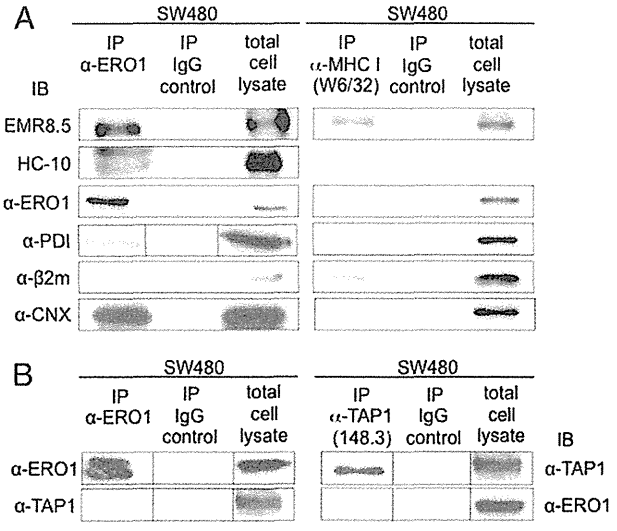

FIGURE 5. ERO1-α forms a complex with PDI, calnexin, and unfolded MHC class I HC but not with TAP. SW480 cells were lysed in 1% digitonin lysis buffer. (**A**) The lysates were immunoprecipitated with anti–ERO1-α mAb, anti-mature MHC class I mAb (W6/32), or control IgG and immunoblotted with indicated Abs. The black lines indicate where parts of the image were joined. (**B**) The lysates were immunoprecipitated with anti–ERO1-α mAb or TAP1 mAb and immunoblotted with indicated Abs. The black lines indicate where parts of the image were joined.

we used BN-PAGE to investigate the subunit composition of the ERO1-α–associated Ag-presenting machinery. BN-PAGE analysis showed that TAP1 formed a complex with MHC class I molecules with a molecular mass of 450 kDa, being consistent with results reported by Rufer et al. (Fig. 6) (13). However, ERO1-α, PDI, and calnexin were not included in the TAP1-associated complexes and, more importantly, these molecules formed a multiprotein complex with a molecular mass of 250 kDa as detected by BN-PAGE. To confirm these findings, we performed two-dimensional SDS-PAGE followed by Western blotting. Apparently, the protein complex with a molecular mass of 250 kDa was constituted with ERO1-α, PDI, calnexin, and MHC class I HC. Moreover, we observed that ERO1-α or MHC class I HC might form a complex with undefined molecules with a molecular mass of ~150–160 kDa. These findings need to be investigated further to identify the associated molecules. It has been shown that ERO1-α exclusively generates disulfide bonds with PDI but not other oxidoreductases, including ERp57 (14, 15), indicating that ERO1-α plays a role through oxidoreductase activity of PDI. Additionally, calnexin has been shown to associate with MHC class I HC for glycosylation at an early stage of MHC class I folding. We show in the present study that ERO1-α generated a complex with PDI, calnexin, and unfolded MHC class I HC, indicating that ERO1-α seemed to act at least in part at an early stage of MHC class I assembly in collaboration with PDI. Based on the results, we concluded that ERO1-α formed a complex with unfolded MHC class I HC, PDI, and calnexin before being incorporated into TAP1-associated PLC. We named this step the "pre-PLC" stage (Supplemental Fig. 3).

Hypoxia augments the expression of ERO1-α and rescues the oxidative folding of MHC class I HC in an ERO1-α–depleted cell

As shown in Fig. 7A, when SW480 cells were cultured under the condition of hypoxia for 24–48 h, expression of ERO1-α was clearly increased. Interestingly, when SW480 cells transfected with shERO1-α were cultured under the condition of hypoxia for 24–48 h, expression of ERO1-α gradually recovered. This indicated that hypoxia augmented *ero1-α* mRNA expression, which was beyond the inhibitory effect of transfected shRNA for ERO1-α, resulting in recovery of ERO1-α protein level. Recently, oxidoreductases such as PRDX IV (16, 17), VKOR (18), and QSOX (19) in the ER other than ERO1-α have been demonstrated. We examined the dynamics of these oxidoreductases under the condition of hypoxia. Notably, among these oxidoreductases, ERO1-α was the only oxidoreductase that was upregulated under the condition of hypoxia. The other oxidoreductases were downregulated or remained at a steady level under the condition of hypoxia, as it is known that protein synthesis is inhibited under such an ER stress condition. PDI was at a steady level for 48 h during hypoxia, but then its protein level increased after 96 h, possibly because hypoxia-augmented ERO1-α reoxidized reduced PDI and it could be stabilized. We examined whether hypoxia-induced ERO1-α also contributes to MHC class I expression on SW480 cells. Ghosh et al. (20) demonstrated that transcription of MHC class I is upregulated in hypoxic glioma cells. Therefore, we first examined whether the gene expression level of MHC class I was affected by hypoxia. Because SW480 cells express HLA-A*2402,

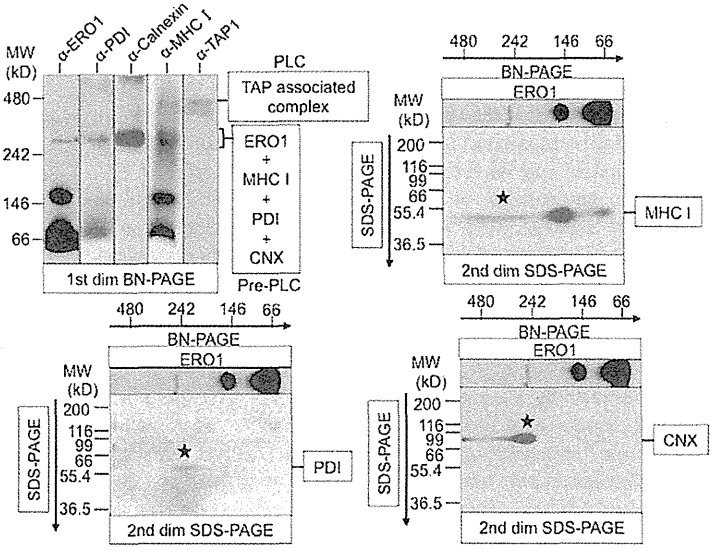

FIGURE 6. Molecular architecture of ERO1-α–associated MHC class I molecules. BN-PAGE and Western blot analysis of MHC class I–related molecules. SW480 cells were lysed in stabilizing BN buffer containing 1% digitonin. Lysates were separated by BN-PAGE and analyzed in Western blots probed for ERO1-α, PDI, calnexin, MHC class I HC, and TAP1 (*upper left*). Second-dimensional SDS-PAGE followed by Western blots probed for MHC class I HC (*upper right*), PDI (*lower left*), and calnexin (*lower right*) in combination with ERO1-α were analyzed.

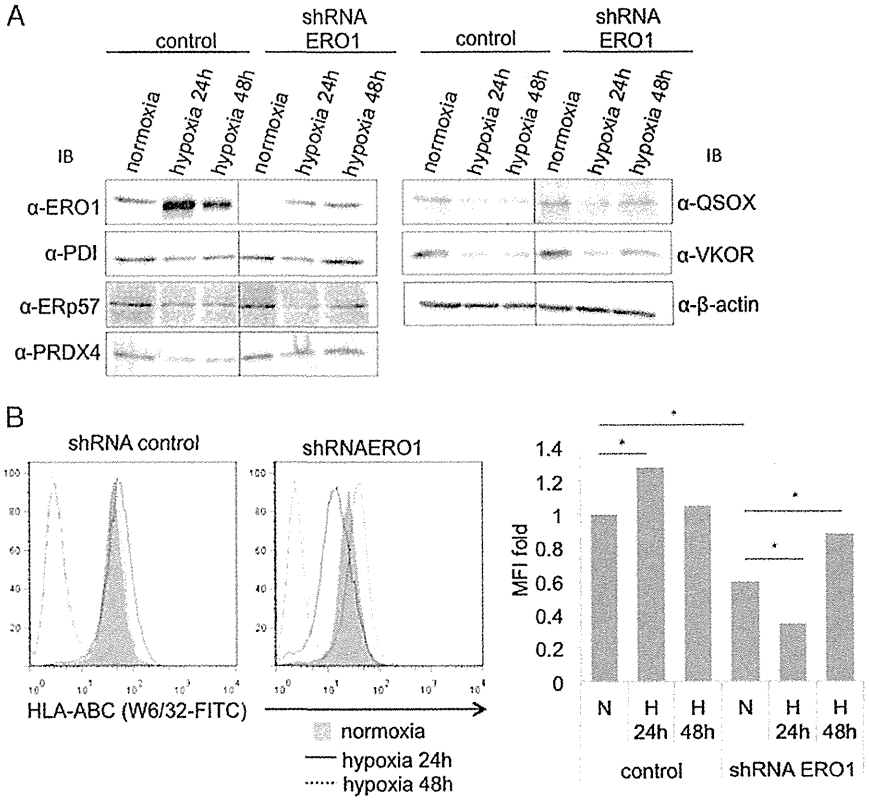

FIGURE 7. Hypoxia augments the expression of ERO1-α and rescues the oxidative folding of MHC class I HC in an ERO1-α–depleted cell. (**A**) SW480 cells were cultured under the condition of hypoxia for 24–48 h. Cells were lysed and separated by SDS-PAGE and analyzed in Western blots probed for ERO1-α, PDI, ERp57, PRDX IV, QSOX, and VKOR. β-actin served as a loading control. The black lines indicate where parts of the image were joined. (**B**) Cell surface expression of MHC class I on SW480 cells with shRNA control or shRNA ERO1-α was compared by flow cytometric analysis (*left*). The fold increases of mean fluorescence intensity (MFI) of each MHC class I are shown (*right*). $*p < 0.05$.

we compared the HLA-A mRNA expression levels of shRNA control cells cultured under normoxia and hypoxia conditions for 24 or 48 h by real-time RT-PCR. The mRNA expression levels of HLA-A were not different under the conditions of normoxia and hypoxia (Supplemental Fig. 2). These results are not compatible with those of Ghosh et al., and the different results are thought to be due to the difference in cell type. In contrast, as shown in Fig. 7B, cell surface expression of MHC class I of SW480 cells with shRNA control was augmented after hypoxia for 24 h. Conversely, SW480 cells with depletion of ERO1-α showed decreased expression of MHC class I on the surface under the condition of 24 h hypoxia. More strikingly, when ERO1-α was increased to a level comparable to that in shRNA control cells by 48 h hypoxia, expression of MHC class I on the surface of cells with ERO1-α depletion also recovered to the level on shRNA control cells after 48 h hypoxia. These results indicated that ERO1-α expressed in cancer cells plays a pivotal role in the generation of disulfide bonds within MHC class I HC at the posttranscriptional level but not at the gene expression level. Thus, cancer-associated ERO1-α plays a crucial role in the proper folding of MHC class I HC, leading to upregulation of MHC class I at the cell surface. In particular, ERO1-α plays a pivotal role in the maintenance of MHC class I expression in such stressful hypoxic circumstances, which is a hallmark of a cancer microenvironment.

Relationship between MHC class I expression and ERO1-α expression in colon cancer tissues

Next, we performed immunohistochemical analysis to determine whether expression of ERO1-α has an impact on the expression of MHC class I molecules of colon cancer cells. Eighty-five patients with colon cancer who underwent surgery between 2005 and 2008 in Sapporo Medical University Hospital were analyzed in this study. The expression of ERO1-α (Fig. 8A) and MHC class I (Fig. 8B) was investigated by immunohistochemistry using an anti–ERO1-α mAb and an anti–pan MHC class I Ab, EMR8.5. We found that the expression level of ERO1-α was significantly correlated with the expression of MHC class I (Spearman's rank correlation coefficient RS = 0.401, $p < 0.01$) (Fig. 8C). In contrast, the ERO1-α–negative group showed significantly reduced expression of MHC class I compared with that in the ERO1-α–positive group (odds ratio = 4.29, $p < 0.01$, 95% CI = 1.61–11.43) (Fig. 8D). Thus, our data indicated that augmented expression of ERO1-α in colon cancer cells is indispensable for the expression of MHC class I molecules via correct oxidative folding.

Discussion

In mammalian cells, proteins that are directed to the plasma membrane or to endomembranes or proteins that will be secreted from cells undergo posttranslational modifications such as disulfide bond formation in the ER. ERO1-α–PDI has been shown to play an important role in the formation of disulfide bond formation. ERO1-α contains two essential cysteine triads, Cys^{85}–Cys^{94}–Cys^{97} and Cys^{391}–Cys^{394}–Cys^{397} (21).The last two cysteines in the N-terminal triad are involved in a direct interaction with PDI, whereas those in the C-terminal triad form an active-site disulfide near the FAD isoalloxazine ring. Thus, the Cys^{94}–Cys^{99} pair functions as a shuttle disulfide, transferring reducing equivalents from PDI to the FAD-containing reaction center in ERO1-α. Moreover, human ERO1-α was observed to oxidize the C-terminal a′ domain of PDI more effectively than the a domain (22, 23). Recently, Inaba et al. (15) demonstrated that specific interaction between the PDI b′ domain and ERO1-α has a role in activation of the oxidase (15). Moreover, they suggested that because ERO1-α and substrates for PDI seem to compete for the PDI-binding site in the b′ domain, oxidative protein folding occurs in a stepwise manner (21). Therefore, based on our data, 1) reduced PDI binds

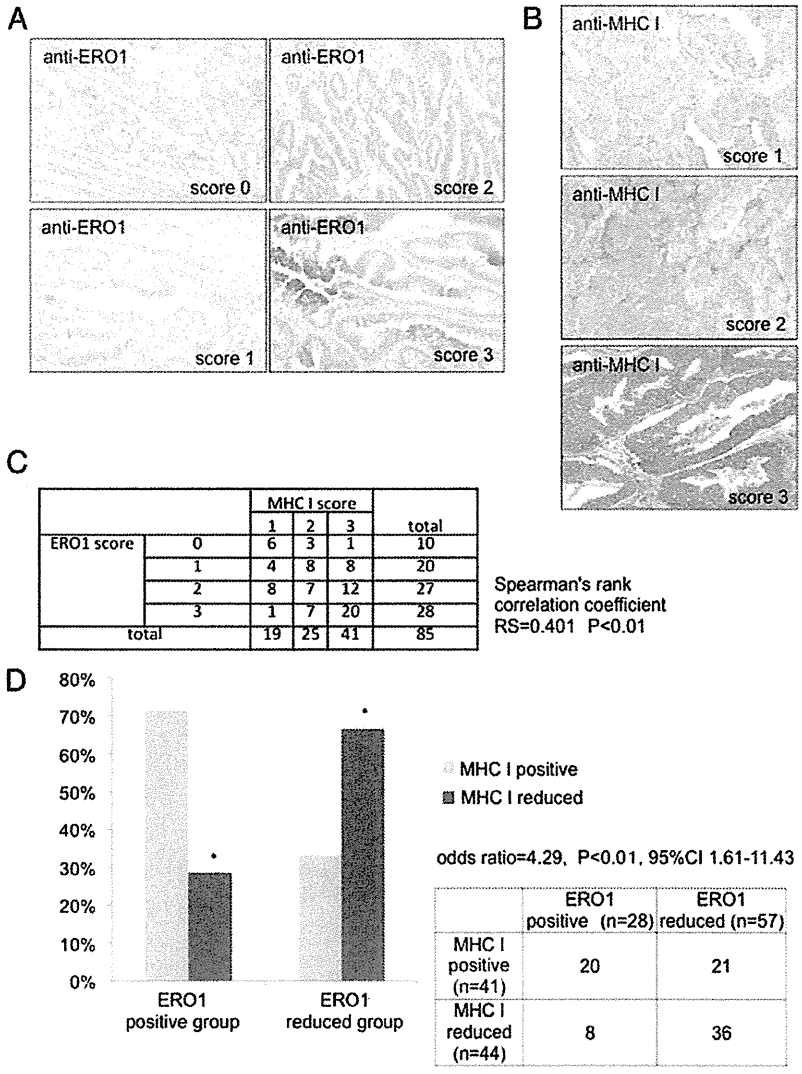

FIGURE 8. Relationship between MHC class I expression and ERO1 expression in colon cancer tissues. Eighty-five patients with colon cancer who underwent surgery were analyzed by immunohistochemistry. Tissue sections were developed using diaminobenzidine. (**A**) Staining pattern of ERO1-α was scored as 0–3 (original magnification ×100). (**B**) Staining pattern of MHC class I was scored as 1–3 (original magnification ×100). (**C**) Expression level of ERO1-α was significantly correlated with expression of MHC class I by a Spearman rank test. (**D**) The ERO1-α–reduced group showed significantly reduced expression of MHC class I compared with that in the ERO1-α–positive group. $^*p < 0.05$.

ERO1-α (at the b′ domain of PDI and Cys^{94} of ERO1-α) to be oxidized, 2) oxidized PDI dissociates from ERO1-α to bind substrate proteins, and 3) finally PDI introduces disulfide bonds into substrate proteins including MHC class I HC (Supplemental Fig. 3). According to the result of immunoprecipitation experiments and BN-PAGE, it seems that ERO1-α associates with HCs through calnexin but not through mixed disulfides. Nössner et al. (24) demonstrated that calnexin binds to free HCs, whereas β2m-assembled MHC class I HCs dissociated from calnexin in human cells. It has been demonstrated that ERO1-α formed disulfide intermediates with PDI but not other oxidoreductases, including ERp57 (14, 15). Therefore, our observations suggested that ERO1-α–PDI-mediated oxidation of MHC class I HC may occur at the calnexin-associated stage and before assembly with β2m. Park et al. (4) demonstrated that PDI forms a disulfide intermediate with calnexin-chaperoned MHC class I HC at the substrate-binding b′ domain. Additionally, we showed that ERO1-α was co-immunoprecipitaed with calnexin, indicating that ERO1-α binds to calnexin via a noncovalent bond. Thus, our results indicate the molecular basis of regulated and specific oxidation of PDI by ERO1-α, and this PDI–ERO1-α pathway plays a pivotal role in the oxidative folding of MHC class I HC at the pre-PLC stage. Because we showed that disulfide bond formation between ERO1-α and PDI was increased in ERO1-α–overexpressing cells, the redox status of PDI seemed to shift to the oxidized PDI, which could oxidize other target molecules. Additionally, we showed in Fig. 2A and 2B that overexpression of ERO1-α resulted in an increased ratio of oxidized/reduced forms of MHC class I HC, indicating a shift to the oxidized form of MHC class I HC. These results suggested that augmented expression of ERO1-α increased activity for oxidative folding via oxidization of PDI. Moreover, our results showed that the expression level of ERO1-α was significantly correlated with the expression of MHC class I. Because many studies have demonstrated that low MHC class I expression is associated with poor prognosis (25–27), we are currently investigating whether low expression of ERO1-α correlates with poor prognosis. In conclusion, the cancer-associated ERO1-α plays a pivotal role in the host's immune response via proper Ag presentation by MHC class I molecules within a hypoxic microenvironment.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References

1. Diedrich, G., N. Bangia, M. Pan, and P. Cresswell. 2001. A role for calnexin in the assembly of the MHC class I loading complex in the endoplasmic reticulum. *J. Immunol.* 166: 1703–1709.
2. Wearsch, P. A., and P. Cresswell. 2008. The quality control of MHC class I peptide loading. *Curr. Opin. Cell Biol.* 20: 624–631.
3. Kienast, A., M. Preuss, M. Winkler, and T. P. Dick. 2007. Redox regulation of peptide receptivity of major histocompatibility complex class I molecules by ERp57 and tapasin. *Nat. Immunol.* 8: 864–872.
4. Park, B., S. Lee, E. Kim, K. Cho, S. R. Riddell, S. Cho, and K. Ahn. 2006. Redox regulation facilitates optimal peptide selection by MHC class I during antigen processing. *Cell* 127: 369–382.
5. Kang, K., B. Park, C. Oh, K. Cho, and K. Ahn. 2009. A role for protein disulfide isomerase in the early folding and assembly of MHC class I molecules. *Antioxid. Redox Signal.* 11: 2553–2561.
6. Sevier, C. S., H. Qu, N. Heldman, E. Gross, D. Fass, and C. A. Kaiser. 2007. Modulation of cellular disulfide-bond formation and the ER redox environment by feedback regulation of Ero1. *Cell* 129: 333–344.
7. Benham, A. M., A. Cabibbo, A. Fassio, N. Bulleid, R. Sitia, and I. Braakman. 2000. The CXXCXXC motif determines the folding, structure and stability of human Ero1-Lα. *EMBO J.* 19: 4493–4502.
8. Cabibbo, A., M. Pagani, M. Fabbri, M. Rocchi, M. R. Farmery, N. J. Bulleid, and R. Sitia. 2000. ERO1-L, a human protein that favors disulfide bond formation in the endoplasmic reticulum. *J. Biol. Chem.* 275: 4827–4833.
9. Frand, A. R., and C. A. Kaiser. 1999. Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. *Mol. Cell* 4: 469–477.
10. Kutomi, G., Y. Tamura, T. Tanaka, T. Kajiwara, K. Kukita, T. Ohmura, H. Shima, T. Takamaru, F. Satomi, Y. Suzuki, et al. 2013. Human endoplasmic reticulum oxidoreductin 1-α is a novel predictor for poor prognosis of breast cancer. *Cancer Sci.* 104: 1091–1096.
11. Idenoue, S., Y. Hirohashi, T. Torigoe, Y. Sato, Y. Tamura, H. Hariu, M. Yamamoto, T. Kurotaki, T. Tsuruma, H. Asanuma, et al. 2005. A potent immunogenic general cancer vaccine that targets survivin, an inhibitor of apoptosis proteins. *Clin. Cancer Res.* 11: 1474–1482.
12. Torigoe, T., H. Asanuma, E. Nakazawa, Y. Tamura, Y. Hirohashi, E. Yamamoto, T. Kanaseki, T. Hasegawa, and N. Sato. 2012. Establishment of a monoclonal anti-pan HLA class I antibody suitable for immunostaining of formalin-fixed tissue: unusually high frequency of down-regulation in breast cancer tissues. *Pathol. Int.* 62: 303–308.
13. Rufer, E., R. M. Leonhardt, and M. R. Knittler. 2007. Molecular architecture of the TAP-associated MHC class I peptide-loading complex. *J. Immunol.* 179: 5717–5727.
14. Mezghrani, A., A. Fassio, A. Benham, T. Simmen, I. Braakman, and R. Sitia. 2001. Manipulation of oxidative protein folding and PDI redox state in mammalian cells. *EMBO J.* 20: 6288–6296.
15. Inaba, K., S. Masui, H. Iida, S. Vavassori, R. Sitia, and M. Suzuki. 2010. Crystal structures of human Ero1α reveal the mechanisms of regulated and targeted oxidation of PDI. *EMBO J.* 29: 3330–3343.
16. Zito, E., E. P. Melo, Y. Yang, Å. Wahlander, T. A. Neubert, and D. Ron. 2010. Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. *Mol. Cell* 40: 787–797.
17. Tavender, T. J., J. J. Springate, and N. J. Bulleid. 2010. Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. *EMBO J.* 29: 4185–4197.
18. Li, W., S. Schulman, R. J. Dutton, D. Boyd, J. Beckwith, and T. A. Rapoport. 2010. Structure of a bacterial homologue of vitamin K epoxide reductase. *Nature* 463: 507–512.
19. Kodali, V. K., and C. Thorpe. 2010. Oxidative protein folding and the quiescin-sulfhydryl oxidase family of flavoproteins. *Antioxid. Redox Signal.* 13: 1217–1230.
20. Ghosh, S., A. Paul, and E. Sen. 2013. Tumor necrosis factor α-induced hypoxia-inducible factor 1α-β-catenin axis regulates major histocompatibility complex class I gene activation through chromatin remodeling. *Mol. Cell. Biol.* 33: 2718–2731.
21. Masui, S., S. Vavassori, C. Fagioli, R. Sitia, and K. Inaba. 2011. Molecular bases of cyclic and specific disulfide interchange between human Ero1α protein and protein-disulfide isomerase (PDI). *J. Biol. Chem.* 286: 16261–16271.
22. Baker, K. M., S. Chakravarthi, K. P. Langton, A. M. Sheppard, H. Lu, and N. J. Bulleid. 2008. Low reduction potential of Ero1α regulatory disulphides ensures tight control of substrate oxidation. *EMBO J.* 27: 2988–2997.
23. Wang, L., S. J. Li, A. Sidhu, L. Zhu, Y. Liang, R. B. Freedman, and C. C. Wang. 2009. Reconstitution of human Ero1-Lα/protein-disulfide isomerase oxidative folding pathway in vitro. Position-dependent differences in role between the a and a′ domains of protein-disulfide isomerase. *J. Biol. Chem.* 284: 199–206.
24. Nössner, E., and P. Parham. 1995. Species-specific differences in chaperone interaction of human and mouse major histocompatibility complex class I molecules. *J. Exp. Med.* 181: 327–337.
25. Tsukahara, T., S. Kawaguchi, T. Torigoe, H. Asanuma, E. Nakazawa, K. Shimozawa, Y. Nabeta, S. Kimura, M. Kaya, S. Nagoya, et al. 2006. Prognostic significance of HLA class I expression in osteosarcoma defined by anti-pan HLA class I monoclonal antibody, EMR8-5. *Cancer Sci.* 97: 1374–1380.
26. Turcotte, S., S. C. Katz, J. Shia, W. R. Jarnagin, T. P. Kingham, P. J. Allen, Y. Fong, M. I. D'Angelica, and R. P. DeMatteo. 2014. Tumor MHC class I expression improves the prognostic value of T-cell density in resected colorectal liver metastases. *Cancer Immunol. Res.* 2: 530–537.
27. Reimers, M. S., C. C. Engels, H. Putter, H. Morreau, G. J. Liefers, C. J. van de Velde, and P. J. Kuppen. 2014. Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: a retrospective cohort study. *BMC Cancer* 14: 486.

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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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References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013;63:11–30.
2. Salman B, Zhou D, Jaffee EM, Edil BH, Zheng L. Vaccine therapy for pancreatic cancer. Oncoimmunology. 2013;2:e26662.
3. Soares KC, Zheng L, Edil B, Jaffee EM. Vaccines for pancreatic cancer. Cancer J. 2012;18:642–52.
4. Hamada T, Nakai Y, Yasunaga H, Isayama H, Matsui H, Takahara N, et al. Prognostic nomogram for nonresectable pancreatic cancer treated with gemcitabine-based chemotherapy. Br J Cancer. 2014;110:1943–9.
5. Saif MW. Controversies in the adjuvant treatment of pancreatic adenocarcinoma. JOP. 2007;8:545–52.
6. Kanda M, Fujii T, Takami H, Suenaga M, Inokawa Y, Yamada S, et al. The combination of the serum carbohydrate antigen 19-9 and carcinoembryonic antigen is a simple and accurate predictor of mortality in pancreatic cancer patients. Surg Today. 2014;44:1692–701.
7. Niccolai E, Prisco D, D'Elios MM, Amedei A. What is recent in pancreatic cancer immunotherapy? Biomed Res Int. 2013;2013:492372.
8. Kitami CE, Kurosaki I, Kawachi Y, Nihei K, Tsuchiya Y, Nomura T, et al. Portal vein infusion chemotherapy with gemcitabine after surgery for pancreatic cancer. Surg Today. 2013;43:33–9.
9. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol. 1997;15:2403–13.
10. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol. 2007;25:1960–6.
11. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013;369:1691–703.
12. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med. 2011;364:1817–25.
13. Shirasaka T. Development history and concept of an oral anticancer agent S-1 (TS-1): its clinical usefulness and future vistas. Jpn J Clin Oncol. 2009;39:2–15.
14. Ueno H, Ioka T, Ikeda M, Ohkawa S, Yanagimoto H, Boku N, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: gEST study. J Clin Oncol. 2013;31:1640–8.
15. Moss DJ, Misko IS, Burrows SR, Burman K, McCarthy R, Sculley TB. Cytotoxic T-cell clones discriminate between A- and B-type Epstein-Barr virus transformants. Nature. 1988;331:719–21.
16. Kimura Y, Tsukada J, Tomoda T, Takahashi H, Imai K, Shimamura K, et al. Clinical and immunologic evaluation of dendritic cell-based immunotherapy in combination with gemcitabine and/or S-1 in patients with advanced pancreatic carcinoma. Pancreas. 2012;41:195–205.
17. Seliger B. Novel insights into the molecular mechanisms of HLA class I abnormalities. Cancer Immunol Immunother. 2012;61:249–54.
18. Plate JM, Harris JE. Immunobiotherapy directed against mutated and aberrantly expressed gene products in pancreas cancer. J Cell Biochem. 2005;94:1069–77.
19. Goydos JS, Elder E, Whiteside TL, Finn OJ, Lotze MT. A phase I trial of a synthetic mucin peptide vaccine. Induction of specific

immune reactivity in patients with adenocarcinoma. J Surg Res. 1996;63:298–304.
20. Karanikas V, Hwang LA, Pearson J, Ong CS, Apostolopoulos V, Vaughan H, et al. Antibody and T cell responses of patients with adenocarcinoma immunized with mannan-MUC1 fusion protein. J Clin Invest. 1997;100:2783–92.
21. Gilewski T, Adluri S, Ragupathi G, Zhang S, Yao TJ, Panageas K, et al. Vaccination of high-risk breast cancer patients with mucin-1 (MUC1) keyhole limpet hemocyanin conjugate plus QS-21. Clin Cancer Res. 2000;6:1693–701.
22. Ramanathan RK, Lee KM, McKolanis J, Hitbold E, Schraut W, Moser AJ, et al. Phase I study of a MUC1 vaccine composed of different doses of MUC1 peptide with SB-AS2 adjuvant in resected and locally advanced pancreatic cancer. Cancer Immunol Immunother. 2005;54:254–64.
23. Yamamoto K, Ueno T, Kawaoka T, Hazama S, Fukui M, Suehiro Y, et al. MUC1 peptide vaccination in patients with advanced pancreas or biliary tract cancer. Anticancer Res. 2005;25:3575–9.
24. Rong Y, Qin X, Jin D, Lou W, Wu L, Wang D, et al. A phase I pilot trial of MUC1-peptide-pulsed dendritic cells in the treatment of advanced pancreatic cancer. Clin Exp Med. 2012;12:173–80.
25. Gjertsen MK, Buanes T, Rosseland AR, Bakka A, Gladhaug I, Soreide O, et al. Intradermal ras peptide vaccination with granulocyte-macrophage colony-stimulating factor as adjuvant: clinical and immunological responses in patients with pancreatic adenocarcinoma. Int J Cancer. 2001;92:441–50.
26. Abou-Alfa GK, Chapman PB, Feilchenfeldt J, Brennan MF, Capanu M, Gansukh B, et al. Targeting mutated K-ras in pancreatic adenocarcinoma using an adjuvant vaccine. Am J Clin Oncol. 2011;34:321–5.
27. Vasef MA, Ross JS, Cohen MB. Telomerase activity in human solid tumors. Diagnostic utility and clinical applications. Am J Clin Pathol. 1999;112:S68–75.
28. Schnabl B, Choi YH, Olsen JC, Hagedorn CH, Brenner DA. Immortal activated human hepatic stellate cells generated by ectopic telomerase expression. Lab Invest. 2002;82:323–33.
29. Bernhardt SL, Gjertsen MK, Trachsel S, Moller M, Eriksen JA, Meo M, et al. Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: a dose escalating phase I/II study. Br J Cancer. 2006;95:1474–82.
30. Miyazawa M, Ohsawa R, Tsunoda T, Hirono S, Kawai M, Tani M, et al. Phase I clinical trial using peptide vaccine for human vascular endothelial growth factor receptor 2 in combination with gemcitabine for patients with advanced pancreatic cancer. Cancer Sci. 2010;101:433–9.
31. Smith JP, Fantaskey AP, Liu G, Zagon IS. Identification of gastrin as a growth peptide in human pancreatic cancer. Am J Physiol. 1995;268:R135–41.
32. Gilliam AD, Watson SA. G17DT: an antigastrin immunogen for the treatment of gastrointestinal malignancy. Expert Opin Biol Ther. 2007;7:397–404.
33. Gilliam AD, Broome P, Topuzov EG, Garin AM, Pulay I, Humphreys J, et al. An international multicenter randomized controlled trial of G17DT in patients with pancreatic cancer. Pancreas. 2012;41:374–9.
34. Srivastava PK. Immunotherapy of human cancer: lessons from mice. Nat Immunol. 2000;1:363–6.
35. Maki RG, Livingston PO, Lewis JJ, Janetzki S, Klimstra D, Desantis D, et al. A phase I pilot study of autologous heat shock protein vaccine HSPPC-96 in patients with resected pancreatic adenocarcinoma. Dig Dis Sci. 2007;52:1964–72.
36. Laheru D, Jaffee EM. Immunotherapy for pancreatic cancer - science driving clinical progress. Nat Rev Cancer. 2005;5:459–67.
37. Hodge JW, Sabzevari H, Yafal AG, Gritz L, Lorenz MG, Schlom J. A triad of costimulatory molecules synergize to amplify T-cell activation. Cancer Res. 1999;59:5800–7.
38. Kaufman HL, Kim-Schulze S, Manson K, DeRaffele G, Mitcham J, Seo KS, et al. Poxvirus-based vaccine therapy for patients with advanced pancreatic cancer. J Transl Med. 2007;5:60.
39. Brockstedt DG, Giedlin MA, Leong ML, Bahjat KS, Gao Y, Luckett W, et al. Listeria-based cancer vaccines that segregate immunogenicity from toxicity. Proc Natl Acad Sci. 2004;101:13832–7.
40. Hassan R, Laszik ZG, Lerner M, Raffeld M, Postier R, Brackett D. Mesothelin is overexpressed in pancreaticobiliary adenocarcinomas but not in normal pancreas and chronic pancreatitis. Am J Clin Pathol. 2005;124:838–45.
41. Le DT, Brockstedt DG, Nir-Paz R, Hampl J, Mathur S, Nemunaitis J, et al. A live-attenuated Listeria vaccine (ANZ-100) and a live-attenuated Listeria vaccine expressing mesothelin (CRS-207) for advanced cancers: phase I studies of safety and immune induction. Clin Cancer Res. 2012;18:858–68.
42. Kobayashi M, Shimodaira S, Nagai K, Ogasawara M, Takahashi H, Abe H, et al. Prognostic factors related to add-on dendritic cell vaccines on patients with inoperable pancreatic cancer receiving chemotherapy: a multicenter analysis. Cancer Immunol Immunother. 2014;63:797–806.
43. Nishida S, Koido S, Takeda Y, Homma S, Komita H, Takahara A, et al. Wilms tumor gene (WT1) peptide-based vaccine combined with gemcitabine for patients with advanced pancreatic cancer. J Immunother. 2014;37:105–14.
44. Taniuchi K, Nakagawa H, Nakamura T, Eguchi H, Ohigashi H, Ishikawa O, et al. Down-regulation of RAB6KIFL/KIF20A, a kinesin involved with membrane trafficking of discs large homologue 5, can attenuate growth of pancreatic cancer cell. Cancer Res. 2005;65:105–12.
45. Asahara S, Takeda K, Yamao K, Maguchi H, Yamaue H. Phase I/II clinical trial using HLA-A24-restricted peptide vaccine derived from KIF20A for patients with advanced pancreatic cancer. J Transl Med. 2013;11:291.
46. Suzuki N, Hazama S, Ueno T, Matsui H, Shindo Y, Iida M, et al. A phase I clinical trial of vaccination with KIF20A-derived peptide in combination with gemcitabine for patients with advanced pancreatic cancer. J Immunother. 2014;37:36–42.
47. Albers GH, Fleuren G, Escribano MJ, Nap M. Immunohistochemistry of CEA in the human pancreas during development, in the adult, chronic pancreatitis, and pancreatic adenocarcinoma. Am J Clin Pathol. 1988;90:17–22.
48. Geynisman DM, Zha Y, Kunnavakkam R, Aklilu M, Catenacci DV, Polite BN, et al. A randomized pilot phase I study of modified carcinoembryonic antigen (CEA) peptide (CAP1-6D)/montanide/GM-CSF-vaccine in patients with pancreatic adenocarcinoma. J Immunother Cancer. 2013;1:8.
49. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res. 1998;58:5315–20.
50. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med. 1997;3:917–21.
51. Hirohashi Y, Torigoe T, Maeda A, Nabeta Y, Kamiguchi K, Sato T, et al. An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. Clin Cancer Res. 2002;8:1731–9.
52. Kameshima H, Tsuruma T, Kutomi G, Shima H, Iwayama Y, Kimura Y, et al. Immunotherapeutic benefit of alpha-interferon (IFNalpha) in survivin2B-derived peptide vaccination for advanced pancreatic cancer patients. Cancer Sci. 2013;104:124–9.
53. Yutani S, Komatsu N, Yoshitomi M, Matsueda S, Yonemoto K, Mine T, et al. A phase II study of a personalized peptide vaccination for chemotherapy-resistant advanced pancreatic cancer patients. Oncol Rep. 2013;30:1094–100.

54. Yanagimoto H, Shiomi H, Satoi S, Mine T, Toyokawa H, Yamamoto T, et al. A phase II study of personalized peptide vaccination combined with gemcitabine for non-resectable pancreatic cancer patients. Oncol Rep. 2010;24:795–801.
55. Le TD, Wang-Gillam A, Picozzi JJV, Greten FT, Crocenzi ST, Springett MG, et al. A phase 2, randomized trial of GVAX pancreas and CRS-207 immunotherapy versus GVAX alone in patients with metastatic pancreatic adenocarcinoma: updated results. J Clin Oncol. 2014;32(suppl 3):177.
56. Brennan GT, Relias V, Saif MW. Novel agents for the treatment of pancreatic cancer. JOP. 2014;15:110–3.
57. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. Cancer. 1981;47:207–14.
58. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst. 2000;92:205–16.
59. Choi JH, Ahn MJ, Rhim HC, Kim JW, Lee GH, Lee YY, et al. Comparison of WHO and RECIST criteria for response in metastatic colorectal carcinoma. Cancer Res Treat. 2005;37:290–3.
60. Hoos A, Parmiani G, Hege K, Sznol M, Loibner H, Eggermont A, et al. A clinical development paradigm for cancer vaccines and related biologics. J Immunother. 2007;30:1–15.
61. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. Clin Cancer Res. 2009;15:7412–20.
62. Myc LA, Gamian A, Myc A. Cancer vaccines. Any future? Arch Immunol Ther Exp (Warsz). 2011;59:249–59.

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Expression of Hepatocyte Growth Factor in Prostate Cancer May Indicate a Biochemical Recurrence After Radical Prostatectomy

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Abstract. *We previously found that prostate cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) express hepatocyte growth factor (HGF) and that the HGF/c-MET proto-oncogene product (c-MET) signal has a role in the maintenance of prostate CSCs/CICs in an autocrine fashion. HGF is, thus, a novel marker for prostate CSCs/CICs. We hypothesized that high expression of HGF might be related to early recurrence of prostate cancer after radical prostatectomy, and the purpose of the present study was to evaluate the relationship between expression of HGF in prostate tissues and biochemical recurrence after radical prostatectomy. One hundred-one patients with prostate cancer who underwent open or laparoscopic radical prostatectomy from November 2008 to October 2011 with an adequate prostate-specific antigen (PSA) follow-up period, were investigated. Immunohistochemical staining of HGF was compared to biochemical recurrence after radical prostatectomy. Patients with tumors exhibiting HGF positivity of 5% or more had a significantly shorter biochemical recurrence-free period than that of patients whose tumor HGF positivity was less than 5% (p=0.001). In multivariate Cox regression, preoperative PSA and HGF positivity were independent predictors of biochemical recurrence following prostatectomy. Our finding suggests a direct link between expression of HGF, a novel prostate marker of CSCs/CICs, and biochemical recurrence after radical prostatectomy in patients with prostate cancer.*

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Prostate cancer is one of the common and lethal types of cancer in males. The behavior of some cancer types can vary regardless of the Gleason score and other clinicopathological factors. Androgen deprivation is the standard therapy for advanced prostate cancer; however, most patients with an aggressive form of prostate cancer, named castration-resistant prostate cancer (CRPC), experience relapse within a few years after initial treatment, and there is no known effective treatment for CRPC. Recent efforts have thus focused on developing biomarkers that provide clinicians with better ability to identify clinically significant cancer and aid in treatment decisions (1).

Signaling of hepatocyte growth factor (HGF) and its receptor c-MET proto-oncogene product(c-MET) is activated in cancer cells and is related to cancer cell growth, cell motility and matrix invasion (2, 3). HGF/c-MET signaling can therefore reasonably be a target for cancer therapy (4). Furthermore, HGF and its receptor c-MET may play important roles in progression of castration-resistant prostate cancer (CRPC). c-MET expression has been shown to be associated with the emergence of a castration-resistant tumor (5). However, HGF and c-MET have not been associated with biochemical recurrence after radical prostatectomy, that is, after treatment of clinically localized prostate cancer.

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) have high tumor-initiating ability and are resistant to chemotherapy and radiotherapy, and they are, therefore, thought to be responsible for cancer recurrence after treatment and for distant metastasis (6, 7). Prostate cancer contains a small population of CSCs/CICs, and we have previously reported successful isolation of prostate

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CSCs/CICs from the human prostate carcinoma cell line 22Rv1 by using aldehyde dehydrogenase (ALDH) activity (8). In addition, gene expression analysis revealed that growth factors, including HGF, were overexpressed in prostate CSCs/CICs, whereas the receptor of HGF, c-MET, was expressed in both CSCs/CICs and non-CSCs/CICs at similar levels. Further analysis revealed that HGF secreted from prostate CSCs/CICs had a role in maintenance of prostate CSCs/CICs in an autocrine fashion (9).

In the present study, we hypothesize that high expression of HGF is related to early recurrence of prostate cancer after radical prostatectomy and investigated the relationship between frequency of prostate CSCs/CICs, which were identified by immunohistochemical staining using an antibody against HGF, and biochemical recurrence after radical prostatectomy.

Patients and Methods

Patients. We reviewed clinical pathology archives of 106 consecutive patients who underwent open or laparoscopic radical prostatectomy and were diagnosed as having localized prostate cancer at Sapporo Medical University Hospital during the period from November 2008 to October 2011. One hundred and one of 106 patients with prostate cancer were followed-up for a minimum of two years. Fifty-one patients were surgically treated with a laparoscopic approach and 50 patients with open approach. Informed consent was obtained from the patients to use surgical specimens remaining after pathological diagnosis for the investigational study, which was approved by the Institutional Review Board for Clinical Research at our university (approval no. 25-36). All hematoxylin- and eosin-stained slides were reviewed, and all of these specimens revealed prostate adenocarcinoma. The median age at operation was 67 years (range=50-78 years). The median follow-up period of patients with no biochemical recurrence was 40 months (range=28-60 months). All hematoxylin- and eosin-stained slides were reviewed, and clinical stage was assigned using the seventh edition of the American Joint Committee on Cancer TNM Staging System for Prostate Cancer (7th edition, 2009) (10).

The patients' characteristics are shown in Table I. Lymph node dissection was performed in all patients. No cases underwent neoadjuvant or adjuvant hormonal therapy.

Immunohistochemistry and scoring. Immunohistochemical staining using formalin-fixed paraffin-embedded sections of surgically-resected prostate carcinoma was performed as described previously (11). Goat monoclonal antibody against HGF (R and D Systems, Minneapolis, MN, USA) was used at 1,000-times dilution. The slides were then counterstained with hematoxylin, rinsed, dehydrated through graded alcohols into a nonaqueous solution, and cover-slipped with mounting media. All specimens were reviewed independently using light microscopy in at least five areas at ×400 magnification by investigators who were blinded to clinicopathological data (TT and YH). Tumors presenting at least one HGF-positive cancer cell per high power field were considered to be HGF-positive. The positivity rate and intensity grade were recorded.

Table I. *Characteristics of 101 patients of the study.*

Characteristics	
Median age in years (range)	67 (50-78)
Median body mass index (kg/m^2) (range)	23.5 (18.7-32.7)
Median preoperative PSA ng/ml (range)	6.4 (2.4-46.7)
Surgical approach N (%)	
Open	50 (49.5)
Laparoscopy	51 (50.5)
Pathological stage N (%)	
2a	21 (20.7)
2b	28 (27.7)
2c	38 (37.6)
3a	7 (6.9)
3b	7 (6.9)
Positive surgical margins N (%)	43 (42.5)
Positive nodes N (%)	3 (2.9)
Gleason sum N (%)	
6	15 (14.8)
7	60 (59.4)
8	7 (6.9)
9	19 (18.8)

Statistical analysis. We investigated the relationships between HGF positivity and other clinicopathological parameters, *i.e.* preoperative PSA, pathological T stage, and Gleason grade, by Fisher's exact tests. Biochemical recurrence-free survival was assessed by the Kaplan–Meier method, and differences between two groups were compared using the log-rank test. Biochemical recurrence after radical prostatectomy was defined as an initial PSA value of 0.2 ng/ml or less followed by subsequent confirmatory PSA value of 0.2 ng/ml or more.

To define HGF overexpression, time-dependent receiver operating characteristic (ROC) curve analysis for biochemical recurrence within 2 years or 5 years after radical prostatectomy was performed using the positivity rate and intensity grade of HGF expression to identify the optimum cut-off point that maximized the sum of sensitivity and specificity (12). Cut-off points of HGF positivity and intensity were defined as the point on the ROC curve closest to the upper left corner. Cox regression analyses were used to assess whether HGF or other conventional factors were associated with biochemical recurrence following prostatectomy. To assess a statistical independence of HGF from the other factors, we perform multivariate Cox regression analysis. However, because the incidence of the event (biochemical recurrence) was very low, we selected a few important variables using the backward elimination methods. In this step, after entering all of the variables assessed by the univariate analysis, the variables were removed one by one if Wald $p>0.1$. SPSS Version 20.0 (IBM, Armonk, NY, USA) was used to perform statistical analyses. All tests were two-tailed and a p-value of less than 0.05 was considered statistically significant.

Results

Expression of HGF in human prostate cancer tissues. HGF-positive cells were detected in 45 out of the 101 cases (Figure 1). In 45 positive cases, the median cell positivity rate was 5% (range=1-50%). Time-dependent ROC curve

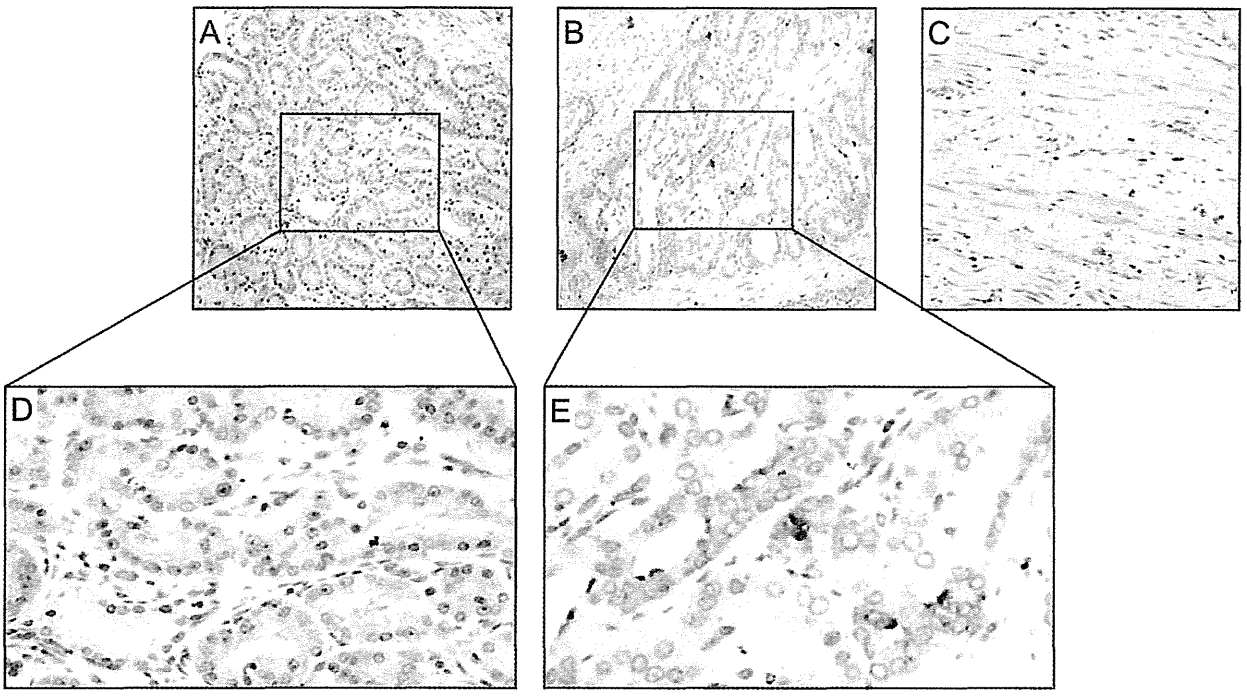

Figure 1. *Representative immunohistochemical staining of hepatocyte growth factor (HGF): negative HGF expression in tumor cells shown in (A) and (D), positive HGF expression in tumor cells shown in (B) and (E), and positive HGF expression in fibroblasts shown in (C). Original magnification ×100.*

analysis for biochemical recurrence within 2 years demonstrated excellent discrimination of HGF-positivity rate (AUC=0.756), with sensitivity and specificity of 72.7% and 81.1%, respectively. The AUC for biochemical recurrence within 2 years was higher than that for within 5 years (data not shown). Using the optimum cut-off of 5% to define overexpression, there was a significantly larger number of patients with biochemical recurrence in the group with HGF staining of 5% or more than in the group with HGF staining of less than 5% (Table II). Hereafter, cases with an expression rate of 5% or more are denoted as HGFP≥5% and those with an expression rate of less than 5% are denoted as HGFP<5%.

Associations between expression rates of HGF and clinicopathological variables. Kaplan–Meier plots and log-rank tests showed that patients with prostate cancer with HGFP ≥5% had a significantly shorter biochemical recurrence-free period than did patients with HGFP<5% (Figure 2) ($p<0.001$). Univariate analysis demonstrated that HGF positivity predicted biochemical recurrence ($p<0.05$). Furthermore, multivariate Cox regression with/without the backward elimination method suggested that preoperative PSA and HGF positivity were independent predictors of

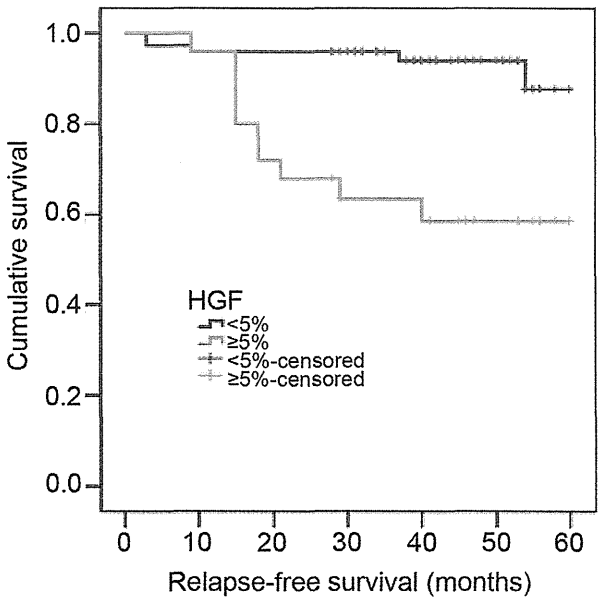

Figure 2. *Kaplan–Meier curve for rates of biochemical recurrence of prostate cancer after radical prostatectomy according to hepatocyte growth factor (HGF) expression status. Patients with HGF ≥5% had a significantly shorter biochemical recurrence-free period than did patients whose tumors exhibited HGF<5% (p<0.001).*

Table II. *Hepatocyte growth factor (HGF) expression and pathological factors in patients underwent radical prostatectomy*

		HGFP <5%		5% or more		Total	
		Mean (N)	SD (%)	Mean (N)	SD (%)	N	*p*-Value
	N	76		25			
Age (years)		66.3	6.1	62.9	6.5		0.021
BMI (kg/m^2)		23.7	2.6	23.7	2.3		0.988
Preoperative PSA		8.8	6.4	9.4	5.7		0.663
Stage	2a	15	14.8%	6	21.9%	21	
	2b	21	27.0%	7	25.5%	28	
	2c	33	42.4%	5	18.2%	38	
	3a	3	3.9%	4	14.6%	7	
	3b	4	5.1%	3	10.9%	7	0.742
Surgical margins	–	44	59.5%	12	48.0%	56	
	+	30	40.5%	13	52.0%	43	0.357
Gleason sum	6	12	14.0%	3	9.4%	15	
	7	44	51.2%	16	50.0%	60	
	8	7	8.1%	0	0.0%	7	
	9	13	15.1%	6	18.8%	19	0.796
Biochemical recurrence within 2 years		3		8			
Biochemical recurrence rate (2 years/1000person-year)		1.70		14.98			
Biochemical recurrence within 5 years		5		10			
Biochemical recurrence rate (5 years/1000 person-year)		1.58		11.07			
Median follow-up period (months)		40.5		40			

PSA: Prostate-specific antigen; MBI: body mass index.

biochemical recurrence following prostatectomy (Table III). Gleason score was not selected as a statistically independent predictor of biochemical recurrence in multivariate analysis.

Discussion

There is a need for more accurate biomarkers to predict the prognosis of patients with prostate cancer, especially for those with intermediate-grade tumors. Few markers that reliably predict treatment failure (*e.g.* PSA recurrence after surgery) have been reported. Recently, heterochromatin protein 1γ (HP1γ) was reported as a marker of treatment failure (13). To the best of our knowledge, this is the first study in which the relationships between expression of putative CSCs/CICs markers and the most clinically relevant features of prostate cancer were evaluated. Our findings suggest that CSCs/CICs are linked to more aggressive behavior of prostate cancer.

HGF is a paracrine factor produced by cells of mesenchymal origin, while the HGF receptor, c-MET, is expressed by epithelial and endothelial cells (14). HGF plays important roles in the progression of many invasive and metastatic types of cancer (15). The interaction between tumor cells and their surrounding stromal environment remains a crucial factor governing tumor invasion and metastasis. HGF and its receptor c-MET may play important roles, in the progression of CRPC. Serum HGF levels are higher in patients with metastatic prostate cancer than in those with localized tumors or benign lesions (16), and high HGF levels have been associated with poorer outcomes (17). HGF further induces cell invasion associated with stem cell features (18); that is, c-MET was considered to be important for patients with CRPC. In our previous study, we showed that both prostate CSCs/CICs and fibroblasts secrete HGF and that HGF has a role in the maintenance of prostate CSCs/CICs in autocrine and paracrine fashions (9). In this study, there was no difference in immunohistochemical c-MET expression in prostate specimens with and without biochemical recurrence (data not shown), and we demonstrated a relationship between early recurrence after radical prostatectomy and HGF positivity. We, therefore, found a role for the expression of HGF in predicting treatment failure. Our results indicate that the existence of prostate CSCs/CICs has a strong influence on biochemical recurrence after radical prostatectomy, and early treatment targeting prostate CSCs/CICs after radical prostatectomy may, therefore, be important for preventing recurrence.

Preclinical studies have shown that c-MET and HGF are highly expressed in advanced prostate cancer and are associated with disease progression. It has recently been reported that c-MET inhibitors demonstrated antiproliferative efficacy when combined with androgen ablation therapy for

Table III. *Univariate and multivariate analysis for predicting biochemical recurrence of prostate cancers.*

Factor	Univariate analysis				Multivariate analysis with all items				Multivariate analysis with backward elimination started with all items			
			95%CI				95%CI				95%CI	
	p-Value	HR	Lower	Upper	*p*-Value	HR	Lower	Upper	*p*-Value	HR	Lower	Upper
2-year RFS												
Age (per 1-year increase)	0.884	1.01	0.92	1.11	0.055	1.18	1.00	1.40	**0.039**	1.2	1.0	1.3
BMI (per 1kg/m2 increase)	0.813	1.03	0.82	1.29	0.504	1.13	0.80	1.59				
Preoperative PSA (per 1 ng/ml increase)	**0.009**	1.07	1.02	1.12	**0.003**	1.17	1.06	1.30	**0.001**	1.2	1.1	1.3
Pathological T stage (per 1-category increase)	0.108	1.53	0.91	2.57	0.716	1.16	0.52	2.61				
Positive margins *vs.* negative margins	0.057	3.63	0.96	13.69	0.059	4.03	0.95	17.11	0.089	3.3	0.8	13.3
Gleason sum (per 1 increase)	0.582	1.18	0.65	2.14	0.743	0.85	0.33	2.20				
HGF≥5%	**0.002**	8.58	2.27	32.40	0.001	18.36	3.41	98.73	**0.001**	17.1	3.4	86.7
Open *vs.* laparoscopy	0.719	1.24	0.38	4.08	0.263	2.226	0.548	9.042				
5-year RFS												
Age (per 1-year increase)	0.529	1.03	0.95	1.11	**0.024**	1.15	1.02	1.29	**0.015**	1.14	1.03	1.26
BMI (per 1kg/m2 increase)	0.823	1.02	0.84	1.24	0.815	1.03	0.80	1.33				
Preoperative PSA (per 1 ng/ml increase)	**0.023**	1.06	1.01	1.11	**0.003**	1.13	1.04	1.23	**0.001**	1.13	1.05	1.22
Pathological T stage (per 1-category increase)	0.071	1.51	0.97	2.36	0.677	1.15	0.60	2.18				
Positive margins *vs.* negative margins	0.160	2.11	0.75	5.94	0.142	2.31	0.76	7.03				
Gleason sum (per 1 increase)	0.459	1.21	0.73	2.01	0.93	0.97	0.46	2.05				
HGF ≥5%	**0.001**	6.62	2.26	19.40	**<0.001**	13.29	3.46	51.04	**<0.001**	14.28	3.88	52.54
Open *vs.* laparoscopy	0.671	0.799	0.284	2.247	0.999	0.999	0.321	3.114				

RFS: Recurrence-free survival; MBI: body mass index; PSA: prostate-specitic antigen; HGF: hepatocyte growth factor; HR: hazord ratio; CI: conficdence interval. Bold indicates statistically significant difference.

advanced prostate cancer (19). Furthermore, targeting the c-MET pathway with rilotumumab, a fully human monoclonal antibody against HGF, in combination with mitoxantrone and prednisone (MP), is a potentially viable therapeutic option in CRPC, and a double-blinded, randomized phase II study for patients with CRPC has been performed (20). Unfortunately, rilotumumab combined with MP did not show a treatment advantage in patients with CRPC. Since, HGF–c-MET signaling has important role for self-renewal of CSCs/CICs, c-Met inhibitor treatment might be better to be started for high risk patients just after radical prostatectomy to eradicate remaining CSCs/CICs before they start to differentiate into non-CICs/CICs. Inhibition of c-MET has potency in blocking stem cell-like transition and is therefore a promising tool for targeted-therapy of prostate cancer.

There are several limitations to our study. Firstly, there are the limitations inherent to any retrospective study. Secondly, open or laparoscopic radical prostatectomy was performed by many surgeons. The relatively high margin-positive rate may be due to immature technical skills. Thirdly, immunohistochemistry has inherent limitations such as reproducibility and reliability. Since it is difficult to stain and correctly evaluate old specimens, specimens used in our study were limited to ones obtained in the period from 2008 to 2011. Finally, the follow-up period was relatively short because of the limitation for specimens. Related to this problem, because the incidence of the biochemical recurrence was very low, the reliability of the result of multivariate regression analysis is relatively unsatisfactory. In addition, although the predictability should have been validated in an independent cohort, it could not be performed with small samples. Further investigation is therefore needed.

In summary, the results of this study indicate a direct link between expression of prostate CSC/CIC markers and biochemical recurrence after radical prostatectomy in patients with prostate cancer. Our data support the current CSC hypothesis for prostate cancer, which suggests that therapeutic targeting of CSCs/CICs in prostate cancer is a future possibility.

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Conflicts of Interest

The Authors declare no conflict of interest.

References

1 Punnen S, Cooperberg MR, D'Amico AV, Karakiewicz PI, Moul JW, Scher HI, Schlomm T and Freedland SJ: Management of biochemical recurrence after primary treatment of prostate cancer: a systematic review of the literature. European urology *64*: 905-915, 2013.

2 Humphrey PA, Zhu X, Zarnegar R, Swanson PE, Ratliff TL, Vollmer RT and Day ML: Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. The American journal of pathology *147*: 386-396, 1995.

3 Kasai S, Sugimura K, Matsumoto K, Nishi N, Kishimoto T and Nakamura T: Hepatocyte growth factor is a paracrine regulator of rat prostate epithelial growth. Biochemical and biophysical research communications *228*: 646-652, 1996.

4 Cecchi F, Rabe DC and Bottaro DP: Targeting the HGF/Met signaling pathway in cancer therapy. Expert Opin Ther Targets *16*: 553-572, 2012.

5 Knudsen BS and Edlund M: Prostate cancer and the met hepatocyte growth factor receptor. Advances in cancer research *91*: 31-67, 2004.

6 Park CY, Tseng D and Weissman IL: Cancer stem cell-directed therapies: recent data from the laboratory and clinic. Molecular therapy: the journal of the American Society of Gene Therapy *17*: 219-230, 2009.

7 Hirohashi Y, Torigoe T, Inoda S, Takahashi A, Morita R, Nishizawa S, Tamura Y, Suzuki H, Toyota M and Sato N: Immune response against tumor antigens expressed on human cancer stem-like cells/tumor-initiating cells. Immunotherapy *2*: 201-211, 2010.

8 Nishida S, Hirohashi Y, Torigoe T, Kitamura H, Takahashi A, Masumori N, Tsukamoto T and Sato N: Gene expression profiles of prostate cancer stem cells isolated by aldehyde dehydrogenase activity assay. The Journal of urology *188*: 294-299, 2012.

9 Nishida S, Hirohashi Y, Torigoe T, Inoue R, Kitamura H, Tanaka T, Takahashi A, Asanuma H, Masumori N, Tsukamoto T, and Sato N: Prostate cancer stem-like cells/cancer-initiating cells have an autocrine system of hepatocyte growth factor. Cancer science *104*: 431-436, 2013.

10 Edge S, Byrd DR, Compton CC, Fritz AG, Greene FL and Trotti A: AJCC Cancer Staging Manual. 2010.

11 Kitamura H, Torigoe T, Hirohashi Y, Asanuma H, Inoue R, Nishida S, Tanaka T, Fukuta F, Masumori N, Sato N and Tsukamoto T: Prognostic impact of the expression of ALDH1 and SOX2 in urothelial cancer of the upper urinary tract. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc *26*: 117-124, 2013.

12 Heagerty PJ, Lumley T and Pepe MS: Time-dependent ROC curves for censored survival data and a diagnostic marker. Biometrics *56*: 337-344, 2000.

13 Slezak J, Truong M, Huang W and Jarrard D: HP1gamma expression is elevated in prostate cancer and is superior to Gleason score as a predictor of biochemical recurrence after radical prostatectomy. BMC Cancer *13*: 148, 2013.

14 Rubin JS, Bottaro DP, and Aaronson SA: Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product. Biochimica et biophysica acta *1155*: 357-371, 1993.

15 Peruzzi B and Bottaro DP: Targeting the c-Met signaling pathway in cancer. Clinical cancer research : an official journal of the American Association for Cancer Research *12*: 3657-3660, 2006.

16 Naughton M, Picus J, Zhu X, Catalona WJ, Vollmer RT and Humphrey PA: Scatter factor-hepatocyte growth factor elevation in the serum of patients with prostate cancer. The Journal of urology *165*: 1325-1328, 2001.

17 Humphrey PA, Halabi S, Picus J, Sanford B, Vogelzang NJ, Small EJ and Kantoff PW: Prognostic significance of plasma scatter factor/hepatocyte growth factor levels in patients with metastatic hormone- refractory prostate cancer: results from cancer and leukemia group B 150005/9480. Clinical genitourinary cancer *4*: 269-274, 2006.

18 van Leenders GJ, Sookhlall R, Teubel WJ, de Ridder CM, Reneman S, Sacchetti A, Vissers KJ, van Weerden W and Jenster G: Activation of c-MET induces a stem-like phenotype in human prostate cancer. PloS one *6*: e26753, 2011.

19 Verras M, Lee J, Xue H, Li TH, Wang Y and Sun Z: The androgen receptor negatively regulates the expression of c-Met: implications for a novel mechanism of prostate cancer progression. Cancer research *67*: 967-975, 2007.

20 Ryan CJ, Rosenthal M, Ng S, Alumkal J, Picus J, Gravis G, Fizazi K, Forget F, Machiels JP, Srinivas S, Zhu M, Tang R, Oliner KS, Jiang Y, Loh E, Dubey S and Gerritsen WR: Targeted MET inhibition in castration-resistant prostate cancer: a randomized phase II study and biomarker analysis with rilotumumab plus mitoxantrone and prednisone. Clinical cancer research: an official journal of the American Association for Cancer Research *19*: 215-224, 2013.

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