Significant Association of Caveolin-1 and Caveolin-2 with Prostate Cancer Progression

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Abstract. *Background/Aim: Up-regulation of caveolin (CAV)-1 is associated with aggressive prostate cancer. Recently, it has been inferred that CAV2, a co-factor sub-type of CAV1, cross-talks with CAV1 and promotes tumor growth. We previously reported that plasma CAV1 levels are elevated in patients with castration-resistant prostate cancer (CRPC), but not in hormone-sensitive prostate cancer (non-CRPC), implying that CAV1 may be a therapeutic target for CRPC. However, a correlation of CAV1 and CAV2 expression in PC has not yet been reported. Herein, we analyzed associations between PC progression and plasma CAV1 and -2 in Japanese men, and expression of CAV1 and -2 in PC3 (CRPC) and LNCaP (non-CRPC) cell lines. Materials and Methods: We investigated plasma samples from 36 patients with CRPC and 22 with non-CRPC. We used enzyme-linked immunosorbent assay (ELISA) to determine plasma levels of CAV1 and -2, and examined correlations with clinicopathological characteristics such as Gleason grade and clinical T stage. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to evaluate CAV1 and CAV2 mRNA in PC cell lines. We also introduced CAV1- and CAV2-specific small interfering (siRNA) into PC3 cells to knock-down (KD) both molecules, and examined its influence on the expression of these genes between PC3 CAV1 and -2 KD cells and control cells. Results: Plasma CAV1 and -2 levels in patients with CRPC were significantly higher than in those with non-CRPC (CAV1, $p=0.003$; CAV2, $p<0.001$). Plasma levels of CAV1 and -2 were significantly correlated ($p<0.001$). However, we did not find any significant relationship between CAV1 or CAV2 expression and clinicopathological factors. ELISA and real-time qRT-PCR showed that both proteins and mRNAs in PC3 cells were significantly over-expressed compared to LNCaP cells ($p<0.001$). In PC3 CAV1 KD cells, expression of CAV2 was suppressed and confirmed the linkage of CAV2 KD and suppression of CAV1 expression. Conclusion: There was a significant correlation between plasma CAV-1 and -2 levels and progression of PC. CAV1 and -2 were highly expressed in the PC3 compared to the LNCaP cell line. Our findings support the potential of these molecules as therapeutic targets for CRPC.*

Prostate cancer (PC) is the sixth leading cause of cancer-related death among Japanese men (1), usually from metastatic disease. Understanding the mechanisms that underlie the progression of PC will facilitate the development of biomarkers and novel therapeutic strategies to control this devastating malignancy.

Caveolin (CAV)-1 and CAV2, encoded by their respective genes, are major structural components of the caveolae that are co-expressed and form a hetero-oligomeric complex in many cell types, with particularly high levels in adipocytes (2). CAV1 expression is increased in cancer of the prostate (3-5), pancreas (6, 7), colon (8, 9), breast (10) and esophagus (11, 12), thus suggesting it plays a positive role in tumor progression. Paradoxically, CAV1 expression is decreased in lung (13), colon (14, 15), ovarian (16), breast (17, 18) and thyroid (19) cancer. These data imply that CAV1 has multiple activities in cancer depending on its interaction with other signaling molecules and the specific cell type or tissue in which it is expressed. Therefore, whether CAV1 promotes or suppresses tumor progression remains controversial.

CAV2 has similar distribution and tissue expression to CAV1, and is also an accessory protein that functions in conjunction with CAV1. CAV2 expression is increased in breast cancer (10), whereas is reduced in lung (13), breast (10, 17) and thyroid cancer (19). However, the clinical significance of CAV2 has been less extensively studied than that of CAV1. Therefore, in the present study, we examined plasma CAV1 and -2 levels and analyzed them with clinical data by enzyme-linked immunosorbent assay (ELISA). In addition, the expression of CAV1 and -2 was investigated

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using PC cell lines PC3 (castration-resistant PC; CRPC) and LNCaP (hormone-sensitive; non-CRPC) by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and ELISA to clarify the significance of CAV1 and -2.

Materials and Methods

Study participants. We retrospectively obtained clinical data from the medical records of 58 Japanese men with PC, including 36 patients with CRPC and 22 with pre-treatment PC as non-CRPC, treated at the Department of Urology, Miyazaki Medical University Hospital and related hospitals between August 2011 and October 2013. All patients were informed of the aims and procedures of the study. Patients were clinically staged according to TNM classification (20), and diagnosed pathologically using biopsy specimens by two pathologists in accordance with the World Health Organization classification of tumors and recommendations published by the International Society of Urological Pathology (21). All patients in the non-CRPC group were treated by radical prostatectomy without androgen-deprivation therapy. Blood samples were collected at the time of prostate-specific antigen (PSA) relapse in patients with CRPC and collected preoperatively in the non-CRPC group. The relapse of PSA was defined by more than two continuous PSA elevations in the process of androgen-deprivation therapy. The study was approved by the Ethics Committee of Miyazaki University and related hospitals (Approval Number 847, August 2011).

ELISA. Blood samples (5 ml in EDTA disodium salt) were collected by venipuncture. Immediately after collection, plasma was obtained by centrifugation at 12,000 × *g* for 15 min at 4˚C and stored at −80˚C until later analysis. We used human Caveolin-1 and Caveolin-2 Kits (Uscn Life Science Inc., Wuhan, China; detection range=0.24-15 ng/ml) to determine plasma CAV1 and -2 levels, respectively. To detect the plasma CAV1and -2 levels using these ELISA kits, 60 ng/ml of the standards was diluted to a concentration range of 0.24-16 ng/ml in duplicate assays.

Cell culture. The androgen-dependent LNCaP and androgen-independent PC3 cancer cell lines (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco). Cells were grown in 9.6-cm^2 cell culture dishes at 37˚C in a humidified atmosphere of 5% CO_2. For real-time qRT-PCR and ELISA, cells were seeded in 9.6-cm^2 dishes with 2.5-ml culture medium for both LNCaP and PC3 cells. Media were changed every day for 5 days. To extract CAV1 and -2 proteins, cells grown to 90% confluence were passaged by trypsinization. After centrifugation, the cell pellet was resuspended in 1 ml protease inhibitor, and cells were counted on a hemocytometer. Aliquots containing 5×10^4 cells in protease inhibitor were frozen at -80˚C until use.

Real-time qRT-PCR and ELISA for PC cell lines. Total RNA was extracted from cells using an RNA Mini kit (Ambion, Paisley, OR, USA). Both genes were amplified from 2 mg DNase-I-treated total RNAs using Thunderbird Reverse Transcriptase (Toyobo, Tokyo, Japan) and random primers. The primers used for real-time qRT-PCR were as follows: *CAV1* forward, 5'-TTCTGGGCTTCATCTG GCAAC-3', reverse, 5'-GCTCAGCCCTATTGGTCCACTTTA-3'; *CAV2* forward, 5'-CACCCTCAGCTGTCTGCACAT-3', reverse, 5'-GGCAGAACCATTAGGCAGGTCTT-3'; and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) forward 5'-GCACCGTC AAGGCTGAGAAC-3', and reverse 5'-TGGTGAAGACGCCAGTG GA-3'. Real-time qRT-PCRs were performed with a Thermal Cycler Dice Real-Time System II (Takara Bio, Shiga, Japan). Reaction mixtures (25 μl) containing 2 μl cDNA template, 1 μl each sense and anti-sense primers, and 1× SYBR Premix Ex Taq II (Takara) were amplified as follows: 95˚C for 30 s and 40 cycles at 95˚C for 5 s, 60˚C for 30 s, and a final dissociation stage (95˚C for 15 s, 60˚C for 30 s, and 95˚C for 15 s). *GAPDH* was used as an internal control. The results were evaluated using the Thermal Cycler Dice Real-Time System software program (Takara Bio). The ΔΔ-Ct algorithm was used to analyze the relative changes in gene expression. To analyze protein expression of PC cell lines, ELISA protocols were used.

Knock-down (KD) of CAV1 and CAV2 in PC3 cells. Twenty-four hours before siRNA transfection, PC3 cells in the logarithmic phase were inoculated in six-well plates at 5×10^4 cells/well. siRNA (20 nM final concentration) was mixed with Lipofectamine RNAiMAX (Life Technologies, Tokyo, Japan) in serum-free medium (Opti-MEM: Life Technologies), and then incubated for 20 min at room temperature and added to the cells. Transfection was carried out using *CAV1*-specific siRNA (Silencer Select siRNA Lot # AS00RPF7; Life Technologies), *CAV2*-specific siRNA (Silencer Select siRNA Lot # AS00RPF6; Life Technologies) and negative control (NC) siRNA (Silencer Select siRNA Lot # AS000AAR; Life Technologies).

Statistical analysis. Statistical analysis was performed using the R i386 2.15.1 software package (Vienna University of Economics and Business, Vienna, Austria). Significance of differences in plasma levels of CAV1 and CAV2 between patients with CRPC and those with non-CRPC, and of expression differences between PC3 (CRPC model) and LNCaP (non-CRPC model) cells was determined by the Mann–Whitney *U*-test. A Spearman rank test was used to analyze the correlation between CAV1 and -2. The significance of expression differences between PC3 (control), PC3 *CAV1* KD cells (siCAV1), and PC3 *CAV2* KD cells (siCAV2) was determined by the Kruskal–Wallis rank sum test. A value of $p<0.05$ was considered statistically significant.

Results

Clinicopathological characteristics of the CRPC and non-CRPC groups are summarized in Table I. The mean patient age for the CRPC group was 68.3±7.4 (range=61-75) years and 66.9±8.3 (range=59-75) years for the non-CRPC group. The mean age did not significantly differ between the groups ($p=0.21$). There was a significant difference in mean serum PSA levels between the CRPC (41.8±28.4 ng/ml) and non-CRPC (8.8±4.1 ng/ml) groups ($p<0.001$). Additionally, there were significant differences in Gleason grade, clinical T-stage, and presence of bone metastasis.

Plasma levels of CAV1 and CAV2 in patients with PC. Because high levels of these proteins were recorded, samples

Table I. *Clinicopathological characteristics*.

	CRPC	Non-CRPC	*p*-Value
Age (years), mean±SD	68.7±7.7	66.2±5.2	0.18
PSA (ng/ml), mean±SD	41.8±28.4	8.8±4.1	<0.001
Gleason score, n (%)			
≤6	2 (5.6)	4 (18.2)	0.045
7	8 (22.2)	9 (40.9)	
≥8	26 (72.2)	9 (40.9)	
Clinical stage, n (%)			
T1-T2	7 (19.4)	13 (59.1)	<0.004
T3-T4	29 (80.6)	9 (40.9)	
N0	16 (44.4)	22 (100)	<0.001
N1	20 (55.6)	0 (0)	
M0	7 (19.4)	22 (100)	<0.001
M1	29 (80.6)	0 (0)	
Total	36	22	

CRPC: Castration-resistant prostate cancer, PSA: Prostate-specific antigen.

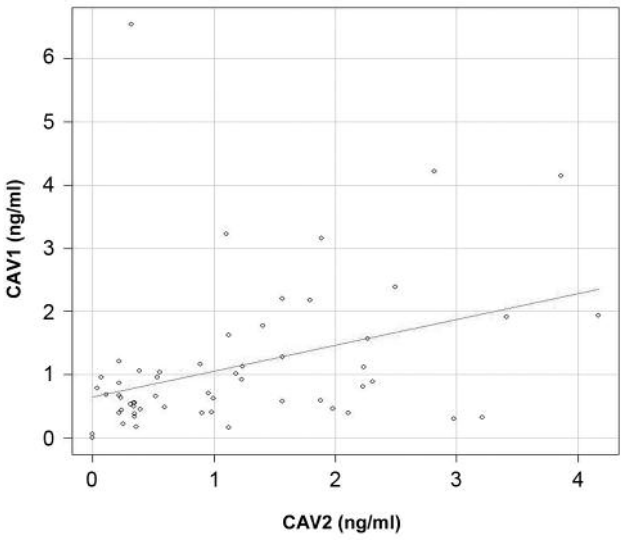

Figure 1. *Plasma levels of caveolin (CAV)-1 and CAV2 were significantly correlated in the castration-resistant prostate cancer group (CRPC): n=36; r=0.45, p<0.001.*

were diluted prior to analysis. Plasma levels of CAV1 and -2 in patients with PC were significantly higher than those in the non-CRPC group (CAV1: 1.46±1.37 ng/ml in the CRPC group *vs*. 0.56±0.32 ng/ml in the non-CRPC group, p=0.003; CAV2: 1.62±1.08 ng/ml in the CRPC group *vs*. 0.39±0.32 ng/ml in the non-CRPC group, p<0.001). In addition, a correlation between CAV1 and -2 levels in plasma was significant in patients with CRPC (r=0.45, p<0.001; Figure 1); however, there was no correlation in the non-CRPC group.

We analyzed the correlation between clinicopathological parameters and plasma CAV1 and -2 levels in patients with CRPC to investigate possible associations between plasma CAV1 or -2 levels and clinicopathological characteristics such as Gleason grade, clinical T-stage, and PSA level; there were no significant associations.

CAV1 and CAV2 mRNA and protein expression in PC cell lines. Strong expression of *CAV1* and *CAV2* mRNA was observed in PC3 compared to LNCaP cells (PC3 *vs*. LNCaP: *CAV1*/*GAPDH*=1 *vs*. 0.013, p<0.001; *CAV2*/*GAPDH*=1 *vs*. 0.014, p<0.001). Because high levels of these proteins were also recorded, samples were diluted prior to analysis. Expression of CAV1 and -2 was significantly higher in PC3 than LNCaP cells (CAV1: 2.67±0.31 ng/ml *vs*. 0.34±0.16 ng/ml, p=0.003; CAV2: 2.69±0.25 ng/ml *vs*. 0.37±0.29 ng/ml, p=0.002).

In PC3 *CAV1* KD cells, we observed suppression of *CAV2* mRNA expression and confirmed the linkage of KD of CAV2 and suppression of *CAV1* mRNA expression (*CAV1*/*GAPDH*: controls *vs*. siCAV1 *vs*. siCAV2=1 *vs*. 0.024 *vs*. 0.611, p=0.006; *CAV2*/*GAPDH*: controls *vs*. siCAV1 *vs*. siCAV2=1 *vs*. 0.603 *vs*. 0.096, p=0.006).

Discussion

In the present study, we investigated the plasma levels of CAV1 and -2 in Japanese patients with PC. Both proteins were highly expressed in patients with CRPC. We also investigated differences in CAV1 and -2 protein and mRNA expression between PC3 cells (CRPC model) and LNCaP cells (non-CRPC model). Our data show that increasing levels of plasma CAV1 and -2 have prognostic potential for PC progression.

CAV1 has been linked to various types of cancer over the past decade, during which it was most extensively studied in breast and prostate cancer. We previously reported that CAV1 levels in tumor tissue and in plasma may be associated with tumor protection or progression (4, 5). In PC, in particular, elevated CAV1 expression was observed in tumor tissues in humans and in a mouse model compared to non-tumor tissues (22-25). Additionally, suppression of CAV1 expression restored sensitivity to androgens in androgen-insensitive tumors (25). Another study implied that CAV1 and cancer-promoting growth factors collaborate in PC progression, although more evidence is needed (26). CAV1 is thought to suppress tumor growth and metastasis in human breast and colon cancer (27-29). However, CAV1 function

may differ among organs, and CAV1 could thus exert opposing effects, resulting in promotion or suppression of tumor progression. For example, CAV1 expression is increased in tumor samples from the kidney, prostate, and stomach, and re-expression has been found in some advanced adenocarcinomas (30, 31). Elevated CAV1 expression is associated with the progression of some adenocarcinomas, such as PC (32), and in adult T-cell leukemia (33). Activated CAV1 expression is associated with higher grades of PC, although few significant relationships have been identified between CAV1 expression and tumor multiplicity, recurrence, progression, or overall survival (28).

Li *et al*. showed that CAV1 is secreted by mouse and human PC cell lines, and secreted CAV1 promotes cancer cell survival and clonal growth *in vitro* (34, 35). They further showed that CAV1 secreted from tumor cells promotes pro-angiogenic activity in PC through the phosphoinositol-3-kinase/protein kinase B (AKT)/endothelial NO synthase (eNOS) signaling pathway (36). Regarding the mechanisms through which CAV1 mediates oncogenic activity, they showed that CAV1 holds AKT in an activated form in PC cells by binding to and inhibiting the serine/threonine protein phosphatases PP1 and PP2A (26). Thus, engagement of CAV1 as a tumor metastasis promoter depends on the cellular context, and at the molecular level, on the signaling molecules and pathways affected and regulated by CAV1. We hypothesize that altered CAV1 expression interferes with homeostasis, and may increase the frequency of PC.

The caveolin family is a class of oligomeric structural proteins that are necessary for caveolar formation. Caveolae are 50-100 nm Ω-shaped invaginations of the plasma membrane that function as signal transduction regulators. A scaffolding amino acid sequence identified in CAV1 allows this protein to interact with signaling molecules, such as epidermal growth factor receptor (EGFR), G-proteins, c-Src-like kinases, HA-Ras, protein kinase C, eNOS, and integrin (37). In several types of cancer cells, CAV1 is associated or co-localized with EGFR and appears to modulate EGFR signaling (38, 39). In contrast, the function of CAV2 remains obscure. CAV2 expression has been studied in several cancer types, in which expression patterns are up- or down-regulated. Therefore, its role in cancer is still debated and controversial (19, 40). Additionally, few studies have clarified the correlation of expression of CAV1 and -2 in plasma samples and cell lines. Herein, we tried for the first time to investigate associations between PC progression and plasma CAV1 and -2 levels in Japanese patients, as well as expression in PC3 (CRPC) and LNCaP (non-CRPC) cell lines. We also introduced CAV1- and -2-specific siRNAs into PC3 to KD both molecules, and examined their influence on expression of these genes. Our results show that CAV1 and -2 are associated with tumor progression and metastasis, and are elevated in androgen-resistant tumors. PC3 cells significantly expressed CAV1 and -2. Therefore, elevation of plasma CAV1 and -2 levels in patients with advanced cancer, such as CRPC, would be expected. Therefore, both molecules could be markers for an aggressive form of cancer (32, 36, 41-44).

In addition, our results revealed an association between plasma expression of CAV1or CAV2 and PC progression, and showed that CAV1 and -2 were markedly elevated in CRPC, suggesting their potential as biomarkers of PC. Our findings, together with data from other studies, suggest that CAV1 and -2 are involved in disease pathogenesis and progression. To the best of our knowledge, this is the first study to show a correlation between plasma CAV1 and -2 levels in patients with CRPC, and to detect strongly linked expression of their genes. Further study of the role of CAV1 and -2 in PC could contribute to our understanding over this disease, and possibly offer novel targeted therapeutic approaches. Although associations between *CAV1* and *CAV2* genes and high-risk tumors were identified in the present study, we still cannot confirm CAV1 and -2 as being markers for high-risk aggressive tumors, even at the stage when tumors are localized in the prostate and thus are still curable. To do so would require monitoring their levels in a follow-up study over the course of PC progression from its initial stages. Our results should also be verified in a larger group of patients, including those with disseminated disease.

Conclusion

Our results showed a relationship of CAV1 and CAV2 expression with PC progression, and support the possibility of using these molecules as therapeutic targets for CRPC.

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