[2–6]. In 2009, the Working Group of WHO-IARC concluded that acetaldehyde associated with alcoholic beverages was carcinogenic to humans and confirmed the group 1 classification of alcohol consumption [7]. In addition, heterozygous traits found in 40% of Asians, who have an inactive alcohol metabolizing enzyme of aldehyde dehydrogenases 2, accumulate acetaldehyde, with higher relative risks of these cancers [7, 8]. Furthermore, the prevalence of multiple Lugol-unstained lesions (LULs) [9, 10], which are caused by repeated exposure to acetaldehyde, was strongly related to the occurrence of synchronous or metachronous cancers in the esophagus and head and neck regions [11].

In contrast, most patients with PSCC are detected at an advanced stage with a poor prognosis. Even in an operable PSCC case, the extensive surgical resection required may cause a loss of function with respect to swallowing and/or speaking and can lead to cosmetic deformities. Thus it is difficult to determine a final treatment from the viewpoints of both curability and retaining organ function. In cancers combining ESCC and PSCC, the selection of treatment is even more critical. Because of this, the ability to detect pharyngeal lesions at an earlier stage, e.g. as carcinoma in situ, would be of clear benefit to patients. Recently, superficial PSCC has been detected by NBI endoscopy [12].

Systemic 5-fluorouracil-cisplatin (5-FU-CDDP) chemotherapy combined with radiotherapy is the standard treatment for ESCC, and the same treatment is also effective for PSCC patients [13, 14]. The radiation field used in radiotherapy for ESCC does not generally reach the region of the larynx and pharynx, while chemotherapy acts systemically. There have been no reports regarding the efficacy of systemic chemotherapy for patients with superficial PSCC. In this study, we examined the effect on superficial PSCC of chemoradiotherapy (CRT) targeted for invasive ESCC.

Patients and Methods

Patients

Between January 2003 and December 2006, concurrent CRT was performed in 348 patients with invasive ESCC who met the following criteria of this study: (1) newly diagnosed thoracic ESCC; (2) aged between 20 and 75 years; (3) clinical stage I to IVA according to the UICC-TNM classification; (4) absence of previous chemotherapy for malignancy; (5) absence of radiation or surgical treatment for head and neck, and esophageal cancers, and (6) absence of active malignancy except ESCC and PSCC. All patients with invasive ESCC visited our hospital to receive treatment after histological diagnosis of ESCC by endoscopy at another hospital.

Endoscopic Observation of the Oral Cavity and Pharynx

Since January 2003, endoscopic screening of the oral region has been performed in all ESCC patients in order to detect synchronously superficial PSCC. In the initial endoscopic observation in our hospital, narrow band imaging (NBI) or conventional endoscopy was used because both evaluation of ESCC and gastroduodenal screening including oral cavity and pharynx are performed in all patients. When a mucosal abnormality in the oral cavity or pharynx, or multiple LULs in the esophagus, were found in initially conventional endosopcy, the oral cavity and pharynx were observed again by magnifying NBI endoscopy within 2 weeks. Figure 1 shows the NBI findings of an oral cavity and pharynx using a video endoscope system (EVIS LUCERA CV-260, Olympus Optical Co. Ltd., Tokyo, Japan). When a brownish area and an enhancement of the intraepithelial papillary capillary loop were found in the pharynx (fig. 2), an endoscopic biopsy was performed to histologically confirm the carcinoma.

Lugol chromoendoscopy was performed in all patients for both diagnosis of the correct cancer region and evaluation of LULs in the background esophageal epithelium. After ordinary endoscopic observation, 5–10 ml of 2.0% glycerin-free Lugol iodine solution, which is a brown liquid consisting of 2.0 g potassium iodine and 4.0 g iodine in 100 ml distilled water, was sprayed from the upper thoracic esophagus to the gastroesophageal junction using a plastic spray catheter passed through the biopsy channel of the endoscope. Multiple LULs were defined as described in our previous study [15].

Definition of Superficial Pharyngeal Cancer

According to the Japan Society for Head and Neck Cancer [16], a superficial pharyngeal lesion is defined as one in which the invasion depth is comparatively limited and visual changes do not indicate an advanced cancer. The pharynx has no muscularis mucosa, so this somewhat vague definition suggests that the depth of invasion is limited to the epithelium or just beneath the epithelium, but does not extend to the muscle layer.

Treatment Schedule of CRT for ESCC

Chemotherapy consisted of a protracted infusion of 5-FU at a dose of 1,000 mg/m^2 per day on days 1–5 and 22–26, combined with a 2-hour infusion of CDDP at 75 mg/m^2 on days 1 and 22. A 10-MV radiation treatment was administered for 6 weeks (5 days/week) at 1.8 Gy/day with a total radiation dose of 50.4 Gy, concomitantly with chemotherapy.

Patients who were evaluated for an objective response to this treatment received additional chemotherapy consisting of a continuous infusion of 5-FU at a dose of 1,000 mg/m^2 on days 1–5 and CDDP at a dose of 75 mg/m^2 on day 1. This treatment schedule was administered for 1 week followed by a 3-week break. All patients receiving CRT were monitored by neck, chest and abdominal computed tomography, and by endoscopy to evaluate the efficacy of the treatment on both ESCC and PSCC.

As for response for ESCC, objective responses of measurable metastatic lesions were evaluated according to the response evaluation criteria in solid tumors (RECIST v 1.0) guideline. Response of the primary tumor was evaluated by the criteria of the Japan Esophageal Society [17, 18].

Evaluation of Response for PSCC

All follow-up evaluations after 5-FU-CDDP chemotherapy for PSCC were performed every 2 months for the first year and every 6 months thereafter by magnifying NBI endoscopy, with the same periods of evaluation as for ESCC. For PSCC, complete response (CR) was defined as the disappearance of all visible tumors (brownish areas), including ulceration, for at least 4 weeks, confirmed by normal endoscopic biopsy specimens. The recurrence was defined as the reappearance of a brownish area accompanied by an enhancement of intraepithelial papillary capillary loop by NBI endoscopy, and was confirmed in histological findings by endoscopic biopsy. Non-CR for PSCC was defined as the remnant of brownish areas and was classified into a partial response, stable disease or progressive disease.

In the case of non-CR for PSCC, the second treatment was selected according to the efficacy of CRT for ESCC. When ESCC reached CR with remnant or recurrence of PSCC, endoscopic resection (ER) was performed for PSCC. When the ESCC was evaluated for non-CR, thereafter treatment for ESCC, such as second-line chemotherapy, salvage surgery or palliation was performed.

ER for PSCC after CRT

The ER involved endoscopic mucosal resection using the cup method or an endoscopic subepithelial dissection method with the patient under general anesthesia. An important consideration was that ER for PSCC should be performed with cooperation from the endoscopists and the head and neck surgeons. Some head and neck surgeons participated in the ER to prepare emergency treatment, such as tracheostomy, with evaluation of the degree of laryngeal edema after the procedure.

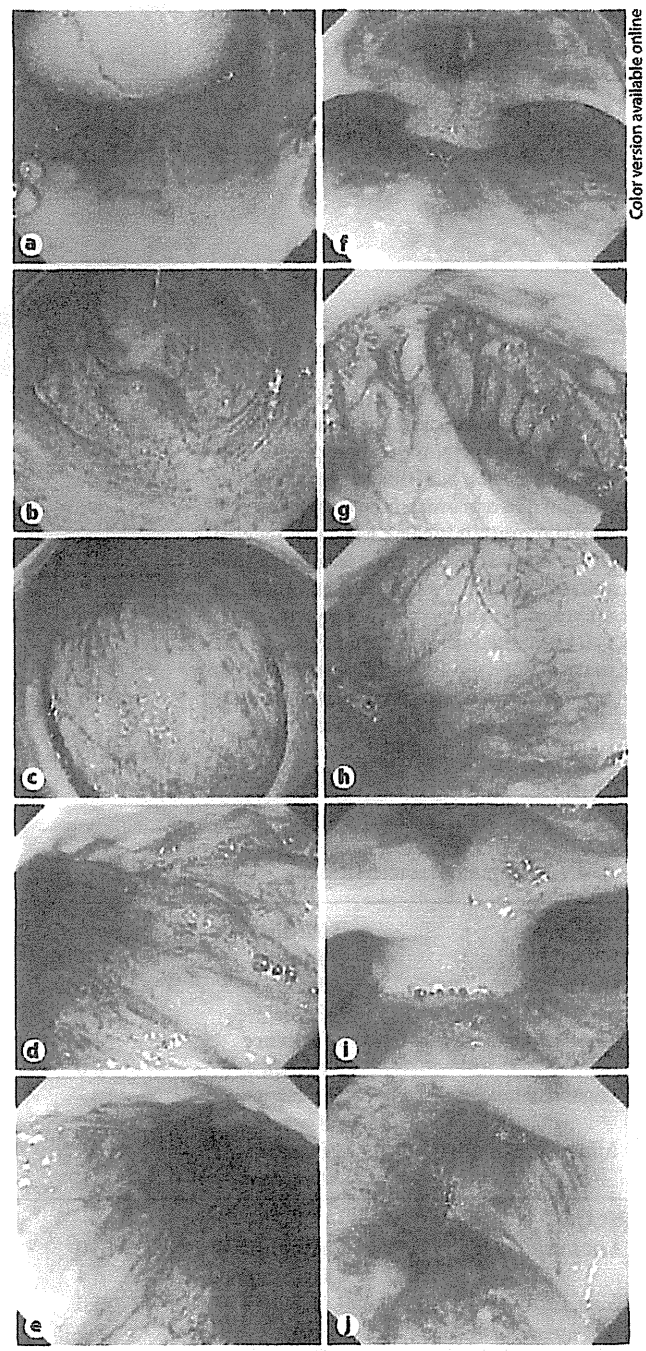
Statistics

All statistical analyses were performed using IBM SPSS Statistics 18 software (SPSS Inc., Tokyo, Japan). Overall survival data were calculated from the date of commencement of CRT to the date of death or the most recent follow-up visit. Survival curves were plotted according to the Kaplan-Meier method. The significance of differences was assessed using the log-rank test. A p value of <0.05 was considered statistically significant.

Results

Patient Characteristics

Fourteen patients (4.0%) with synchronous superficial PSCC were found among the 348 patients with invasive ESCC (table 1). Of the 14 patients, 13 (93%) were male and the median age was 62 years. The number of patients for ESCC clinical stage I, II, III, and IVA were 5, 2, 6 and 1, respectively. All 14 patients had both daily alcohol consumption and multiple LULs of the esophagus. All PSCC lesions were detected at our institute with no prior detection in other hospitals. Twelve (86%) PSCC lesions were detected using magnifying NBI endoscopy and the other 2 (14%) by conventional endoscopy. The latter 2 lesions were reevaluated with magnifying NBI endoscopy before



Color version available online

Fig. 1. Narrow Band Imaging observations in individual regions from the oral cavity to the pharynx. **a** The view seen from the entrance of the oral cavity: dorsal side of tongue, hard palate and soft palate. **b** Uvula, palatoglossal arch and lateral walls of oropharynx. **c** The posterior wall of oropharynx. **d** The right side of base of tongue and lateral wall of oropharynx. **e** The left side of base of tongue and lateral wall of oropharynx. **f** Posterior wall of hypopharynx and larynx. **g** Vallecula of epiglottis, median glossoepiglottic fold. **h** The lateral wall and apex of right piriform sinus. **i** Arytenoids. **j** The lateral wall and apex of left piriform sinus.

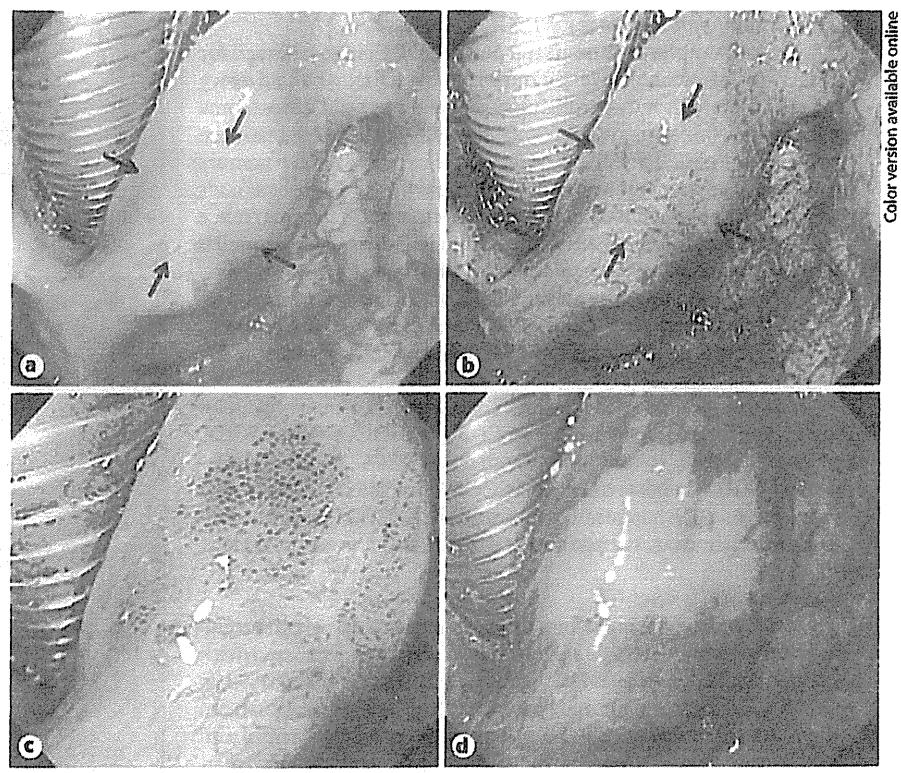


Fig. 2. Superficial cancer of the right arytenoid. **a** Conventional endoscopic observation. The margin of the cancer is unclear (black arrows). **b** NBI observation. Cancer is shown as a brownish area (black arrows) and the margin is clear. **c** Magnifying NBI observation. The enhanced intraepithelial papillary capillary loop is seen in the cancer area. **d** The view of Lugol staining. Lugol-unstained lesion coincided with the cancer area. Lugol staining method was used to improve lesion visualization during endoscopic treatment. Color refers to the online version only.

Table 1. Patient characteristics

Age, years	Median	62
	Range	47–71
Gender	Male	13
	Female	1
Alcohol consumption	Presence	14
	Absence	0
Cigarette smoking	Presence	12
	Absence	2
Multiple LULs	Presence	14
	Absence	0
PSCC		
Location	Hypopharynx	10
	Oropharynx	4
Size, mm	Median	20
	Range	5–50
Macroscopic findings	Elevated type	5
	Flat type	4
	Depressed type	5
ESCC		
Clinical stage	I	5
	II	2
	III	6
	IVA	1

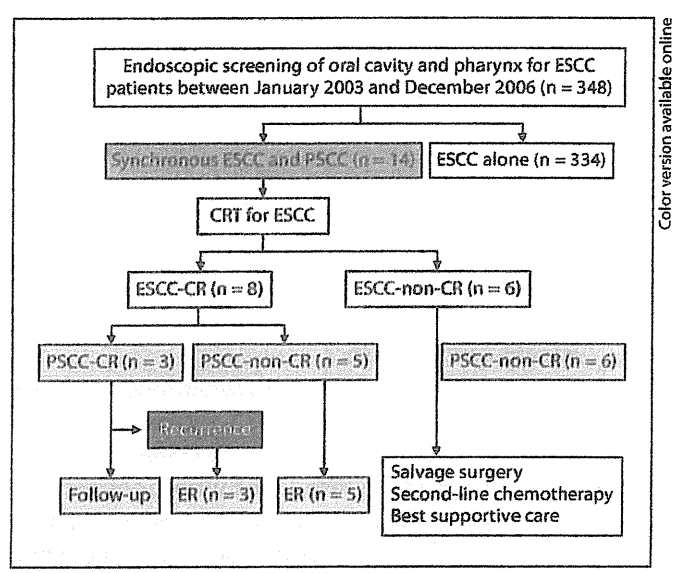


Fig. 3. Flow chart of this study.

CRT. The majority of PSCCs were located in the hypopharynx. In macroscopic findings, there were various lesion types. The median lesion diameter was 20 mm, ranging from 5 to 50 mm. All PSCC lesions were superficial cancers with no advanced cancers.

Efficacy of 5-FU-CDDP Chemotherapy for PSCC

The treatment for PSCC was determined according to response to CRT for primary ESCC (fig. 3). CRT for ESCC resulted in CR in 8 of the 14 patients. In contrast, only 3 of 14 PSCC lesions were evaluated as CR. The 3 PSCC-CR lesions (38%) were found in the ESCC-CR patients (fig. 3). However, the 3 PSCC-CR lesions were only transiently disappeared, and local recurrence was found in the same region. In the 6 ESCC-non-CR patients, there were no PSCC-CR lesions. Of the 6 patients, 2 who were finally evaluated as partial response for ESCC had transformation of their superficial PSCC to invasive lesions. Therefore, active salvage surgery with laryngopharyngeal and esophageal resection was undertaken in these 2 patients. Of the remaining 4 patients, 2 lesions had no change in size and shape while the other 2 were evaluated as partial response because of decreased tumor size.

ER for PSCC and Complications

ER for PSCC was performed in the 8 patients with ESCC-CR. Histologic findings showed the depth of infiltration was invasive PSCC in 2 patients and cancer in situ in 6 patients. However, no lymphovascular involvement was found in any of the 8 cases with PSCC.

Major complications associated with ER included 1 case of aspiration pneumonia. There were no severe complications such as subcutaneous emphysema, post-ER stricture or delayed bleeding. Of the 8 PSCC lesions, 1 was recurrent 4 months after ER. Because the recurrent lesion was superficial and small, an additional ER was performed with complete resection. The median duration of follow-up after ER was 28 months ranging from 12 to 39 months, and no more recurrences of the PSCC were found.

Survival

The 8 ESCC-CR patients received ER for PSCC and the remaining 6 ESCC-non-CR patients did not. The pretreatment clinical stages of ESCC in 8 ER and 6 non-ER patients were 4 and 1 patient in stage I, 1 and 1 in stage II, 2 and 4 in stage III and 1 and 0 in stage IVA, respectively. There were no differences in clinical staging variety in the ESCC pretreatment evaluation between ER and non-ER patients. Median survivals of ER and non-ER patients were 51 and 14 months, respectively (p = 0.002; log-rank test; fig. 4). The 3-year survival rates of ER and non-ER patients were 63 and 0%, respectively. In contrast, 4 of the 8 ER patients died during follow-up periods. Preclinical stages of the 4 patients were 2 patients in stage I, 1 in stage II and 1 in stage IVA, respectively. The 2 patients in stage I died of radiation-induced pneumonia and cerebral infarction. The patient in stage II died of ESCC progression with lymph node metastases and the remaining patient in stage IVA died of multiple lung metastases.

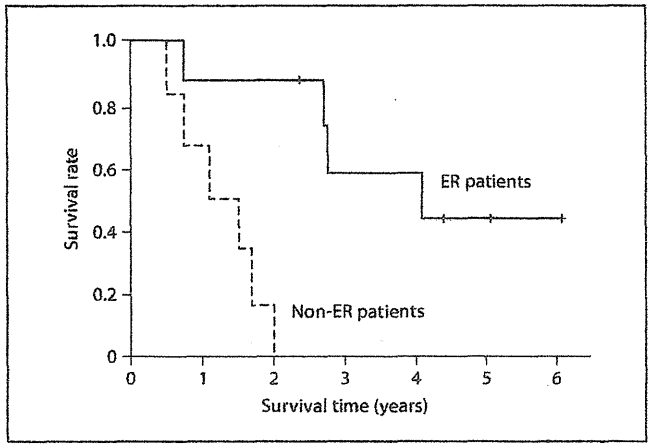


Fig. 4. Overall survival. Median survivals of ER and non-ER patients were 51 and 14 months, respectively (p = 0.002; log-rank test).

After CR confirmation in ESCC, ER was performed in PSCC. The median duration from commencement of CRT to ER in the 8 patients receiving ER was 5.4 months, ranging from 3.8 to 18.9 months. ER was performed in 5 of the 8 patients immediately after CRT since PSCC lesions of the 5 patients were not evaluated as CR. However, the time periods to perform ER after CRT were extended in the remaining 3 PSCC-CR patients from 10 to 18.9 months due to following-up for PSCC-CR. There were no cases in which superficial PSCC transformed to an advanced stage during the follow-up periods. Thus, no functional disorder caused by progression of PSCC, such as difficulty swallowing or speaking, were found in ER patients during all follow-up periods.

Discussion

Of 348 patients with invasive ESCC, 14 (4%) had superficial PSCC detected through endoscopic screening of the oral cavity and pharynx. Standard 5-FU-CDDP CRT targeted for invasive ESCC was administered to the 14

patients with synchronous superficial PSCC and invasive ESCC. After CRT, 8 (57%) were evaluated as CR for invasive ESCC, while only 3 patients with superficial PSCC (21%) achieved transient CR despite receiving 5-FU-CDDP chemotherapy. Therefore, systemic 5-FU-CDDP chemotherapy had no CR potential for superficial PSCC. In contrast, ER for superficial PSCC is quite effective even in a situation after chemotherapy because of minimally invasive treatment with no functional disorder in the pharyngeal region. We propose using novel treatment strategies for synchronous superficial PSCC and invasive ESCC.

Acetaldehyde associated with alcoholic beverage and aldehyde dehydrogenases 2 heterozygous traits can cause pharyngeal and esophageal cancers [7]. According to recent reports regarding multiple cancers, the prevalence of multiple LULs is a biomarker of synchronous or metachronous cancers in the esophagus and head and neck regions [19–21]. In our present study, all 14 patients with synchronous ESCC and PSCC had both daily alcohol consumption and multiple LULs in their esophageal background epithelium. Lugol chromoendoscopy is useful not only to detect superficial ESCC but also to understand the risk of multiple cancers. However, the Lugol solution cannot be routinely sprayed in the region of the pharynx and larynx of patients under conscious sedation because of the stimulation caused by the application of the solution. Thus, we suggest that detecting superficial PSCC by NBI is useful in ESCC patients, especially those with both an alcohol drinking habit and multiple LULs in their esophagus.

5-FU-CDDP treatment has been performed in PSCC patients since the 1980s. The CR rate of this therapy without radiotherapy was 17–20% of locally advanced or metastatic PSCC cases in phase I–II studies [13, 14], and was 5–7% of metastatic or recurrent cases in phase III studies [22, 23]. 5-FU-CDDP treatment alone is likely to be more effective in locally advanced PSCC than in metastatic PSCC. In contrast, there has been no study of 5-FU-CDDP alone in PSCC of early clinical stage, especially stage 0–I. Therefore, the 5-FU-CDDP treatment efficacy in superficial PSCC is uncertain. If the therapy had a high efficacy for superficial PSCC, overlooked superficial PSCC would be cured by the systemic 5-FU-CDDP therapy given to treat ESCC. This is quite a benefit for the patients with these synchronous cancers. As a result, PSCC-CR was found, while no efficacy in continuing CR for superficial PSCC was found in 5-FU-CDDP treatment. In contrast, no progression of PSCC was found in patients having excellent efficacy with CRT for ESCC, although the time periods until CR confirmation for ESCC were required to be at least several months. A good correlation in treatment efficacy between PSCC and ESCC was indicated. It seems that 5-FU-CDDP chemotherapy has a potential in restraining the progression of PSCC. In some recent reports, platinum-based chemotherapy or CRT plus cetuximab were more effective in esophageal and the head and neck cancers [24–26]. CRT combined with cetuximab, a molecular targeted drug, may contribute to a novel treatment strategy for patients with synchronous PSCC and ESCC.

The outcomes of ER for superficial PSCC have been reported [27]. Complications, such as laryngeal edema requiring overnight intubation, aspiration pneumonia and sustained dermatitis around the mouth caused by backflow of Lugol solution from the pharynx, were found in 13% of patients after ER [27]. Complications are transient and tolerable in most of cases, and feasibility is confirmed with no functional disorder. In our study, there were no severe complications, with high treatment efficacy for ER during long follow-up periods, although ER was performed in the condition after 5-FU-CDDP chemotherapy. It is important to maintain function with respect to swallowing and/or speaking, and to perform ER under cooperation with head and neck surgeons.

Regarding the treatment strategy, CRT for ESCC should be the initial therapy in patients with both superficial PSCC and ESCC. As the second step, ER for PSCC should be determined after evaluation of CRT for ESCC. A factor deciding the prognosis of patients with the synchronous cancers depends on the CRT effects for ESCC. In our previous study, the prognosis between CR and non-CR cases of ESCC was quite different [28]. In our present study, the median survival time of ER (ESCC-CR) patients was also significantly longer than that of non-ER (ESCC-non-CR) patients. Furthermore, 5-FU-CDDP chemotherapy showed potential in restraining the progression of PSCC including transient CR. If ER was performed initially, the period before commencement of CRT would be delayed. In addition, when complications occurred in ER, the commencement would be further delayed. Therefore, ER for superficial PSCC should be secondary to CRT for invasive ESCC. We suggest that the ER for PSCC contributed to the beneficial prognosis in patients with synchronous superficial PSCC and invasive ESCC. It is uncertain whether all superficial PSCC lesions progress to an advanced stage in the natural history. However, if superficial PSCC was overlooked and progressed to an advanced stage in ESCC-CR patients, it would be difficult to achieve long survival. Furthermore,

13 Gibson MK, Li Y, Murphy B, Hussain MHA, DeConti RC, Ensley J, Arlene A: Randomized phase III evaluation of cisplatin plus fluorouracil versus cisplatin plus paclitaxel in advanced head and neck cancer (E1395): an Intergroup Trial of the Eastern Cooperative Oncology Group. J Clin Oncol 2005;23: 3562–3567.
14 Jacobs C, Lyman G, Velez-Garcia E, Sridhar KS, Knight W, Hochster H, Goodnough LT, Mortimer JE, Einhorn LH, Schacter L, Cherng N, Dalton T, Burroughs J, Rozencweig M: A phase III randomized study comparing cisplatin and fluorouracil as single agents and in combination for advanced squamous cell carcinoma of the head and neck. J Clin Oncol 1992;10:257–263.
15 Kaneko K, Katagiri A, Konishi K, Kurahashi T, Ito H, Kumekawa Y, Yamamoto T, Muramoto T, Kubota Y, Nozawa H, Makino R, Kushima M, Imawri M: Study of *p53* gene alteration as a biomarker to evaluate the malignant risk of Lugol-unstained lesion with non-dysplasia in the oesophagus. Br J Cancer 2007;96:492–498.
16 Japan Society for Head and Neck Cancer: General Rules for Clinical Studies on Head and Neck Cancer. Tokyo, Kanehara, 2005.
17 Diseases JSFE: Guidelines for the Clinical and Pathological Studies on Carcinoma of the Esophagus (in Japanese), ed 9. Tokyo, Kanehara Shuppan, 1999, pp 59–79.
18 Takubo K, Makuuchi H, Fujita H: Japanese Classification of Esophageal Cancer, tenth edition: part 1. Esophagus 2009;6:1–25.
19 Hori K, Okada H, Kawahara Y, Takenaka R, Shimizu S, Ohno Y, Onoda T, Shirakawa Y, NaomotoY, Yamamto K: Lugol-voiding lesions are an important risk factor for a second primary squamous cell carcinoma in patients with esophageal cancer or head and neck cancer. Am J Gastroenterol 2011;106: 858–866.
20 Muto M, Hitomi Y, Ohtsu Y, Ebihara S, Yoshida S, Esumi H: Association of aldehyde dehydrogenase 2 gene polymorphism with multiple esophageal dysplasia in head and neck cancer patients. Gut 2000;47:256–261.
21 Muto M, Hironaka S, Nakane M, Boku N, Ohtsu A, Yoshida S: Association of multiple Lugol-voiding lesions with synchronous and metachronous esophageal squamous cell carcinoma in patients with head and neck cancer. Gastrointest Endosc 2002;56:517–521.
22 Rowland KM Jr, Taylor SG 4th, Spiers AS, DeConti RC, O'Donnell MR, Showel J, Stott PB, Milner LM, Marsh JC: Cisplatin and 5-FU infusion chemotherapy in advanced, recurrent cancer of the head and neck: an Eastern Cooperative Oncology Group Pilot Study. Cancer Treat Rep 1986;70:461–464.
23 Caponigro F, Comella P, Rivellini F, Avallone A, Budillon A, Di Gennaro E, Mozzillo N, Ionna F, De Rosa V, Manzione L, Comella G: Cisplatin, raltitrexed, levofolinic acid and 5-fluorouracil in locally advanced or metastatic squamous cell carcinoma of the head and neck: a phase I–II trial of the Southern Italy Cooperative Oncology Group (SICOG). Ann Oncol 2000;11:575–580.
24 Vermorken JB, Mesia R, Rivera F, Remenar E, Kawecki A, Rottey S, Erfan J, Zabolotnyy D, Kienzer HR, Cupissol D, Peyrade F, Benasso M, Vynnychenko I, Raucourt DD, Bokemeyer C, Schueler A, Amellal N, Hitt R: Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008;359:1116–1127.
25 Safran H, Suntharanlingam M, Dipetrillo T, Ng T, Doyle A, Krasna M, Plette A, Evans D, Wanebo H, Akerman P, Spector J, Kennedy N, Kennedy T: Cetuximab with concurrent chemoradioation for esophagogastric cancer: assessment of toxicity. Int J Radiat Oncol Biol Phys 2008;70:391–395.
26 Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, Jones CU, Sur R, Raben D, Jassem J, Ove R, Kies MS, Baselga J, Youssoufian H, Amellal N, Rowinsky ER, Ang KK: Radiotherapy plus cetuximab for squamous cell carcinoma of the head and neck. N Engl J Med 2006;354:567–578.
27 Suzuki H, Saito Y, Oda I, Nonaka S, Nakanishi Y: Feasibility of endoscopic mucosal resection for superficial pharyngeal cancer: a minimally invasive treatment. Endoscopy 2010;42:1–7.
28 Ohtsu A, Boku N, Muro K, Chin K, Muto M, Yoshida S, Satake M, Ishikura S, Ogino T, Miyata Y, Seki S, Kaneko K, Nakamura A: Definitive chemoradiotherapy for T4 and/or M1 lymph node squamous cell carcinoma of the esophagus. J Clin Oncol 1999;17:2915–2921.
29 Shimizu Y, Tsukagoshi H, Fujita M, Hosokawa M, Watanabe A, Kawabori S, Kato M, Sugiyama T, Asaka M: Head and neck cancer arising after endoscopic mucosal resection for squamous cell carcinoma of the esophagus. Endoscopy 2003;35:322–326.
30 Katada C, Tanabe S, Koizumi W, Higuchi K, Sasaki T, Azuma M, Katada N, Masaki T, Nakayama M, Okamoto M, Muto M: Narrow band imaging for detecting superficial squamous cell carcinoma of the head and neck in patients with esophageal squamous cell carcinoma. Endoscopy 2010;42:185–190.

Int J Clin Oncol
DOI 10.1007/s10147-012-0402-z

ORIGINAL ARTICLE

Clinical significance of *KRAS* gene mutation and epidermal growth factor receptor expression in Japanese patients with squamous cell carcinoma of the larynx, oropharynx and hypopharynx

Satoshi Fujii · Hideoki Uryu · Ken Akashi · Kensuke Suzuki · Manabu Yamazaki · Makoto Tahara · Ryuichi Hayashi · Atsushi Ochiai

Received: 8 December 2011 / Accepted: 1 March 2012

Abstract

Purpose The significance of epidermal growth factor receptor (EGFR) signaling has been recognized in various cancers and anti-EGFR therapies in Japan are currently under consideration in squamous cell carcinoma of the head and neck (SCCHN) similar to colorectal cancer. However, there was no established survey regarding heterogeneous EGFR protein expression in Japanese SCCHN patients. The purpose of this study is to examine the relationship between EGFR expression or *KRAS* mutation (related to the alteration of EGFR pathway) and the clinicopathological characteristics of SCCHN.

Materials and methods We retrospectively examined the expression of EGFR protein by immunohistochemistry and *KRAS* gene mutation at codons 12 and 13 by using paraffin-embedded and formalin-fixed primary tumor tissues from 205 patients with SCCHN who underwent surgery at National Cancer Center Hospital East.

Results In 200 of the 205 patients (97.6 %), EGFR protein was expressed despite intratumoral heterogeneity. No patients had *KRAS* mutation at codons 12 or 13, and all 183 tumors showed wild-type *KRAS*. Positive rate of EGFR protein expression was significantly associated with better disease free survival (DFS) ($P = 0.0471$) and the intensity of EGFR protein expression showed a tendency for better DFS ($P = 0.1034$). Both higher EGFR positive rate and more intense EGFR expression were significantly associated with well differentiated subtype of squamous cell carcinoma ($P = 0.0003$ and $P = 0.0007$, respectively).

Conclusion Most SCCHN patients may be good candidates for the anti-EGFR therapies.

Keywords Squamous cell carcinoma · KRAS · EGFR · Larynx · Pharynx

Electronic supplementary material The online version of this article (doi:10.1007/s10147-012-0402-z) contains supplementary material, which is available to authorized users.

S. Fujii · M. Yamazaki · A. Ochiai (✉)
Pathology Division, Research Center for Innovative Oncology, National Cancer Center at Kashiwa, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan
e-mail: aochiai@east.ncc.go.jp

H. Uryu · K. Akashi · K. Suzuki · R. Hayashi
Department of Head and Neck Surgery, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan

M. Yamazaki
Division of Oral Pathology, Department of Tissue Regeneration and Reconstruction, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

M. Tahara
Department of Head and Neck Medicine, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan

Introduction

Head and neck carcinoma (HNC) is the seventh most common malignancy and around 600,000 cases are diagnosed annually [1]. Its incidence increases with aging and HNC occurs predominantly from 40 to 60 years of age, affecting at least twice as many men as women. Tobacco smoking and alcohol consumption are risk factors for HCN, including squamous cell carcinoma of the head and neck (SCCHN), as is known for esophageal squamous cell carcinoma [2]. In Europe, 132,000 new cases of HNC were diagnosed and 63,000 patients died of HNC in 2008 [3]. In Japan, 13,026 cases of SCCHN were diagnosed and deaths

Published online: 24 March 2012

caused by SCCHN have increased recently [4], with 6,255 and 6,768 deaths from SCCHN in 2001 and 2005, respectively [4]. As this increase continues, it is estimated that the annual number of deaths from SCCHN will rise to 10,700 by 2020 [5, 6]. Also, only 40 % of SCCHN patients have early disease (Stages I or II) that can benefit from surgical intervention and/or radiation therapy. The five-year survival rate of those patients ranges from 60 to 90 % [2], but over 50 % of SCCHN patients are in Stages III or IVA/B with locally advanced cancer [7, 8]. Local recurrence affects 60 % of patients with locally advanced cancer, while distant metastases and second cancers occur in about 30 and 5–13 %, respectively [9]. There is an urgent need for novel chemotherapy regimens to treat locally advanced cancer.

Erbitux® (cetuximab) is a chimeric IgG1 mAb that binds specifically and with high affinity to the extracellular domain of human EGFR. Preclinical studies demonstrated synergy of cetuximab with topoisomerase I inhibitors against human colorectal cancer (CRC) cell lines in vivo [10]. Subsequent clinical trials revealed that cetuximab can overcome resistance to topoisomerase I inhibitors and has modest activity as monotherapy, resulting in accelerated provisional FDA approval for the treatment of irinotecan-refractory CRC [10]. Full FDA approval was subsequently granted to cetuximab after a randomized clinical trial (The National Canadian Institute of Cancer Study CO.17) demonstrated improvement of overall survival (OS) in patients receiving cetuximab plus best supportive care compared with best supportive care alone [11]. Cetuximab is the second mAb approved for treating CRC and the first anti-EGFR antibody approved for clinical use in Japan. Clinical utility has been improved by the discovery of negative biomarkers since cetuximab is less effective against tumors harboring *KRAS* mutation [12]. Thus, CRC patients with tumors bearing *KRAS* mutations do not benefit from cetuximab, unlike patients whose tumors have wild-type *KRAS*. In contrast, *KRAS* mutation has no influence on the survival of patients receiving best supportive care alone [12]. Although *KRAS* mutation was reported to be rare in SCCHN overseas, there have been no reports about its influence on survival in SCCHN patients receiving cetuximab.

The EGFR is a 170,000 dalton cell surface tyrosine kinase transmembrane receptor from the ErbB family [13]. It is normally expressed by various epithelial cells, and is overexpressed in the epithelium of the bronchial tree, gastrointestinal tract, skin, and gynecologic tract. After the extracellular component of the receptor binds to a natural ligand, such as TGFα, amphiregulin, or epiregulin, homodimerization or heterodimerization occurs with other members of the ErbB family including ErbB2 or HER2/neu, ErbB3, and ErbB4 [14]. Binding and activation of the receptor lead to phosphorylation of tyrosine kinase and subsequent downstream activation of multiple cellular signaling pathways, such as the Ras-Raf-MAP kinase pathway, PI3 K pathway, and protein-serine/threonine kinase Akt pathway. These signal transduction pathways regulate various cellular activities, including replication, invasion, repair, and protection against exogenous insults [15]. The EGFR is an attractive target for anticancer therapy because it is activated in various cancers, including CRC [16, 17]. In fact, CRC patients with a quantitative increase of EGFR expression demonstrated by immunohistochemistry (IHC) have a worse prognosis [18, 19]. Therefore, interruption of this signaling pathway could potentially abolish the growth advantage of cancer cells and/or promote tumor cell death. EGFR protein is expressed in normal epithelial cells, such as epidermal cells and hair follicle cells, as well as in various cancers (SCCHN, pancreatic, renal, colorectal, and non-small cell lung cancer) [14, 20]. An increase of the *EGFR* gene copy number is frequent in SCCHN and indicates a poor prognosis, suggesting the potential significance of EGFR inhibitor therapy for SCCHN patients [20].

Immunohistochemical staining of tumor tissue specimens with anti-EGFR antibody is usually done before treating CRC with cetuximab. The prevalence of EGFR expression by CRC was reported as about 80 % [21], although a study of 91 SCCHNs found EGFR expression is 100 % [22]. Therefore, immunohistochemical staining for EGFR protein is not required before treating SCCHN with cetuximab overseas and it is necessary to examine the prevalence of EGFR protein expression among Japanese SCCHN patients. *KRAS* gene mutation has been recognized as a negative predictor for responsiveness of CRC to cetuximab. However, *KRAS* gene mutation was estimated to occur in <3 % of SCCHNs, making it of doubtful value to investigate *KRAS* in all candidates for cetuximab treatment, especially from the perspective of cost versus benefit. Therefore, it is important to examine *KRAS* mutation and EGFR protein expression in Japanese patients with SCCHN.

The present study of EGFR protein expression and *KRAS* gene mutation in Japanese SCCHN patients was therefore expected to assist in avoiding unnecessary examinations before starting anti-EGFR therapy such as cetuximab, which could help to reduce health care costs.

Materials and methods

Tissue samples and patients

Two-hundred and five SCCHN tumor samples surgically resected were collected at the National Cancer Center Hospital East between 1994 and 2006. The tumors were located in the larynx ($n = 51$), oropharynx ($n = 71$), and

hypopharynx ($n = 83$) of 135 males and 70 females. Genomic DNA was isolated from formalin-fixed and paraffin-embedded tissue sections with the maximum amount of cancer tissue as microscopically confirmed by pathologists (non-cancerous tissue accounted for <30 %). Genomic DNA was extracted using a DNA extraction kit from QIAGEN (QIAamp FFPE DNA kit, Valencia, CA, USA). This study received institutional review board approval.

KRAS gene mutation analysis

Tumor samples were analyzed for exon 2 mutations of *KRAS* located within codons 12 and 13. DNA was amplified by the polymerase chain reaction (PCR) using the following sense and antisense primers: 5′-TGTGTGACA TGTTCTAATATAGTCACATTT-3′ and 5′-TTAAAACA AGATTTACCTCTATTGTTGGAT-3′ [23]. PCR was carried out in a reaction mixture (25 μL) with 250 ng of genomic DNA. After purification using Illustra GFX DNA and a Gel Band Purification Kit or Agencourt AMPure XPkit, the PCR products were subjected to direct sequencing using a BigDye V1.1 terminator sequencing kit (Applied Biosystems, Foster, CA, USA), ethanol precipitation, and an automated sequencer (ABI Prism 3100: Applied Biosystems). The sensitivity of direct sequencing has been validated since the mutation contamination rate is kept to be lower than 20 %.

Immunohistochemical staining for EGFR protein

EGFR protein expression was evaluated by immunohistochemical staining of 4 μm sections obtained from paraffin-embedded specimens fixed in 20 % (v/v) formalin [24]. Sections were deparaffinized in xylene and then rehydrated in an alcohol series. Endogenous peroxidase activity was blocked by immersion in methanol containing 0.3 % hydrogen peroxide for 5 min. Then the sections were incubated with protease K, followed by immunohistochemical staining using an EGFR pharmDx kit and Autostainer (Dako, Tokyo, Japan), counterstaining with Mayer's hematoxylin, and mounting. A positive control (HT-29 human colon cancer cell line) and a negative control (CAMA-1 human breast cancer cell line) for EGFR protein expression were included in each staining series. Membranous staining of EGFR was assessed according to the EGFR pharmDx kit protocol and EGFR positivity was judged by two pathologists blinded to the clinical data.

Assessment of immunohistochemical staining

Sections were examined at 400× magnification and staining intensity was evaluated by comparison with noncancerous squamous epithelium, which served as a reference for moderate intensity (M). Tumor staining less intense than the basal layer of noncancerous squamous epithelium was categorized as weak intensity (W), more intense staining was categorized as strong intensity (S), and no staining was categorized as negative (N). We also calculated the percentages of cells with different staining intensities and the predominant intensity was recorded for each tumor. Based on the percentage of cells with EGFR protein expression, tumors were classified into three groups: grade 0 (0–10 %), grade 1 (11–50 %), and grade 2 (51–100 %). We then examined the relationship between EGFR protein expression and overall survival (OS) or disease-free survival (DFS).

Statistical analysis

OS and DFS were analyzed by the Kaplan–Meier method. OS was defined as the period from the operation date to the date of death. DFS is the percentage of individuals in the group who are likely to be free of disease after a specified duration of time from the day of surgical operation to the day of termination of observation without the event such as death or the first recurrence. Comparison between survival functions for different strata was assessed with the log-rank test. Multivariate analysis of prognostic factors was performed by Cox's regression model. Statistical significance was accepted at $P < 0.05$. The software used in this study is JMP9, SAS.

Results

Clinicopathological factors

The clinicopathological characteristics of the patients are shown in Table 1. They were aged from 36 to 85 years, with the mean and median age being 62 and 61 years, respectively. The pT and pN factors, and pStage of patients are shown according to the UICC classification (7th edition). This study did not include any patient with a distant metastasis (M1) and any patient who had received preoperative therapy. The number of patients who had received postoperative therapy such as radiation or chemotherapy was 19 (9.2 %). Tobacco and alcohol consumption (major risk factors for laryngeal, hypopharyngeal, and oropharyngeal squamous cell carcinoma) were reported by 159 (77.6 %) and 139 (87.4 %) patients, respectively. Multiple cancers were present in 69 patients (33.7 %).

KRAS gene mutational analysis

KRAS mutational analysis was done in 183 of 205 patients (89.3 %) including 113 males (83.7 %) and 70 females

Table 1 Clinicopathological factors of patients with SCCHN

Variables	Number		
Gender			
Male	135		
Female	70		
Age			
Range	36–85		
Mean	62		
Median	61		
Tumor site			
Larynx	51		
Oropharynx	71		
Hypopharynx	83		
TNM classification	Larynx	Oropharynx	Hypopharynx
pT[a]			
T1	5	7	10
T2	21	32	28
T3	17	21	23
T4	8	11	22
pN[a]			
N0	31	22	20
N1	7	12	20
N2	13	36	43
N3	0	1	0
pStage[a]			
I	3	2	6
II	12	10	8
III	18	15	16
IV	18	44	53
Post-operative therapy			
Yes		19	15 (RT[b]) 4 (CT[c])
No		186	
Alcohol consumption			
Yes		139	
No		55	
Unknown		11	
Smoking			
Yes		159	
No		38	
Unknown		8	
Multiple cancers			
Yes		69	
No		136	

[a] UICC classification 7th edition

[b] Radiotherapy

[c] Chemotherapy

Table 2 KRAS gene status and EGFR protein expression by immunohistochemistry

KRAS gene status		EGFR protein expression	
Wild cases (%)	Mutant cases (%)	Positive cases (%)	Negative cases (%)
183 (100 %)	0 (0 %)	200 (97.6 %)	5 (2.4 %)

(100 %) (Table 2). Wild-type *KRAS* was found in all patients examined (100 %). Two representative cases are shown in Fig. 1.

EGFR protein expression

EGFR positivity

All 205 patients underwent immunohistochemical analysis of EGFR protein expression. Negative controls did not show any EGFR staining. Five male patients (2.4 %) had EGFR-negative SCCHN, while 200 patients (97.6 %) had EGFR-positive tumors (Table 2). Two representative cases are shown in Fig. 1. All major clinicopathological characteristics were similar among different levels of positivity or intensity of EGFR expression. Although all EGFR-negative patients were male, no difference was noted for the age at diagnosis (mean age 65.5 years). Four EGFR-negative tumors were located in the hypopharynx and one was in the oropharynx.

Heterogeneity of EGFR expression and clinicopathological factors

There was marked intratumoral heterogeneity of EGFR positivity (%) and staining intensity. Staining pattern was qualified as 'diffuse' when intensity was alike in more than 99 % of carcinoma cells, and termed as 'mosaic' in the case with heterogeneous intensity of staining or positivity less than 99 % of carcinoma cells. As shown in Table 3, there was no significant association between EGFR positivity and intensity. We used Kaplan–Meier analysis to detect differences in the survival of patients with higher EGFR positivity or more intense EGFR staining versus those with normal or low EGFR expression. We found that increased positivity of EGFR protein expression was not associated with overall survival (OS) (Fig. 2a), although it was significantly associated with better DFS ($P = 0.0471$) (Fig. 2b). In contrast, there was no significant association between the intensity of EGFR expression and either OS or DFS (Fig. 2c, d).

Next, we explored the relationship between the positivity or intensity of EGFR protein expression and various clinicopathological factors. The relationship between the

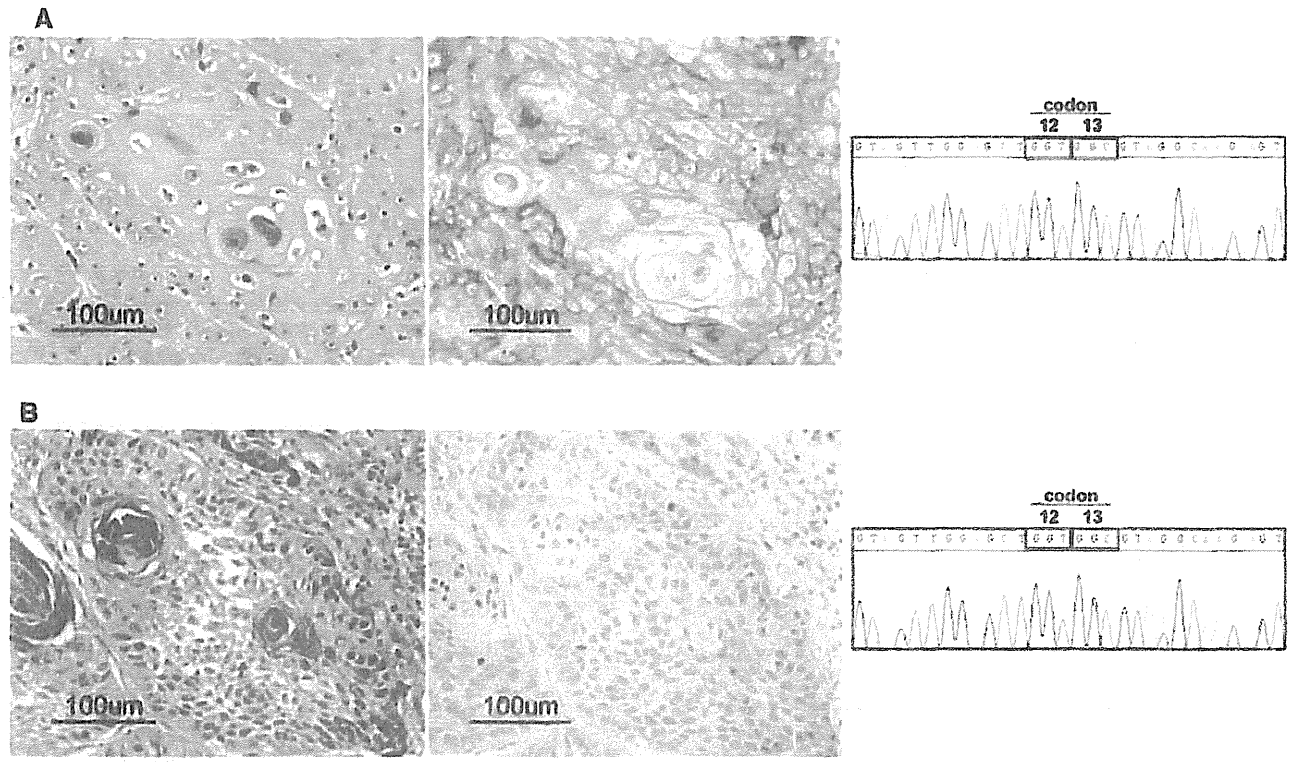


Fig. 1 Representative cases with and without EGFR expression. a Case no. 50 (55 years old, male, tumor site: larynx) shows EGFR expression and *KRAS* wild type. b The case no. 155 (69 years old, male, tumor site: hypopharynx) showed no EGFR expression and *KRAS* wild type

Table 3 Relationship between positivity and intensity of EGFR protein expression

Intensity	Positivity			
	0–10 % (grade 0)	11–50 % (grade 1)	51–100 % (grade 2)	Total
Negative	5	0	0	5
Weak	1	3	0	44
Moderate	14	22	5	41
Strong	16	77	62	155
Total	36	102	67	205

positivity of EGFR protein expression and the intensity of EGFR protein expression was shown in Table 3. However, there was no significant association among these two factors. Higher positivity and intensity of EGFR expression were significantly associated with well differentiated cancer ($P = 0.0003$ and 0.0007, respectively) (Fig. 3a, b). Laryngeal SCC is reported to have a better prognosis than oropharyngeal or hypopharyngeal SCC [25], as confirmed by the OS and DFS data in this study (Fig. 4a, b). However, we found no significant difference in the prevalence of well differentiated SCC between laryngeal, oropharyngeal, and hypopharyngeal cancer (Fig. 4c). There was no significant correlation between tumor differentiation and either OS or DFS for cancer of the hypopharynx and larynx, unlike cancer of the oropharynx (Supplementary Fig. 1). These findings suggested that EGFR positivity may predict DFS independently of tumor location.

EGFR expression and gender

We also examined whether gender was related to the positivity and intensity of EGFR protein expression. There was a weak correlation between gender and both EGFR positivity and intensity in the 205 patients ($P = 0.0630$ and 0.0699, respectively) (Fig. 5a, b). Male patients with hypopharyngeal SCC had significantly higher positivity for EGFR protein expression than female patients ($P = 0.0169$) (Fig. 5c), but there was no significant difference in the intensity of EGFR expression ($P = 0.0828$) (Fig. 5d).

EGFR expression and clinicopathological factors in hypopharyngeal SCC

As shown in Fig. 4a, patients with hypopharyngeal SCC had the worst prognosis among hypopharyngeal, oropharyngeal, and laryngeal cancer. When we examined the relationship between EGFR protein expression and various clinicopathological factors, patients with lower EGFR positivity had the worst prognosis (Fig. 6a, b), but there was no significant correlation between the positivity or intensity of EGFR

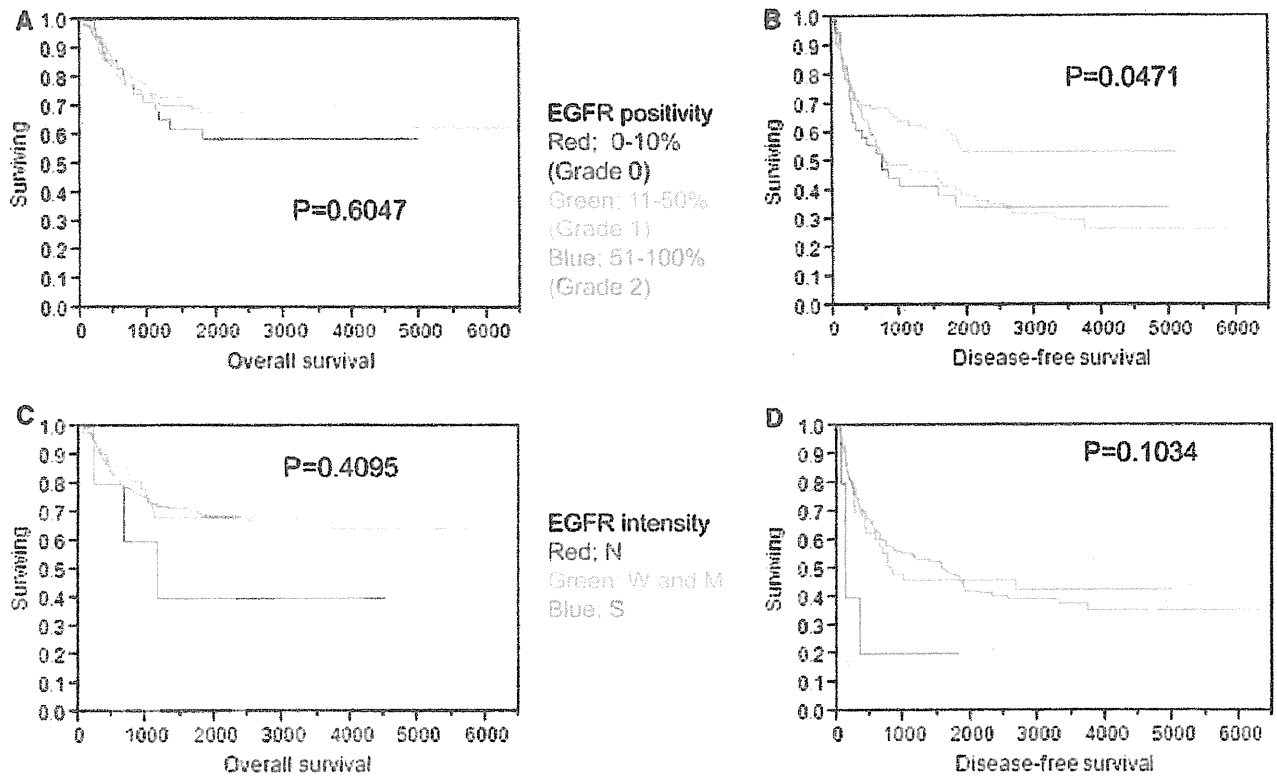


Fig. 2 Relationship between positive rate or intensity of EGFR protein expression and OS or DFS. Immunohistochemical analysis of SCCHNs using epitope-specific antibody for EGFR: **a** positive rate versus OS, **b** positive rate versus DFS, **c** intensity versus OS, **d** intensity versus DFS. *N* negative, *W* weak, *M* moderate, *S* strong

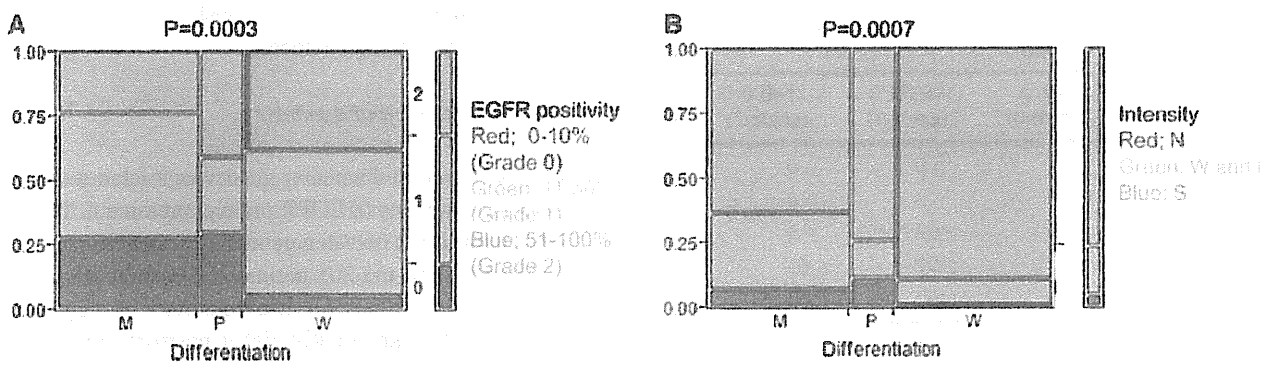


Fig. 3 Relationship between positive rate or intensity of EGFR protein expression and differentiation of SCCHNs. **a** EGFR positive rate versus tumor differentiation. *W* well differentiated, *M* moderately differentiated, *P* poorly differentiated. **b** EGFR intensity versus tumor differentiation. *N* negative, *W* weak, *M* moderate, *S* strong

expression and either OS or DFS (Fig. 6a–d). Multivariate analysis (EGFR positivity, EGFR intensity, gender, tumor, and nodal status) showed that lymph node metastasis was an independent predictor of OS and DFS (Tables 4, 5).

Discussion

The association between members of the EGFR expression or *KRAS* gene mutation-driven pathway and the clinical outcome has been extensively investigated mainly as a predictor of prognoses and sensitivity to cetuximab in CRC. However, the results obtained have been often controversial and not easily applicable in clinical practice. Data obtained for CRC have suggested the association between a worse prognosis of patients and abnormal expression of EGFR, but the findings have not shown reproducible results across different studies [26]. The omission into the analysis of other known factors with an established prognostic role, the small number of patients

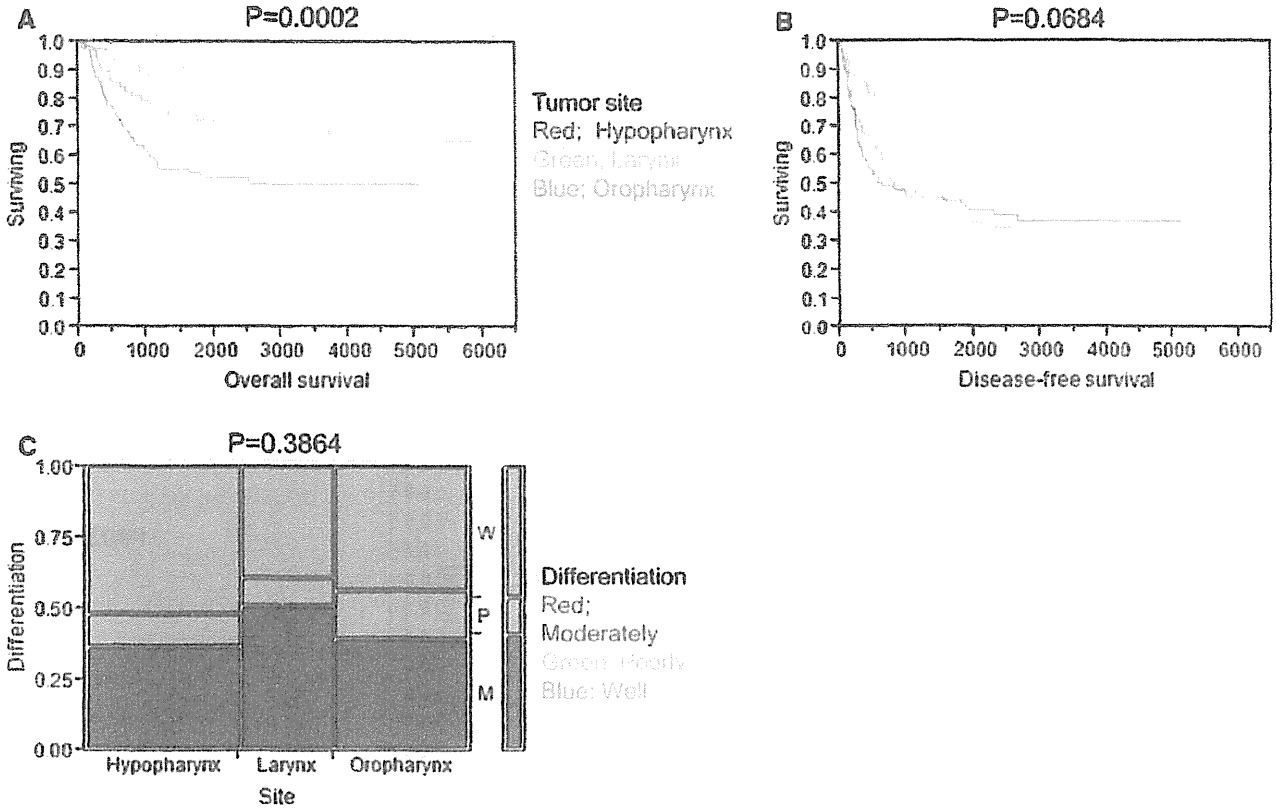


Fig. 4 Relationship between tumor site and OS or DFS (a, b), and relationship between tumor differentiation and sites (c)

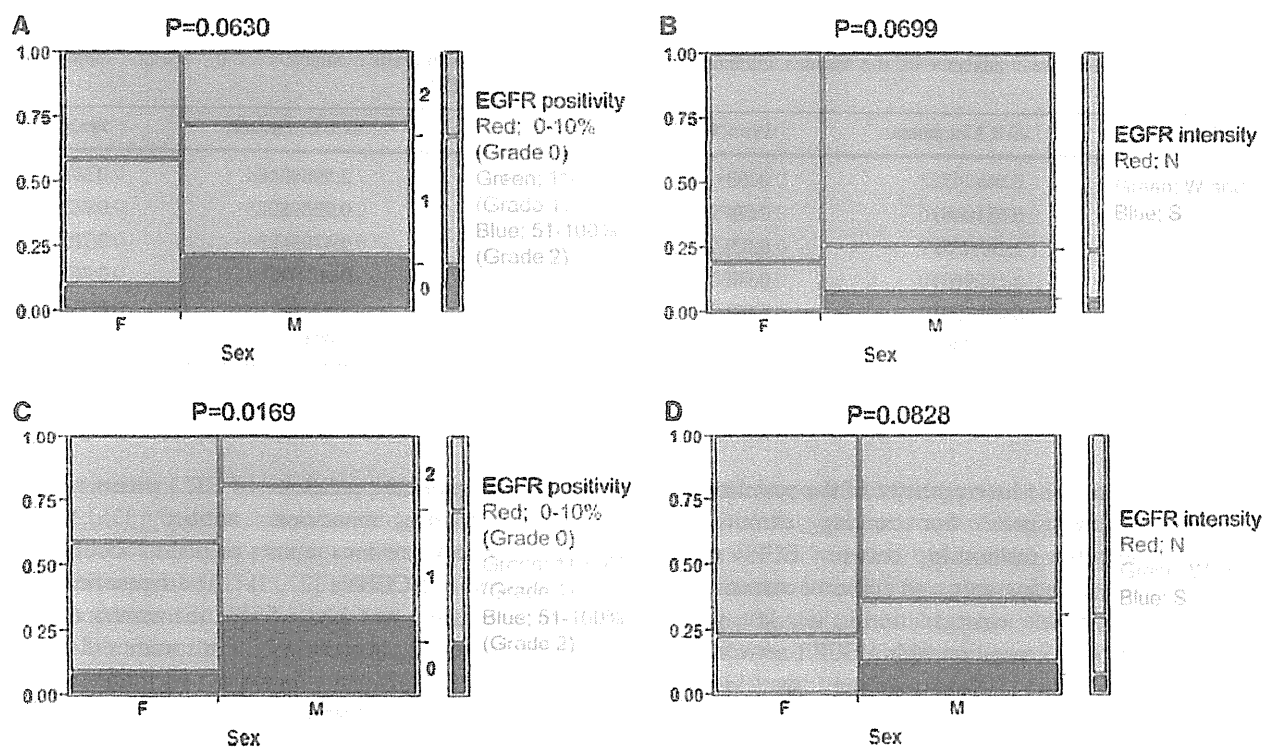


Fig. 5 Relationship between positive rate or intensity of EGFR protein expression and gender. a, b All cases c, d hypopharyngeal cases. *N* negative, *W* weak, *M* moderate, *S* strong

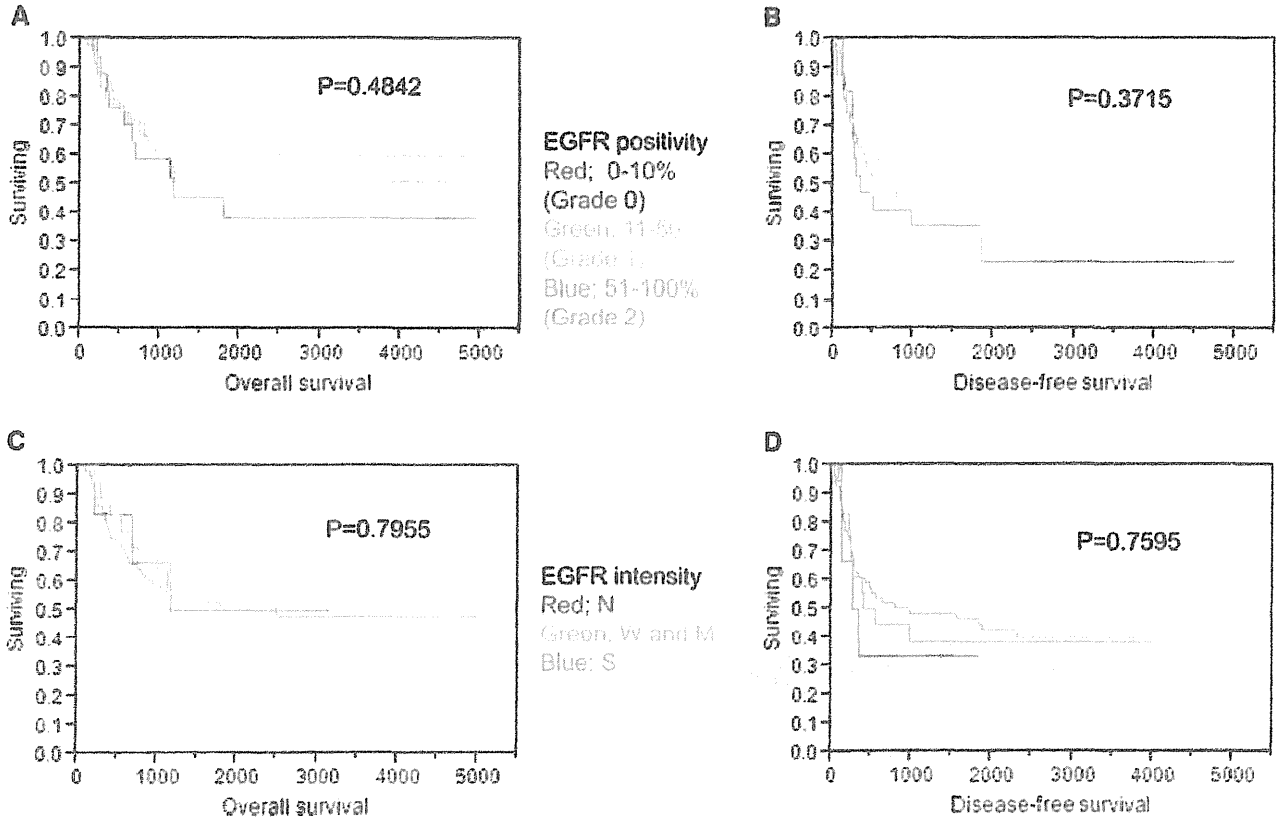


Fig. 6 Relationship between positive rate or intensity of EGFR protein expression and OS or DFS in hypopharyngeal cases. **a** Positive rate versus OS, **b** positive rate versus DFS, **c** intensity versus OS, **d** intensity versus DFS. *N* negative, *W* weak, *M* moderate, *S* strong

Table 4 Multivariate analysis of the various clinicopathological factors for OS

Source	L-R Chi square	Prob > Chi square
Positivity	2.64814728	0.2660
Intensity	0.58104816	0.7479
Diff	1.26395599	0.5315
Sex	0.18296970	0.6688
T	0.85633419	0.8360
N	8.54583035	0.0139*

Diff differentiation

* $P < 0.05$

Table 5 Multivariate analysis of the various clinicopathological factors for DFS

Source	L-R Chi square	Prob > Chi square
Positivity	2.59842484	0.2727
Intensity	0.03598025	0.9822
Diff	0.49056834	0.7825
Sex	0.06567847	0.7977
T	0.79049711	0.8517
N	14.0217666	0.0009*

Diff differentiation

* $P < 0.01$

examined, and the heterogeneity of the population studied prevented investigators from reaching definitive conclusions about the relationship between EGFR expression, *KRAS* gene mutation status, and clinical outcome. A strong scientific rationale emerged during the last years for a position of *KRAS* mutation within EGFR networks in CRC. Preclinical findings have suggested that blocking the EGFR-activated downstream signaling network in presence of *KRAS* gene mutation may result in an ineffective therapeutic outcome. This scientific rationale has been postulated for CRC. *KRAS* gene status has largely been known to be an appropriate predictor of CRC patients treated with EGFR-targeting monoclonal antibody [26]. There have been several previous reports published about KRAS gene mutation of SCCHNs [27, 28]. The frequencies of KRAS gene mutation was 6 and 4.5 %, the numbers of SCCHNs were 89 and 22, respectively. Both studies did not include Japanese SCCHNs, the tumor site was limited to tonsil in the assay of Van Damme et al. [28]. In Japanese cohort study, 2 % of SCCHNs showed KRAS gene mutation (codon 12) [29]. However, the number of case was 102 and the sites of SCCHNs were unknown in detail [29]. Thus,

data about the prevalance of *KRAS* mutation has not been published in a large number of SCCHN patients, there has been no report about the data in more than 200 of Japanese SCCHNs like our study. For the ethnic difference, further studies using a huge number of cases are necessary. In present study, we found that all 205 SCCHNs had *KRAS* wild type and may be identified as candidates for responders to EGFR-targeting monoclonal antibody.

The value of EGFR expression for predicting the response to EGFR-targeting monoclonal antibody such as cetuximab has remained unclear. A recent study showed that EGFR expression [fluorescence in situ hybridization (FISH) and immunohistochemistry] was useful for determining resistance to anti-EGFR therapies [30]. The author stated that FISH and CISH *EGFR* gene copy number may both represent effective tools for a further patients' selection in *KRAS* wild-type CRC treated with cetuximab [22]. EGFR amplification detected by FISH and EGFR protein expression detected by immunohistochemistry have also been observed in SCCHN [31]. However, their relationship with clinical response to treatment with anti-EGFR monoclonal anti-bodies has not been examined for Japanese SCCHN patients. EGFR protein expression by immunohistochemistry was heterogeneous in almost all of the SCCHNs investigated in the current study. It remains unclear if all SCCHNs have an aberrant stimulation of EGFR pathway deriving from heterogeneous expression of EGFR. A study published has suggested that there is no association between the EGFR expression and clinical response to EGFR inhibitor in SCCHN [32]. Further analyses are necessary to find a key regulator for the responder or non-responder of EGFR targeted therapy.

It has also been remained unclear whether heterogeneous EGFR expression observed in SCCHNs represents a stronger determinant for the biological behavior of the tumor. There have been no reports about the prognostic significance of heterogeneous EGFR expression detected by immunohistochemistry in a large series of SCCHNs, except for the present study involving tumors in the larynx, oropharynx and hypopharynx. In the two previous studies on the relationship between EGFR expression and the prognosis of SCCHN [22, 28], only 65 and 71 patients were examined, respectively. Also SCC of oral cavity and metastatic SCC were included and the small number of SCC of the larynx, oropharynx and hypopharynx were examined in these studies. One of the two reports indicated that EGFR expression was not significantly related to DFS or OS [22], while the other study showed that EGFR protein expression with high intensity was associated with a significantly worse survival, although interestingly increased expression of phospho-EGFR protein (activated EGFR expression) showed a tendency for better survival compared with patients with normal or decreased EGFR activation [28]. It has also been reported that the OS and DFS rates of patients with high EGFR-expressing SCCHNs were highly significant lower and local–regional relapse rate was highly significantly higher compared with those of patients with low EGFR-expressing HNSCCs in the most of cases with stage III or IV [33]. However, none of these reports examined the relationship between the percentages of positive area of EGFR protein expression which reflects heterogeneity of tumor, and OS or DFS such as performed in the present study. We found that a higher positive rate of EGFR protein expression was significantly associated with better DFS ($P = 0.0471$), and more intense EGFR protein expression tended to be associated with better DFS ($P = 0.1034$) (Fig. 2b, d). Both higher positive rate and stronger intensity of EGFR protein expression were significantly associated with well differentiated SCC ($P = 0.0003$ and $P = 0.0007$, respectively) (Fig. 3a, b). These results suggest that well differentiated SCCHN with higher positive rate and stronger intensity EGFR protein expression may be a good target population for anti-EGFR antibody therapies. However, further studies are necessary to investigate the efficacy of EGFR targeted therapy for these patients. On the other hand, the patients with lower positive rate of EGFR protein expression showed a significantly poorer DFS ($P = 0.0471$) and the cases with weaker intensity of EGFR protein expression showed a tendency for poorer DFS ($P = 0.1034$) (Fig. 2b, d). Both lower positive rate and weaker intensity of EGFR protein expression were associated with a significantly poorly differentiated SCC ($P = 0.0003$ and $P = 0.0007$, respectively) (Fig. 3a, b). However, these findings should be considered with caution and further studies including novel anti-cancer therapies to confirm them are needed in the future.

This study included 70 women with SCCHN. Interestingly, females with SCCHN tended to higher positive rate or stronger intensity of EGFR protein expression compared with those of males ($P = 0.0630$ and 0.0699, respectively) (Fig. 5a, b).

In conclusion, this is the first investigation of *KRAS* gene mutation and EGFR protein expression in more than 200 Japanese patients with SCCHN. No *KRAS* gene mutation was detected and only five of the 205 patients (2.4 %) were negativity for EGFR protein expression. This investigation is necessary and primary work before treatment with anti-EGFR antibody therapies to SCCHN in Japan. On the basis of our findings, most Japanese patients with SCCHN will be a good target for anti-EGFR antibody therapies such as cetuximab.

Acknowledgments We thank Mr. Shinya Yanagi, Mr. Takuya Aiba and Ms. Mari Takahashi for preparing tissue thin sections for genomic DNA extraction and immunohistochemistry, Mr. Masaya Mizushima, Mr. Yoshiteru Ishikawa and Ms. Naomi Komatsuzaki from Merck Serono Co., Ltd. for secretarial assistance.

Conflict of interest This work was supported by a research grant from MerckSerono Co. and reviewed by Merck Serono.

References

1. Ferlay J, Shin HR, Bray F et al (2010) Estimates of worldwide burden of cancer in 2008. GLOBOCAN 2008. Int J Cancer 127:2893–2917
2. Diaz Jr EM, Sturges EM, Laramore GE (2003) Neoplasms of the head and neck. Holland JF, Frei E (eds) Cancer Medicine. BC Decker Inc., London, pp 1325–1371
3. Ferlay J, Parkin DM, Steliarova-Foucher E (2010) Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer 46:765–781
4. Matsuda T, Marugame T, Kamo KI et al (2011) The Japan Cancer Surveillance Research Group. Cancer incidence and incidence rates in Japan in 2005: Based on data from 12 population-based cancer registries in the Monitoring of Cancer Incidence in Japan (MCIJ) Project. Jpn J Clin Oncol 41:139–147
5. The Research Group for Population-based Cancer Registration in Japan (2004) Cancer incidence and incidence rates in Japan in 1999: estimates based on data from 11 population-based cancer registries. Jpn J Clin Oncol 34(6):352
6. Ohshima A et al (2004) Cancer statistics morbidity/death/prognosis 2004. Shinohara Shuppan Shinsha, Tokyo
7. Iro H, Waldfahrer F (1998) Evaluation of the newly updated TNM classification of head and neck carcinoma with data from 3247 patients. Cancer 83:2201–2207
8. Vokes E, Weichselbaum R, Lippman S et al (1993) Head and neck cancer. New Engl J Med 328:184–194
9. Argiris A, Bruce E (2004) Competing causes of death and second primary tumors in patients with locoregionally advanced head and neck cancer treated with chemotherapy. Clin Cancer Res 10:1956–1962
10. Garrett CR, Eng C (2011) Cetuximab in the treatment of patients with colorectal cancer. Expert Opin Biol Ther 11(7):937–949
11. Jonker DJ, O'Callaghan CJ, Karapetis CS et al (2007) Cetuximab for the treatment of colorectal cancer. N Engl J Med 357(20): 2040–2048
12. Karapetis CS, Khambata-Ford S, Jonker DJ et al (2008) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 359(17):1757–1765
13. Carpenter G (1983) The biochemistry and physiology of the receptor-kinase for epidermal growth factor. Mol Cell Endocrinol 31:1–19
14. Salomon DS, Brandt R, Ciardiello F et al (1995) Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 19:183–232
15. Mendelsohn J, Baselga J (2003) Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol 21:2787–2799
16. Mendelsohn J, Baselga J (2006) Epidermal growth factor receptor targeting in cancer. Semin Oncol 33(4):369–385
17. Lockhart AC, Berlin JD (2005) The epidermal growth factor receptor as a target for colorectal cancer therapy. Semin Oncol 32(1):52–60
18. Steele RJ, Kelly P, Ellul B et al (1990) Epidermal growth factor receptor expression in colorectal cancer. Br J Surg 77(12):1352–1354
19. Kluftinger AM, Robinson BW, Quenville NF et al (1992) Correlation of epidermal growth factor receptor and c-erbB2 oncogene product to known prognostic indicators of colorectal cancer. Surg Oncol 1(1):97–105
20. Chung CH, Ely K, McGavran L et al (2006) Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol 24:4170–4176
21. Cunningham D, Humblet Y, Siena S et al (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 351(4): 337–345
22. Sheikh Ali MA, Gunduz M, Nagatsuka H et al (2008) Expression and mutation analysis of epidermal growth factor receptor in head and neck squamous cell carcinoma. Cancer Sci 99(8):1589–1594
23. Karapetis CS, Khambata-Ford S, Jonker DJ et al (2004) K-ras mutations and benefit from cetuximab in advanced colorectal cancer. J Histochem Cytochem 52(7):893–901
24. Atkins D, Reiffen KA, Tegtmeier CL et al (2008) Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: variation in staining intensity due to choice of fixative and storage time of tissue sections. J Histochem Cytochem 52(7):893–901
25. Japanese Society of Medical Oncology (2009) Clinical oncology update-essentials for medical oncologists, 2nd edn. Nankodo, Tokyo
26. Dahabreh IJ, Terasawa T, Castaldi PJ et al (2011) Systematic review: anti-epidermal growth factor receptor treatment effect. Ann Intern Med 154(1):37–49
27. Weber A, Langhanki L, Sommerer F et al (2003) Mutations of the *BRAF* gene in squamous cell carcinoma of the head and neck. Oncogene 22(30):4757–4759
28. Van Damme N, Deron P, Van Roy N et al (2010) Epidermal growth factor receptor and K-RAS status in two cohorts of squamous cell carcinomas 10:189
29. Hama T, Kato T (2010) Molecular targeted therapy for EGFR and VEGF in head and neck squamous cell carcinomas. Toukeibu-Gan 36(4):436–441
30. Scartozzi M, Bearzi I, Mandolesi A et al (2009) Epidermal growth factor receptor (EGFR) gene copy number (GCN) correlates with clinical activity of irinotecan-cetuximab in K-RAS wild-type colorectal cancer: a fluorescence in situ (FISH) and chromogenic in situ hybridization (CISH) analysis. BMC Cancer 9:303
31. Szabó B, Nelhűbel GA, Kárpáti A et al (2011) Clinical significance of genetic alterations and expression of epidermal growth factor receptor (EGFR) in head and neck squamous cell carcinomas. Oral Oncol 47(6):487–496
32. Sharafinski ME, Ferris RL, Ferrone S et al (2010) Epidermal growth factor receptor targeted therapy of squamous cell carcinoma of the head and neck. Head Neck 32(10):1412–1421
33. Ang KK, Berkey BA, Tu X et al (2002) Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Res 62(24):7350–7356

Breast Cancer Res Treat (2013) 138:817–827
DOI 10.1007/s10549-013-2468-3

CLINICAL TRIAL

International multicenter tool to predict the risk of four or more tumor-positive axillary lymph nodes in breast cancer patients with sentinel node macrometastases

Tuomo J. Meretoja · R. A. Audisio · P. S. Heikkilä · R. Bori · I. Sejben · P. Regitnig · G. Luschin-Ebengreuth · J. Zgajnar · A. Perhavec · B. Gazic · G. Lázár · T. Takács · B. Kővári · Z. A. Saidan · R. M. Nadeem · I. Castellano · A. Sapino · S. Bianchi · V. Vezzosi · E. Barranger · R. Lousquy · R. Arisio · M. P. Foschini · S. Imoto · H. Kamma · T. F. Tvedskov · M.-B. Jensen · G. Cserni · M. H. K. Leidenius

Received: 2 January 2013 / Accepted: 25 February 2013 / Published online: 5 April 2013

Abstract Recently, many centers have omitted routine axillary lymph node dissection (ALND) after metastatic sentinel node biopsy in breast cancer due to a growing body of literature. However, existing guidelines of adjuvant treatment planning are strongly based on axillary nodal stage. In this study, we aim to develop a novel international multicenter predictive tool to estimate a patient-specific risk of having four or more tumor-positive axillary lymph nodes (ALN) in patients with macrometastatic sentinel node(s) (SN). A series of 675 patients with macrometastatic SN and completion ALND from five European centers were analyzed by logistic regression analysis. A multivariate predictive model was created and validated internally by 367 additional patients and then externally by 760 additional patients from eight different centers. All statistical tests were two-sided. Prevalence of four or more tumor-positive ALN in each center's series ($P = 0.010$), number of metastatic SNs ($P < 0.0001$), number of negative SNs ($P = 0.003$), histological size of the primary tumor ($P = 0.020$), and extra-capsular extension of

T. J. Meretoja (✉) · M. H. K. Leidenius
Breast Surgery Unit, Helsinki University Central Hospital, P.O. Box 140, 00029 HUS Helsinki, Finland
e-mail: tuomo.meretoja@fimnet.fi

R. A. Audisio
Department of Surgery, St Helens Teaching Hospital, St Helens, UK

P. S. Heikkilä
Department of Pathology, Helsinki University Central Hospital, Helsinki, Finland

R. Bori · I. Sejben · G. Cserni
Department of Pathology, Bács-Kiskun County Teaching Hospital, Kecskemet, Hungary

P. Regitnig
Department of Pathology, Medical University of Graz, Graz, Austria

G. Luschin-Ebengreuth
Department of Obstetrics and Gynecology, Medical University of Graz, Graz, Austria

J. Zgajnar · A. Perhavec
Department of Surgical Oncology, Institute of Oncology, Ljubljana , Slovenia

B. Gazic
Department of Pathology, Institute of Oncology, Ljubljana, Slovenia

G. Lázár · T. Takács
Department of Surgery, University of Szeged, Szeged, Hungary

B. Kővári · G. Cserni
Department of Pathology, University of Szeged, Szeged, Hungary

Z. A. Saidan · R. M. Nadeem
Department of Breast Surgery, Lancashire Teaching Hospitals, Chorley, UK

I. Castellano · A. Sapino
Breast Unit Azienda Ospedaliera Città della Salute e della Scienza of Torino, Department of Medical Sciences, University of Torino, Turin, Italy

S. Bianchi · V. Vezzosi
Section of Pathological Anatomy, Department of Medical and Surgical Critical Care, University of Florence, Florence, Italy

E. Barranger · R. Lousquy
Department of Gynecology and Obstetrics, Lariboisiere Hospital, Paris , France

SN metastasis ($P < 0.0001$) were included in the predictive model. The model's area under the receiver operating characteristics curve was 0.766 in the internal validation and 0.774 in external validation. Our novel international multicenter-based predictive tool reliably estimates the risk of four or more axillary metastases after identifying macrometastatic SN(s) in breast cancer. Our tool performs well in internal and external validation, but needs to be further validated in each center before application to clinical use.

Keywords Breast cancer · Sentinel node biopsy · Axillary lymph node dissection · Tumor staging

Introduction

Completion axillary lymph node dissection (ALND) has been the standard treatment in the management of breast cancer patients with a tumor-positive sentinel node (SN). Completion ALND reveals the number of metastatic as well as tumor-free axillary lymph nodes (ALNs) and thus provides prognostic information beyond the SN status [1, 2]. However, patients receiving ALND are exposed to an increased morbidity when compared with sole SN biopsy (SNB). [3, 4]

During recent years, many centers have modified their axillary treatment protocols due to a growing body of literature indicating that completion ALND after the identification of a metastatic SN might neither provide survival benefit nor reduce axillary recurrence rates [5–8]. Although the debate generated by the ACOSOG Z0011 trial is still ongoing, many centers have opted for the omission of ALND after the detection of metastatic SNs [9]. Nonetheless, omitting completion ALND after positive SNB leads to less accurate axillary staging, as it remains unknown whether and how many additional ALN metastases remain in the axilla.

The existing guidelines of adjuvant treatment planning of breast cancer are largely based on axillary nodal stage as the main prognostic factor. The American Society of Clinical Oncology (ASCO) and the European Society for Medical Oncology (ESMO) both recommend postmastectomy radiotherapy (PMRT) in the presence of four or more metastatic ALNs (pN2 disease) [10, 11]. This has also implications on the choice of immediate breast reconstruction, since several surgeons and radiation oncologists do not recommend immediate reconstruction if PMRT is likely to be required. Furthermore, the ESMO guidelines recommend the inclusion of supraclavicular lymph nodes within the target radiotherapy field in the presence of pN2 disease, after mastectomy as well as breast-conserving surgery.

Therefore, the knowledge of both the total axillary tumor burden and the extent of residual axillary disease are important in case of one or more metastatic SN. To our knowledge, only three centers have developed predictive tools to estimate the risk of having four or more metastatic ALNs in the constellation of a tumor-positive SN. These were developed from relatively small series and were scarcely validated. Furthermore, these models included patients with micrometastatic disease in their SNs although these patients very rarely had four or more tumor-positive ALNs [12–15].

In this study, we aim to develop a novel international multicenter predictive tool to estimate a patient-specific risk of having four or more tumor-positive ALN in patients with macrometastatic SN. We thus aim to examine whether it is possible to recognize patients with macrometastatic SN(s) who are at risk of a substantial axillary tumor burden and who could therefore benefit from completion ALND. We further aim to validate the predictive tool both internally and externally.

Patients and methods

Original patient series

Retrospective data was collected in five European centers, each on 200 consecutive women with invasive breast cancer and one or more tumor-positive SNs and a completion ALND. Altogether a series of 1,000 patients was surgically treated between January 2004 and January 2011. Patients who received neoadjuvant treatment or previous axillary surgery were excluded. This data was originally collected in order to assess how the differences in SNB procedures and

R. Arisio
Department of Pathology, O.I.R.M.-Sant'Anna Hospital, Turin, Italy

M. P. Foschini
Section of Anatomic Pathology at Bellaria Hospital, Department of Biomedical Sciences and Neuromotory Disorders, University of Bologna, Bologna, Italy

S. Imoto
Department of Breast Surgery, Kyorin University School of Medicine, Tokyo, Japan

H. Kamma
Department of Pathology, Kyorin University School of Medicine, Tokyo, Japan

T. F. Tvedskov
Department of Breast Surgery, Copenhagen University Hospital, Copenhagen, Denmark

M.-B. Jensen
Danish Breast Cancer Cooperative Group, Copenhagen University Hospital, Copenhagen, Denmark

pathology practices would impact the performance of existing predictive models for nonsentinel node involvement [16, 17].

Twelve patients had four tumor-positive SNs and were excluded from analysis. Patients with isolated tumor cells (ITC) or micrometastasis as the largest tumor-positive SN finding [18] were also excluded from the original 1,000 patient as their probability of having four or more tumor-positive ALNs was very low. 68 patients had ITC as their largest SN finding and only one (1.5 %) of them had four tumor-positive ALNs. Similarly 245 patients had micrometastatic SN finding and only seven (2.9 %) of them had four or more tumor-positive ALNs. The remaining 675 patients with macrometastases in their SNs were included in the analysis.

The centers who contributed to the collection of the original series are: Bács-Kiskun County Teaching Hospital, Kecskemét, Hungary; Helsinki University Central Hospital, Finland; Medical University of Graz, Austria; Institute of Oncology, Ljubljana, Slovenia and University of Szeged, Hungary.

The data gathered was based on known risk factors for additional axillary metastases in patients with tumor-positive SNs [19–33]. The collected data included both primary tumor and SN-specific variables (Table 1). The detection method of the SN metastases was categorized as: intraoperative (frozen section/imprint cytology), paraffin standard staining, serial sectioning or immunohistochemistry. Patient, tumor and lymph node characteristics of the original patient series are given in Table 1.

Breast and axillary surgery as well as pathological work-up of the primary tumors and the axillary specimen were conducted according to each center's own protocols [16].

Internal validation patients

Internal validation series included additional series of consecutive patients treated at the same centers with similar inclusion and exclusion criteria. Consequently, the surgical technique and the methods of pathological assessment were the same as in the original series. The internal validation series included 367 additional patients with macrometastatic SNs operated between 2003 and 2011.

External validation patients

The external validation was performed on consecutive patients from eight European centers plus one unit from Japan. Each center in the external validation provided consecutive series with similar inclusion and exclusion criteria to the original series. The performance of the predictive tool was examined separately for each external validation center in addition to pooled data; the number of patients included from each center was hence unrestricted. A total of 760 patients with macrometastatic SNs were collected to form the series for external validation. These patients received surgery between 2003 and 2011.

The centers contributing to the external validation were: Lariboisiere Hospital, Paris, France; Lancashire Teaching Hospitals, Chorley, UK; AziendaOspedalieraCittàdella Salute e dellaScienza of Torino, Italy; Careggi Hospital and University of Florence, Florence, Italy; Sant'Anna Hospital, Turin, Italy; Bellaria Hospital, University of Bologna, Bologna, Italy; Kyorin University Hospital, Tokyo, Japan; Copenhagen University Hospital, Copenhagen, Denmark.

Statistical analyses

The individual risk factors for four or more tumor-positive ALNs associated with a macrometastatic SN were examined by univariate analysis of the original series. Distribution of continuous variables was analyzed with the Mann–Whitney *U*-test and the Chi-squared test was used for categorical variables.

Variables with a P value of less than 0.05 in the univariate analysis were included into a binary logistic regression analysis using a backward stepwise likelihood ratio method. Variables with a P value of less than 0.05 were included in the final predictive model.

The subsequent multivariate predictive model was then validated first internally and then externally by the independent patient series. Discrimination of the model was evaluated by area under the receiver operating characteristic curve (AUC) and the calibration of the model by Hosmer–Lemeshow goodness-of-fit test. A cutoff value of less than 20 % risk of four or more metastatic ALNs was considered as low-risk. Sensitivity and specificity were determined for various risk estimates. All statistical tests were two-sided.

IBM® SPSS® Statistics Version 20 (SPSS Inc., Chicago, IL) software was used to conduct the statistical analyses.

Ethical considerations

The patients' treatment was not influenced by our study as the data was collected retrospectively and anonymously. Institutional review boards and ethical committees were consulted as required in each center with no ethical objections raised.

Results

130 (19.1 %) of the 675 patients had four or more tumor-positive ALNs after SNB and completion ALND. The proportion of patients with four or more tumor-positive ALNs varied between centers (from 11.4 to 25.0 %).

Table 1 Patient, tumor and lymph node characteristics in the original patient series of 675 patients

	Center A (Bács-Kiskun) $n = 135$	Center B (Helsinki) $n = 113$	Center C (Graz) $n = 124$	Center D (Ljubljana) $n = 137$	Center E (Szeged) $n = 166$
Patient age (years)					
Mean	58	58	58	57	56
Standard deviation	13	12	13	10	11
Histological size of the primary tumor (mm)					
Mean	20	21	18	22	22
Standard deviation	14	18	9	11	11
Multifocal primary tumor	43 (31.9 %)	30 (26.5 %)	14 (11.3 %)	38 (27.7 %)	18 (10.8 %)
Lymphovascular invasion in the primary tumor	56 (41.5 %)	34 (30.1 %)	35 (28.2 %)	57 (41.6 %)	46 (27.7 %)
Estrogen receptor positive	121 (89.6 %)	107 (94.7 %)	98 (79.0 %)	122 (89.1 %)	124 (74.7 %)
Progesterone receptor positive	108 (80.0 %)	85 (75.2 %)	92 (74.2 %)	107 (78.1 %)	120 (72.3 %)
HER-2 positive	13 (9.6 %)	10 (8.8 %)	15 (12.1 %)	11 (8.0 %)	29 (17.5 %)
Nuclear grade of the primary tumor					
I	10 (7.4 %)	9 (8.0 %)	25 (20.2 %)	4 (2.9 %)	10 (6.0 %)
II	52 (38.5 %)	66 (58.4 %)	50 (40.3 %)	84 (61.3 %)	64 (38.6 %)
III	73 (54.1 %)	38 (33.6 %)	49 (39.5 %)	49 (35.8 %)	92 (55.4 %)
Histological grade of the primary tumor					
I	34 (25.2 %)	25 (22.1 %)	14 (11.3 %)	18 (13.1 %)	18 (10.8 %)
II	55 (40.7 %)	56 (49.6 %)	46 (37.1 %)	73 (53.3 %)	83 (50.0 %)
III	46 (34.1 %)	32 (28.3 %)	64 (51.6 %)	46 (33.6 %)	65 (39.2 %)
Histology of the primary tumor					
Ductal	98 (72.6 %)	82 (72.6 %)	100 (80.6 %)	111 (81.0 %)	140 (84.3 %)
Lobular	12 (8.9 %)	22 (19.5 %)	6 (4.8 %)	17 (12.4 %)	12 (7.2 %)
Mixed	8 (5.9 %)	4 (3.5 %)	14 (11.3 %)	7 (5.1 %)	3 (1.8 %)
Other	17 (12.6 %)	5 (4.4 %)	4 (3.2 %)	2 (1.5 %)	11 (6.6 %)
Detection method of the sentinel node metastasis					
Intraoperative analysis	88 (65.2 %)	113 (100 %)	95 (76.6 %)	51 (37.2 %)	Not done
Paraffin standard staining	45 (33.3 %)	0	2 (1.6 %)	49 (35.8 %)	166 (100 %)
Paraffin immunohistochemistry	2 (1.5 %)	0	4 (3.2 %)	28 (20.4 %)	0
Serial sectioning	0	Not done	23 (18.5 %)	9 (6.6 %)	Not done
ECE of sentinel node metastasis present	77 (57.0 %)	52 (46.0 %)	36 (29.0 %)	46 (33.6 %)	34 (20.5 %)
Sentinel nodes harvested					
Mean	1.8	2.5	1.7	1.8	1.9
Standard deviation	0.9	1.4	1.0	0.9	1.0
Nonsentinel nodes harvested					
Mean	11.7	19.8	15.2	17.4	10.0
Standard deviation	4.8	6.3	6.0	6.3	4.6
Patients with four or more tumor-positive axillary lymph nodes	33 (24.4 %)	28 (24.8 %)	31 (25.0 %)	19 (13.9 %)	19 (11.4 %)

HER-2 human epidermal growth factor receptor 2, *ECE* extra-capsular extension

Univariate analysis comparing the two patient groups with less than four versus four or more tumor-positive ALNs is given in Table 2. Prevalence of four or more tumor-positive ALNs in each center's series, histological tumor size, multifocality of the primary tumor, lymphovascular invasion of the primary tumor, histological type of the primary tumor, SN metastasis detection method, extracapsular extension (ECE) of the SN metastasis and number of positive and negative SNs all had a P value of less than 0.05 and were included in the multivariate analysis.

The prevalence of four or more tumor-positive ALNs in each center ($P = 0.010$), the number of tumor-positive SNs