, the overexpression of p16, cyclin D1 and Notch1 was not significantly correlated with clinicopathologic features, such as age, gender, smoking status and alcohol consumption, T and N classifications, and histopathological differentiation (Table 3).

Survival analysis with regard to the immunohistochemistry expression of p53, p16, cyclin D1, EGFR and Notch1, and clinicopathologic features

Univariable analysis indicated that the immunohistochemistry expression of EGFR and Notch1 ($P=0.015$ and $P=0.025$) as well as T and N classifications ($P=0.0004$ and $P=0.0083$) are significant prognostic factors for TBSCC (Table 4). The 5-year OS rates were 92.9% and 75.7% for patients negative for the expression of EGFR and Notch1, and 48.1% and 25.0% for those positive, respectively (Fig. 2d, e). Meanwhile, no correlation was found between the expression of p53, p16 or cyclin D1 and prognosis (Fig. 2a-c). The 5-year OS rate was 93.3% for patients with T1-2 disease and 35.9% for those with T3-4 (Fig. 3a). Those for patients with N0 and N1-3 disease were

85.7% and 33.3%, respectively (Fig. 3b). However, other clinicopathologic features, such as age, gender, smoking status and alcohol consumption, and histopathological differentiation, were not significantly correlated with survival outcomes (Table 4).

Stepwise regression analysis and multivariable analysis using the Cox proportional hazards model did not demonstrate that these factors (EGFR, Notch1, and T and N classifications) were independent predictors of OS rate.

Discussion

There is a consensus that patients with early-stage TBSCC have good survival outcomes, whereas advanced-stage patients have a poor prognosis [1, 2, 15]. This analysis confirmed that the prognosis for patients with TBSCC was good for those with T1-2 disease and negative lymph node metastasis but poor for those with T3-4 disease and/or positive lymph node metastasis, suggesting that T and N classifications are potential prognostic factors. Meanwhile, no studies are yet to focus on biomarkers for TBSCC and their relationship to prognosis. To date, it has been assumed that TBSCC is biologically similar to HNSCC based on its histologic appearance. However, HNSCC arise from the mucosa of the nasal cavity, paranasal sinus, oral cavity, pharynx or larynx, whereas the majority of cases of TBSCC occur in the skin of external auditory canal. Thus, it is necessary to analyze directly the molecular biological mechanism in TBSCC and explore the potential biomarkers with regard to prognosis for the development of an appropriate treatment [16].

This analysis found that the positive expression of p53 in TBSCC tissues was significantly associated with T classification. The tumor-suppressor gene p53 has been reported to be involved in cell cycle regulation, proliferation and apoptosis [17]. The mutated p53 has a longer half-life and deactivates the trigger of p53-dependent apoptosis, resulting in tumor development and enlargement in more than 50% of human cancers [17, 18]. As the mutated p53 has been reported to be detected by the IHC method and correlates with T classification in other forms of HNSCC [19], the

overexpression of p53 may be a practical and valuable biomarker for identifying individuals at high risk of developing tumors in TBSCC. Meanwhile, the positive expression of p53 in TBSCC tissues was not significantly correlated with N classification or prognosis, in which metastasis and recurrence, in contrast to carcinogenesis and tumor development, was not seemingly implicated. However, these findings may be attributed to differences in gene mutation site and biochemical properties of the new proteins. Whereas missense mutations that lead to the production of aberrant functional proteins are detected by IHC staining, null mutations that lead to no p53 protein expression at all are identified by the complete absence of IHC staining [19]. Thus, differences between the analysis of genetic mutations and protein expression by IHC affects the corresponding cancer risk [20].

This analysis also demonstrated that positive EGFR expression in TBSCC tissues was significantly associated with both T and N classification. EGFR is a transmembrane glycoprotein expressed in the majority of epithelial malignancies, and the activation of EGFR leads to the initiation of intracellular signaling pathways which regulate the activation of cell proliferation, invasion, angiogenesis, and metastasis [21]. The overexpression of EGFR, as well as p53, may be a predictive marker for the development of tumors in TBSCC. Furthermore, the positive expression of EGFR was significantly associated with a poor survival outcome in TBSCC. Patients with T3-4 disease were compelled to undergo additional RT, while RT does not appear to improve survival outcome or afford an alternative to

the complete resection of the tumor [22]. This result may be explained by the observation that EGFR activation plays a role in resistance of tumor cells to RT and chemotherapy [23]. Thus, the role of EGFR-targeted therapy for patients with unresectable TBSCC remains a matter of debate. EGFR protein is highly overexpressed in both mucosal and cutaneous HNSCC [16], and subsequent downstream signaling events activate tumor proliferative pathways [24]. Inhibition of downstream EGFR signaling, such as EGFR-ligand binding blockade by monoclonal antibodies and the competitive binding to the intracellular tyrosine kinase domain of EGFR by small molecule inhibitors, are important mechanisms for EGFR-targeted therapy [25]. However, complete inhibition is difficult due to a wide range of mechanisms and escape pathways. EGFR inhibition is known to be ineffective in tumor entities with KRAS mutations which activate the downstream signaling of EGFR [26]. As KRAS mutations are rarely detected in either mucosal or cutaneous HNSCC [27], further investigation would be valuable for evaluating whether TBSCC patients positive for EGFR expression are candidates for EGFR-targeted therapy as is currently the case for patients with other forms of HNSCC.

The Notch signaling pathway is an evolutionarily conserved, intercellular signaling cascade, which has been considered to be a key integrator in the mediation of a series of cellular biological behaviors in neoplasms, such as migration, metastasis, and angiogenesis [28]. The Notch1 protein is strongly expressed in a variety of malignancies, including HNSCC [8, 14, 29-31], and could play an important role in the estimation of disease progression,

metastasis and prognosis [8, 29, 30]. The current study found that Notch1 expression in TBSCC was significantly correlated with poor survival outcomes, supporting the possibility that Notch1 expression could be used as a potential biomarker reflecting the prognosis of TBSCC patients. However, this analysis also indicated that the rate of Notch1 protein expression in TBSCC was only 13.3%, although none of the adjacent normal skin samples were positive for Notch1. These results may be attributed to the small number of TBSCC samples. Thus, a sequencing project is necessary to determine the mutations in the Notch1 gene by its detection in a large number of tissues and define the function and mechanisms of the Notch1 protein in the invasion and metastasis of TBSCC.

The relationship among survival outcomes and potential factors (the expression of EGFR and Notch1, and T and N classifications) were not statistically significant in the stepwise regression analysis or the multivariable Cox analysis. The results of this analysis might be affected by the small number of samples of TBSCC, potential confounding factors, the sensitivity of antibodies, antigen retrieval methods, incubation time, detection system and the IHC assessment methods. Another limitation is the discrepancy between the analysis of genetic mutations and protein expression by IHC, with the latter method more widely used as it is more convenient and practical in a clinical setting [32]. As the rarity of TBSCC limits our ability to clarify its biological behavior, thereby preventing more directed therapy, further multi-institutional investigations as well as gene sequencing analysis are required for the evaluation of a large number of

samples.

Conclusion

The positive expression of p53 was significantly associated with T classification. Furthermore, the overexpression of EGFR showed a significant correlation with the T and N classifications as well as poor survival outcome. These results suggested that the expression of EGFR may be a valuable biomarker for identifying individuals at high risk of developing tumors and predicting survival outcome in TBSCC. As there is no high-level evidence-based data supporting postoperative RT or definitive CRT, clinical trials of anti-EGFR therapy in advanced-stage TBSCC may be sufficient for the evaluation of its potential benefit as is currently the case for other forms of HNSCC.

Acknowledgement

This study received financial support from a Health and Labour Sciences Research Grant for Clinical Cancer Research (H26-141) from the Ministry of Health, Labour and Welfare of Japan, and the National Cancer Center Research and Development Fund (26-A-4) of Japan.

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Table headings

Table 1. Characteristics of patients with temporal bone squamous cell carcinoma

Table 2. Immunohistochemistry expression of p53, p16, cyclin D1, EGFR and Notch1 in temporal bone squamous cell carcinoma and adjacent normal tissues

Table 3. Relationships among clinicopathologic features and immunohistochemistry expression of p53, p16, cyclin D1, EGFR and Notch1

Table 4. Univariable analysis of various potential prognostic factors for overall survival

Figure legends

Fig. 1a-e. Immunohistochemical staining of p53 (a), p16 (b), cyclin D1 (c), EGFR (d), and Notch1 (e) in temporal bone squamous cell carcinoma samples under high magnification (×400)

Fig. 2a-e. The overall survival rates according to the immunohistochemistry expression of p53 (a), p16 (b), cyclin D1 (c), EGFR (d), and Notch1 (e) in patients with temporal bone squamous cell carcinoma

-; negative expression, +; positive expression

Fig. 3a-b. The overall survival rates by T (a) and N (b) classifications in patients with temporal bone squamous cell carcinoma

Table 1. Characteristics of patients with temporal bone squamous cell carcinoma

Parameter		
Age (years)		
range		39 - 86
median		67.5
Gender (n)		
Female		15
Male		15
Follow-up period (months)		
range		9 - 112
median		36
Smoking (n)		
Absent		13
Present		17
Alcohol (n)		
Absent		15
Present		15
T classification (n)		
1		10
2		7
3		5
4		8
N classification (n)		
0		21
1-3		9
Differentiation (n)		
Well		21
Moderate		8
Poor		1
Treatment (n)		
Surgery alone		14
Surgery + RT		11
Definitive CRT		5
RT; radiotherapy, CRT; chemoradiotherapy		

Table 2. Immunohistochemistry expression of p53, p16, cyclin D1, EGFR and Notch1 in temporal bone squamous cell carcinoma and adjacent normal tissues

Parameter	TBSCC tissues, n (%)	Adjacent normal tissues, n (%)
p53		
Positive	21 (70.0%)	0 (0%)
Negative	9 (30.0%)	30 (100%)
p16		
Positive	8 (26.7%)	0 (0%)
Negative	22 (73.3%)	30 (100%)
Cyclin D1		
Positive	18 (60.0%)	0 (0%)
Negative	12 (40.0%)	30 (100%)
EGFR		
Positive	16 (53.3%)	0 (0%)
Negative	14 (46.7%)	30 (100%)
Notch1		
Positive	4 (13.3%)	0 (0%)
Negative	26 (86.7%)	30 (100%)
TBSCC; temporal bone squamous cell carcinoma		

Table 3. Relationships among clinicopathologic features and immunohistochemistry expression of p53, p16, cyclin D1, EGFR and Notch1

	n	p53+, n (%)	p16+, n (%)	Cyclin D1+, n (%)	EGFR+, n (%)	Notch1+, n (%)
Age						
≤ 60 years	6	3 (50.0%)	1 (16.7%)	3 (50.0%)	2 (33.3%)	1 (16.7%)
> 60 years	24	18 (75.0%)	7 (29.2%)	15 (62.5%)	14 (58.3%)	3 (4.2%)
<i>P</i> value		0.33	0.66	0.66	0.38	>0.99
Gender						
Female	15	8 (53.3%)	5 (33.3%)	9 (60.0%)	8 (53.3%)	2 (13.3%)
Male	15	13 (86.7%)	3 (20.0%)	9 (60.0%)	8 (53.3%)	2 (13.3%)
<i>P</i> value		0.11	0.68	>0.99	>0.99	>0.99
Smoking						
Absent	13	8 (61.5%)	5 (38.5%)	8 (61.5%)	9 (69.2%)	3 (23.1%)
Present	17	13 (76.5%)	3 (17.6%)	10 (58.8%)	7 (41.2%)	1 (5.9%)
<i>P</i> value		0.44	0.24	>0.99	0.16	0.29
Alcohol						
Absent	15	11 (73.3%)	5 (33.3%)	9 (60.0%)	11 (73.3%)	2 (13.3%)
Present	15	10 (66.7%)	3 (20.0%)	9 (60.0%)	5 (33.3%)	2 (13.3%)
<i>P</i> value		>0.99	0.68	>0.99	0.066	>0.99
T classification						
1-2	17	9 (52.9%)	5 (29.4%)	10 (58.8%)	5 (29.4%)	1 (5.9%)
3-4	13	12 (92.3%)	3 (23.1%)	8 (61.5%)	11 (84.6%)	3 (23.1%)
<i>P</i> value		0.042	>0.99	>0.99	0.0039	0.29
N classification						

0	21	13 (61.9%)	6 (28.6%)	13 (61.9%)	8 (38.1%)	2 (9.5%)
1-3	9	8 (88.9%)	2 (22.2%)	5 (55.6%)	8 (88.9%)	2 (22.2%)
<i>P</i> value		0.21	>0.99	>0.99	0.017	0.56
Differentiation						
Well	21	15 (71.4%)	7 (33.3%)	13 (61.9%)	10 (47.6%)	3 (14.3%)
Moderate-Poor	9	6 (66.7%)	1 (11.1%)	5 (55.6%)	6 (66.7%)	1 (11.1%)
<i>P</i> value		>0.99	0.37	>0.99	0.44	>0.99

+; positive expression

Statistical differences were analyzed using the Mann-Whitney U-test.

Table 4. Univariable analysis of various potential prognostic factors for overall survival

	Univariable analysis		
	HR	95% CI	<i>P</i> value
Age			
≤ 60 years	Ref.		
> 60 years	0.80	0.15-4.22	0.78
Gender			
Female	Ref.		
Male	3.83	0.91-12.5	0.071
Smoking			
Absent	Ref.		
Present	1.58	0.42-5.79	0.42
Alcohol			
Absent	Ref.		
Present	0.81	0.22-3.00	0.76
T classification			
1-2	Ref.		
3-4	15.7	3.20-50.7	0.0004
N classification			
0	Ref.		
1-3	5.25	1.68-31.3	0.0083
Differentiation			
Well	Ref.		
Moderate-Poor	1.28	0.30-5.53	0.73
p53			
Negative	Ref.		
Positive	3.73	0.64-10.7	0.18
p16			
Negative	Ref.		
Positive	1.26	0.30-5.42	0.74
Cyclin D1			
Negative	Ref.		
Positive	0.78	0.20-2.94	0.71
EGFR			

	Negative	Ref.		
	Positive	5.11	1.37-19.1	0.015
Notch1				
	Negative	Ref.		
	Positive	4.22	1.39-96.9	0.025
HR; hazard ratio, CI; confidence interval, Ref.; reference (HR=1.0)				
Statistical differences were analyzed using the log rank test.				

Figure 1

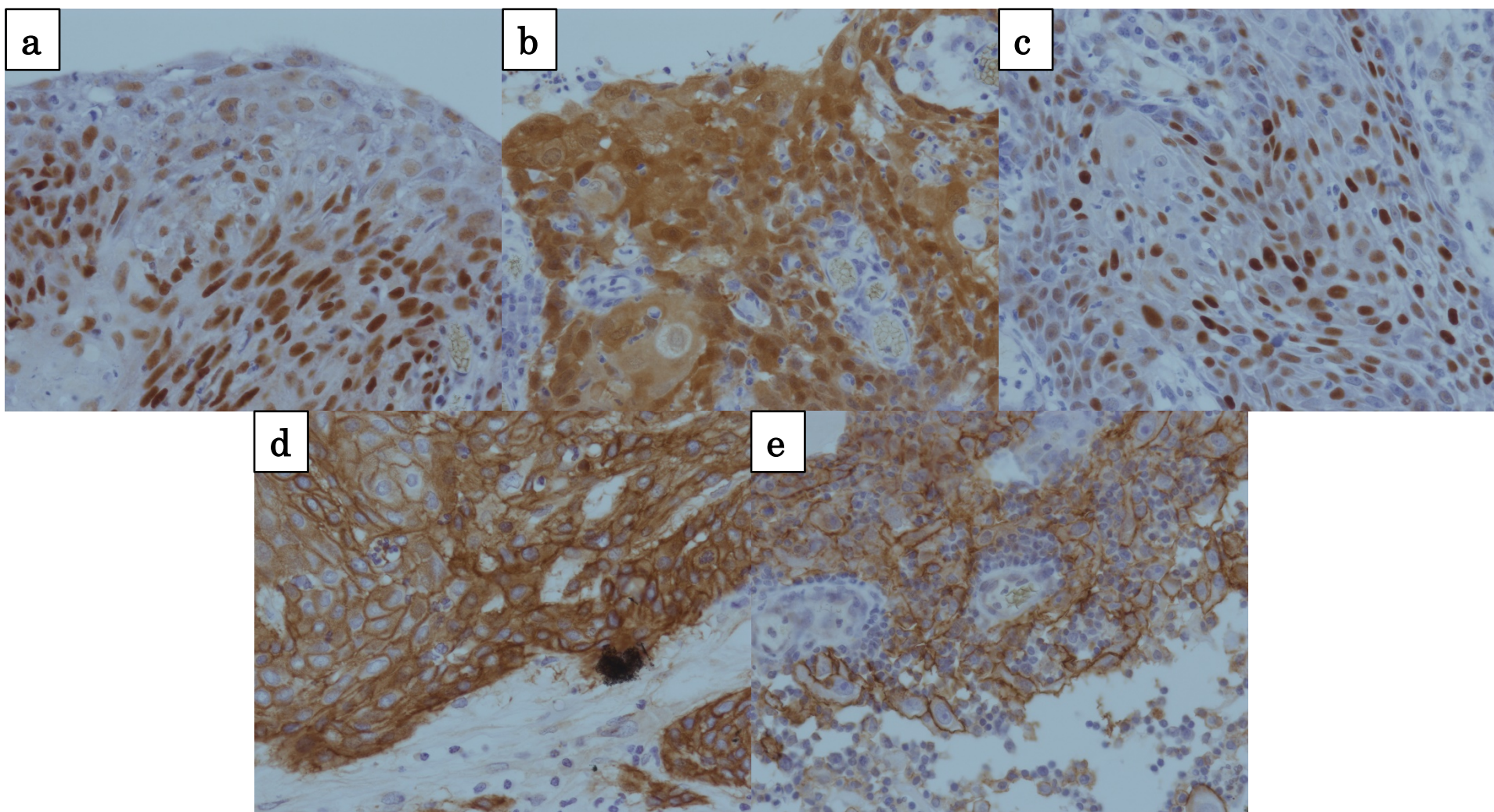
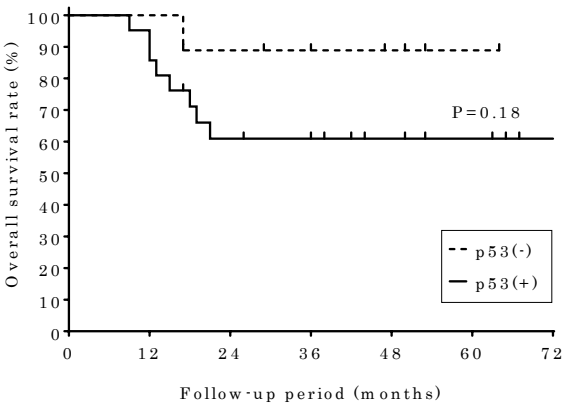
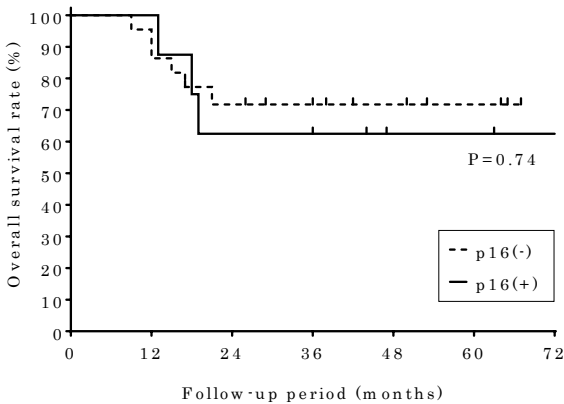


Figure 2

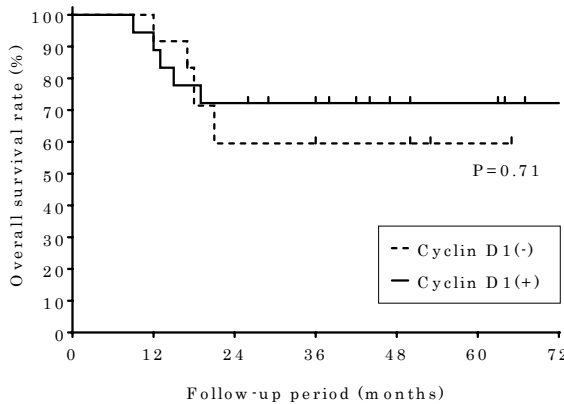
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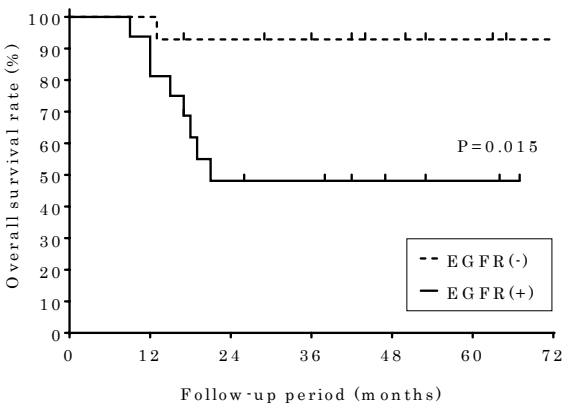
b



c



d



e

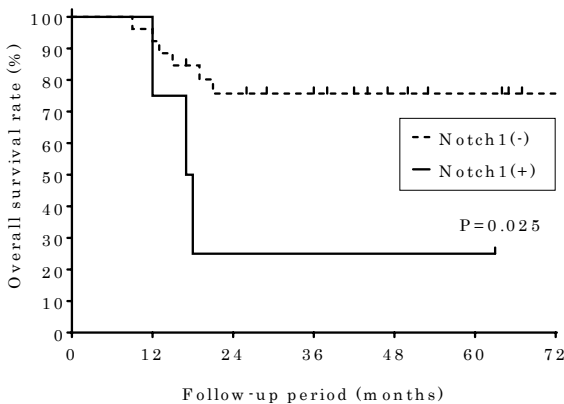


Figure 3

