space in the flank of five-week old female NOD/SCID mice under anaesthetization. Tumorigenic capacity was judged 8 weeks after injection.

cDNA Preparation and Quantitative Real-time RT-PCR for Gene Expression Assay

After SP and NSP cells in ACHN and KRC/Y were isolated, total RNA was extracted using an RNeasy Plus Micro Kit (Qiagen, Valencia, CA, USA), and complementary DNA (cDNA) was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed to examine the expression of CSC-LC property-related genes (e.g., ABC transporter genes (ABCB1 and ABCG2), self-replication genes (BMI1 and c-MYC), anti-apoptosis genes (BCL2 and CFLAR), hypoxia-related genes (hypoxia inducible factor 1α (HIF1α) and vascular endothelial growth factor-A (VEGFA)), and epithelial-mesenchymal transition (EMT)-related genes (Snail and Twist)) with an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Gene expression assays and primer and probe mixes were used for ABCB1, ABCG2, ALDH1A1, BMI1, c-MYC, BCL2, CFLAR, HIF1α, VEGFA, Snail, Twist, and β-actin (assay IDs (Hs 00184500_m1, Hs00184979_m1, Hs00946916_m1, Hs00180411_m1, Hs00153408_m1, Hs00608023_m1, Hs00153439_m1, Hs00153153_m1, Hs00900055_m1, Hs00195591_m1, Hs01675818_s1, and Hs99999903_m1, respectively; Applied Biosystems),and thermal cycle conditions were as follows: initial incubation at 95°C for 10 min, then 40 cycles alternating in turn with 95°C for 10 s, 60°C for 20 s, and 72°C for 15 s, and then maintained at 72°C for 10 min.

Comparative gene expression analysis was performed using the $2^{(-\Delta\Delta Ct)}$ methods with normalization to the level of internal control gene, β-actin.

ALDH1 Expression in SP and NSP Cells and in Cells under Pathologic Conditions

ALDH1 expression was investigated in samples prepared from SP and NSP cells, drug-treated cells, and cells cultured under hypoxic conditions. Briefly, SP and NSP cells were isolated from ACHN and KRC/Y cells cultured for 72 hours using the method described above. Parental cells and isolated SP and NSP cells were used as samples. ACHN cells cultured with EMEM containing Sorafenib (1 μM) or IFNα (4,000 IU/m) for 48, 72 or 96 hours and the cells cultured under hypoxic (1% O_2) conditions for 48, 72 or 96 hours were also used as samples. Samples were suspended in ALDEFLUOR assay buffer containing ALDH substrate, BAAA (Bodipy-aminoacetaldehyde) (50 μg dry reagent), with or without 5 μl of the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB 1.5 mM in 95% ethanol stock solution), as a negative control, and incubated for 60 min at 37°C (ALDEFLOUR KIT, Stem cells technologies, Vancouver, BC, Canada), and analyzed using flow cytometry (FCM).

Biologic Characteristics of ALDH1-positive and ALDH1-negative Cells

Sphere formation assay was performed in ACHN and KRC/Y cells. Tumorigenicity assay and gene expression assay were performed to examine biological features of ALDH1-positive and ALDH1-negative ACHN cells. To compare the self-renewal capacity between ALDH1-positive and ALDH1-negative ACHN cells, we examined a sphere-forming ability by three consecutive serial passages of single-dissociated cells according to the method of Lim et al. [29]. Briefly, after dissociating the first passage sphere with 0.25% trypsin, single-dissociated cells in ALDH1-positive and ALDH1-negative cells of ACHN were plated in 6-well plates. One week later, the number of spheres was counted and the same procedure was repeated once again. Tumorigenicity assay and gene expression assay were performed as described above except the comparison at 1×10^3 cells was not performed in the tumorigenicity assay.

Statistical Analysis

Comparison of cell growth assay was performed using two-factor factorial ANOVA, and those of colony formation assay, sphere formation assay, and drug resistance assay were performed using Student's *t*-test. The other data comparisons were performed using the Mann-Whitney U test. A value of $P<0.05$ was considered significant.

Results

Expression of CSC Markers

ACHN expressed CD90 (96.9%) and EpCAM (87.7%), but expression of CD105 (1.5%), CD133 (1.3%) and ABCG2 (0.9%) remained at very low levels. On the other hand, KRC/Y expressed CD105 (28.9%) and EpCAM (93.0%), but expression of CD90 (1.7%), CD133 (1.7%), and ABCG2 (2.9%) was very low.

SP Cells Analysis and Expression of CSC Markers in SP and NSP Cells

The SP cell fractions in ACHN and KRC/Y were 1.4% and 1.7%, respectively (Fig. 1A). Subsequently, we examined the expression of CSC markers, such as CD90 and EpCAM in ACHN, and CD105 and EpCAM in KRC/Y, in SP and NSP cells. There was no apparent difference in CD90 and EpCAM expression between SP and NSP cells in ACHN. Although there was no difference in EpCAM expression between SP and NSP cells in KRC/Y, CD105 expression in SP cells (24.6%) was much higher than in NSP cells (4.6%) (Fig. 1B).

Biological Features of SP and NSP Cells in ACHN and KRC/Y in vitro

There was no significant difference in the cell proliferative ability and clonogenicity between SP and NSP cells in ACHN. On the other hand, after culturing for 48 hours, SP cells in KRC/Y had a significantly higher proliferative ability than NSP cells ($P<0.0001$) (Fig. 2A). Although SP cells in KRC/Y had a significantly higher clonogenicity than NSP cells ($P<0.01$) (Fig. 2B), there was no significant difference in sphere forming ability between SP and NSP cells in KRC/Y. Conversely, SP cells in ACHN had a significantly higher sphere forming ability than NSP cells (Fig. 2C). After 72, 96 or 144 hours treatment with Sorafenib or IFNα, the sensitivity to each drug was assessed with the MTT assay. There was no difference in sensitivity between SP cells and NSP cells in KRC/Y against Sorafenib or IFNα treatment. However, the SP cells in ACHN had a significantly higher IFNα resistance ($P<0.0001$) (Fig. 2D).

Tumorigenicity Assays in vivo in SP and NSP Cells

Both SP and NSP cells showed tumor forming ability in each of the two RCC cell lines. The ratio of tumorigenicity between SP and NSP cells in ACHN and KRC/Y was not significantly different, but the tumorigenicity of SP cells was slightly higher than that of NSP cells in ACHN (Table 1).

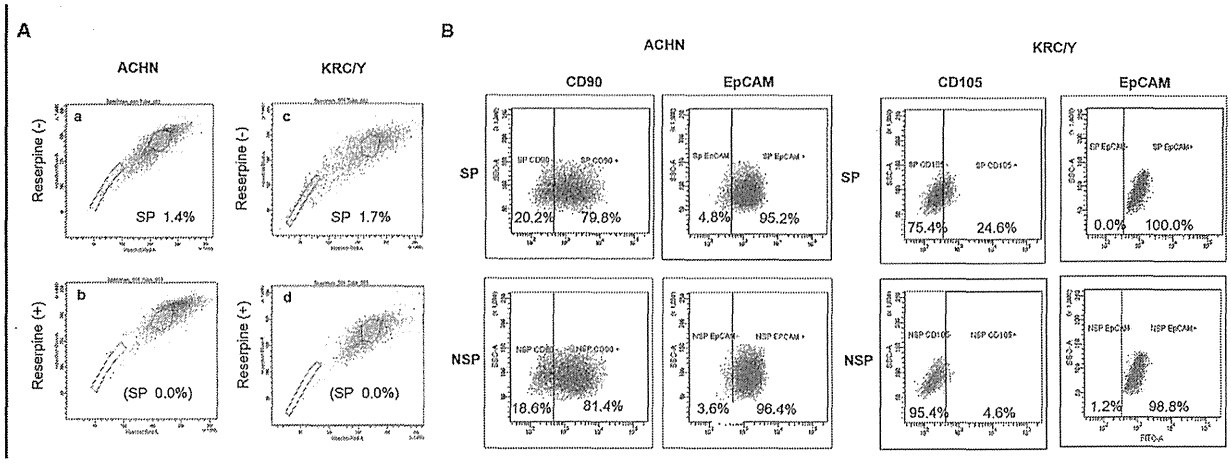

Figure 1. SP cells analysis and expression of CSC markers in SP and NSP cells. (A) ACHN and KRC/Y were labeled with Hoechst 33342, and then analyzed by FCM. The SP cell rates in ACHN and KRC/Y were 1.4% (A–a) and 1.7% (A–c), respectively, which decreased significantly in the presence of reserpine (A–b, A–d). The experiment was repeated at least three times for each cell line and almost identical results were obtained. A representative figure of our experiments is shown. (B) There was no apparent difference in CD90 and EpCAM expression between SP and NSP cells in ACHN. In the KRC/Y cell line, although there was no difference in EpCAM expression, SP cells expressed a higher CD105-positive cell rate than NSP cells (SP vs NSP : 24.6% vs 4.6%). The experiments were repeated twice, and almost identical results were obtained. A representative figure of our experiments is shown.
doi:10.1371/journal.pone.0075463.g001

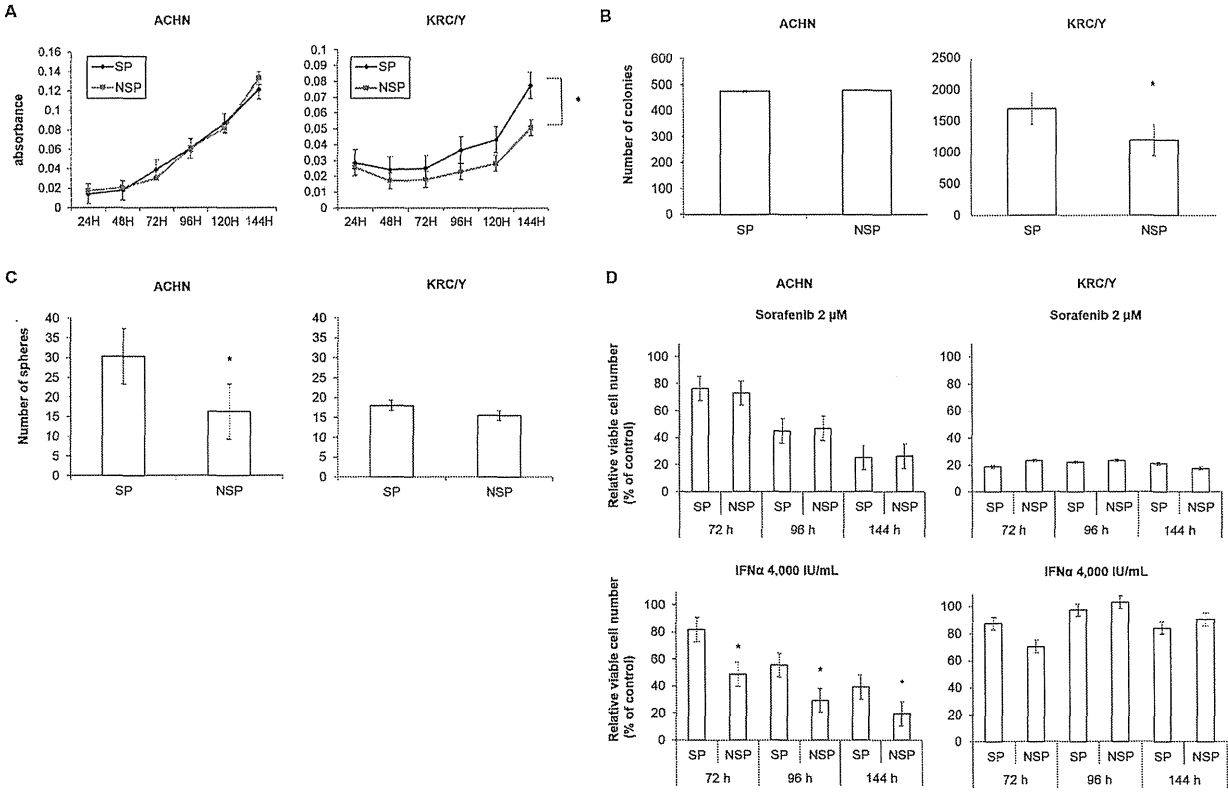

Figure 2. Biological features of SP and NSP cells in ACHN and KRC/Y in vitro. (A) Growth curves of SP and NSP cells. SP cells in KRC/Y showed a higher proliferative ability compared to NSP cells (* $P<0.0001$). (B) The clonogenity was significantly increased in SP cells in KRC/Y (* $P<0.01$). (C) Sphere forming ability was significantly higher in SP cells in ACHN (* $P<0.05$). (D) Drug resistance of SP and NSP cells treated with Sorafenib or IFNα. SP cells in ACHN had higher IFNα resistance (* $P<0.0001$). The experiments were repeated twice, and almost identical results were obtained.
doi:10.1371/journal.pone.0075463.g002

Table 1. Tumorigenicity of side population (SP) and Non-SP (NSP) cells in ACHN and KRC/Y.

		Injected cell number		
		1×10^3	1×10^4	1×10^5
ACHN	SP	0/5	1/5	3/5
	NSP	0/5	0/5	1/5
KRC/Y	SP	0/5	0/5	3/5
	NSP	0/5	0/5	2/5

doi:10.1371/journal.pone.0075463.t001

Analysis of CSC-LC Property-related Gene Expression in SP and NSP Cells by qRT-PCR

There were no significant differences in mRNA expressions of ABC transporter genes (ABCB1 and ABCG2), self-replication genes (BMI-1 and c-MYC), anti-apoptosis genes (BCL2 and CFLAR), hypoxia-related genes (VEGFA and HIF1α), and EMT-related genes (Snail and Twist) between SP and NSP cells in the 2 cell lines. SP cells in ACHN expressed a slightly higher level of ALDH1A1 mRNA than NSP cells, but no apparent difference was observed in KRC/Y (Fig. 3).

ALDH1 Expression, and Biological Features of ALDH1-positive and ALDH1-negative RCC Cells

The ALDH1-positive cell rate in KRC/Y cells was 6.5%. There was no difference in ALDH1 expression between SP and NSP cells. In ACHN cells, the ALDH1-positive cell rate was 15.3%. Also, the number of ALDH1-positive SP cells (32.7%) was higher than that of NSP cells (14.6%) (Fig. 4A). Cell growth was significantly suppressed in cells treated with Sorafenib or IFNα and in cells exposed to hypoxia, as compared with control cells (Fig. 4B). Regarding ALDH1 expression, there was no apparent difference in ALDH1-positive cell rates among control cells, cells treated with Sorafenib or IFNα, and cells exposed to hypoxic condition for 48 hours. However, the percentage of ALDH1-positive cells increased chronologically, especially in cells treated with Sorafenib or exposed to hypoxic conditions. In particular, after exposure to Sorafenib or IFNα, or hypoxia for 96 hours, the percentages of ALDH1-positive cells were 40.0%, 19.2% and 37.1%, respectively (Fig. 4C).

The sphere forming ability of ALDH1-positive cells in both ACHN and KRC/Y was higher than that of ALDH1-negative cells. Moreover, ALDH1-positive cells in ACHN generated significantly larger sphere sizes than ALDH1-negative cells (Fig. 4D). Also, we found that single-dissociated sphere cells plated at a density of 4,000 cells per well gave rise to secondary and tertiary spheres within 1 week of seeding. Although the number of spheres was shown to decrease in the second and third passages compared to the first passage, the sphere forming ability of ALDH1-positive cells in ACHN was maintained during the second and third passages. On the other hand, ALDH1-negative cells formed a few secondary spheres (Fig. 5).

Tumor formation was observed in three of five and five of five mice injected with 10×10^3 and 100×10^3 ALDH1-positive cells, respectively, at 8 weeks. However, ALDH1-negative cell injection developed no visible tumors in all mice by this time (Table 2).

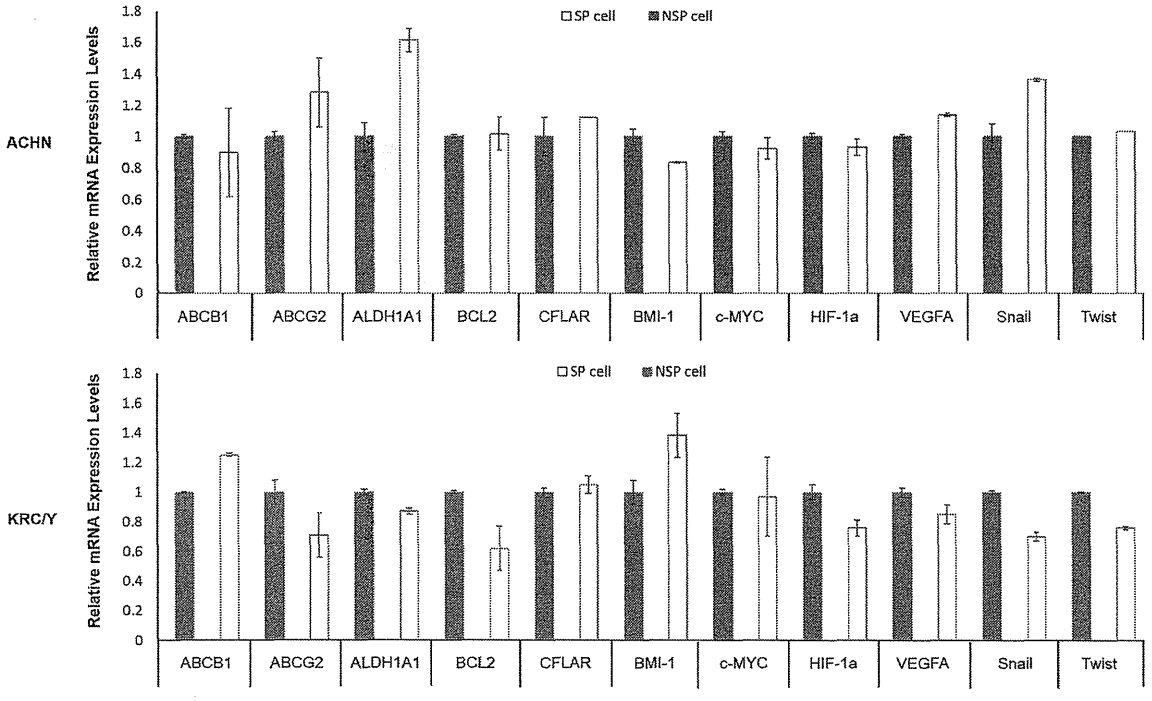

Figure 3. Quantification of mRNA expression of CSC-LC property-related genes in SP and NSP cells by real-time PCR. There were no significant differences in mRNA expressions of ABC transporter genes (ABCB1 and ABCG2), self-replication genes (BMI-1 and c-MYC), anti-apoptosis genes (BCL2 and CFLAR), hypoxia-related genes (VEGFA and HIF1α), and EMT-related genes (Snail and Twist) between SP and NSP cells in the 2 cell lines. SP cells in ACHN expressed a slightly higher level of ALDH1A1 mRNA than NSP cells, but no apparent difference was observed in KRC/Y. The experiment was repeated at least four times for each cell line and almost identical results were obtained.
doi:10.1371/journal.pone.0075463.g003

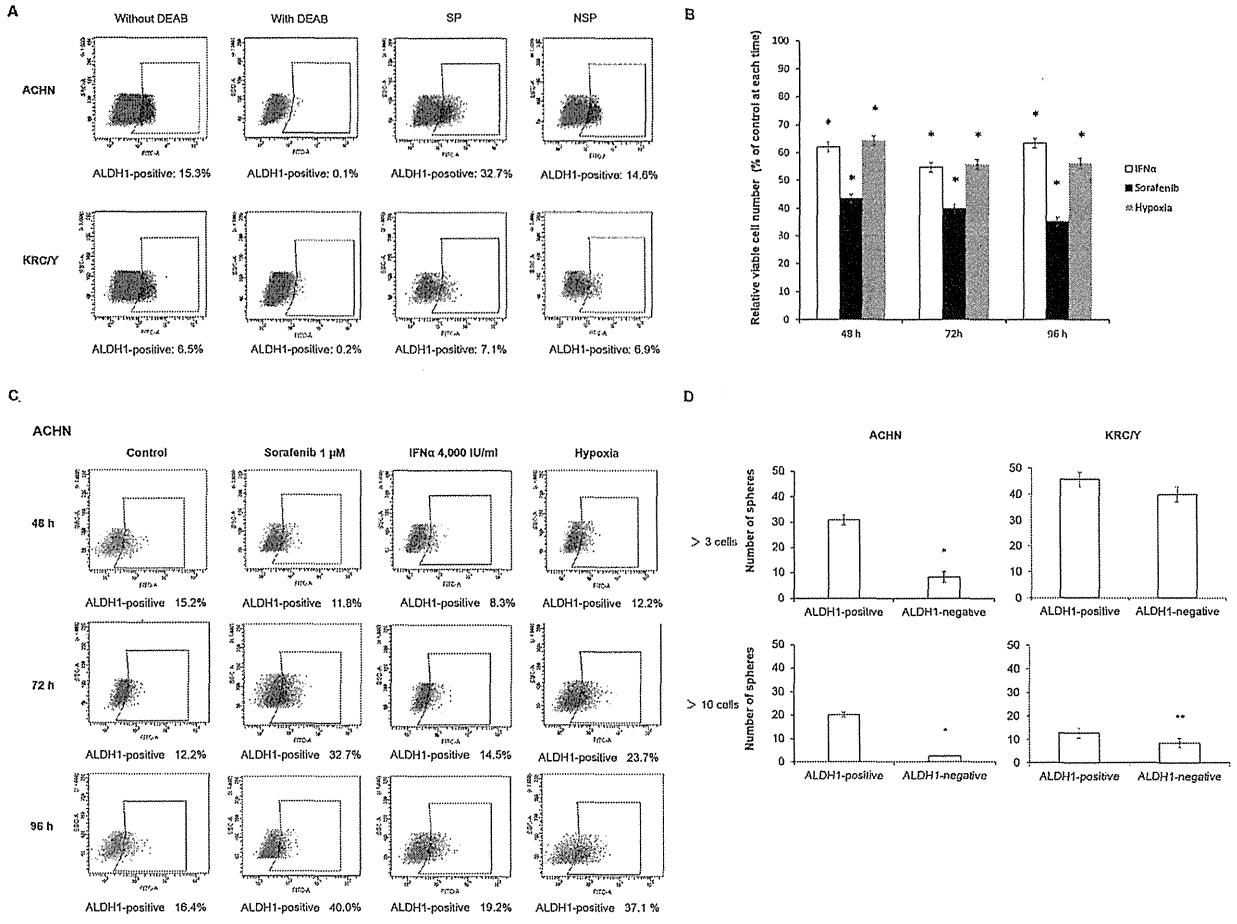

Figure 4. ALDH1 expression, and biological features of ALDH1-positive and ALDH1-negative RCC cells. (A) The expression of ALDH1 in SP cells and NSP cells in ACHN and KRC/Y. The ALDH1-positive cell rates in ACHN and KRC/Y were 15.3% and 6.5%, respectively. (B) Comparison of cell growth among control cells, cells treated with Sorafenib or IFNα, and cells exposed to hypoxia in ACHN. Cell growth was measured at 48, 72 or 96 hours after drug treatment or exposure to hypoxia. Cell growth after drug treatment or exposure to hypoxia was significantly suppressed as compared with control (* $P<0.005$, ** $P<0.0001$ vs. control). (C) The percentage of ALDH1-positive cells in cells treated with Sorafenib or IFNα, or cells exposed to hypoxia for 48, 72 or 96 hours. The percentage of ALDH1-positive cells in cells treated with Sorafenib or IFNα, or cells exposed to hypoxia for 96 hours was higher as compared with the normal condition. The experiments were repeated twice, and almost identical results were obtained. A representative figure of our experiments is shown. (D) Sphere forming ability between ALDH1-positive cells and ALDH1-negative cells. The sphere formation of ALDH1-positive cells in ACHN and KRC/Y was higher than that of ALDH1-negative cells. The experiments were repeated twice, and almost identical results were obtained.
doi:10.1371/journal.pone.0075463.g004

qRT-PCR in ALDH1-positive and ALDH1-negative ACHN Cells

We performed qRT-PCR analysis to compare CSC-LC property-related gene expression in ALDH1-positive and ALDH1-negative ACHN cells. ALDH1-positive cells expressed significantly higher levels of mRNA in all genes except Snail than ALDH1-negative cells. The levels of the increase were as follows: ABCB1, 4.9-fold; ABCG2, 2.5-fold, ALDH1A1, 4.8-fold; BCL2, 5.0-fold; CFLAR, 4.1-fold; BMI-1, 3.9-fold; c-MYC, 3.9-fold; HIF1α, 3.4-fold; VEGFA, 2.7-fold; Twist, 4.0-fold (Fig. 6).

Discussion

Since the CSC concept was proposed to explain the heterogeneity of tumor cells, CSCs or CSC-LCs have been identified in many types of cancer. In general, CSCs possess both self-renewal and differentiation capabilities allowing CSC to partially recreate the cellular heterogeneity of the parental tumor. A number of studies have reported that the inability of conventional therapies to prevent recurrence or metastases is due to the presence of small subsets of resistant cells, namely CSCs [8,30]. In recent years the SP technique has become one of the most widely used methods of isolating CSC-LCs. Since the detailed staining and measurement method of Goodell et al. was first introduced, many researchers have reported that SP cells are a subset of cells with higher grade malignancy, and CSC-LCs characteristics [15,16,31]. With regard to RCC, Addla et al. reported that SP cells accounted for 4–6% of total cancer cells. However the cellular characteristics of SP cells are not well understood [17].

In our present study we found that the SP fractions in ACHN and KRC/Y were 1.4% and 1.7%, respectively. There was no difference between KRC/Y SP and NSP cells in tumorigenicity, sphere forming ability, or in resistance to Sorafenib or IFNα, which are conventionally used to treat advanced RCC. These

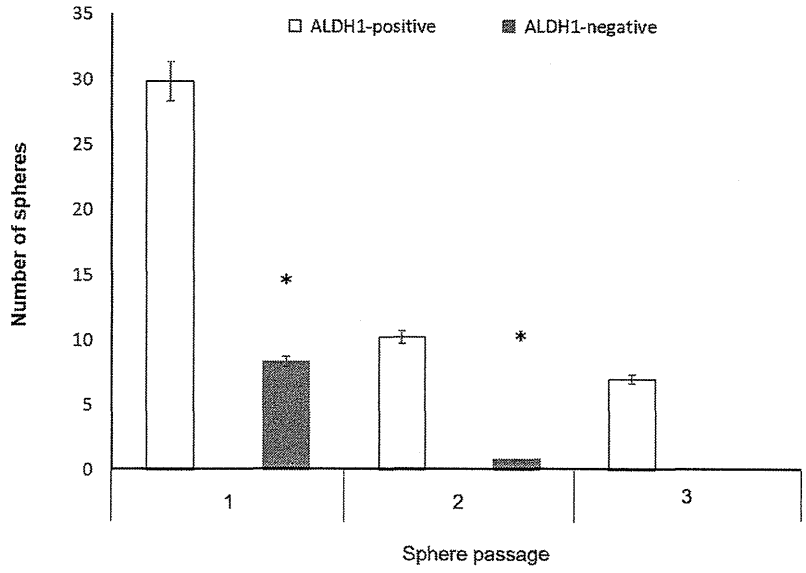

Figure 5. A self-renewal capacity between ALDH1-positive and ALDH1-negative ACHN cells. The sphere forming ability of ALDH1-positive cells in ACHN was maintained during the second and third passages (* $P<0.0001$).
doi:10.1371/journal.pone.0075463.g005

findings indicate that KRC/Y SP cells lack the characteristics of CSCs-LCs. In contrast, whereas there were no significant differences between ACHN SP and NSP cells in the in vitro cell growth or colony formation assays, SP cells did show a higher sphere forming ability, higher IFNα resistance and higher tumorigenicity in NOD/SCID mice than NSP cells, suggestive of cells with CSC-LC properties are included in ACHN SP cells. At present the SP approach is the most widely used method to identify CSC markers, however many researchers still question the relationship between SP cells and CSCs [32–34]. In addition, Ibrahim et al. studied the relationship between Hoechst staining concentration and incubation time and reported that Hoechst staining concentration had an effect on cell damage [35]. In our present study, in order to identify the SP cells in ACHN and KRC/Y we used Hoechst staining at a concentration of 5 μg/mL and 10 μg/mL, respectively. Hoechst staining is generally carried out at a concentration of 5 μg/mL, but in the present study we used a higher concentration in KRC/Y cells [36]. Thus, we cannot completely rule out the possibility that cellular damage due to Hoechst staining was responsible for the difference in biological characteristics observed between KRC/Y cells in vivo and in vitro in our current study.

Table 2. Tumorigenicity of aldehyde dehydrogenase 1 (ALDH1)-positive and ALDH1-negative cells in ACHN.

		Injected cell number	
		1×10^4	1×10^5
ACHN	ALDH1-positive	3/5	5/5
	ALDH1-negative	0/5	0/5

doi:10.1371/journal.pone.0075463.t002

Bussolati et al. previously reported in a human RCC cell line that CD105-positive cells represented a cell group with high clonogenicity and high tumorigenicity; however, our present study found that while KRC/Y SP cells contained about five times as many CD105-positive cells as KRC/Y NSP cells, there were no differences in CSC-LC properties between KRC/Y SP and NSP cells. In addition, CD105 expression was found in a few cells in the ACHN. Thus, our current results conflict with the findings of Bussolati et al., and suggest the possibility that CD105 may not be a universal CSC marker in RCC.

Many recent studies have reported that SP cells show a higher expression of ABC transporters, especially ABCG2, than NSP cells in many solid tumors and cell lines, and that this may play a role in drug efflux and drug resistance. The expression of drug transporters via ABCG2 is an important marker in the identification and analysis of SP cells [19,20,31,37]. In our present study, we observed no difference in ABCG2 expression at the mRNA level between SP and NSP cells in either of the two RCC cell lines studied. However, in the past few years several studies have reported that SP cells express other transporters, such as ABCB1 and ABCB5, in addition to ABCG2 [38,39]. Therefore, this result may be due to the expression of the other transporters in SP cells, or it may be because the functions of ABCB1 and ABCG2 were not reflected by mRNA expression of these genes. This point needs to be further studied.

Next, in order to study other CSC markers, we performed an Aldefluor assay. ALDH1 enzymatic activity has been recognized in recent years as a general marker of both normal stem cells and CSCs [40,41]. ALDH1-positive cells have CSC-LC characteristics, such as the ability to self-replicate and to form tumors, so a number of researchers have used ALDH1 enzymatic activity as a CSC marker in many different types of cancer, including lung,

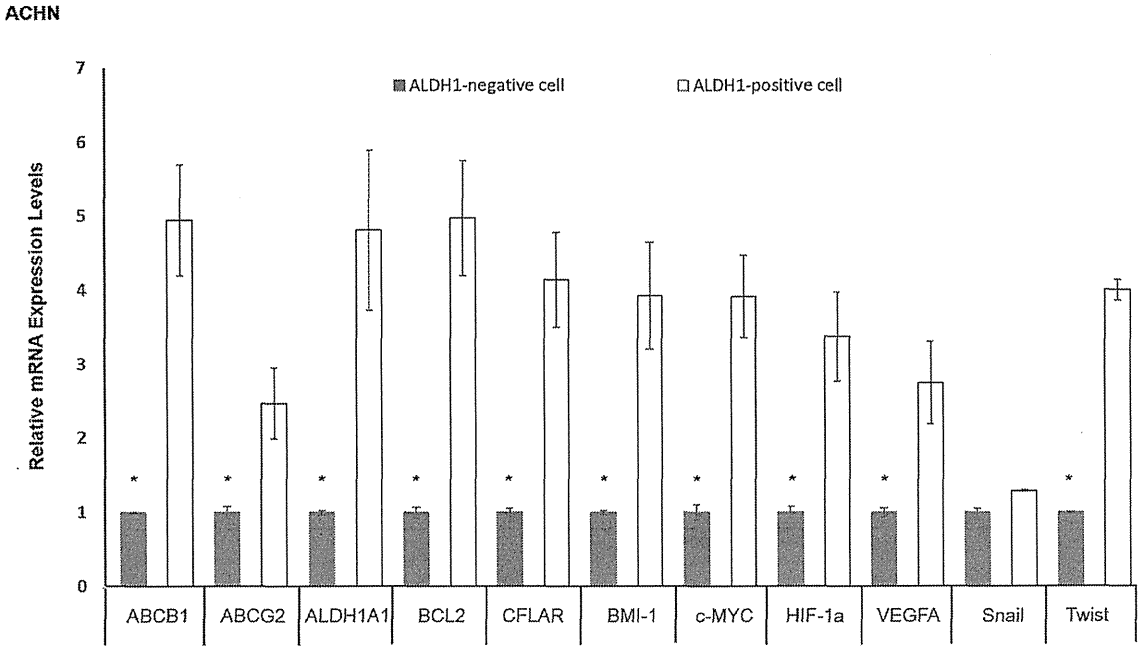

Figure 6. Quantification of mRNA expression of CSC-LC property-related genes in ALDH1-positive and ALDH1-negative ACHN cells by real-time PCR. ALDH1-positive cells showed significantly higher mRNA expression of ALDH1A1, transporter-related genes (ABCB1 and ABCG2), self-replication genes (BMI-1 and c-MYC), anti-apoptosis genes (BCL2 and CFLAR), hypoxia-related genes (HIF1α and VEGFA) and EMT-related genes (Twist) than ALDH1-negative cells in ACHN. However, there was no significant difference in mRNA expression of Snail between ALDH1-positive and ALDH1-negative cells. The experiments were repeated at least four times, and almost identical results were obtained.
doi:10.1371/journal.pone.0075463.g006

liver, pancreas, prostate, bladder, breast and malignant melanoma [42–46]. It has also been reported in breast and several other cancers that high ALDH1 expression is closely associated with poor clinical prognosis [23]. Recently, sphere formation assays have been widely used to assess the self-renewal capacity of CSC-LCs. Our present study revealed that ACHN SP cells contain more ALDH1-positive cells than NSP cells and that not only ACHN but also KRC/Y ALDH1-positive cells had greater sphere forming ability. In order to elucidate our results, we performed subsequent generations of sphere forming assays. The self-renewal capacity of ALDH1-positive cells in ACHN, but not ALDH1-negative cells, was maintained for at least three generations. Furthermore, the tumorigenicity of ALDH1-positive cells was significantly higher than ALDH1-negative cells. These results indicate that ALDH1 could be a CSC marker in RCC. According to some recent reports, the VEGF-neutralizing antibody Bevacizumab, and anti-angiogenesis drugs such as Sorafenib and Sunitinib, which are VEGF receptor tyrosine kinase inhibitors, suppressed tumor proliferation, but at the same time promoted invasion and metastasis [47,48]. Also, Conley et al. found in a breast cancer cell line that anti-angiogenesis therapy caused an increase in ALDH1-positive cells, indicating that these cells were associated with resistance to therapy [49]. The present study found that ALDH1-positive cells expanded chronologically under hypoxic conditions and after exposure to drugs. These findings indicate that ALDH1-positive cells are resistant to conventional therapies for RCC, and that they represent a cell fraction that can survive under hypoxic conditions and can replicate in adverse environments. Previous studies have reported that CSC-LCs have anti-apoptotic and drug resistant properties due to expression of anti-apoptosis genes such as BCL2 and CFLAR [50]. Moreover, recent studies have found that CSC-LCs occupy a hypoxic niche, that they can survive treatment with VEGFR2 inhibitors, and that they are involved in resistance to therapy [49,51,52]. Our real time PCR assays also found that self-replication markers such as BMI-1 and c-MYC were highly expressed in ALDH1-positive cells, along with a variety of drug efflux transporters. Moreover, anti-apoptosis genes such as BCL2 or CFLAR were also highly expressed in ALDH1-positive cells, along with HIF1α. These findings suggest that ALDH1-positive cells not only have anti-apoptotic effects, but also that they can survive under hypoxic conditions and could represent a cell population that is resistant to current conventional therapies. Our present study also found that ALDH1 expression was increased after drug treatment or exposure to hypoxia, which suggests the involvement of ALDH1-positive cells in drug resistance. Several recent reports have suggested that EMT also results in the acquisition of other properties involved in carcinoma progression, such as increased resistance to apoptosis and the acquisition of CSC-LC properties [52]. In our study, although Snail mRNA level was not significant different between ALDH1-positive and ALDH1-negative cells, Twist mRNA level was significantly increased in ALDH1-positive cells. These results suggest that ALDH1-positive cells may be related to EMT phenomenon. However, this finding needs to be further studied.

In conclusion, the results suggest that the ALDH1-positive cell population rather than SP cells shows CSC-LC properties in human RCC cells. Further studies are needed to determine the relationship between these findings and the clinical prognosis in RCC.

Acknowledgments

We thank Ms. Akemi Fujiyoshi for her assistance in our experiments.

Author Contributions

Conceived and designed the experiments: Kosuke Ueda SO JA M. Nakayama KT Keiko Ueda S. Sanada S. Suekane M. Noguchi KM HY. Performed the experiments: Kosuke Ueda SO JA M. Nakayama KT Keiko Ueda S. Sanada S. Suekane M. Noguchi KM HY. Analyzed the data: Kosuke Ueda SO JA M. Nakayama KT Keiko Ueda S. Sanada S. Suekane M. Noguchi KM HY. Contributed reagents/materials/analysis tools: Kosuke Ueda SO JA M. Nakayama KT Keiko Ueda S. Sanada S. Suekane M. Noguchi KM HY. Wrote the paper: Kosuke Ueda SO JA M. Nakayama KT Keiko Ueda S. Sanada S. Suekane M. Noguchi KM HY.

References

1. Kroeger N, Seligson DB, Klatte T, Rampersaud EN, Birkhauser FD, et al. (2012) Clinical, molecular, and genetic correlates of lymphatic spread in clear cell renal cell carcinoma. Eur Urol 61: 888–895.
2. Costa LJ, Drabkin HA (2007) Renal cell carcinoma: new developments in molecular biology and potential for targeted therapies. Oncologist 12: 1404–1415.
3. Flanigan RC, Campbell SC, Clark JI, Picken MM (2003) Metastatic renal cell carcinoma. Curr Treat Options Oncol 4: 385–390.
4. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, et al. (2007) Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 356: 115–124.
5. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, et al. (2007) Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med 356: 125–134.
6. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, et al. (2008) Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet 372: 449–456.
7. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730–737.
8. Nowell PC (1976) The clonal evolution of tumor cell populations. Science 194: 23–28.
9. Dalerba P, Cho RW, Clarke MF (2007) Cancer stem cells: models and concepts. Annu Rev Med 58: 267–284.
10. Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755–768.
11. Bussolati B, Bruno S, Grange C, Ferrando U, Camussi G (2008) Identification of a tumor-initiating stem cell population in human renal carcinomas. FASEB J 22: 3696–3705.
12. Bussolati B, Brossa A, Camussi G (2011) Resident stem cells and renal carcinoma. Int J Nephrol 2011: 286985.
13. Kim K, Ro JY, Kim S, Cho YM (2012) Expression of stem-cell markers OCT-4 and CD133: important prognostic factors in papillary renal cell carcinoma. Hum Pathol 43: 2109–2116.
14. Kim K, Ihm H, Ro JY, Cho YM (2011) High-level expression of stem cell marker CD133 in clear cell renal cell carcinoma with favorable prognosis. Oncol Lett 2: 1095–1100.
15. Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, et al. (2004) A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci U S A 101: 14228–14233.
16. Hulspas R, Quesenberry PJ (2000) Characterization of neurosphere cell phenotypes by flow cytometry. Cytometry 40: 245–250.
17. Addla SK, Brown MD, Hart CA, Ramani VA, Clarke NW (2008) Characterization of the Hoechst 33342 side population from normal and malignant human renal epithelial cells. Am J Physiol Renal Physiol 295: F680–687.
18. Nishizawa S, Hirohashi Y, Torigoe T, Takahashi A, Tamura Y, et al. (2012) HSP DNAJB8 controls tumor-initiating ability in renal cancer stem-like cells. Cancer Res 72: 2844–2854.
19. Kondo T, Setoguchi T, Taga T (2004) Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A 101: 781–786.
20. Loebinger MR, Giangreco A, Groot KR, Prichard L, Allen K, et al. (2008) Squamous cell cancers contain a side population of stem-like cells that are made chemosensitive by ABC transporter blockade. Br J Cancer 98: 380–387.
21. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, et al. (1999) Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. Hepatology 29: 1124–1130.
22. Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, et al. (2011) Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells 29: 32–45.
23. Resetkova E, Reis-Filho JS, Jain RK, Mehta R, Thorat MA, et al. (2010) Prognostic impact of ALDH1 in breast cancer: a story of stem cells and tumor microenvironment. Breast Cancer Res Treat 123: 97–108.
24. Huang CP, Tsai MF, Chang TH, Tang WC, Chen SY, et al. (2012) ALDH-positive lung cancer stem cells confer resistance to epidermal growth factor receptor tyrosine kinase inhibitors. Cancer Lett 328: 144–151.
25. Ozbek E, Calik G, Otunctemur A, Aliskan T, Cakir S, et al. (2012) Stem cell markers aldehyde dehydrogenase type 1 and nestin expressions in renal cell cancer. Arch Ital Urol Androl 84: 7–11.
26. Yano H, Maruiwa M, Sugihara S, Kojiro M, Noda S, et al. (1988) Establishment and characterization of a new human renal cell carcinoma cell line (KRC/Y). In Vitro Cell Dev Biol 24: 9–16.
27. Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, et al. (2006) Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology 44: 240–251.
28. Hisaka T, Yano H, Ogasawara S, Momosaki S, Nishida N, et al. (2004) Interferon-alphaCon1 suppresses proliferation of liver cancer cell lines in vitro and in vivo. J Hepatol 41: 782–789.
29. Lim YC, Oh SY, Cha YY, Kim SH, Jin X, et al. (2011) Cancer stem cell traits in squamospheres derived from primary head and neck squamous cell carcinomas. Oral Oncol 47: 83–91.
30. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414: 105–111.
31. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183: 1797–1806.
32. Burkert J, Otto WR, Wright NA (2008) Side populations of gastrointestinal cancers are not enriched in stem cells. J Pathol 214: 564–573.
33. Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, et al. (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27: 1006–1020.
34. Broadley KW, Hunn MK, Farrand KJ, Price KM, Grasso C, et al. (2011) Side population is not necessary or sufficient for a cancer stem cell phenotype in glioblastoma multiforme. Stem Cells 29: 452–461.
35. Ibrahim SF, Diercks AH, Petersen TW, van den Engh G (2007) Kinetic analyses as a critical parameter in defining the side population (SP) phenotype. Exp Cell Res 313: 1921–1926.
36. Ooka H, Kanda S, Okazaki H, Suzuki H, Mishima K, et al. (2012) Characterization of side population (SP) cells in murine cochlear nucleus. Acta Otolaryngol 132: 693–701.
37. Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, et al. (1997) Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3: 1337–1345.
38. Luo Y, Ellis LZ, Dallaglio K, Takeda M, Robinson WA, et al. (2012) Side population cells from human melanoma tumors reveal diverse mechanisms for chemoresistance. J Invest Dermatol 132: 2440–2450.
39. Smith PJ, Wiltshire M, Chappell SC, Cosentino L, Burns PA, et al. (2012) Kinetic analysis of intracellular Hoechst 33342-DNA interactions by flow cytometry: Misinterpretation of side population status? Cytometry A 83: 161–169.
40. Ma I, Allan AL (2011) The role of human aldehyde dehydrogenase in normal and cancer stem cells. Stem Cell Rev 7: 292–306.
41. Marcato P, Dean CA, Giacomantonio CA, Lee PW (2011) Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform. Cell Cycle 10: 1378–1384.
42. Ma S, Chan KW, Lee TK, Tang KH, Wo JY, et al. (2008) Aldehyde dehydrogenase discriminates the CD133 liver cancer stem cell populations. Mol Cancer Res 6: 1146–1153.
43. Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, et al. (2009) Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. Mol Cancer Res 7: 330–338.
44. Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, et al. (2007) ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1: 555–567.
45. Kim MP, Fleming JB, Wang H, Abbruzzese JL, Choi W, et al. (2011) ALDH activity selectively defines an enhanced tumor-initiating cell population relative to CD133 expression in human pancreatic adenocarcinoma. PLoS One 6: e20636.
46. van den Hoogen C, van der Horst G, Cheung H, Buijs JT, Lippitt JM, et al. (2010) High aldehyde dehydrogenase activity identifies tumor-initiating and metastasis-initiating cells in human prostate cancer. Cancer Res 70: 5163–5173.
47. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, et al. (2009) Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 15: 232–239.
48. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, et al. (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 15: 220–231.
49. Conley SJ, Gheordunescu E, Kakarala P, Newman B, Korkaya H, et al. (2012) Antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia. Proc Natl Acad Sci U S A 109: 2784–2789.
50. Yajima T, Ochiai H, Uchiyama T, Takano N, Shibahara T, et al. (2009) Resistance to cytotoxic chemotherapy-induced apoptosis in side population cells of human oral squamous cell carcinoma cell line Ho-1-N-1. Int J Oncol 35: 273–280.

51. Borovski T, De Sousa EMF, Vermeulen L, Medema JP (2012) Cancer stem cell niche: the place to be. Cancer Res 71: 634–639.

52. Heddleston JM, Li Z, McLendon RE, Hjelmeland AB, Rich JN (2009) The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype. Cell Cycle 8: 3274–3284.

Noguchi et al. BMC Cancer 2013, 13:613
http://www.biomedcentral.com/1471-2407/13/613

BMC Cancer

RESEARCH ARTICLE Open Access

A phase II trial of personalized peptide vaccination in castration-resistant prostate cancer patients: prolongation of prostate-specific antigen doubling time

Masanori Noguchi[1,2*], Fukuko Moriya[2], Shigetaka Suekane[2], Rei Ohnishi[2], Satoko Matsueda[3], Tetsuro Sasada[3], Akira Yamada[4] and Kyogo Itoh[3]

Abstract

Background: Cancer vaccine is one of the attractive treatment modalities for patients with castration-resistant prostate cancer (CRPC). However, because of delayed immune responses, its clinical benefits, besides for overall survival (OS), are not well captured by the World Health Organization (WHO) and Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Several surrogate markers for evaluation of cancer vaccine, including prostate-specific antigen doubling time (PSADT), are currently sought. The purpose of this study was to assess prospectively the PSA kinetics and immune responses, as well as the efficacy, safety, and biomarkers of personalized peptide vaccination (PPV) in progressive CRPC.

Methods: One hundred patients with progressive CRPC were treated with PPV using 2–4 positive peptides from 31 candidate peptides determined by both human leukocyte antigen (HLA) class IA types and the levels of immunoglobulin G (IgG) against each peptide. The association between immune responses and PSADT as well as overall survival (OS) was studied.

Results: PPV was safe and well tolerated in all patients with a median survival time of 18.8 months. Peptide-specific IgG and T-cell responses strongly correlated with PSADT ($p < 0.0001$ and $p = 0.0007$, respectively), which in turn showed correlation with OS ($p = 0.018$). Positive IgG responses and prolongation of PSADT during PPV were also significantly associated with OS ($p = 0.001$ and $p = 0.004$) by multivariate analysis.

Conclusions: PSADT could be an appropriate surrogate marker for evaluation of the clinical benefit of cancer vaccine. Further randomized trials are needed to confirm these results.

Trial registration: UMIN000001850

Keywords: Prostate-specific antigen doubling time, Personalized peptide vaccine, Prostate cancer, Surrogate marker, Overall survival

Background

Changes in serum prostate-specific antigen (PSA) can reflect the burden of disease and clinical benefit in patients with castration-resistant prostate cancer (CRPC) with cytotoxic chemotherapy or hormonal agents known to kill tumor cells; these changes can have practical utility by providing and updating prognostic information on an individual patient over time [1-4]. As observed in many clinical trials, however, immunotherapy can induce novel patterns of antitumor responses distinct from those of chemotherapy [5]. For example, an autologous dendritic-cell-based vaccine (sipuleucel-T) is known to improve survival without having an impact on early PSA decline [6], whereas docetaxel's improvement in overall survival (OS) correlates for the most part with a PSA decline within the first 3 months of therapy [7,8]. Thus, interpreting PSA decline in the context of novel immunotherapy must be

* Correspondence: noguchi@med.kurume-u.ac.jp
[1]Clinical Research Division of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan
[2]Departments of Urology, Kurume University School of Medicine, Kurume, Japan
Full list of author information is available at the end of the article

carried out with caution on the basis of the mechanism of action, and may also depend on the time of sampling [9].

Personalized peptide vaccine (PPV) uses multiple peptides based on the pre-existing immunity. Under PPV treatment, each patient with human leukocyte antigen (HLA)-class IA types positive was tested for their immunological reactivity to 31 different peptides capable of inducing T-cell responses. The 31 peptides were derived from a number of tumor associated antigens: PSA, prostatic acid phosphatase (PAP), prostate-specific membrane antigen (PSMA), multidrug resistance protein and a variety of other epithelial tumor antigens. We previously demonstrated that PPV was safe and improved OS with immune responses in phase I, I/II, and II clinical trials in patients with CRPC [10-16]. However, it was not addressed whether PSADT could be an appropriate surrogate marker for evaluation of the clinical benefit of cancer vaccine. To address this, we evaluated data from a phase II clinical trial for CRPC using PPV.

Methods

Patient Eligibility

Eligibility required a histological diagnosis of prostate adenocarcinoma and progressive disease (PD) defined as at least two consecutive increases in PSA, new metastatic lesion on radionuclide bone scan, or progressive tumor lesions on cross-sectional imaging, despite adequate androgen ablative therapy. Patients showed positive IgG

Table 1 Peptide candidates for personalized peptide vaccination

Symbol for peptide	Origin protein	Position of peptide	Amino acid sequence	HLA type
CypB-129	Cyclophilin B	129-138	V	A2,A3sup[a]
Lck-246	p56 lck	246-254	KLVERLGAA	A2
Lck-422	p56 lck	422-430	DVWSFGILL	A2,A3sup
MAP-432	ppMAPkkk	432-440	DLLSHAFFA	A2,A26
WHSC2-103	WHSC2	103-111	ASLDSDPWV	A2,A3sup[a],A26
HNRPL-501	HNRPL	501-510	NVLHFFNAPL	A2,A26
UBE-43	UBE2V	43-51	RLQEWCSVI	A2
UBE-85	UBE2V	85-93	LIADFLSGL	A2
WHSC2-141	WHSC2	141-149	ILGELREKV	A2
HNRPL-140	HNRPL	140-148	ALVEFEDVL	A2
SART3-302	SART3	302-317	LLQAEAPRL	A2
SART3-309	SART3	309-317	RLAEYQAYI	A2
SART2-93	SART2	93-101	DYSARWNEI	A24
SART3-109	SART3	109-118	VYDYNCHVDL	A24,A3sup[a],A26
Lck-208	p56 lck	208-216	HYTNASDGL	A24
PAP-213	PAP	213-221	LYCESVHNF	A24
PSA-248	PSA	248-257	HYRKWIKDTI	A24
EGFR-800	EGF-R	800-809	DYVREHKDNI	A24
MRP3-503	MRP3	503-511	LYAWEPSFL	A24
MRP3-1293	MRP3	1293-1302	NYSVRYRPGL	A24
SART2-161	SART2	161-169	AYDFLYNYL	A24
Lck-486	p56 lck	486-494	TFDYLRSVL	A24
Lck-488	p56 lck	488-497	DYLRSVLEDF	A24
PSMA-624	PSMA	624-632	TYSVSFDSL	A24
EZH2-735	EZH2	735-743	KYVGIEREM	A24
PTHrP-102	PTHrP	102-111	RYLTQETNKV	A24
SART3-511	SART3	511-519	WLEYYNLER	A3sup[a]
SART3-734	SART3	734-742	QIRPIFSNR	A3sup[a]
Lck-90	p56 lck	90-99	ILEQSGEWWK	A3sup[a]
Lck-449	p56 lck	449-458	VIQNLERGYR	A3sup[a]
PAP-248	PAP	248-257	GIHKQKEKSR	A3sup[a]

[a]A3sup, HLA-A3 supertype (A3, A11, A31, and A33).

Table 2 Patient characteristics

Characteristics	Patients (N = 100) No.
Age, years	
Median	69
Range	51-92
ECOG performance status	
0	91
1	9
HLA typing	
A24	66
A2	21
A3 supertype	11
A26	2
Baseline PSA, ng/ml	
Median	29.8
Range	0.2-2481
PSADT, months	
Median	2
Range	0.3-36+
Lymphocyte, 1300/μL	
Low	41
High	59
CRP, 3 μg/mL	
Low	53
High	47
SAA, 8 μg/mL	
Low	27
High	76
IL6, 2.4 pg/mL	
Low	84
High	16
Gleason score	
≤7	34
≥8	57
Unknown	9
Site of metastasis	
no	14
Bone only	33
Bone and nodal/organ	40
Nodal/organ	13
Prior chemotherapy	
(-)	60
(+)	40

Abbreviations: PPV, personalized peptide vaccination; ECOG, Eastern Cooperative Oncology Group; HLA, human leucocyte antigen; PSA, prostate-specific antigen; PSADT, PSA doubling time; CRP, C-reactive protein; SAA, serum amyloid A; IL6, interleukin 6.

responses to at least two of the 31 different candidate peptides (Table 1). Any number of previous hormonal therapies was allowed. Patients were required to wait at least four weeks for entry into the study after the completion of prior radiation therapy, chemotherapy, or a change in hormonal therapy. Other inclusion criteria included age ≥ 20 years; Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1; life expectancy of at least 12 weeks; positive status for HLA-A2, -A24, -A3 supertype (–A3, -A11, -A31, and -A33), or -A26; adequate hematologic, hepatic, and renal function; and negative status for hepatitis virus B and C. Exclusion criteria included an acute infection; a history of severe allergic reactions; pulmonary, cardiac, or other systemic diseases; and other inappropriate conditions for enrollment as judged by clinicians.

Study design and treatment

This was a single institution, single arm, open-label, phase II study. The endpoints of this study were primarily safety and feasibility of PPV in patients with CRPC. Secondary endpoints were to assess the PSA kinetics and immune responses. In addition, we identified potential factors for predicting OS and selecting suitable patients for this treatment. This study protocol was approved by Kurume University Ethical Committee. Written informed consent was obtained from all patients before any study procedures.

In this study, 31 peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies [10-18], were employed for vaccination [12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for the HLA-A3 supertype (A3, A11, A31, or A33), and 4 peptides for HLA-A26] (Table 1). All peptides were prepared under conditions of Good Manufacturing Practice using a Multiple Peptide System (San Diego, CA). The selection of 2 to 4 peptides for vaccination to each patient was based on HLA typing and high titer level of peptide-specific IgG to candidate peptides. Each of the selected peptides was mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France) and emulsified in the 5 ml plastic syringe, and a maximum of four peptides of 1.5 ml emulsion (3 mg/peptide) were injected subcutaneously into the lateral thigh area once a week for 6 weeks. The

peptides were re-selected according to peptide-specific IgG levels at every cycle of 6 vaccinations and administered at 2-, 3-, or 4-week intervals until withdrawal of consent or unacceptable toxicity.

Assessment of clinical activity

Patients were monitored at each visit by history and physical examinations. Serum PSA test and routine laboratory studies were performed every 6 vaccinations for any adverse effects. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTCAE Ver3).

All patients underwent relevant radiologic studies and bone scans every 6 months or at the progression of symptoms. PD was defined as radiographic progression evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) criteria [19] or clinical progression.

To assess the PSA response for each patient, percent PSA change from baseline was calculated for each phase of the study (pre- and during vaccination). In addition, PSA doubling time (PSADT) was calculated using all serum PSA values for a specified period, and using a minimum of three PSA values by the formula $\log_2/b$, where b denotes the least square estimate of the linear regression model of the log-transformed PSA values on time. For analytical purposes, negative PSADT estimates and high positive PSADT estimates (>36 months) were censored at 36 months.

To investigate biomarkers for OS that may allow patient selection and prediction of a response to PPV, serum amyloid A (SAA), C-reactive protein (CRP), and interleukin (IL)-6 in plasma at baseline were additionally examined by enzyme-linked immunosorbent assay (ELISA), respectively.

Measurement of humoral and T-cell responses specific to the vaccinated peptides

To study the humoral responses specific to the vaccinated peptides, peptide-specific IgG levels were measured by a Luminex system (Luminex, Austin, TX), as reported previously [20]. If the total titers of selected peptide-specific IgG in any cycles of post-vaccination plasma were more than 2-fold higher than those in the pre-vaccination plasma, the changes were considered to be a positive response.

Although T-cell subsets using flowcytometry was not analyzed in this study, T-cell responses specific to the vaccinated peptides were evaluated by IFN-γ ELISPOT assay using peripheral blood mononuclear cells (PBMCs), as reported previously [18]. Peptide-specific T-cell responses were evaluated by the differences between the numbers of spots per 10^5 x PBMCs in response to the vaccine peptides and those to the control peptide at pre- and 6th vaccination; at least 2-fold more spots at the 6th vaccination than at pre-vaccination was considered positive.

Statistical analysis

All patients who received more than 6 vaccinations were considered evaluable for tumor response, and all patients entered were included in the survival analysis. Data were

Table 3 Adverse events during peptide vaccination

	Grade 1	Grade 2	Grade 3	Total
Injection site reaction	73	24	13	43
Constitutional symptoms				
Bone pain	16	14	13	43
Appetite loss	29	5	1	35
Fatigue	23	11	0	34
Edema peripheral	10	3	0	10
Blood/bone marrow				
Lymphocytopenia	17	13	5	35
Anemia	7	7	16	30
White blood cell count decreased	6	6	5	17
Laboratory				
Hypoalbuminemia	27	13	0	40
ALP increased	20	8	6	34
AST increased	24	4	1	29
Hyponatremia	24	1	0	25
ALT increased	13	2	1	16
Blood triglycerides increased	10	2	0	12
Creatinine increased	6	1	2	8

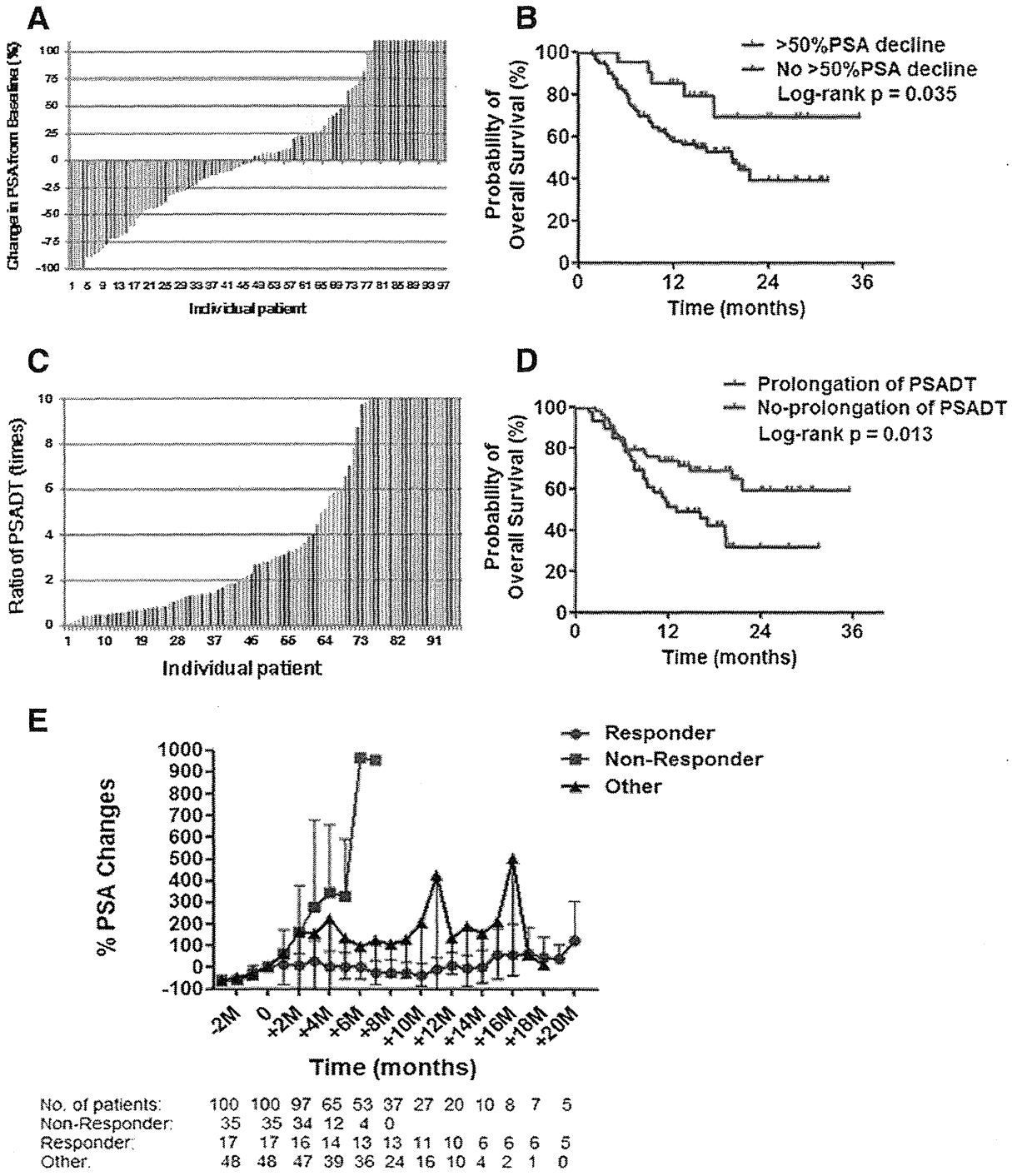

Figure 1 PSA kinetics and overall survival. **(A)** Waterfall plot showing the maximal PSA changes (%) from baseline during personalized peptide vaccination (PPV) at any time point. **(B)** Overall survival by >50% PSA decline. **(C)** The ratio of PSADT changes for each patient pre- and during PPV is plotted. The ratio of PSADT changes was calculated by dividing PSADT during treatment by pre-treatment PSADT. A ratio greater than 2 indicates prolongation of PSADT. **(D)** Overall survival by prolongation of PSDT. **(E)** Longitudinal average PSA changes (%) before and during PPV. Green histograms: Responder group (alive for more than 20 months). Red histograms: Non-responder group (death within 12 months). Gray histograms: Other group.

analyzed at the end of November, 2012 using commercially available computer software. The Student's t-test and the chi-square test were used to compare quantitative and categorical variables, respectively. Survival was calculated from the date of first treatment until the date of any cause of death. Patients lost to follow-up were censored at the last known date of survival. The Kaplan-Meier method was used to estimate actuarial survival curves, and groups were compared using a log-rank test. Cox proportional hazards regression model was used for univariate and multivariate analyses to identify factors that had a significant impact on survival. All baseline parameters in the survival and proportional hazards regression analysis were analyzed as dichotomous variables using median or cut-off values. A two-sided significance level of 5% was considered statistically significant.

Results

Characteristics of the patients

Between April 2009 and August 2011, 100 patients with CRPC were enrolled in this trial at Kurume University Hospital. All 100 patients received at least one vaccination with a median of 16 vaccinations (range, 1 to 40) and were included in the safety assessment and survival analysis. Three patients did not complete 6 vaccinations (1 cycle) and were excluded from the assessment of PSA response and immune responses. The reason for these failures to complete 6 vaccinations was withdrawal of consent. The

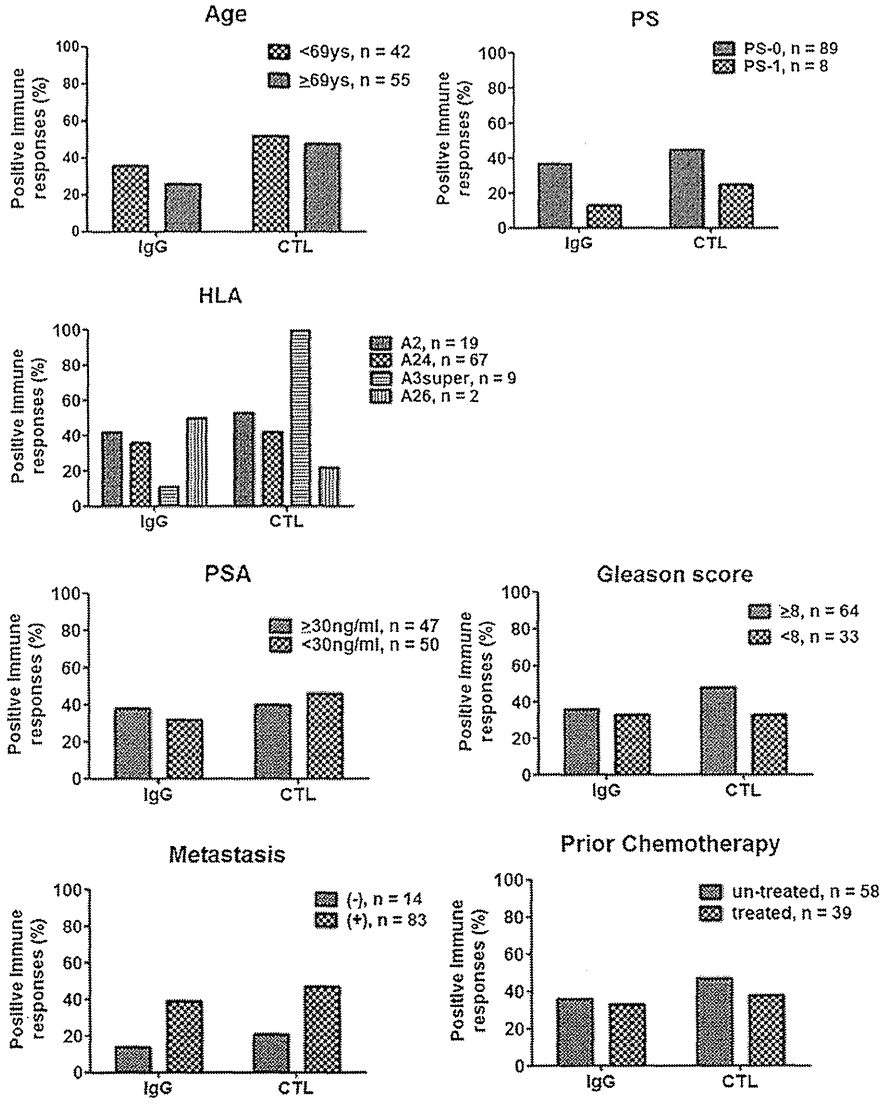

Figure 2 Positive immune responses of IgG and CTL based on baseline characteristics.

median age of participants was 69 years (range, 51 to 92 years), and the ECOG performance status was 0 in 91of the patients and 1 in the remaining 9. The median PSA and pre-vaccination PSADT at the entry to the study was 29.8 ng/ml (range, 0.2 to 2481 ng/ml) and 2 months (range, 0.3 to 36+ months), respectively. Fifty-seven patients had a Gleason score of ≥ 8 and 86 patients had metastasis. All patients had experienced progression after androgen deprivation therapy as an initial or secondary therapy. Forty patients had received docetaxel based chemotherapy with a median cycle of 6.5 as a third line treatment. Baseline patient characteristics are shown in Table 2.

Adverse events

The overall toxicities are shown in Table 3. The most frequent adverse events were local redness and swelling at injection sites, bone pain, hypoalbuminemia, lymphocytopenia, appetite loss, fatigue, increased ALP, and anemia, which were grade 1 or 2 in most cases. There were no grade 4 toxicities and no treatment-related deaths. A total of 51 grade 3 toxicities including anemia, bone pain, increased ALP, lymphocytopenia, decreased white blood cells, increased creatinine, injection site reaction, and increased AST and ALT were observed during the study. All of these severe adverse events were concluded to be not directly associated with the vaccinations, but with cancer progression or other causes by the independent safety evaluation committee in this trial.

Clinical outcome

Forty-eight (49%) patients exhibited some decrease in PSA from baseline, ranging from 1.9% to 99.6% (Figure 1A). Confirmed ≥50% PSA decline at any point during PPV was observed in 21 patients (22%), with a median time of 4 months to ≥50% PSA decline and a median duration of ≥50% PSA decline of 3 months. Delayed PSA response was observed. Patients with ≥50% PSA decline during PPV showed longer survival than remaining patients ($p = 0.035$) (Figure 1B). The median estimated PSADT pre- and during PPV were 2 and 3.89 months, respectively. Fifty-four (56%) patients displayed at least 2-fold increase over the pre-treatment PSADT (range, 2.1- to 75-fold), and these patients with a prolongation of PSADT showed longer survival than patients without a prolongation of PSADT ($p = 0.013$) (Figure 1C and D). To compare the difference in PSA responses with clinical outcomes, patients were divided into three groups: responder group with survival longer than 20 months after PPV, non-responder group with death within 12 months after PPV, and another group with the remaining patients. Average% PSA changes in the responder group were significantly lower than those in the non-responder group at 2 to 5 months ($p < 0.005$) and those in the other group at 5 to 10 months ($p < 0.005$) during the PPV. In addition, average% PSA changes in the responder group showed a trend of PSA plateau. Average% PSA changes from baseline among three groups before and during PPV are shown in Figure 1E.

There was no complete response or partial response in terms of measurable disease. The median time to disease progression, as defined by clinical and/or radiologic criteria, was 10.9 months (95% CI, 6 to 19 months). At the time of analysis with a median follow-up of 18 months (95% CI, 14.1 to 24 months), 64 deaths had occurred. Median survival time was 18.8 months (95% CI, 14.9 to 28.6 months) in all patients. Median survival time in chemotherapy naive patients and in patients after docetaxel chemotherapy were 21.6 months and 11.6 months, respectively.

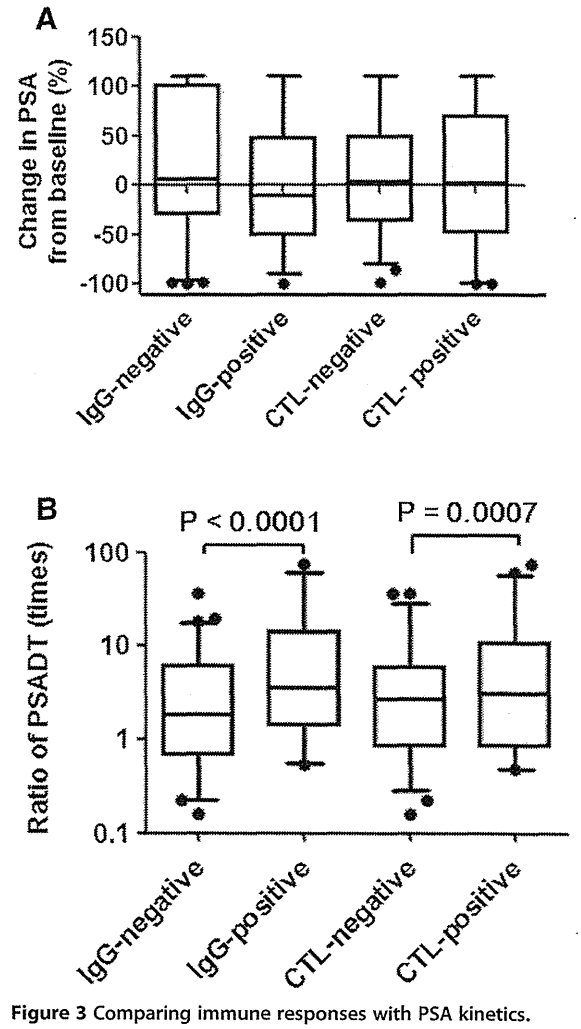

Figure 3 Comparing immune responses with PSA kinetics. **(A)** Change in PSA from baseline (%) based on immune responses. **(B)** Ratio of PSADT based on immune responses.

Immunological response

The number of selected peptides were 4 peptides in 62 patients, 3 peptides in 17 patients and 2 peptides in 21 patients at the first screening. Same peptide at the first screening were only selected in 29 of 97 (30%) patients at second screening and in 10 of 66 (15%) patients at the third screening, remaining patients received at least 1 different peptide during the study. The most frequently selected peptides were Lck486 (40 patients), CypB129 (31 patients), PAP213 (24 patients), SART2-93 (21 patients), PSA248 (20 patients), Lck488 (17 patients) and WHSC2-123 (16 patients) at the first screening. All 31 peptides were selected at any screening in the study.

Total IgG responses specific to the vaccinated peptide were augmented in 42 of 97 (43%) patients, 62 of 66 (94%) patients, 36 of 36 (100%) patients, 16 of 16 (100%) patients, and 7 of 7 (100%) patients at the 6th, 12th, 18th, 24th, and 30th vaccinations, respectively. Finally, positive IgG responses during PPV were observed in 76/97 (79%) patients. PBMCs from 97 patients were available for IFN-γ Elispot assay at the pre- and 6th vaccination. Peptide-specific T-cell responses were detectable in 42 patients (43%) at the 6th vaccination. There was no obvious correlation between IgG and CTL responses. Positive immune responses of both IgG and CTL based on baseline characteristics including age, PS, HLA typing, PSA, Gleason score, presence of metastasis and prior chemotherapy are shown in Figure 2. There was no difference in positive immune responses among baseline characteristics. In comparing immune responses with PSA kinetics, although average PSA changes did not correlate with immune responses, average ratio of PSADT was significantly higher in patients with positive IgG (8 vs. 4, $p < 0.0001$) and CTL (8.8 vs. 6.1, $p = 0.0007$) responses (Figure 3).

Survival analysis

Cox proportional hazards regression analysis was performed to determine factors that would predict disease death (Table 4). Univariate Cox analysis showed that good performance status ($p < 0.0001$), positive IgG response ($p < 0.0001$), low CRP ($p = 0.012$), prolongation of PSADT ($p = 0.018$), low PSA ($p = 0.004$), prior chemotherapy status ($p = 0.037$), positive T-cell response ($p = 0.039$), and presentation of ≥50% PSA decline ($p = 0.046$) were significantly associated with survival.

The factors showing p less than 0.05 in the univariate analysis were included in multivariate analysis of the model. Finally, positive IgG response ($p = 0.001$) and prolongation of PSADT ($p = 0.004$) during PPV, as well as baseline good performance status ($p = 0.004$), low CRP levels ($p = 0.006$), and low PSA levels ($p = 0.008$), were significantly favorable factors for OS (Table 4).

Discussion

As observed in several clinical trials, immunotherapy can induce novel patterns of antitumor responses distinct from those of chemotherapy, which are consequently not captured by the WHO or RECIST criteria [5]. On the other hand, there is debate regarding the utility of PSA changes, especially with immunotherapy, and the PSA Working Group 2 has advocated using radiographic progression-free survival as a preferred endpoint for phase

Table 4 Cox proportional hazards regression analysis of association between potential factors and death after PPV in the 100 CRPC patients

Factors	Cut-offs[a]	Univariate			Multivariate		
		p value	Hazard ratio	95% CI	p value	Hazard ratio	95% CI
IgG response	Positive vs. negative	<0.0001	0.19	0.101-0.355	0.001	0.272	0.125-0.592
ECOG performance status	0 vs. 1	<0.0001	0.073	0.031-0.174	0.004	0.179	0.056-0.569
CRP	Low (<3000 ng/mL) vs. high	0.012	0.461	0.252-0.842	0.006	0.389	0.199-0.759
PSADT	Increase (2 times) vs. no	0.018	0.477	0.258-0.881	0.004	0.357	0.176-0.725
PSA	Low (<30 ng/mL) vs. high	0.004	0.407	0.221-0.749	0.008	0.361	0.171-0.762
Prior chemotherapy	Untreated vs. treated	0.037	0.536	0.298-0.962	0.329	0.695	0.335-1.445
T-cell response	Positive vs. negative	0.039	0.51	0.269-0.967	0.273	0.679	0.340-1.357
>50% PSA decline	Positive vs. negative	0.046	0.387	0.152-0.984	0.553	0.733	0.263-2.042
Number of lymphocytes	High (>1300/μL) vs. low	0.054	0.562	0.313-1.009	-	-	-
IL6	Low (<2.4 pg/mL) vs. high	0.057	0.491	0.236-1.021	-	-	-
Pts. age	Low (<69 years) vs. high	0.186	0.666	0.364-1.218	-	-	-
Gleason score	Low (<8) vs. high	0.623	1.162	0.637-2.128	-	-	-
SAA	Low (<8 μg/mL) vs. high	0.709	0.875	0.433-1.767	-	-	-

Of the 100 men, 64 died.
[a]Lymphocyte, PSA, and patient age are based on median values.
Abbreviations: PPV, personalized peptide vaccination; CRPC, castration-resistant prostate cancer; CI, confidence intervals; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen; PSADT, PSA doubling time; CRP, C-reactive protein; SAA, serum amyloid A; IL6, interleukin 6.

II trials [21]. Others have argued that changes in PSADT may be a marker of drug effect, understanding that shorter PSADT corresponds to worse prognosis and, thus, a favorable change in PSADT suggests drug activity [22,23]. However, clinical trials of recently developed drugs, such as sipuleucel-T [6], cabazitaxel [24], and abiraterone acetate [25], for the treatment of progressive CRPC patients did not analyze the usefulness of PSADT as a surrogate marker of response in CRPC patients. In the current study, we attempted careful and stringent collection of multiple PSA values in order to calculate PSADT changes before and during PPV accurately. While delayed PSA responses were observed, we did see a statistically significant increase in PSADT. Importantly, patients with prolongation of PSADT showed statistically longer survival ($p = 0.018$). These results suggest that the development of late immune responses is associated with changes in PSADT.

The evaluation of T-cell immune responses to target self antigens after vaccine clinical trials presents several challenges. Antigen-specific T-cells can be evaluated by their peptide target specificity, proliferative capacity, cytokine secretion, cytolytic activity, and membrane markers of activation. At present, the best measure of antigen-specific T-cells is unknown, as is the optimal time to evaluate immune responses. In our current analysis, we evaluated both humoral responses determined by peptide-specific IgG levels using a Luminex system and antigen-specific CD8+ T-cell responses by using IFN-γ ELISPOT assays, to provide a more direct quantitative assessment after immunization. Delayed 50% PSA decline and prolongation of PSADT were observed in patients with positive IgG and T-cell respkonses, and these immune responses were associated with OS. These results suggest that further immunological analysis at multiple time points might be needed to determine whether T-cell response or the development of late immune responses is associated with clinical responses.

Cancer vaccinations do not always extract good immune and/or clinical responses in vaccinated patients. This study showed that IgG responses and prolongation of PSADT during PPV, along with baseline performance status, CRP, and PSA levels, were well correlated with OS in patients with CRPC treated by PPV. These results suggest that risk stratification based on these factors could be helpful for estimating the OS in patients with CRPC treated by immunotherapy.

Despite these encouraging observations, the current study must be interpreted as hypothesis-generating due to several limitations. This single-arm phase II study without a concurrent control arm did not allow estimation of the potential clinical or immune effects of this treatment. Another potential limitation of this study regarding OS is the lack of treatment data after the treatment phase of the trial. Imbalances due to chance may have occurred in treatments after progression. However, only docetaxel has been shown to affect survival in this population of patients, and only by a few months. The median survival of 18.8 months (95% CI, 14.1 to 24 months) observed in this study surpassed the survival that was observed from docetaxel-based clinical trials in a similar population by TAX-327 (median survival, 19.2 months) and South West Oncology Group 9906 (median survival, 17.5 months) [7,8]. Thus, we think it unlikely that a potential imbalance in post-study treatments could explain the survival results.

Conclusions

This study showed that PPV in patients with CRPC was active and well tolerated, improving survival with immune responses, delayed PSA responses, and prolongation of PSADT. Further randomized trials are needed to confirm these preliminary results.

Abbreviations

CR: Complete response; CT: Computed tomography; CRPC: Castration-resistant prostate cancer; CTL: Cytotoxic T lymphocytes; EOCG: Eastern cooperative oncology group; HLA: Human leukocyte antigen; IFN- γ: Interferon-γ; IgG: Immunoglobulin G; OS: Overall survival; PBMC: Peripheral blood mononuclear cells; PPV: Personalized peptide vaccination; PSA: Prostate specific antigen; PSADT: Prostate specific antigen doubling time.

Competing interests

K. Itoh is a consultant/advisory board member in Green Peptide Co. A. Yamada is a part-time executive of Green Peptide Co. No potential conflicts of interest were disclosed by other authors.

Authors' contributions

NM conceived of the study, and participated in its design and coordination and drafted the manuscript. KI and AY participated in its design and helped to draft the manuscipt. FM, SS, RO performed the clinical trial and collected the data. SM and TS carried out the immunoassays. All authors read and approved the final manuscript.

Details of all funding sources

This study was supported in part by Grants-in-Aid (KAKENHI) (no.22591782 to M. Noguchi), and by the grants from the Regional Innovation Cluster Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Author details

[1]Clinical Research Division of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan. [2]Departments of Urology, Kurume University School of Medicine, Kurume, Japan. [3]Immunology and Immunotherapy, Kurume University School of Medicine, Kurume, Japan. [4]Cancer Vaccine of the Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume, Japan.

Received: 7 June 2013 Accepted: 3 September 2013
Published: 30 December 2013

References

1. Vollmer RT, Dawson NA, Vogelzang NJ: **The dynamics of prostate specific antigen in hormone refractory prostate carcinoma: an analysis of cancer and leukemia group B study 9181 of megestrol acetate.** *Cancer* 1998, 83:1989–1994.
2. Armstrong AJ, Garrett-Mayer E, Ou Yang YC, Carducci MA, Tannock I, de Wit R, Eisenberger M: **Prostate-specific antigen and pain surrogacy analysis in metastatic hormone-refractory prostate cancer.** *J Clin Oncol* 2007, 25:3965–3970.
3. Scher HI, Kelly WM, Zhang ZF, Ouyang P, Sun M, Schwartz M, Ding C, Wang W, Horak ID, Kremer AB: **Post-therapy serum prostate-specific antigen**

level and survival in patients with androgen-independent prostate cancer. *J Natl Cancer Inst* 1999, 91:244–251.
4. Petrylak DP, Ankerst DP, Jiang CS, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED: Evaluation of prostate-specific antigen declines for surrogacy in patients treated on SWOG 99–16. *Natl Cancer Inst* 2006, 98:516–521.
5. Hoos A, Eggermont AM, Janetzki S, Hodi FS, Ibrahim R, Anderson A, Humphrey R, Blumenstein B, Old L, Wolchok J: Improved endpoints for cancer immunotherapy trials. *J Natl Cancer Inst* 2010, 102:1388–1397.
6. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF: IMPACT Study Investigators: Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010, 363:411–422.
7. Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Théodore C, James ND, Turesson I, Rosenthal MA, Eisenberger MA: TAX 327 Investigators: Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004, 351:1488–1490.
8. Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED: Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004, 351:1513–1520.
9. Stein WD, Gulley JL, Schlom J, Madan RA, Dahut W, Figg WD, Ning YM, Arlen PM, Price D, Bates SE, Fojo T: Tumor regression and growth rates determined in five intramural NCI prostate cancer trials: the growth rate constant as an indicator of therapeutic efficacy. *Clin Cancer Res* 2011, 17:907–917.
10. Noguchi M, Kobayashi K, Suetsugu N, Tomiyasu K, Suekane S, Yamada A, Itoh K, Noda S: Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. *Prostate* 2003, 57:80–92.
11. Noguchi M, Itoh K, Suekane S, Yao A, Suetsugu N, Katagiri K, Yamada A, Yamana H, Noda S: Phase I trial of patient-oriented vaccination in HLA-A2-positive patients with metastatic hormone-refractory prostate cancer. *Cancer Sci* 2004, 95:77–84.
12. Noguchi M, Itoh K, Suekane S, Morinaga A, Sukehiro A, Suetsugu N, Katagiri K, Yamada A, Noda S: Immunological monitoring during combination of patient-oriented peptide vaccination and estramustine phosphate in patients with metastatic hormone refractory prostate cancer. *Prostate* 2004, 60:32–45.
13. Noguchi M, Itoh K, Yao A, Mine T, Yamada A, Obata Y, Furuta M, Harada M, Suekane S, Matsuoka K: Immunological evaluation of individualized peptide vaccination with a low dose of estramustine for HLA-A24+ HRPC patients. *Prostate* 2005, 63:1–12.
14. Noguchi M, Mine T, Yamada A, Obata Y, Yoshida K, Mizoguchi J, Harada M, Suekane S, Itoh K, Matsuoka K: Combination therapy of personalized peptide vaccination and low-dose estramustine phosphate for metastatic hormone refractory prostate cancer patients: an analysis of prognostic factors in the treatment. *Oncol Res* 2007, 16:341–349.
15. Noguchi M, Kakuma T, Uemura H, Nasu Y, Kumon H, Hirao Y, Moriya F, Suekane S, Matsuoka K, Komatsu N, Shichijo S, Yamada A, Itoh K: A randomized phase II trial of personalized peptide vaccine plus low dose estramustine phosphate (EMP) versus standard dose EMP in patients with castration resistant prostate cancer. *Cancer Immunol Immunother* 2010, 59:1001–1009.
16. Noguchi M, Uemura H, Naito S, Akaza H, Yamada A, Itoh K: A phase I study of personalized peptide vaccination using 14 kinds of vaccine in combination with low-dose estramustine in HLA-A24-positive patients with castration-resistant prostate cancer. *Prostate* 2011, 71:470–479.
17. Matsumoto K, Noguchi M, Satoh T, Tabata K, Fujita T, Iwamura M, Yamada A, Komatsu N, Baba S, Itoh K: A phase I study of personalized peptide vaccination for advanced urothelial carcinoma patients who failed treatment with methotrexate, vinblastine, adriamycin and cisplatin. *BJU Int* 2011, 108:831–838.
18. Yoshiyama K, Terazaki Y, Matsueda S, Shichijo S, Noguchi M, Yamada A, Mine T, Ioji T, Itoh K, Shirouzu K, Sasada T, Takamori S: Personalized peptide vaccination in patients with refractory non-small cell lung cancer. *Int J Oncol* 2012, 24:795–801.
19. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG: New guidelines to evaluate the response to treatment in solid tumor: 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, 92:205–216.
20. Komatsu N, Shichijo S, Nakagawa M, Itoh K: New multiplexed flow cytometric assay to measure anti-peptide antibody: a novel tool for monitoring immune responses to peptides used for immunization. *Scand J Cin Lab Invest* 2004, 64:1–11.
21. Scher HI, Halabi S, Tannoch I, Morris M, Sternberg CN, Carducci MA, Eisenberger MA, Higano C, Bubley GJ, Dreicer R, Petrylak D, Kantoff P, Basch E, Kelly WK, Figg WD, Small EJ, Beer TM, Wilding G, Martin A, Hussain M, Prostate Cancer Clinical Trials Working Group: Design and end points of clinical trials for patients with progressive prostate cancer and castrate levels of testosterone: recommendations of the Prostate Cancer Clinical Trials Working Group. *J Clin Oncol* 2008, 226:1148–1159.
22. McNeel DG, Dunphy E, Davies JG, Frye TP, Johnson LE, Staab MJ, Horvath DL, Straus J, Alberti D, Marnocha R, Liu G, Eickhoff JC, Wilding G: Safety and immunological efficacy of a DNA vaccine encoding prostatic and phosphatase in patients with D0 prostate cancer. *J Clin Oncol* 2009, 27:4047–4054.
23. Sweeney C, Liu G, Yiannoutsos C, Kolesar J, Horvath D, Staab MJ, Fife K, Armstrong V, Treston A, Sidor C, Wilding G: A phase II, multicenter, randomized, double-blind, safety trial assessing the pharmacokinetics, pharmacodynamics, and efficacy of oral 2-methoxyestradiol capsules in hormone-refractory prostate cancer. *Clin Cancer Res* 2005, 11:6625–6633.
24. De Bono JS, Oudard S, Ozguroglu M, Hansen S, Machiels JP, Kocak I, Gravis G, Bodrogi I, Mackenzie MJ, Shen L, Roessner M, Gupta S, Sartor AO: TROPIC Investigators: Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomized open-label trial. *Lancet* 2010, 376:1147–1154.
25. De Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones RJ, Goodman OB Jr, Saad F, Staffurth JN, Mainwaring P, Harland S, Flaig TW, Hutson TE, Cheng T, Patterson H, Hainsworth JD, Ryan CJ, Sternberg CN, Ellard SL, Fléchon A, Saleh M, Scholz M, Efstathiou E, Zivi A, Bianchini D, Loriot Y, Chieffo N, Kheoh T, *et al.*: Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med* 2011, 364:1995–2005.

doi:10.1186/1471-2407-13-613
Cite this article as: Noguchi *et al.*: A phase II trial of personalized peptide vaccination in castration-resistant prostate cancer patients: prolongation of prostate-specific antigen doubling time. *BMC Cancer* 2013 13:613.

Send Orders for Reprints to reprints@benthamscience.net

2332 *Current Medicinal Chemistry*, 2014, *21*, 2332-2345

Personalized Peptide Vaccine for Treatment of Advanced Cancer

Tetsuro Sasada[1,*], Akira Yamada[2], Masanori Noguchi[2,3] and Kyogo Itoh[1]

[1]*Department of Immunology and Immunotherapy, Kurume University School of Medicine, Kurume, Japan;* [2]*Research Center for Innovative Cancer Therapy, Kurume University, Kurume, Japan;* [3]*Department of Urology, Kurume University School of Medicine, Kurume, Japan*

Abstract: The field of cancer immunotherapy has moved forward drastically in the past 20 years, since many tumor-associated antigens (TAA) have been identified. Although various approaches for therapeutic cancer immunotherapies, including peptide-based vaccines, have been developed and clinically examined, the complexity and diversity of tumor cell characteristics and host immune cell repertoires seem to limit the therapeutic efficacy of this treatment modality. Considering the diversity of immune responses against heterogeneous tumor cells, tailored selections of vaccine antigens appropriate for individual patients could be a rational approach for developing effective cancer vaccines. We have developed a novel immunotherapeutic approach called personalized peptide vaccine (PPV), in which a maximum of four human leukocyte antigen (HLA)-matched vaccine peptides were selected based on the pre-existing host immunity before vaccination. We conducted a series of phase I and phase II clinical trials of PPV, which have shown better antigen-specific immune responses and promising clinical outcomes in patients with various types of advanced cancers. Further randomized phase III trials would be recommended to prove the clinical benefits of PPV. In addition, novel biomarkers for selecting patients who would benefit most from PPV remain to be identified.

Keywords: Advanced cancer, biomarker, cancer immunotherapy, clinical trial, peptide epitope, personalized peptide vaccine.

1. INTRODUCTION

The field of cancer immunology and immunotherapy has moved forward drastically in the past 20 years, since many different tumor-associated antigens (TAA) have been identified [1-5]. Various approaches for therapeutic cancer immunotherapies have been developed and clinically examined, including cancer vaccines using tumor cells, proteins, peptides, viral vectors, DNA, or dendritic cells, and great advances have been made in the clinical efficacy of cancer immunotherapy [1-5]. Notably, two novel immunotherapeutic agents have recently been approved by the US Food and Drug Administration (FDA) for patients with advanced cancer [6, 7]. In April 2010, sipuleucel-T (Provenge; Dendreon Corporation, Seattle, WA), an autologous antigen-presenting cell (APC) product designed to stimulate antigen-specific immune responses against human prostatic acid phosphatase (PAP), was approved for the first time by the US FDA for the treatment of patients with castration-resistant prostate cancer (CRPC). The FDA granted this approval after treatment with sipuleucel-T improved overall survival by 4.1 months [mean survival time (MST), 25.8 months vs 21.7 months] in the largest phase 3 randomized controlled trial (the IMPACT study) [6]. In addition, in March 2011 the FDA approved ipilimumab (Yervoy; Bristol-Meyers Squibb, Princeton, NJ), an immunomodulating antibody that blocks cytotoxic T-lymphocyte antigen 4 (CTLA-4), one of the immune checkpoint molecules in T cells, to treat advanced melanoma patients. In the phase III randomized controlled trial, this agent resulted in a 3-month improvement in overall survival with a disease control rate of 28.5%, where 60% of the responding patients maintained disease control for more than 2 years [7].

Moreover, there have been promising results in immunotherapeutic approaches to the treatment of various types of advanced cancers, although they have not yet been officially approved. For example, blocking antibodies against a T-cell co-inhibitory receptor, programmed death 1 (PD-1), and one of its ligands, PD-ligand 1 (PD-L1), which have been reported to contribute to tumor cell escape from host immune surveillance, have shown feasible results against various types of cancers [8, 9]. Topalian *et al.* demonstrated that anti-PD-1 antibody revealed objective responses in approximately 20 to 25% of patients with non-small-cell lung cancer (NSCLC), melanoma, or renal-cell cancer [8]. Brahmer *et al.* reported that anti-PD-L1 antibody, which blocks the interaction between PD-1 and PD-L1, could induce durable tumor regression (objective response rates of 6% to 17%) and prolonged stabilization of disease (12% to 41% of patients at 24 weeks) in patients with advanced cancers, including NSCLC, melanoma, and renal-cell cancer [9]. Currently, these promising advancements are generating great optimism and heightened enthusiasm for the further development of cancer immunotherapies.

In addition to these significant advances, many other clinical trials of cancer immunotherapies have been under way to show beneficial therapeutic effects in patients compared to existing treatments [1-5]. In this review, we discuss the recent advances in peptide-based cancer vaccines. In par-

*Address correspondence by this authors at the Department of Immunology and Immunotherapy, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan; Tel: +81-942-31-7551; Fax: +81-942-31-7699; E-mail: tsasada@med.kurume-u.ac.jp.

1875-533X/14 $58.00+.00

ticular, we describe the details of our novel immunotherapeutic approach, called the personalized peptide vaccine (PPV), which has demonstrated promising results for advanced cancer patients in a series of clinical trials.

2. PERSONALIZED PEPTIDE VACCINE (PPV)

In 1991, Boon *et al.* for the first time reported a cDNA-expression cloning technique to identify TAA [10]. Subsequently, serologic analysis of recombinant cDNA expression libraries (SEREX), another technique for detecting TAA using autologous antibodies, was introduced for the identification of genes recognized by the host immune system [11]. Such advancement of molecular biological and immunological techniques has helped identify a large number of TAA and peptide epitopes applicable as cancer vaccines [12-14]. Since 1995, when Hu *et al.* reported the first clinical trial of the vaccination of a peptide derived from melanoma antigen gene-1 (MAGE-1) [15], many clinical trials of peptide vaccines have been reported [16, 17]. In earlier stages of clinical trials of peptide vaccines, one to several human leukocyte antigen (HLA) class I-restricted peptides emulsified with Montanide ISA51, a clinical grade of Freund's incomplete adjuvant, were employed. Although the early phase clinical trials demonstrated the feasibility and good toxicity profile of this approach, most of the late-phase randomized trials, other than few exceptions [18], failed to show beneficial therapeutic effects in patients compared to existing treatments [16, 17]. Therefore, a variety of new types of peptide-based vaccines have been developed [19, 20] (Fig. **1**). We first discuss our novel peptide-based approach, PPV, in which multiple vaccine antigens appropriate for each patient are selected from a panel of vaccine candidates based on pre-existing host immunity.

2.1. Rationale for Personalized Selections of Vaccine Peptides

Cancer patients possess anti-tumor immunity, which may depend strongly on both the tumor cell characteristics and the immunological status of the host [21-24]. The anti-tumor immunity might differ widely among individuals, since the tumor cell characteristics and the host immune cell repertoires are quite diverse and heterogeneous among patients, even among those with identical HLA types and the same pathological types of cancer. Nevertheless, before patients are enrolled in clinical trials of cancer vaccines, the expressions of vaccine antigens in tumor cells are sometimes confirmed, but the immunological statuses of the hosts are rarely evaluated. Considering the complexity and diversity of the host immune cell repertoires, it is likely that vaccine antigens that are selected and administered without considering the host immunological status might not efficiently induce beneficial anti-tumor immune responses [24]. Since, in most clinical trials of therapeutic cancer vaccines, common antigens are employed for vaccination independently of the immunological status of patients [16, 17], the low clinical efficacies might be explained at least in part by mismatches between the vaccine antigens and the host immune cell repertoires.

To evaluate the host immune cell repertoires, we examine patients' pre-existing immunity to a panel of vaccine candidates before vaccination and select appropriate vaccine antigens with immunological memory in each patient [25]. Vaccine antigens, to which patients already possess antigen-specific immunological memory, are expected to cause quick and strong secondary immune responses after vaccination (Fig. **2**). In contrast, vaccinations with inadequate antigens without immunological memory could not easily provide

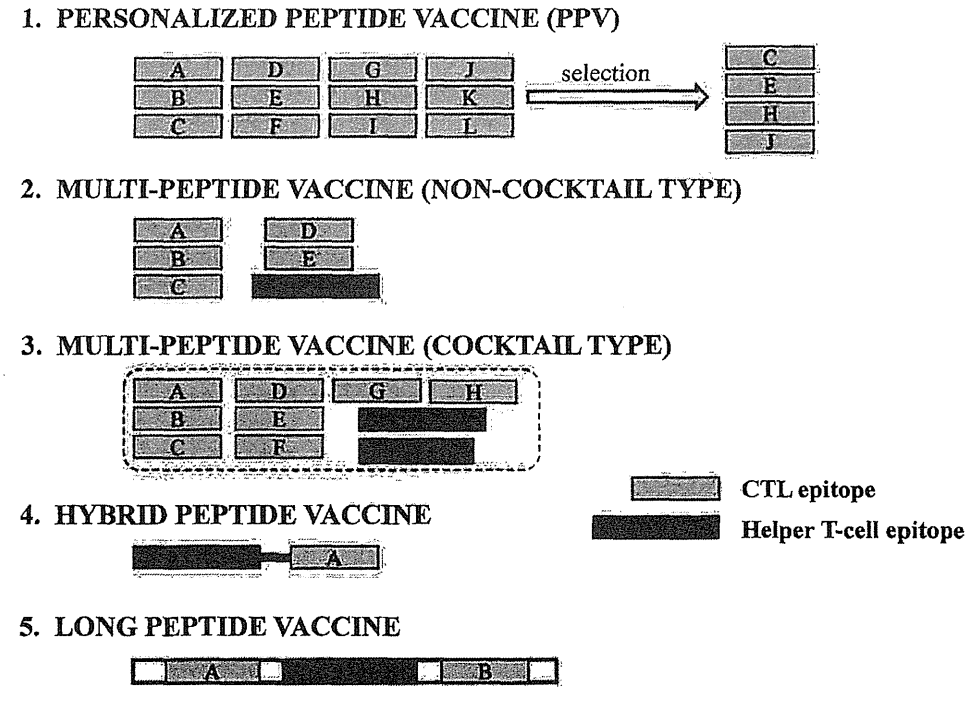

Fig. (1). Recent development of new types of peptide-based vaccines. Examples of new types of peptide-based vaccines are shown. Gray and black boxes indicate CTL and helper T-cell epitopes, respectively.