Table 2: Diagnostic performance of ^{18}F-FDG PET/CT and ultrasonography in axillary staging

^{18}F-FDG uptake	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Visual assessment	34	118	6	25	57.6	95.2	85	82.5	83.1
SUV cutoff point									
0.8	30	118	6	29	50.8	95.2	83.3	80.3	80.9
1.3	24	122	2	35	40.7	98.4	92.3	77.7	79.8
1.5	21	123	1	38	35.6	99.2	95.5	76.4	78.7
1.8	21	124	0	38	35.6	100	100	76.5	79.2
2	20	124	0	39	33.9	100	100	76.1	78.7
3	16	124	0	43	27.1	100	100	74.3	76.5
AUS	32	123	1	27	54.2	99.2	97	82	84.7
Visual assessment of ^{18}F-FDG uptake Combined with AUS	38	117	7	21	64.4	94.4	84.4	84.8	84.7

AUS, Axillary ultrasonography; TP, True positive; TN, True negative; FP, False positive; FN, False negative; PPV, Positive predictive value; NPV, Negative Predictive value;

The accuracy of diagnosis of ^{18}F-FDG PET/CT was compared among various SUV cut-off points ranging from 0.8 to 3.0, using entire data set of 183 patients.

When a SUV cut-off points were set from 0.8 up to 1.8, specificity increased from 95% to 100%, but sensitivity decreased from 51% to 36%. As SUV increased over 1.8, specificity of 100% did not vary, but sensitivity further decreased. When the SUV was 1.8, PPV, NPV and accuracy were 100%, 77%, and 79%, respectively. Therefore, the SUV of 1.8 achieved excellent specificity and PPV, but low sensitivity in comparison with visual assessment.

Ultrasonography detected 33 (18%) AUS-positive patients and 150 (82%) AUS-negative patients. Of the 33 AUS-positive patients, thirty-two patients (97%) were truely positive, and one patient (3%) was false-positive. Of the 150 AUS-negative patients, 123 (82%) were truely negative, whereas 27 (18%) were false-negative. Sensitivity, specificity, PPV, NPV, and accuracy were 54, 99, 97, 82, and 85%, respectively.

Combined with visual assessment of ^{18}F-FDG uptake and AUS, 138 (75%) patients with double-negative ^{18}F-FDG uptake and AUS were considered to be nodal negative, and 45 (25%) patients with positive finding in the visual assessment of ^{18}F-FDG uptake and/or AUS were considered to be nodal positive. Sensitivity, specificity, PPV, NPV, and accuracy of the combination were 64, 94, 84, 85, and 85%, respectively.

Feasibility of SNB for patients having negative AUS

Of the 150 patients having negative AUS, 125 (83%) consented and underwent SNB. Table 3 shows diagnostic performance of SNB in axillary staging in AUS-negative patients. SNB identification rate was 99.2% (124 of 125 patients). Twenty five patients (20%) had axillary nodal metastasis in permanent pathology. Intraoperative pathological diagnosis of metastasis in SNB was accurately performed in 123 (99%) of 124 patients. One patient (1%) was false negative by frozen section intraoperatively, but a micrometastatic deposit was postoperatively detected in one of sentinel nodes by permanent histology. With regard to intraoperative pathological diagnosis of metastasis in SNB, sensitivity, specificity, PPV, NPV and overall accuracy were 96, 100, 100, 99, and 99% respectively in Table 3A.

In the 12 AUS-negative but ^{18}F-FDG uptake positive patients who consented and recieved SNB, 6 (50%) had ALN involvement. One (8%) patient was false negative. With regard to intraoperative pathological diagnosis of SLN, sensitivity, specificity, PPV, NPV, and overall accuracy were 83, 100, 100, 86, and 92%, respectively in Table 3B.

In the 112 AUS-negative and ^{18}F-FDG uptake negative patients, who consented and underwent SNB, 19 (17%) had ALN involvement. With regard to intraoperative pathological diagnosis of SNB, sensitivity, specificity, PPV, NPV, and overall accuracy were all 100% in Table 3C.

Axillary nodal clinicopathological factors correlated with ^{18}F-FDG uptake

Table 4 shows correlation of axillary ^{18}F-FDG uptake with nodal clinicopathological factors of 59 patients having ALN involvement. The maximum size and nuclear grade of involved ALN were significantly correlated with ^{18}F-FDG uptake at SUV cut-off point 1.8 (p = 0.006 and 0.03, respectively). The number of involved ALNs was not cor-

Table 3: Diagnostic performance of SNB for axillary staging in AUS-negative patients

		No. of patients		
			Permanent histopathology	
Intraoperative frozen Histopathology		Total	Metastasis positive	Metastasis negative
A.	Total (n = 124)			
	Metastasis positive	24	24	0
	Metastasis negative	100	1	99
	Total	124	25	99
B.	^{18}F-FDG uptake positive (n = 12)			
	Metastasis positive	5	5	0
	Metastasis negative	7	1	6
	Total	12	6	6
C.	^{18}F-FDG uptake negative (n = 112)			
	Metastasis positive	19	19	0
	Metastasis negative	93	0	93
	Total	112	19	93

Note; SNB, sentinel node biopsy; AUS, axillary ultrasound; No., Number; SNB identification rate 99.2% (124 of 125 cases)
Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPN), and accuracy overall were 96, 100, 100, 99, and 99%, respectively in A. Sensitivity, specificity, PPV, NPV, and overall accuracy were 83, 100, 100, 86, and 92%, respectively in B.
Sensitivity, specificity, PPV, NPV, and overall accuracy were 100, 100, 100, 100, and 100%, respectively in C.

related with ^{18}F-FDG uptake at SUV cut-off point 1.8 (p = 0.15).

Categories of ^{18}F-FDG PET/CT combined with ultrasound for indications of ALND and PSC

Table 5 indicates four categories which were divided by clinical findings of ^{18}F-FDG PET/CT and AUS to the axilla. We used a visual assessment of ^{18}F-FDG uptake which proved to be a reproducible method and have acceptable sensitivity and specificity [6]. Of all 183 patients, 138 (75%) patients who had negative AUS with negative axillary ^{18}F-FDG uptake were classified as category 1. Frequency of ALNs involvement was only 21 (15%), 12 (57%) of these 21 patients had single involved ALNs. The maximum size of involved ALNs was 10 mm or smaller in 17 (81%) of 21 patients. The nuclear grade of involved ALNs was grade 1 or 2 in 16 (76%), and grade 3 in only 5 (24%).

Twenteen (7%) patients who had negative AUS but positive axillary ^{18}F-FDG uptake were classified as category 2. In category 2, ALNs involvement was detected in 6 (50%)

Table 4: Axillary nodal clinicopathological factors correlated with ^{18} F-FDG uptake

Clinicopathological factors	SUV cut-off 1.8	No. of pts	Average	SD	p-value
No. of involved ALNs	Low	38	3.6	5	0.15
	High	21	6.9	7	
The maximum size (mm)	Low	38	8.6	6.4	0.006
	High	21	14.6	7.9	
	SUV	No. of pts	Grade	No. ofpts	p-value
Nuclear grade	Low	38	Grade1/2	27	0.03
			Grade3	11	
	High	21	Grade1/2	9	
			Grade3	12	

SUV, Standardized uptake value; No, Number; pts, patients; SD, Standard derivation

Table 5: Categories of ^{18}F-FDG PET/CT combined with ultrasonography for indications of ALND/PSC

Category	1	2	3	4
AUS	-	-	+	+
^{18}F-FDG-uptake	-	+	-	+
No. of involved ALNs				
0	117 (85)	6 (50)	1 (20)	0 (0)
1	12 (9)	5 (42)	2 (40)	9 (32)
2 ≦ Involved ALNs ≦ 5	6 (4)	1 (8)	1 (20)	9 (32)
6 ≦	3 (2)	0 (0)	1 (20)	10 (36)
The maximum size of involved ALNs				
≦ 5 mm	10(48)	5 (83)	0 (0)	0 (0)
5 mm < metastasis < 10 mm	7(33)	1 (17)	2 (50)	6 (21)
10 mm ≦	4(19)	0 (0)	2 (50)	22(79)
Nuclear grade of involved ALNs				
Grade 1 and 2	16(76)	3 (50)	4 (100)	13(46)
Grade 3	5(24)	3 (50)	0 (0)	15(54)
Frequency of involved ALNs	15%	50%	80%	100%
Indications of ALND/PSC	SNB	FNAC/Bx is needed	FNAC/Bx is needed	Acceptable
Total	138 (100)	12 (100)	5 (100)	28 (100)

AUS, Axillary ultrasound; ALN, Axillary lymph node; ALND, ALN dissection; PSC, Primary systemic chemotherapy; No., Number; pts, patients; FNAC, Fine needle aspiration

of 12. A single ALN involvement was present in 5 (83%) of these 6. All 6 patients had ALNs involvement with the maximum size of less than 10 mm in category 2. Nuclear grade was 1 or 2 in 3 (50%) patients, whereas nuclear grade was 3 in 2 (50%).

Five (3%) patients who had positive AUS but negative axillary ^{18}F-FDG uptake were classified as category 3. Twenty-eight (15%) patients who had double-positive nodal status of AUS and ^{18}F-FDG uptake were classified as category 4. Four (80%) of 5 patients in category 3 had ALNs involvement and all patients in category 4 had ALNs involvement. Especially 2-or-more involved ALNs were 2 (50%) of 4 cases in category 3, and 19 (68%) of 28 cases in category 4. The maximum size of involved ALNs was 10 mm or more in 2 (50%) of 4 cases in category 3 and in 22 (79%) of 24 cases in category 4.

Nuclear grade was 1 or 2 in all 4 (100%) cases in category 3, whereas nuclear grade was 1 or 2 in 13 (46%) of 28 cases in category 4.

Diagnostic performance of SNB in ^{18}F-FDG-positive and AUS-negative patients

Table 6 shows diagnostic performance of SNB for axillary staging in ^{18}F-FDG-positive and AUS-negative patients of category 2. Six (50%) of 12 patients had involved SNs and others (50%) had no involved SNs in spite of ^{18}F-FDG uptake. No metastases were found in non-SNs in all patients that had involved SNs and received aubsequent axillary dissection.

Discussion

Visual assessment of ^{18}F-FDG PET/CT for the axillary staging

In visual assessment of ^{18}F-FDG PET/CT to the axilla, we demonstrated that diagnostic accuracy of ^{18}F-FDG PET/CT was almost equivalent to that of AUS for detecting of ALN involvement in patients with primary breast cancer. Visual assessment of ^{18}F-FDG uptake to the axilla achieved higher sensitivity than AUS, and the specificity and PPV of ^{18}F-FDG PET/CT were acceptably high, 95%, and 85%, respectively.

There were 40 (22%) of 183 patients having axillary uptake of ^{18}F-FDG. Six (15%) of these patients had no metastasis of ALNs. The reason of these false positive for the ^{18}F-FDG uptake is not known, but reactive lymphadenopathy caused by breast biopsy would lead to false positive results [9,14].

AUS showed limited sensitivity equal to ^{18}F-FDG PET/CT for detecting ALN involvement, and showed almost perfect specificity and PPV. According to diagnostic perform-

Table 6: Diagnostic performance of SNB for axillary staging in ^{18}F-FDG-positive and AUS-negative patients of category 2

Patients	SUV	Involved SNs/resected SNs	Involved non-SNs/resected non-SNs	Ax dissection
1	8.1	1/1	0/10	Performed
2	2.5	1/1	0/17	Performed
3	2.4	1/2	0/12	Performed
4	1.4	1/3	0/21	performed
5	1.3	4/4	0/12	performed
6	0.7	1/4	0/6	performed
7	1.5	0/1	-	not performed
8	1	0/4	-	not performed
9	1	0/1	0/1	not performed
10	1	0/4	0/1	not performed
11	0.9	0/1	0/2	not performed
12	0.9	0/4	0/1	not performed

AUS, Axillary ultrasound;SUV, Standardized uptake value; SNs, Sentinel nodes, Ax, Axillary

ance of axillary ultrasonography, the present results showed higher outcome than others' previous studies [15-17]. In our previous study, AUS was performed using an SSD-650CL (Aloka, Tokyo, Japan), an old model of SSD-6500, and indicated sensitivity, specificity, PPV, and overall accuracy of 45, 97, 92.6, and 75%, respectively [5]. Furthermore an ultrasound specialist performed axillary investigation in the present study. From these reasons, we considered the present results were superior to those in our previous study.

Differences in criteria for judgment of axillary status or in the type of ultrasound device might have given rise to such inconsistency. Furthermore, although ultrasonography is less-invasive and relatively easy to apply, experienced skills are required to judge AUS-positive nodes. We sometimes wavered in our judgement whether ALNs were positive or not when ultrasound image of lymph nodes was less than 10 mm in diameter but homogeneously hypoechoic in centric area. From these reasons, AUS alone might be difficult to determine axillary staging.

We classified patients into 4 categories of axillary status according to ^{18}F-FDG PET/CT and ultrasonography (Table 5).

Category 1 showed the patients who have AUS-negative lymph nodes without axillary ^{18}F-FDG uptake. Fifteen percent of these 138 patients had ALNs involvement. Characteristics of ALN involvement were lesser number, smaller sizes, and lower nuclear grade of metastatic foci (Table 5). For these patients, SNB was successfully performed as shown in Table 3, and SNB is recommended to assess axillary nodal status.

Category 2 and 3 showed the patients having discrepancy between the axillary examinations of AUS and ^{18}F-FDG uptake.

Category 2 showed the patients having ^{18}F-FDG uptake but negative AUS. Half of these patients have metastatic foci in their axilla. The reason of the discrepancy was related to the fact that metastatic foci of small size (5 mm or less) and/or higher nuclear grade was detected by ^{18}F-FDG uptake but were not by AUS.

Category 3 showed the patients having positive AUS without axillary ^{18}F-FDG uptake. We found 4 (80%) of 5 patients had ALN involvement. The characteristic of these metastastic foci was lower nuclear grade. This result are in keeping with previous reports [8-10].

The conclusions could not be determined because the number of patients in categories 2 and 3 have been limited, but we could indicate lymph nodes having discrepancy in diagnosis between AUS and axillary ^{18}F-FDG uptake were found to be frequently metastasized. When the discrepancy occurred between these two modalities, therefore, we suggest further axillary investigations such as core-needle biopsy, or fine needle aspiration cytology to evaluate precisely axillary nodal status.

We confirmed the positive lymph nodes found by ^{18}F-FDG-PET matched the SNB results in all patients of category 2 that had involved SNs and received subsequent axillary dissection (shown in Table 6).

Category 4 showed the patients had double-positive ALNs of AUS and ^{18}F-FDG uptake. PPV for detecting ALN involvement was 100%. These patients were recommended to undergo ALND without SLN. In addition, it might be rational to consider that patients having AUS positive nodes and axillary ^{18}F-FDG uptake will have PSC without biopsy or fine needle aspiration cytology to the axilla.

Semiquantitative assessment of ^{18}F-FDG PET/CT for the axilla

Table 4 showed higher SUV were significantly correlated with nuclear grade 3 and maximum size of metastatic foci but not with number of involved ALNs. These results also appear to reveal biological significance of axillary ^{18}F-FDG accumulated to metastasized cancer cells.

When the cut-off of SUV exceeded 1.8, specificity and PPV of ^{18}F-FDG PET/CT were almost 100%, but sensitivity notably decreased to 36% or lower.

From the present results, appropriate determination of the cut-off of SUV appeared possible to evaluate ALN involvement by means of ^{18}F-FDG uptake. Especially by setting of cutoff of SUV, we could predict ALN involvement with excellent specificity and PPV. The cut-off of SUV for ALN involvement varies from 1.2 to 2.3 among reports previously published [9,11]. The inter-institutional standardization of the cut-off value-off SUV for ALN evaluation remains to be settled.

Thus, we found that axillary ^{18}F-FDG uptake added incremental diagnostic confidence to AUS. Richard L et al reported that ^{18}F-FDG PET may have a role in assessing patients with medially or superiorly situated breast cancers that may drain preferentially or exclusively to internal mammary or supraclavicular nodes[10]. A. Gil-Rendo et al also described that an advantage of ^{18}F-FDG PET was to be able to detect internal mammary node metastasis, which is often clinically occult and poorly visualized by conventional modality including ultrasonography [8].

We experienced a patient having ^{18}F-FDG uptake in a parasternal lymph node in spite of double-negativity in AUS and axillary ^{18}F-FDG uptake. The lymph node has been proven to be metastasized by fine-needle aspiration cytology. Another patient who have double-positivity in AUS and axillary ^{18}F-FDG uptake, also showed ^{18}F-FDG uptake in infraclavicular lymph nodes. These two patients had chosen PSC, having been ineligible for this study protocol.

Thus, we considered the whole-body ^{18}F-FDG PET/CT would be informative for imaging investigations for regional nodes involvement as well as distant metastasis [12,18].

Conclusion

In conclusion, the diagnostic accuracy of visual assessment of ^{18}F-FDG PET/CT was almost equivalent to that of AUS in sensitivity, specificity, and overall accuracy. When cut-off of SUV was set at 1.8 or more, specificity and PPV was each 100%. However, there are numerous factors that will influence SUV results and we should take into consideration the limited value of SUV in breast.

To our knowledge, this is the first study to compare between ^{18}F-FDG PET/CT and ultrasonography for detecting of ALN involvement.

Considering their limited sensitivities, the high radiation exposure by ^{18}F-FDG PET/CT and also costs of the examination, it is likely that AUS will be more cost-effective in detecting massive axillary tumor burden. However, when we cannot judge the axillary staging using AUS alone, metabolic approach of ^{18}F-FDG PET/CT for axillary staging would enable us a much more confident diagnosis.

Abbreviations

ALN: axillary lymph node; ALND: ALN dissection; AUS: axillary ultrasonography; NPV: negative predictive value; ^{18}F-FDG PET/CT: positron emission tomography/computed tomography with ^{18}F-fluorodeoxyglucose; SNB: sentinel node biopsy; SUV: standardized uptake value; PPV: positive predictive value.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SU performed the planning, acquisition of data, analysis of data, and writing of the manuscript. HT (pathologist) performed the planning, interpretation of data, and the manuscript in co-operation with SU. HA, JO, and KF (breast surgeons) performed surgery and the statistic analysis. YH, KT, JI, and YA (radiologists) performed the evaluation of tumoral SUV levels and data acquisition. NK (biochemist) performed the statistic analysis. TK (ultrasonographer) carried out axillary assessment. HM participated in its design and coordination in co-operation with SU and HT. All authors read and approved the final manuscript.

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Molecular Detection of Lymph Node Metastases in Breast Cancer Patients: Results of a Multicenter Trial Using the One-Step Nucleic Acid Amplification Assay

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Abstract

Purpose: Accurate assessment of metastasis in sentinel lymph nodes (SLN) of breast cancer is important but involves a heavy workload for the pathologist. We conducted a multicenter clinical trial in Japan to evaluate a new automated assay system for cytokeratin 19 mRNA, the one-step nucleic acid amplification (OSNA) assay (Sysmex), to detect lymph node metastasis of breast cancer.

Experimental Design: Surgically obtained axillary lymph nodes were sectioned into four pieces, two of which were examined with the OSNA assay. The other two adjacent pieces were examined with H&E and immunohistochemical staining for cytokeratin 19. Serial sections at 0.2-mm intervals were used in trial 1 to determine the specificity of the OSNA assay, and three pairs of sections cut from the sliced surfaces of the pieces were used in trial 2 to compare the accuracy of the OSNA assay with that of a routine pathologic examination for SLNs in Japan.

Results: In trial 1, the sensitivity and specificity were 95.0% [95% confidence interval (95% CI), 75.1-99.9%] and 97.1% (95% CI, 91.8-99.4%), respectively, for 124 axillary lymph nodes obtained from 34 patients. In trial 2, the agreement between findings of the assay and of the pathologic examination was 92.9% (95% CI, 90.1-95.1%) for 450 axillary lymph nodes obtained from 164 patients.

Conclusion: The OSNA assay can detect lymph node metastasis as accurately as can conventional pathology and thus can be an effective addition to or alternative for rapid intraoperative examination of SLNs.

Sentinel lymph node (SLN) biopsy for breast cancer is expected to become a standard surgical procedure in the near future, and accurate assessment of metastasis of SLNs is essential for making decisions about the avoidance of unnecessary axillary dissection and the provision of appropriate adjuvant treatment for patients. However, methods for the pathologic examination of SLNs to detect metastasis remain controversial (1-4). Although more detailed examination of SLNs can provide more accurate information about metastasis (5), to obtain more accurate results, a comparatively greater number of pathologic specimens need to be examined (6). This involves much time for preparation of the specimens and a heavy workload for pathologists to examine them, especially intraoperatively.

To overcome these problems, molecular detection of metastasis has been developed as one of the most promising methods for SLN examination. With this procedure, the whole lymph node can be examined during a short time

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Translational Relevance

Sentinel lymph node biopsy will most probably become a standard surgical procedure for early breast cancer patients; therefore, an accurate assessment of metastasis in sentinel node is required to avoid unnecessary axillary dissection. However, detailed examinations involve a heavy workload for the pathologists. Recently, several molecular detection procedures for lymph node metastasis have been developed as the most promising solution for this problem and now are commercially available. This paper reports the results of a Japanese multicenter clinical trial comparing a molecular-based method using a new automated assay system, the one-step nucleic acid amplification assay, with a routine pathologic examination for detection of lymph node metastasis of breast cancer. A high concordance rate was observed between the assay and the pathologic examination. The assay provided results in a short time and was easy to do. The one-step nucleic acid amplification assay may thus become an effective addition to or alternative for rapid intraoperative examination of sentinel lymph nodes.

without requiring much work for the pathologist. Very recently, several molecular-based metastasis detection procedures with proper calibration for clinical use have been developed and are expected to become alternatives to conventional pathologic examinations (7–10). The one-step nucleic acid amplification (OSNA) assay (Sysmex), an automated system for rapid and quantitative detection of cytokeratin 19 (CK19) mRNA with the reverse transcription loop-mediated isothermal amplification (RT-LAMP) method (11), has been shown to feature high specificity with a low false-positive rate (12). To assess the validity of this assay in clinical use, a multicenter clinical trial was conducted in Japan, concurrently with some single-institute studies in the Netherlands and Germany (13).

In this article, we report the results of the Japanese trial of the OSNA assay for detection of lymph node metastasis in breast cancer and discuss its validity for clinical use, especially for intraoperative SLN examination.

Materials and Methods

The two trials. The materials consisting of axillary lymph nodes were obtained from patients who underwent surgery for breast cancer between October 2005 and May 2006 at seven Japanese institutes and hospitals that had joined this study. Two of these institutes participated in trial 1, three in trial 2, and two in both. Patients were given the necessary information about the trial, and only the lymph nodes from patients who had given their consent were used. The trial consisted of two different protocols. In trial 1, designed to determine the specificity of the OSNA assay for detection of metastasis in comparison with that of detailed pathologic examination, clinically metastasis-negative (N0) lymph nodes with a maximum size between 4 and 8 mm were examined. Sampled lymph nodes were immediately divided into four pieces (a, b, c, and d) of 1 or 2 mm thickness with cutting devices developed by Tsujimoto et al. (12), and two of the four pieces (a and c) were examined with the OSNA assay in the laboratory of the participating institute concerned (Fig. 1). The two adjacent pieces (b and d) were sent to one of the three pathologists of the central committee, who examined them in the following manner. After the samples were fixed with formalin and embedded in paraffin, the two pieces were sliced sequentially at 0.2-mm intervals and a pair of sections with a thickness of 5 μm each was obtained from each level of slice. One of the paired sections was then stained with H&E, and the other was examined immunohistochemically for CK19 by using monoclonal antibody RCK108 (Dako). The pathologists examined the preps without access to information about the results of the OSNA assay. The pathologic diagnosis was negative when the specimen contained no tumor cells or only isolated tumor cells (ITC) and positive when micrometastasis or macrometastasis was found according to the criteria of the sixth edition of the tumor-node-metastasis classification of the International Union Against Cancer and the classification of the American Joint Committee on Cancer.

In trial 2, designed to determine the accuracy of the OSNA assay for detection of metastasis compared with that of routine pathologic examination, randomly sampled lymph nodes or SLNs with a maximum size between 4 and 8 mm were analyzed. The sampled lymph nodes were immediately divided into four pieces in the same manner as described above. Two pieces were examined with the OSNA assay, and the other two adjacent pieces were examined pathologically. Frozen sections of the SLNs were prepared and examined by a

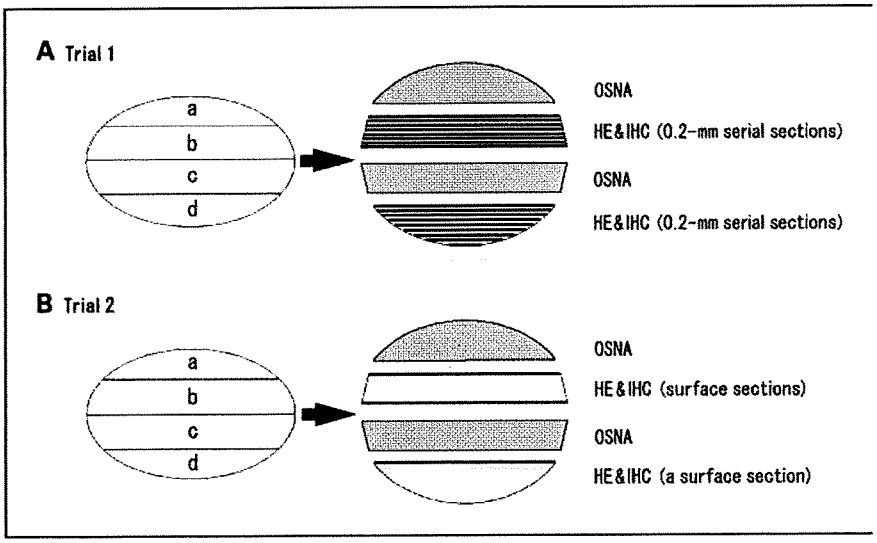

Fig. 1. Preparation of lymph nodes for the OSNA assay and pathologic examination. Lymph nodes were divided into four pieces (a, b, c, and d) 1 to 2 mm thick, and two pieces (a and c) were homogenized and subjected to the OSNA assay. In trial 1, the remaining two pieces (b and d) were serially sectioned at 0.2-mm intervals, and a pair of sections obtained at each level of the slice was stained with H&E and immunohistochemistry (*IHC*) for pathologic examination. In trial 2, a pair of sections obtained from the cut surface of the pieces was examined with H&E and immunohistochemical staining.

Table 1. Patient characteristics

	No. patients (%)	
	Trial 1	Trial 2
Enrolled	36	185
Excluded	2*	21†
Analyzed	34	164
Average age (y)	55.9	54.7
Clinical stage		
0	2 (6)	14 (9)
I	8 (24)	51 (31)
IIA	14 (41)	64 (40)
IIB	3 (9)	28 (17)
III	5 (15)	7 (4)
IV	0 (0)	0 (0)
Unknown	2 (6)	0 (0)
Histologic type		
DCIS	0 (0)	18 (11)
Invasive ductal	32 (94)	130 (79)
Invasive lobular	1 (3)	7 (4)
Special type	1 (3)	9 (5)

Abbreviation: DCIS, ductal carcinoma *in situ*.
*Consent was withdrawn by one patient and samples from another patient did not contain lymph node tissue.
† Consent was withdrawn by three patients and samples from four patients did not contain lymph node tissue. Six patients received neoadjuvant chemotherapy and the assay process was deemed invalid for eight.

pathologist at the institute concerned. The remains of the SLN specimens and intact non-SLN specimens were sent to the central committee where three pairs of sections were obtained at the cut surface of each piece (Fig. 1) and examined with H&E and immunohistochemistry for CK19 in the same manner as in trial 1.

The OSNA assay. The OSNA assay for lymph nodes has been described in detail in a previous report (12). Briefly, pieces obtained from axillary lymph nodes were homogenized with 4 mL of a lysis buffer solution and centrifuged at 10,000 × *g* at room temperature. Two microliters of the supernatant were analyzed with the RD-100i system (Sysmex), an automated molecular detection system using a RT-LAMP method. A standard positive control sample containing 5×10^3 copies/μL of CK19 mRNA and a negative control sample containing 0 copy/μL of CK19 mRNA were used for calibration in every assay. The results of the assay were expressed as the numbers of CK19 mRNA copies per microliter, and metastasis was assessed in accordance with the cutoff level determined by Tsujimoto et al. (12). That is, the lymph node was assessed negative when there were less than 2.5×10^2 copies/μL of CK19 mRNA and positive when there were 2.5×10^2 copies/μL or more.

Further examination for cases showing discrepancies between the OSNA assay and pathologic examination results. In trial 2, several nodes showed discrepant results for the OSNA assay and pathologic examination. After the trial period, such lymph nodes were subjected to further examination to determine the existence and localization of metastatic cells. For this purpose, the remaining pathologic specimen blocks were sectioned at 0.2-mm intervals and examined with H&E and immunohistochemistry for CK19 in the same manner as in trial 1. Furthermore, the lysate sample used for the OSNA assay was examined for CK19 protein expression by means of Western blotting analysis.

Statistical analysis. Sensitivity, specificity, and accuracy were determined by comparing the results of the OSNA assay and pathologic examination. The statistical program R 2.4.1[18] for binomial distribution analysis with 95% confidence interval (95% CI) was used for all statistical analyses.

[18] http://www.r-project.org/

Results

Trial 1. A total of 149 axillary lymph nodes surgically obtained from 36 patients with early breast cancer (Table 1) constituted the materials for this study. Five nodes from one patient were excluded from the analysis because consent was withdrawn by the patient, as were 19 nodes in which at least one of the four pieces did not contain lymphatic tissue and one involving a technical error. The remaining 124 nodes from 34 N0 patients were then analyzed. Of the 104 nodes pathologically identified as negative, 101 were assessed as negative by the OSNA assay for a specificity of 97.1% (95% CI, 91.8-99.4%; Table 2). Of the three nodes that were pathologically identified as negative but assessed as positive in the OSNA assay, one was found to contain ITCs and another to have >0.3 ng/μL of CK19 protein in the remaining sample solution used for the OSNA assay. Of the 20 pathologically positive nodes, 19 were assessed as positive by the OSNA assay for a sensitivity of 95% (95% CI, 75.1-99.9%). One node assessed as negative by the OSNA assay was found to contain a micrometastasis.

Trial 2. A total of 551 axillary lymph nodes surgically obtained from 185 patients with early breast cancer (Table 1) constituted the materials of this study. Eight of the nodes from three patients were excluded because their consent was withdrawn. Twenty-six nodes from six patients who received neoadjuvant chemotherapy were also excluded, as well as 36 in which at least one of the four pieces of a node did not contain lymphatic tissue and 31 that did not meet the specifications of this study, such as the use of frozen materials for the assay. Of the 450 lymph nodes eligible for the analysis, 70 were assessed as positive and 348 as negative by both the OSNA assay and pathologic examination for an accuracy of 92.9% (95% CI, 90.1-95.1%; Table 3). Seventy of the 80 pathologically positive nodes were detected by the OSNA assay (sensitivity, 87.5%; 95% CI, 78.5-93.8%). On the other hand, 348 of the 370 (94.1%) pathologically negative nodes were assessed as negative in the OSNA assay, whereas 5.9% of the pathologically negative nodes were positive. These lymph nodes showing discrepant results were subjected to further analysis.

Further examination of discrepant cases from trial 2. The 10 nodes that were pathologically positive but negative in the OSNA assay (false negative) and the 22 nodes that were

Table 2. Results of trial 1

	Pathology		
	Positive		Negative
	Macrometastasis	Micrometastasis	
OSNA			
Positive*	16	3	3
Negative†	0	1	101

NOTE: Specificity of the OSNA assay for pathology was 97.1% (101 of 104; 95% CI, 91.8-99.4%). Sensitivity of the OSNA assay for pathology was 95.0% (19 of 20; 95% CI, 75.1-99.9%).
*CK19 mRNA $\geq 2.5 \times 10^2$ copies/μL.
† CK19 mRNA $< 2.5 \times 10^2$ copies/μL.

Table 3. Results of trial 2

	Pathology		
	Positive		Negative
	Macrometastasis	Micrometastasis	
OSNA			
Positive*	64	6	22
Negative †	4	6	348

NOTE: Sensitivity of the OSNA assay for pathology was 87.5% (70 of 80; 95% CI, 78.2-93.8%). Specificity of the OSNA assay for pathology was 94.1% (348 of 370; 95% CI, 91.0-96.3%). Accuracy of the OSNA assay for pathology was 92.9% (418 of 450; 95% CI, 90.1-95.1%).
*CK19 mRNA $\geq 2.5 \times 10^2$ copies/μL.
†CK19 mRNA $< 2.5 \times 10^2$ copies/μL.

pathologically negative but positive in the OSNA assay (false positive) were subjected to further analysis. The results are summarized in Table 4. In eight of the 10 false-negative nodes, uneven localization of the tumor cells was found in the remnants of the nodes. However, two nodes with pathologically identified macrometastasis remained negative in the OSNA assay because tumor cells in these nodes showed faint expression of CK19, as was confirmed by immunohistochemical staining for CK19.

In 5 of the 22 false-positive nodes, some foci of tumor cells were found in the remnants of the nodes. Another eight of these nodes were not found to contain tumor cells in the pieces remaining after the pathologic examination, but lymphatic vascular invasions were detected in the main tumors of these nodes, whereas the lysate of two of them preserved for the OSNA assay contained a significant amount of CK19 protein. However, further analysis of the remaining nine nodes showed no pathologic or clinical signs of metastasis after additional sectioning.

The results based on the further analysis are shown in Table 5. The final accuracy of the OSNA assay based on the results of further examination was 93.1% (95% CI, 90.0-95.5%), the final sensitivity was 87.7% (95% CI, 78.5-93.9%), the final specificity was 94.3% (95% CI, 95.3-98.8%), the final positive predictive value was 77.2% (95% CI, 67.2-85.3%), and the final negative predictive value was 97.2% (95% CI, 95.5-98.9%). The OSNA assay detected 94.1% of the lymph node metastases larger than 2 mm (macrometastasis).

Table 4. Results of further analysis of lymph nodes with discrepant results in trial 2

No. LN	Result of trial 2		Result of further analysis	
	OSNA*	Pathology	CK19 protein (ng/μL)	Pathology
1	-	Ma	0.05	CK19 negative
2	-	Ma	0.14	CK19 negative
3	-	Mi	0.2	Mi in one of two pieces
4	-	Mi	0.13	Mi in one of two pieces
5	-	Mi	0.03	Mi in one of two pieces
6	-	Mi	0.14	Mi in one of two pieces
7	-	Ma	0.02	Ma in one and Mi in the other piece
8	-	Ma	0.26	Ma in one and Mi in the other piece
9	-	Mi	0.28	Mi in both pieces
10	-	Mi	0.04	Mi in both pieces
11	+	Neg (ITC)	0.32	Mi in one of two pieces
12	+	Neg (ITC)	0.3	ITC in one of two pieces
13	+	Neg (ITC)	0.04	ITC in one of two pieces
14	+	Neg (ITC)	0.08	ITC in one of two pieces
15	+	Neg (none)	0.04	ITC in one of two pieces
16	+	Neg (none)	0.38	None, ly+
17	+	Neg (none)	0.58	None, ly+
18	+	Neg (none)	0.08	None, ly+
19	+	Neg (none)	0.02	None, ly+
20	+	Neg (none)	0.12	None, ly+
21	+	Neg (none)	0.02	None, ly+
22	+	Neg (none)	0.05	None, ly+
23	+	Neg (none)	0.04	None, ly+
24	+	Neg (none)	0.1	None
25	+	Neg (none)	0.15	None
26	+	Neg (none)	0.02	None
27	+	Neg (none)	0.02	None
28	+	Neg (none)	0.1	None
29	+	Neg (none)	0.09	None
30	+	Neg (none)	0.15	None
31	+	Neg (none)	0.15	None
32	+	Neg (none)	0.03	None

Abbreviations: Ma, macrometastasis; Mi, micrometastasis; Neg, negative; none, no tumor cells; ly+, lymphatic vessel invasion observed in the main tumor.
*++: CK19 mRNA $\geq 5 \times 10^3$ copies/μL; +: $2.5 \times 10^2 \leq$ CK19 mRNA $< 5 \times 10^3$ copies/μL; -: CK19 mRNA $< 2.5 \times 10^2$ copies/μL.

Table 5. The final results of trial 2

	Pathology		
	Macrometastasis	Micrometastasis	Negative
OSNA			
Positive*	64	7	21
Negative†	4	6	348

NOTE: The final sensitivity of the OSNA assay for pathology was 87.7% (71 of 81; 95% CI, 78.5-93.9%). The final specificity of the OSNA assay for pathology was 94.3% (348 of 369; 95% CI, 95.3-98.8%). The final accuracy of the OSNA assay for pathology was 93.1% (419 of 450; 95% CI, 90.0-95.5%). The final sensitivity of the OSNA assay for pathologic macrometastasis (>2 mm) was 94.1% (64 of 68; 95% CI, 85.6-98.4%).
*CK19 mRNA $\geq 2.5 \times 10^2$ copies/μL.
†CK19 mRNA $< 2.5 \times 10^2$ copies/μL.

Discussion

Because several studies have shown the feasibility of molecular detection of micrometastasis in the lymph nodes using reverse transcription-PCR (14–17), a lot of markers, such as CK19, mammaglobin, carcinoembryonic acid, MUC1, prolactin-induced protein, have been examined for their sensitivity and specificity for detection of metastasis, and a variety of combinations of these markers have been proposed for clinical use (18–23). One of these, a combination of CK19 and mammaglobin, showed both high sensitivity and specificity for detection of lymph node metastasis of breast cancer (23), and the system using these two markers showed high reliability and is now being used clinically (7–10).

The OSNA assay is also a molecular-based metastasis detection system, which uses CK19 as a single marker. CK19, a representative epithelial marker widely expressed in human cancers, is considered to be a promising marker with high sensitivity for detection of lymph node metastasis from various cancers. Reverse transcription-PCR for CK19, on the other hand, is sometimes unreliable because of the presence of pseudogenes (24) and contamination of benign epithelial cells (25). To overcome this problem, the OSNA assay adopted the RT-LAMP method developed by Notomi et al. (11). The amplification is processed isothermally by means of six primers and can detect mRNA of CK19 quantitatively without interference by pseudogenes. The assay can differentiate contamination of a few benign epithelial cells and the presence of ITCs from clinically significant tumor metastasis by using a verified cutoff value (12). The assay can be done with on-the-spot preparation and easy operation because homogenization of a lymph node in the lysis buffer takes only 90 seconds and centrifugation of the sample 1 minute, whereas placement of the supernatant and reagents into the detector does not require extraction and purification of mRNA to synthesize cDNA, both of which are necessary for the reverse transcription-PCR method. The mRNA is automatically amplified in a RD-100i gene amplification detector (Sysmex) in 16 minutes, and stable results are provided without being affected by the size of the sample (maximum of 600 mg for one assay; ref. 12).

Trial 1 was designed to determine its specificity because high specificity is needed to avoid unnecessary axillary dissection when the assay is used for SLN examination. The specificity of the assay in our study was 97.1% (95% CI, 91.8-99.4%), with only three nodes judged positive in the OSNA assay but negative pathologically, but two of these nodes were found to contain ITCs in the specimen used for pathology. The specificity of the OSNA assay thus reaches 99.0% when these cases with ITCs are excluded.

In trial 2, the accuracy of the assay was examined in comparison with a routine method for pathologic examination for SLNs used in Japan, which is similar to a protocol with three sections from each 2- to 3-mm slice of the node recommended by the consensus meeting (26). The rate of concordance between the assay and the pathologic examination was 92.9% (418 of 450; 95% CI, 90.1-95.1%), and the results indicated that the lymph node metastasis detection capability of the OSNA assay was statistically equal to that of the pathologic examination for three sections cut from slices obtained at 2-mm intervals when discrepancies caused by differences in the samples used for the assay and the pathologic examination are taken into consideration. This is so because a sample for molecular examination needs to be homogenized, it cannot be used for pathologic examination, so that studies comparing the two modalities using different pieces of a sample must therefore of necessity include some cases producing discrepant results caused by uneven localization of tumor foci. Actually, 32 nodes (7.1%) with discrepant results were found. In eight of the 10 false-negative nodes, only a few of the serial sections contained metastasized foci of the tumor. These small metastases might have been detected by the OSNA assay if the whole lymph node had been used for the assay. In addition, some tumor cell clusters or ITCs were found in the remaining specimens of 5 of the 22 false-positive nodes after additional sectioning, which had not been detected by routine pathologic examination using 2-mm interval sections. In another eight false-positive nodes, no tumor cells were found in the pieces remaining after the pathologic examination, but lymphatic vascular invasions were observed in the main tumors of these nodes. In addition, the lysate of two of them preserved for the OSNA assay contained a significant amount of CK19 protein. These nodes may thus have harbored some foci of tumor cells in the piece used for the OSNA assay.

On the other hand, two nodes from different patients accounted for the false-negative cases with a very weak expression of CK19 mRNA. The primary tumors of the patients also showed negative staining for CK19 as confirmed by immunohistochemistry. The actual incidence of tumors with low CK19 expression remains unclear. Bartek et al. (27) reported an incidence for breast cancer of 0%, but Parikh et al. (28) of 20.5% for young patients. The OSNA assay is more sensitive than immunohistochemistry so that the incidence of false-negative results caused by low expression of CK19 can be expected to become exceptional in clinical use. In fact, another lymph node obtained from one of the two patients was positive in the OSNA assay, so that only 1 of the 185 patients (0.5%) was identified as negative by the assay. However, this ratio should be confirmed with more lymph nodes and patients.

The findings of the further analysis of discrepant cases in trial 2 prompted a reanalysis of the data, resulting in the final specificity of the OSNA assay compared with the pathologic examination becoming 94.3%, the final accuracy 93.1%, and the final negative predictive value 97.2%. These ratios indicate that the OSNA assay can accurately detect node-negative cases.

As for pathologically positive lymph nodes (diameter of metastasis, >0.2 mm), 87.7% could be detected by the OSNA assay, whereas 94.1% of macrometastases (>2 mm) were assessed as positive. Our results were similar to recently reported results reported by Visser et al. (13), who compared the OSNA assay with pathologic examination at five levels at 0.25-mm intervals of each piece used for pathology.

In conclusion, the OSNA assay showed high specificity, accuracy, and negative predictive value compared with conventional pathologic examination for the detection of lymph node metastasis of breast cancer. It provides satisfactory results in a short time and with easy procedure. The OSNA assay can thus be used as an alternative tool for examining metastasis in SLNs. However, for the time being, it is recommended to use the assay together with pathologic examination for minimal numbers of specimens until additional clinical trials with more lymph nodes and patients show that prognostic outcomes determined by the assay are equal or superior to those determined by conventional pathology.

Disclosure of Potential Conflicts of Interest

Y. Tamaki, F. Akiyama, T. Kaneko, K. Tsugawa, M. Tsujimoto, and N. Matsuura: honorarium, Sysmex Corp. S. Noguchi and N. Matsuura: Advisory Board, Sysmex Corp. M. Tsujimoto, T. Kaneko, and N. Matsuura: travel grant, Sysmex Corp.

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An individual patient data meta-analysis of adjuvant therapy with uracil–tegafur (UFT) in patients with curatively resected rectal cancer

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Clinical Studies

An individual patient data meta-analysis of adjuvant therapy with uracil–tegafur (UFT) in patients with curatively resected rectal cancer

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Uracil–Tegafur (UFT), an oral fluorinated pyrimidine chemotherapeutic agent, has been used for adjuvant chemotherapy in curatively resected colorectal cancer patients. Past trials and meta-analyses indicate that it is somewhat effective in extending survival of patients with rectal cancer. The objective of this study was to perform a reappraisal of randomised clinical trials conducted in this field. We designed an individual patient-based meta-analysis of relevant clinical trials to examine the benefit of UFT for curatively resected rectal cancer in terms of overall survival (OS), disease-free survival (DFS), and local relapse-free survival (LRFS). We analysed individual patient data of five adjuvant therapy randomised clinical trials for rectal cancer, which met the predetermined inclusion criteria. These five trials had a combined total of 2091 patients, UFT as adjuvant chemotherapy compared to surgery-alone, 5-year follow-up, intention-to-treat-based analytic strategy, and similar endpoints (OS and DFS). In a pooled analysis, UFT had significant advantage over surgery-alone in terms of both OS (hazard ratio, 0.82; 95% confidence interval (CI), 0.70–0.97; $P = 0.02$) and DFS (hazard ratio, 0.73; 95%CI, 0.63–0.84; $P < 0.0001$). This individual patient-based meta-analysis demonstrated that oral UFT significantly improves both OS and DFS in patients with curatively resected rectal cancer.
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Colorectal cancer accounts for 10–15% of all cancers and is the second leading cause of cancer deaths in developed countries (Pisani *et al*, 1993). In Japan alone, nearly 56 000 new cases are diagnosed and this disease causes 36 000 deaths every year (Statistics and information department, Ministry of Health and Welfare, 1996). Surgical treatment is the primary management of colorectal cancers, with 75–80% of the patients being operable at the time of diagnosis (Boring *et al*, 1991; Vernaba *et al*, 1994). However, even if a curative resection is performed, those patients with regional lymph node involvement (Dukes' C, Stage III) have a 40–50% 5-year survival rate.

Recently, in the field of Stage III colon cancer treatment, adjuvant chemotherapy by 5-fluorouracil (5-FU)/levamisole was proved to be superior to surgery-alone therapy, and then various 5-FU/leucovorin (LV) regimens were confirmed to be effective from the results of numerous large-scale randomised trials and from the pooled analysis of clinical trials (Wolmark *et al*, 1993; International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators, 1995; O'Connell *et al*, 1997). In 2004, results from the Multicenter International Study of Oxaliplatin/5-FU/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC) trial demonstrated that combination chemotherapy with 5-FU/LV (de Gramont regimen) plus oxaliplatin was significantly superior to 5-FU/LV alone (André *et al*, 2004). With regard to adjuvant chemotherapy for colon cancer, therefore, solid evidence has been accumulated from relevant clinical trials, and steady evolution of the new treatment modalities has been achieved.

However, the situation is still uncertain focusing on adjuvant therapy for rectal cancer. Despite apparently curative surgery, rectal cancer recurs in more than 55% of the patients, including local recurrence rates of 25% (Vernaba *et al*, 1994). Despite the recommendation of the consensus conference by the National Institute of Health (NIH consensus conference, 1990) that concluded that adjuvant radiotherapy and chemotherapy should be given to all patients with locally advanced rectal cancer, recent findings by a large-scale randomised trial and meta-analysis have failed to prove significant benefit of radiotherapy for survival (Fisher *et al*, 1988; Vernaba *et al*, 1994). In this regard, the quest for an effective adjuvant treatment with a robust advantage on the

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outcome of resected rectal cancer remain an important task for gastrointestinal oncologists.

In Japan, mesorectal excision is standard surgical procedure. Radiotherapy is not routinely performed as adjuvant therapy.

In Japan, adjuvant therapy after resection of colorectal cancer was developed primarily using oral fluorinated pyrimidines (O-FPs). A meta-analysis of three old trials (Sakamoto *et al*, 1999) and a more sophisticated analysis of four recent pivotal randomised trials (Sakamoto *et al*, 2004) demonstrated a statistically significant benefit of O-FPs on the outcome of colorectal cancers over surgery alone. However, the survival benefit shown in that meta-analysis was more pronounced in colon cancers. The risk reduction in terms of rectal cancer was only 8% and the result of those previous meta-analyses that analysed various types of oral fluorinated pyrimidine clinical trials was not sufficient to show a significant effect on survival.

Uracil–tegafur (UFT) is one of the O-FPs. In colon cancer, the majority of recurrences occurred in the liver, whereas in rectal cancer many recurrences occurred in the lung and locally in addition to the liver. Treatment effect may thus differ between colon cancer and rectal cancer. As the previous meta-analysis, two trials of UFT in patients with rectal cancer have been reported. The present study focused on rectal cancer, which lacked a clear-cut survival benefit in our previous meta-analysis. Unlike oral fluoropyrimidines such as carmofur and capecitabine, the formulation of UFT includes a dihydropyrimidine dehydrogenase inhibitor (Diasio, 1999), designed to enhance the bioavailability of FU. This combination of uracil and tegafur was shown, in an animal tumour system, to increase the anti-tumour activity compared with tegafur alone (Ooi *et al*, 2001). UFT also produced an enhanced intratumoural concentration of fluorinated pyrimidine, 5–10 times greater than that achieved with Tegafur alone (Fukunaga *et al*, 1987). Preclinical studies established that the optimal molar ratio of uracil to Tegafur is 4 : 1, which resulted in the highest 5-FU tumour: blood and tumour: normal tissue partition coefficients (Kawaguchi *et al*, 1980). UFT has now been clinically tested for lung cancer (Kato *et al*, 2004), breast cancer (Noguchi *et al*, 2005), and for gastric cancer (Kinoshita *et al*, 2005) in an adjuvant setting in Japan. Recently, UFT has also been tested in Western countries, regarding its efficacy for both advanced and curatively resected colon cancer (Carmichael *et al*, 2002; Douillard *et al*, 2002; Lembersky *et al*, 2006).

Here, we present an individual patient data meta-analysis of five centrally randomised trials recently performed in Japan to compare rectal cancer patients treated with UFT, with the surgery-alone control group. This meta-analysis includes data from more than 2000 patients and therefore provides a more reliable assessment of the effect of UFT on the survival, disease-free survival (DFS), and local relapse-free survival (LRFS) of the patients with rectal cancer than is available from any of the individual studies.

PATIENTS AND METHODS

Selection of trials

Trials that randomly assigned patients to either long-term (12 months) administration of UFT or surgery-alone treatment after curative resection of rectal cancer were eligible for meta-analysis. The randomisation technique used in these trials was the centralised randomisation that precluded the possibility of prior knowledge of the treatment to be allocated.

Five relevant trials identified as Japanese Foundation for Multidisciplinary Treatment of Cancer (JFMC) 7-1 (Kodaira *et al*, 1998), JFMC15-1, JFMC15-2 (Watanabe *et al*, 2004), Tokai Adjuvant Chemotherapy Study Group for Colorectal Cancer (TAC–CR) (Kato *et al*, 2002), and National Surgical Adjuvant Study of Colorectal Cancer (NSAS-CC) (Akasu *et al*, 2006) were included in the meta-analysis involving a total of 2091 patients. In trials JFMC7-1, JFMC15-1, and JFMC 15-2, patients who were randomly assigned to the experimental group received intravenous mitomycin C ($6\,mg\,m^{-2}$) at 1 week and once monthly for 6 months. In the JFMC15-1 and 15-2 trials, patients who were randomly assigned to the experimental group additionally received an induction course of intravenous 5-FU ($250\,mg\,daily^{-1}$) during 7 postoperative days (Table 1).

Protocol and data collection for the meta-analysis

In December 2003, a protocol for the meta-analysis, describing the study rationale, statistical methods, and guidelines for publication, was distributed to the principal investigators of the five trials. Investigators were asked to provide individual data for every randomised patient, whether eligible or not, assessable or not, and

Table 1 Details of the randomized controlled trials included in the individual patient data meta-analysis

Category	JFMC7-1	JFMC15-1	JFMC15-2	TAC-CR	NSAS-CC	Total
Additional chemotherapy	Mitomycin C	Mitomycin C+FU IV	Mitomycin C+FU IV	None	None	—
Radiotherapy	None	None	None	None	None	—
UFT dose/day	400 mg	400 mg	400 mg	400 mg	600 mg[a]	—
Period	12 months	12 months	12 months	24 months	12 months	—
Dates of accrual	1986–1988	1989	1990	1991–1994	1996–2001	—
No. of patients	834	447	391	143	276	—
Duration of accrual, months	35	24	24	36	54	—
Sex, No. of patients (male–female ratio)						
Male	521 (62.4%)	260 (58.1%)	244 (62.4%)	93 (65.0%)	167 (60.5%)	1285 (61.4%)
Female	313 (37.6%)	187 (41.9%)	147 (37.6%)	50 (35.0%)	109 (39.5%)	806 (38.9%)
Duke's stage, No. of patients						
A	135	67	62	12	0	276
B	326	175	139	53	0	693
C	373	205	189	78	276	1121
Median age	57	60	59	62	58	58
Upper age limit, years	70	75	75	75	75	—

JFMC = Japanese Foundation for Multidisciplinary Treatment of Cancer; NSAS-CC = National Surgical Adjuvant Study of Colorectal Cancer; TAC–CR = Tokai Adjuvant Chemotherapy for Colorectal Cancer; UFT = Uracil–Tegafur. [a]$400\,mg\,m^{-2}\,day^{-1}$ for 5 days every 7 days.

properly followed up or not. Items requested for every patient were as follows: patient identification, date of surgery, eligibility, allocated treatment by random assignment, age, sex, primary tumour site, Dukes' stage, induction chemotherapy, dates of recurrence, death, or last visit. Disease-free survival was calculated from the date of surgery to the date of recurrence, second primary cancer or death, whichever occurred first. Survival was calculated from the date of surgery to the date of death, regardless of the cause of death. Local relapse-free survival was calculated from the date of surgery to the date of local recurrence. Data from patients with only distant recurrence and those who were died without recurrence were censored. Patients enrolled in these trials had been followed up for 5–7 years. Toxicity data were not collected, because detailed analysis of side effects can be found in the published reports of the individual trials (Kodaira *et al*, 1998; Kato *et al*, 2002; Watanabe *et al*, 2004; Akasu *et al*, 2006).

All investigators and the Clinical Trial Committee of all the trials agreed to join in the meta-analysis. Individual patient data were received by the independent secretariat by February 2004 and October 2006.

Pretreatment patient characteristics

All 2091 patients had curatively resected rectal cancer without evidence of distant metastasis by diagnostic imaging criteria or by macroscopic examination of the abdominal organs during surgery. Patients with severe postoperative complications were excluded from all trials, as were patients with any previous chemotherapy or radiotherapy or with a synchronous or metachronous second cancer. Median patient age was 61 years at the time of random assignment. The male/female ratio was approximately 3:2. Performance status was less than 2 on the Japan Clinical Oncology Group scale for all patients.

Statistical analysis

The method used for the meta-analysis and the format for the presentation of the results have been described in detail elsewhere (Advanced Colorectal Cancer Meta-Analysis Project, 1992). All analyses were based on individual patient data. Treatment effects on DFS, LRFS, and survival were first estimated within each trial and then combined using classical meta-analytic methods (Colorectal Cancer Collaborative Group, 2001). Treatment effects were displayed as hazard ratios. These ratios were estimated by univariate Cox's proportional model as relative risks of having an event in the UFT group as compared with having the same event in the surgery-alone control group. A ratio less than unity indicates benefit from UFT, and this benefit is statistically significant when the 95% confidence interval (CI) of the ratio does not include unity. The overall effect of treatment was assessed through a χ^2_1 d.f. and the heterogeneity between five trials through a χ^2_4 d.f. (Colorectal Cancer Collaborative Group, 2001). Additional analyses were carried out to determine which of the following prognostic features, if any, were predictive of the treatment effect: Dukes' stage (A *vs* B *vs* C), sex (male *vs* female), and age (three groups of increasing age). Tests for interaction were applied to detect departures from the homogeneity of treatment effects. Multivariate analyses were performed with the use of the Cox proportional hazards regression model for DFS, LRFS, and survival to assess the robustness of the observed effects to adjustments for important covariates and the magnitude of interaction between treatment effect and covariate (Advanced Colorectal Cancer Meta-Analysis Project, 1992). All *P*-values resulted from use of two-sided statistical tests. The significance level was set at 5% for all tests.

RESULTS

Survival

Survival hazard ratios for all the trials are presented in Figure 1. The overall hazard ratio was 0.82 (95% CI, 0.70–0.97; $P=0.02$) with no significant heterogeneity between the treatment effects in different trials (χ^2_4 for heterogeneity = 4.31; $P=0.37$). UFT showed significant effect on survival of curatively resected rectal cancers with a 5-year survival benefit of approximately 5%.

Figure 2 shows the breakdown of the survival hazard ratio stratified by various patient characteristics. There was a slight trend toward larger treatment benefits in earlier Dukes' stages (Hazard ratio; Dukes' $A=0.60$, Dukes' $B=0.79$, Dukes' $C=0.86$) but heterogeneity tests did not show any significant difference ($\chi^2_2=1.41$; $P=0.495$). There was no statistically significant difference in sex (χ^2_1 for interaction = 1.62; $P=0.204$) or age (χ^2_2 for interaction = 0.22; $P=0.898$).

Figure 3 shows survival curves by treatment and disease stage. These curves confirm the hazard ratio analysis shown in Figure 2 and point to favourable effects of UFT in all Dukes' stages.

Disease-free survival

Disease-free survival hazard ratios are presented in Figure 4 for all the trials. These figure show a somewhat larger effect of treatment on DFS than on survival, with an overall DFS ratio of 0.73 (95%CI,

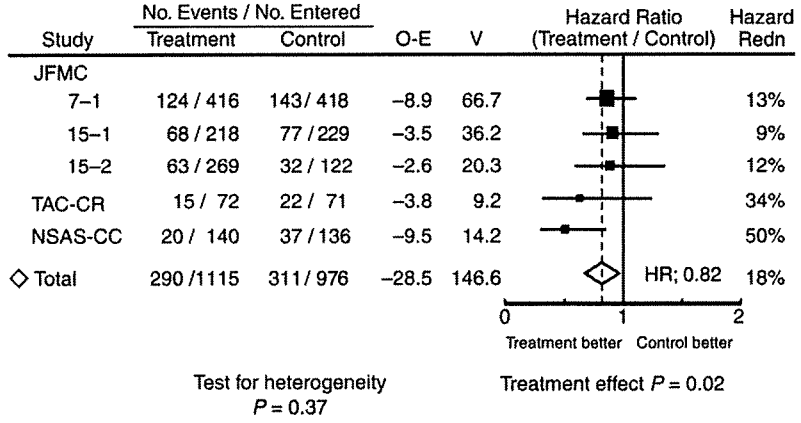


Figure 1 Survival hazard ratios by individual trial (Abbreviations: O/N = observed number of events/number of patients; O−E = Observed minus Expected number of events; V = variance of (O−E); Hazard Redn = hazard reduction; SE = standard error of hazard reduction).

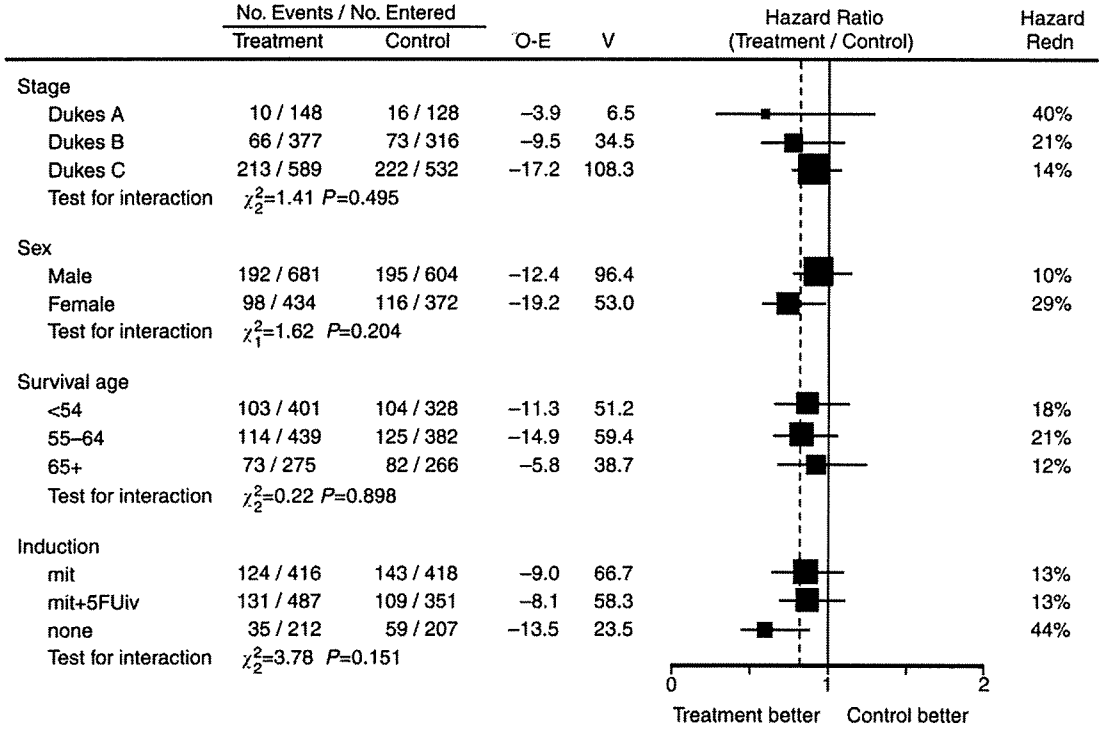
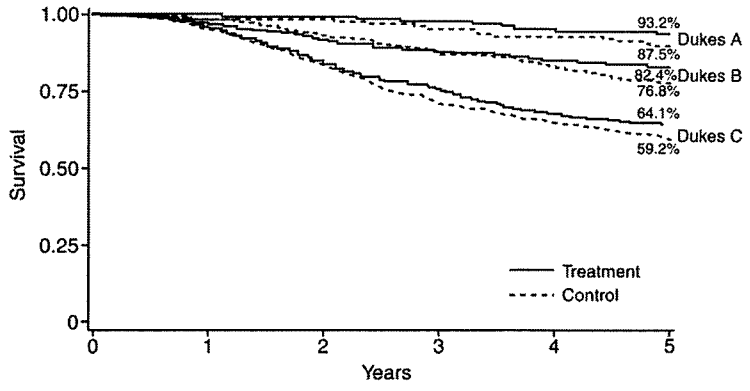


Figure 2 Survival hazard ratios by patient and treatment characteristics (Abbreviations as in Figure 1).



		No. at risk					
Dukes A	Treatment	148	148	147	145	139	137
	Control	128	125	125	121	118	114
Dukes B	Treatment	377	364	343	328	316	304
	Control	316	310	291	273	258	236
Dukes C	Treatment	589	560	494	438	388	357
	Control	532	507	442	374	339	299

Figure 3 Survival curves by tumour stage and by treatment.

0.63–0.84; $P<0.0001$) with a 5-year DFS benefit of 9.7%, but demonstrating some heterogeneity among the treatment effects in different trials (χ^2_4 for heterogeneity = 7.85; $P=0.097$). Additionally, random effect model assuming the variation between trials was applied. The results of the random effect model still revealed highly significant differences owing to the relatively high effect in TAC–CR and NSAS-CC trials.

Figure 5 lists the DFS hazard ratios by various patient and treatment characteristics.

Figure 6 shows DFS curves by treatment and disease stage. These curves again point to benefits of UFT in Dukes' A, B and C stages. Roughly identical effect extended across all Dukes' stages: the DFS benefits at 5 years in terms of risk reduction were 0.42, 0.33, 0.23.

Local relapse free survival

The overall hazard ratio was 0.68 (95%CI, 0.53–0.87; $P=0.0026$), and demonstrating some heterogeneity among the treatment

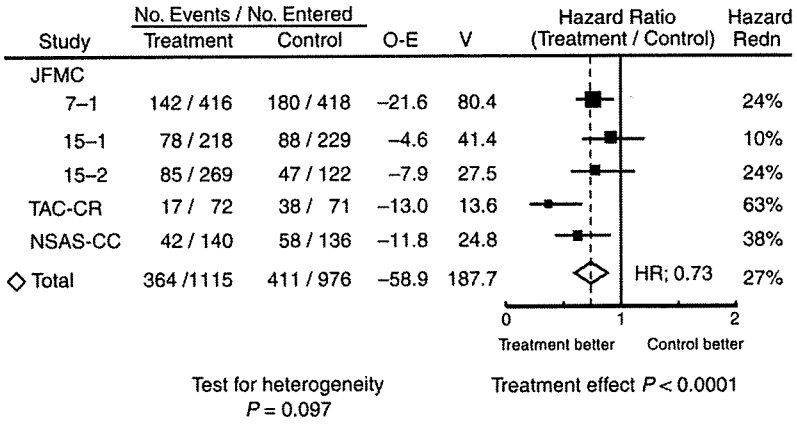


Figure 4 Disease-free survival hazard ratios by individual trial (Abbreviations as in Figure 1).

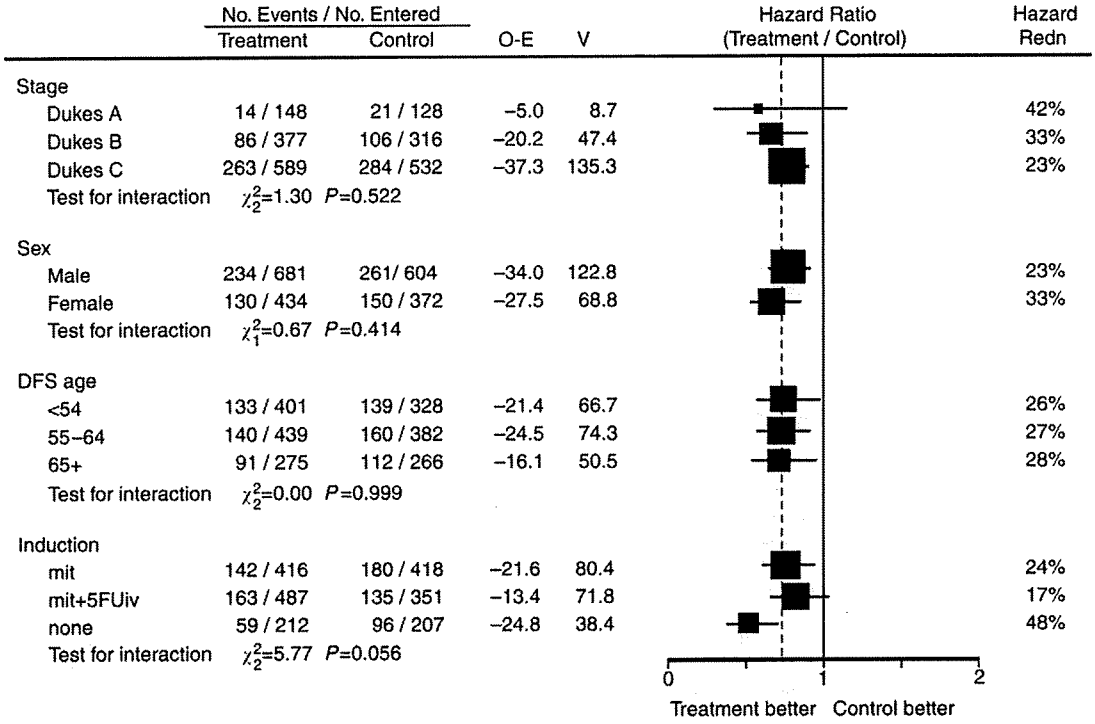


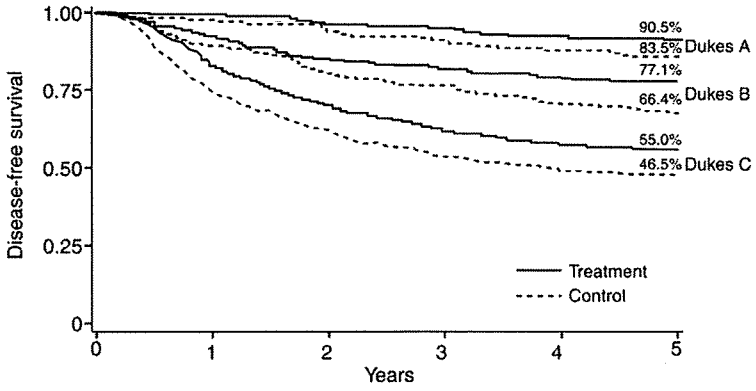
Figure 5 Disease-free survival hazard ratios by patient and treatment characteristics (Abbreviations as in Figure 1).

effects in different trials (χ^2_4 for heterogeneity = 8.82; $P = 0.0658$). UFT also showed significant effect on LRFS of curatively resected rectal cancers.

DISCUSSION

Extensive preclinical and clinical research led to the optimisation of 5-FU administration, with 5-FU bolus in combination with LV as standard therapy both in metastatic disease (Advanced Colorectal Cancer Meta-Analysis Project, 1992) and after curative resection of Stage III (Dukes' C) colon cancer (International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators, 1995; O'Connell *et al*, 1997; Wolmark *et al*, 1999). However, the toxicity of bolus 5-FU/LV regimen, especially the risk of haematologic toxicity and mucositis, could not have been negligible.

Continuous-infusion 5-FU modulated by LV, utilised mostly in European countries, showed somewhat better efficacy and definitely better tolerance than bolus 5-FU in advanced diseases (de Gramont *et al*, 1997; Meta-Analysis Group In Cancer, 1998a, b; Schmoll *et al*, 2000). In the adjuvant setting, one of the continuous regimens (LV5-FU2) was shown to have low toxicity than the bolus regimen, but no difference was shown in terms of survival (André *et al*, 2003). Recently, combination of continuous 5-FU/LV and oxaliplatin (FOLFOX 4) was demonstrated to have significant effect on DFS, and is now considered as the standard adjuvant regimen for colon cancer in the Western world.



		No. at risk					
Dukes A	Treatment	148	147	143	140	134	132
	Control	128	124	119	115	111	108
Dukes B	Treatment	377	347	315	303	291	282
	Control	316	280	250	237	217	206
Dukes C	Treatment	589	487	403	354	320	298
	Control	532	396	323	277	251	227

Figure 6 Disease-free survival curves by tumour stage and by treatment.

The recent development of O-FPs has therefore opened new perspectives. Oral fluorinated pyrimidines may mimic continuous regimens without its technical inconvenience and deterring patients' quality of life. In patients with advanced colorectal cancer, the efficacy of UFT (typical and most prescribed O-FP) plus oral LV (Carmichael *et al*, 2002; Douillard *et al*, 2002) or of capecitabine alone (Hoff *et al*, 2001; Van Cutsem *et al*, 2001) seems comparable in terms of the efficacy with significantly less significant severe haematologic toxicities and/or stomatitis. The risk of severe hand-foot syndrome is lower in UFT than with capecitabine, but the risk of severe diarrhoea and other gastrointestinal symptoms is higher in UFT and in UFT/oral LV treatment for Western patients.

In Japan, UFT have been administered for many years especially for patients with curatively resected colorectal cancers. For some unknown reason, severe gastrointestinal toxicities are much less frequent in Japanese patients, and patients usually prefer oral chemotherapy especially in an adjuvant setting (Borner *et al*, 2002).

Furthermore, with regard to rectal cancer, it is a difficult objective for a clinical trial to accrue enough patients, compared to colon cancer, and despite the fact that several attempts of determining a standard adjuvant treatment for rectal cancer, almost no clinical trial has succeeded in showing a relevant survival benefit of adjuvant treatment, except one with preoperative radiotherapy (Swedish Rectal Cancer Trial, 1997).

In this context, several Japanese groups conducted randomised clinical trials comparing UFT with surgery alone for curatively resected rectal cancers. Five such trials were identified after a meticulous search, and are included in the present meta-analysis. This meta-analysis was restricted to trials that had been randomised centrally and from which no patient had been excluded for any reason. It represents the largest series of properly randomly assigned patients receiving the single oral adjuvant O-FP agent, that is, UFT, for rectal cancer comparing with patients receiving no therapy after curative tumour resection.

This meta-analysis found a statistically significant benefit of UFT with regard to overall survival (OS) (hazard ratio = 0.82; $P = 0.02$) as well as DFS (hazard ratio = 0.73; $P < 0.0001$), and LRFS (hazard ratio = 0.68; $P = 0.0026$). As can seen by comparing the data in Figures 1 and 4, the data from the NSAS-CC and TAC-CR study show benefits that are, apparently, larger than the others. As shown in Table 1, the dosage and duration of treatment with UFT in the NSAS-CC and TAC-CR trials differed from those in the other three trials; the dose intensity of UFT was higher in the former two trials. Several studies have reported that a high-dose intensity of UFT improves survival in patients given postoperative adjuvant chemotherapy for gastric cancer (Sugimachi *et al*, 1997; Danno *et al*, 2001). The higher dose intensity of UFT in the NSAS-CC and TAC-CR trials may have influenced the outcomes.

Most of the Japanese rectal cancer patients did not receive pre- or postoperative radiotherapy in any of the trials. Although radiotherapy has been considered one of the standard adjuvant treatments in the Western countries, significant survival benefit has not been shown with reproducibility (Wolmark *et al*, 2000; Colorectal Cancer Collaborative Group, 2001). The ostensible advantage of adjuvant radiotherapy is to decrease local recurrence of rectal cancers. As compared with postoperative chemoradiotherapy, preoperative chemoradiotherapy does not improve OS, but inhibits local recurrence and reduces toxicity (Sauer *et al*, 2004). In our study, however, LRFS was also significantly better in the UFT group compared to surgery alone group. As far as our results are concerned, UFT might also be useful in preventing local recurrence in Japanese patients who usually do not receive radiotherapy in an adjuvant setting.

Also, there is still a debate whether adjuvant chemotherapy for early stage rectal cancer is feasible (Buyse and Piedbois, 2001). In terms of numbers needed to treat, these benefits imply that approximately 20 patients need to be treated for one more patient to survive 5 years, and approximately 10 to be treated for one fewer patient to suffer a cancer recurrence within 5 years, regardless of disease stage. Our results show that the therapy is beneficial in Stage II patients not only Stage III patients with nodal involvement (Mamounas *et al*, 1999; Gray *et al*, 2004). As for early stage disease, further investigations are needed to assess potential benefits of treatment because events were infrequent and hazard ratios were small.

Regardless of the disease stage and patient background characteristics, there is a need for further trials involving UFT and new agents that are effective in advanced disease, such as irinotecan, oxaliplatin, and monoclonal antibodies.

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