complications and mortality. Takeshita et al. [10] suggested that limited lymph node dissection should be considered for patients between 80 and 84 years of age, since the prognostic significance of radical surgery is uncertain in patients 85 years old or over. However, Orsenigo et al. [19] showed that the rates of postoperative complications and mortality in elderly patients were comparable to those in younger patients despite their comorbidities, and they did not have a worse overall survival.

Since the surgical outcomes in gastric cancer patients sometimes vary by hospital or country, the optimal procedure for elderly patients remains unclear. Furthermore, most of the previous studies did not use a common criterion for evaluating surgical complications [20–22]. Recently, the Clavien–Dindo classification has become a worldwide standard classification system for surgical complications [13]. In our study, we evaluated Grade II or higher complications according to the Clavien–Dindo classification in more than 400 consecutive patients with gastric cancer. To confirm the results obtained in our study, multicenter cohort studies using the same criteria for age and postoperative complications are warranted.

Among the patients who underwent total gastrectomy, 34 (28.6 %) younger patients and one octogenarian patient (6.7 %) underwent splenectomy. This appears to indicate that we tended to avoid extensive lymphadenectomy for the elderly patients compared with the younger patients. Nevertheless, the postoperative complication rate was higher in the octogenarian group than in the younger group. Furthermore, total gastrectomy significantly affected the postoperative complication rate. A previous study reported that the quality of life declines after surgery for gastric cancer, especially total gastrectomy [23, 24]. Since long-term survival can be expected with radical surgery even in elderly patients, radical gastrectomy should not be ruled out as a treatment for gastric cancer. However, in elderly patients, the type of gastrectomy should be determined after a comprehensive evaluation of each individual patient, including his or her performance status and lifestyle.

Conflict of interest Jota Mikami and co-authors have no conflicts of interest to declare.

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Eur Radiol (2015) 25:368–374
DOI 10.1007/s00330-014-3373-9

GASTROINTESTINAL

Accuracy of multidetector-row CT in diagnosing lymph node metastasis in patients with gastric cancer

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Received: 18 April 2014 / Accepted: 22 July 2014 / Published online: 6 August 2014

Abstract

Objectives The purpose of this study was to determine the optimal cut-off value of lymph node size for diagnosing metastasis in gastric cancer with multidetector-row computed tomography (MDCT) after categorizing perigastric lymph nodes into three regions.

Methods The study included 90 gastric cancer patients who underwent gastrectomy. The long-axis diameter (LAD) and short-axis diameter (SAD) of all visualized lymph nodes were measured with transverse MDCT images. The locations of lymph nodes were categorized into three regions: lesser curvature, greater curvature, and suprapancreatic. The diagnostic value of lymph node metastasis was assessed with receiver operating characteristic (ROC) analysis.

Results The area under the curve was larger for SAD than LAD in all groups. The optimal cut-off values of SAD were determined as follows: overall, 9 mm; differentiated type, 9 mm; undifferentiated type, 8 mm; lesser curvature region, 7 mm; greater curvature region, 6 mm; and suprapancreatic region, 9 mm. The diagnostic accuracies for lymph node metastasis using individual cut-off values were 71.1 % based on histological type and 76.6 % based on region of lymph node location.

Conclusions The diagnostic accuracy of lymph node metastasis in gastric cancer was improved by using individual cut-off values for each lymph node region.

Key points

- *Multidetector-row computed tomography is widely used to predict pathological nodal status.*
- *An optimal cut-off value of lymph node size has not been determined.*
- *Cut-off values were assessed according to histology and nodal location.*
- *The optimal cut-off values differed based on histology and nodal location.*
- *Diagnostic accuracy was improved by using individual cut-off values for each region.*

Keywords Gastric cancer · Stomach · Multidetector-row CT · Lymph node metastasis · Staging accuracy

Abbreviations

MDCT	multidetector-row computed tomography
LAD	long-axis diameter
SAD	short-axis diameter
ROC	receiver operating characteristics
AUC	area under the curve

Introduction

Gastric cancer is a major cause of cancer-related deaths worldwide, and it is the most common cause of cancer-related mortality in eastern Asia [1]. Lymph node metastasis is one of the most important factors affecting the prognosis of gastric cancer [2, 3]. Locally advanced tumours usually require preoperative chemotherapy to improve curative resection rates and long-term survival. In European countries, perioperative chemotherapy using a regimen of epirubicin, cisplatin, and fluorouracil is a standard treatment for localized gastric cancer [4, 5]. The National Comprehensive Cancer Network (NCCN) guidelines state that perioperative chemotherapy or preoperative chemoradiation is the preferred approach for T2 or more advanced gastric cancer [6]. Although accurate

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staging of lymph node metastasis is desirable for preoperative treatment, the rate of accuracy in detecting lymph node metastasis with conventional diagnostic tools is only around 60 % [7–13]. The use of multidetector-row computed tomography (MDCT) has recently gained wide adoption worldwide, allowing for more detailed imaging with thinner section collimation. However, an optimal cut-off value for lymph node size to diagnose pathological metastasis has not yet been determined. Although some previous studies have used different criteria for long-axis diameter (LAD) or short-axis diameter (SAD), diagnostic accuracies remain around 70 % for T2 or more advanced gastric cancer, even using MDCT [14, 15].

The mean size of benign lymph nodes on MDCT differs according to the specific location of lymph nodes in the abdomen and mediastinum [16–18]. However, no studies have diagnosed lymph node metastasis using different cut-off values based on the location of lymph nodes in gastric cancer patients. Thus, this retrospective study was conducted to assess the diagnostic accuracy of nodal size after categorizing perigastric lymph nodes into three regions.

Materials and methods

Patient population

The present study included 90 gastric cancer patients who underwent gastrectomy between January 2010 and December 2012 at Osaka University Hospital. All tumours were histologically diagnosed as adenocarcinoma of the stomach. Patients who had pathological T1 cancer or who underwent preoperative chemotherapy were excluded. Since patients with T2 or more advanced gastric cancers are candidates for preoperative treatment according to NCCN guidelines [6], we included only gastric cancer of T2 or a more advanced stage in this study. Patients underwent extended lymphadenectomy, either D2 or D2 minus splenic hilum node (station no. 10) dissection, according to the Japanese Gastric Cancer Association treatment guidelines [19]. Pathological tumour depth, nodal status, and surgical curability were classified according to the seventh edition of the International Union Against Cancer (UICC) classification system [20].

Preoperative examination

The MDCT protocol has previously been described in detail [21, 22]. All 90 patients underwent enhanced MDCT after overnight fasting, with an MDCT system (Discovery CT750 HD; GE Healthcare, Milwaukee, WI, USA). Each patient was placed in a prone position on the imaging table to avoid artefacts caused by air in the stomach. Pre-contrast imaging was not performed. A total of 100 mL of non-ionic contrast material (iopromide; Proscope, Tanabe Seiyaku, Osaka, Japan) containing 300 mg of iodine per mL was administered intravenously at 3 mL/second using a power injector (Auto-Enhance A-50; Nemoto Kyorindou, Tokyo, Japan). Imaging was performed 30 seconds and 75 seconds after initiation of contrast material injection, corresponding to the arterial and venous phases. Imaging began at the level of the dome of the right hemidiaphragm and ended at the caudal edge of the stomach, so as to include the entire liver. CT parameters were as follows: 64 detector rows; section thickness, 0.625 mm; pitch, 1.375 mm; reconstruction interval, 0.625 mm; 200 milliamperes; 120 kilovolts; and tube rotation time, 0.4 seconds. Transverse images with a section thickness of 2.5 mm were created using volumetric data obtained during MDCT. A written informed consent for preoperative staging with MDCT was obtained from all patients.

Evaluation

Transverse CT images were reviewed without knowledge of the surgical or histopathological findings of the resected lymph nodes. The mediastinal window settings consisted of a window level (WL) of 60 and a window width (WW) of 300, with standard function. The LAD and SAD of all visualized lymph nodes were measured on MDCT images (Fig. 1). Diameters of less than 5 mm were rounded down to 0 mm in this study. The locations of regional lymph nodes, identified on preoperative MDCT and confirmed at the time of surgery, were recorded based on nodal grouping according to the Japanese Gastric Cancer Association (JGCA) classification system [23]. In addition, this study categorized locations into three regions: the lesser curvature region (Nos. 1, 3, 5, 7), greater curvature region (Nos. 2, 4, 6), and suprapancreatic region (Nos. 8, 9, 10, 11, and 12).

Statistical analysis

The diagnostic value of lymph node metastasis was assessed by calculating the area under the receiver operating characteristic (ROC) curve, not only for the overall patient population but also for each histological and regional group. The cut-off value was based on the ROC curve with Youden's index (J), calculated using the equation J = sensitivity + specificity − 1. Statistical analyses were performed using the SPSS statistical package, version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Overall patient characteristics are shown in Table 1. The median number of dissected lymph nodes was 39, and the

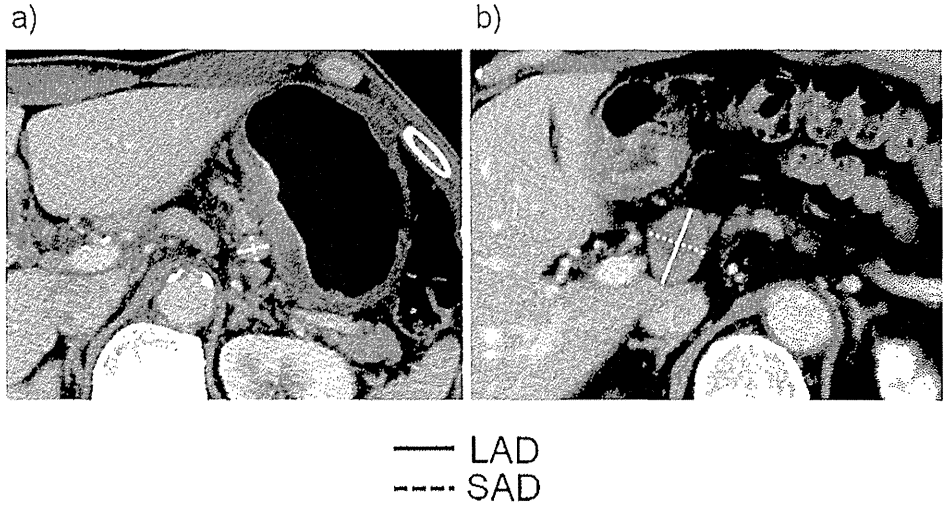

Fig. 1 A transverse CT image of a 64-year-old man shows an enlarged lymph node in the lesser curvature region (a). A transverse CT image of a 65-year-old man shows lymph node enlargement in the suprapancreatic region (b). The long-axis diameter (*LAD, solid line*) and short-axis diameter (*SAD, dotted line*) of each lymph node were measured, as shown

pathological node-positive rate was 52 % (47/90). More than half of the patients had differentiated-type tumours.

The mean nodal sizes detected on MDCT were 12 mm for LAD and 8 mm for SAD. Nodes associated with differentiated tumours (mean LAD, 13.6 mm; mean SAD, 8.5 mm) were larger than those associated with undifferentiated tumours (mean LAD, 10.9 mm; mean SAD, 7.4 mm). Regarding lymph node location, the greater curvature region (mean LAD, 6.1 mm; mean SAD, 3.8 mm) had smaller nodes than the region of lesser curvature (mean LAD, 9.6 mm; mean SAD, 6.3 mm) and the suprapancreatic region (mean LAD, 9.4 mm; mean SAD, 5.4 mm).

We analysed the detectability of lymph node metastasis on MDCT with ROC curves (Fig. 2). The area under the curve (AUC) was larger for SAD than LAD in all groups. Based on the ROC curves, the optimal cut-off values of SAD were as follows: overall, 9 mm; differentiated type, 9 mm; undifferentiated type, 8 mm; lesser curvature region, 7 mm; greater curvature region, 6 mm; and suprapancreatic region, 9 mm. With these cut-off values, all parameters – including accuracy, sensitivity, and specificity – were higher for the differentiated type than for the undifferentiated type (Table 2). The three regions showed similar accuracy, but the sensitivity in the suprapancreatic region was much lower than in the lesser curvature or greater curvature regions (Table 2).

In the MDCT diagnosis of clinical N status with a single cut-off value (SAD 9 mm), the overall accuracy, sensitivity, and specificity were 70.6 %, 55.3 %, and 86.0 %, respectively. When we used individual cut-off values according to histological type (SAD 9 mm for the differentiated type, SAD 8 mm for the undifferentiated type), the accuracy (71.1 %) was similar to overall values (Table 3). On the other hand, after categorizing lymph node locations into three regions, accuracy could be increased to 76.6 % with individual cut-off values (SAD 7 mm for the lesser curvature region, SAD 6 mm for the greater curvature region, SAD 9 mm for the suprapancreatic region) (Table 3).

Discussion

The present study showed that SAD was superior to LAD as an indicator for diagnosing lymph node metastasis. This result is in accordance with the revised version (ver.1.1) of the

Table 1 Patient Backgrounds

Characteristics		n
Sex	Male / Female	67 / 23
Age (years)	Median (range)	69 (32–90)
Location	Upper / Middle / Lower	22 / 37 / 31
Gastrectomy	Total / Subtotal	32 / 58
Number of dissected lymph nodes	Median (range)	39 (13–89)
Number of lymph nodes with metastasis	Median (range)	1 (0–27)
pT	T2 / T3 / T4	27 / 42 / 21
pN	N0 / N1 / N2 / N3	43 / 14 / 14 / 19
pStage	I / II / III / IV	15 / 41 / 27 / 7
Histology	Differentiated / Undifferentiated	47 / 43

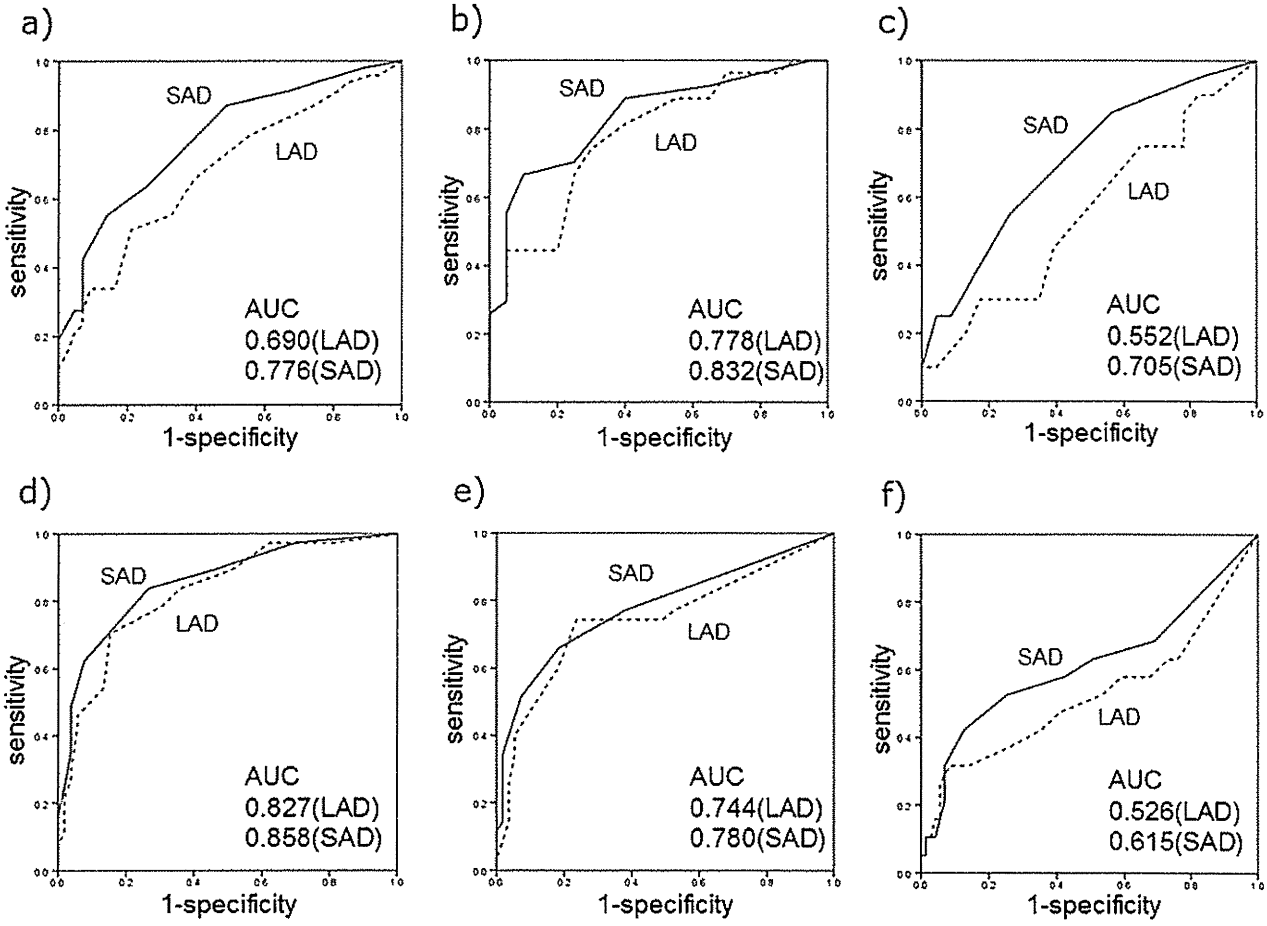

Fig. 2 ROC curve for the detectability of lymph node metastasis on MDCT: overall (**a**), differentiated type (**b**), undifferentiated type (**c**), lesser curvature region (**d**), greater curvature region (**e**), and suprapancreatic region (**f**)

Response Evaluation Criteria in Solid Tumours (RECIST), which adopted SAD as a criterion for lymph node metastasis [24]. Compared with the conventional method using a single cut-off value, diagnostic accuracy was improved by using individual cut-off values for each lymph node region. Specificities were high in all categorized regions, but the sensitivity decreased to 42 % in the suprapancreatic region. Indeed, the size of benign lymph nodes located in the suprapancreatic region is usually larger than those in other regions as identified during gastric cancer surgery. Radiologists as well as gastric surgeons should keep in mind that cut-off values for diagnosing nodal metastasis differ according to the region of lymph node location.

Accurate preoperative staging of regional lymph node metastasis in gastric cancer is very important in planning therapeutic strategies, especially for preoperative chemotherapy. Although there are a number of different criteria and methods for assessing nodal status, no solid criteria exist for appropriately diagnosing metastatic lymph nodes. The definition of metastatic lymph nodes differs among studies using MDCT, and various cut-off values have been applied [14, 15, 25–30]. Ahn et al. defined metastatic lymph nodes as having SAD of

Table 2 Optimal cut-off values and diagnostic accuracy of lymph node metastasis, overall and for subgroups, based on histological type and lymph node region

	n	Optimal cut-off value	Accuracy (%)	Sensitivity (%)	Specificity (%)
Overall	90	SAD 9 mm	70.6	55.3	86.0
Differentiated type	47	SAD 9 mm	76.6	66.7	90.0
Undifferentiated type	43	SAD 8 mm	65.1	55.0	73.9
Lesser curvature region	90	SAD 7 mm	77.8	83.8	73.6
Greater curvature region	90	SAD 6 mm	75.6	65.7	81.8
Suprapancreatic region	90	SAD 9 mm	77.8	42.1	87.3

SAD short-axis diameter

Table 3 The diagnostic accuracy of clinical N status on MDCT with a single cut-off value overall (a), and with individual cut-off values based on histological type (b) and lymph node region (c)

(a)		Pathological N status		
		N(−)	N(+)	Accuracy 70.6 %
Clinical N status on MDCT	N(−)	37	21	Sensitivity 55.3 %
	N(+)	6	26	Specificity 86.0 %
(b)		Pathological N status		
		N(−)	N(+)	Accuracy 71.1 %
Clinical N status on MDCT	N(−)	35	18	Sensitivity 61.7 %
	N(+)	8	29	Specificity 81.3 %
(c)		Pathological N status		
		N(−)	N(+)	Accuracy 76.6 %
Clinical N status on MDCT	N(−)	27	5	Sensitivity 89.4 %
	N(+)	16	42	Specificity 62.8 %

≥8 mm [29], while Chen et al. used a definition of ≥8 mm for LAD [30]. Previous studies have reported diagnostic accuracy of lymph node metastasis in gastric cancer that has varied from 54 % to 84 % [14, 15, 25–35]. When comparing accuracy among studies, differences in eligibility criteria must be considered. Most previous studies have included patients with any stage of gastric cancer. Particularly in Japan and Korea, more than half of patients with gastric cancer have T1 stage (mucosal or submucosal) tumours. If the eligibility criteria include such early-stage cancers, the diagnostic accuracy of lymph node metastasis is usually inflated, because these cancers are associated with a low incidence of lymph node metastasis [14, 15]. Furthermore, since NCCN guidelines indicate preoperative treatment for gastric cancer of stage T2 or greater[6], preoperative diagnosis of N status is more relevant for T2-or-higher tumours than for T1 tumours. As such, we included only T2 or higher-stage gastric cancer in this study. The accuracy (76.6 %), sensitivity (89.4 %), and specificity (62.8 %) in our study were similar or superior to those in previous studies also including early-stage cancer.

This study also showed that the diagnostic accuracy of lymph node metastasis differed between the differentiated and the undifferentiated types. Noda et al. reported that the mean size of metastatic lymph nodes in differentiated-type tumours was significantly larger than in undifferentiated-type tumours [36]. However, no previous study has investigated the influence of differences in metastatic lymph node size on MDCT findings between histological types. Our results, which showed that the cut-off value for the differentiated type was larger than for the undifferentiated type, were consistent with the report of Noda et al. As undifferentiated tumours grow diffusely, tumour invasion does not directly affect the size of metastatic lymph nodes [37]. This may explain why the mean size of metastatic lymph nodes in undifferentiated tumours is smaller than in differentiated tumours. In our study, all diagnostic parameters – including accuracy, sensitivity, and specificity – were higher in the differentiated type than in the undifferentiated type. This implies that high diagnostic accuracy for metastatic lymph nodes can be expected in the differentiated type, while surgeons and radiologists should consider the difficulty in diagnosing nodal metastasis in the undifferentiated type.

At present, the diagnosis of lymph node abnormalities on MDCT is based primarily on size criteria. In addition to size, other CT features of the lymph node, such as an almost circular shape (longitudinal/transverse diameter ratio <1.5), central necrosis, marked or heterogeneous enhancement (>85 HU in the enhanced scan), and clustered nodes regardless of size, can also be used to differentiate positive from negative lymph nodes [15, 30, 38, 39]. Furthermore, multiplanar reformation images, which enable us to measure the longitudinal diameter of lymph nodes, have been reported as superior to transverse images in assessing lymph node metastasis, although some reports were unable to demonstrate the superiority of these images [15, 26, 30]. Regarding other modalities, Kwee and Kwee reviewed the diagnostic accuracy of preoperative N-staging by comparing endoscopic ultrasound (EUS), MDCT, MRI, and PET-CT [40]. In their study, the accuracy of N status diagnosis ranged from 40 % to 90 % for EUS, 54 % to 80 % for MDCT, 50 % to 65 % for MRI, and 55.1 % for PET-CT. Although EUS showed accuracy similar to MDCT, EUS is not objective, and there is some difficulty in evaluating lymph nodes that are located at a greater distance from the gastric wall. MRI does not involve any radiation exposure, but its diagnostic accuracy is low. Low accuracy was also reported in PET-CT studies due to the low sensitivity in detecting lymph node metastases with FDG-PET [41]. Considering the convenience and the objectivity of MDCT, it seems that this is a useful modality for determining firm MDCT criteria in the diagnosis of lymph node metastasis.

One of the limitations of our study was the small number of patients and thus the lack of validation using other datasets. A large-scale study is needed to verify the clinical usefulness of our findings for preoperative N-staging in gastric cancer. Second, we did not evaluate the reproducibility of lymph node size measurement between reviewers, and therefore future studies of reproducibility are desirable. Third, lymph node metastasis was evaluated in each region, not for each lymph node individually, because it is impossible to match the lymph nodes dissected during surgery with those evaluated on MDCT. We believe that matching the regions of dissected lymph nodes with the regions evaluated preoperatively is the most practical way to evaluate the diagnostic accuracy of lymph node metastasis. Furthermore, the most important point for clinical use is the accurate diagnosis of clinical N status, not individual nodal metastasis. Thus, the improved accuracy of clinical N status observed in our study is beneficial for

decision-making regarding preoperative treatment for gastric cancer patients.

In conclusion, the optimal cut-off values of lymph node size for diagnosing metastasis differed with histological type and location. The diagnostic accuracy of lymph node metastasis can be improved by using individual cut-off values based on the regions of lymph node location.

Acknowledgements The scientific guarantor of this publication is Yuichiro Doki. The authors of this manuscript declare no relationships with any companies whose products or services may be related to the subject matter of the article. The authors state that this work has not received any funding. No complex statistical methods were necessary for this paper. Institutional Review Board approval and written informed consent were not required because this is a retrospective diagnostic study.. A written informed consent for preoperative staging with MDCT was obtained from all patients. Methodology: retrospective diagnostic or prognostic study, performed at one institution

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FULL PAPER

BJC

British Journal of Cancer (2015) 112, 352–356 | doi: 10.1038/bjc.2014.609

Keywords: gastric cancer; circulating tumour DNA; next-generation sequencing; deep sequencing

Monitoring gastric cancer progression with circulating tumour DNA

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Background: Circulating tumour DNA (ctDNA) is an emerging candidate biomarker for malignancies and may be useful for monitoring the disease status of gastric cancer.

Methods: We performed targeted deep sequencing of plasma cell-free DNA (cfDNA) by massively parallel sequencing in patients with tumours harbouring *TP53* mutations. The quantitative values of *TP53*-ctDNA during the clinical course were compared with the tumour status.

Results: Three out of ten patients with *TP53* mutations in primary tumours showed detectable *TP53* mutation levels in preoperative cfDNA. Although the cfDNA concentrations were not always reflective of the disease course, the ctDNA fraction correlated with the disease status.

Conclusions: ctDNA may serve as a useful biomarker to monitor gastric cancer progression and residual disease.

Computed tomography (CT) is the major diagnostic tool for clinical management of gastric cancer. However, minimal residual disease and peritoneal dissemination, the most frequent recurrent patterns in gastric cancer, are typically difficult to detect with CT. Circulating tumour DNA (ctDNA)—fragmented DNA released from cancer cells into the bloodstream—is an emerging candidate marker to follow disease progression (Schwarzenbach *et al*, 2011). Previous studies have indicated that ctDNA levels correlate with tumour burden in colon cancer (Diehl *et al*, 2008) and breast cancer (Dawson *et al*, 2013). However, no comparative studies have been conducted in gastric cancer, and it is necessary to examine the possibility that ctDNA can be used for monitoring disease status, especially minimal residual disease and peritoneal dissemination. We previously developed a system to detect *EGFR* mutations in the plasma of lung cancer patients using a massively parallel sequencer (Kukita *et al*, 2013) and applied it to monitor advanced gastric cancer.

MATERIALS AND METHODS

Sample collection. Gastric cancer tissue and blood were prospectively collected from 42 patients who underwent gastrectomy for advanced gastric cancer at the Osaka University Hospital from April 2011 to November 2012. Tumour tissue was obtained from the primary lesion of the resected specimen immediately after surgery and stored at −80 °C as a fresh-frozen sample. Peripheral blood (5 ml) was drawn using an EDTA-2Na tube at admission (before surgery), before discharge (after surgery), and, in three patients, after recurrence. After removal of cellular debris and platelets by centrifugation, the plasma samples were stored at −80 °C until DNA extraction. Written informed consent for this study was obtained from all patients. The study was approved by the Ethics Committees of the Osaka University and the Osaka Medical Center for Cancer and Cardiovascular Diseases.

Mutation detection in the primary tumours. DNA was extracted using a DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, using serial 40-nm sections. *TP53* exons 4–10 were amplified by PCR (Supplementary Table 1), and direct sequencing was performed with an ABI3730xl/ABI3100 using BD v3.1, following the EXO-1/SAP procedure.

Mutations in other genes were searched using Ion AmpliSeq Cancer Panel, version 1 (Life Technologies, Carlsbad, CA, USA),

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Received 17 March 2014; revised 18 August 2014; accepted 4 November 2014; published online 9 December 2014

targeted for 739 hot-spot mutations in 604 loci in the 46 cancer-related genes, following the supplier's protocol.

Deep sequencing of the amplified target regions. Cell-free DNA was extracted from 1.2 ml of plasma using a Circulating Nucleic Acids Kit (Qiagen). DNA was eluted in a final volume of 40 μl. A volume of 10 μl was used for a single sequencing reaction. DNA concentrations were measured using a method based on LINE-1 copy number (Rago *et al*, 2007). The PCR primers were designed to generate final products ~100 base pairs in length (Supplementary Table 2).

TP53 target regions were amplified from patient cell-free DNA or genomic DNA purified from the white blood cells obtained from normal individuals. They were then sequenced using a semiconductor sequencer as previously described (Kukita *et al*, 2013), maintaining the number of reads as >100 000. Data analysis was performed as described.

RESULTS

Selection of patients for the assessment of gastric cancer progression. Given that gastric cancer somatic mutations occur most frequently in *TP53*, we chose *TP53* as the target gene. Direct sequencing of the *TP53* gene revealed that 10 (24%) of the 42 tumours analysed harboured mutations (Supplementary Table 3). Because cell-free DNA was available before surgical resection in 6 of these 10 cases, we performed cell-free DNA deep sequencing to evaluate the mutations identified in each primary tumour (Supplementary Figure 1, Supplementary Table 4). Given that one plasma sample corresponding to 1 ml of whole blood typically includes cell-free DNA corresponding to ~5000 genomes deduced from the mean concentration in our lung cancer study (Kukita *et al*, 2013), base changes of <0.02% are considered background. Mutations in three of the six cases fulfilled this criterion. It may be noted that the number of reads with base changes of excluded cases did not exceed that corresponding to a single molecule estimated from the amount of DNA used for sequencing.

Detection threshold values were based on deep sequencing data from 24 normal samples as described previously (Kukita *et al*, 2013). We used DNA purified from white blood cells, because the error profile of white blood cells was not substantially different from that of cell-free DNA in our previous study. The threshold values obtained for base changes in 100 000 reads were as follows: 603 for c.103delT (patient 4), 11 for c.747G>C (patient 5), and 39 for c.166G>T (patient 8; Supplementary Table 5). Because the initial levels of all three cases exceeded the threshold, these cases were used for the follow-up study.

Comparison of cell-free DNA and ctDNA as indicators of disease progression. The median concentration of extracted cell-free DNA of all samples from the three cases was 43.1 ng ml^{-1} plasma (range 9.5–1338 ng ml^{-1}). The mutant fraction range was 0.001–77.8% (median 0.90%).

First, we compared cell-free DNA and ctDNA dynamics for the ability to monitor the treatment response (Figure 1). The cell-free DNA values fluctuated in all but one of the patients, regardless of the disease course. In one patient (patient 8), cell-free DNA increased along with the rapid progression of multiple liver metastases. In this patient, the majority of the cell-free DNA was derived from the tumour. By contrast, ctDNA dynamics were concordant with disease progression.

ctDNA validation using additional mutant genes. For the three cases, mutations in genes other than *TP53* were surveyed with the Ion AmpliSeq Cancer Panel. The results are shown in Supplementary Table 6. We focused on *PIK3CA* c.1633G>A (found in patients 4 and 5) and *FBXW7* c.1177C>T (found in patient 8) and confirmed these mutations by direct sequencing (Supplementary Table 1). We analysed the same plasma samples, which were subjected to *TP53* analysis, by deep sequencing of the new genes. ctDNA fraction values estimated from the assay

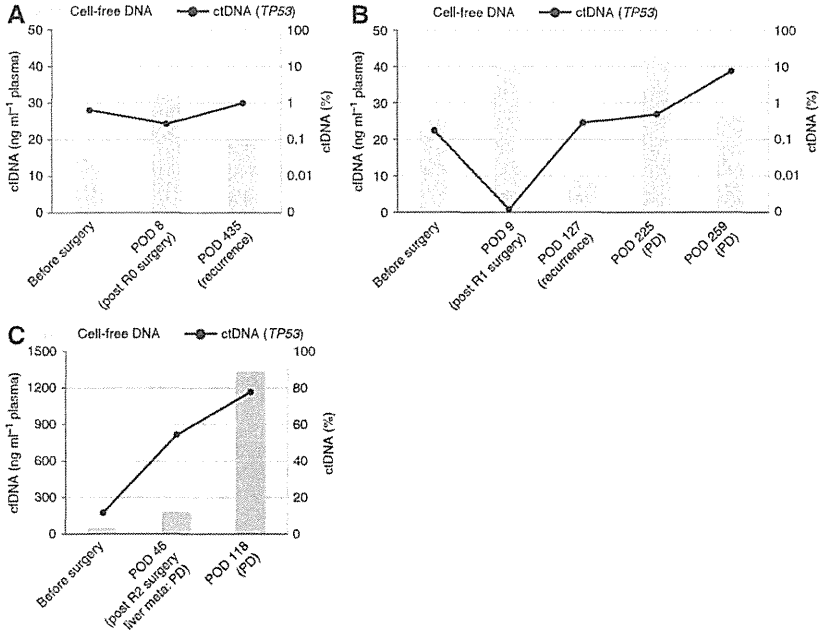

Figure 1. Comparison of cell-free DNA and ctDNA with regard to treatment response. The levels of cell-free DNA (cfDNA; grey bar) and ctDNA (line graph) at various time points in patients 4 (**A**), 5 (**B**), and 8 (**C**) are displayed. The vertical axis on the left represents the concentration of cfDNA, and the vertical axis on the right represents the *TP53*-mutant fraction in cfDNA. The horizontal axis labels represent the time points of plasma sampling and tumour status (in parenthesis). The presence or absence of tumour after surgery is described by the R status (R0: no residual tumour, R1: microscopic residual tumour, R2: macroscopic residual tumour). Abbreviations: POD: postoperative day; PD: progressive disease.

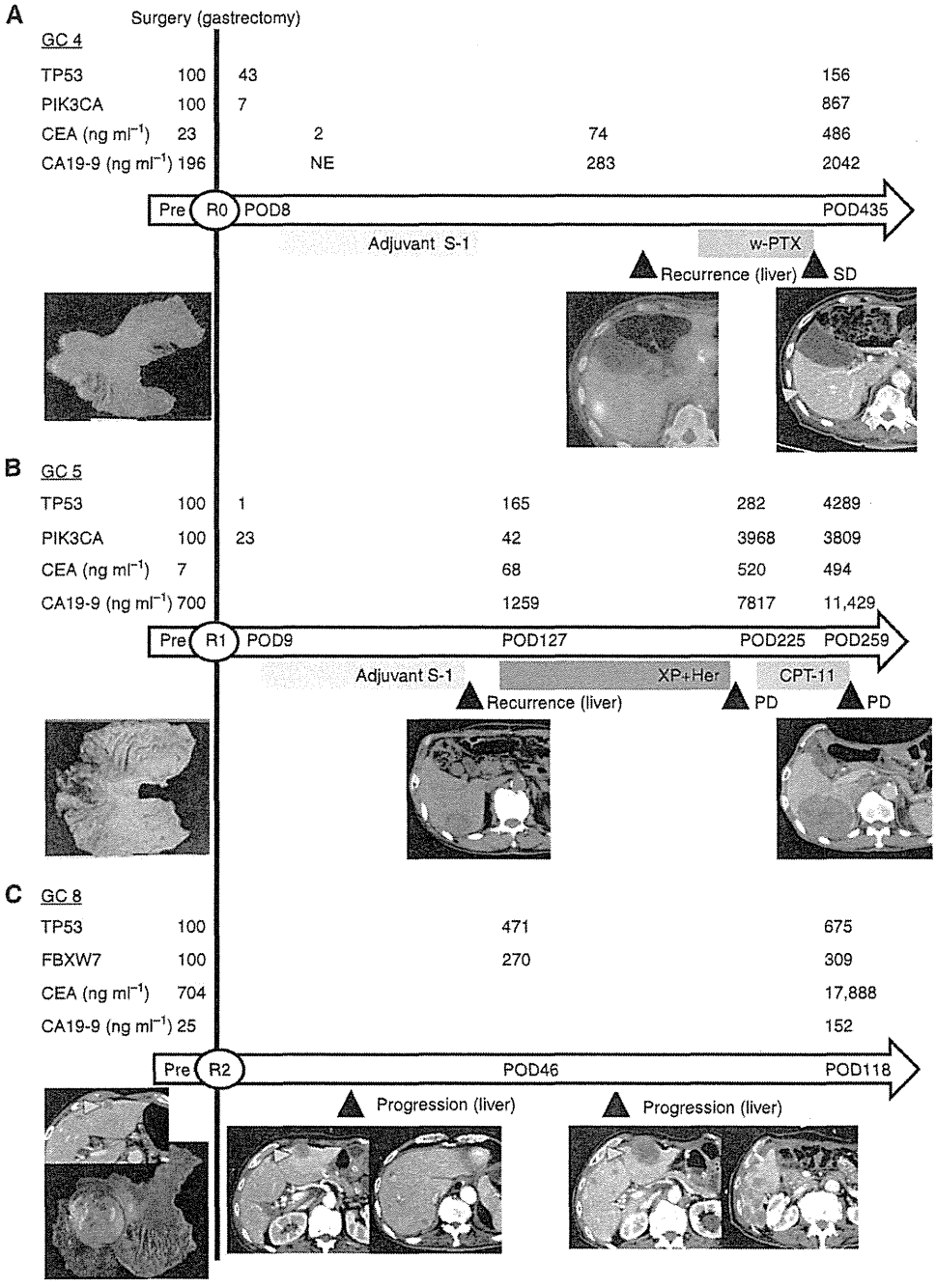

Figure 2. The relationship between ctDNA and clinical observations during the treatment course. ctDNA values during the clinical course are depicted in comparison with serum tumour markers, treatment, response, and tumour burden (specimen photos and radiological images). The horizontal arrows indicate the time scale for each case. The day of gastrectomy is represented by the vertical line. The presence or absence of tumour after surgery is described by the R status (R0: no residual tumour, R1: microscopic residual tumour, R2: macroscopic residual tumour). The intervals from surgery are indicated inside the arrows. Chemotherapy is depicted by colour bars under the time scale. The specimen photos depict the resected stomach, representing the tumour burden before surgery. CT (**A**, **B**, and **C**) and positron emission tomography (PET)-CT (**A**) images display the status of metastatic liver tumours at each assessment point, indicated by triangle markers. In each case, ctDNA is expressed as the percent relative to the ctDNA values before surgery. POD: postoperative day, PD: progressive disease, XP: capecitabine + cisplatin, Her: trastuzumab, CPT-11: irinotecan, w-PTX: weekly administration of paclitaxel. (**A**) The ctDNA fraction was measured for both *TP53* (c.103delT) and *PIK3CA* (c.1633G>A). (**B**) The ctDNA fraction was measured for both *TP53* (c.747G>C) and *PIK3CA* (c.1633G>A). (**C**) The ctDNA fraction was measured for both *TP53* (c.155G>T) and *FBXW7* (c.1177C>T).

results of two different genes (*TP53* and *PIK3CA/FBXW7*) were within one order of magnitude in 8 (73%) of the 11 samples, suggesting a good dynamic concordance of the different genes (Figure 2).

The relationship between ctDNA and clinical findings during the clinical course of gastric cancer. The clinical and genetic characteristics of the three cases are summarised in Table 1. CT images were evaluated using the Response Evaluation Criteria in

Table 1. Clinical and genetic characteristics of three cases assessed by follow-up study

Patient number.	Age	Gender	Histological type	p-Stage	Residual tumour after surgery	Gene mutation 1	Gene mutation 2	Postoperative chemotherapy	Metastasis site, number, timing	Treatment for metastasis	Response[a]
4	86	Male	Differentiated	IIIC	R0	TP53 c.103delT	PIK3CA c.1633G>A	Yes	Liver, single, POD275	Chemotherapy	SD
5	73	Male	Differentiated	IV[b]	R1	TP53 c.747G>C	PIK3CA c.1633G>A	Yes	Liver, multiple, POD93	Chemotherapy molecular-target drug	PD
8	80	Male	Differentiated	IV[c]	R2	TP53 c.166G>T	FBXW7 c.1177C>T	No	Liver, multiple, (residue)	None	PD

Abbreviations: PD = progressive disease; POD = postoperative day; R1 = microscopic residual tumour; R2 = macroscopic residual tumour; R0 = no residual tumour (curative resection); SD = stable disease.
[a]According to the Response Evaluation Criteria in Solid Tumors (RECIST).
[b]Peritoneal cytology positive for carcinoma cells, and
[c]Hepatic metastasis.

Solid Tumors (RECIST; Eisenhauer *et al*, 2009). The alterations in the ctDNA levels and clinical disease course are presented in Figure 2. Details of individual cases are provided below.

Case 1 (patient 4). Curative distal gastrectomy was performed for a 55-mm tumour with multiple lymph node metastases, but the patient suffered from a single liver metastasis. ctDNA of *TP53* c.103delT that was 0.63% before surgery, decreased to 0.27% (below the background noise level) after surgery, and increased to 0.98% after recurrence. Similar alterations were observed in the *PIK3CA* c.1633G>A ctDNA.

Case 2 (patient 5). Distal gastrectomy was performed for a 65-mm tumour with multiple lymph node metastases. Intraoperative peritoneal cytology was positive. Despite postoperative S-1 chemotherapy, the patient developed multiple liver metastases. Recurrent tumours were treated with chemotherapy, but the disease progressed. ctDNA levels of *TP53* c.747G>C and *PIK3CA* c.1633G>A were 0.17% and 0.84%, respectively, before surgery. ctDNA levels decreased after resection and increased after recurrence and tumour progression in both the genes. However, there was some discrepancy between the two genes after the use of trastuzumab.

Case 3 (patient 8). A Stage IV gastric cancer with a 100-mm lymph node metastasis was removed by palliative gastrectomy. A 17-mm liver metastasis was left unresected. The liver tumours grew rapidly in size and number after surgery. The ctDNA levels of *TP53* c.166G>T and *FBXW7* c.1177C>T were 11% before surgery and continually increased with tumour progression, reaching 77.8%.

DISCUSSION

In this report, we demonstrated that concentrations of ctDNA, not total plasma DNA, exhibited good correlation with the disease status of gastric cancer. We observed a decrease in ctDNA levels after surgical resection, indicating its potential application for the detection of residual disease. Tie *et al* (2014) demonstrated the possibility that recurrence of stage II colorectal cancer after surgical resection may be predicted by ctDNA. Similar applications may be possible for gastric cancer cases. Serological biomarkers such as CA19-9 and CEA are used to assist in the diagnosis of CT, but they have limitations owing to their low sensitivity (Shimada *et al*, 2013). In our study, ctDNA levels were parallel with these markers, suggesting a comparable ability to monitor the disease progression. However, from our analysis, it is still unknown whether ctDNA is advantageous over serological biomarkers because of our small number of patients.

There have been several studies on cell-free DNA obtained from the blood of gastric cancer patients, using concentration (Park *et al*, 2012), *MYC/GAPDH* ratio (Park *et al*, 2009), and promoter methylation status of *MGMT*, *p15*, and *hMLH1* (Kolesnikova *et al*, 2008). These markers are not necessarily specific to cancer cells and therefore lack theoretical backgrounds for their validity. By contrast, cancer-related somatic mutations such as those in *TP53* are specific to malignancies, and the DNA with these mutations is indicative for the presence of malignancies. Our results also agree with this reasoning; levels of the somatic mutations were correlated with surgical resection and disease progression, but concentrations of cell-free DNA were not.

The recent attention to ctDNA is due mainly to technical developments, especially that of digital PCR pioneered by BEAMing (Dressman *et al*, 2003). It is important to note that massively parallel sequencers may be regarded as digital PCR because they employ digital PCR in the template preparation step after global amplification of target DNA. Because of the much higher throughput of data production than with other digital PCR technologies and the considerable investment, the massively parallel sequencers should be the method of choice if several technical problems, such as read error (Junemann *et al*, 2013), are resolved.

ACKNOWLEDGEMENTS

This work was partly supported by KAKENHI 23591928.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)

Gastric Cancer (2014) 17:200–205
DOI 10.1007/s10120-013-0300-8

REVIEW ARTICLE

Clinical application of ghrelin administration for gastric cancer patients undergoing gastrectomy

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Received: 25 March 2013 / Accepted: 30 August 2013 / Published online: 20 September 2013

Abstract Loss of body weight is a common (and the most serious) sequela after gastrectomy. It impairs quality of life, increases various diseases including infection, and may affect long-term survival. Ghrelin, an intrinsic ligand of the growth hormone secretagogue receptor, was discovered in the stomach in 1999. In addition to growth hormone secretion, ghrelin has pleiotropic functions including appetite stimulation, increasing bowel movement and absorption, and anti-inflammatory reactions. In consequence, ghrelin comprehensively leads positive energy balance and weight gain. The fundic gland of the stomach produces the majority of ghrelin, and plasma ghrelin declines to 10–30 % of the preoperative level after total gastrectomy and 50–70 % after distal gastrectomy. Although plasma ghrelin is never restored after total gastrectomy, it gradually recovers to the preoperative level within a few years after distal gastrectomy. Chronic gastritis due to *Helicobacter pylori* infection and vagotomy are additional factors that perturb the ghrelin secretion of gastric cancer patients after gastrectomy. A randomized clinical trial that revealed that recombinant ghrelin administration successfully increased both food intake and appetite, and ameliorated weight loss after total gastrectomy. Ghrelin administration could thus be a promising strategy to transiently improve the nutritional status of patients who who have undergone gastrectomy, but its effect in the long term remains unclear. Further studies are warranted to elucidate the mechanism of ghrelin and to create and evaluate the analogs that could be administered orally or subcutaneously.

Keywords Ghrelin · Gastrectomy · Gastric cancer · Weight loss

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Introduction

Loss of body weight is a common, serious outcome in patients with gastric cancer who have undergone gastrectomy. It correlates well with a decline in postoperative quality of life and is the most reliable indicator of malnutrition, which impairs immune function, infection susceptibility, and survival [1–3]. Although various mechanisms have been considered, such as the perturbation of absorption due to reduced pancreatic excretion [4, 5], a decrease in the gastric acid level [6], reflux esophagitis [7], intestinal floral alteration [8], and increased peristalsis and diarrhea [9], reduced food intake [10, 11] is the most conceivable explanation for weight loss after gastrectomy. To combat loss of appetite, surgeons dealing with gastric cancers have tried to increase food intake by producing a gastric substitute, such as a jejunal pouch, with limited success [12]. However, we frequently observe that patients do not exhibit significant weight loss after total gastrectomy when they resort to small but frequent meals. Another study indicated that the majority of patients with total gastrectomy were able to eat as much food as healthy subjects under a regulated program [13].

Taken together, we can conclude that (1) patients who have undergone gastrectomy have the ability to maintain body weight when food intake is adequately performed; (2) only loss of storage volume cannot account for reduced

food intake after gastrectomy; (3) there is a relatively large change in eating behavior after gastrectomy that is controlled by an unknown mechanism. In this review, we discuss ghrelin and research about its clinical applications.

The discovery of ghrelin and its features

Ghrelin is a peptide hormone that was discovered in 1999 as an endogenous ligand for the growth hormone (GH)-secretagogue receptor (GHS-R). The 28-amino-acid ghrelin peptide is the endogenous ligand for GHS-R1a, which stimulates GH release from the pituitary gland [14]. X/A-like cells of the oxyntic glands in the stomach produce the majority of ghrelin, and smaller amounts are secreted by other organs, such as the intestine, pancreas, kidney, and hypothalamus [15, 16]. Ghrelin has several physiological functions in addition to the secretion of GH, including the promotion of the appetite signal that antagonizes leptin in the hypothalamus [17], stimulation of gastrointestinal activity (e.g., peristalsis, gastric acid secretion, and pancreatic excretion through the vagal nerves) [18], and regulation of fat metabolism [19] (Table 1). Ghrelin also mitigates proinflammatory cytokine production and attenuates the stress signal [20]. Ghrelin exists as two major molecular forms: acyl ghrelin and des-acyl ghrelin. Ghrelin is octanoylated at Ser3, an unusual post-translational modification that is catalyzed by the enzyme ghrelin O-acyltransferase (GOAT) [21, 22]. Des-acyl ghrelin, which lacks the Ser3 residue octanoylation, is unable to release GH or bind to the classic GHS-R1a receptor [23]. These characteristics indicate that octanoic acid plays an important role in physiological activity via GHS-R1a, and des-acyl ghrelin has been considered an inactive form of ghrelin.

Ghrelin peptide is the only gastrointestinal hormone known to stimulate appetite. A randomized double-blind study of healthy volunteers demonstrated that ghrelin enhances appetite and increases food intake [24, 25]. Several clinical trials of patients with heart failure [26], pulmonary disease [27], cancer cachexia [28], or undergoing chemotherapy [29] concluded that ghrelin successfully improved their diseases along with increased oral food intake and body weight. In the field of surgical treatment for obesity, reduced ghrelin levels after sleeve gastrectomy are associated with successful weight loss and appetite suppression [30]. Taken together, the discovery of ghrelin allows the proposal of a new concept, body weight regulation by the stomach, which can be applied to various diseases with malnutrition.

Gastrectomy and ghrelin secretion

Fundic glands in the stomach produce the majority of ghrelin. Patients with resected gastric cancer experience low plasma ghrelin concentrations. Table 2 lists studies of the change in ghrelin concentration after gastrectomies [31–37]. In total gastrectomy patients, ghrelin concentrations were immediately reduced to 12–29 % of the preoperative concentration. In contrast, ghrelin concentrations decreased to 39–71 % of the preoperative concentration immediately after distal gastrectomy. These reductions in ghrelin concentration are a direct result of the fact that most ghrelin is produced by A-like cells in the fundic gland of the stomach. In fact, sleeve gastrectomy for bariatric surgery immediately results in a 67 % reduction in the concentration of ghrelin [38]. *H. pylori* infection also markedly reduces ghrelin-producing cells and plasma ghrelin.

Table 1 Physiological functions of ghrelin

Orexigenic effect via the hypothalamus [17, 58, 59]
Ghrelin, which increases c-fos expression in the arcuate nucleus, also activates hypothalamic neuropeptide Y (NPY)/Y1 receptors and agouti-related peptide (AgRP) pathways
Stimulation of GH secretion from the pituitary gland [17, 60–62]
Ghrelin is involved in GH release in a non-acute setting. GH regulates IGF-I levels, promotes anabolism, and increases muscle strength. Ghrelin enhances lipolysis via GH and stimulates protein synthesis, myoblast differentiation, and muscle growth via IGF-1
Antiinflammatory action [20, 63, 64]
Ghrelin inhibits the activation of NF-κB, a transcription factor known to control the production of multiple proinflammatory cytokines during inflammatory insults
Stimulation of gastrointestinal peristalsis [18]
Ghrelin acts on motor neurons in the myenteric plexus, activates a vago-vagal reflex, or may stimulate central pathways
Augmentation of cardiac output and reduction of blood pressure [26]
Ghrelin improves myocardial structure and function in chronic heart failure (CHF) via its GH-releasing effects
Inhibition of insulin secretion [65, 66]
Ghrelin has obesogenic/diabetogenic properties. These properties may be direct effects of ghrelin on pancreatic islet function and/or indirect effects through the modulation of GH secretion

Table 2 Representative reports of changes in ghrelin concentration in patients who have undergone gastrectomy

References	Procedure	Number of cases	Preoperative ghrelin level	% Postoperative decline of ghrelin concentration from baseline	
				Short term (%)	Long term (%)
Jeon et al. [32]	DG	24	Active 276 pg/ml (82.7 fmol/ml[a])	Day 1 51 Day 7 88	
Takachi et al. [35]	DG	38	Total 95 fmol/ml	Day 3 39	3 years 77
Wang et al. [36]	DG (B-I)	23	Active 468 pg/ml (138.8 fmol/ml[a])	Day 1 37 Day 7 51	1 year 93
Wang et al. [36]	DG (B-II)	19	Active 460 pg/ml (136.5 fmol/ml[a])	Day 1 36 Day 7 51	1 year 82
Kim et al. [34]	DG	45	Total 310 pg/ml (92 fmol/ml[a])	Day 2 71	3 months 81
Kamiji et al. [33]	DG	14	Active 993 pg/ml (294.2 fmol/ml[a])	–	6 years 77
Jeon et al. [31]	DG	18	Active 113 pg/ml (33.5 fmol/ml[a])	Day 1 50 Day 7 85	1 year 57
Zub-Pokrowieckae al. [37]	DG	10	Active 293 pg/ml (86.9 fmol/ml[a])	–	4–5 years 82
Jeon et al. [31]	TG	12	Active 390 pg/ml (115.7 fmol/ml[a])	Day 1 29 Day 7 30	–
Takachi et al. [35]	TG	26	Total 95 fmol/ml	Day 3 12	3 years 20
Kamiji et al. [33]	TG	7	Active 993 pg/ml (294 fmol/ml[a])	–	3–5 years 51
Zub-Pokrowiecka et al. [37]	TG	10	Active 293 pg/ml (86.9 fmol/ml[a])	–	4–5 years 46
Jeon et al. [32]	PG	4	Active 427 pg/ml(126.7 fmol/ml[a])	Day 1 25 Day 7 48	–

TG total gastrectomy, *DG* distal gastrectomy, *PG* proximal gastrectomy, *B-I* Billroth-I reconstruction, *B-II* Billroth-II reconstruction

[a] x pg/l was converted to x/3.3709 fmol/ml

Generally, patients with gastric cancer and atrophic gastritis have a low basal level of ghrelin. Therefore, the degree of decline caused by gastrectomy can be considered low. Ghrelin concentrations recover relatively soon after surgery; many studies have shown that at 7 days after surgery, the ghrelin concentrations of patients with distal gastrectomy were 51–88 % of preoperative levels. In the long term, postoperative plasma ghrelin levels sometimes approach preoperative levels in patients who have undergone distal gastrectomy. It has been reported that the number of ghrelin-producing cells does not increase after gastrectomy [39]. Persistent low body weight after gastrectomy might stimulate ghrelin secretion from individual ghrelin-producing cells in a negative feedback manner. In contrast, the plasma ghrelin concentrations of patients who have undergone total gastrectomy do not rebound to normal levels if the patients suffer from continuous malnutrition [35]. Although ghrelin is produced by organs other than the stomach, those sources cannot sufficiently compensate for the disappearance of ghrelin-producing cells in the stomach.

Vagotomy and ghrelin response

Both anterior and posterior vagal trunks were usually resected during gastrectomy for gastric cancer, especially in order to complete D2 lymph node dissection. Therefore, we should consider the influence of truncal vagotomy on ghrelin signals in both afferent and efferent pathways. In the rodent, vagotomy alone has led to the significant reduction of the baseline of fasting plasma ghrelin [40]. After radical esophagectomy for esophageal cancers (which includes truncal vagotomy and reconstruction of the whole gastric tube), ghrelin secretion in human patients was reduced by one-half compared to preoperative levels and gradually recovered within a few years [41, 42].

Vagotomy also perturbs the normal ghrelin secretion response (i.e., significant decline immediately after oral food intake). Pekic et al. [43] performed an oral glucose tolerance test (OGTT) in gastrectomized/vagotomized patients and BMI-matched control patients. Plasma ghrelin levels decreased significantly during the OGTT in control subjects, while no reduction was detected in gastrectomized-vagotomized patients. We frequently employ distal gastrectomy, which preserves the celiac branch of the vagal nerve. The downregulation of plasma ghrelin by food intake was significantly greater in patients with vagal nerve preservation than in patients with complete vagotomy (unpublished observation).

With respect to the efferent pathway, there is a report that the administration of exogenous ghrelin stimulated GH

secretion in vagotomized patients as much as in normal subjects [44]. Increases in appetite and amount of food intake after ghrelin administration are reportedly less significant in vagotomized patients than in control patients [45]. However, other studies in rats reported that ghrelin successfully stimulated food intake after vagotomy when administered intraperitoneally [46]. Moreover, in our previous study, intravenous administration of exogenous ghrelin successfully stimulated food intake and appetite immediately after total gastrectomy and esophagectomy [47, 48]. Our findings suggested that the administered ghrelin crossed the blood-brain barrier to the central nervous system, likely increasing the appetite signal through both the vagal pathway and the circulatory system.

As a whole, vagotomy definitely damages the normal control of ghrelin secretion. However, the relationship between ghrelin and vagotomy remains poorly defined in the output system of endogenous and exogenous ghrelin. Therefore, we cannot draw conclusions about the influence of vagotomy on the biological effects of ghrelin, although GH secretion and appetite stimulation may be differently involved with the vagal nerve. Further observation and experiments are required to clarify this issue.

Effects of ghrelin administration after total gastrectomy

Because the anabolic effect of ghrelin is apparent, the possible clinical applications of ghrelin in the context of various cachexic states (e.g., anorexia nervosa, heart failure, chronic obstructive pulmonary disease, and the terminal stage of unresectable cancers) should be considered. These studies have demonstrated increases of oral food intake and body weight in both humans and rats. The two species do differ with regard to body composition. For example, ghrelin administration tended to increase fat volume in the rat, while muscle weight and muscle power have been increased more than fat volume in humans.

There are two large differences in the rationale of ghrelin administration with respect to the cachexic states listed above and the post-gastrectomy state. By various means, cachexia has consistently exhibited high plasma ghrelin concentrations combined with weight loss as the result of negative feedback; the effect of exogenous ghrelin may be restricted if the ghrelin signals are already saturated by endogenous ghrelin. In contrast, the post-gastrectomy state is associated with low plasma ghrelin combined with significant weight loss. Therefore, in the latter context it appears reasonable to administer exogenous ghrelin to compensate for reduced endogenous ghrelin. In this respect, we can expect more significant ghrelin effects in gastrectomy patients than in cachexic patients. Another concern is the influence of vagotomy, which, as described in the previous section, might minimize the effect of ghrelin in gastrectomy patients.

There is a randomized, phase II study [47] in which 21 patients undergoing total gastrectomy were assigned to groups receiving ghrelin ($n = 11$) or a placebo ($n = 10$). In the 10 days after starting oral food intake (postoperative days 5–7), an intravenous drip infusion of synthetic human ghrelin (3 μg/kg) or placebo (pure saline) was administered twice daily (before breakfast and before dinner). The mean intake over the 10-day period represented a 32.7 % increase in the ghrelin group compared with the placebo group (13.8 vs. 10.4 kcal/kg/day). At the end of the study period, weight loss was 3.7 % for the placebo group compared with 1.4 % for the ghrelin group. They used dual-energy X-ray absorptiometry to measure body composition. Fat mass, lean body mass and basal metabolic rate decreased significantly in the placebo group; however, the reductions in lean body mass and basal metabolic rate were not significant in the ghrelin group, although the reduction of fat mass was significant. Therefore, exogenous ghrelin lessened weight loss, especially the loss of lean body mass. There were no significant side effects; however, one patient experienced grade 1 diaphoresis. Several months after the trial, there was no between-group difference in weight or appetite. The most critical drawback is that they are currently only able to administer ghrelin intravenously. For long-term administration, another delivery system (e.g., subcutaneous injection or inhalation) should be developed [49]. Oral ghrelin analog, which is already in clinical trials, is a possible ghrelin substitute.

As ghrelin is also a potent GH secretagogue, there are concerns about GH-mediated stimulation of tumor growth, especially regarding treatment of cancer patients. In vitro studies suggest that ghrelin may enhance the proliferation of prostate [50] and pancreatic [51] cancer cells, but not of a lung cancer cell line, where it induced dose-dependent inhibition of cell proliferation and increased apoptosis [52]. Some tumors from archival samples express ghrelin [53], whereas others (gastric cancer and esophageal cancer) do not [54]. According to a review that analyzed ghrelin administration studies, there was no report of anyone suffering from new cancer as an adverse event among 1,850 participants who were registered to 121 studies. [55–57].

Conclusion

Although our prospective randomized study had a limited number of patients and short-term observation periods, it revealed the beneficial effects of the administration of exogenous ghrelin on body weight and oral intake after total gastrectomy. Although there are issues that must be resolved before clinical application, including elucidation of the

duration of administration and adequate assessment of clinical benefits, surgeons dealing with gastric cancers should be encouraged by the availability of ghrelin. Although decline of ghrelin is certain to play a major role in appetite loss after gastrectomy, it cannot account for all causes that lead to body weight loss. Some patients continue to weigh less even after the amount of food intake has recovered, possibly because of vagotomy, defective fat absorption due to pancreatic insufficiency, bacterial overgrowth, and shortened small bowel transit time [13]. Although surgery is essentially non-physiological and highly invasive, it remains the most reliable therapeutic option to cure cancer. Therefore, it is our obligation to invent new procedures to minimize postoperative side effects.

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