in the scores among adenocarcinomas (all histological types; data not shown).

p53 expression in surgical specimens

p53 was expressed in the nucleus in some adenocarcinomas and a few specimens with BilIN-2 and BilIN-3 (Fig. 1). Multiplication scores in specimens with adenocarcinoma, BilIN-3, BilIN-2, BilIN-1, and non-neoplastic epithelium were (mean±SD/median) 7.4±9.5/3, 4.3±6.3/1.5, 2.0±5.1/0, 2.9±6.9/0, and 0/0, respectively (Table 2). For each neoplasm, the multiplication scores for p53 were lower than those for cldn18 and maspin, but p53 was the most specific of the three markers (Table 2). Multiplication scores in well, moderately, and poorly differentiated adenocarcinoma were (mean±SD/median) 7.8±9.6/3, 7.5±9.3/2.5, and 5.8±8.7/2, respectively (Table 3 and Supplementary Fig. S1).

Diagnostic value of cldn18, maspin, and p53 in surgical specimens

To distinguish neoplasms from non-neoplastic epithelium in the surgical specimens from patients with biliary tract cancers, we calculated the AUC for cldn18, maspin, and p53, as described in the "Materials and methods" (Fig. 2a). The AUC for cldn18 was 0.992 [95 % CI, 98.3 to 100] (Fig. 2b). A cutoff value of 6 produced the highest accuracy (minimal number of false-negative and false-positive results); sensitivity and specificity were 95.6 and 96.8 %, respectively. There was no significant difference in the scores between BilIN-1–3, so BilIN-1–3 were referred to collectively as "BilIN" (Fig. 2c). As shown in the upper panel of Fig. 2c, 87 of 91 (95.1 %) specimens with adenocarcinoma/BilIN (63 of 66 with adenocarcinoma and 24 of 25 with BilIN) were detected as neoplastic. Among specimens with non-neoplastic epithelium, 61 of 63 (96.8 %) were detected as non-neoplastic. The AUC for maspin (N) was 0.879 [95 % CI, 82.5 to 93.3] (Fig. 2b). A cutoff value of 1 gave the highest accuracy; sensitivity and specificity were 85.6 and 77.8 %, respectively. As shown in the third panel of Fig. 2c, 78 of 91 (85.7 %) specimens with adenocarcinoma/BilIN (58 of 66 with adenocarcinoma and 20 of 25 with BilIN) were detected as neoplastic. Among the specimens with non-neoplastic epithelium, 49 of 63 (77.8 %) were detected as non-neoplastic. The AUC for p53 was 0.806 [95 % CI, 73.7 to 87.4] (Fig. 2b). A cutoff value of 1 produced the highest accuracy; sensitivity and specificity were 61.1 and 100 %, respectively. As shown in the bottom panel of Fig. 2c, 56 of 91 (61.5 %) specimens with adenocarcinoma/BilIN (47 of 66 with adenocarcinoma and 9 of 25 with BilIN) were detected as neoplastic. Among specimens with non-neoplastic epithelium, all 63 were detected as non-neoplastic. For distinguishing adenocarcinoma from non-neoplastic epithelium or BilIN from non-neoplastic epithelium, ROC curves showed that all the three markers were highly accurate (Supplementary Figs. S2A and S2B).

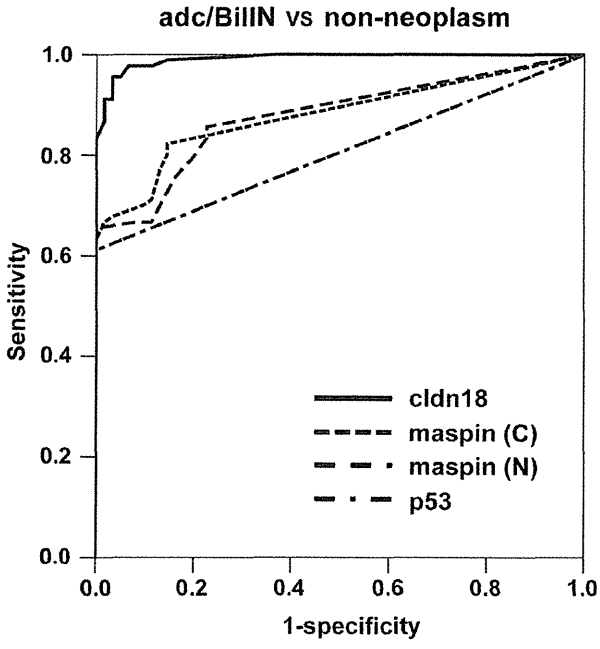
Next, we obtained additional scores by combining the multiplication scores for cldn18, maspin (N), and p53 and analyzed the new scores to distinguish neoplastic from non-neoplastic tissues. The AUC for the combined multiplication score was 0.996 [95 % CI, 98.8 to 100] (Supplementary Fig. S3A). A cutoff value of 15 had the highest specificity; sensitivity and specificity were 96.6 and 100 %, respectively. A cutoff value of 12 had the highest sensitivity; sensitivity and specificity were 100 and 96.8 %, respectively (Supplementary Fig. S3A). When all specimens were differentiated by a cutoff value of 15, 88 of 91 (96.7 %) specimens with adenocarcinoma/BilIN were detected as neoplastic, and all 63 specimens with histologically diagnosed non-neoplastic epithelium were detected as non-neoplastic (Supplementary Fig. S3B). These results indicate that the strategy of combining the three multiplication scores successfully distinguished neoplasms from non-neoplastic epithelia in the surgical specimens of biliary tract carcinoma.

For convenience, we employed an additional step. Before combining the three parameters for cldn18, maspin (N), and p53, we converted each of the multiplication scores to binary values (0 for immuno-negative and 1 for immuno-positive) on the basis of the best cutoff values calculated above. With the binary values for cldn18, maspin (N), and p53, we calculated the AUC and analyzed the new scores to distinguish neoplasms from non-neoplastic tissues (Fig. 3). After these processes, the AUC was 0.989 [95 % CI, 97.8 to 99.9] (Fig. 3a). As shown in Fig. 3b, the best cutoff score was 2, 81 of 91 (89.0 %) specimens with adenocarcinoma/BilIN and none with non-neoplastic epithelium were distinguished as neoplastic, and sensitivity and specificity were 91.1 and 100 %, respectively. At a cutoff value of 1, all 91 specimens with adenocarcinoma/BilIN and 15 of 63 (23.8 %) with non-neoplastic epithelium were distinguished as neoplastic; sensitivity and specificity were 100 and 74.6 %, respectively. At a cutoff value of 3, 41 of 91 (45.1 %) specimens with adenocarcinoma/BilIN and none with non-neoplastic epithelium were distinguished as neoplastic; sensitivity and specificity were 51.1 and 100 %, respectively.

Diagnostic value of cldn18, maspin, and p53 in presurgical endobiliary forceps biopsy specimens

Next, we examined whether this analysis is applicable to presurgical endobiliary forceps biopsy specimens because they are the most important source for both clinical diagnosis and rapid intraoperative diagnosis. The immunostaining patterns of the presurgical endobiliary forceps biopsy specimens were similar to those of the surgical specimens (Fig. 4a). In biopsy specimens, we observed that some epithelia without apparent dysplasia, including intestinal epithelium, were

a

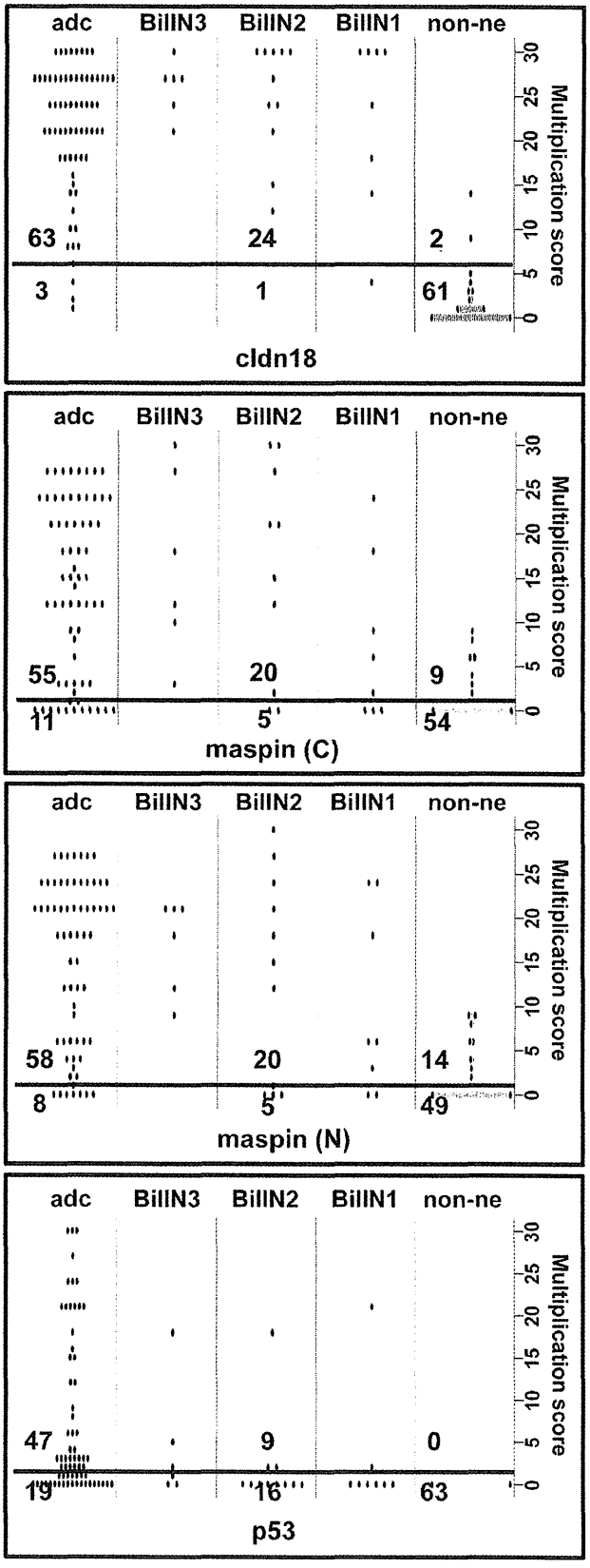


b

	AUC	95%CI Lower	95%CI Upper
cldn18	0.992	0.983	1.000
maspin (C)	0.881	0.826	0.935
maspin (N)	0.879	0.825	0.933
p53	0.806	0.737	0.874

score	Best cutoff	Sensitivity	Specificity
cldn18	≥6	0.956	0.968
maspin (C)	≥1	0.822	0.857
maspin (N)	≥1	0.856	0.778
p53	≥1	0.611	1.000

c



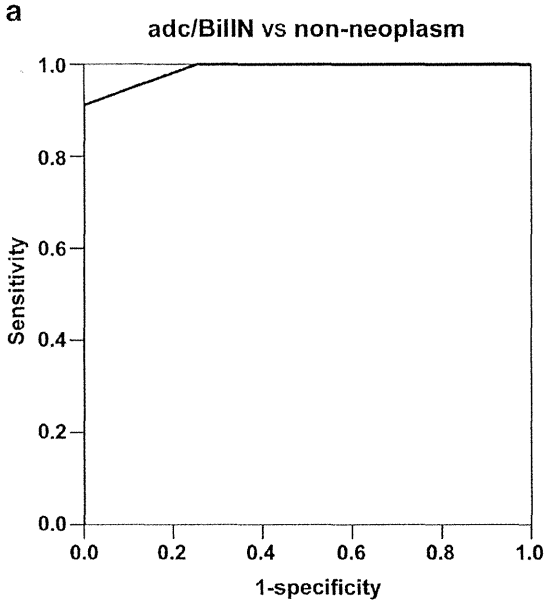
◀ **Fig. 2** ROC curve analysis of cldn18, maspin, and p53 in surgical specimens. **a** ROC curves were calculated according to multiplication scores that represented the degrees of immunoreactivity with a scale of 0 to 30 for each antibody as described in "Materials and methods." Immunoreactivity against maspin was separately evaluated in the cytoplasm (C) and nucleus (N). Cutoff values were calculated from the ROC curves to distinguish adc/BilIN from non-neoplastic epithelium (*non-ne*). **b** Accuracy was measured by the AUC. The 95 % CIs for AUC and best cutoff values with the highest accuracy are summarized in the tables. **c** *Dot plots* show the distribution of multiplication scores for each antibody. In each panel, the *bold horizontal line* represents the best cutoff value to divide the cases into two groups, adc/BilIN (*above the line*) and non-ne (*below the line*). *CI* confidence interval

positive for maspin (5 of 22 non-neoplastic cases). However, we classified these as negative for maspin because H&E staining clearly demonstrated them as non-neoplastic (data not shown). To distinguish neoplasms from non-neoplastic epithelia, we analyzed the combined scores from the binary values for cldn18, maspin (N), and p53, as described for the surgical specimens. The AUC was 0.990 [95 % CI, 0.964–100] (data not shown). As shown in Figs. 4b, c, and d, at a cutoff value of 1, all 18 specimens with adenocarcinoma/BilIN and 4 of 19 with non-neoplastic epithelium were distinguished as neoplastic. The sensitivity and specificity were 100 and 78.9 %, respectively. At a cutoff value of 2, all 18 specimens with adenocarcinoma/BilIN and 1 of 19 with non-neoplastic epithelium were distinguished as neoplastic. The sensitivity and specificity were 100 and 94.7 %. When the cutoff value was set at 3, 11 of 18 specimens with adenocarcinoma/BilIN and none of those with non-neoplastic epithelium were distinguished as neoplastic. The sensitivity and specificity were 61.1 and 100 %, respectively. A cutoff value of 2 had the highest sensitivity and specificity, and the score provided good separation of neoplasm from malignancy-undetermined atypical epithelium (18/21, 85.7 %) in cases that showed nuclear atypia but could not be proven as adenocarcinoma with a biopsy. We examined seven percutaneous liver biopsy specimens and found that a cutoff value of 2 had the highest sensitivity and specificity (Supplementary Fig. S4).

Double staining of cldn18 and maspin

We examined 14 surgical specimens of biliary tract cancer by double staining for cldn18 and maspin. The staining pattern was coincident with those expected from the single staining patterns of cldn18 and maspin (Fig. 4e). Some specimens of neoplastic epithelium were immunoreactive for both cldn18 and maspin (12/14, 86 %); others were reactive only for cldn18 (2/14, 14 %), indicating that all examined biliary tract adenocarcinomas were positive for at least cldn18 or maspin. Among the 14 specimens, 9 had BilINs and all were positive for at least one marker. All of the specimens with BilIN-1 (3/3) and BilIN-3 (3/3) and 1/3 of those with BilIN-2 were double-

a



	95%CI	
AUC	Lower	Upper
0.989	0.978	0.999

b

		Sensitivity	Specificity
Score (binary value)	≥1	1.000	0.746
	≥2	0.911	1.000
	≥3	0.511	1.000

		adc/BilIN	non-neoplasm
Score (binary value)	0	0	48
	1	10	15
	2	40	0
	3	41	0
Total		91	63

Fig. 3 ROC curve analysis in surgical specimens performed by using combined parameters of cldn18, maspin, and p53. **a** The ROC curve was calculated according to the combined binary values of the multiplication scores for each antibody. Briefly, each of the multiplication scores was converted to a binary value (0 for immuno-negative and 1 for immuno-positive) on the basis of the best cutoff values for each antibody (refer to Fig. 2b). Then, the binary values were combined, to give scores of 0: all immuno-negative to 3: all immuno-positive. Cutoff values were calculated from the ROC curve to distinguish adc/BilIN from non-neoplasm. Accuracy was measured by AUC. **b** Sensitivity and specificity for each cutoff value are summarized. With a cutoff value of 2, where any two antibodies produced immuno-positive staining, 81/91 adc/BilIN and none of non-neoplasm were evaluated as neoplastic with the highest sensitivity and specificity

positive, and 2/3 of those with BiliN-2 were positive only for cldn18. In contrast, most non-neoplastic specimens were negative for both cldn18 and maspin, except for one that was positive for maspin (1/14, 7 %).

Discussion

The aim of this study is to provide a new approach to distinguish biliary tract carcinoma and BilIN from non-neoplastic epithelia with high sensitivity and specificity. We achieved this by a combination of immunohistochemical staining for cldn18, maspin, and p53. Expression of these three antigens has been reported in association with biliary tract carcinomas [20, 25, 32, 34–36], but immunostaining of each individual marker seems to be insufficient to distinguish biliary tract carcinoma/BilIN from non-neoplastic epithelia with certainty. For example, some diagnoses remained controversial if staining for cldn18 or maspin was weakly positive or if cldn18 was detected only in the cytoplasm, even though biliary tract carcinoma/BilIN is usually thought to overexpress cldn18 and maspin [20, 32, 34]. In addition, the prevalence of p53 expression in biliary tract carcinoma/BilIN is relatively low (approximately 30 % at most) with heterogeneous staining patterns even in a single specimen, although the p53 nuclear staining pattern is conspicuous and easy to evaluate in adenocarcinoma. Considering these advantages and disadvantages, we used a panel of all three immunohistochemical markers to reliably detect biliary tract carcinoma/BilIN.

First, we examined surgical specimens of biliary tract cancer by immunochemical staining for each marker, independently. Staining with each of the three antibodies seemed to demonstrate lack of homogeneity within an individual adenocarcinoma. In particular, almost every adenocarcinoma in the present study showed variable intensity and proportion of staining for cldn18 and maspin, not in association with histological differences. This may be attributed to genetic heterogeneity as neoplasms are genetically heterogeneous (interindividually and intraindividually), which is closely related to their progression and treatment response [34].

In the present study, we defined a parameter to maximize the reproducibility and accuracy of the immunohistochemical evaluation. This parameter, designated as the multiplication score, was calculated by multiplying the intensity (4 grades) and proportion (11 grades) of immunoreactivities for each antibody. All ROC curves revealed that sensitivity and specificity of the three markers are high enough to distinguish biliary tract adenocarcinomas/BilIN from non-neoplastic epithelia. Individually, maspin (N) immunostaining showed relatively high sensitivity (85.6 %) and specificity (77.8 %) in detecting adenocarcinoma and BilIN. p53 immunostaining showed the highest specificity (100 %), but the sensitivity was low (61 %). Cldn18 immunostaining also showed markedly high sensitivity (95.6 %) with a high specificity of 96.8 %, which would result from the high cutoff value of 6. Focal and weak cldn18-positive staining was detected in a small number of non-neoplastic epithelia, especially in the presence of reactive changes. Therefore, use of cldn18 or maspin alone may lead to an inadequate differentiation between non-neoplastic epithelia and neoplasms. Because both false-positive and false-negative results should be avoided as much as possible in medical practice, a panel of antibodies against all three markers, cldn18, maspin, and p53, should be used to increase diagnostic sensitivity and specificity. In fact, the combined multiplication score of the three markers improved the accuracy, as compared to the three individual scores (Fig. 2 and Supplementary Fig. S3B).

Fig. 4 **a** H&E staining and immunohistochemical staining in three presurgical endobiliary forceps biopsy specimens with adenocarcinoma (adc)/BilIN and non-neoplasm. The immunostaining patterns were almost identical to those in the surgical specimens. **b** Immunohistochemical staining results based on the binary values to give scores of 0: all immuno-negative to 3: all immuno-positive. **c** The sensitivity and specificity for each cutoff value to distinguish adenocarcinoma (adc)/BilIN from non-neoplasm are summarized. **d** The sensitivity and specificity for each cutoff value to distinguish atypical epithelium from non-neoplasm are summarized. **e** Double immunohistochemical staining of cldn18 and maspin in the surgical specimens. The staining patterns of cldn18 (*brown*) and maspin (*blue*) were coincident with those expected from staining for each individually. A non-neoplastic gland was negative for both cldn18 and maspin (*asterisk*)

The combined multiplication score successfully distinguished neoplasms from non-neoplastic epithelia in the surgical specimens of the biliary tract; however, the scores were complicated to calculate and inconvenient for clinical application. Therefore, before combining the three parameters for cldn18, maspin (N), and p53, we converted each of the multiplication scores to binary values (0 for immuno-negative and 1 for immuno-positive) on the basis of the cutoff values that were calculated from the respective ROC curves. The binary values of staining for cldn18, maspin (N), and p53 were then combined to obtain an additional score. Binary processing of multiplication scores did not impair the high sensitivity and specificity of the original scores. For the combined binary values, cutoff values of 2 or 3, denoting immune-positivity for two or three antibodies, provided the highest sensitivity and specificity (91.1 and 100 %, respectively).

In the analysis of presurgical biopsy specimens, we divided the specimens into three groups on the basis of histology (adenocarcinoma/BilIN, malignancy-undetermined atypical epithelium, and non-neoplastic epithelium), because the condition of the biopsy specimens varied as described above which made it difficult to classify them precisely into multiple stages as we did with the surgical specimens. Among the groups, the patients with a final diagnosis of "malignancy-

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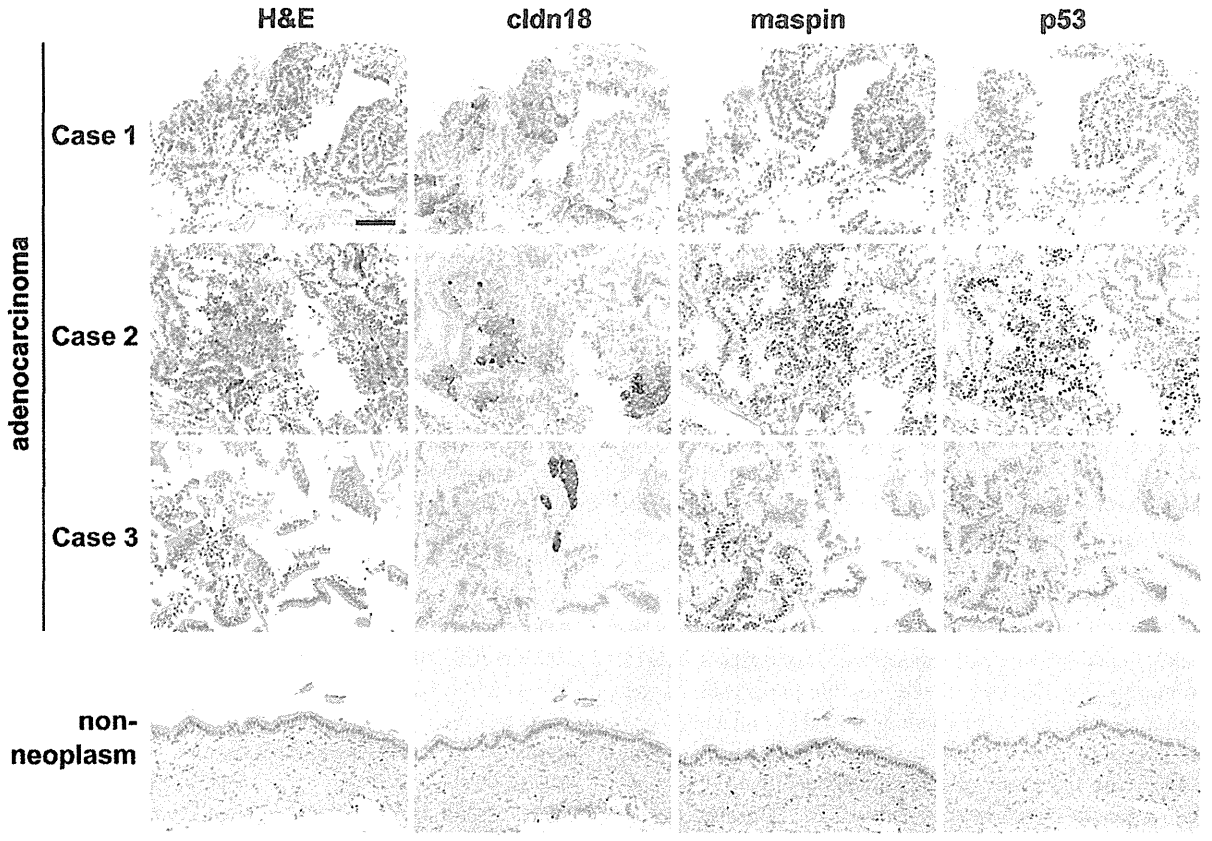


b

		adc/BilIN	atypical epithelium	non-neoplasm
Score (binary value)	0	0	1	15
	1	0	2	3
	2	7	6	1
	3	11	12	0
Total		18	21	19

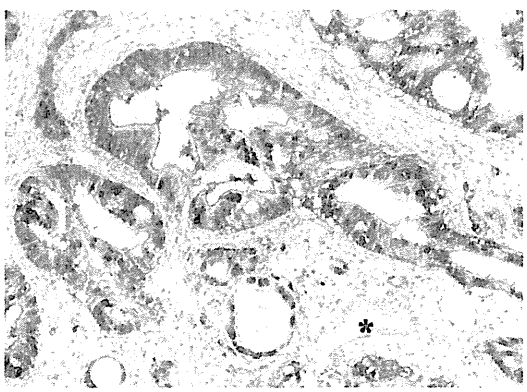
c

adc vs non-neoplasm		Sensitivity	Specificity
Score (binary value)	≥1	1.000	0.789
	≥2	1.000	0.947
	≥3	0.611	1.000

d

atypical vs non-neoplasm		Sensitivity	Specificity
Score (binary value)	≥1	0.952	0.789
	≥2	0.857	0.947
	≥3	0.571	1.000

e



undetermined atypical epithelium" were not biopsy-proven but were strongly suspected of having malignant tumors based on their clinical presentation, clinical course, and imaging and examination findings. Those cases resulted in an indication for surgery and then received a definitive diagnosis of adenocarcinoma based on pathological examinations. We believe that these cases should be discussed separately from typical biopsy-proven adenocarcinoma, because a definitive diagnosis on suspected adenocarcinoma biopsy is important for treatment selection including surgical resection as early as possible. In this study, 85.7 % of malignancy-undetermined atypical epithelium (18/21) was differentiated as neoplastic.

In the present study, we included presurgical biopsy specimens with reactive/regenerative epithelium into the non-neoplastic epithelium category. We also included those with superficial spread lesions in the BilIN category as intraepithelial neoplastic lesions even though they are not usually classified this way. In presurgical endobiliary forceps biopsy specimens, a binary cutoff value of 2 was used for analysis and all 18 of the specimens with adenocarcinoma/BilIN and 1 of 19 with non-neoplastic epithelium were distinguished as neoplasms. The sensitivity and specificity were 100 and 94.7 %. These results reveal that even tiny biopsy specimens can be analyzed with high sensitivity. Of special note is the extremely high specificity, which could help to minimize indeterminate diagnoses in biopsy specimens.

In the statistical analysis of this study, there was no significant difference between BilIN-2 and BilIN-3 by using either each single marker or the panel of the three markers. To date, a number of candidate markers have been examined, some of which are expressed as early as BilIN-1, while others are only expressed from BilIN-2. For example, 25 % of BilIN-1 has been reported to carry a mutation in RAS genes [17]. BilIN-1/BilIN-2 is a neoplastic proliferation that should be followed as a precursor lesion that may progress to BilIN-3 and adenocarcinoma through multiple stages [15]. The detection of BilIN-1/BilIN-2 can be expected to identify high-risk cases, which might increase its clinical importance in the future even though further study is necessary.

We went on to perform the first reported evaluation of the efficiency of dual staining for cldn18/maspin in the diagnosis of bile duct adenocarcinoma in the surgical specimens. The difference in localization (membranous for cldn18; nuclear and cytoplasmic for maspin) and the two-colored chromogenic reaction (brown for cldn18 and blue for maspin) enables easy recognition of the markers in a single slide. The use of the cldn18/maspin double-staining strategy in our study confirmed that these markers are useful in differentiating neoplasms from non-neoplastic epithelia in surgical specimens from the biliary tract.

In conclusion, we show that immunohistochemical staining cldn18, maspin, and p53 as a panel achieves reliable distinction of biliary tract cancers and BilINs from non-neoplastic epithelia in both surgical and biopsy specimens. This panel can serve to improve diagnostic accuracy and might aid the early diagnosis of biliary tract carcinoma and BilIN in presurgical biopsy specimens. This study is limited because it is retrospective; however, the high sensitivity and specificity observed suggests that this approach would support a diagnosis even in indeterminate cases. Further prospective and retrospective studies are needed to evaluate the practical value of this diagnostic method for clinical application.

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Conflict of interest The authors declare that they have no conflict of interest.

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REVIEW ARTICLE

Trials of vaccines for pancreatic ductal adenocarcinoma: Is there any hope of an improved prognosis?

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Abstract Pancreatic tumors are chemoresistant and malignant, and there are very few therapeutic options for pancreatic cancer, as the disease is normally diagnosed at an advanced stage. Although attempts have been made to develop vaccine therapies for pancreatic cancer for a couple of decades, none of the resultant protocols or regimens have succeeded in improving the clinical outcomes of patients. We herein review vaccines tested within the past few years, including peptide, biological and multiple vaccines, and describe the three sets of criteria used to evaluate the therapeutic activity of vaccines in solid tumors.

Keywords Pancreatic cancer · Vaccine · Immunomodulation

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States [1–3] and the fifth most common cause of such deaths in Japan [4]. Although surgical resection is considered to be the only curative therapy for pancreatic cancer, only 20 % of patients have resectable disease at the time of diagnosis [5, 6]. In addition, advanced pancreatic cancer patients exhibit a median survival time (MST) of approximately six months and a 5-year overall survival rate of less than 5 %, despite efforts to manage the tumors with chemotherapy, radiotherapy and other treatments [3, 5–8].

In 1997, Burris et al. reported that gemcitabine monotherapy is superior to fluorouracil (5-FU) monotherapy for

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Table 1 Chemotherapy for advanced pancreatic cancer

	Median survival time (months)	Overall response rate (%)	Trial name	References
Gemcitabine	5.65	5.4		J Clin Oncol 1997;15: 2403–13.
Gemcitabine + erlotinib	6.24	8.6	NCIC CTG PA.3	J Clin Oncol 2007;25: 1960–6.
FOLFIRINOX	11.1	31.6	ACCORD 11	N Engl J Med 2011;364: 1817–25.
Nab-paclitaxel + gemcitabine	8.7	29.2	MPACT trial NCT00844649	N Engl J Med 2013;369: 1691–703.
Gemcitabine +TS-1	10.1	29.3	GEST trial	J Clin Oncol 2013; 31:640–8.

treating pancreatic ductal adenocarcinoma (PDAC) [9]. Gemcitabine monotherapy has subsequently become the standard chemotherapy for PDAC, resulting in an MST of 5.65 months (Table 1). Currently, three protocols have proven to be superior to gemcitabine monotherapy. Combining gemcitabine with erlotinib improved the MST of PDAC to 6.24 months in the NCIC CTG PA3 trial [10], while combining gemcitabine with nab-paclitaxel improved the MST to 8.7 months in the MPACT trial [11]. FOLFIRINOX achieved the longest MST for PDAC (11.1 months) in the ACCORD11 trial [12], and the GEST study obtained similar clinical outcomes. S-1 is an oral fluoropyrimidine derivative that has been shown to be effective against various cancers, and a previous study found that it is at least as effective as gemcitabine against PDAC [13]. In addition, treatment with a combination of gemcitabine + S-1 has been demonstrated to result in an MST of 10.1 months [14]. Although these chemotherapies extend the survival period among PDAC patients, they also result in serious adverse events. Therefore, the optimal chemotherapy regimen for PDAC depends on the patient's performance status.

There have been numerous attempts to develop vaccine therapies for cancer over the past century [2, 3]. Although clinical trials of such vaccines have obtained promising results in specific patients, none of the tested vaccines has exhibited significant improvements in efficacy compared with established therapies. In addition, several issues must be resolved before vaccine therapies can be used in the clinical setting. Tumor-associated antigens (TAA) have been demonstrated to recognize specific human leukocyte antigens (HLA) [15]. Theoretically, the tumor lysate contains all of the antigens expressed by the tumor, and cytotoxic T lymphocytes (CTL) are capable of recognizing some of these antigens [16]. All vaccines for pancreatic cancer are based on the fact that CTL recognize TAA expressed on tumor cells and subsequently attack these cells. The question is how strongly and specifically each TAA stimulates CTL in vivo in the clinical setting. Immune tolerance can develop via various mechanisms, including the downregulation of the major histocompatibility complex (MHC) molecule expression, induction of T cell anergy, reductions in the number of immune effectors and increases in the number of regulatory T cells [17, 18], which may explain why no cancer vaccine therapy has been established as a standard treatment for advanced PDAC. Therefore, in this study, we comprehensively reviewed the clinical outcomes of vaccine therapy against advanced PDAC.

Peptide-based vaccines developed within the past few years

MUC1

Mucin 1, cell surface associated, (MUC1) is a type I transmembrane protein containing multiple tandem repeats of a 20-amino acid sequence. Several MUC1 peptides have been tested as vaccines in the clinical setting; however, most of them have failed to activate CTL [19–21]. Ramanathan et al. [22]; Yamamoto et al. [23] injected pancreatic patients with a vaccine containing a 100-mer extracellular tandem repeat domain of MUC1 and Montanide ISA-51, and both studies obtained similar clinical responses; i.e., the authors detected cytokines (interferon (IFN)-γ or interleukin (IL)-4) and anti-MUC1 antibodies in the patients' sera but did not observe any significant clinical effects. Another recent study involving a vaccine based on a different MUC1 epitope showed similar clinical outcomes, i.e., all seven patients had progressive disease (PD), although some of the patients exhibited immunological responses, such as IFN-γ and granzyme B secretion [24].

K-RAS mutants

K-RAS mutations are frequently found in patients with PDAC. Vaccines targeting mutations in codon 12 of the K-RAS gene have been tested as treatments for advanced [25] or postoperative [26] PDAC in the clinical setting. Gjertsen et al. [[21]] investigated the utility of a K-RAS peptide vaccine containing granulocyte–macrophage colony-stimulating factor (GM-CSF) in 10 patients who had undergone potentially curative

resection (CTN RAS 95002) and 38 patients with advanced disease (CTN RAS 97004). In that study, one patient achieved a partial response (PR), which lasted for 28 months, and the MST of the immunological responders was 4.9 months, compared to 2.0 months for the non-responders.

Human telomerase reverse transcriptase (hTERT)

Human telomerase reverse transcriptase (hTERT) is frequently expressed in cancer cells [27]. hTERT maintains functional telomeres at the end of chromosomes, which protect against cell senescence [28]. A vaccine against pancreatic cancer containing the telomerase peptide GV1001: hTERT (611-626) and GM-CSF was examined by Bernhardt et al. [29], who found the MST of the immunological responders and non-responders to be 7.2 and 2.9 months, respectively.

Vascular endothelial growth factor receptor 2 (VEGFR2)

Vascular endothelial growth factor (VEGF) plays an important role in the progression of PDAC. The type 2 VEGF receptor (VEGFR2) is expressed in PDAC and associated with tumor neovascularization. Miyazawa et al. [[30]] investigated the efficacy of combined treatment consisting of PDAC with a VEGFR2-169 peptide-based vaccine and gemcitabine chemotherapy and reported that one patient achieved a PR, while the disease control rate was 67 %. In addition, the MST was 7.7 months, although 15/18 patients were chemotherapy naive.

G17DT (gastrimmune)

Gastrin is expressed in PDAC and plays a role in regulating the autocrine, paracrine and endocrine systems [31]. The administration of the anti-gastrin immunogen G17DT results in increased serum antibody levels and reduced tumor growth in patients with gastrointestinal malignancies [32]. A randomized, double-blind, placebo-controlled multicenter trial of G17DT was also recently performed [33]. Although, among the intention to treat (ITT) population, no significant differences in MST were detected between the PDAC patients treated with G17DT and those given the placebo, the MST of the two groups differed significantly after excluding major protocol violators and censoring for chemotherapy.

Heat shock protein (HSP)

Heat shock protein (HSP) itself is not an immunogen; however, it acts as a chaperone or carrier of antigenic peptides and possesses a repertoire of cellular peptides for pancreatic cancer [34]. Furthermore, HSPPC-96 (Oncophage) has been tested as a vaccine in the adjuvant setting after complete resection of PDAC [35]. In the latter study, the MST of PDAC was reported to be 2.9 months after surgery; however, this did not result in further clinical studies because only two of 10 patients exhibited increased enzyme-linked immunospot (ELISPOT) reactivity.

Biological vaccines

Fowlpox viral vaccine

Carcinoembryonic antigen (CEA) and MUC1 are highly expressed in PDAC [36]. Viral vectors carrying CEA, MUC1 and TRICOM [a triad of costimulatory molecules: B7.1, intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 3 (LFA-3)] have been investigated as vaccines against advanced PDAC [37]. In one study, a vaccinia viral vector was used for the initial T cell priming, and a fowlpox viral vector was used for immune boosting. Although this treatment resulted in an MST of 6.3 months (1.5–21.1 months), the five patients who showed T cell responses achieved a longer survival period than the five patients who did not (15.1 and 3.9 months, respectively; $P = 0.002$) [38]. It should be noted that GM-CSF was used as a vaccine adjuvant in the latter trial (Table 2).

Live-attenuated, double-deleted (LADD) Listeria monocytogene vaccine

ANZ-100 is a live-attenuated double-deleted Listeria monocytogene strain (LADD; Lm ΔactA/ΔinlB) found to induce a local proinflammatory response, resulting in the activation of innate and adaptive effector cells [39]. Mesothelin is expressed in PDAC and plays an important role in tumor progression [40]. CRS-207 is a LADD Lm strain that delivers mesothelin antigens into class I and II antigen-processing pathways [41]. In a study examining the utility of CRS-207 as a treatment for advanced cancer, three of the seven subjects with PDAC were long-term survivors, although the detection of a mesothelin-specific T cell response was not correlated with survival [41].

Recent vaccine therapies

WT1

Kobayashi et al. reported a retrospective analysis of 255 advanced PDAC patients who were treated with dendritic

Table 2 Peptide-based vaccines and biological vaccines for advanced pancreatic cancer

Author	Journal	Antigen peptide	Sequences	Combination	Patients	Outcome/MST
Yamamoto	Anticancer Res. 2005;25:3575–9	MUC1	10-mer extracellular tandem repeat domain: $(GVTSAPDTRPAPGSTAPPAH)_5$	Montanide ISA-51	6	1/6 SD
Rong	Clin Exp Med. 2012;12:173–80	MUC1	PDTRPAPGSTAPPAHGVTSA	DC cells	7	All PD
Gjertsen	Int J Cancer. 2001;92:441–50	K-ras	KLVVVGAGGVGKSALTI Asp: D Arg: R Val: V Cyc: C	GM-CSF	38	1 PR 10 SD (10.2 M; 3-23 M) 27 PD 4.9 M responders 2.0 M non-responders
Abou-Alfa	Am J Clin Oncol. 2011;34:321–5	ras12R ras12 V ras12D Wild-type ras	TEYKLVWGARGVGKSALTIQ TEYKLVWGAVGVGKSALTIQ TEYKLVWGADGVGKSALTIQ TEYKLVWGAGGVGKSALTIQ	hGM-CSF	24	Postoperative adjuvant treatment
Bernhardt	Br J Cancer. 2006;95:1474–82	Telomerase hTERT (611–626)	GV1001; EARPALLTSRLRFIPK	GM-CSF	38	7.2 M (24 responders) 2.9 M (14 non-responders)
Miyazawa	Cancer Sci. 2010;101:433–9	VEGFR2-169	RFVPDGNRI	Gemcitabine	18	7.7 M
Gilliam	Pancreas. 2012;41:374–9	Anti-gastrin G17DT Gastrimmune	EGPWLEEEEEAYGWMDF-DT (diphtheria toxoid)	G17DT vs. placebo	152	5.0 M vs 2.8 M
Maki	Dig Dis Sci. 2007;52:1964–72	HSP HSPPC-96 (gp96, Oncophage)			10	Postoperative adjuvant treatment 2.7 Y
Kaufman	J Transl Med. 2007;5:60	MUC1 and CEA	CEA agonist peptide CAP1-6D (YLSGADLNL) MUC-1 agonist peptide P-93L (ALWGQDVTSV)	B7.1, ICAM-1, LFA-3 (TRICOM) Vaccinia virus: PANVAC-V Fowlpox virus: PANVAC-F GM-CSF	10	6.3 M
Le	Clin Cancer Res. 2012;18:858–68	Listeria vaccine ANZ-100, CRS-207			9 vs. 17	NA

Table 3 Recently developed peptide-based vaccines and multiple vaccines for advanced pancreatic cancer

Author	Journal	Antigen peptide	Sequences	Restricted HLA	Combination	Patients	Outcome/MST
Kobayashi	Cancer Immunol Immunother. 2014;63:797–806	WT1 MUC1	CYTWNQMNL RMFPNAPYL TRPAPGSTAPPAHG-VTSAP DTRPAPGSTAP	A24:02 A02:01/02:06 Any A	DC cells OK432	255	9.9 M 10.4 M (erythema)
Nishida	J Immunother. 2014;37:105–14	WT1	CYTWNQMNL	A24:02	Weekly 1000 mg/m^2 GEM	31	8.1 M 10.9 M (DTH)
Asahara	J Translation Res. 2013;11:291	KIF20A-66	KVYLRVRPLL	A2402	Montanide ISA51VG	31	4.7 M 6.1 M (reaction)
Suzuki	J Immunother. 2014;37:36–42	KIF20A-10-66	KVYLRVRPLL	A2402	Montanide ISA51VG	9	5.8 M
Geynisman	J ImmunoThera Cancer. 2013;1:8	CEA CAP1-6D	YLSGADLNL	A2	Montanide/GM-CSF	19	11.1 M
Kameshima	Cancer Sci. 2013;104:124–9	SVN2B	AYACNTSTL	A2402	Montanide/IFN-oc	6	(9.6 M)
Yutani	Oncology Reports. 2013;30:1094–100	31 vaccine peptides		A2, A24, A3, A26	Mono: 8 Chemo: 33	41	7.9 M 9.6 M (chemo)
Kimura	Pancreas. 2012;41:195–205	WT1, Her2, CEA, MUC1, CA125, autologous tumor lysate			DC cells plus LAK plus GEM and S1 OK432	49	S: 8.0 M G: 12.0 M GS + LAK: 16.9 M
Le	J Clin Oncol. 2014;32(suppl 3):Abstract 177	GVAX pancreas and CRS-207 vs. GVAX pancreas alone	Irradiated GM-CSF-secreting allogeneic pancreatic tumor vaccine (GVAX pancreas)		Cyclophosphamide	90	6.1 M vs. 3.9 M 9.7 M (3 or more rounds of vaccine therapy)

cell (DC) vaccines containing Wilms tumor 1 (WT1) and MUC1 after being recruited from seven institutions that followed a unified standard operating procedure. The MST of these patients was 9.9 months [42]. Nishida et al. also examined the utility of chemo-vaccine therapy in which a WT1-based vaccine was used in combination with the administration of 1,000 mg/m^2 of gemcitabine weekly. The latter regimen resulted in an MST of 8.1 months among 31 advanced PDAC patients [43]. In addition, the MST of the immunological responders in these two studies was very similar (10.4 and 10.9 months, respectively) (Table 3).

KIF20A

Kinesin family member 20A (KIF20A) plays an important role in the trafficking of molecules and organelles [44] and is one of the molecules targeted by vaccines against PDAC. A KIF20A vaccine was recently tested using different regimens, including vaccine monotherapy [45] and chemo-vaccine therapy involving gemcitabine [46], and similar MST values were reported in both studies (4.7 and 5.8 months, respectively).

Carcinoembryonic antigen (CEA)

CEA is a 180-kDa immunoglobulin-like molecule expressed on the surface of 90 % of PDAC tumor cells [47]. CAP1-6D, a modified CEA peptide, was combined with Montanide/GM-CSF to produce a vaccine against pancreatic cancer that was subsequently tested in advanced PDAC patients [48]. The MST of the 19 patients was 11.1 months, and one patient, randomized into the 0.01 mg arm, achieved a complete response (CR).

Survivin2B

Survivin is a member of the inhibitors of apoptosis (IAP) family of proteins that protect apoptotic signals by inhibiting the caspase activity [49, 50]. Hence, survivin-expressing cancer cells escape from apoptosis and do not die. Using a peptide-binding assay, we found that the survivin2B 80–88 peptide induces a strong CTL response [51]. We also examined the effects of a survivin2B 80–88 peptide-based vaccine on various cancers in the clinical setting and obtained promising outcomes. In particular, the anti-tumor effect of the survivin2B 80–88 peptide was enhanced by combining it with incomplete Freud's adjuvant and IFN-α injection. Our preliminary clinical study demonstrated a 66.6 % disease control rate in advanced PDAC patients (four of six patients) [52]. Moreover, the PDAC patients in our recent clinical phase I study exhibited an MST of 9.6 months.

Table 4 Evaluation of therapeutic activity in solid tumors

Method	WHO	RECIST	IrRC
	Sum of the products of the two longest perpendicular dimensions (bidimensional)	Sum of the longest dimensions (unidimensional)	Sum of the products of the two longest perpendicular dimensions (SPD) of all index lesions. (bidimensional)
No. of measured lesions	All lesions	Five per organ, 10 in total	Five per organ, 10 in total, and five cutaneous index lesions
CR	Disappearance of all known disease, confirmed at 4 weeks	Disappearance of all known disease, confirmed at 4 weeks	Disappearance of all known disease, confirmed at 4 weeks apart
PR	>50 % decrease in total tumor size, confirmed at 4 weeks	>30 % decrease in total tumor size, confirmed at 4 weeks	>50 % decrease in tumor burden compared with baseline in two observations at least 4 weeks apart
SD	CR, PR, and PD criteria not met	CR, PR, and PD criteria not met	CR, PR, and PD criteria not met
PD	>25 % increase in total tumor size; no CR, PR, or SD documented before increase in tumor size; new lesion (s); > 25 % increase in size of one lesion	>20 % increase in total tumor size; no CR, PR, or SD documented before increase in tumor burden; new lesion (s)	>25 % increase in tumor burden compared with nadir (at any single time point) in two consecutive observations at least 4 weeks apart

Tumor burden = $SPD_{index\ lesions}$ +SPD new, measurable lesions

Multiple vaccines

Personalized peptides

In a previous study, a set of 31 peptides was used to create personalized vaccines for advanced PDAC [53]. A maximum of four peptides were selected from among the 31-peptide set based on the results of HLA typing and the patients' peptide-specific IgG titers. Eight patients received vaccine monotherapy, and 31 patients received chemo-vaccine therapy. In the chemo-vaccine therapy group, gemcitabine was administered in eight patients, S-1 was administered in six patients and gemcitabine + S-1 was given in eight patients. The overall MST was 9.6 months, although that of the patients who underwent monotherapy was 7.9 months. Yanagimoto et al. reported similar clinical outcomes for chemo-vaccine therapy involving personalized vaccines and gemcitabine based on the same regimen [54]. The MST of the patients in the latter study was 9.0 months, although that of the immunological responders was 15.5 months. None of the patients in either study achieved CR (Table 3).

Autologous tumor lysate combined with lymphokine-activated killer cell therapy

Kimura et al. treated 49 PDAC patients with vaccines based on five different peptides and autologous tumor lysate, although the vaccine preparation regimens and anti-tumor therapies varied in each case [16]. Two patients achieved CR after treatment with a combination of DC cell and lymphokine-activated killer cell (LAK) therapy. The MST of the patients treated with LAK + gemcitabine and S-1 was 16.9 months, whereas that of all patients was 12.0 months. It should be noted that the survival time was calculated from the day after the first vaccination, which may have resulted in a shorter survival time (by a couple of months) than would have been obtained using the methods employed in other studies. It is very difficult to evaluate the clinical results of this study due to the effects of the different therapeutic strategies used in each case. However, the fact that multiple patients achieved CR will encourage researchers to pursue this approach further.

GVAX pancreas with CRS-207

GVAX is a series of irradiated GM-CSF-secreting allogeneic pancreatic cell lines that elicit broad antigenic responses. CRS-207 is a LADD Lm strain (Lm ΔactA/ΔinlB) that expresses mesothelin and stimulates the innate and adaptive immune systems. A phase II randomized control trial of GVAX pancreas combined with CRS-207 versus GVAX pancreas alone was presented at the 2014 American Society of Clinical Oncology (ASCO) Gastrointestinal Cancers Symposium [55]. Interestingly, the clinical results demonstrated that both treatments had dose-dependent survival benefits. The MST of the patients who received three or more rounds of vaccine therapy was 9.7 months, and the MST of the GVAX with CRS-207 arm was longer than that of the GVAX-alone arm (6.1 vs. 3.9 months; $P = 0.01$) [56].

Evaluation of therapeutic activity in solid tumors

The response of solid tumors is evaluated using either the WHO [57] or RECIST criteria [58]. The RECIST criteria were developed because the WHO criteria are quite complex and measuring all visible lesions in two dimensions is both time consuming and subject to measuring bias [59]. However, the use of immunotherapeutic agents in cancer patients is associated with the following problems: (a) The measurable anti-tumor activity can take longer to appear during immunotherapy than during cytotoxic therapy; (b) Responses to immunotherapy can occur after the standard criteria for progressive disease (PD) have been met; (c) Discontinuing immunotherapy may not be appropriate in some cases, unless PD is confirmed; (d) Allowing for "clinically insignificant" PD (e.g., small new lesions developing in the presence of other responsive lesions) is recommended; and (e) Durable stable disease (SD) may represent the anti-tumor activity [60]. Therefore, the immune-related response criteria (irRC) were developed to evaluate the immunotherapeutic activity in solid tumors [61]. The most important aspects of the irRC criteria are that (a) new lesions are not classified as PD and (b) two consecutive observations obtained at least four weeks apart are required to diagnose PD. However, the clinical utility of the irRC remains unclear and these criteria may require further optimization [61] (Table 4).

Future research topics

Initial time point for survival assessments

The initial time point for survival assessments should be unified to allow clinical outcomes to be compared between studies. Most PDAC patients already have advanced disease at the time of diagnosis [6]. In addition, the adverse effects of chemotherapies differ markedly among the various regimens [8]. Therefore, the status of PDAC patients at the time point at which they are registered can differ both within and between clinical studies. Kobayashi et al. reported that the MST from the date of diagnosis and the MST from the first vaccination are very different (16.5 vs.

9.9 months) [42]. Therefore, MST data must be interpreted carefully.

Vaccine therapy and chemotherapy

The goal of vaccine therapy for cancer is to increase the native immunity of cancer patients. However, chemotherapy causes irreversible damage to proliferating cancer cells as well as immune cells, including T and B cells. Therefore, there is a conflict between the fundamental principles of these two treatments. Chemotherapy is currently the gold standard treatment for advanced PDAC. Although the biological mechanisms of vaccine therapy and chemotherapy conflict with each other, the anti-cancer activity of vaccine monotherapy or chemo-vaccine combination therapy should be greater than that of chemotherapy alone.

Slow clinical response to vaccine therapy

It is very hard to achieve a complete response (CR) with vaccine therapy alone. We reviewed 19 studies involving a total of 860 patients and found that CR responses were obtained in only three cases. Although none of these studies involved a large number of patients, the poor reported response rates are a concern. One of the patients who achieved a CR was administered CEA CAP1-6D + Montanide/GM-CSF therapy, while the other two were treated with WT1, Her2, CEA, MUC1, cancer antigen 125 and autologous tumor lysate vaccines combined with DC cell-based LAK therapy and chemotherapy. Immunological responses require a long time to control tumor growth and achieve remission. The primary goal of vaccine therapy is to achieve long-term SD [62]. Most previous clinical studies of PDAC involved patients with advanced disease for whom no other therapies were available. Therefore, vaccine therapy may be suitable for patients in other clinical stages or possibly a useful postoperative adjuvant therapy. The main advantage of vaccine therapy is that it has few adverse effects, although it has also demonstrated minimal clinical effects in previous trials. We are currently conducting a phase II study of the survivin2B 80–88 peptide + Montanide + IFN-β as a treatment for PDAC (SUCCESS, Study of Unresectable CanCEr with Survivin-2B peptide vaccine in Sapporo: UMIN000012146), in which half of the required patients have been recruited. The clinical results of the SUCCESS phase II study will be reported by the end of next year.

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Phase I Clinical Study of Survivin-Derived Peptide Vaccine for Patients with Advanced Gastrointestinal Cancers

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Abstract

Survivin is a member of the Inhibitor of Apoptosis Protein (IAP) family. It is expressed in fetal tissues but not in normal adult tissues. Since Survivin is over expressed in various types of tumor tissues as well as tumor cell lines, it is considered to be suitable as a target antigen for cancer vaccine therapy. We identified an HLA-A24-restricted antigenic peptide, SVN-2B (AYACNTSTL), derived from a splicing variant of Survivin-2B. In the present study, we carried out a phase I clinical study assessing the safety and efficacy of vaccination with the peptide in patients having advanced gastrointestinal cancer. Vaccinations with 0.1mg, 1.0mg, or 3.0mg doses of the SVN-2B peptide were given subcutaneously four times at 14-day intervals. In 20 patients who received at least one vaccination, grade 1 and grade 2 treatment-related adverse events were observed, including injection site extravasation (grade 2), injection site reaction (grade 1), skin induration (grade 1) and fever (grade 1). No severe adverse event was observed in any patient. Based on tumor size evaluated by computed tomography, eight of the 15 patients who completed the vaccination schedule were considered to have stable disease as assessed by the RECIST criteria. Analysis of peripheral blood lymphocytes using HLA-A24/peptide tetramers revealed the highest increase of SVN-2B-specific cytotoxic T lymphocyte frequency in the 1.0mg dose group. The present clinical study indicates that SVN-2B peptide vaccination is safe and can be considered a potent immunotherapy for HLA-A24-positive gastrointestinal cancer patients.

Keywords

Survivin, Cancer vaccine, Gastrointestinal cancer, Tetramer, Phase I trial

Abbreviations

IAP: Inhibitor of Apoptosis Protein, CTLs: Cytotoxic T lymphocytes, HLA: Human Leukocyte Antigen, CT: Computed Tomography, PBLs: Peripheral Blood Lymphocytes, AEs: Adverse Events, HIV: Human Immunodeficiency Virus, PD: Progressive Disease, SD: Stable Disease, IFN: Interferon

Introduction

Cytotoxic T lymphocytes (CTLs) can recognize MHC class I-bound peptides derived from tumor antigens in cancer cells. Following the first report of the identification of a human tumor antigen, melanoma antigen-1 (MAGE-1), in 1991 [1] a large number of antigenic peptides from various human cancers have been identified [2-7]. They have been employed in immunotherapy for cancer and clinical trials of peptide-based vaccine therapies have taken place [8-11].

We have identified a human leukocyte antigen (HLA)-A24-restricted antigenic peptide, SVN-2B (AYACNTSTL), which was derived from the exon 2B-encoded region of Survivin-2B, a splicing variant of Survivin [12]. Survivin is a member of the inhibitor of apoptosis protein (IAP) family with a single baculovirus IAP repeat domain [13]. It is expressed during fetal development but undetectable in terminally differentiated normal adult tissues. In contrast to normal tissues, Survivin and Survivin-2B are expressed in transformed cell lines and in most common cancers, including gastrointestinal cancer and pancreatic cancer [13,14]. We reported previously that SVN-2B peptide-specific CTLs were increased by

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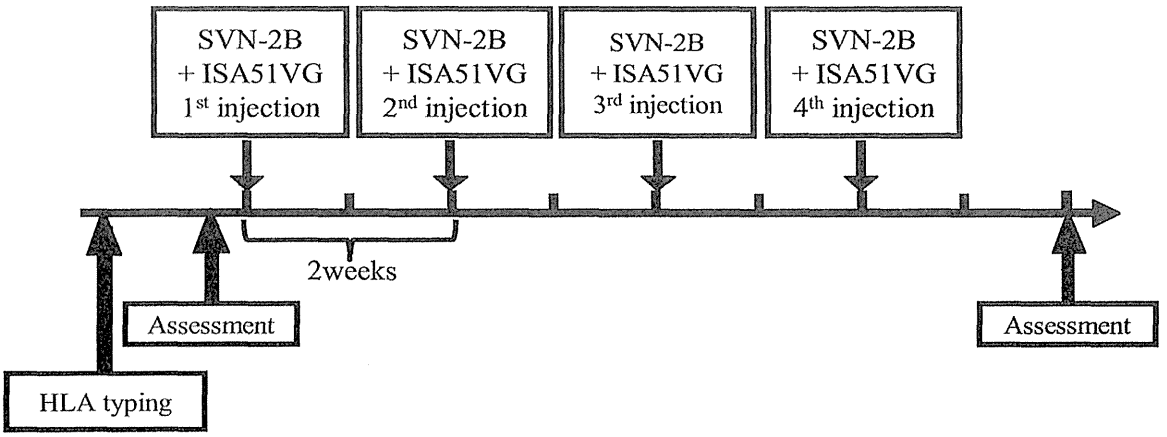

Figure 1: Protocols of the clinical study

The SVN-2B peptide at a dose of 0.1mg/1mL, 1mg/1mL, or 3mg/1mL was emulsified with Montanide ISA51VG at a volume of 0.8mL immediately before vaccination. The patients were then vaccinated subcutaneously (s.c.) four times at 14-day intervals. Tumor size and the immunological response were evaluated before treatment and at two weeks after the 4th vaccination.

stimulating peripheral blood lymphocytes (PBLs) of cancer patients with the peptide *in vitro* [15]. The induced CTLs showed specific cytotoxicity against HLA-A24-positive cancer cells [15-17]. We have carried out clinical trials of SVN-2B vaccination. The SVN-2B peptide was given subcutaneously to patients six times or more at biweekly intervals for colon, breast, oral cavity, and urinary bladder cancer patients [18-24]. There were no severe adverse effects and, clinically, certain patients showed reductions in tumor markers and tumor size as assessed by Computed Tomography (CT). In the present clinical study, we reevaluated the safety and efficacy of SVN-2B vaccination in accordance with good clinical practice guidelines and evaluated the optimal dose of the peptide.

Methods

Patient selection

The study protocol was approved by the Institutional Review Board of Sapporo Medical University. All patients gave informed consent before being enrolled. This study was conducted in accordance with the International Conference on Harmonisation E6 requirements for Good Clinical Practice and with the ethical principles outlined in the Declaration of Helsinki.

Patients enrolled in this study were required to conform to the following criteria: (1) to have histologically confirmed gastrointestinal, bile duct, or pancreatic cancer, (2) to be HLA-A*2402 positive, (3) to have Survivin-positive cancer tissue confirmed by immunohistochemical staining, (4) to be between 20 and 85 years old, (5) to have lesions measureable by CT at the time of registration, (6) to have a history of standard chemotherapy, (7) to have grade 0 or 1 in Eastern Cooperative Oncology Group (ECOG) performance status, and (8) to have no serious organ failure within 30 days at the time of registration.

Exclusion criteria included: (1) prior cancer therapy such as chemotherapy, radiation therapy or other immunotherapy within the previous 4 weeks, (2) presence of other cancers that might influence the prognosis, (3) administration of immunosuppressive drugs such as systemic steroid therapy, (4) severe cardiac insufficiency, acute infection, or hematopoietic failure, (5) uncontrollable diabetes or hypertension, (6) pregnancy or ongoing breast-feeding, and (7) unsuitability for the trial based on clinical judgment. In addition, patients with a high frequency of the peptide-specific CTLs at the time of registration were excluded since such patients were poor responders to the vaccine in our previous studies [23,24]. The number of the HLA-A24/SVN-2B peptide tetramer-positive CTLs per 10,000 CD8-positive T cells (CTLpre) was analyzed at the time of registration and patients who had a value of log10 (1+CTLpre) higher than 1.6 were excluded.

Peptide preparation

The peptide SVN-2B with the sequence **AYACNTSTL** was prepared under good manufacturing practice conditions by PolyPeptide Laboratories San Diego (San Diego, CA, USA). The identity of the peptide was confirmed by mass spectral analysis, and the purity was shown to be more than 98% as assessed by high pressure liquid chromatography analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 ml of physiological saline (Ohtsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and stored at -80°C until just before use.

Patient treatment

This study was carried out as an open-label, randomized parallel group study at the Department of Surgery, Surgical Oncology and Science of Sapporo Medical University Hospital to evaluate the safety and efficacy of the SVN-2B peptide vaccine for patients who had advanced or recurrent gastrointestinal or pancreatic cancer (UMIN000008611). The patients were randomly assigned into the following three dosage groups: group 1 patients received 0.1mg, group 2 received 1.0mg and group 3 received 3mg. Each group included five patients. SVN-2B at a dose of 0.1mg/1mL, 1mg/1mL, or 3mg/1mL was emulsified with Montanide ISA51VG (Seppic, Paris, France) at a volume of 0.8mL immediately before vaccination. The patients were then vaccinated subcutaneously (s.c.) four times at 14-day intervals (Figure 1).

Toxicity evaluation

Patients were examined closely for signs of toxicity during and after vaccination. Adverse events (AEs) were recorded using CTCAE (version 4.03) criteria and graded for severity.

Clinical response evaluation

Physical and hematological examinations were conducted before and after each vaccination. Changes in tumor marker levels (CEA and CA19-9) were evaluated by comparison of the serum levels before the first vaccination and those after the fourth vaccination. Tumor size was evaluated by CT scans before treatment and at two weeks after the fourth vaccination (Figure 1). The antitumor response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST: version 1.1) guideline [25]. Briefly, a complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response (PR) was defined as a >=30%

Table1: Profiles of patients in the full analysis set for safety assessment (N=20)

Clinical variables		0.3 mg (n=7)	1.0 mg (n=7)	3.0 mg (n=6)	Total (n=20)
Gender	Men: Women	2:5	5:2	3:3	10:10
Age	Median (min-max)	69.5 (53-80)	63.0 (51-84)	64.4 (41-66)	65.1(41-84)
Type of cancer	Pancreatic cancer	5	2	5	12
	Colon cancer	2	3	1	6
	Gastric cancer	0	1	0	1
	Bile duct cancer	0	1	0	1
Metastasis	(positive: negative)	7:0	5:2	5:1	17:3
Prior surgery	(positive: negative)	4:3	5:2	3:3	12:8
Prior radiation therapy	(positive: negative)	2:5	2:5	3:3	7:13
Prior chemotherapy	(positive: negative)	6:1	6:1	6:0	18:2
ECOG PS	(0:1)	1:6	1:6	2:4	4:16
Treatment-related AEs					
Fever	Grade 1	1			1
Injection site extravasation	Grade 2			1	1
Injection site reaction	Grade 1	1		1	2
Skin induration	Grade 1	1	1		2

decrease from baseline in the size of all measurable lesions (sum of maximal diameters). Progressive disease (PD) was defined as an increase in the sum of maximal diameters by at least 20% or the appearance of new lesions. Stable disease (SD) was defined as the absence of criteria matching those for CR, PR or PD. Patients who received fewer than four vaccinations were excluded from clinical response evaluations in this study.

In vitro stimulation of PBLs

PBLs were isolated by Ficoll-Conray density gradient centrifugation using Lymphoprep (AXIS-SHIELD, Oslo, Norway). They were then frozen and stored at -80°C. The frozen PBLs were thawed and incubated in the presence of 40μg/mL SVN-2B in AIM-V medium (Life Technologies, Carlsbad, CA, USA) containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 hour, 2 days and 4 days after addition of the peptide. On day 7 of culture, the PBLs were analyzed by tetramer staining assay and ELISPOT assay.

Tetramer staining

FITC-labeled HLA-A*2402/human immunodeficiency virus (HIV)-derived peptide (RYLRDQQLL) and PE-labeled HLA-A*2402/SVN-2B peptide tetramers were purchased from MBL, Inc. (Nagoya, Japan). For flow cytometric analysis, PBLs, which were stimulated *in vitro* as above, were stained with the FITC-labeled tetramer and PE-labeled tetramer at 37°C for 20 min, followed by staining with a PC5-labeled anti-CD8 monoclonal antibody (Beckton Dickinson Biosciences, San Jose, CA, USA) at 4°C for 30 min. The cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was performed using FACSCalibur and CellQuest software (Beckton Dickinson Biosciences). The frequency of CTL precursors was calculated as the number of HLA-A24/SVN-2B tetramer-positive cells per 10,000 CD8-positive cells.

ELISPOT assay

ELISPOT plates were coated sterilely overnight with an IFN-γ capture antibody (Beckton Dickinson Biosciences) at 4°C. The plates were then washed once and blocked with AIM-V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients' PBLs ($5x10^3$ cells/well), which were stimulated *in vitro* as above, were then added to each well along with HLA-A24-transfected CIR cells (CIR-A24) ($5x10^4$ cells/well) preincubated with SVN-2B (10ng/mL, 100ng/mL, 10μg/mL) or the HIV peptide (RYLRDQQLL) as a negative control. After incubation in a 5% CO_2 humidified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-γ detection antibody (Beckton Dickinson Biosciences) and horseradish peroxidase-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany).

Immunohistochemistry

Immunohistochemical study of the HLA class I expression in the patients' primary cancer tissues was done with anti-HLA class I heavy chain monoclonal antibody EMR8-5 according to the standard methods described previously [26].

Statistical analysis

All statistical analyses were done using SAS Version 9.3 and JMP Version 11.0 (SAS Institute, Inc.). For the tetramer assay, statistical analysis was performed using a one-sided t-test. Statistical analysis of ELISPOT assay was performed using the student t-test.

Results

Patient profiles

From August 2012 to May 2013, 38 patients were assessed for eligibility and 21 patients were initially enrolled in this trial (Figure 2). However, one patient was withdrawn before the first vaccination due to deterioration of the systemic condition. Twenty patients who received at least one vaccination were evaluated for safety as a full analysis set (FAS). Five patients discontinued halfway through the protocol due to progression of the disease. None of the interruptions was due to treatment-related AEs. Fifteen patients received the complete regimen including four vaccinations and were evaluated for efficacy of the vaccine (Figure 2). The patient profiles are shown in Table 1. The primary malignant tumors of the 20 patients were 12 pancreatic cancers, 6 colon cancers (including 2 appendix cancers), 1 gastric cancer and 1 bile duct cancer.

Safety

Peptide vaccination was well tolerated in all patients. The treatment-related AEs are listed in Table 1. They included injection site extravasation (grade 2), injection site reaction (grade 1), skin induration (grade 1) and fever (grade 1). No serious toxicity-associated adverse event was observed during or after the vaccination.

Clinical responses

Table 2 summarizes the clinical outcomes of the 15 patients who received the complete regimen. CT evaluation of tumor size showed that 8 patients had SD and 7 patients PD, although none had PR or