Declaration of Financial Disclosure

Hideo Takasu is an employee of Dainippon Sumitomo Pharma Co., Ltd.

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Immunotherapeutic benefit of α-interferon (IFNα) in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients

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Survivin, a member of the inhibitor of apoptosis protein (IAP) family containing a single baculovirus IAP repeat domain, is highly expressed in cancerous tissues but not in normal counterparts. Our group identified an HLA-A24-restricted antigenic peptide, survivin-2B80–88 (AYACNTSTL), that is recognized by CD8 + CTLs and functions as an immunogenic molecule in patients with cancers of various histological origins such as colon, breast, lung, oral, and urogenital malignancies. Subsequent clinical trials with this epitope peptide alone resulted in clinical and immunological responses. However, these were not strong enough for routine clinical use as a therapeutic cancer vaccine, and our previous study of colon cancer patients indicated that treatment with a vaccination protocol of survivin-2B80–88 plus incomplete Freund's adjuvant (IFA) and α-interferon (IFNα) conferred overt clinical improvement and enhanced the immunological responses of patients. In the current study, we further investigated whether this vaccination protocol could efficiently provide not only improved immune responses but also better clinical outcomes for advanced pancreatic cancers. Tetramer and enzyme-linked immunosorbent spot analysis data indicated that more than 50% of the patients had positive clinical and immunological responses. In contrast, assessment of treatment with IFNα only to another group of cancer patients resulted in no obvious increase in the frequency of survivin-2B80-88 peptide-specific CTLs. Taken together, our data clearly indicate that a vaccination protocol of survivin-2B80-88 plus IFA and IFNα is very effective and useful in immunotherapy for this type of poor-prognosis neoplasm. This trial was registered with the UMIN Clinical Trials Registry, no. UMIN000000905. (*Cancer Sci* 2013; 104: 124–129)

Recent progress in human tumor immunology research has presented us with the possibility that immunotherapy could be established as an effective cancer therapy in the very near future.[1–6] Indeed, since the first discovery of a human tumor antigen in 1992,[7] many clinical trials for cancer vaccines have been carried out, and these studies have suggested that active immunization using HLA class I restricted tumor antigenic peptides and the whole or part of the tumor antigenic protein could work as activators of antigen-specific CTLs, at least in some cancer patients.[8–16] However, even in effective cases, vaccination with these molecules alone is not sufficient to evoke a potent and stable immune response and subsequent strong clinical effect. Thus, it is crucial to develop various methods for enhancing the immunological efficacy of tumor antigens.

We have studied how tumor antigenicity can be efficiently enhanced in cancer patients since 2003. In our studies, the HLA-A24-restricted peptide survivin-2B80-88 was given s.c. to patients six times or more at biweekly intervals for colon, breast, lung, oral cavity, and urinary bladder cancers, and lymphomas. Clinically, certain patients with colon, lung, and urinary bladder cancers showed reductions in tumor markers and growth arrest as assessed by computed tomography (CT).[8–12] These effects, however, were not strong enough for the clinical requirements as decided by the criteria for cancer chemotherapy. When assessed with the Response Evaluation Criteria in Solid Tumors, which requires more than 30% regression of tumors on CT, only one patient each of 15 with colon cancers and three with urinary bladder cancers had a positive clinical response, indicating that the therapeutic potential was obviously not strong enough for routine clinical use as a cancer treatment.

In a previous study,[8] to determine if the immunogenicity of the survivin-2B80-88 peptide could be enhanced with other vaccination protocols, we carried out and compared clinical trials in advanced colon cancer patients with two vaccination protocols: (i) survivin-2B80-88 plus incomplete Freund's adjuvant (IFA); and (ii) survivin-2B80-88 plus IFA and a type-I interferon (IFN), IFNα. Our data clearly indicated that, although the effect of survivin-2B80-88 plus IFA was not significantly different from that with survivin-2B80-88 alone, treatment with survivin-2B80-88 plus IFA and IFNα resulted in clear clinical improvement and enhanced the immunological responses of patients. We also analyzed CTLs of these patients by single-cell sorting, and found that each CTL clone from vaccinated patients was indeed not only peptide-specific but also cytotoxic against human cancer cells in the context of the expression of both HLA-A24 and survivin molecules.

Pancreatic cancer is still one of most difficult malignant neoplasms to treat, so in the current study we investigated whether the most effective protocol for colon cancer patients, namely survivin-2B80-88 plus IFA and IFNα, could work similarly in pancreatic cancers as in colon cancers. Furthermore, we carried out frequency monitoring of survivin-2B80-88 peptide-specific CTL in cases of cancer patients treated with IFNα alone, and found no overt increase of these CTLs. Once the survivin-2B80-88 peptide was administered with IFNα, patients showed strong clinical and immunological responses as assessed by tetramer and enzyme-linked immunosorbent spot (ELISPOT) analyses. Taken together, our current data strongly suggest that vaccination using survivin-2B80-88 plus IFA and IFNα is actually very effective in patients with advanced pancreatic cancers from both the clinical and immunological points of view.

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Materials and Methods

Patients. Patient selection was done as reported in our previously published work. The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University (Sapporo, Japan).[8–12] All patients gave informed consent before being enrolled. Patients who participated in this study were required to: (i) have histologically confirmed pancreatic cancer; (ii) be HLA-A*2402 positive; (iii) have survivin-positive carcinomatous lesions by immunohistochemistry; (iv) be between 20 and 85 years old; (v) have unresectable advanced cancer or recurrent cancer; and (vi) have Eastern Cooperative Oncology Group performance status between 0 and 2. Exclusion criteria included: (i) prior cancer therapy such as chemotherapy, radiation therapy, steroid therapy, or other immunotherapy within the past 4 weeks; (ii) the presence of other cancers that might influence the prognosis; (iii) immunodeficiency or a history of splenectomy; (iv) severe cardiac insufficiency, acute infection, or hematopoietic failure; (v) use of anticoagulants; and (vi) unsuitability for the trial based on clinical judgment. This study was carried out at the Department of Surgery, in the Sapporo Medical University Primary Hospital from December 2005 through to November 2010.

Peptide, IFA, and IFNα preparation. The peptide, survivin-2B80-88 with the sequence AYACNTSTL, was prepared under good manufacturing practice conditions by Multiple Peptide Systems (San Diego, CA, USA).[8–10,12] The identity of the peptide was confirmed by mass spectrometry analysis, and the purity was shown to be more than 98% as assessed by HPLC analysis. The peptide was supplied as a freeze-dried, sterile white powder. It was dissolved in 1.0 mL physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) and stored at −80°C until just before use. Montanide ISA 51 (Seppic, Paris, France) was used as IFA. Human IFNα was purchased from Dainippon-Sumitomo Pharmaceutical (Osaka, Japan).

Patient treatment. In this clinical study, we used the protocol illustrated in Fig. 1, with the survivin-2B80-88 peptide plus IFA and IFNα. In this trial, the primary endpoint was safety. The second endpoint was investigation of the antitumor effects and clinical and immunological monitoring.

In this protocol, survivin-2B80-88 at a dose of 1 mg/1 mL plus IFA at a dose of 1 mL were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, IFNα at a dose of 3 000 000 IU was given s.c. twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination (Fig. 1).

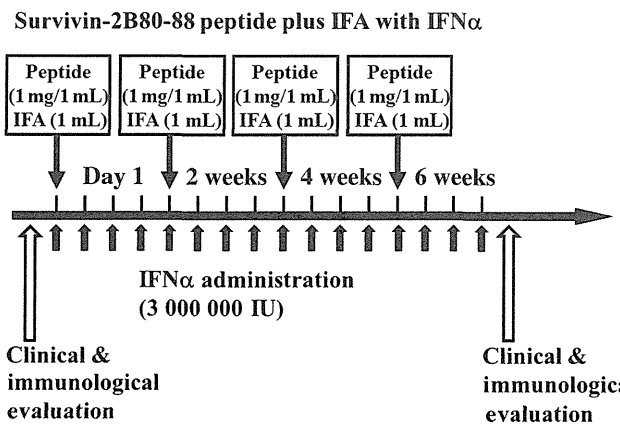

Fig. 1. Clinical protocol of study. Survivin-2B80-88 and incomplete Freund's adjuvant (IFA) were mixed immediately before vaccination. The patients were then vaccinated s.c. four times at 14-day intervals. In addition, α-interferon (IFNα) was given twice a week close to the site of vaccination. For this, IFNα was mixed with the peptide and IFA immediately before vaccination and given at the time of peptide and IFA biweekly vaccination.

Toxicity evaluation. Patients were examined closely for signs of toxicity during and after vaccination. Adverse events were recorded using the National Cancer Institute Common Toxicity Criteria.[8–10]

Clinical response evaluation. Physical examinations and hematological examinations were carried out before and after each vaccination.[8–10] A tumor marker (Ca19-9) was examined. Changes in the tumor marker levels were evaluated by comparison of the serum level before the first vaccination and that after the fourth vaccination. Immunohistochemical study of the HLA class I expression in patients' primary pancreatic cancer tissues was done with anti-HLA class I heavy chain mAb EMR-8-5[13] (Funakoshi, Tokyo, Japan).

Tumor size was evaluated by CT scans or MRI by comparing the size before the first vaccination with that after the fourth vaccination. A complete response (CR) was defined as complete disappearance of all measurable and evaluable disease. A partial response was defined as a $\geq 30\%$ decrease from the 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 complete response, partial response, or PD.[8–10] Patients who received fewer than four vaccinations were excluded from all evaluations in this study.

***In vitro* stimulation of PBMC, tetramer staining, and ELISPOT assay.** The samples for tetramer analysis and ELISPOT analysis were simultaneously obtained at the time of the hematological examination before and after each vaccination. These experiments were carried out as in our previous report. The PBMCs were isolated from blood samples by Ficoll–Conray density gradient centrifugation. Then they were frozen and stored at −80°C. As needed, frozen PBMCs were thawed and incubated in the presence of 30 μg/mL survivin-2B80-88 in AIM V (Life Technologies Corp, Grand Island, NY, USA) medium containing 10% human serum at room temperature. Next, interleukin-2 was added at a final concentration of 50 U/mL 1 h, 2 days, 4 days, and 6 days after the addition of the peptide. On day 7 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

The FITC-labeled HLA-A*2402-HIV peptide (RYLRDQQLL) and phycoerythrin (PE)-labeled HLA-A*2402-survivin-2B8-88 peptide tetramers were purchased from Medical and Biological Laboratories (MBL) Co., Ltd (Nagoya, Japan). For flow cytometric analysis, PBMCs, stimulated *in vitro* as above, were stained with the PE-labeled tetramer at 37°C for 20 min, followed by staining with a PE-Cy5-conjugated anti-CD8 mAb (BD Biosciences, San Jose, CA, USA) at 4°C for 30 min. Cells were washed twice with PBS before fixation in 1% formaldehyde. Flow cytometric analysis was carried out using FACSCalibur and CellQuest software (BD Biosciences). The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8-positive cells.[8,10,12]

The ELISPOT plates were coated overnight in a sterile environment with an IFNγ capture antibody (BD 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' PBMCs (5×10^3 cells/well) that were stimulated *in vitro* as above were then added to each well along with HLA-A24-transfected T2 cells (T2-A24) (5×10^4 cells/well) that had been preincubated with or without survivin-2B80-88 (10 mg/mL) or

with an HIV peptide 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 IFNγ antibody and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Oberkochen, Germany). In this study, positive (+) ELISPOT represents a more than twofold increase of survivin-2B80-88 peptide-specific CD8 T cell IFNγ-postive spots as compared with HIV peptide-specific CD8 T cell spots, whereas negative (−) means a less than twofold increase.

Single-cell cloning and functional assessment of tetramer-positive CTLs. Survivin-2B80-88 peptide tetramer-positive CTLs were sorted and subsequently cloned to single cells using FACS (Aria II Special Order; BD Biosciences). The peptide-specific cytotoxicity of each of these CTLs was determined by pulsing T2A24 cells[8,17] with survivin-2B80-88 or HLA-A*2402 HIV (RYLRDQQLL) peptides, as previously described.

Results

Patient profiles, safety, and clinical responses. In the present protocol with the survivin-2B80-88 peptide plus IFA and IFNα, six patients were enrolled in the study (Table 1). None dropped out because of adverse events due to the vaccination. They consisted of three men and three women, whose age range was 50–80 years.

With respect to the safety, vaccination was well tolerated in all patients. Four patients had fever reaching nearly 39°C after the vaccination, possibly due to the action of IFNα. No other severe adverse events were observed during or after vaccination except for induration at the injection site, which was conduced by IFA.

The clinical outcomes for the six patients treated with survivin-2B80-88 plus IFA and IFNα are summarized in Table 1. In some patients, particularly No. 1, the postvaccination Ca19-9 value was clearly decreased as compared with prevaccination, and was within the normal limit. Other patients (Nos. 2, 4, and 6) also had decreased or stable postvaccination levels of Ca19-9, although not as large. As for tumor size evaluated by CT, four patients (Nos. 1, 2, 4, and 6) were considered to have SD, but the other two patients (Nos. 3 and 5) had PD. Consequently, it appeared that there was a close correlation between clinical SD outcomes and a reduced or stable Ca19-9 level.

Immune responses, single-cell cloning, and subsequent functional assessment of tetramer-positive CTLs. As in our previous study with colon cancer patients, we determined if the survivin-2B80-88 peptide vaccination could actually induce specific immune responses in the patients enrolled. The peptide-specific CTL frequency was analyzed using the HLA-A24/peptide tetramer. The CTL frequencies before the first vaccination (prevaccination) and after the last vaccination (postvaccination) were assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer, compared with an HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer as a negative tetramer control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CD8 T cells in 10^4 CD8 T cells was determined. In the current study, ELISPOT was also carried out using these peptides.

As summarized in Table 1, four of the six patients (Nos. 1, 2, 4, and 6) had enhanced frequency with a more than 200% increase. It was also interesting that all four of these patients were also positive in the ELISPOT study, and all four had SD by CT evaluation, suggesting that immune responses might appropriately reflect clinical responses with the current vaccination protocol.

As in our previous work, we also analyzed tetramer-positive CD8 T cells at the single-cell level, and determined whether these T cells had specificity for the survivin-2B80-88 peptide and cytotoxic potential against live survivin-2B-positive tumor cells in the context of HLA-A*2402. As shown in Fig. 2, patient No. 1 (62 years old, female) had a reduced serum Ca19-9 level, and obvious immune responses as assessed by the survivin-2B80-88 ELISPOT and tetramer analyses (Fig. 3) after vaccination.

Subsequently, CD8 T cells of the tetramer-positive fraction were sorted by FACS, then cultured with 1, 3, and 10 cells/well for 7–10 days. Almost all growing T cells were survivin-2B peptide-specific T cells (data not shown), and we next assessed peptide-specific cytotoxicity by using these T cells. As Fig. 4 clearly shows, all T cells had very high peptide-specific cytotoxic potential. Taken together, these data clearly indicated that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα was capable of inducing a strong CTL response and for some pancreatic cancer patients might result in clinical effectiveness.

Assessment of treatment effect with IFNα alone. The above data strongly suggested that the current vaccination protocol

Table 1. Profiles of patients with advanced pancreatic cancer enrolled in the study and their clinical and immunological responses to vaccination with survivin-2B80-88 peptide, incomplete Freund's adjuvant and IFNα

Patient no.	Age/sex	Adverse effects	Tumor markers pre/post (CA19-9 U/mL)	CT eval.	Tetramer staining†		ELISPOT‡	
					Pre/post	% Increase	Pre/post	% Increase
1	62/F	Induration	136.5/31.4	SD	23/246	1069.6	27/294	1088.9
2	61/F	Induration Fever	63.6/60.6	SD	1/157	15700.0	25/71	284.0
3	56/M	Induration Fever Thrombopenia	171.4/978.8	PD	22/19	86.3	19/525	2763.2
4	80/F	Induration Fever	30.0/22.7	SD	9/1030	11444.4	1/101	10100.0
5	58/M	Induration Fever	436.0/2885.0	PD	3/0	0.0	34/20	58.8
6	50/M	Induration	4389.0/4295.0	SD	2/7	350.0	27/85	314.8

†Cytotoxic T-lymphocyte frequency of prevaccinated (pre) and postvaccinated (post) patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The numbers of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 × CD8 T cells are shown. ‡γ-Interferon (IFNγ) secretion of pre- and postvaccinated patients' CD8 T cells was assesed with enzyme-linked immunosorbent spot (ELISPOT) assay using T2-A24 cells pulsed with survivin-2B80-88 peptide. The numbers of spots in 5 × 10^3 CD8 T cells are shown. CT eval., evaluation by computed tomography; IFNα, α-interferon; PD, progressive disease; SD, stable disease.

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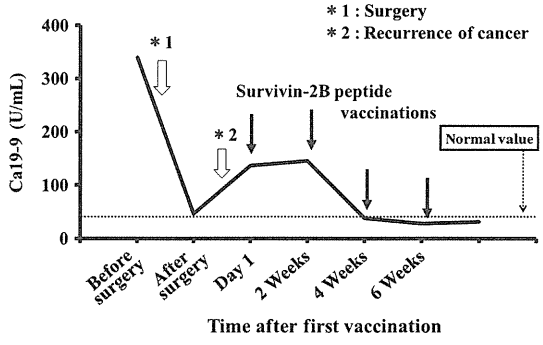

Fig. 2. Representative illustration of the clinical effect in patient No. 1 as assessed by the serum Ca19-9 level. Arrows indicate vaccinations with survivin-2B80-88 plus incomplete Freund's adjuvant with α-interferon (IFNα).

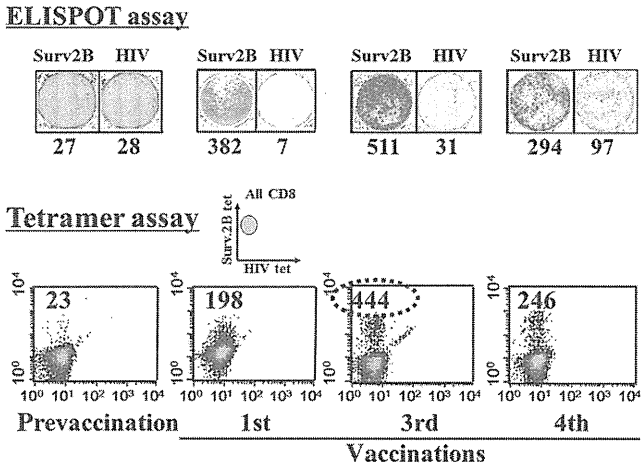

Fig. 3. Immunological analysis of CTL responses against HLA-A24 restricted survivin-2B80-88 peptide (surv2B) before and after vaccinations as assessed by enzyme-linked immunosorbent spot (ELISPOT) and tetramer (tet) analyses. Numbers in the ELISPOT assay indicate γ-interferon (IFNγ) secretion against survivin2B80-88 or HIV peptide pulsed T2-A24 cells in $10^4\times$ $CD8^+$ T cells. Numbers in tetramer analysis indicate survivin-2B80-88 peptide-specific $CD8^+$ T cells among $10^4\times$ $CD8^+$ T cells.

with the survivin-2B80-88 peptide plus IFA and IFNα could work as a potential therapeutic regimen in pancreatic cancers. However, it remained to be clarified if IFNα alone without the peptide could function in a similar manner, at least to some extent, as this cytokine is considered to be the most potent for the activation and maturation of dendritic cells (DCs) as well as upregulation of HLA class I in tumor cells. To this end, we studied this profile in three patients with colon cancer, not pancreatic cancer, whose condition was similar to those in this study, that is, patients with unresectable advanced or recurrent cancer. This was done because patients with the latter cancer had highly advanced clinical cases, making this type of study impossible. As shown Table 2, all three patients showed no obvious increases, but rather reductions, in the frequency of survivin-2B peptide-specific T cells as assessed by tetramer analysis before and after two to four treatments with IFNα alone. Furthermore, this was also true for ELISPOT analysis. These data supported the idea that IFNα alone did not actively participate in the activation of survivin-2B peptide-specific T cells.

Discussion

Our group previously showed that the vaccination protocol of survivin-2B80-88 plus IFA and IFNα could work as a potent

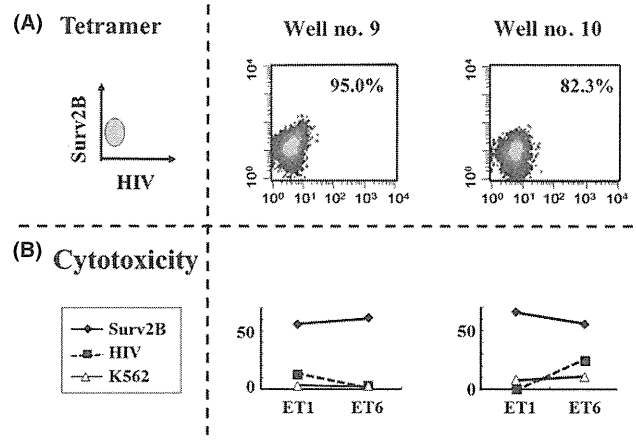

Fig. 4. Single-cell analysis of survivin-2B80-88 peptide tetramer-positive CD8 CTL cells). Survivin-2B80-88 peptide tetramer-positive CD8 T cells in Fig. 3 (circled) were sorted and cultured at 1, 3, and 10 cells /well for 7–10 days. Subsequently, clonal CTL cells were examined for their reactivity to the survivin-2B80-88 peptide tetramer (Surv2B) (A) and against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide and against control K562 cells (B). ET, effector/target ratio.

immunotherapeutic regimen in colon cancers.[8] In addition to colon cancer, survivin2B protein is expressed in most tumor cells of various tissue origins, such as those in the gastrointestinal and biliary tracts and pancreas, therefore, there is a possibility that the survivin2B peptide could work as a potential therapeutic tumor vaccine in cancer patients with these neoplasms.

In this present study, we assessed whether the vaccination protocol using survivin-2B80-88 plus IFA and IFNα could be effective in pancreatic cancer patients from immunological and clinical points of views. Consequently, our data strongly suggested that this protocol was very effective and useful in immunotherapy for advanced pancreatic cancers as in colon cancers. Actually it was shown that more than 50% of patients with pancreatic cancers showed positive clinical and immunological responses in tetramer and ELISPOT analyses. In some cases, the immunological response of survivin-2B80-88 peptide-specific CTLs was elucidated at the single-cell level. Taken together, the current data implied that our vaccination protocol was very useful in immunotherapy for pancreatic cancers.

As shown in Fig. 3, the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced from the third to the fourth vaccination. We speculate that there could be various reasons for this reduction. One might be immune escape by the downregulation of HLA expression, cytokines, or regulatory T cells. Another might be an activity of the stored samples, or differences between the environment of the peripheral circulation and the tumor. In other words, the peptide-specific CTL responses were reduced in immunological monitoring in the peripheral circulation, but maintained in the local cancer environment. In this case, the clinical responses, such as tumor marker (CA19-9) level and tumor size evaluated by CT, had been maintained also after that, even though the number of tetramer-positive populations and IFNγ-positive spots in the ELISPOT assay was reduced between the third and fourth vaccinations. Therefore, CA19-9 levels had been kept within normal limits and new cancer lesions had not appeared.

We evaluated immunological monitoring of this clinical protocol by tetramer staining and IFNγ ELISPOT assay. Tetramer staining recognizes the structure of the T cell receptor, and

Table 2. Frequency monitoring of the number of survivin-2B80-88 peptide tetramer-positive CTLs in cancer patients treated with IFNα alone

Patient no.	Tumor	Age/sex	Number of treatment	Tetramer staining†		ELISPOT‡	
				Pre/post	% Increase	Pre/post	% Increase
1	Colon	60/M	3	1/0	0.0	111/75	67.6
2	Colon	63/M	4	11/9	81.8	44/20	45.5
3	Colon	77/F	2	13/3	23.1	26/40	153.8

†CTL frequency before and after treatment with IFNα alone in patients was assessed with an HLA-A24-restricted survivin-2B80-88 (AYACNTSTL) peptide tetramer. An HLA-A24-restricted HIV peptide (RYLRDQQLL) tetramer was used as a negative control. The number of survivin-2B80-88 peptide tetramer-positive but HIV peptide-negative CTLs in 10^4 CD8 T cells is shown. ‡γ-Interferon (IFNγ) secretion of pre and post IFNα treatmnet were assesed with ELISPOT assay using T2-A24 cells pulsed with survivin2B80-88 peptide. The number of spots in 5×10^3 CD8 T cells are shown. IFNα, α-interferon.

detects naive T cells, memory T cells, and activated CTLs. The ELISPOT assay detects more the functional aspects of T cells by IFNγ release, therefore, ELISPOT detects memory T cells and CTLs. In this study, the tetramer-positive cases are also positive in the ELISPOT study. Therefore, these results indicate that memory T cells and CTLs can be effectively induced by this peptide vaccination protocol.

In this present study, we also assessed evidence concerning the extent to which peptide-specific CTL responses in pancreatic cancer patients treated with peptide vaccines could occur at the single-cell level. To assess this, CTLs of patients were sorted to the single-cell level, and we confirmed that each CTL obtained from vaccinated patients was indeed peptide-specific in the context of the expression of HLA-A24.

Type-I interferons such as IFNα are known to work in various immunological manners to activate T cell responses.[18–25] The maturation of DCs and their effect on the expression of HLA molecules seems to be the main action of this cytokine. Although we could not actually compare these features of patients' DCs and primary pancreatic tumor tissues before and after treatment with IFNα, the obvious enhancement of CTL responses and improvement of clinical responses in our previous and current studies favors the two main actions described above. These observations strongly suggest that the action of IFNα is remarkable from the aspect of being an immunogenic enhancer for human cancer peptide vaccines.

It is widely known that IFNα is involved in DC maturation and activation.[18,21] This particular cytokine is also potent for increasing the expression of MHC class I molecules.[26–29] Indeed, our previous study of the expression of HLA class I molecules in pancreatic cancer indicated that many tumor tissues heterogeneously expressed such molecules, with some tumor cells showing high expression, whereas others had only weak expression. Interferon-α is presumed to actually enhance their expression even in those tumor tissues with weak expression. Moreover, because tumor patients generally show overt expression of survivin protein in their tumor tissues and, although in small numbers, survivin-2B peptide-specific T cells in peripheral blood, it is considered that IFNα alone may increase the frequency of these T cells in peripheral blood as well. These features of this particular cytokine lead to the possibility that treatment with IFNα alone could result in, at least to some extent, certain immunological and clinical effects of survivin-2B peptide-specific T cells in tumor-bearing patients. However, we analyzed three colon cancer patients, and our data strongly suggested that there was no increase of these T cells as assessed by tetramer and ELISPOT analyses.

Taken together, our results highly suggest that the vaccination protocol with survivin-2B80-88 plus IFA and IFNα is very effective for pancreatic and colon cancers, and that this protocol might be useful as a standard, general immunotherapy modality for human cancers. However, further clinical studies involving many patients are necessary in order to consolidate the immunotherapeutic benefit of this vaccination protocol.

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Disclosure Statement

The authors have no conflict of interest.

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ORIGINAL ARTICLE

Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer

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Abstract

AIM: To investigate human epidermal growth factor receptor 2 (HER2)-phosphatidylinositol 3-kinase (PI3K)-v-Akt murine thymoma viral oncogene homolog signaling pathway.

METHODS: We analyzed 231 formalin-fixed, paraffin-embedded gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of the medical records. Expression of HER2 was analyzed by immunohistochemistry (IHC) using the HercepTest™ kit. Standard criteria for HER2 positivity (0, 1+, 2+, and 3+) were used. Tumors that scored 3+ were considered HER2-positive. Expression of phospho Akt (pAkt) was also analyzed by IHC. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more. PI3K, catalytic, alpha polypeptide (PIK3CA) mutations in exons 1, 9 and 20 were analyzed by pyrosequencing. Epstein-Barr virus (EBV) infection was analyzed by *in situ* hybridization targeting EBV-encoded small RNA (EBER) with an EBER-RNA probe. Microsatellite instability (MSI) was analyzed by polymerase chain reaction using the mononucleotide markers BAT25 and BAT26.

RESULTS: HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively. HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type (15/20 *vs* 98 /205, $P = 0.05$). PIK3CA mutations were present in 20 cases (8.7%) and significantly correlated with MSI (10/20 *vs* 9/211, $P < 0.01$).

The mutation frequency was high (21%) in T4 cancers and very low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively. Two new types of PIK3CA mutation, R88Q and R108H, were found in exon1. All PIK3CA mutations were heterozygous missense single-base substitutions, the most common being H1047R (6/20, 30%) in exon20. Eighteen cancers (8%) were EBV-positive and this positivity significantly correlated with a diffuse histological type (13/18 *vs* 93/198, P = 0.04). There were 7 cases of lymphoepithelioma-like carcinomas (LELC) and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). pAkt expression was positive in 119 (53%) cases but showed no correlation with clinicopathological characteristics. pAkt expression was significantly correlated with HER2 overexpression (16/20 *vs* 103/211, $P < 0.01$) but not with PIK3CA mutations (12/20 *vs* 107/211, P = 0.37) or EBV infection (8/18 *vs* 103/211, P = 0.69). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations. One case showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However, no cases showed both PIK3CA mutations and HER2 overexpression. One EBV-positive cancer with PIK3CA mutation (H1047R) was MSI-positive. Three of these 4 cases were positive for pAkt expression. In survival analysis, pAkt expression significantly correlated with a poor prognosis (hazard ratio 1.75; 95%CI: 1.12-2.80, P = 0.02).

CONCLUSION: HER2 expression, PIK3CA mutations and EBV infection in gastric cancer were characterized. pAkt expression significantly correlates with HER2 expression and with a poor prognosis.

Key words: Human epidermal growth factor receptor 2; Phosphatidylinositol 3-kinase; Catalytic; Alpha polypeptide; Epstein-Barr virus; Akt; Gastric cancer

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INTRODUCTION

Gastric cancer is one of the most common cancer types and the second leading cause of cancer-related deaths worldwide[1]. Genetic and epigenetic alterations play important roles in the development and progression of these tumors[1,2]. Considerable attention has been given to the potential role of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway in gastric cancer[3,4]. Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and inactivation of phosphatase and tensin homolog (PTEN) lead to activation of the PI3K-Akt signaling pathway. With regards to growth factor receptors, there is growing evidence that human epidermal growth factor receptor 2 (HER2) is a key driver of tumorigenesis and an important biomarker in gastric cancer. The amplification or overexpression of HER2 is observed in 7%-34% of these cases[5-9].

PIK3CA is mutated in a wide variety of human tumor types[10,11], including gastric cancers[12-15]. Activating mutations in this gene up-regulate the PI3K-Akt signaling pathway, making it a potentially useful therapeutic target. For example, oncogenic mutations of PIK3CA reportedly render breast cancers more resistant to treatment with the anti-HER2 receptor antibody trastuzumab[16]. Thus, this signaling pathway is thought to be one of the mechanisms underlying resistance to trastuzumab. Trastuzumab has recently been approved for treatment of advanced gastric cancers[5,6].

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17]. PIK3CA mutations have been detected in 4%-25% of gastric cancers[12-15]. However, in most previous studies, only exons 9 and 20 hot spot mutations in PIK3CA were analyzed by DNA sequencing. Moreover, the association between HER2 expression and PIK3CA mutations in gastric cancer has not been reported.

A significant correlation has been found between Epstein-Barr virus (EBV) and the methylation of multiple genes in gastric cancers[18-20]. EBV infection reportedly induces PTEN expression loss through CpG island methylation of its promoter, leading to activation of the PI3K-Akt signaling pathway, in EBV-associated gastric cancer[21].

The aim of our present study was to systematically characterize HER2 expression, PIK3CA mutations, and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of gastric cancers (n = 231). We wished to determine the prevalence of each of these factors with a high precision and thereby correlate them with clinicopathological and molecular features of gastric lesions, including microsatellite instability (MSI) and phospho Akt (pAkt) expression.

MATERIALS AND METHODS

Tissue samples

A total of 231 formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment was analyzed in

Table 1 Clinicopathological characteristics of patients with gastric cancer

Variables (n = 231)		n (%)
Sex	Male	157 (68)
	Female	74 (32)
Age (yr)	Median (range)	71 (25-91)
Location	Cardias	82 (35)
	Body	62 (27)
	Antrum	83 (36)
	Unknown	4 (2)
Depth of invasion	T2	125 (54)
	T3	92 (40)
	T4	14 (6)
Lymph node metastasis	N0	65 (28)
	N+	158 (68)
	N1	73 (32)
	N2	56 (24)
	N3	29 (13)
	Unknown	8 (3)
Stage	I B	49 (21)
	II	45 (19)
	IIIA + IIIB	82 (35)
	IV	51 (22)
	Unknown	4 (2)
Lauren histotype	Intestinal	113 (49)
	Diffuse	112 (48)
	Others	6 (3)

this study. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of their medical records. Clinicopathological findings were determined according to the criteria of the Japanese Research Society for Gastric Cancer (Table 1). Our institutional review committee approved the study.

Immunohistochemistry

HER2 expression was analyzed using the HercepTest™ kit (DAKO, Carpinteria, CA) by manual sample processing in accordance with the manufacturer's instructions. Standard criteria for HER2 positivity (0, 1+, 2+ and 3+) were used. Tumors that scored 3+ were considered HER2-positive. For the immunohistochemical analysis of pAkt, FFPE specimens were processed using SignalStain Boost Detection Reagent (Cell Signaling Technology, Beverly, MA). Briefly, 5-μm-thick sections were dewaxed in xylene, rehydrated in ethanol, and heated with target retrieval solution (DAKO) in an autoclave for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in methanol for 10 min. The tissue sections were then washed twice with tris-buffered saline (TBS) and preblocked with 10% goat serum in TBS for 60 min. After washing with TBS, the sections were incubated with an anti-phospho-Akt (Ser473) polyclonal antibody (D9E, Cell Signaling Technology) at a dilution of 1:100 for 30 h at 4 ℃. The sections were washed three times in TBS and incubated with SignalStain Boost Detection Reagent for 45 min. After three further washes in TBS, a diamino-benzidine tetrahydrochloride working solution was applied. Finally, the sections were counterstained with hematoxylin. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more[22]. Only clear staining of the tumor cell nucleus and/or cytoplasm was considered positive.

Mutation analysis of the PIK3CA gene by pyrosequencing

Genomic DNA was extracted from tumor specimens and mutations in exon9 and exon20 of the *PIK3CA* gene were analyzed by pyrosequencing as described previously[23,24]. We also developed a pyrosequencing assay to detect PIK-3CA exon1 mutations using the primer sets exon1-RS1 (5'-GGGAAGAATTTTTTGATGAAACA-3' for the biotinylated forward primer and 5'-GGTTGCCTACT-GGTTCAATTACTT-3' for the reverse primer) and exon1-RS2 (5'-CGGCTTTTTCAACCCTTTTT-3' for the forward primer and 5'-ATTTCTCGATTGAG-GATCTTTTCT-3' for the biotinylated reverse primer). Each polymerase chain reaction (PCR) mix contained the forward and reverse primers (each 10 μmol/L), a 25 mmol/L dNTP mix with dUTP, 75 mmol/L $MgCl_2$, 1 × PCR buffer, 1.0 U of exTaq, and 2 μL of template DNA in a total volume of 25 μL. PCR conditions were as follows: initial denaturing at 95 ℃ for 5 min; 50 cycles of 94 ℃ for 20 s, 50 ℃ for 20 s and 74 ℃ for 40 s; and a final extension at 72 ℃ for 1 min. The PCR products (each 25 μL) were sequenced using the PyroMark kit and Pyrosequencing PSQ96 HS System (Qiagen, Valencia, CA).

In situ hybridization for EBER1

The presence of EBV in the carcinoma tissues was evaluated by *in situ* hybridization (ISH) targeting of EBV-encoded small RNA (EBER-ISH) with an EBER-RNA probe (Dako Cytomation).

Microsatellite instability analysis

MSI was analyzed by PCR using the mononucleotide markers (BAT25 and BAT26). Based on the number of markers showing instability per tumor sample, cancers were divided into two groups; those with one or more of the two markers displaying MSI and those with no instability (microsatellite stable).

Statistical analysis

For all statistical analysis, the JMP program was used. All P values were two-sided and statistical significance was set at $P \leq 0.05$. For categorical data, the χ^2 test was used. For survival analysis, Kaplan-Meier method and log-rank test were used. For analysis of cancer-specific mortality, we excluded surgery-related deaths (deaths within one month of surgery).

RESULTS

HER2 expression in gastric cancer tissues

HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively (Figure 1). HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type

Table 2 Clinicopathological characteristics of patients with gastric cancer based on human epidermal growth factor receptor 2 expression, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations and Epstein-Barr virus infection *n* (%)

		HER2			PIK3CA			EBV		
		Positive (*n* = 20)	Negative (*n* = 211)	*P* value	Mutation (*n* = 20)	Wild type (*n* = 211)	*P* value	Positive (*n* = 18)	Negative (*n* = 204)	*P* value
Sex	Male	15 (75)	142 (67)	0.48	13 (65)	144 (68)	0.77	14 (78)	138 (68)	0.38
	Female	5 (25)	69 (33)		7 (35)	70 (32)		4 (22)	66 (32)	
Age	Median	69 (50-84)	71 (25-91)	0.26	71 (25-85)	70 (38-91)	0.40	72 (48-90)	70 (38-91)	0.41
Location	Cardias	10 (50)	72 (34)	0.49	5 (25)	77 (36)	0.31	8 (44)	73 (36)	0.70
	Body	5 (25)	57 (27)		4 (20)	58 (27)		5 (28)	55 (27)	
	Antrum	5 (25)	78 (37)		10 (50)	73 (35)		5 (28)	75 (37)	
	Unknown	0	4 (2)		1 (5)	2 (1)		0	1 (0)	
Depth	T2	12 (60)	113 (54)	0.48	8 (40)	117 (55)	0.15	12 (67)	106 (52)	0.35
	T3	8 (40)	84 (40)		9 (45)	83 (39)		6 (33)	85 (42)	
	T4	0	14 (6)		3 (15)	11 (5)		0	13 (6)	
L/N meta	N0	5 (25)	60 (28)	0.71	4 (20)	61 (29)	0.37	3 (17)	57 (28)	0.28
	N+	14 (70)	144 (68)		16 (80)	142 (67)		14 (77)	140 (69)	
	N1	5 (25)	68 (32)		8 (40)	65 (31)		8 (44)	63 (31)	
	N2	6 (30)	50 (24)		6 (30)	50 (24)		2 (11)	53 (26)	
	N3	3 (15)	26 (12)		2 (10)	27 (13)		4 (22)	24 (12)	
	Unknown	1 (5)	7 (3)		0	8 (4)		1 (6)	7 (3)	
Stage	I	5 (25)	44 (21)	0.89	1 (5)	48 (23)	0.14	3 (17)	41 (20)	0.98
	II	3 (15)	42 (20)		7 (35)	38 (18)		4 (22)	39 (19)	
	III	6 (30)	76 (36)		8 (40)	74 (35)		6 (33)	75 (37)	
	IV	5 (25)	46 (22)		4 (20)	47 (22)		4 (22)	46 (23)	
	Unknown	1 (5)	3 (1)		0	4 (2)		1 (6)	3 (1)	
Lauren	Intestinal	15 (75)	98 (46)	0.05	14 (70)	99 (47)	0.13	5 (28)	105 (51)	0.04
histotype	Diffuse	5 (25)	107 (51)		6 (30)	106 (50)		13 (72)	93 (46)	
	LELC	0	6 (3)		2 (10)	4 (2)		5 (28)	0	
	Others	0	6 (3)		0	6 (3)		0	6 (3)	
MSI		2 (10)	28 (13)	0.72	10 (50)	20 (9)	< 0.01	1 (6)	26 (13)	0.36
pAkt		16 (84)	103 (51)	< 0.01	12 (63)	107 (53)	0.37	8 (47)	103 (52)	0.69
3 yr OS (%)		29.4	59.2	0.24	57.3	56.8	0.59	57.4	57.3	0.98

MSI: Microsatellite instability; LELC: Lymphoepithelioma-like carcinoma; HER2: Human epidermal growth factor receptor 2; PIK3CA: Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations; EBV: Epstein-Barr virus; pAkt: Phospho Akt; OS: Overall survival.

Table 3 Frequencies of phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected in gastric cancer tissues

	Mutation	Overall frequency	Percent/ total cases	Percent/ mutated cases	Microsatellite instability
Exon1	R88Q	1	0.4	5	1
	R108H	4	1.7	20	1
	Total	5	2.2		2
Exon9	E542K	5	2.2	25	1
	E545K	2	0.9	10	1
	E545G	2	0.9	10	1
	Total	9	4.0		3
Exon20	H1047Y	1	0.4	5	1
	H1047R	6	2.6	30	4
	Total	7	3.0		5

(15/20 *vs* 98/205, $P = 0.05$, Table 2). Three-year survival rates were 29% in patients with HER2 overexpression and 59% in cases without HER2 overexpression, respectively [hazard ratio (HR) 1.73; 95%CI: 0.87-3.14, $P = 0.24$].

Mutations of the PIK3CA gene in gastric cancer tissues

PIK3CA mutations were present in 20 cases (8.7%) (Table 2 and Figure 2). The mutation frequency was high (21%) in T4 cancers and low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 of PIK3CA were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively (Table 3). One case had multiple PIK3CA mutations (R108H and E542K). The exon20/exon9 prevalence ratio was 0.78 (7/9). Two new types of PIK3CA mutations, R88Q and R108H, were detected in exon1. All mutations were heterozygous missense single-base substitutions and the most common mutation was H1047R (6/20; 30%) in exon20. PIK3CA mutations were also found to significantly correlate with MSI (10/20 *vs* 9/211, $P < 0.01$) but not with other clinicopathological characteristics. The three-year survival rates were 57% in patients with PIK3CA mutations and 57% in cases without PIK3CA mutations, respectively (HR 1.37; 95%CI: 0.68-3.26, $P = 0.59$).

EBV infection

Eighteen samples in our cohort (8%) were EBV-positive and this positivity significantly correlated with diffuse histological type (13/18 *vs* 93/198, $P = 0.04$) (Table 2 and Figure 3). There were 7 cases of LELC and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). The three-year survival rates were 57% in patients with EBV infection and 57% in those without EBV infection (HR 0.81; 95%CI:

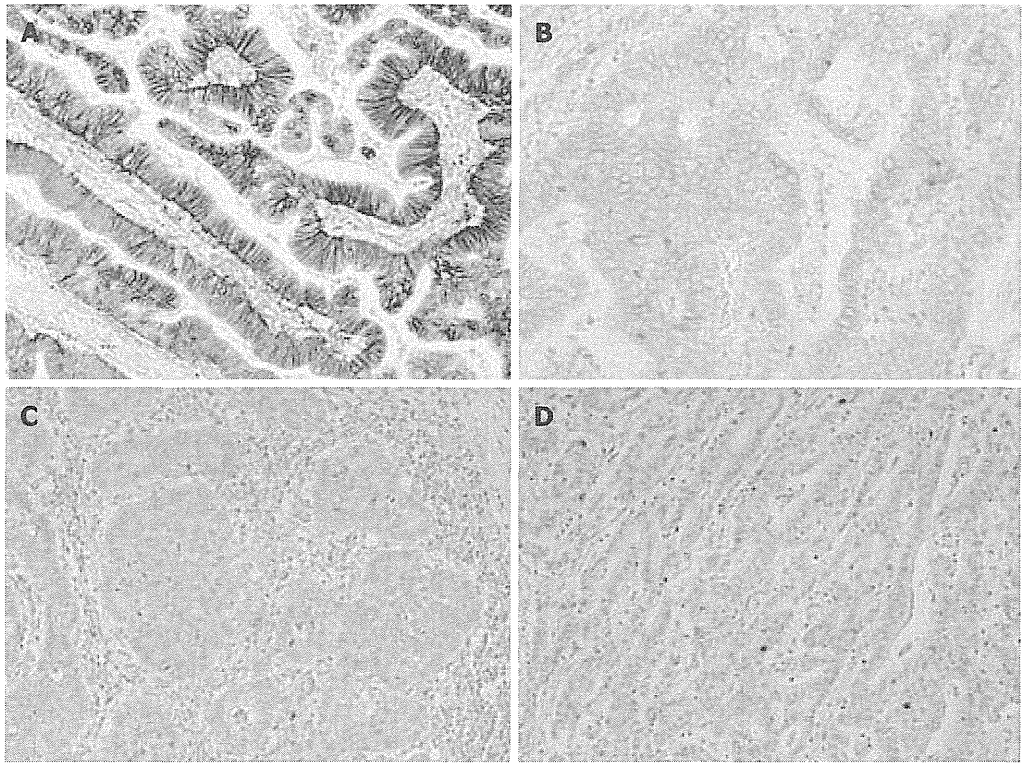

Figure 1 Immunohistochemical analysis of human epidermal growth factor receptor 2 in gastric cancer tissues. A: Human epidermal growth factor receptor 2 (HER2) 3+; B: HER2 2+; C: HER2 1+; D: HER2 0. Original magnification, ×200.

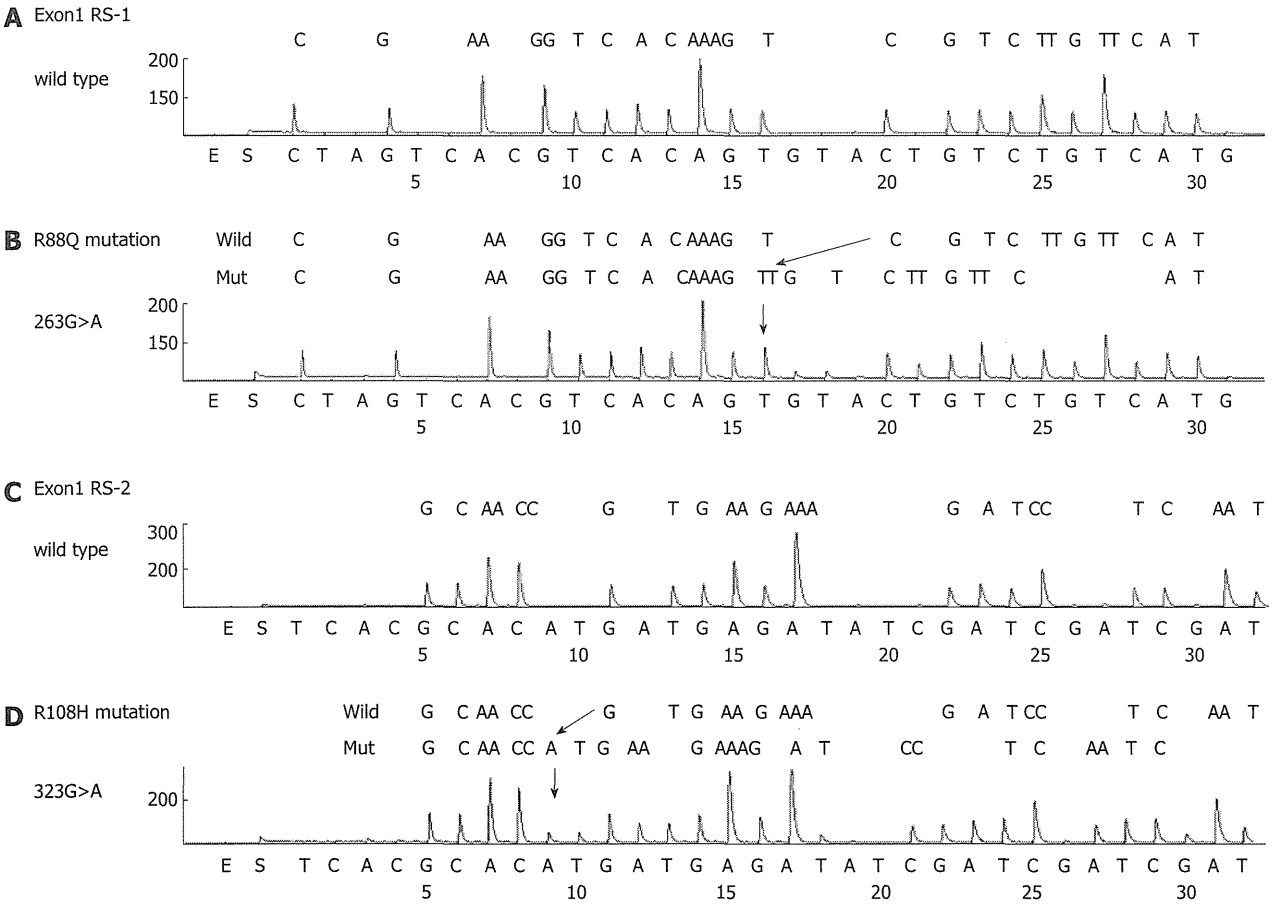

Figure 2 Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected by pyrosequencing in gastric cancer tissues. A: Exon1 RS1 wild type; B: 263G>A (R88Q) mutation; C: Exon1 RS2 wild type; D: 323G>A (R108H) mutation.

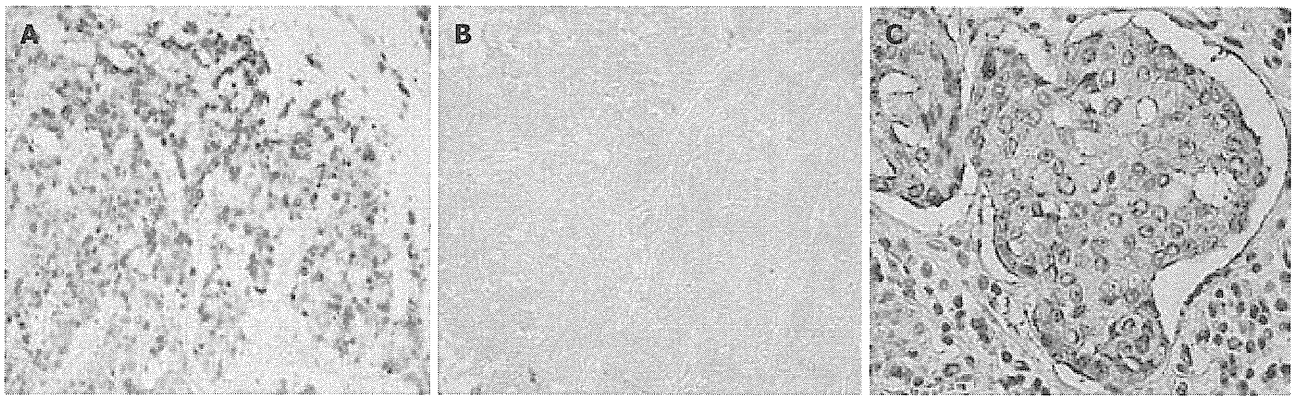

Figure 3 *In situ* hybridization analysis of Epstein-Barr virus-encoded small RNA-1 and human epidermal growth factor receptor 2 immunohistochemical expression in gastric cancer tissues. A: Gastric adenocarcinoma positive for Epstein-Barr virus-encoded small RNA-1 (EBER-1); B: Gastric adenocarcinoma negative for EBER-1; C: Immunohistochemical analysis of human epidermal growth factor receptor 2 (HER2) in an Epstein-Barr virus-positive and HER2-positive case. Original magnification, ×200.

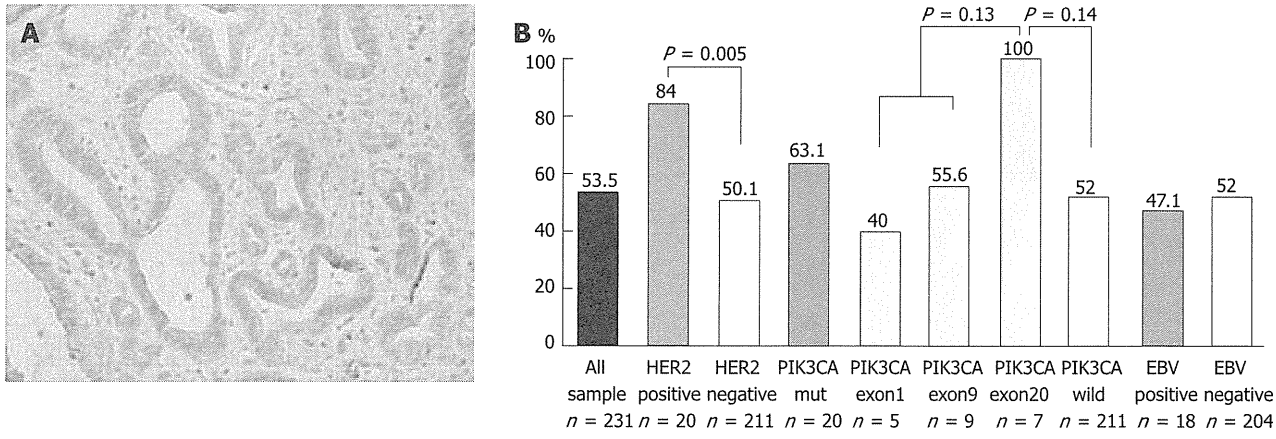

Figure 4 Immunohistochemical analysis and assessment of phospho Akt positivity based on molecular alterations in gastric cancer tissues. A: Gastric adenocarcinoma showing phospho Akt (pAkt) positivity. Original magnification, ×200; B: pAkt expression significantly correlates with human epidermal growth factor receptor 2 (HER2) overexpression ($P < 0.01$) but not with phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutations ($P = 0.37$) or Epstein-Barr virus (EBV) infection ($P = 0.69$).

0.36-2.31, $P = 0.98$).

Association of HER2 overexpression, PIK3CA mutations and EBV infection

One of our cases showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However there were no cases showing both PIK3CA mutations and HER2 overexpression. Three of the 4 cases were positive also for pAkt expression. PIK3CA mutations were present in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). One EBV-positive cancer with a PIK3CA mutation (H1047R) was MSI-positive.

pAkt expression

pAkt expression was positive in 119 (53%) of our cases but this showed no correlation with clinicopathological characteristics (Figure 4A). On the other hand, pAkt expression was found to be significantly correlated with HER2 overexpression (16/19 *vs* 103/204, $P < 0.01$) but not with PIK3CA mutations (12/19 *vs* 107/204, $P = 0.37$) or EBV infection (8/17 *vs* 103/198, $P = 0.69$) (Table 2). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations of PIK3CA, although this difference did not reach statistical significance (Figure 4B). The five-year survival rates were 37% in patients with pAkt expression and 59% in those without pAkt expression (HR 1.75; 95%CI: 1.12-2.80, $P = 0.02$) (Figure 5). Hence, pAkt expression significantly correlates with a poor prognosis in gastric cancer.

DISCUSSION

In our present study, we systematically characterized HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer ($n = 231$). We aimed to determine the prevalence of these characteristics with a high level of precision and to correlate them with clinicopathological and molecular features, such as MSI and pAkt expression.

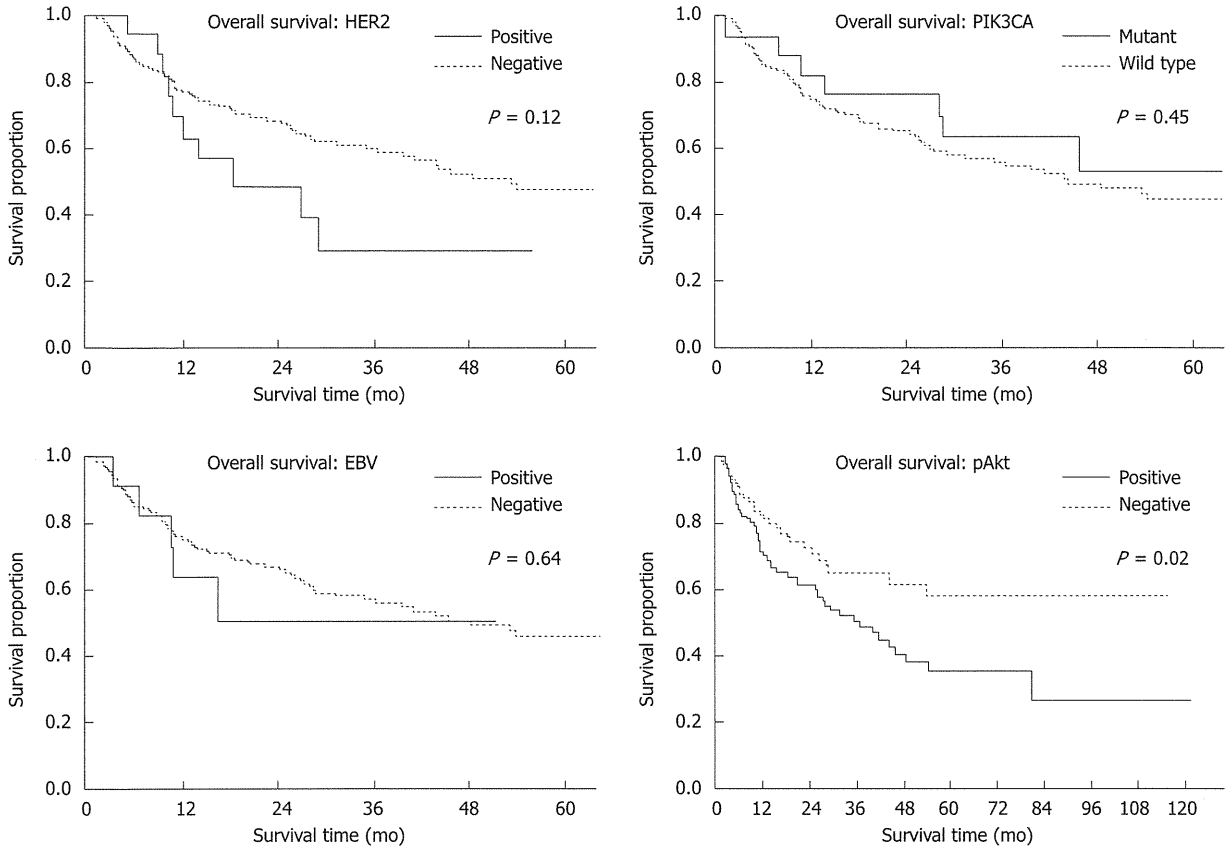

Figure 5 Survival analysis of gastric cancer patients. Three year survival of human epidermal growth factor receptor 2 (HER2)-positive *vs* HER2-negative, 29.1 mo *vs* 59.4 mo; Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutation *vs* wild type, 63.7 mo *vs* 56.3 mo; Epstein-Barr virus (EBV)-positive *vs* EBV-negative, 51.3 mo *vs* 57.6 mo; And phospho Akt (pAkt)-positive *vs* pAkt-negative, 50.7 mo *vs* 64.8 mo. Five year survival of pAkt-positive *vs* pAkt-negative cases, 35.5 mo *vs* 58.1 mo.

HER2 overexpression (IHC 3+) was present in 20 samples (8.4%), a value that is within the range (7%-34%) reported in the current literature[5-9]. HER2 overexpression was found to significantly correlate with the intestinal histological type. Hence, the frequency of HER2 expression may depend on, at least in part, the distribution of histology in a cohort of gastric cancer samples. Some studies have suggested that HER2 positivity in gastric cancer is associated with poor outcomes and aggressive disease, but the results are conflicting. We found for the first time in our present analyses that HER2 overexpression significantly correlates with pAkt expression in gastric cancer tissues. Moreover, pAkt expression correlated with a poor prognosis in these patients. Thus, the HER2-Akt axis may play an important role in gastric cancer.

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17,23,24]. We characterized PIK3CA mutations in gastric cancer tissues using pyrosequencing for the first time. The overall prevalence of PIK3CA mutations was found in our analysis to be 8.7%, a value that is within the previously reported range (4% to 25%)[10,12-15]. The mutation frequency was found to be high (21.4%) in T4 cancers and low (6.4%) in T2 cancers in our sample cohort. Thus, PIK3CA mutations appear to be late events in gastric carcinogenesis, leading to tumor progression. These patients might therefore be appropriate for targeted therapies directed against the PI3K pathway.

The most common PIK3CA mutation found in our analysis was H1047R, which was also found previously[15]. Importantly, two new types of mutations were found in exon1. To our knowledge, PIK3CA mutations involving residues 88 and 108 (R88Q and R108H) have been never reported previously in gastric cancer, nor described in the COSMIC database, despite the large number of previous studies in which this region was investigated. These mutations have been detected in several other types of cancer tissues[25]. Importantly also, these mutations have been reported to be gain-of-function[26-28]. Our present results thus have potential clinical implications since the mutational status of PIK3CA could stratify patients for genotype-based molecular therapies targeting the PI3K pathway. Hence, exon1 of PIK3CA should be analyzed in gastric cancer patients in these clinical settings.

PIK3CA mutations were found to be significantly associated with the MSI phenotype in our experiments. An association between PIK3CA mutations and MSI has been reported, or at least suggested, for both gastric and colon cancers[12,13,29]. We found in our present study that PIK3CA mutations in cancers with MSI are distributed in exon1, exon9 and exon20. These results further sup-

port the notion that PIK3CA is one of the most important oncogenes activated by missense mutations in MSI-positive gastric cancers.

The frequency of pAkt expression was found to be higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations in PIK3CA. These results further support the notion that the functional significance of PIK3CA mutations depends on the mutation type and that the H1047R hotspot mutation has high oncogenic activity.

The previous ToGA study has shown that the addition of trastuzumab to the chemotherapeutic regimen improves survival in patients with advanced gastric or gastroesophageal junction cancer[5,6]. PIK3CA mutation is one of the mechanisms underlying the resistance to trastuzumab in breast cancer[30]. Trastuzumab is likely to be effective for HER2-overexpressing breast cancers with no PIK3CA mutations, with possible rescue using HER2-TKIs in cases of relapse[31]. For HER2-overexpressing breast cancer with PIK3CA mutations, inhibitors against molecules of the PI3K pathway are possibly more effective than anti-HER2 agents, which are unlikely to be beneficial[32]. In our present study, PIK3CA mutations were not found in gastric cancers with HER2 overexpression. Thus, it is unlikely that PIK3CA mutation is a major mechanism underlying the resistance to trastuzumab in gastric cancer.

HER2 overexpression was found in only one of the 18 EBV-positive gastric cancers in our sample cohort. This result can be explained, at least in part, by the fact that HER2 overexpression and EBV infection significantly correlate with intestinal and diffuse histological types, respectively. On the other hand, PIK3CA mutations were identified in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). Although not analyzed in our current study, EBV infection reportedly inactivates PTEN through the CpG island methylation of its promoter in EBV-associated gastric cancer[21]. Thus, alterations in the PI3K-Akt signaling pathway in EBV-positive gastric cancers may differ from those in EBV-negative cancers.

Finally, pAkt expression was found to correlate with a poor prognosis in gastric cancer. A significant association between increased pAkt expression and poor prognosis has been reported previously in patients with T3/T4 gastric cancer but not in those with T1/T2 cancer[33]. It has been reported also that pAkt expression is associated with increased resistance to multiple chemotherapeutic agents in gastric cancer patients, when chemotherapeutic sensitivities were tested using MTT assays[34]. Thus, Akt activation appears to lead to a poor prognosis and resistance to chemotherapeutic agents in gastric cancer. A positive correlation between a decrease in the pAkt levels after gefitinib administration and tumor apoptotic index in gastric cancer has also been reported[35]. Further analyses regarding the pAkt status in cancer tissues before and after chemotherapy and molecular targeted therapy will be necessary. Not all Akt activation events can be explained by HER2 expression, PIK3CA mutations, and EBV infection in gastric cancer. We have reported previously that a dominant negative insulin-like growth factor (IGF)-1 receptor blocks the Akt-1 activation induced by IGF-1 and IGF-2 in gastric cancer cell lines[36]. Thus, molecular alterations, such as the overexpression of IGF-1 receptor, might be involved in the activation of Akt in gastric cancer and this issue needs to be clarified in the near future.

COMMENTS

Background

Personalized therapy has begun also in advanced gastric cancer through the use of trastuzumab, an anti-human epidermal growth factor receptor 2 (HER2) antibody. Many drugs targeting the phosphatidylinositol 3-kinase (PI3K)-Akt pathway have now been developed and clinical trials are ongoing. An appropriate biomarker is necessary for successful molecular targeted therapy. The alterations of molecules in the PI3K-Akt pathway could be a good biomarker for such drugs.

Research frontiers

Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and Epstein-Barr virus (EBV) infection lead to activation of the PI3K-Akt signaling pathway. However, clinicopathological and molecular correlates among such alterations have not been clearly addressed. In the present study, the authors identify new clinicopathological and molecular correlations between HER2 expression, PIK3CA mutations, EBV infection and phospho Akt (pAkt) expression in gastric cancer.

Innovations and breakthroughs

This is the first study to systematically characterize HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer. The prevalence of these characteristics was thereby determined with a high level of precision and correlations with the clinicopathological and molecular features of gastric cancers, such as microsatellite instability and pAkt expression, could be assessed accurately for the first time.

Applications

The results have potentially important clinical implications since the mutational status of PIK3CA can be used to stratify cancer patients for genotype-based molecular therapies that target the HERs-PI3K pathway.

Terminology

PI3K-Akt pathway: Akt is believed to transduce the major downstream PI3K signals in cancer. Akt regulates cell growth and survival pathways by phosphorylating substrates such as GSK3, forkhead transcription factors, and the TSC2 tumor suppressor protein; PIK3CA: PIK3CA encodes a key enzymatic subunit of PI3K. Gain of function mutations in PIK3CA occur frequently in several cancer types. Hotspots of PIK3CA mutations are located in exons 9 and 20.

Peer review

The authors investigated HER2 expression, PIK3CA mutations and EBV infection in patients with gastric cancer. The results demonstrated that pAkt expression significantly correlates with the prognosis and the HER2 expression status in gastric cancer. This article is important for the further development of molecular targeted therapy in patients with advanced gastric cancer.

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ORIGINAL ARTICLE

Treatment of pancreatic fibrosis with siRNA against a collagen-specific chaperone in vitamin A-coupled liposomes

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ABSTRACT

Background and objective Fibrosis associated with chronic pancreatitis is an irreversible lesion that can disrupt pancreatic exocrine and endocrine function. Currently, there are no approved treatments for this disease. We previously showed that siRNA against collagen-specific chaperone protein gp46, encapsulated in vitamin A-coupled liposomes (VA-lip-siRNAgp46), resolved fibrosis in a model of liver cirrhosis. This treatment was investigated for pancreatic fibrosis induced by dibutyltin dichloride (DBTC) and cerulein in rats.

Methods Specific uptake of VA-lip-siRNAgp46, conjugated with 6′-carboxyfluorescein (FAM) by activated pancreatic stellate cells (aPSCs), was analysed by fluorescence activated cell sorting (FACS). Intracellular distribution of VA-lip-siRNAgp46-FAM was examined by fluorescent microscopy. Suppression of gp46 expression by VA-lip-siRNAgp46 was assessed by immunoblotting. Collagen synthesis in aPSCs was assayed by dye-binding. Specific delivery of VA-lip-siRNAgp46 to aPSCs in DBTC rats was verified following intravenous VA-lip-siRNA-FAM and ^{3}H-VA-lip-siRNAgp46. The effect of VA-lip-siRNA on pancreatic histology in DBTC- and cerulein-treated rats was determined by Azan-Mallory staining and hydroxyproline content.

Results FACS analysis revealed specific uptake of VA-lip-siRNAgp46-FAM through the retinol binding protein receptor by aPSCs in vitro. Immunoblotting and collagen assay verified knockdown of gp46 and suppression of collagen secretion, respectively, by aPSCs after transduction of VA-lip-siRNAgp46. Specific delivery of VA-lip-siRNAgp46 to aPSCs in fibrotic areas in DBTC rats was confirmed by fluorescence and radioactivity 24 h after the final injection. 10 systemic VA-lip-siRNAgp46 treatments resolved pancreatic fibrosis, and suppressed tissue hydroxyproline levels in DBTC- and cerulein-treated rats.

Conclusion These data suggest the therapeutic potential of the present approach for reversing pancreatic fibrosis.

Significance of this study

What is already known on this subject?

- Pancreatic fibrosis is an irreversible lesion that can disrupt pancreatic function; there are no approved treatments.
- The accumulation of fibrotic tissue results from sustained activation of pancreatic stellate cells (PSCs).
- The characteristics of PSCs resemble those of hepatic stellate cells (HSCs).
- We have previously succeeded in resolving liver fibrosis in cirrhotic rat models by targeting HSCs with vitamin A coupled liposomes which carried siRNA against the collagen specific chaperone protein gp46.

What are the new findings?

- Rat activated PSCs take up vitamin A-coupled liposomes (VA-lip-siRNAgp46) in a retinol binding protein-mediated fashion, similarly to activated HSCs.
- Transduction of siRNAgp46 in activated PSCs caused suppression of collagen secretion.
- Activated PSCs specifically took up siRNAgp46 encapsulated in vitamin A liposomes in the pancreas of dibutyltin dichloride (DBTC)-treated rats.
- VA-lip-siRNAgp46 treatments resolved pancreatic fibrosis, and suppressed tissue hydroxyproline levels in DBTC- and cerulein-treated rats.
- This is the first demonstration of successful targeting of antifibrotic drug to both cells and molecule which are responsible for pancreatic fibrosis.

How might it impact on clinical practice in the foreseeable future?

- Results suggest the therapeutic potential of the present approach for reversing pancreatic fibrosis.

INTRODUCTION

Chronic pancreatitis is characterised by inflammation and replacement of parenchymal cells with fibrotic tissue leading to functional alterations, such as debilitating exocrine and occasional endocrine insufficiency. The accumulation of fibrotic tissue results from sustained activation of pancreatic stellate cells (PSCs), which proliferate and secrete collagen in response to stimulation by cytokines, growth factors, and reactive oxygen species from inflammatory cells and damaged pancreatic tissue.[1 2] Various approaches to suppress the

activation of PSCs and to eradicate activated PSCs (aPSCs) have been explored for the prevention and treatment of fibrosis associated with chronic pancreatitis.[3–5] However, so far no clinically applicable agents have been developed, mainly because of the inability for specific drug delivery to aPSCs.

We previously demonstrated complete resolution of liver cirrhosis in rat models using vitamin A-coupled liposomes to specifically deliver siRNA against the collagen-specific chaperone, gp46 (VA-lip-siRNAgp46), to hepatic stellate cells (HSCs) via the circulating receptor for retinol binding protein (RBP).[6] The characteristics of PSCs, including collagen synthesis, storage of vitamin A, expression of gp46, etc, resemble those of HSCs.[7] Therefore, in the present study, we examined whether our previous therapeutic approach for liver cirrhosis could be successfully applied for the treatment of pancreatic fibrosis.

MATERIALS AND METHODS

Preparation of siRNAgp46 and its conjugate with 6′-carboxyfluorescein

A formulation of siRNA directed against gp46, a rat homologue of human HSP47, was purchased from Hokkaido System Science (Sapporo, Japan). The sense and anti-sense strands of siRNAs have been described in detail previously.[6] For fluorescence activated cell sorting (FACS) analyses and in vivo tracing of gp46siRNA, gp46 siRNA with 6′-carboxyfluorescein (6-FAM) -coupled to the 5′ end of the sense strand was used.

A formulation of siRNA directed against HSP47 (GenBank accession no. 50454) was purchased from Hokkaido System Science. The sense and anti-sense strands of siRNAs were: HSP47, 5′-ggacaggccucuac aacuaTT-3′ (sense); 5′-uaguuguagagg ccuguccTT-3′ (antisense).

Preparation of vitamin A-coupled liposomes carrying siRNAgp46

Vitamin A-coupled liposomes carrying siRNAgp46 (VA-lip-siRNAgp46) were prepared as described previously.[6]

Animals

Male Lewis rats (Charles River, Tokyo, Japan), weighing 150–200 g, and male Wistar rats (Charles River), weighing 250–300 g, were used for dibutyltin dichloride (DBTC) and cerulein experiments, respectively. All animal procedures were approved by the Sapporo Medical College Institutional Animal Care and Use Committee.

Isolation and cultivation of rat PSCs

Rat PSCs were isolated by density gradient centrifugation, as detailed previously.[8] All experiments were performed with culture-activated cells (aPSCs, passage 1–3).

Isolation and cultivation of human PSCs

Human pancreases were obtained during surgery for chronic pancreatitis. All these patients were seen at Sapporo Medical University. Informed consent in writing was obtained from each patient. Human PSCs were isolated by outgrowth, using explant techniques from the pancreas as described previously.[1] In this study, experiments were performed on activated α-smooth muscle actin (SMA)-positive cells between the first and third serial passages using lines.

FACS analysis of VA-lip-siRNAgp46-FAM

Rat aPSCs were cultivated with VA-lip-siRNAgp46-FAM (50 nM of siRNA) for 30 min. For the blocking assay, 1×10^4 cells were treated with mouse anti-RBP antibody (10 μg/ml, BD Pharmingen, San Diego, California, USA) for 30 min before adding VA-lip-siRNAgp46-FAM. The mean fluorescence intensity of VA-lip-siRNAgp46-FAM-treated cells was assessed on a FACScalibur with CellQuest software (Becton Dickinson, San Jose, California, USA).

Intracellular distribution analysis of VA-lip-siRNAgp46-FAM

Distribution of VA-lip-siRNAgp46-FAM in aPSCs after transduction was analysed as described previously.[6]

Western blot analysis

Protein extracts of cells and pancreas specimens were resolved over 4/20 sodium dodecylsulphate–polyacrylamide gels, transferred onto nitrocellulose membranes, probed with antibodies against HSP47 (gp46) (Stressgen Biotechnologies, Victoria, BC, Canada) or β-actin (Cell Signaling, Beverly, Massachusetts, USA), and then probed with peroxidase-coupled antibodies as the secondary antibody (Oncogene Research Product, Boston, Massachusetts, USA). Lastly, the cells were visualised with ECL (Amersham Life Science, Arlington Heights, Illinois, USA). Western blots were quantified using ImageJ 1.43u (NIH, Bethesda, Maryland, USA).

Quantification of collagen production

Collagen production by rat aPSCs was measured according to the method described previously for aHSCs, except for cell cultivation period after transduction; 24 h for aHSCs and 72 h for aPSCs.[6 9]

Quantitative RT–PCR

Total RNA was isolated using RNeasy mini kits (Qiagen, Hilden, Germany). Total RNA (1 μg) was used for reverse transcription with SuperScript II (Invitrogen, Carlsbad, California, USA) plus RNaseOUT (Invitrogen) using random primers (Invitrogen) according to the manufacturer's instructions. All TaqMan primers mixed with probes (GAPDH, Rn 99999916_s1; MMP2, Rn 02532334_s1; COL1A1, Rn00801649_g1; TIMP-1, Rn 00587558_m1; Gp46, Rn 00367777_m1; TGFβ, Rn01475963_m1) were purchased from Applied Biosystems (Foster City, California, USA). The TaqMan reactions were performed using 7300 Fast Real Time PCR System (Applied Biosystems). The results were expressed as the ratio of the number of copies of the product gene to the number of copies of the housekeeping gene (GAPDH) from the same RNA (respective cDNA) sample and PCR run.

Induction of pancreatic and hepatic fibrosis by DBTC

Among several models of pancreatic fibrosis,[10–18] we chose the DBTC model in which the common bile duct is obstructed by a plug formed with necrotic biliopancreatic ductal epithelium, because the procedure is relatively simple to perform as compared with other models and irreversible pancreatitis can be induced by a single injection of DBTC. DBTC (Sigma, St Louis, Missouri, USA) was first dissolved in ethanol (1 part) and then mixed with glycerol (2 parts) and dimethyl sulfoxide (2 parts).[10] For administration of DBTC, we selected the right jugular vein route to avoid any possible damage of the tail vein caused by the tail vein route for subsequent application of VA-lip-siRNAgp46.[10] In preliminary experiments, we found the lethality rates of dosages 8.0, 7.0 and 5.0 mg/kg body weight to be 4/4 (100%), 7/16 (44%) and 5/27 (19%), respectively. Thus, we selected the dosage of 5.0 mg/kg for the main experiments (see online supplementary figure S1).

Fibrosis, as revealed by Azan-Mallory staining, was evident in pancreatic and hepatic specimens on days 29 and 43 of DBTC injection (see online supplementary figure S1A–C).

Induction of pancreatic fibrosis by cerulein

Male Wistar rats received two intraperitoneal injections of 50 μg/kg cerulein (Sigma) 1 h apart every week for 6 weeks, as described by Ishibashi (see online supplementary figure S2A,B).[18]

Collagenase activity in pancreas homogenates

Collagenase activity (collagen type I) in pancreas homogenates was measured as described previously.[19]

In vivo localisation of VA-lip-siRNArandom-FAM in rat pancreas

From day 43 of DBTC administration, rats were injected intravenously with 1 μl/g body weight of VA-lip-siRNArandom-FAM or Lip-siRNArandom-FAM (0.75 mg/kg siRNA) three times on alternating days. At 24 h after the last injection, the rats were sacrificed by saline perfusion. Pancreatic tissue was immediately embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) medium and cryogenically sectioned. Multicoloured fluorescent staining of sections and their analysis were carried out as described previously.[6]

Tissue distribution of radiolabelled VA-lip-siRNArandom

^{3}H-VA-lip-siRNArandom (200 μCi), prepared as described previously, was administered via the tail vein under normal pressure in either DBTC-treated rats (day 43) or normal rats. After 24 h, the rats were sacrificed under anaesthesia, and radioactivity of each tissue was assayed as described previously.[6]

Treatment of DBTC rats and cerulein rats with VA-lip-siRNAgp46 and measurement of hydroxyproline content

Three groups of rats (n=10 per group) were used for histological evaluations. From day 43 of DBTC or cerulein administration, Lip-siRNAgp46, VA-lip-siRNAgp46 (0.75 mg/kg siRNA) or phosphate buffered saline (PBS; three times a week every other day) were injected for a total of 10 times via the tail vein under normal pressure in a volume of 1 μl/g body weight.[6] Hydroxyproline content in the pancreas was measured as previously described.[20]

Immunohistochemical staining for α-SMA

Pancreas was fixed with 10% paraformaldehyde. Then, immunohistochemical staining for α-SMA was performed by the dextran polymer method using monoclonal anti α-SMA antibody (1 : 1000, Sigma) and an Envision Kit (Dako), followed by colouring with 3, 3'-diaminobenzidine (DAB) and nuclear staining with Gill's haematoxylin solution. To accurately quantitate areas stained with α-SMA, slides from six randomly selected low-power fields (×100) per pancreas section from each rat were viewed by microscopy (Axioplan 2; Carl Zeiss) and the percentage of areas stained with α-SMA was quantified as previously described.[6]

In vitro apoptosis assay

Rat and human aPSCs under going apoptosis were stained with an in situ Cell Death Detection kit (Roche) according to the manufacturer's protocol. Slides were washed with PBS, and exposed to Prolong Gold Antifade Reagent with 4′, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) to stain nuclei. The number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL)-positive cells (green) in aPSCs were counted in 10 random high-power fields (×800) using fluorescent microscopy (Keyence, BZ-8000) for each sample.

Double staining of pancreas specimen for TUNEL and α-SMA

Double staining for TUNEL and α-SMA was undertaken and the number of TUNEL-positive apoptotic cells within the fibrotic bands was determined using a method modified from that described by Iredale *et al.*[19] Briefly, the specimen was first stained with an in situ Cell Death Detection kit (Roche) according to the manufacturer's protocol, followed by immunostaining for α-SMA (Sigma), with an alkaline phosphatase-conjugated anti-mouse second antibody (KPL, Gaithersburg, Maryland, USA) using a Vector Red alkaline phosphatase substrate kit 1 (Vector Lab, Peterborough, UK). The number of TUNEL-positive cells (brown) in α-SMA-positive areas (red), but not those in parenchymal area, were counted in 10 random high-power fields (×800).

Ethics approval

This research follows the tenets and regulations of the Declaration of Helsinki and has been approved by the Animal Care and the Institutional Review Board at Sapporo Medical University.

Statistics

Results are presented as mean±SD for each sample. Multiple comparisons between control groups and other groups were performed by Dunnet's test.

RESULTS

Specific uptake of RBP bound VA-lip-siRNAgp46 by rat aPSCs

Rat aPSCs, which were stained positive for α-SMA (figure 1A), were incubated with VA-lip-siRNAgp46-FAM or Lip-siRNAgp46-FAM in the presence of 10% fetal calf serum, and were observed under a fluorescence microscope. In VA-lip-siRNAgp46-FAM treated aPSCs, fluorescence appeared as a fine granular pattern in the cytoplasm at 30 min and as denser granular patterns in the perinuclear region at 2 h (figure 1B). In contrast, in Lip-siRNAgp46-FAM treated aPSCs, no green fluorescence was seen at 30 min and perinuclear fluorescence at 2 h was very faint (figure 1B). The fluorescence intensity of VA-lip-siRNAgp46-FAM aPSCs as revealed by FACS was clearly suppressed by RBP antibody to nearly the same level as Lip-siRNAgp46-FAM aPSCs (figure 1C).

Suppression of gp46 expression and collagen secretion of rat aPSCs by VA-lip-siRNAgp46

Treatment of aPSCs with VA-lip-siRNAgp46 brought about dose-dependent suppression of gp46 with almost complete suppression at 50 nM, which lasted at least 72 h (figure 1D,E) while treatment with VA-lip-siRNA random or Lip-siRNAgp46 did not cause any suppression. In the culture plate of VA-lip-siRNAgp46-treated aPSCs, significantly less collagen than that of VA-lip-siRNA random-treated or non-treated aPSCs was found (figure 1F).

Delivery of VA-lip-siRNArandom-FAM to aPSCs in vivo

The specific delivery of VA-lip-siRNArandom-FAM to aPSCs in the fibrotic pancreas was examined by fluorescent emission 24 h after three injections (figure 2A). Specimens were prepared from head (figure 2B,D) and body portions (figure 2C,E) of the pancreas. In both portions, fluorescence of VA-lip-siRNArandom-FAM (green) was identified predominantly in the