A retrospective study was performed at the authors' institution to investigate the outcome of patients with sarcopenia who underwent hepatic resection for HCC. The outcome of these patients was compared with that of patients without sarcopenia undergoing hepatic resection during the same period.

Methods

All patients who underwent hepatic resection with curative intent as the initial treatment in the Department of Surgery II, Kyushu University Hospital, between January 2004 and December 2009 were enrolled in the study. Curative resection was defined as complete macroscopic removal of the tumour. All patients had preoperative computed tomography (CT). A transverse CT image at the third lumbar vertebra (L3) in the inferior direction was assessed from each scan. Skeletal muscle was identified and quantified by Hounsfield unit (HU) thresholds of −29 to +150 (water is defined as 0 HU, air as 1000 HU). Multiple muscles were quantified, including the psoas, erector spinae, quadratus lumborum, transversus abdominis, external and internal oblique abdominal muscle, and rectus abdominis muscle (*Fig. 1*). CT measurements were calibrated with water and air at fixed intervals. Cross-sectional areas (cm^2) of skeletal muscles in the L3 region were measured by manual outlining on the CT images, and checked by the radiologist. The cross-sectional areas were then normalized for height (cm^2/m^2).

Cut-off values for skeletal muscle associated with overall survival were defined as 43·75 cm^2/m^2 for men and 41·10 cm^2/m^2 for women[10]. Based on this cut-off, patients were assigned to one of two groups, depending on the presence or absence of sarcopenia. The clinicopathological background and rates of overall and recurrence-free survival were compared between the two groups.

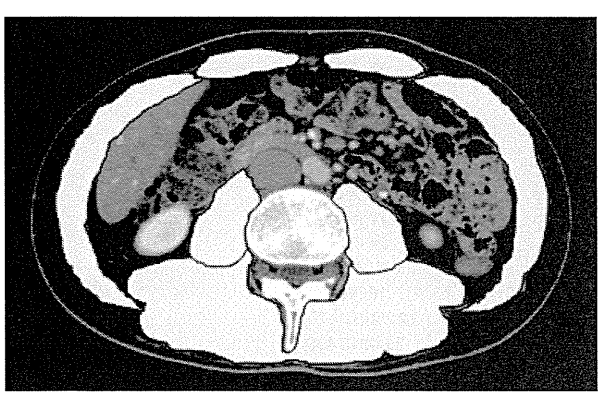


Fig. 1 Computed tomogram showing the area of skeletal muscle mass in the L3 region (highlighted yellow)

The prognostic factors were examined with respect to overall and recurrence-free survival on the basis of the following variables: sarcopenia (absence *versus* presence); skeletal muscle mass; age; sex (male *versus* female); body mass index (BMI); hepatitis B surface antigen (positive *versus* negative), hepatitis C virus antibody (positive *versus* negative); serum albumin level; serum total bilirubin level; serum aspartate aminotransferase level; platelet number; indocyanine green retention test at 15 min (ICGR15); Child–Pugh grade (A *versus* B); Model for End-Stage Liver Disease (MELD) score; histological liver cirrhosis (normal liver + chronic hepatitis *versus* liver fibrosis and liver cirrhosis); tumour size; tumour number (solitary *versus* multiple); tumour node metastasis (TNM) stage according to the Liver Cancer Study Group of Japan[17] (I + II *versus* III + IV); tumour differentiation (well differentiated + moderately differentiated *versus* poorly differentiated); microvascular invasion (MVI) (absence *versus* presence); intrahepatic metastases (absence *versus* presence); serum α-fetoprotein level (AFP); des-γ-carboxyprothrombin (DCP) level; operative procedure (anatomical *versus* non-anatomical resection); duration of surgery; estimated blood loss; and postoperative complications (absence *versus* presence). Patients with diabetes were defined as those using an oral hypoglycaemic agent or insulin. The MELD score was calculated in accordance with a previous report[18]. Postoperative complications within 1 month after partial hepatectomy included liver failure, encephalopathy, gastrointestinal bleeding, intraperitoneal abscess, abdominal haemorrhage, bile leakage, pleural effusion, intractable ascites and wound infection. Complications were classified according to Clavien–Dindo[19]; grade III complications (those requiring surgical intervention) were considered to indicate the presence of a postoperative complication.

Surgical procedures

Details of surgical techniques and patient selection criteria have been reported previously[7]. Selection criteria for hepatic resection were: ascites not detected, or controllable by diuretics; serum total bilirubin level lower than 2·0 mg/ml; and ICGR15 value below 40 per cent. The surgical approach included a J-shaped incision for routine abdominal access, hepatic dissection using an ultrasonic dissector with a coagulator (CUSA EXcel®; Integra, Plainsboro, New Jersey, USA), with systematic ligation of all sizable vessels, and close ultrasonographic guidance along the transection line. Cholecystectomy was performed

in all patients if applicable. An intraoperative bile leak test was performed routinely[20]. Small bile leaks on the cut liver surface were repaired by Z-suturing with 6-0 polydioxanone (PDS II; Johnson and Johnson, Tokyo, Japan). Intraoperative vascular control was achieved with the Pringle manoeuvre[21].

Follow-up strategy and recurrence pattern

After discharge, all patients were examined monthly for recurrence by ultrasonography and estimation of tumour markers, such as AFP and DCP, and by CT every 6 months. When recurrence was suspected, additional examinations such as hepatic angiography were performed. Recurrent HCC was treated by repeat hepatectomy, ablation therapy and lipiodolization, as described previously[22].

Histological assessment

All resected specimens were cut into serial 5–10-mm thick slices and fixed in 10 per cent formalin. After macroscopic examination, the slice with the greatest dimensions was trimmed for embedding in paraffin and cut into 4-μm microscopic sections. The sections were stained with haematoxylin and eosin. Tumour differentiation, MVI, intrahepatic metastases and histological liver cirrhosis were assessed by the pathologist in accordance with the rules of the Liver Cancer Study Group of Japan[17].

Table 1 Clinicopathological factors in patients with, and without sarcopenia

	Sarcopenia ($n = 75$)	No sarcopenia ($n = 111$)	P†
Age (years)	67(11)	66(10)	0·553
Sex ratio (M : F)	50 : 25	95 : 16	0·004‡
Skeletal muscle mass (cm^2/m^2)	37·8(3·7)	49·7(6·5)	<0·001
Body mass index (kg/m^2)	20·5(2·4)	24·0(2·8)	<0·001
Diabetes mellitus	22 (29)	35 (31·6)	0·999‡
Albumin (g/dl)	3·8(0·4)	4·0(0·4)	0·002
Total bilirubin (mg/dl)	0·9(0·4)	0·8(0·3)	0·096
Platelet count ($\times 10^4/\mu l$)	15·5(7·5)	16·3(6·2)	0·454
ICGR15 (%)	15·7(8·2)	13·6(6·2)	0·049
Child–Pugh grade			0·190‡
A	68 (91)	107 (96·4)	
B	7 (9)	4 (3·6)	
MELD score	7·7(2·1)	7·9(1·8)	0·591
Hepatitis grade			0·652‡
None	11 (15)	13 (11·7)	
Mild	55 (73)	80 (72·1)	
Severe	9 (12)	18 (16·2)	
Liver cirrhosis			0·290‡
Normal liver + chronic hepatitis	32 (43)	55 (49·5)	
Liver fibrosis + liver cirrhosis	43 (57)	56 (50·5)	
Tumour size (cm)	4·0(3·2)	3·9(2·8)	0·770
No. of tumours			0·171‡
Solitary	52 (69)	88 (79·3)	
Multiple	23 (31)	23 (20·7)	
TNM stage			0·967‡
I	11 (15)	18 (16·2)	
II	38 (51)	57 (51·4)	
III	20 (27)	29 (26·1)	
IV	6 (8)	7 (6·3)	
Differentiation of HCC			0·690‡
Well	9 (12)	10 (9·0)	
Moderate	50 (67)	77 (69·4)	
Poor	16 (21)	24 (21·6)	
Microvascular invasion	24 (32)	37 (33·3)	0·890‡
Intrahepatic metastases	12 (16)	18 (16·2)	0·978‡
α-Fetoprotein level (ng/ml)	3459(18 300)	12 250(70 470)	0·297
DCP (munits/l)	4318(13 627)	2942(12 499)	0·480
Postoperative complications	24 (32)	56 (50·5)	0·613‡

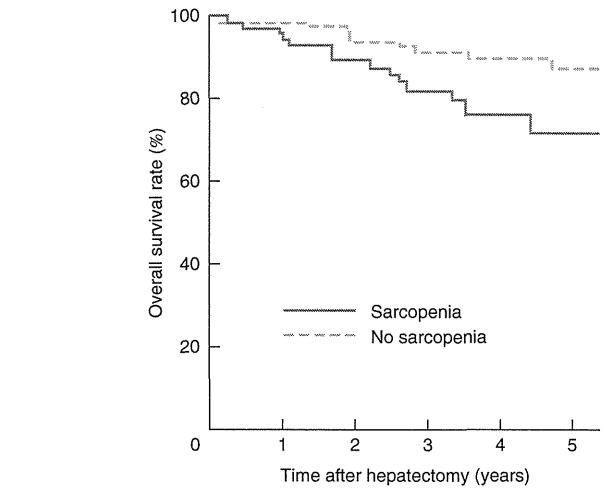
Values are mean(s.d.) unless indicated otherwise: *values in parentheses are percentages. ICGR15, indocyanine green dye retention test at 15 min; MELD, Model for End-Stage Liver Disease; TNM, tumour node metastasis (stage defined by the Liver Cancer Study Group of Japan); HCC, hepatocellular carcinoma; DCP, des-γ-carboxyprothrombin. †Mann–Whitney U test, except ‡Fisher's exact test or χ^2 test.

Statistical analysis

Associations of continuous and categorical variables with relevant outcome variables were assessed using the Mann–Whitney *U* test and Fisher's exact test respectively. The variable skeletal muscle was not *a priori* categorized into a binary variable (sarcopenia present or not), because categorizing a continuous predictor would result in an inevitable loss of information. Instead, the multivariable fractional polynomial (MFP) approach was adopted. In the polynomial fractional model, for each continuous variable X, one or two terms of the form X^p were fitted with powers, p, chosen from (-2, -1, $-0{\cdot}5$, 0, 0·5, 1, 2 and 3). The results of the MFP analysis revealed that the most appropriate power for skeletal muscle mass in the MFP model was given in the form of X (that is, $p = 1$), allowing expression of a final multivariable model in terms of the usual Cox regression model. Therefore, the results of the usual Cox model are reported here, giving the results of the log rank tests for the association between the presence of sarcopenia (as defined by dichotomizing skeletal muscle mass) and overall or disease-free survival[23]. To identify prognostic factors after hepatectomy, all variables were included in the overall multivariable Cox proportional model in the analyses of both overall and recurrence-free survival using the backward selection method. The overall and recurrence-free survival curves were analysed by the Kaplan-Meier method and compared with the log rank test. All analyses were performed with StatView® 5.0 software (Abacus Concepts, Berkeley, California, USA). $P < 0{\cdot}050$ was considered statistically significant.

Results

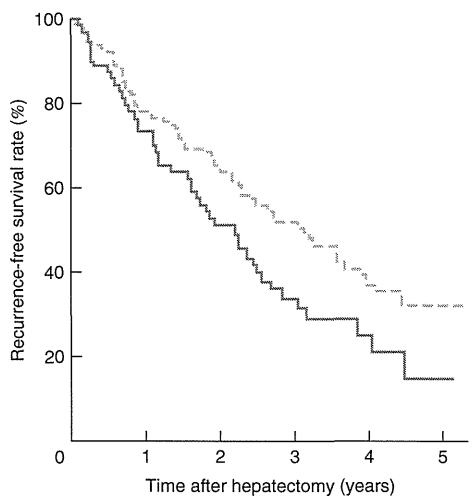
In total, 186 patients with HCC were identified from the database, of whom 75 (40·3 per cent; 50 men and 25 women) had sarcopenia. Clinicopathological characteristics of patients with and without sarcopenia are shown in *Table 1*. Women were more likely to have sarcopenia than men. Patients with sarcopenia had a significantly lower BMI than those without. Regarding liver function, serum albumin levels were significantly lower and ICGR15 values were significantly higher in patients with sarcopenia than in those without. Other host-related factors such as age, hepatitis, diabetes mellitus, Child–Pugh grade, MELD score and liver cirrhosis were not related to the presence of sarcopenia. There were no significant differences in tumour-related factors or surgical outcomes between the two groups. Operative details are shown in *Table S1* (supporting information).



No. at risk

Sarcopenia	75	66	53	35	23	12
No sarcopenia	111	102	84	64	50	35

a Overall survival



No. at risk

Sarcopenia	75	45	30	14	7	2
No sarcopenia	111	80	61	40	22	12

b Recurrence-free survival

Fig. 2 **a** Overall and **b** recurrence-free survival curves after liver resection in patients with, and without sarcopenia. **a** $P = 0{\cdot}001$, **b** $P = 0{\cdot}013$ (log rank test)

Overall and recurrence-free survival curves for patients with and without sarcopenia are shown in *Fig. 2*. Overall and recurrence-free 5-year survival rates were 71 and 13 per cent respectively in patients with sarcopenia, and 83·7 and 33·2 per cent in patients without sarcopenia (*Fig. 2*). Patients with sarcopenia had a significantly worse prognosis

Table 2 Univariable and multivariable analysis of clinicopathological factors and overall survival following partial hepatectomy with curative intent for hepatocellular carcinoma

	Univariable analysis		Multivariable analysis	
	Hazard ratio	*P**	Hazard ratio	*P*†
Age	1·02 (0·98, 1·07)	0·323		
Female sex	1·17 (0·42, 2·79)	0·746		
Skeletal muscle mass	0·92 (0·86, 0·97)	0·004	0·90 (0·84, 0·96)	0·002
Body mass index	0·92 (0·81, 1·04)	0·199		
Albumin	0·47 (0·21, 1·14)	0·092		
ICGR15	1·02 (0·97, 1·07)	0·512		
MELD score	1·08 (0·86, 1·25)	0·460		
Liver fibrosis + cirrhosis	3·97 (1·50, 13·67)	0·004		
Tumour size	1·10 (0·98, 1·22)	0·906		
Multiple tumours	1·60 (0·65, 3·64)	0·292		
TNM stage III + IV	1·62 (0·70, 3·62)	0·255		
Poor differentiation	2·26 (0·98, 5·16)	0·055	2·47 (1·05, 5·81)	0·021
Microvascular invasion	2·39 (1·05, 5·41)	0·038	3·21 (1·29, 7·94)	0·018
Intrahepatic metastases	1·67 (0·55, 4·15)	0·333		
α-Fetoprotein	1·00 (1·00, 1·00)	0·335		
DCP	1·00 (1·00, 1·00)	0·267		
Postoperative complications	2·76 (1·23, 6·28)	0·014	3·27 (1·39, 7·69)	0·007

Values in parentheses are 95 per cent confidence intervals. ICGR15, indocyanine green dye retention test at 15 min; MELD, Model for End-Stage Liver Disease; TNM, tumour node metastasis; DCP, des-γ-carboxyprothrombin. *Log rank test; †Cox proportional model.

Table 3 Univariable and multivariable analysis of clinicopathological factors and recurrence-free survival following partial hepatectomy with curative intent for hepatocellular carcinoma

	Univariable analysis		Multivariable analysis	
	Hazard ratio	*P**	Hazard ratio	*P*†
Age	1·01 (1·00, 1·04)	0·139		
Female sex	1·02 (0·63, 1·59)	0·918		
Skeletal muscle mass	0·98 (0·95, 1·00)	0·049	0·97 (0·95, 1·00)	0·016
Body mass index	0·94 (0·88, 1·02)	0·076		
Albumin	0·49 (0·33, 0·75)	0·001		
ICGR15	1·03 (1·01, 1·06)	0·048	1·02 (1·02, 1·07)	0·001
MELD score	1·03 (0·93, 1·12)	0·526		
Liver fibrosis + cirrhosis	1·98 (1·32, 3·01)	0·001		
Tumour size	1·00 (0·98, 1·11)	0·141		
Multiple tumours	1·89 (1·22, 2·84)	0·005		
TNM stage III + IV	2·44 (1·64, 3·61)	0·001	2·13 (1·38, 3·29)	0·001
Poor differentiation	1·58 (1·04, 2·35)	0·033		
Microvascular invasion	2·39 (1·05, 5·41)	0·038		
Intrahepatic metastases	2·14 (1·30, 3·38)	0·003	2·37 (1·38, 4·06)	0·018
α-Fetoprotein	1·00 (1·00, 1·00)	0·001		
DCP	1·00 (1·00, 1·00)	0·006	1·00 (1·00, 1·00)	0·001
Postoperative complications	1·11 (0·73, 1·67)	0·617		

Values in parentheses are 95 per cent confidence intervals. ICGR15, indocyanine green dye retention test at 15 min; MELD, Model for End-Stage Liver Disease; TNM, tumour node metastasis; DCP, des-γ-carboxyprothrombin. *Log rank test; †Cox proportional model.

than those without in terms of both overall ($P = 0·001$) and recurrence-free survival ($P = 0·013$).

In univariable analysis, significant prognostic factors for overall survival were low skeletal muscle mass, and presence of liver cirrhosis, MVI and postoperative complications (*Table 2*). Significant prognostic factors for recurrence-free survival were lower skeletal muscle mass, serum albumin level, liver cirrhosis, tumour number, tumour stage, poorly differentiated HCC, MVI, intrahepatic metastases, and serum AFP and DCP levels (*Table 3*). Multivariable analysis identified four poor prognostic factors (low skeletal muscle mass, poorly differentiated HCC, MVI and postoperative complications) that influenced overall survival, and five poor prognostic factors (low skeletal muscle mass, high

ICGR15 value, high serum DCP level, presence of intrahepatic metastases, and stage III + IV disease) that influenced recurrence-free survival (*Tables 2* and *3*).

Discussion

The findings of this retrospective single-centre study suggest that sarcopenia is an independent prognostic factor for overall and recurrence-free survival in patients with HCC following partial hepatectomy. The Child–Pugh classification was the first systematic and conventional approach used to determine the severity of cirrhosis and select patients who might tolerate hepatic resection. However, it is not always a reliable indicator of hepatic reserve, and has a limited role in predicting postoperative outcome[24]. The MELD score is a reliable measure of mortality risk in patients with end-stage liver disease and is suitable for use as a disease severity index to determine organ allocation priorities. No useful, objective, easily obtained and precise marker has yet been identified to evaluate the general condition of patients before hepatectomy. The ASA grade gives an estimation of organ disease and functional status, and has been suggested as a useful prognostic factor for preoperative patients with HCC[8]. However, it has been criticized for being subjective and imprecise[16].

Sarcopenia is defined as muscle mass two standard deviations below the mean in healthy young adults[25]. Although sarcopenia is associated with ageing, it can also develop as a consequence of chronic disease and malignancy. The European Working Group on Sarcopenia in Older People[15] recommended using the presence of both low muscle mass and low muscle function for the diagnosis of sarcopenia. However, muscle function is difficult to evaluate, and thus low muscle mass was investigated in the present study. There was no correlation between sarcopenia and age, but sarcopenia was significantly correlated with liver dysfunction as indicated by abnormal serum albumin levels and ICGR15 values, as well as with reduced BMI values. There was no correlation between sarcopenia and the Child–Pugh classification, MELD score or liver cirrhosis. There are some reports that serum albumin levels are decreased in patients with sarcopenia[26], which could be an early warning sign of subclinical conditions and impending disease and disability. Montano-Loza and colleagues[12] reported that, of patients with cirrhosis, those with sarcopenia had a significantly lower BMI than patients without sarcopenia. Liver cirrhosis was observed in 50 per cent of patients in their study, in line with the present findings. There is no report concerning the relationship between ICGR15 values and sarcopenia. In one study[12], skeletal muscle area was correlated with MELD score, which would seem to contradict the present findings; however, the mean MELD score was better in the present study, perhaps explaining these findings.

CT is the standard procedure for quantifying skeletal muscle mass, enabling objective and detailed nutritional and metabolic assessment of patients. Moreover, CT is always performed before hepatectomy, allowing precise assessment of sarcopenia. There are some reports that muscle mass as measured by CT is associated with the prognosis of sarcopenia.

It has been suggested previously that surgical outcomes are worse for obese patients[27]; however, there are few reports concerning the effect of being underweight on patient outcomes following hepatectomy for HCC. In this study, lower BMI was correlated with sarcopenia but not with the prognosis. BMI was significantly lower in sarcopenic patients, although only five patients were considered to be underweight (BMI below 18·5 kg/m^2). Thus, sarcopenia is not present exclusively in underweight patients.

The molecular mechanism of sarcopenia remains poorly understood. Skeletal muscle was recently identified as an endocrine organ[28]. It has therefore been suggested that cytokines and other peptides are produced, expressed and released by muscle fibres. For example, interleukin (IL) 6 is released from skeletal muscle[28], which may subsequently affect liver metabolism. Both the level and timing of IL-6 release appear to be determining factors for the biological effect in patients with liver fibrosis and HCC[28]. Furthermore, levels of insulin-like growth factor (IGF) 1, which plays a stimulatory role in the development and regulation of skeletal muscle mass[28], are decreased in patients with sarcopenia. In some reports, serum IGF-1 levels were significantly lower in patients with cirrhosis than in healthy subjects, and were correlated with the degree of liver dysfunction. Low serum IGF-1 levels were significantly correlated with advanced clinicopathological parameters, and indicative of poor overall survival in HCC[29]. IGF-1 is produced mainly by the liver, and it may be that serum IGF-1 levels are lower in patients with sarcopenia and that low IGF-1 levels promote the progression of HCC. Further study is needed to clarify the molecular mechanism concerning muscle–liver cross-talk.

It is important to note that, among the significant prognostic factors for overall survival, skeletal muscle mass can be evaluated before hepatectomy. Similarly, skeletal muscle mass, ICGR15, serum DCP level and stage can be evaluated before hepatectomy to prognosticate recurrence-free survival. The identification of patients with sarcopenia before hepatectomy might permit early

preventive strategies to maintain muscle mass, in order to improve prognosis and patient selection for hepatectomy. A recent study indicated that a late evening snack, as an intervention to reduce the fasting phase in patients with cirrhosis, has the potential to improve skeletal muscle proteolysis[30].

Disclosure

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Operative details in patients with hepatocellular carcinoma with, and without sarcopenia (Word document)

Snapshot quiz

Snapshot quiz 13/36

Answer: The computed tomography angiogram shows a large right popliteal aneurysm. The options for management are: radiological stenting using a covered stent; and a bypass procedure to exclude the aneurysm. The patient was managed with a bypass procedure from the superficial femoral artery to the below-knee popliteal artery using reversed saphenous vein. The aneurysm was ligated proximally and distally. This aneurysm was deemed unsuitable for radiological stenting owing to the tortuosity of the vessel. The right leg was swollen due to thrombosis of the popliteal vein caused by the pressure effect from the popliteal aneurysm. As this was at least 6 weeks old, the patient did not receive warfarin therapy.

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BMC Cancer

TECHNICAL ADVANCE Open Access

Simultaneous identification of 36 mutations in *KRAS* codons 61and 146, *BRAF*, *NRAS*, and *PIK3CA* in a single reaction by multiplex assay kit

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Abstract

Background: Retrospective analyses in the West suggest that mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* are negative predictive factors for cetuximab treatment in colorectal cancer patients. We developed a novel multiplex kit detecting 36 mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* using Luminex (xMAP) assay in a single reaction.

Methods: Tumor samples and clinical data from Asian colorectal cancer patients treated with cetuximab were collected. We investigated *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations using both the multiplex kit and direct sequencing methods, and evaluated the concordance between the 2 methods. Objective response, progression-free survival (PFS), and overall survival (OS) were also evaluated according to mutational status.

Results: In total, 82 of 83 samples (78 surgically resected specimens and 5 biopsy specimens) were analyzed using both methods. All multiplex assays were performed using 50 ng of template DNA. The concordance rate between the methods was 100%. Overall, 49 (59.8%) patients had all wild-type tumors, 21 (25.6%) had tumors harboring *KRAS* codon 12 or 13 mutations, and 12 (14.6%) had tumors harboring *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations. The response rates in these patient groups were 38.8%, 4.8%, and 0%, respectively. Median PFS in these groups was 6.1 months (95% confidence interval (CI): 3.1–9.2), 2.7 months (1.2–4.2), and 1.6 months (1.5–1.7); median OS was 13.8 months (9.2–18.4), 8.2 months (5.7–10.7), and 6.3 months (1.3–11.3), respectively. Statistically significant differences in both PFS and OS were found between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (PFS: 95% CI, 0.11–0.44; $P < 0.0001$; OS: 95% CI, 0.15–0.61; $P < 0.0001$).

Conclusions: Our newly developed multiplex kit is practical and feasible for investigation of a range of sample types. Moreover, mutations in *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* detected in Asian patients were not predictive of clinical benefits from cetuximab treatment, similar to the result obtained in European studies.

Keywords: Luminex assay, *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, Epidermal growth factor

Background

The clinical significance of *KRAS* codon 12 and 13 mutation tests in the selection of patients with colorectal cancer who might benefit from anti-epidermal growth factor receptor (EGFR) antibodies is well established, and regulatory authorities in Europe, the United States, and Japan have recommended compulsory *KRAS* mutation testing before treatment [1-6]. Although conventional *KRAS* tests are useful to decrease treatment to nonbeneficiary populations, the efficacy of determining beneficiary populations requires improvement. The response rate to anti-EGFR antibody monotherapy among pretreated patients with tumors harboring *KRAS* codons 12 and 13 wild-type is 13%–17% [1,2], and that of combination anti-EGFR antibody and cytotoxic agent therapy is 11%–35% [5,7]. One explanation for such relatively low efficacy is that molecular alterations other than *KRAS* codon

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12 and 13 mutations might confer resistance to anti-EGFR antibody therapies. Recent retrospective studies have revealed that mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* are also related to resistance to anti-EGFR antibodies [8-13].

Several issues should also be considered to establish the clinical utility of expanded genome biomarker tests for anti-EGFR antibodies. First, information about the relation between mutation status and efficacy of treatment, especially among Asian populations, is still limited. Second, efficacious quality-controlled *in vitro* diagnostic kits and systems suitable for multiple genome biomarker detection are needed.

In Japan, a *KRAS* mutation assay kit based on the ARMS–scorpion method that detects seven frequently observed mutations in *KRAS* codons 12 and 13 (TheraScreen® K-RAS Mutation Kit; QIAGEN) was first approved for *in vitro* diagnostic use, and a kit using Luminex (xMAP) assay (MEBGEN KRAS Mutation Detection Kit, MBL) followed [14,15]. We recently developed another Luminex-based research-use kit, GENOSEARCH Mu-PACK, which simultaneously detects 36 mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA*. In addition to the hitherto approved *KRAS* codon 12 and 13 mutation kit, the multiplex kit identifies mutations by a single tube reaction using 50 ng of template DNA from formalin-fixed paraffin-embedded (FFPE) specimens.

In this study, we examined the feasibility and robustness of this multiplex kit using routine clinical samples collected from multiple hospitals. Meanwhile, we collected precise clinical data for these cases and retrospectively analyzed the relation of the mutation profiles of expanded markers to clinical outcomes following cetuximab therapy.

Methods

Patients

We screened and selected clinical and pathological data from consecutive patients who were administered either cetuximab monotherapy or cetuximab plus irinotecan between July 2008 and April 2010.

Patients who met all of the following inclusion criteria were retrospectively included in the analyses: (1) age ≥20 years; (2) histologically confirmed adenocarcinoma of the colon or rectum; (3) presence of unresectable metastatic disease; (4) baseline computed tomography (CT) performed within 28 days of initial cetuximab administration; (5) initial CT evaluation performed within 3 months of initial cetuximab administration; (6) previously documented as refractory or intolerant to fluoropyrimidines, oxaliplatin, and irinotecan; (7) Eastern Cooperative Oncology Group performance status score ≤2; and (8) adequate hematological, hepatic, and renal functions.

In the monotherapy regimen, cetuximab was administered at an initial dose of 400 mg/m^2 followed by weekly infusions of 250 mg/m^2. In the cetuximab plus irinotecan regimen, cetuximab was administered at the same dose as for monotherapy and followed by biweekly infusions of 150 mg/m^2 irinotecan, as per the manufacturer's instructions for irinotecan in Japan.

The study was conducted with the approval of the National Cancer Center Institutional Review Board, Cancer Institute Hospital of Japanese Foundation for Cancer Research Review Board, National Hospital Organization Shikoku Cancer Center Review Board, Shizuoka Cancer Center Review Board, Saitama Cancer Center Review Board, Hokkaido University Review Board, and the Ethics Committee of the University of Toyama. Written informed consent was obtained from as much patients who were alive as possible. For the deceased patients and their relatives, we also disclosed the study design at the website of National Cancer Center and gave them chances to express their wills in accordance with Epidemiological Study Guideline of Ministry of Health, Labour and Welfare in Japan.

Tissue samples and DNA extraction

Genomic DNA was obtained from primary and metastatic colorectal cancer tissues of all patients treated with cetuximab. Tissue samples harvested by biopsy or surgical resection at the participating hospitals were collected and sent to the research institution (MBL, Japan). A 2-μm hematoxylin-eosin (HE) slide and a 10-μm unstained slide were obtained from the FFPE tissue blocks; the latter was subsequently sliced into 3–10 sections. Pathological diagnoses were confirmed by a pathologist (Satoshi Fujii), with reference to the 4th edition of the WHO classification. The tumor area, determined by examining HE slides, was macroscopically dissected. Genomic DNA was isolated as described previously [16].

Luminex (xMAP) tests

A total of 36 mutations of *KRAS* codon 61 (Q61K, Q61E, Q61L, Q61P, Q61R, Q61H), *KRAS* codon 146 (A146T, A146S, A146P, A146E, A146V, A146G), *BRAF* codon 600 (V600E), *NRAS* codon 12 (G12S, G12C, G12R, G12D, G12V, G12A), codon 13 (G13S, G13C, G13R, G13D, G13V, G13A), codon 61 (Q61K, Q61E, Q61L, Q61P, Q61R, Q61H), *PIK3CA* exon 9 codon 542 (E542K), codon 545 (E545K), codon 546 (E546K), and exon 20 codon 1047 (H1047R, H1047L) were analyzed using Luminex (xMAP) technology (GENOSEARCH Mu-PACK, MBL, Japan).

First, 50 ng of template DNA collected from FFPE tissue samples was amplified by polymerase chain reaction (PCR) using a biotin-labeled primer. Thereafter, the PCR products and fluorescent Luminex beads (oligonucleotide probes complementary to wild and mutant genes were bound to the beads) were hybridized and labeled with streptavidin–phycoerythrin. Subsequently, the products

were processed by Luminex assay and the collected data analyzed using UniMAG software (MBL, Japan). The procedure time was approximately 4.5 h.

We also used the Luminex assay kit (MEBGEN KRAS Mutation Detection Kit, MBL, Japan) currently approved for clinical use by the Ministry of Health, Labour and Welfare of Japan [16] to detect *KRAS* codon 12 and 13 mutations.

Direct sequencing methods

In addition, to confirm the mutations detected by the Luminex assays, the same mutations of *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* were analyzed by direct sequencing. A total of 700 ng of template DNA was used for these PCR reactions and the PCR products were directly sequenced with the same primers used for PCR. A BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3730xl DNA Analyzer (Life Technologies) were used. Analyses of DNA sequences were performed using Sequencher (GeneCodes).

Statistical analysis

Response rates (RRs) and disease control rates (DCRs) (including complete or partial response and stable disease) were evaluated as per the Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.0). Progression-free survival (PFS) was defined as the time from initial administration of a cetuximab-containing regimen to either the first objective evidence of disease progression or death from any cause. Overall survival (OS) was defined as the time from initial administration of a cetuximab-containing regimen to death from any cause. RRs, DCRs, PFS, and OS of all patients were re-evaluated by the principal investigators at each institution. The relative dose intensity was defined as the ratio of the actual dose administered to the planned dose.

Fisher's exact test and the Kruskal–Wallis test were used to compare patient characteristics, relative dose intensity, and treatment response. PFS and OS data were plotted as Kaplan–Meier curves, and differences among the groups according to *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* gene status were compared using the log-rank test and hazard ratio calculated from a Cox regression model with a single covariate. All analyses were performed by a biostatistician (Takeharu Yamanaka), using IBM SPSS® Statistics 21 package software (SPSS Inc., Tokyo, Japan).

Results

Concordance between Luminex and direct sequencing

From September 2008 to April 2010, 376 patients were treated with a cetuximab-containing regimen at seven institutions. Of these, 83 patients met the inclusion criteria and specimens were collected from them for analysis (232 patients did not meet the inclusion criteria and 61 specimens could not be collected). We collected 78 surgically resected specimens and 5 biopsy specimens, from which the median amount of template DNA collected was 25,114 ng (range: 2740–84,738) and 1691 ng (range:1469–2668), respectively (Table 1).

One patient's gene status could not be detected by either Luminex or direct sequencing because DNA harvested from the resected metastatic liver specimens could not be amplified by PCR. In the remaining 82 patients, the concordance rate for mutations between the 2 methods was 100% (Table 2).

Among the 82 specimens, 3 *KRAS* codon 61 mutations (3.6%), 2 *KRAS* codon 146 mutations (2.4%), 4 *BRAF* mutations (4.9%), 2 *NRAS* mutations (2.4%), and 4 *PIK3CA* mutations (4.9%) (1 in exon 9 and 3 in exon 20) were detected using both the expanded kit and direct sequencing. Moreover, we identified 15 *KRAS* codon 12 mutations (18.3%) and 6 *KRAS* codon 13 mutations (7.3%); in total, 21 samples (25.6%) with *KRAS* codon 12 or 13 mutations were detected by using the *KRAS* Luminex assay kit. All mutations except for *PIK3CA* were mutually exclusive (Table 2, Figure 1).

Patient characteristics

Clinical data were collected from 83 patients. We used data from 82 patients whose genomic DNA could be successfully examined using both the expanded kit and direct sequencing. Six of the 82 patients were treated with cetuximab monotherapy, while the remaining 76 were treated with a regimen of cetuximab plus irinotecan.

Of these 82 patients, 49 had tumors with no mutation (all wild type), 21 had tumors with mutation of either *KRAS* codon 12 or 13, and 12 had tumors with mutation of either *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA*. No significant difference was observed in the characteristics of these three groups except for the ratio of refractoriness to intolerance of prior oxaliplatin (Table 3).

Table 1 Template DNA harvested from FFPE specimens

	Surgically resected	Biopsy	Total
Number of specimens	78	5	83
Total amount of template DNA (ng) [median (range)]	25,114 (2,740–84,738)	1,691 (1,469–2,668)	22,591 (1,469–84,738)
Amount of template DNA per slice (ng) [median (range)]	8,371 (914–28,246)	370 (154–889)	7,530 (154–28,246)

Table 2 Concordance between Luminex and direct sequencing

Gene	Direct sequencing (DS)	Luminex	Concordance rate	Mutation rate
***KRAS* codon 61**	**3**	**3**	**100%**	**3.6%**
Q61K	0	0	100%	0%
Q61E	0	0	100%	0%
Q61L	0	0	100%	0%
Q61P	0	0	100%	0%
Q61R	0	0	100%	0%
Q61H	3	3	100%	3.6%
***KRAS* codon 146**	**2**	**2**	**100%**	**2.4%**
A146T	2	2	100%	2.4%
A146S	0	0	100%	0%
A146P	0	0	100%	0%
A146E	0	0	100%	0%
A146V	0	0	100%	0%
A146G	0	0	100%	0%
***BRAF* codon 600**	**4**	**4**	**100%**	**4.9%**
V600E	4	4	100%	4.9%
***NRAS* codon 12**	**2**	**2**	**100%**	**2.4%**
G12S	0	0	100%	0%
G12C	0	0	100%	0%
G12R	0	0	100%	0%
G12D	2	2	100%	2.4%
G12V	0	0	100%	0%
G12A	0	0	100%	0%
***NRAS* codon 13**	**0**	**0**	**100%**	**0%**
G13S	0	0	100%	0%
G13C	0	0	100%	0%
G13R	0	0	100%	0%
G13D	0	0	100%	0%
G13V	0	0	100%	0%
G13A	0	0	100%	0%
***NRAS* codon 61**	**0**	**0**	**100%**	**0%**
Q61K	0	0	100%	0%
Q61E	0	0	100%	0%
Q61L	0	0	100%	0%
Q61P	0	0	100%	0%
Q61R	0	0	100%	0%
Q61H	0	0	100%	0%
***PIK3CA* Exon 9**	**1**	**1**	**100%**	**1.2%**
E542K	1	1	100%	1.2%
E545K	0	0	100%	0%
E546K	0	0	100%	0%
***PIK3CA* Exon 20**	**3**	**3**	**100%**	**3.7%**
H1047R	1	1	100%	1.2%
H1047L	2	2	100%	2.4%

Response to treatment

RRs of patients with all wild-type tumors ($N = 49$), *KRAS* codon 12 or 13 mutations ($N = 21$), and mutations of *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* ($N = 12$) were 38.8%, 4.8%, and 0%, respectively (Table 4). Partial response was observed in one patient with a *KRAS* codon G12C mutation. In addition, DCRs were 77.6%, 57.1%, and 33.3%, respectively, for these patient groups (Table 4). Differences for both RRs and DCRs between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations were statistically significant (Fisher's exact test, RRs: $P = 0.006$, DCRs: $P = 0.006$). On the other hand, there were no statistically significant differences between patients with *KRAS* codon 12 or 13 mutations and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (Fisher's exact test, RRs: $P = 0.636$, DCRs: $P = 0.170$).

The relative dose intensity of cetuximab was significantly higher among patients with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations. However, the number of treatment cycles was significantly greater among patients with all wild-type tumors (Table 4).

RR for all patients included in the study was 24.4%, whereas that for patients with *KRAS* codon 12 or 13 wild-type tumors was 31.1%. Furthermore, RR for patients with all wild-type tumors was 38.8%.

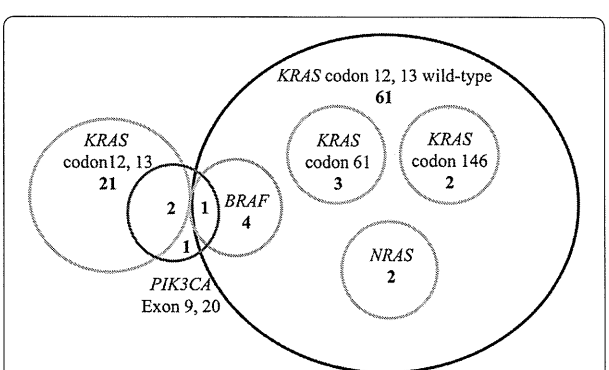

Figure 1 Associations among *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations. *KRAS* codon 12 and 13, *KRAS* codon 61 and 146, *BRAF*, and *NRAS* mutations were mutually exclusive. Only *PIK3CA* Exon 9 and 20 mutations overlapped *KRAS* codon 12 and 13 and BRAF mutations.

Table 3 Baseline patient characteristics

	All wild-type (*N* = 49)	*KRAS* codon 12, 13 mutations (*N* = 21)	*KRAS* codon 61, codon 146, *BRAF*, *NRAS* or *PIK3CA* mutations (any other mutations) (*N* = 12)	
Treatment				
Cetuximab + irinotecan (%)	47 (96)	19 (90)	10 (83)	P = 0.212†
Cetuximab monotherapy (%)	2 (4)	2 (10)	2 (17)	
Age				
Median (range)	61 (29–78)	65 (51–80)	65 (43–76)	P = 0.605‡
Gender				
Male (%)	31 (63)	16 (76)	6 (50)	P = 0.312†
Female (%)	18 (37)	5 (24)	6 (50)	
ECOG PS				
0 (%)	34 (69)	13 (62)	5 (42)	P = 0.185†
1–2 (%)	15 (31)	8 (38)	7 (58)	
Primary lesion				
Colon (%)	28 (57)	15 (71)	9 (75)	P = 0.416†
Rectum (%)	21 (43)	6 (29)	3 (25)	
Site of Metastasis				
Liver				
Yes (%)	33 (67)	13 (62)	8 (67)	P = 0.945†
No (%)	16 (33)	8 (38)	3 (33)	
Lung				
Yes (%)	34 (69)	15 (71)	9 (75)	P = 1.000†
No (%)	15 (31)	6 (29)	3 (25)	
Lymph node				
Yes (%)	26 (53)	7 (33)	9 (75)	P = 0.068†
No (%)	23 (47)	14 (67)	3 (25)	
Peritoneum				
Yes (%)	11 (22)	3 (14)	2 (17)	P = 0.791†
No (%)	38 (78)	18 (86)	9 (83)	
No. of metastatic sites				
1 (%)	9 (18)	9 (42)	3 (25)	P = 0.106†
>2 (%)	40 (82)	12 (58)	9 (75)	
Prior chemotherapy				
Fluoropyrimidine				
Refractory (%)	49 (100)	21 (100)	12 (100)	
Intolerant (%)	0 (0)	0 (0)	0 (0)	
Oxaliplatin				
Refractory (%)	40 (82)	10 (48)	9 (75)	P = 0.017†
Intolerant (%)	9 (18)	11 (52)	3 (25)	
Irinotecan				P = 1.000†
Refractory (%)	48 (98)	21 (100)	12 (100)	
Intolerant (%)	1 (2)	0 (0)	0 (0)	P = 0.669†

Table 3 Baseline patient characteristics *(Continued)*

Before bevacizumab therapy	25 (51)	9 (43)	7 (58)	
Yes (%)	24 (49)	12 (57)	5 (42)	P = 0.236[‡]
No (%)	12	5	25	
Response rate for prior irinotecan-containing therapies (%)				
Pathological classification				
G1, G2 (%)	42 (86)	20 (95)	11 (92)	P = 0.481[†]
G3, G4 (%)	7 (14)	1 (5)	1 (8)	

ECOG PS Eastern Cooperative Oncology Group performance status.
[†]: Fisher's exact test.
[‡]: Kruskal–Wallis test.

Survival

The median PFS among patients with all wild-type tumors ($N = 49$), *KRAS* codon 12 or 13 mutations ($N = 21$), and *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations ($N = 12$) was 6.1 months (95% confidence interval (CI) 3.1–9.2), 2.7 months (1.2–4.2), and 1.6 months (1.5–1.7), respectively (Table 4, Figure 2A). Median OS was 13.8 months (9.2–18.4), 8.2 months (5.7–10.7), and 6.3 months (1.3–11.3), respectively (Table 4, Figure 2B).

We observed statistically significant differences in both PFS and OS between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations [PFS: hazard ratio (HR), 0.22; 95% CI, 0.11–0.44; $P < 0.0001$] (OS: HR, 0.30; 95% CI, 0.15–0.61; $P < 0.0001$) (Figure 2A and 2B). Differences in PFS and OS between patients with wild-type mutations and the 8 patients with *KRAS* codon 61, *KRAS* codon 146, *NRAS*, or *PIK3CA* mutations were statistically significant (PFS: P = 0.001, OS: P = 0.001), but this was not the case for the 4 patients with *BRAF* mutations. The median PFS and OS for these 4 patients were 0.9 months and 11.4 months, respectively.

On the other hand, there were no statistically significant differences between patients with *KRAS* codon 12 or 13 mutations and those with *KRAS* codon 61, *KRAS*

Table 4 Efficacy in the test population determined on the basis of gene status

	All wild-type ($N = 49$)	*KRAS* codon 12, 13 mutations ($N = 21$)	*KRAS* codon 61, codon 146, *BRAF*, *NRAS* or *PIK3CA* mutations (any other mutations) ($N = 12$)	
Complete response	1	0	0	
Partial response	18	1	0	
Stable disease	19	11	4	
Progressive disease	11	9	8	
Total	49	21	12	
Response rate (%)	38.8	4.8	0	P = 0.006[*] (All wild-type vs. Any other mutations)
Disease control rate (%)	77.6	57.1	33.3	P = 0.006[*] (All wild-type vs. Any other mutations)
Progression-free survival [Median (95% CI) (months)]	6.1 (3.1, 9.2)	2.7 (1.2, 4.2)	1.6 (1.5, 1.7)	P < 0.0001[**] (All wild-type vs. Any other mutations)
Overall survival [Median (95% CI) (months)]	13.8 (9.2, 18.4)	8.2 (5.7, 10.7)	6.3 (1.3, 11.3)	P < 0.0001[**] (All wild-type vs. Any other mutations)
Relative dose intensity				
Irinotecan [Median (range) (%)]	72.8 (13.0–100)	81.0 (38.4–100)	98.0 (49.3–100)	P = 0.108[***]
Cetuximab [Median (range) (%)]	86.0 (35.7–100)	86.3 (11.1–100)	100 (80.0–100)	P = 0.042[***]
Number of treatment cycles [Median (range)]	12 (1–86)	5 (1–23)	3 (1–12)	P < 0.0001[***]

[*]: Fisher's exact test.
[**]: log rank test.
[***]: Kruskal–Wallis test.

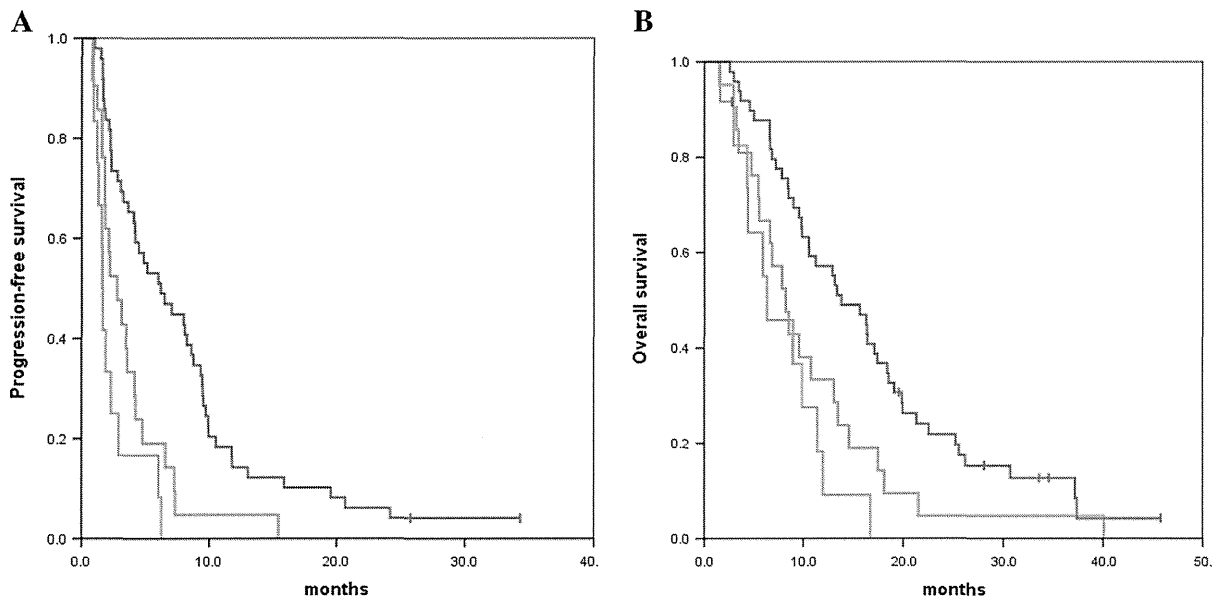

Figure 2 Kaplan–Meier plots of progression-free survival (PFS) and overall survival (OS) according to *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* gene status. Figure 2A. PFS: Median PFS values were 6.1 months [95% confidence interval (CI): 3.1–9.2], 2.7 months (1.2–4.2), and 1.6 months (1.5–1.7) among patients with all wild-type tumors (N = 49, blue line), *KRAS* codon 12 or 13 mutant tumors (N = 21, green line), and *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutant tumors (N = 12, gray-line), respectively. Differences in PFS values between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutant tumors were statistically significant (hazard ratio, 0.22; 95% CI, 0.11–0.44; $P < 0.0001$). **Figure 2B. OS:** Median OS values were 13.8 months [95% confidence interval (CI): 9.2–18.4], 8.2 months (5.7–10.7), and 6.3 months (1.3–11.3) among patients with all wild-type tumors (N = 49, blue line), with *KRAS* codon 12 or 13 mutant tumors (N = 21, green line), and with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (N = 12, gray-line), respectively. Differences in OS values between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutant tumors were statistically significant (hazard ratio, 0.30; 95% CI, 0.15–0.61; $P < 0.0001$).

codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (PFS: $P = 0.091$, OS: $P = 0.236$) (Figure 2A and 2B).

We also analyzed the differences in PFS and OS between patients with *KRAS* codon 12 mutations and those with *KRAS* codon 13 mutations. Similar to our previous study in a different population [17], there were no statistically significant differences between these groups (median PFS: *KRAS* codon 12, 2.1 months vs. *KRAS* codon 13, 3.4 months, P = 0.682; median OS: *KRAS* codon 12, 6.8 months vs. *KRAS* codon 13, 9.6 months, P = 0.147).

Discussion

This study is the first to verify the relevance of the mutation status of *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA*to the clinical efficacy of anti-EGFR antibody therapy among Asian patients. As reported in a pooled analysis from a European population, patients with the aforementioned less-frequent mutations exhibited statistically significant worse outcomes equivalent to those of *KRAS* codon 12 and 13 mutants [8]. Though systemically analyzed studies have not been reported since the first European analysis, our results strongly support the usefulness of the expanded pretreatment test for anti-EGFR therapies.

Because our aim was to compare the outcomes of *KRAS* codon 12 and 13 mutant cases with those characterized by other mutations, clinical data and FFPE specimens of the patients treated with cetuximab-containing regimens at seven Japanese cancer centers from July 2008 to April 2010 were collected. At that time, the Japanese authorities did not require pretreatment *KRAS* tests, and patients with *KRAS* codon 12 and 13 mutations were eventually treated with cetuximab. However, the proportion of patients with *KRAS* codon 12 or 13 mutant tumors in this study (25.6%) was slightly lower than that in previous reports of Western and Asian study populations [18], supposedly because several participating institutions had established lab-based tests and used the data for selecting nonbeneficiary populations. Among *KRAS* codon 12 and 13 wild-type cases, the proportion with mutations of overall tested genes (12/61, 19.7%) was similar to that of previous reports, suggesting that such expanded testing would be equally useful in Western and Asian countries.

Because the potential usefulness of multiplex mutation analyses is demonstrated, the development of robust *in vitro* diagnostic systems is needed for clinical application. The application of multiplex mutation detection systems in colorectal cancer specimens has been reported.

Lurkin I. et al. reported the validity of multiplex assays using a SNaPshot® Multiplex kit (Life Technologies), which detects 22 mutations in *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* [19]. Here we evaluated a quality-controlled kit detecting 36 mutations of *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* using Luminex (xMAP) technology. Data obtained by this kit were fully concordant with those by conventional direct sequencing, regardless of any variation in fixation methods between participating institutes (unpublished data).

This kit has several advantages with regard to its development for routine clinical use. It is manufactured under the same quality as the hitherto approved *in vitro* diagnostic kit detecting mutations in *KRAS* codons 12 and 13. Design of the hands-on operations is simple and easy; detection of the 36 mutations is performed in a single reaction of multiplex PCR followed by Luminex bead assay, with an overall hands-on time of 4.5 h. In addition, the requirement for template DNA is as low as 50 ng. We collected a median of 370 ng (range: 154–889) DNA per 10-μm biopsy slice in this study, which is sufficiently large to perform the test and to reserve backup DNA. Meanwhile, the ARMS–Scorpion assay, another approved *in vitro* diagnostic kit, requires larger amounts of template DNA. The currently approved *KRAS* codons 12 and 13 kit consists of 8 (1 control and 7 mutations) PCR reactions. A total of 80–160 ng of template DNA (10–20 ng for each PCR reaction) are needed to examine a sample [20], and it would be difficult to expand the PCR reactions because of the limitation of template DNA.

It has been estimated that approximately 10%–20% of all patients with colorectal cancer have either *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* gene mutations, suggesting that approximately 60,000–120,000 patients (10%–20% of the 600,000 who die annually from colorectal cancer) worldwide could be screened by this expanded mutation test. Furthermore, because the usefulness of regular administration of aspirin for patients with mutated *PIK3CA* colorectal cancer and the possibility of combining EGFR and BRAF inhibitors for patients with mutated *BRAF* colorectal cancer have been reported, detection of those mutations could become of greater importance in many ways [21,22]. Once further studies with larger sample sizes and a range of clinical samples provide evidence of its clinical utility, this technique might advance the precision of colorectal cancer treatment.

Conclusions

Our newly developed multiplex kit is practical and feasible for investigating various types of FFPE samples. Moreover, mutations in *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* detected in Asian patients were not predictive of clinical benefits from cetuximab treatment, similar to the result obtained in European studies.

Abbreviations

EGFR: Anti-epidermal growth factor receptor; PFS: Progression-free survival; OS: Overall survival; CI: Confidence interval; FFPE: Formalin-fixed, paraffin-embedded; CT: Computed tomography; H-E: Hematoxylin–eosin; PCR: Polymerase chain reaction; RR: Response rate; DCR: Disease control rate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TY and KT conceived the study design. HB carried out the majority of molecular genetic studies and analyses of the clinical data. ES, TN, KY, KY, SY, and SK provided clinical data and helped collect tumor tissues. SF carried out the pathological diagnoses. TY statistically analyzed the clinical data. AO coordinated the study and helped to draft the manuscript. All authors have read and approved the final manuscript.

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

Prognostic Impact of Body Mass Index in Patients with Squamous Cell Carcinoma of the Esophagus

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ABSTRACT

Purpose. To clarify the prognostic impact of body mass index (BMI) in patients with esophageal squamous cell carcinoma (ESCC).

Methods. Two hundred forty-three patients who underwent esophagectomy for ESCC from April 2005 through December 2010 were eligible. Prognoses of the patients were compared between groups stratified according to BMI. We also analyzed the survival difference using propensity score matching to adjust differences in staging and treatment.

Results. Low, normal, and high BMI groups had 35, 177, and 31 patients, respectively. The low BMI group included more advanced cases than did the normal BMI group, while tumor stage was equivalent in the normal and high BMI groups. Disease-free survival of the low and high BMI groups was significantly worse than that of the normal BMI group ($P < 0.0001$ between the low and normal BMI groups; $P = 0.0076$ between the normal and high BMI groups). Disease-free survival of the high BMI group was significantly worse than that of the normal BMI group in the propensity score-matched cohort ($P = 0.0020$). Multivariate analysis in this cohort demonstrated that high BMI was an independent prognostic factor (hazard ratio 2.949, 95 % confidence interval, 1.132–7.683).

Conclusions. High BMI was an independent prognostic factor after curative esophagectomy for ESCC. Although further analysis is required to clarify the influence of overweight on the biological features of ESCC, glucose metabolism may be a therapeutic target for ESCC.

Obesity is a risk factor for several cancers, such as breast, colon, and gynecologic carcinomas.[1] Recently, it has been clarified that obesity not only correlates with carcinogenesis but also influences the prognosis of several cancer patients. For example, obesity correlates with poor prognosis in breast and colon cancers, while favorable prognoses have been reported for obese patients with gastric or renal cancers.[2–5]

The effect of obesity on the prognosis of patients who undergo esophagectomy for esophageal cancer is still unknown. Because increased body mass index (BMI) is also a known risk factor for both Barrett esophagus and esophageal adenocarcinoma, many previous studies focused on adenocarcinoma.[6] Several studies have reported that high BMI does not affect survival.[7,8] However, recent large-scale cohort study demonstrated that obesity is associated with twofold increase in death owing to esophageal adenocarcinoma in patients who never smoked.[9]

As patients with esophageal squamous cell carcinoma (ESCC) were typically had a low BMI because of dysphagia from advanced cancer, little is known about the correlation between obesity and prognosis of ESCC. However, owing to the increasing prevalence of obesity and the development of diagnostic tools facilitating early detection of this disease, the number of overweight patients with ESCC is increasing. In addition, more than 90 % of esophageal cancer patients in Japan have squamous cell carcinoma, which is epidemiologically and clinically

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distinct from adenocarcinoma. Therefore, we sought to clarify the prognostic impact of BMI in patients with ESCC.

In this study, we retrospectively analyzed the correlation between BMI and prognosis of patients who underwent esophagectomy for ESCC.

METHODS

Patients

Two-hundred sixty-eight consecutive patients underwent esophagectomy for esophageal cancer at the Department of Gastroenterological Surgery, Kumamoto University Hospital, from April 2005 through December 2010. Among these, 11 patients with simultaneous head and neck cancers and 14 patients with histologic types of esophageal cancer other than squamous cell carcinoma (12 adenocarcinomas and 2 small cell carcinomas) were excluded. The remaining 243 patients were eligible. The local ethics committee of Kumamoto University approved this study.

Staging of ESCC

Staging of ESCC was based on endoscopy, esophagogram and 18-F-fluorodeoxyglucose (FDG)-positron emission tomography (PET)/CT. Endoscopic ultrasonography (EUS) was performed only when the tumor was limited within the submucosal layer or when tumor invasion into the neighboring organ was suspected by CT. Clinical and pathological findings were classified according to tumor, node, metastasis classification system (7th edition) of the Union for International Cancer Control and the American Joint Committee.[10] When the patient achieved complete response by neoadjuvant treatment, pathologic staging was considered as stage 0.

Collection of BMI Data

Height and weight of all patients were measured at the time of esophagectomy. BMI was calculated by weight (kg) divided by height (m^2). World Health Organization cut points were used to categorize patients as having high BMI ($\geq$25.0 kg/m^2), normal BMI (18.5–24.9 kg/m^2), or low BMI (<18.5 kg/m^2).[11]

Follow-up of Patients

Follow-up of the patients was carried out in our clinic every 3 months until 5 years after surgery. The median follow-up period from the surgery to death or the last visit was 25.7 months. Seven patients were lost to follow-up.

Statistical Analysis

All quantitative data are expressed as mean $\pm$ 1 standard deviation. Statistical analyses were performed by JMP 10 software (SAS Institute, Cary, NC, USA). Differences in clinicopathologic features were determined by Student's *t* test for age, duration of operation, blood loss, number of retrieved nodes, and number of metastatic nodes. Fisher's exact test was used for other variables. Survival rates after esophagectomy were calculated by the Kaplan–Meier method, and statistical significance was determined by the log-rank test. Overall survival was defined as the time from surgery to death resulting from any cause, and disease-free survival was defined as the time from surgery to first recurrence or to death from any cause. The Cox proportional hazard model was used for multivariate analysis of the disease-free survival of 220 patients who underwent R0 esophagectomy.

We created a nonparsimonious logistic regression model to derive a propensity score for patients who belong to the normal and high BMI groups. We matched each patient in the high BMI group with one patient in the normal BMI group. The matched cohort was evaluated for differences in clinicopathologic characteristics between the normal and high BMI groups, using paired *t* test for continuous outcome and McNemar's test for proportions. Survival analysis was performed in this cohort using Kaplan–Meier and Cox proportional hazard model. A P value of <0.05 was considered an indication of statistical significance.

RESULTS

The low, normal, and high BMI groups had 35, 177, and 31 patients, respectively. Differences in clinicopathologic factors among the groups stratified by BMI are summarized in Table 1. Variables were compared between the low and normal BMI groups and between the normal and high BMI groups. There were more women in the low BMI group than in the normal BMI group. Clinical stage of cancer was significantly greater in the low BMI group than in the normal BMI group. Cardiac comorbidity was significantly lesser in the low BMI group than in the normal BMI group. The prevalence of diabetes mellitus was greater in the high BMI group than in the normal BMI group. Clinical stage was comparable between the normal and high BMI groups. Type of esophagectomy, operative time, blood loss, and the incidence of postoperative complications were comparable among the groups. The number of retrieved nodes was significantly less in the low BMI group than in the normal BMI group. Pathologic stage was significantly higher in the low BMI group than in the normal BMI group, and R0 resection was less frequent in the low BMI group than in the normal BMI group. In contrast, pathologic stage was

comparable between the normal and high BMI groups, and no significant difference was observed in the number of retrieved nodes or R0 resection rate between these two groups. Percentage of patients who underwent postoperative chemotherapy was comparable among the groups. These results suggest that there were more advanced cases in the low BMI group than in the normal BMI group, while tumor stage was equivalent in the normal and high BMI groups.

Overall survival and disease-free survival are provided in Fig. 1. Overall survival of both the low BMI group and the high BMI group were significantly worse than that of the normal BMI group ($P = 0.0003$ between the low and normal BMI groups; $P = 0.0014$ between the normal and high BMI groups). Similarly, disease-free survival of the low and high BMI groups was significantly worse than that of the normal BMI group ($P < 0.0001$ between the low and normal BMI groups; $P = 0.0076$ between the normal and high BMI groups).

In order to evaluate influence of preoperative treatment on BMI, we compared BMIs measured before and after treatment (Supplemental Fig. 1). We collected pretreatment BMI data of 81 among 96 patients who underwent preoperative treatment, while we missed the data of 15 cases who underwent preoperative treatment in the other institutes. Significant decrease in BMI was observed in patients who underwent definitive chemoradiotherapy, although there was no significant change in BMI during neoadjuvant treatment. When we compared outcomes among groups stratified by pretreatment BMI, survival of the high BMI group was also significantly worse than that of the normal BMI group.

In order to control the differences in stage of disease and treatment types between the normal BMI group and the high BMI group, propensity score-matching cohort was selected. There were 27 pairs in the cohort. Characteristics of patients are shown in Table 2. There was no significant difference in the stage of disease and types of treatment between the pairs. Disease-free survival of the high BMI group was also significantly worse than that of the normal BMI group in this cohort (Fig. 2, $P = 0.020$). Cox proportional hazard model revealed that high BMI was an independent prognostic factor in this cohort (Table 3, hazard ratio 2.949, 95 % confidence interval, 1.132–7.683).

DISCUSSION

In this study, we clarified that both undernutrition and overweight negatively affected prognosis for patients with ESCC. Advanced disease was the explanation for poor prognosis in the low BMI group, whereas biologically aggressive tumors might be responsible for poor prognosis in the high BMI group. Several authors have reported that an elevated BMI did not reduce survival of patients who underwent esophagectomy for cancer.[6–8, 12,13] However, recent large-scale cohort study demonstrated that obesity among never smokers was independently associated with poor prognosis in patients with esophageal adenocarcinoma.[9] Subjects of these previous studies are mainly adenocarcinoma, and even in the studies which targeted both histologic subtypes, percentage of patients with ESCC was as low as 10–47 %. Therefore, this is the first report to demonstrate the prognostic impact of BMI in ESCC.

More advanced diseases, more salvage esophagectomies after definitive chemoradiotherapy, fewer retrieved lymph nodes, and fewer R0 resections were features of the low BMI group. These findings indicate that advanced cancer is the obvious reason for poor prognosis in this group. Advanced esophageal cancer is often accompanied by malignant stricture and thus can result in poor nutrition. In addition, preoperative treatment for advanced cancer may induce malnutrition. Han-Geurts et al.[14] reported that nutritional parameters are significantly worse in patients with neoadjuvant treatment compared to those who receive no such treatment. In particular, mucosal injuries of the upper digestive tract induced by chemoradiotherapy can decrease caloric intake. The reason of the fewer retrieved lymph nodes was that significantly more salvage surgeries were included in this group. Salvage esophagectomy is well known as high risk surgery, and therefore preventive lymph node dissection is usually minimized to decrease postoperative morbidity and mortality in this type of surgery.[15]

The normal and high BMI groups, however, did not differ in preoperative factors, except the prevalence of diabetes. Although tumor stage and surgical curability were similar between the groups, prognosis was significantly worse in the high BMI group. In addition, disease-free survival of the high BMI group was also significantly worse than that of the normal BMI group in the propensity score-matching cohort. These findings strongly suggest that tumors in overweight patients are more aggressive than those in patients of normal weight. We tried to identify morphologic difference between the high BMI group and the others but failed to find any differences.

Obesity has been known for many years to increase the risk of type 2 diabetes and is itself associated with cancer risk.[1] In this study, the prevalence of diabetes was significantly higher in the high BMI group (16.1 %) than in the normal (7.9 %) and low (2.9 %) BMI groups. Epidemiological studies have reported that patients with diabetes who develop cancer have a worse prognosis after treatment with chemotherapy or surgery and have greater mortality than do those without diabetes.[16,17] Both hyperglycemia and hyperinsulinemia induced by type 2 diabetes have been

TABLE 1 Differences in clinicopathologic factors among the groups stratified by BMI

Characteristic	Variable	Low BMI	Normal BMI	High BMI	*P* value	
		($n = 35$)	($n = 177$)	($n = 31$)	L vs. N	N vs. H
Age	Mean ± SD	64.9 ± 9.6	66.2 ± 8.7	68.6 ± 8.0	0.43	0.14
Sex	Male	26 (74.3)	160 (90.4)	28 (90.3)	0.0079	0.99
	Female	9 (25.7)	17 (9.6)	3 (9.7)		
BMI	Mean ± SD	17.3 ± 1.2	21.7 ± 1.7	26.5 ± 1.1	<0.0001	<0.0001
Location	Upper	6 (17.1)	26 (14.7)	4 (12.9)	0.72	0.97
	Middle	15 (42.9)	89 (50.3)	16 (51.6)		
	Lower	14 (40.0)	62 (35.0)	11 (35.5)		
cT	1	7 (20.0)	88 (49.7)	11 (35.5)	0.0044	0.23
	2	6 (17.1)	27 (15.3)	9 (29.0)		
	3	18 (51.4)	56 (31.6)	10 (32.3)		
	4	4 (11.4)	6 (3.4)	1 (3.2)		
cN	0	11 (31.4)	91 (51.4)	14 (45.2)	0.11	0.81
	1	19 (54.3)	61 (34.5)	13 (41.9)		
	2	4 (11.4)	23 (13.0)	4 (12.9)		
	3	1 (2.9)	2 (1.1)	0		
cStage	IA	4 (11.4)	70 (39.5)	8 (25.8)	0.022	0.63
	IB	2 (5.7)	10 (5.6)	4 (12.9)		
	IIA	5 (14.3)	12 (6.8)	2 (6.5)		
	IIB	6 (17.1)	24 (13.6)	6 (19.4)		
	IIIA	11 (31.4)	39 (22.0)	8 (25.8)		
	IIIB	2 (5.7)	14 (7.9)	2 (6.5)		
	IIIC	5 (14.3)	8 (4.5)	1 (3.2)		
	IV	0	0	0		
Neoadjuvant treatment	None	17 (48.6)	113 (63.8)	17 (54.8)	0.027	0.68
	CT	5 (14.3)	37 (20.9)	9 (29.0)		
	CRT	4 (11.4)	9 (5.1)	1 (3.2)		
	dCRT	9 (25.7)	18 (10.2)	4 (12.9)		
Comorbidity	Present	19 (54.3)	69 (39.0)	9 (29.0)	0.093	0.29
Pulmonary	Present	5 (14.3)	24 (13.6)	4 (12.9)	0.91	0.92
Cardiac	Present	3 (8.6)	57 (32.2)	13 (41.9)	0.0046	0.29
Hepatic	Present	2 (5.7)	12 (6.8)	4 (12.9)	0.82	0.24
Diabetes	Present	1 (2.9)	10 (5.6)	5 (16.1)	0.50	0.038
CVD	Present	1 (2.9)	14 (7.9)	2 (6.5)	0.29	0.78
Type of esophagectomy	TT	31 (88.6)	168 (92.7)	27 (87.0)	0.64	0.12
	IL	2 (5.7)	8 (4.5)	4 (12.9)		
	TH	2 (5.7)	5 (2.8)	0		
Duration of operation (min)	Mean ± SD	533 ± 146	523 ± 122	563 ± 193	0.67	0.13
Blood loss (g)	Mean ± SD	568 ± 388	540 ± 435	676 ± 328	0.72	0.098
Postoperative complication	Present	13 (37.1)	50 (28.2)	9 (29.0)	0.92	0.94
Pulmonary	Present	8 (22.9)	33 (18.6)	8 (25.8)	0.64	0.33
Anastomotic leak	Present	8 (22.9)	27 (15.3)	6 (19.4)	0.32	0.60
Recurrent nerve palsy	Present	7 (20.0)	34 (19.2)	8 (25.8)	>0.99	0.47
No. of retrieved nodes	Mean ± SD	36.2 ± 22.4	44.4 ± 20.5	40.4 ± 18.3	0.033	0.30
No. of metastatic nodes	Mean ± SD	1.9 ± 2.6	1.6 ± 2.8	2.0 ± 2.8	0.67	0.47
pT	0	2 (5.7)	5 (2.8)	3 (9.7)	0.0002	0.22
	1	9 (25.7)	98 (55.4)	12 (38.7)		
	2	3 (8.6)	28 (15.8)	4 (12.9)		
	3	16 (45.7)	43 (24.3)	11 (35.5)		
	4a	2 (5.7)	1 (0.6)	0		
	4b	3 (8.6)	2 (1.1)	1 (3.2)		