hotspots in high-magnification fields (× 200). Four randomly selected regions were analysed and averaged.

Statistical analysis. Values are presented as the means and standard deviation. Statistical analyses were conducted using Dr SPSS II for Windows software (SPSS Inc., Chicago, IL, USA). Differences between groups were assessed statistically using Student's *t*-test or Tukey–Kramer test for parametric data, or the Mann–Whitney *U*-test for non-parametric data. Overall and disease-free survival curves were drawn according to the Kaplan–Meier method, and differences between the curves were analysed by applying the log-rank test. Multivariate Cox regression analysis was used to analyse the independent prognostic factors related to survival. Differences at $P<0.05$ were considered to be statistically significant.

RESULTS

Correlation of NRP1 overexpression with poor outcome in clinical cases of pancreatic cancer. Neuropilin-1 immunostaining in clinical specimens of pancreatic ductal adenocarcinoma from 48 patients was predominantly identified in the cytoplasm and on the cell surfaces of malignant ductal cells (Figure 1). According to the semi-quantitative assessment criteria employed, the specimens were divided into 4 groups: 9 cases (19.7%) that were negative (IHC-0, Figure 1A), 17 (35.4%) that were weakly immunostained (IHC-1 + , Figure 1B), 12 (25.0%) that were moderately immunostained (IHC-2 + , Figure 1C), and 10 (20.8%) that showed strongly positive immunostaining (IHC-3 + , Figure 1D). Comparisons between low NRP1 expression (IHC-0/1 +) and high NRP1 expression (IHC-2 + /3 +) demonstrated similar clinicopathological features, except for MVD within the tumour ($P=0.018$, Table 1). The 3- and 5-year survival rates for the 48 patients overall were 54.2% and 12.5%, respectively. Kaplan–Meier analysis demonstrated a significant difference in overall survival between the groups showing high NRP1 expression (median 16.7 months, range 0.5–46.6 months) and low NRP1 expression (median 34.9 months, range 5.6–94.3 months) ($P=0.0278$, Figure 1E). Disease-free survival also differed significantly between high (median 8.2 months, range 0.5–46.6 months) and low NRP1 expression (median 16.1 months, range 3.2–94.3 months) ($P=0.017$, Figure 1F). Multivariate analysis using the Cox proportional hazards model indicated that, apart from positivity for lymph-node metastasis (hazard ratio (HR) = 5.620, 95% confidence interval (CI): 1.312–24.071, $P=0.020$), involvement of the resection margin (HR = 5.394, 95% CI: 2.227–13.068, $P<0.001$) and high expression of NRP1 (HR = 2.391, 95% CI: 1.045–5.469, $P=0.039$) were significantly correlated with poor overall survival, and were independent prognostic factors for pancreatic cancer (Table 2).

iRGD peptide facilitates drug penetration into murine pancreatic cancer models showing NRP1 overexpression. To investigate the activity of iRGD peptide, co-injection of Evans blue dye, and fluorochrome-labelled dextran was performed. Evans blue dye accumulation was enhanced 1.9-fold in two CXs (BxPC-3 and MIA PaCa-2) by co-administration of iRGD; however, enhanced dye accumulation was not observed in the remaining three CXs (Figure 2A; Supplementary Figure S1).

To evaluate the correlation between the iRGD inducing drug penetration effect and NRP1 expression in cancer cells, IHC was performed. Two CXs that showed enhanced dye accumulation were strongly positive (3 +), and the remaining three CXs were weakly positive (1 +) (Figure 2B; Supplementary Figure S2).

Three CXs (BxPC-3, MIA PaCa-2, and SUIT-2) and three TGs that showed NRP1 overexpression (Supplementary Figure S2) were employed in dextran experiments. Co-administration of iRGD peptide induced the dextran penetration into tumour parenchyma compared with dextran single administration (Figures 2C and D). Extended dextran-positive areas in CXs were 1.8-fold in BxPC-3 ($P=0.001$) and 2.1-fold in MIA PaCa-2 ($P=0.024$), but had no effect in SUIT-2 (Figure 2E). Penetration of dextran by co-administration of iRGD peptide was also observed in TGs, the areas of dextran distribution were extended 1.7-fold in PC-03 ($P=0.008$), 3.0-fold in PC-09 ($P=0.001$), and 1.9-fold in PC-10 ($P=0.040$) (Figure 2E).

Enhancement of the anticancer effect by co-administration of GEM and iRGD peptide in comparison with GEM monotherapy. Enhanced drug penetration into tumour was evaluated by Evans blue dye as a drug substitute tracer (Figure 3A). Drug accumulations in two CXs and one TG (PC-03) were significantly enhanced by iRGD co-administration; however, the effects in other two TGs (PC-09 and 10) were not remarkable. Similarly, co-administration of iRGD with GEM significantly decreased tumour growth in comparison with GEM monotherapy in BxPC-3 ($P=0.046$, Figures 3B and C) and MIA PaCa-2 ($P=0.037$, Figure 3C). One TG model (PC-03) also showed a significant tumour growth

Table 2. Cox proportinal hazards model of prognostic factors in patients with pancreatic cancer (n = 48)

	Univariate analysis			Multivariate analysis		
Factor	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (≥60 years)	0.934	0.418–2.084	0.867	NA	NA	NA
Gender (male)	0.885	0.415–1.887	0.752	NA	NA	NA
T factor (T4)	1.433	0.642–3.199	0.380	NA	NA	NA
Tumour size (≥40 mm)	2.166	1.010–4.646	0.047	1.738	0.748–4.042	0.199
Lymph-node status (positive)	6.712	1.585–28.421	0.010	5.620	1.312–24.071	0.020
Vascular invasion (positive)	1.768	0.769–4.068	0.180	NA	NA	NA
Perineural invasion (positive)	1.726	0.797–3.740	0.166	NA	NA	NA
Resection margin (positive)	5.757	2.501–13.251	<0.001	5.394	2.227–13.068	<0.001
Microvessel density[a] (≥25)	1.214	0.543–2.715	0.637	NA	NA	NA
Neuropilin-1 expression (high)	2.334	1.072–5.083	0.033	2.391	1.045–5.469	0.039

Abbreviations: CI = confidence interval; HR = hazard ratio; NA = not applicable.
[a]Number of vessels per 100 magnification field.

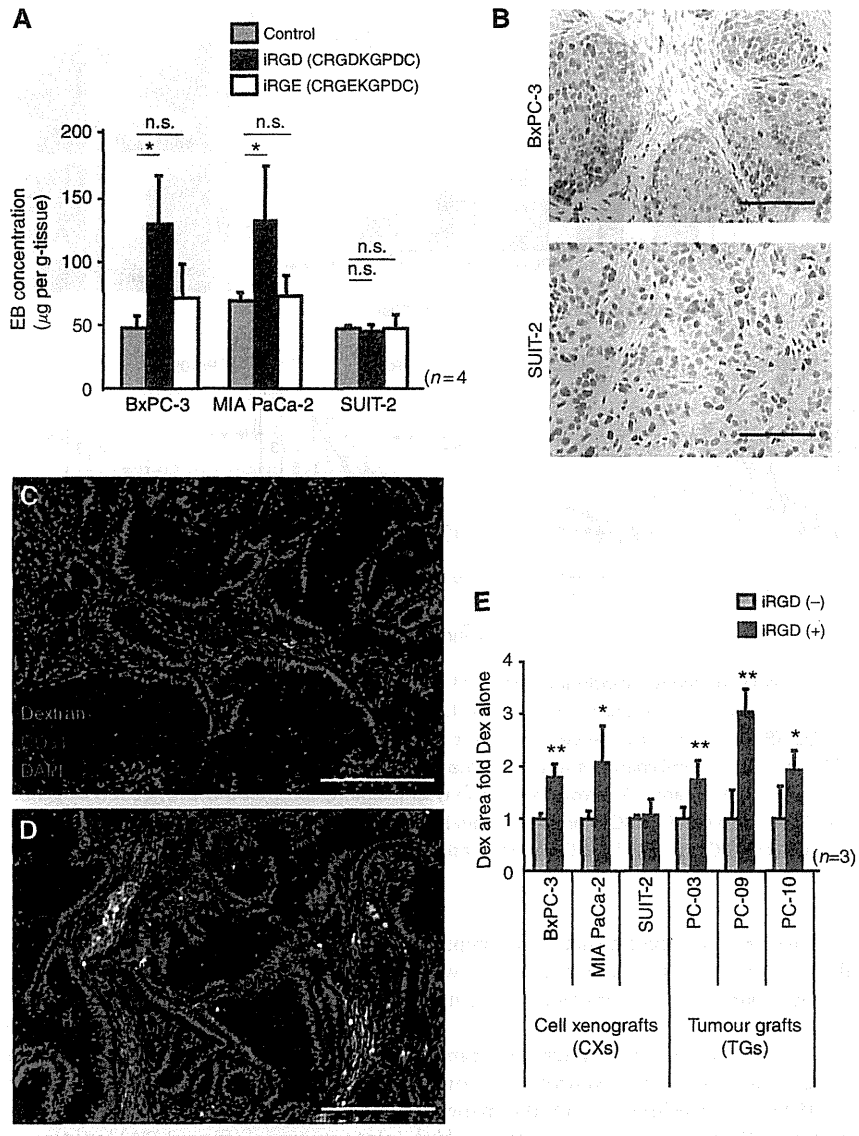

Figure 2. **(A)** Evans blue dye accumulation assay for cell line-based xenograft models (cell xenografts: CXs). Co-administration of iRGD peptide with a drug (black bar) was compared with administration of a single drug alone (grey bar) or iRGE peptide (white bar). Dye accumulation was enhanced 1.9-fold in BxPC-3 and MIA PaCa-2, but not in SUIT-2. Control peptide (iRGE) showed no enhancement of dye accumulation ($N=4$ per group). **(B)** Neuropilin-1 (NRP1) expression in animal models (× 400 magnification; scale bar, 100 μm). Overexpression of NRP1 was shown in BxPC-3 (upper) and MIA PaCa-2 (not shown), but not in SUIT-2 (lower). **(C** and **D)** Distribution of fluorochrome (Alexa-488, green)-labelled dextran in frozen sections of PC-09 (× 200 magnification; scale bar, 200 μm). Tumour vasculature was stained with anti-CD31 antibody (red), and nuclei were stained with DAPI (blue). Dextran appeared faintly after single administration **(C)**, and it was more widely distributed with a stronger intensity after co-administration with iRGD peptide **(D)**. **(E)** The areas of dextran distribution were calculated and compared. iRGD co-administration increased the area of dextran distribution 1.8-fold in BxPC-3 ($P=0.001$), 2.1-fold in MIA PaCa-2 ($P=0.024$), 1.7-fold in PC-03 ($P=0.008$), 3.0-fold in PC-09 ($P=0.001$), and 1.9-fold in PC-10 ($P=0.040$). $^*P<0.05$, $^{**}P<0.01$.

suppression by the combination therapy ($P=0.048$), but the other two TGs (PC-09 and 10) exhibited no significant differences (Figure 3C). The body weight of mice was decreased on days 12 and 15 in the GEM administered groups relative to the control group, but there was no significant difference in body weight between the iRGD co-administration group and the GEM monotherapy group (data not shown).

DISCUSSION

The present study conducted to re-evaluate the use of iRGD peptide demonstrated that it boosted the accumulation of drugs in two of five pancreatic cancer CXs that showed high expression of NRP1, and the anticancer effects of GEM were also enhanced by iRGD co-administration in these two CXs. We concluded that iRGD co-administration therapy would be indicated for nearly half of all patients with pancreatic cancer showing NRP1 overexpression, and we further evaluated this possibility using clinically relevant murine pancreatic cancer TG models. Enhancement of drug accumulation by iRGD was also observed in TGs, but the effects were less marked than those in CXs. Additionally, a significant anticancer booster effect of GEM plus iRGD combination therapy was observed in only one model.

First, our experiments using five CXs reconfirmed the enhanced drug accumulation effect of iRGD peptide. It was noteworthy that our experiments demonstrated that the effect of iRGD was dependent on the level of NRP1 expression; that is, the effects of iRGD were marked in two high-NRP1 CXs, but not significant in

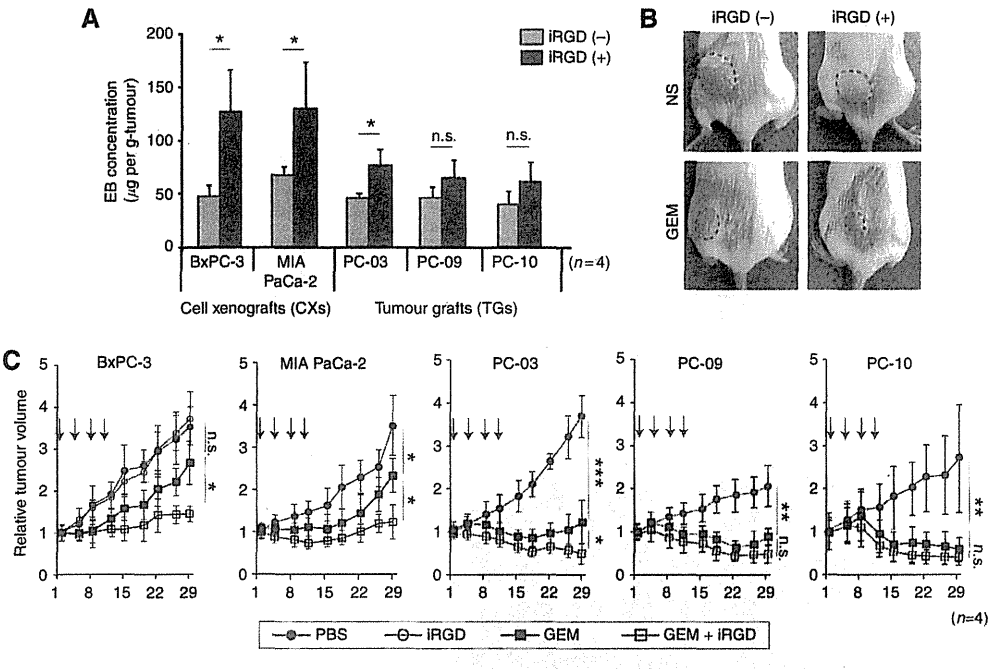

Figure 3. Tumour treatment studies involving co-administration of iRGD peptide. (**A**) Enhanced drug accumulation into tumour using Evans blue dye as a drug substitute tracer. Drug accumulations were enhanced about twofolds by iRGD in two CXs and one TG; however, the effects were not significant in another two TGs. (**B**) Tumour appearances in ectopic BxPC3 models at 28 days after the treatment initiation. (**C**) Tumour growth curves of gemcitabine (GEM) and iRGD combination therapy. Tumour-bearing mice were injected with 100 mg kg^{-1} gemcitabine or 100 μl of PBS (twice per week for 2 weeks; days 1, 4, 8, and 11; arrows show the day of infusion) combined with injection of PBS (Control, and GEM groups) or 8 μmol kg^{-1} iRGD peptide (iRGD, and GEM + iRGD group) 10 min beforehand ($N = 4$ per group). Tumour growth curves of two CXs (BxPC-3 and MIA PaCa-2) and three TGs (PC-03, PC-09, and PC-10) are indicated. *$P<0.05$, **$P<0.01$, ***$P<0.001$; n.s., not statistically significant.

three low-NRP1 models. These results suggest that iRGD peptide co-administration would be beneficial for subset patients with NRP1-overexpressing tumours, similarly to a molecular targeting agent.

We then evaluated the populations of pancreatic cancer overexpressing NRP1 in clinical specimens. Neuropilin-1 overexpression was seen in 45.8% of specimens, and the patients concerned had worse outcomes than patients whose tumours had low NRP1 expression (Figures 1E and F). Neuropilin-1 is known to be a co-receptor that enhances the binding of VEGF-A to the VEGF receptor (Soker *et al*, 1998), and NRP1 overexpression induces upregulation of VEGF signalling that is associated with angiogenesis and cancer metastases (Poon *et al*, 2001). Our finding that NRP1 overexpression was associated with higher MVD (Table 1) was considered to reflect upregulation of VEGF signalling. However, it should also be noted that resistance to GEM might have contributed to the differences in outcome. Neuropilin-1-overexpressing pancreatic cancer cell lines showed chemoresistance to GEM *in vitro* (Wey *et al*, 2005). All of the patients included in our study had received GEM-based adjuvant chemotherapy, and the effects of drug resistance should therefore be considered.

The effect of iRGD peptide in enhancing drug penetration is expected to improve the efficacy of treatment for dismal solid tumours, including pancreatic cancer. However, CXs do not adequately represent the clinical features of pancreatic cancer. We therefore employed pancreatic TGs, which have been reported to show better clinical predictive ability (Dong *et al*, 2010; Hidalgo *et al*, 2011; Morelli *et al*, 2012), and our previous evaluation had indicated that the drug delivery characteristics of TGs were more clinically reproducible. Histological findings of pancreatic TGs were more similar to clinical pancreatic cancer compared with CXs, as showing the atypical cancer glands and stromal tissues (Supplementary Figure S2). Internalised-RGD peptide certainly induced drug penetration in all TGs that were overexpressed NRP1, combination therapy using iRGD peptide was considered to be applicable for clinical pancreatic cancer.

Finally, the efficacy of iRGD peptide co-administration with GEM was verified using five pancreatic cancer models that showed enhanced drug accumulation in dextran experiments. In three models, iRGD peptide significantly enhanced drug accumulation into tumour (Figure 3A). Anticancer effect of GEM was also enhanced by iRGD co-administration in these three models (Figure 3C). Therefore, it seems possible to co-administer GEM, which is a key first-line drug for pancreatic cancer, with iRGD peptide, though the molecular size of GEM (0.3 kDa) is smaller than that of agents previously validated (0.6 kDa–130 nm, Sugahara *et al*, 2010). On the other hand, enhanced drug accumulations by iRGD peptide in the remaining two TGs were limited (Figure 3A), and the anticancer effects were also not significantly enhanced (Figure 3C). A major factor influencing the results was considered to be different histological findings among these models. Drug penetrations around the perfusing tumour vessels were not so different between CXs and TGs as shown in dextran experiments (Figure 2E). However, TGs included relative fewer blood vessels and more stromal tissues like clinical pancreatic cancer, as we previously reported (Akashi *et al*, 2013). We therefore considered that the efficacy of iRGD co-administration might be limited in clinical pancreatic cancer, characterised as poor vascularity and prominent desmoplastic reaction. A TG model (PC-03) that showed significant effect of iRGD peptide was established by transplantation of liver metastatic cancer tissue, whereas the other two TGs (PC-09 and 10) were established from primary pancreatic cancer. Histological findings of PC-03 (Supplementary Figure S2) were relatively similar to CXs, as characterised as hypervascular and fewer stromal tissues (Supplementary Figure S2; Akashi *et al*, 2013). Paradoxically, it is presumed that the impact of histological features on the iRGD efficacy is greater. Though, the difference of

drug susceptibility between CXs and TGs should be accounted for the interpretation of our results. As shown in Figure 3C, GEM monotherapy showed <50% inhibition of tumour growth in two CXs. In contrast, tumour growths in both TGs were significantly supressed by GEM monotherapy. Therefore, further validations, such as experiments using GEM-resistant TGs, might be required.

Our re-evaluations of iRGD peptide demonstrated a substantial booster accumulation effect of drugs in mouse pancreatic cancer models with high NRP1 expression, and this effect may be exploitable in nearly half of all patients with pancreatic cancer showing high NRP1 expression. Since the booster anticancer effects of iRGD co-administration with GEM were marked only in cell line-based models but not so great in TGs, the possible clinical application of iRGD peptide should be considered carefully.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Practical Utility and Objectivity: Does Evaluation of Peritoneal Elastic Laminal Invasion in Colorectal Cancer Overcome These Contrary Problems?

To the Editor:

The presence of peritoneal invasion separates pT3 and pT4a disease, and pT4a is known to be associated with a poor prognosis among patients with colorectal cancer (CRC).[1] In Japan, pathologists belonging to the Japanese Society for Cancer of the Colon and Rectum have worked together to review the current practice of pathologic assessments in different medical institutions.[2] In this review, in addition to the current status of many pathologic factors, tumor spread was also reviewed, and the distributions of pT1 to pT4 cases in 8 institutions were compared (Fig. 1). As a result, substantial differences in the frequency of pT3 and pT4 cases were found among the 8 institutions. Therefore, the previously indicated variability suggested by a review of previous reports on peritoneal invasion was confirmed for Japanese institutions.[3] As shown in a recently published, meticulous study by Liang et al,[1] elastic laminal invasion (ELI) in CRC can be used for the objective diagnosis of peritoneal invasion. These authors investigated ELI in 244 consecutively resected cases of pT3N0M0 CRC and found a strong association with the prognosis. An outstanding point in their study was the use of a single section to determine ELI, and they elucidated the availability of ELI in routine practice. Such results must be based on the proper selection of the section used for ELI evaluation. Liang and colleagues selected sections in which the tumor was closest to the peritoneal surface. Regarding this point, we would like to request a more precise description, which could be useful for concordant selections at multiple pathologic laboratories. Next, as they proposed, we agree with the use of ELI in discriminating pT3 cancers, on the basis of the results associated with the clinical outcome.[4,5] However, we still feel that some problems need to be answered. The most important problem is that immunohistochemistry or histochemistry may not always contribute to an improvement in concordance, as was confirmed in the D2-40 or elastic staining for the diagnosis of vascular invasion.[2] This problem must be assessed and solved in the future. Practical utility and objectivity must be considered together in future diagnostic pathology.

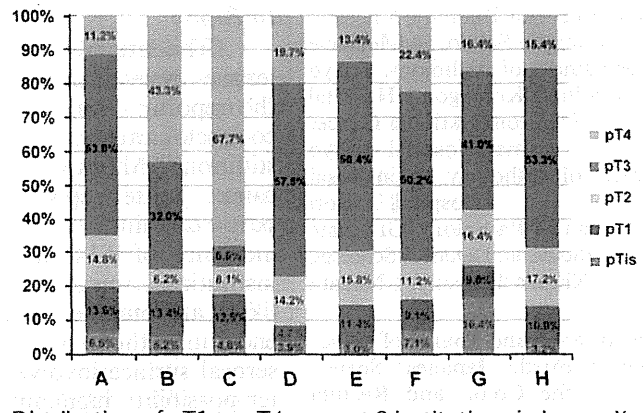

FIGURE 1. Distribution of pT1 to pT4 cases at 8 institutions in Japan. *X*-axis shows the institution, whereas the *Y*-axis shows the frequency of pT1 to pT4 cases. Substantial differences in the frequencies of pT3 and pT4 cases were observed among the 8 institutions.

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Re: Practical Utility and Objectivity

Does Evaluation of Peritoneal Elastic Laminal Invasion in Colorectal Cancer Overcome These Contrary Problems?

In Reply:

The letter by Kojima and colleagues reveals marked variation in the reporting rate of pT3 and pT4 colorectal cancers at 8 Japanese institutions. Although factors such as cancer center specialization might partly explain the markedly increased incidence of pT4 disease at certain institutions, some of this variation is likely attributable to regional differences in pathologist interpretation of serosal surface involvement. The latter possibility highlights the need for more objective techniques to confirm or exclude serosal involvement by colorectal carcinoma. Assessment for peritoneal elastic lamina invasion, studied earlier by Kojima and colleagues and more recently in our study using a single section of tumor tissue is an approach that may help address this issue.[1,2] As Kojima and colleagues point out in their letter, with our method of applying an elastic stain to a single tumor section, the choice of the section is important. In response to their specific question requesting a more precise description of how to choose this section, we would offer the following. First, our selection criteria were defined in very simple terms—the section in which the tumor appears to be closest to the peritoneal surface, following review of all sections of the tumor. Admittedly, there is a degree of subjectivity in this assessment, and in some cases it is unclear whether the surface of the adipose tissue is serosal or not. However, from our experience, the choice of section closest to the surface is obvious in many cases. Pathologists should be aware that the closest serosal surface to the tumor may be an area of serosal retraction or invagination. Occasionally, there is fibrosis in the adipose tissue between the tumor and the closest peritoneal surface. Importantly, the section of tumor closest to the peritoneal surface is not necessarily the section with the greatest depth of invasion beyond the muscularis propria. Often the choice of section will come down to 2 sections for direct comparison, and, although we did not physically measure the distance to the surface of the adipose tissue, that is a practical option which some pathologists might consider. It is important to be aware that, with the single-section approach, the elastic lamina will not be identified in the majority of cases (59% in our study). However, failure to identify the elastic lamina on the most suspicious section does not seem to matter, as the cases with no elastic lamina identified have a similar prognosis to those cases in which the elastic lamina is identified but elastic lamina invasion is not present. Elastic lamina invasion appears promising as a more objective measure of peritoneal involvement. However, we fully agree with Kojima and colleagues that further study is needed to determine whether use of this technique can actually improve interobserver agreement in reporting of serosal involvement.

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OPEN ACCESS

Pathological diagnostic criterion of blood and lymphatic vessel invasion in colorectal cancer: a framework for developing an objective pathological diagnostic system using the Delphi method, from the Pathology Working Group of the Japanese Society for Cancer of the Colon and Rectum

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ABSTRACT

Aims The goal of this study is to create an objective pathological diagnostic system for blood and lymphatic vessel invasion (BLI).

Methods 1450 surgically resected colorectal cancer specimens from eight hospitals were reviewed. Our first step was to compare the current practice of pathology assessment among eight hospitals. Then, H&E stained slides with or without histochemical/immunohistochemical staining were assessed by eight pathologists and concordance of BLI diagnosis was checked. In addition, histological findings associated with BLI having good concordance were reviewed. Based on these results, framework for developing diagnostic criterion was developed, using the Delphi method. The new criterion was evaluated using 40 colorectal cancer specimens.

Results Frequency of BLI diagnoses, number of blocks obtained and stained for assessment of BLI varied among eight hospitals. Concordance was low for BLI diagnosis and was not any better when histochemical/immunohistochemical staining was provided. All histological findings associated with BLI from H&E staining were poor in agreement. However, observation of elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster as well as the presence of D2-40-stained endothelial cells covering more than half of the circumference surrounding the tumour cluster showed high concordance. Based on this observation, we developed a framework for pathological diagnostic criterion, using the Delphi method. This criterion was found to be useful in improving concordance of BLI diagnosis.

Conclusions A framework for pathological diagnostic criterion was developed by reviewing concordance and using the Delphi method. The criterion developed may serve as the basis for creating a standardised procedure for pathological diagnosis.

INTRODUCTION

Blood and lymphatic vessel invasion (BLI) in colorectal cancer (CRC) are known to be strong risk factors correlated with poor outcome. Since it was first reported by Brown *et al* in 1938, numerous studies have been conducted on BLI. BLI is adopted in TNM Classification of Malignant Tumours and College of American Pathologists Consensus Statement in pathology reports.[1–4] Assessment of BLI enables identifying patients with high risk within the same TNM stage and therapeutic strategy can be tailored accordingly, especially for patients with Stage II CRC and patients with endoscopically resected pT1.[5–7] Observation of BLI, however, is also known for its weakness, which is high interobserver variability. Many articles report poor interobserver concordance of BLI assessment and no solution has been offered so far.[8–10] One solution may be to use elastica for histochemical staining or D2-40 for immunohistochemical staining of internal elastic lamina of vessel and lymphatic endothelium.[11–15] Another solution may be to take a conventional approach to develop a framework for diagnostic criterion, through formal procedure to reach consensus, gain support and understanding of pathologists worldwide.[16] It was under this concept that pathologists belonging to the Japanese Society for Cancer of the Colon and Rectum decided to join hands and took a comprehensive approach as follows: (1) review current practice of pathologists' assessment including sampling methods, staining methods and BLI in different medical institutions (2) evaluate concordance of BLI diagnosis and histological findings associated with BLI (3) develop a framework for diagnostic criterion using the Delphi method, with data from current practice and (4) conduct a concordance study to evaluate the usefulness of the new criterion. Our attempt was to develop a framework for an objective criterion to assess BLI, in order to improve concordance in all settings.

MATERIALS AND METHODS

Multicentre, retrospective review of pathological assessment at different departments of pathology

A total of 1450 patients with CRC who underwent surgical resection in 2003 from eight institutions under the Japanese Society for Cancer of the Colon and Rectum were reviewed. Clinicopathological factors including the TNM stage according to the

fifth edition of TNM classification,[3] the presence of BLI, number of paraffin blocks taken to examine primary tumours, use of megablock, tangential tissue sectioning, histochemical staining and immunohistochemical staining were reviewed and compared.[17] The range of histochemical staining and antibody used for immunohistochemical staining were also reviewed.

Interobserver study

Eighty consecutive, surgically resected specimens of Stage II CRC according to the seventh edition of TNM classification[4] between 2003 and 2005 from the National Cancer Center Hospital East were used for the interobserver study. Eight pathologists from eight institutions assessed the slides. Specimens for pathological assessment were divided into six cohorts as follows (table 1) and concordance of diagnosis was reviewed. Cohort 1: H&E-stained slides without any guiding criteria. Cohort 2: H&E-stained slides without any criteria, but focus on designated area of lesion. Assessment was later checked to see which histological findings associated with BLI had good agreement. Concordance of assessment for designated area was also reviewed (figure 1). Cohort 3: H&E-stained, elastica-stained and D2-40-stained slides. Histochemical and immunohistochemical staining without any guiding criteria. Cohort 4: H&E-stained, elastica-stained and D2-40-stained slides. Histochemical and immunohistochemical staining without any guiding criteria but focus on designated area of lesion (figure 1). Observation was later checked to determine which slides of H&E, histochemical or immunohistochemical staining associated with BLI diagnosis had good concordance. Concordance of assessment for designated area of lesion was also reviewed. Cohort 5: H&E-stained, elastic-stained and D2-40-stained slides. Histochemical and immunohistochemical staining and our new criterion were used for assessment. Finally, for Cohort 6, the same H&E-stained, elastic-stained and D2-40-stained slides used in Cohort 3 were assessed by the pathologists (who were unaware of reviewing the same slides) to check diagnostic agreement of histochemical and immunohistochemical staining using our new criterion. Cohorts 1, 3, 5 and 6 each consisted of 20 CRC specimens and Cohorts 2 and 4 each consisted of 10 CRC specimens. H&E-stained slides and slides of largest slice from blocks of specimen including the deepest invasive area of tumour were used in Cohorts 1, 3, 5 and 6, while one representative slide of the tumour was used in Cohorts 2 and 4. In Cohorts 2 and 4, assessment of designated area was reviewed to evaluate the agreement of BLI diagnosis. Three areas of lesion containing histological tumour cluster surrounded by some space or fibrous rim-like vascular structure were chosen randomly and marked with ink. Since the designated area was very small, virtual slides were used to indicate with an arrow where assessment should be made (figure 1). In Cohorts 1, 3, 5 and 6 the 'presence' or 'absence' of BLI was reported. In Cohorts 2 and 4, assessment of designated area was made as 'blood vessel invasion', 'lymphatic vessel invasion' or 'neither'. In Cohorts 2 and 4, histological findings associated with the diagnosis of BLI were collected. This was reviewed thoroughly in a meeting and was recorded in the survey sheet, as either 'present' or 'absent' (table 2).

Developing diagnostic criteria using the Delphi method

Four rounds of consensus meetings, participated by eight pathologists were held as shown in figure 2. Before the meeting, a survey on histological, histochemical and immunohistochemical diagnostic criteria of BLI was prepared. All pathologists were requested to answer the survey anonymously and send it by mail after the meeting.[16] There were a total of 34 questions: 2 on the definition of BLI, 7 on the assessment of BLI, 4 on the use of histochemical and immunohistochemical staining, 8 on assessment of blood vessel invasion and 13 on the assessment of lymphatic vessel invasion (table 3). Scoring was based on 1 to 6 Likert scale (1=strong disagreement, 2=moderate disagreement, 3=some disagreement, 4=some agreement, 5=moderate agreement, 6=strong agreement), maximum score being 6 points. Scores of 5 and 6 were regarded as 'agreement'. Consensus was considered to be achieved when over 80% of the participants' scores resulted in 'agreement', based on the previously described scoring method.[18–20] Four rounds of meetings with active discussion took place and surveys were conducted three times, after the second and third rounds of meetings, as shown in figure 2. At the beginning of the second and third rounds of meetings, interim results of survey were reported to the

Table 1 Details of cohorts and concordance of blood and lymphatic vessel invasion (BLI)

H&E staining	D2-40 and elastica staining	Designated area of lesion	Criterion		κ Value	95% CI	Positive ratio (%)
Cohort 1							
+	−	−	−	Agreement in blood vessel invasion	0.524	0.441 to 0.606	28.1
+	−	−	−	Agreement in lymphatic vessel invasion	0.216	0.133 to 0.299	32.5
Cohort 2							
+	−	+	−	Agreement in BLI	0.466		
Cohort 3							
+	+	−	−	Agreement in blood vessel invasion	0.502	0.419 to 0.584	73.8
+	+	−	−	Agreement in lymphatic vessel invasion	0.153	0.071 to 0.236	33.8
Cohort 4							
+	+	+	−	Agreement in BLI	0.622		
Cohort 5							
+	+	−	+	Agreement in blood vessel invasion	0.547	0.464 to 0.630	42.5
+	+	−	+	Agreement in lymphatic vessel invasion	0.492	0.409 to 0.575	26.9
Cohort 6							
+	+	−	+	Agreement in blood vessel invasion	0.617	0.534 to 0.700	75.6
+	+	−	+	Agreement in lymphatic vessel invasion	0.618	0.534 to 0.700	31.9

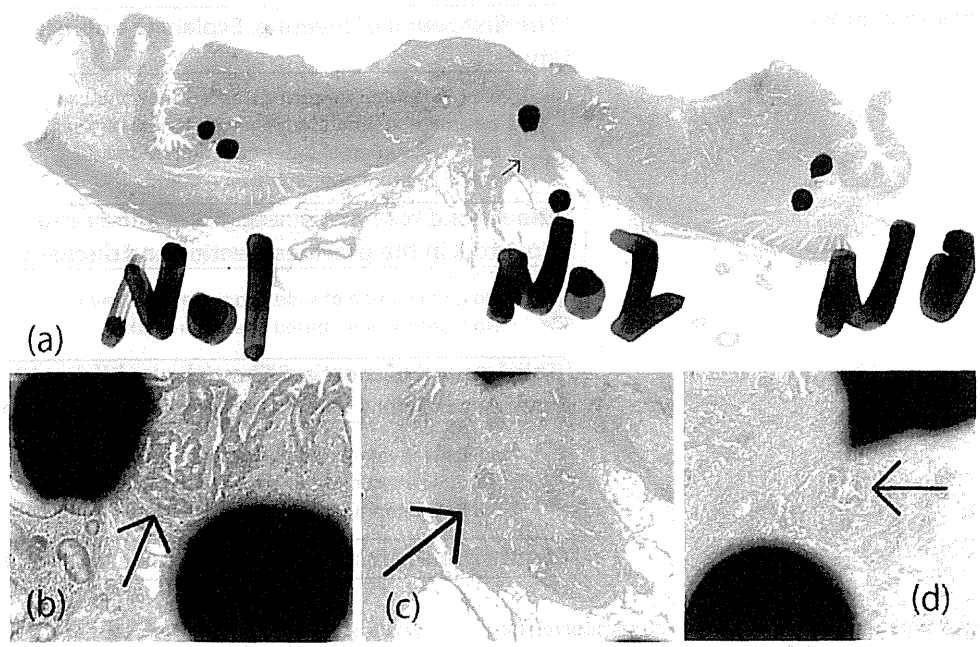

Figure 1 (A–D) A case from Cohort 2. In Cohorts 2, 4, three regions of interest within the histological tumour cluster with surrounding space or fibrous rim-like vascular structure were chosen randomly and marked with ink near the lesion (A). The lesion to be reviewed was indicated with an arrow on the virtual slides (B–D). Eight pathologists reviewed the slides. The assessment for each of the indicated lesions was reported as 'blood vessel invasion', 'lymphatic vessel invasion' or 'neither'. Furthermore, pathological findings associated with the diagnosis of blood and lymphatic vessel invasion were studied. Reviewers recorded their interpretations of the indicated lesion using the query sheet, answering the questions as 'present' or 'absent'.

participants to facilitate building consensus on key histological findings with high concordance. For findings that failed to present immediate agreement, further discussion took place and the next vote was performed. Consistent with the Delphi method, some questions in the survey were modified to enable building general agreement.[18] After the third round of meetings, the findings for which consensus had been reached were summarised and new diagnostic criterion was developed.

Statistical analysis

The concordance reached by pathologists on rating tumour invasion was evaluated using κ coefficients. Using %mκ SAS macro, we

Table 2 Concordance of histological findings associated with the blood and lymphatic vessel invasion

Histological findings	κ Value	95% CI
Cohort 2		
Presence of space around tumour nests	0.492	0.424 to 0.560
Presence of endothelium around tumour nests	0.518	0.451 to 0.586
Presence of vascular smooth muscle around tumour nests	0.412	0.344 to 0.479
Presence of spicula at the periphery of tumour nests	0.308	0.240 to 0.376
Presence of lymphatic fluid in peritumoural space	0.454	0.386 to 0.522
Presence of blood cells in peritumoural space	0.395	0.327 to 0.462
Difficult to distinguish blood and lymphatic vessel	0.064	−0.033 to 0.132
Presence of continuity in normal blood or lymphatic vessel	0.276	0.209 to 0.344
Cohort 4		
Presence of space around tumour nests	0.471	0.404 to 0.539
Presence of endothelium around tumour nests	0.269	0.201 to 0.336
Presence of vascular smooth muscle around tumour nests	0.372	0.305 to 0.440
Presence of spicula at the periphery of tumour nests	0.002	−0.066 to 0.069
Presence of lymphatic fluid in peritumoural space	0.142	0.075 to 0.210
Presence of blood cells in peritumoural space	0.055	−0.013 to 0.122
Difficult to distinguish blood and lymphatic vessel	0.055	−0.013 to 0.123
Presence of continuity in normal blood or lymphatic vessel	0.150	0.082 to 0.217
Growth along with normal artery	0.491	0.424 to 0.559
Presence of elastica-stained internal elastic membrane covering more than half of the circumference surrounding the tumour cluster	0.801	0.734 to 0.869
Presence of D2-40 positive cells covering more than half of the circumference surrounding the tumour cluster	0.451	0.383 to 0.518
Presence of D2-40 positive endothelial cells covering more than half of the circumference surrounding the tumour cluster	0.682	0.615 to 0.750

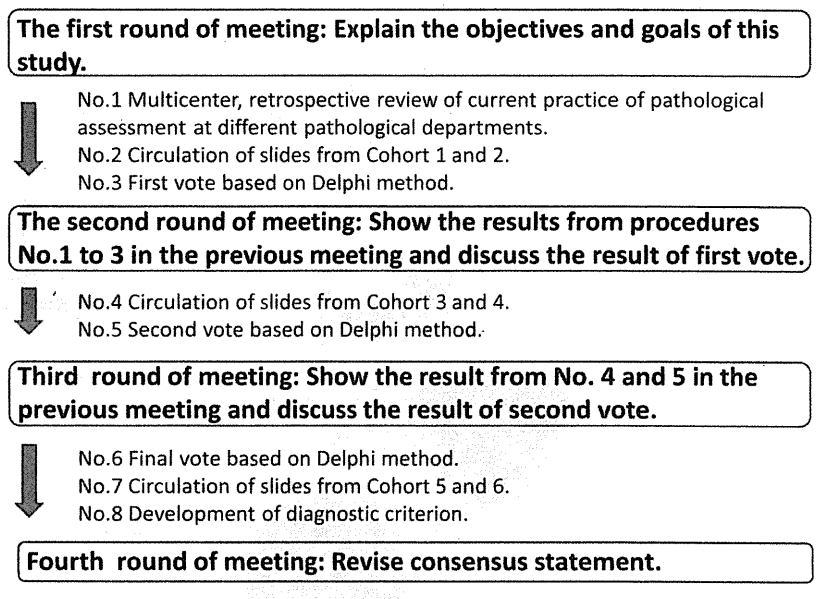

Figure 2 Time flow for consensus development.

estimated the Fleiss type multi-rater κ coefficient and corresponding 95% CI.[21] All statistical analyses was performed with SAS Release V.9.3 (SAS Institute, Inc, Cary, North Carolina, USA).

RESULTS

Multicentre retrospective review of current practice of pathological assessments

The result of the study is shown in table 4 and figure 3. The total number of cases reviewed in this study by eight institutions ranged from 54 to 441, of which 51.0–72.5% were colon cancer, 53.5–68.0% were male patients, and their average age was between 63 years and 68 years. The average number of paraffin blocks used for primary tumour pathological assessment varied widely among institutions, ranging from 4.8 blocks to 34.4 blocks. Use of histochemical staining and immunostaining was also different among eight institutions. And the range of histochemical staining performed was also different. Antibody used in immunohistochemical staining was D2-40 in three institutions. And one institution used D2-40 and alpha-smooth muscle actin (SMA). Figure 3 shows the results of a retrospective review of the stage and assessment of BLI. Although the stage distributions were similar among the eight institutions (Stage 0, I; 19.8–28.0%, Stage II; 10.2–17.4%, Stage III; 12.1–21.5% and Stage IV; 3.0–9.9%), substantial difference was noted on the presence of BLI (lymphatic vessel invasion; 18.9–74.8%, blood vessel invasion; 17.7–66.7%). None of the institutions in this study used megablock or tangential sectioning.

Interobserver study and development of diagnostic criterion using the Delphi method

The result of the interobserver study is shown in table 1. After the first round of meeting, Cohorts 1 and 2 were given only H&E stained slides without any additional staining or guiding criteria. The concordance of assessment of blood vessel invasion was moderate and it was low for lymphatic vessel invasion. This was not any better when pathologists in Cohort 2 were asked to focus on the designated area. Furthermore, the concordance of all histological findings associated with BLI was low in Cohort 2 (table 2). Diagnosis of BLI is based on multiple histological findings considered to be associated with BLI, most of which were included in this study as shown in table 2. Consistent diagnosis of BLI only with H&E-stained slides seemed to be difficult to achieve and this was informed to the pathologists during the second round of meetings. It was then decided to distribute H&E-stained slides as well as the histochemical and immunohistochemical staining, without any guiding criteria (Cohorts 3 and 4). Although this increased positive findings of blood vessel invasion in Cohort 3, it did not improve the concordance of BLI diagnosis. In Cohort 4, concordance improved for the designated area. Interestingly, we found few histochemical and immunohistochemical findings associated with the diagnosis of BLI having good agreement (table 2). This was reported to pathologists during the third round of meetings and it was agreed that they should be included in the diagnostic criteria of BLI (table 3). Pathologists summarised the findings which they were able to agree upon and new diagnostic criterion was developed with active discussion (box 1). Finally, the use of the new criterion was evaluated with Cohorts 5 and 6. There was a remarkable improvement in concordance in Cohort 5 (κ=0.547 for blood vessel invasion, κ=0.492 for lymphatic vessel invasion), as well as in Cohort 6 (κ=0.617 for blood vessel invasion, κ=0.618 for lymphatic vessel invasion), which used the same slide as in Cohort 3 (κ=0.502 for blood vessel invasion, κ=0.153 for lymphatic vessel invasion). This serves as direct evidence on the usefulness of new criterion in improving agreement in BLI diagnosis (table 1).

DISCUSSION

The need for a diagnostic criterion which enables a standardised and objective diagnosis is clear. Our attempt was to provide a framework for developing a consensus-based criterion to be used in different pathological settings through this study. BLI are distinct pathological factors and have different clinicopathological implications.[22] There are, however, some common morphologies such as the presence of endothelium or cavity, and distinct identification of small venules and lymphatic vessel is often difficult. There is also the problem of interobserver variability.[2] BLIs are strong risk factors in many types of cancer. In CRC, BLIs can be used as a criterion to determine the need for adjuvant therapy in Stage II cases, which account for a significant proportion of CRC cases (27.1% in this study). For endscopically resected pT1 cases, BLIs are reported to be risk factors of lymph node metastasis and can be used as a criterion for making a decision of surgical resection. In this study, we tried to solve interobserver variabilities in BLI. The criterion that we developed may be useful for achieving