# Isolation of Prostate Cancer-related Exosomes

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**Abstract.** *Background/Aim: Exosomes have been demonstrated to be useful non-invasive biomarkers for several cancers including prostate cancer. Since normal cells also secrete exosomes, isolation of cancer-derived exosomes from blood is a prerequisite for their better understanding. The aim of this study is to establish the method for isolation of prostate cancer-related exosomes from blood. Materials and Methods: Exosomes were collected from prostate cancer LNCaP and PC-3 cell lines by ultracentrifugation and by using magnetic beads conjugated with anti-CD9 antibody and anti-prostate-specific membrane antigen (PSMA) antibody. Prostate cancer-related exosomes were also isolated from the plasma of prostate cancer patients by anti-PSMA beads. Isolated exosomes were analyzed by western blotting. Results: Exosomes were isolated from LNCaP cells by ultracentrifugation, contained PSMA and androgen receptor (AR). AR was also detected in exosomes isolated from LNCaP cells by anti-PSMA and anti-CD9 beads, showing that AR is present in prostate cancer-related exosomes. The amount of CD9 in isolated exosomes was much higher in advanced and chemo-resistant prostate cancer patients than in prostate cancer patients without metastasis and healthy volunteers, indicating that patients with aggressive prostate cancer exhibit higher levels of prostate cancer-related exosomes in blood. Conclusion: The immunoaffinity-based method we developed is capable of isolating prostate cancer-related exosomes from blood, the use of which will enhance investigation processes on exosomes in prostate cancer.*

Prostate cancer-caused death reached more than 250,000 worldwide during 2008 (1). Although prostate cancer is eminently treatable at the early stage by castration and anti-androgen therapy, it becomes castration-resistant at the advanced stage because of the emergence of androgen-independent cells. Taxane-based chemotherapy is used for castration-resistant prostate cancer (CRPC), but which eventually becomes chemo-resistant with a median survival benefit of 2 to 3 months (2). Thus, there is a great need of developing novel diagnostic and treatment strategies that target the castration-resistant and chemo-resistant prostate cancer. One of the potential strategies is the use of microvesicles including exosomes secreted from prostate cancer cells as a tool for diagnosis.

It has been well-accepted that intracellular signaling pathways are potential therapeutic targets for solid tumors since they play important roles in gene expression, cell survival and apoptosis (3-5). Drugs targeting the intracellular signaling pathways have been used to treat several types of cancer (*e.g.* tyrosine kinase inhibitors, raf kinase inhibitors and mammalian target of rapamycin (mTOR) inhibitors). Once cancer acquires resistance, drug selection has to be reconsidered based on its molecular information. Surgical and biopsy specimens provide valuable information for decision making on treatment but serial biopsy is not usually performed on prostate cancer patients due to its invasiveness. Information on the characteristics of cancer cells, by analyzing exosomes in the peripheral blood, could provide more treatment choices using appropriate drugs such as those targeting intracellular signaling pathways.

Exosomes are membrane vesicles with a diameter of 40-150 nm that are secreted by various types of mammalian cells. Exosomes contain various molecular constituents of

*Abbreviations:* AR: androgen receptor; PC: prostate cancer; CRPC: castration-resistant prostate cancer; CAB: combined androgen blockade; RT: radiation therapy; PSA: prostate-specific antigen; PSMA: prostate-specific membrane antigen.

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their origin such as proteins and nucleic acids (6, 7). Over the years, researchers have been trying to identify the roles of exosomes, contained proteins, their release mechanisms, *etc*. and their studies suggested that exosomes could be considered as novel diagnostic markers and therapeutic targets for cancer. Since exosomes are also released by normal cells and play physiologically important roles, isolation of cancer-specific exosomes is required to better understand their functioanal capabilities. In the present study, we developed a novel immunoaffinity-based method to isolate prostate cancer-related exosomes.

## Materials and Methods

*Cell culture*. Human prostate cancer LNCaP and PC-3 cells obtained from ATCC (Manassas, VA, USA) were maintained in RPMI1640 (Wako Pure Chemical Industries, Osaka Japan), 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) under a humidified atmosphere of 5% $CO_2$ at 37˚C.

*Isolation of exosomes by sequential centrifugation*. All experiments were performed with exosome-free FBS. Exosome-free FBS was prepared by ultracentrifugation at 110,000 × *g* for 16 hours. Exosomes were collected according to a previous report with minor modification (8). Briefly, cells were seeded at $4\times10^6$ cells in 10 $cm^2$ flasks. One hundred and twenty hours after seeding, conditioned medium was collected for exosome isolation. The medium was centrifuged at 1,800 × *g* for 10 min to eliminate cells and debris. The second centrifugation was at 16,500 × *g* for 20 min followed by passage through a 0.20 μm PVDF filter (Sartorius AG, Goettingen, Germany). Then exosomes were pelleted by ultracentrifugation at 110,000 × *g* for 70 min (Hitachi Koki, Tokyo, Japan). Pellets were washed with PBS, re-pelleted and suspended in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Sigma-Aldrich, MO, USA). Samples were stored at –20˚C until use.

*Isolation of exosomes by using antibody-conjugated beads*. The antibody-conjugated magnetic microbeads were prepared with Dynabeads® M-270 epoxy magnetic beads (Life technologies AS, Oslo, Norway), mouse IgGκ isotype control (BioLegend, San Diego, CA, USA), mouse monoclonal anti-CD9 antibody (BioLegend) and mouse monoclonal anti-PSMA antibody (Medical and Biological Lab, Nagoya, Japan), according to the protocols supplied by the manufacturers. The conditioned medium was centrifuged at 1,800 g for 10 min to eliminate cells and debris. The second centrifugation was at 16,500 × *g* for 20 min followed by passage through a 0.20 μm PVDF filter. Then 20 ml medium was co-incubated with 5 mg of antibody-conjugated beads for 90 min with rotation at 4˚C. Beads were washed three times with ice-cold PBS and suspended in RIPA buffer containing the protease inhibitor cocktail. The resultant supernatants were used in the following experiments.

*Western blotting*. LNCaP and PC-3 cells ($3\times10^4$ and $1\times10^4$ cells, respectively) were seeded onto 6 well-plates and cultured in complete medium with normal FBS. Forty eight hours later, whole cell lysates were harvested and suspended in RIPA buffer containing the protease inhibitor cocktail. The cell lysates and isolated exosomes were subjected to SDS-PAGE followed by Western blotting. The PVDF membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween for 60 min at room temperature. The membranes were incubated overnight at 4˚C with the following primary antibodies: rabbit anti-AR antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-CD9 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-PSMA antibody (Cell Signaling Technology), rabbit anti-CD63 antibody (Santa Cruz) or rabbit anti-GAPDH antibody (Cell Signaling Technology). The immunoreactive bands were detected with horseradish peroxidase-conjugated anti-rabbit and -mouse IgG (GE Healthcare, Buckinghamshire, UK) and the enhanced chemiluminescence (ECL) detection system (GE Healthcare).

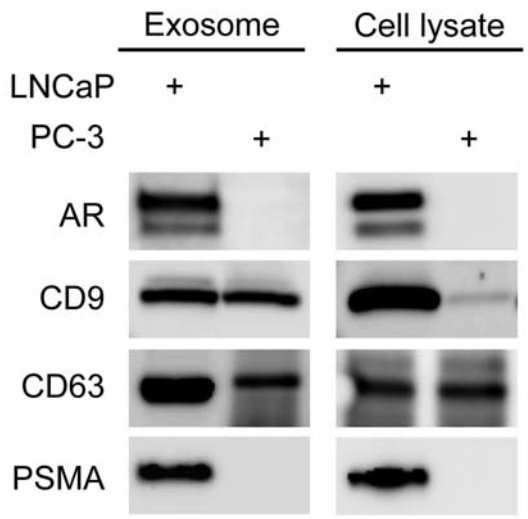


Figure 1. *Purification of exosomes from the conditioned media of prostate cancer cell lines by sequential centrifugation. Whole-cell lysates (5 μg of protein) of LNCaP and PC-3 cells were subjected to western blotting with antibodies indicated (right panels). Total exosome fraction (10 μg of protein) that was isolated from the conditioned media of both cell lines by sequential centrifugation was also analyzed by western blotting (left panels).*

*Isolation of exosomes from human plasma*. This experiment was approved by the Medical Review Board of Gifu University, Graduate School of Medicine. Written informed consent was obtained from all patients and healthy volunteers. Ten ml of peripheral blood was drawn from prostate cancer patients and healthy volunteers in containers with heparin. Plasma was separated by centrifugation at 1,800 × *g* for 10 min and stored at –80˚C until use. For exosome isolation, 2 ml of plasma was diluted with 4 ml of PBS. The diluted plasma was centrifuged at 16,500 × *g* for 20 min followed by passage through a 0.20 μm PVDF filter. Then samples were co-incubated with 2.5 mg of anti-PSMA antibody-conjugated beads for 90 min with rotation at 4˚C. Beads were washed three times with PBS and suspended in RIPA buffer containing the protease inhibitor cocktail. Total amount of the resultant supernatant was used for each experiment.

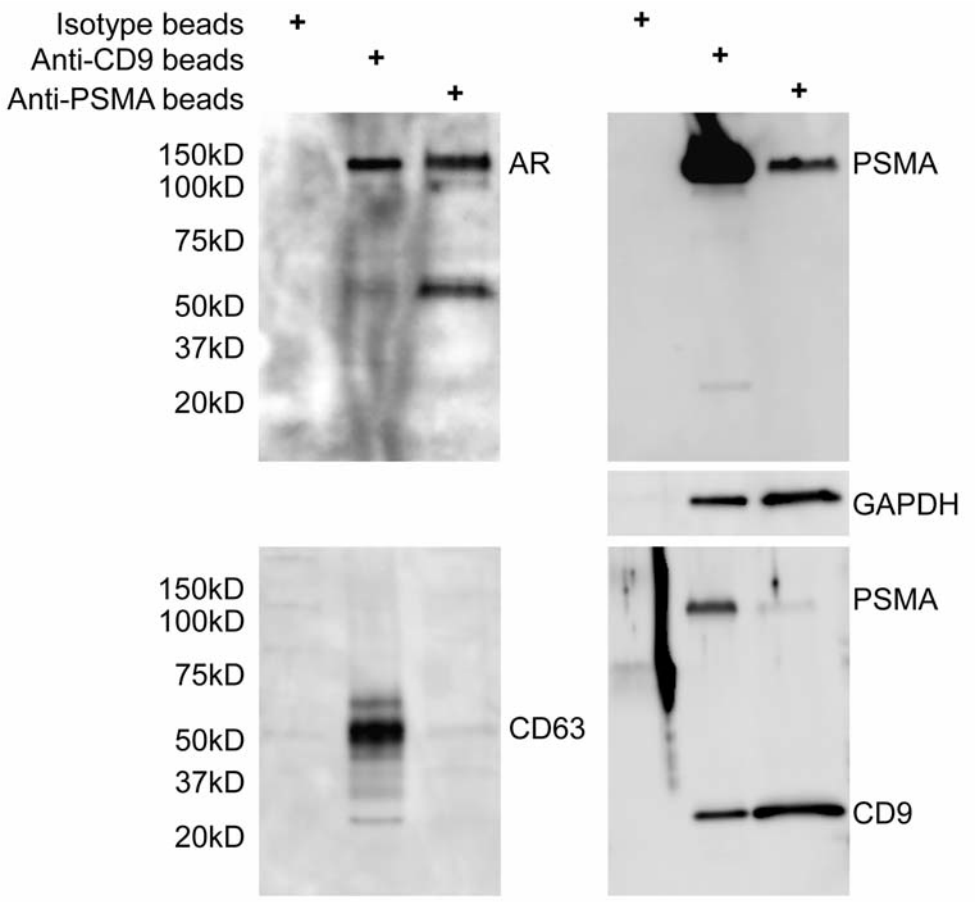


Figure 2. *Isolation of exosomes from the conditioned media of prostate cancer cell lines by antibody-conjugated beads. Total proteins that were obtained from the reaction of anti-CD9 or anti-PSMA beads and conditioned medium were subjected to western blotting with anti-AR antibody and anti-PSMA antibody (upper panels). The blots were re-probed with anti-GAPDH antibody, anti-CD63 antibody and anti-CD9 antibody (middle and lower panels).*

## Results

*Analysis of exosomes isolated from LNCaP and PC-3 cells by ultracentrifugation*. We isolated total exosomes from the conditioned media of LNCaP and PC-3 cells by sequential centrifugation followed by ultracentrifugation. In order to confirm the isolation of exosomes, we analysed the presence of exosomal proteins in whole-cell lysates as well as in exosomes by western blotting (Figure 1). Two well-known exosomal markers (9), CD9 and CD63, were found to be present in both LNCaP and PC-3 exosomes. The expression of CD9 in whole cell lysates was lower in PC-3 cells than in LNCaP cells, but that in exosomes was similar between the two cell lines. We also examined if androgen receptor (AR) is present in exosomes. AR is known to be present in the cytosol and transported to the nucleus by androgen stimulation, resulting in transactivation of androgen-responsive genes (10, 11). In LNCaP cells, AR was detected in exosomes as well as in whole-cell lysates. It is not surprising that PC-3 exosomes did not contain AR, because PC-3 cells express no or very little AR. To isolate prostate cancer-related exosomes, it was necessary to identify proteins expressed on the surface of exosomes. Prostate-specific membrane antigen (PSMA) is a cell surface antigen highly expressed in prostate especially in advanced prostate cancer compared to other organs (12-14). Our results showed that PSMA present in LNCaP cells and also in its exosomes, suggesting that PSMA could be an excellent target for isolation of prostate cancer-specific exosomes by the immunoaffinity-based method.

*Analysis of exosomes isolated from LNCaP cells by the immunoaffinity method*. In order to develop an immunoaffinity system for isolation of prostate cancer-related exosomes, we made magnetic beads conjugated with anti-CD9 or anti-PSMA antibody. The exosomes isolated by

Table I. *Clinical characteristics of the prostate cancer patients. RT, RP and CAB denote radiation therapy, radical prostatectomy and combined androgen blockade, respectively, N/A: not applicable.*

| | Clinical stage | Gleason score | PSA (ng/ml) | Past treatment |
|---|---|---|---|---|
| P1 | cT2aN0M0 | 4+3 | 17.2 | None |
| P2 | cT3aN0M0 | 4+5 | 130.9 | None |
| P3 | cT4N1M1b | 4+5 | 1031.2 | Bicalutamide (for 2 weeks) |
| P4 | Castration- and chemotherapy-resistant with multiple bone metastasis | 4+5 | 50.6 | RT, CAB and Docetaxel |
| P5 | Castration- and chemotherapy-resistant with multiple lymph node and bone metastasis | N/A | 776.5 | RP, RT, CAB and Docetaxel |

the anti-CD9 and -PSMA antibody beads contained CD9, PSMA, AR and GAPDH in common (Figure 2). However, CD63, another exosome marker, was only detected in exosomes isolated by the anti-CD9 beads, but not in those isolated by the anti-PSMA beads. It is noteworthy that exosomes isolated by both bead preparations contained AR, showing that AR is present in prostate cancer-related exosomes. These results suggest that the immunoaffinity system we developed with anti-PSMA antibody is capable of isolating prostate cancer-related exosomes.

*Analysis of prostate cancer-related exosomes isolated from human plasma*. Several researches have demonstrated that PSMA is highly expressed in prostate cancer, metastatic and castration-resistant prostate cancer (12-14). Herein we examined if microbeads conjugated with the anti-PMSA antibody were capable of isolating prostate cancer-related exosomes from human blood. We isolated exosomes from the plasma (2 ml) of healthy individuals and prostate cancer patients, and the amounts of prostate cancer-related exosomes were determined by western blotting using the anti-CD9 antibody. The clinical information of the prostate cancer patients is shown in Table I. C1 and C2 are healthy volunteers, P1 and P2 are prostate cancer patients without metastasis without any treatment and P3 is a patient with metastasis under anti-androgen treatment for two weeks before combined androgen blockade (CAB). Both P4 and P5 are prostate cancer patients who undertook surgery or radiation therapy, eventually became castration- and chemotherapy (docetaxel)-resistant. Patients with advanced prostate cancer (P3) and castration- and chemotherapy-resistant prostate cancer (P4 and P5) exhibited higher CD9 expression in isolated exosomes than healthy volunteers (C1 and C2) and prostate cancer patients without metastasis (P1 and P2) (Figure 3). These results indicate that patients with aggressive prostate cancer exhibit higher levels of exosomes in blood, suggesting that the anti-PMSA antibody-conjugated beads we developed are capable of isolating prostate cancer-related exosomes from blood.

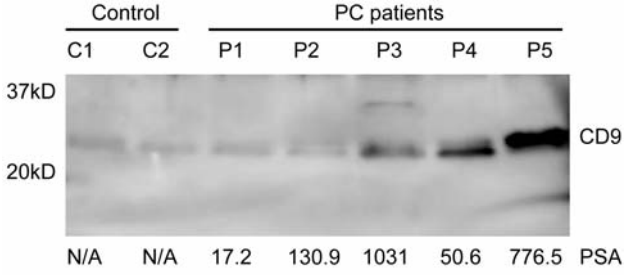


Figure 3. *Analysis of prostate cancer-related exosomes isolated from the plasma. Exosomes isolated with anti-PSMA beads from the plasma of healthy volunteers (C1 and 2), prostate cancer patients without metastasis (P1 and P2), patients with advanced prostate cancer (P3) and those with castration- and chemotherapy-resistant prostate cancer (P4 and P5) were subjected to western blotting with an anti-CD9 antibody.*

## Discussion

In the present study, we developed a novel immunoaffinity-based method to isolate prostate cancer-related exosomes from peripheral blood. Various purification methods to isolate microvesicles have been reported for the isolation of microvesicles including sequential centrifugation followed by ultracentrifugation, filtration, immunoaffinity-based isolation and microfluidics techniques (9, 15). Among them, we used sequential centrifugation and immunoaffinity isolation. Exosomes isolated by sequential centrifugation included the representative exosome markers, CD9 and CD63. In addition, PSMA and AR, prostate-related markers, were also detected. CD63 was detected in the LNCaP exosomes that were isolated with anti-CD9 beads but not in those isolated with anti-PSMA beads. These results suggest that exosomes may vary depending on the isolation method and show different protein profiling. It is also suggested that that centrifuge-purified exosomes may include several types of exosomes. To study exosomes in details, however, it would be advisable to use more than one method for their isolation such as ultracentrifugation and immunoaffinity method.

Interestingly, AR was detected in exosomes isolated from LNCaP cells by ultracentrifugation. In order to exclude the possibility of protein precipitation caused by ultracentrifugation, we isolated exosomes by the immunoaffinity method using antibody-conjugated beads and confirmed AR expression in exosomes from LNCaP cells. AR is one of the most important therapeutic targets for prostate cancer. For decades, various types of agents targeting AR have been used for advanced prostate cancer (16, 17). A challenge in AR research, using clinical samples, is that AR has been thought to be expressed within the cells. Our study is the first report demonstrating that exosomes secreted from prostate cancer cells contain AR *in vitro*. However, AR expression was hardly detected in prostate cancer-related exosomes isolated from the plasma of patients. This discrepancy may be, in part, due to the amount of exosomes isolated from blood and of AR contained in exosomes. The immunoaffinity-based isolation system has some disadvantages compared to the centrifugation method including the reactivity and sensitivity of antibody used and the presence of inhibitors of the reaction. The development of more effective immunoaffinity methods for isolating exosomes may overcome the discrepancy observed in the present study, for which further investigation will be required.

Nevertheless, the present study demonstrated higher expression of exosomes in the plasma of patients with advanced prostate cancer and castration- and chemotherapy-resistant prostate cancer than in the plasma of healthy volunteers and prostate cancer patients without metastasis. The results presented here, in combination with previous studies showing higher expression of exosomes in advanced cancer, strongly support that our the anti-PSMA beads isolated prostate cancer-related exosomes. Analysis of intra-cellular molecules in prostate cancer-related exosomes is ongoing.

In summary, this is the first report on the isolation of prostate cancer-related exosomes from the blood of prostate cancer patients by an immunoaffinity-based method using an anti-PSMA antibody. This is a novel and useful method, which will promote further research on exosomes in prostate cancer.

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