Ad5/35E1aPSESE4: A novel approach to marking circulating prostate tumor cells with a replication competent adenovirus controlled by PSA/PSMA transcription regulatory elements

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ABSTRACT

Circulating tumor cells serve as useful biomarkers with which to identify disease status associated with survival, metastasis and drug sensitivity. Here, we established a novel application for detecting PSA/PSMA-positive prostate cancer cells circulating in peripheral blood employing an adenovirus called Ad5/35E1aPSESE4. Ad5/35E1aPSESE4 utilized PSES, a chimeric enhancer derived from PSA/PSMA promoters that is highly active with and without androgen. A fluorescence signal mediated by GFP expression upon Ad5/35E1aPSESE4 infection was selectively amplified in PSA/PSMA-positive prostate cancer cells in vitro and ex vivo. Furthermore, for the in vivo model, blood drawn from TRAMP was tested for CTCs with Ad5/35E1aPSESE4 infection and was positive for CTCs at week 16. Validation was performed on patient blood at various clinical stages and found out 1–100 CTCs expressing GFP upon Ad5/35E1aPSESE4 infection. Interestingly, CTC from one patient was confirmed to be sensitive to docetaxel chemotherapeutic reagent and to abundantly express metastasis-related genes like MMP9, Cofilin1, and FCER1G through RNA-seq. Our study established that the usage of Ad5/35E1aPSESE4 is effective in marking PSA/PSMA-positive prostate cancer cells in patient blood to improve the efficacy of utilizing CTCs as a biomarker.

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Introduction

Prostate cancer is the second most common cancer among men worldwide, with the highest incidence in Western countries such as Australia, New Zealand, North America and Western Europe [1]. Its metastasis to bone, lymph node, liver and lung tissue is a major cause of cancer-related death. Patients have relatively high 5-year survival rates, partially due to the early detection of prostate cancer, but many prostate cancer patients still die of this condition. Prostate-specific antigen (PSA), which is a protein produced exclusively by the prostate gland, is widely used as a serum marker because its level in blood becomes elevated upon prostate cancer development and progression. PSA has several limitations to its accuracy for the diagnosis of prostate cancer because the PSA level can be elevated regardless of the occurrence of prostate cancer and its progression. With the increase in life span, there are often higher levels of PSA due to the development of benign prostatic hyperplasia (BPH). Furthermore, PSA levels vary by race and Asians have a relatively lower PSA level than Caucasians [2]. Often, men have a high level of PSA with no sign of cancer or a man with prostate cancer may have blood PSA levels within the normal range. Among cases with elevated PSA levels, only 25% are found to have prostate cancer as verified by prostate biopsy [3], suggesting that we need alternative strategies to complement PSA levels to assess disease progression status, such as the monitoring of circulating tumor cells (CTCs).

A CTC is defined as a cell that escapes from the primary tumor site and circulates in the bloodstream. For metastasis to distant sites, tumor cells can travel through the circulation of the body; hence, CTCs are indicative of the potential growth of tumors in distant organ sites, so-called ‘metastasis’ [4]. In 1869, it was found that cancer cells observed in the blood may shed light upon the mode of origin of multiple tumors existing in the same person. Since then, a number of reports have demonstrated that CTCs can be used as a prognostic marker and can be indicative of metastatic relapse or progression...
in tumors [5]. In addition to their biological relevance, CTCs in the blood are advantageous because they provide a so-called “liquid biopsy.” The harvesting of blood samples in the clinic is a simple procedure. There are a number of clinical trials registered at www.clinicaltrials.gov using CTCs as a biomarker, but there are a few hurdles to be resolved before this approach becomes a routine procedure in the clinic. First, CTCs are present at such low numbers in the peripheral blood, ranging from 1 to 10 cells per 10 mL of blood in cancer patients [6], making their detection difficult. Second, most methods developed thus far lack specificity for the detection of CTCs. Some researchers utilize a specific antibody to detect cancer-associated antigens expressed on CTCs, but patient antigen levels vary. Not all of the CTCs in a single patient express a target antigen [7]. In the case of prostate cancer, the presence of CTCs was related to poor prognosis. Patients with high numbers of CTCs presented poor outcomes of survival as analyzed by the CellSearch™ system (Raritan, NJ, USA) [8]. The CellSearch™ system utilizes the strategy of catching EpCAM expressed on a CTC membrane. EpCAM is a marker for viral replication, E1a/b, is placed in a tumor or tissue-conditional replication of an adenovirus in a tumor, a key regulator for the lytic process of the viral life cycle and thus causing cell death in a directed fashion. For the prostate tumor by controlling the lytic process of the viral life cycle and thus causing cell death in a directed fashion. The lytic process of the viral life cycle and thus causing cell death in a directed fashion. For the clinical purpose of developing adenovirus-mediated gene therapy, an adenovirus has a double-stranded DNA genome and utilizes the Coxackievirus and adenovirus receptor (CAR) on the surface of host cells [9]. Upon entry into the host cell, the adenovirus replicates in the nuclei of host cells utilizing a host replication system. Once the virus successfully replicates, its viroid particles are released through cell lysis. With the clinical purpose of developing adenovirus-mediated gene therapy for cancer, adenovirus replication has been restricted to a prostate tumor by controlling the lytic process of the viral life cycle and thus causing cell death in a directed fashion. For the conditional replication of an adenovirus in a tumor, a key regulator for viral replication, E1a/b, is placed in a tumor or tissue-specific promotor [10,11]. Interestingly, a replication-competent adenovirus (RCA) under the hTERT promotor was also harnessed for the detection of CTCs with high effectiveness, demonstrating this as an effective alternative strategy to overcome deficiencies in marker-based CTC detection [12]. With respect to prostate cancer, the rat probasin promoter, which becomes active in the presence of androgen, was employed to construct an RCA for CTCs. A probasin-controlled RCA was designed to express and secrete luciferase protein out of the cell during its replication [13]. Nevertheless, there is still a need to detect CTCs in prostate cancer patients with a very low level of androgen as a result of hormone therapy. In a previous report, we introduced a prostate-specific RCA, Ad5Se1aPSESE4, and the replication of Ad5Se1aPSESE4 was controlled by PSA/PSMA transcription regulatory elements (PSES) [14,15]. Here, we describe a novel approach to detecting circulating prostate tumor cells to enhance the efficacy of the prediction of disease stage and progression in prostate cancer using Ad5/35E1aPSESE4 modified from Ad5Se1aPSESE4.

Adenovirus construction

Adenovirus Ad5/35E1aPSESE4 was constructed on the basis of the previous report ligating three vectors: 1) pEd1020CMVGFSP6FA, containing an adenovirus left inverted terminal repeat and packaging signal [14]; 2) E1HSF/35E4(Aor1-4) for serotype 5 genome-based backbone vector [15], which contains the fiber knob replaced with that of serotype 35; and 3) p304SFI, containing the right-sided inverted terminal repeat of the adenoviral genome [15]. All vectors were digested with Sfi I restriction enzyme and were ligated to make pAd5/35E1aPSESE4. The adenoviral vector pAd5/35E1aPSESE4 was digested with PstI and was transfected into 911E4 cells for virus production. For high-titer adenovirus purification, Ad5/35E1aPSESE4 was purified using an ultras centrifugation method with CCl4, followed by dialysis using a membrane exchange method.

Infection of Ad5/35E1aPSESE4 in cancer cells or cells from TRAMP

An indicated number of each cell type was plated in a 12-well plate for 1 day. Cells seeded in a 12-well plate were infected with 1 MOI of Ad5/35E1aPSESE4 and were observed under a fluorescence microscope for 24 h, 48 h and 72 h. For experiments involving BALB/c 1 x 104 of C4-2 cells that were prepared in PBS were injected through the mouse tail vein of a BALB/c nude mouse. Six hours post-injection of the cells; 200 μL of blood was withdrawn from each mouse and analyzed for circulating C-4-2 cells. For TRAMP experiments, a 1118F/PET-CT scan was employed to trace tumor growth in prostate TRAMP in a non-invasive manner. Every 2 weeks, 200 μL of blood was withdrawn and was exposed to 1 MOI of adenovirus Ad5/35E1aPSESE4 for detecting CTCs. For the PET monitoring of prostate tumors in a TRAMP mouse, 1118F/PET/FDG was produced by a standard method using our on-site cyclotron (RDS-111, Siemens, Germany) and automatic radiosynthesis modules (CPCU, Siemens, Germany). Mice were supplied with water and without caloric food materials for 6 h. Before the mice were anesthetized with 2% isoflurane in 100% oxygen for the FDG-scan, their blood glucose levels were verified as being below 100 μg/mL before every 1118F/PET/FDG injection. We administered 14.8 MBq of 1118F/PET/FDG through IV. The 1118F/PET/FDG PET-CT scan was performed in a three-dimensional acquisition mode (eXplore Vistct, GE Healthcare Life Science, Piscataway, USA). PET scans were acquired for 10 min per bed position for all studies and were processed through normalization but without scatter correction and attenuation correction. All images were reconstructed with iterative reconstruction (OSEM 2-D, 32 subsets, two interactions). For CT scans, X-ray sources were used with 300 μA and 40 kV for 6 min. The CT resolution was 290 μm, and the number of acquired projections was 360.

Quantitative PCR analysis

A total of 1 x 104 of C-4-2 cells was plated into a 6-well plate. One day after seeding, the cells were infected with 1 MOI of Ad5/35E1aPSESE4. The cells were harvested at indicated time points, after which the total DNA was extracted using the Qiagen Blood Kit (QIAGEN Sciences, Gaithersburg, MD, USA). For counting Ad5/35E1aPSESE4 particles, quantitative PCR was performed using a QGreen Master Mix kit (Cellsafe, Suwon, Korea) and pre-designed primer/probe pairs for adenovirus (5’-CCT GTG AGT ACT CCT TAG TAT CAT C-3’ and 5’-GCC ACC TTC GAC TGT CTT GT-3’). Relative expression levels were normalized to GAPDH using the LightCycler software (Roche, Indianapolis, IN, USA).

Counting CTCs in prostate cancer blood using Ad5/35E1aPSESE4

For the separation of PBMCs (peripheral blood mononuclear cells) from whole blood, 5 mL of whole blood in a K2 EDTA tube was added to a 50 mL conical tube containing 4 mL of Ficoll-Paque PLUS (GE Healthcare Life Science) and was gently mixed with PBS to reach a total volume of 10 mL. The mixture in a 50 mL conical tube was centrifuged at 400 x g for 10 min at room temperature (RT). The PBMC layer was harvested, placed into a new 50 mL conical tube and washed with PBS. After the PBMC sample was pelleted at 200 x g for 20 min at RT, the sample was extensively washed with PBS and re-suspended in a microcentrifuge tube containing 1 mL PBS. The PBMC sample in the microcentrifuge tube was centrifuged at 6000 x g for 1 min and was re-suspended with 1 mL of DMEM supplemented (GE Healthcare Life Science) with 10% (v/v) FBS and 1% (v/v) antibiotics. Finally, the PBMC sample was plated in a 12-well plate for 1 day. PBMCs and CTCs were infected with 0.01 MOI of Ad5/35E1aPSESE4 and were observed under a fluorescence microscope for 24 h, 48 h and 72 h. Blood was determined to contain CTCs when large GFP-expressing cells were found. Tumor cells were maintained in Dulbecco’s High Glucose MEM medium supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics.

mRNAseq experiments and data analysis

For control and test RNAs, the construction of library was performed using SENSE mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer’s instructions. Briefly, 2 μg of each total RNA was prepared and incubated with magnetic beads decorated with oligo-dT, and then other RNAs except mRNA was removed by washing solution. Library production was initiated by the random hybridization of starter/stopper heterodimers to the polyA RNA still bound to the magnetic
beads. These starter/stopper heterodimers contained Illumina-compatible linker sequences. A single-tube reverse transcription and ligation reaction extended the starter to the next hybridized heterodimer, where the newly-synthesized cDNA insert was ligated to the stopper. Second strand synthesis was performed to release the library from the beads, and the library was then amplified. Bar codes were introduced when the library was amplified. High-throughput sequencing was performed as paired-end 100 sequencing using HiSeq 2000 (Illumina, Inc., San Diego, CA, USA). For data analysis, RNA-Seq reads were mapped using TopHat software tool in order to obtain the alignment file. The alignment file was used for assembling transcripts, estimating their abundances and detecting differential expression of genes or isoforms using cufflinks.

Results

Establishment of an in vitro assay to detect circulating prostate cancer cells using Ad5/35E1aPSESE4

The transcription regulatory element controlling rat probasin protein expression has been used to construct a replication-competent adenovirus and to detect CTCs. However, it is doubtful whether this method could be applied to most prostate cancer patients regardless of androgen/androgen receptor status. In the previous study, we introduced a chimeric transcription enhancer PSEs that was composed of PSA and PSMA transcription regulatory elements. When PSES was used to control the E1a and E4 genes, prostate-restricted RCA Ad5E1aPSESE4 was active selectively in PSA/PSMA-positive prostate cancer cells and active in the absence of androgen [14]. For better infectivity of cancer cells, we replaced a serotype 5 fiber knob with a serotype 35 fiber knob, resulting in a chimeric adenovirus Ad5/35E1aPSESE4 (Fig. 1A). Here, we explored whether Ad5/35E1aPSESE4 could infect prostate CTCs and label CTCs with fluorescence in blood drawn from a prostate cancer patient. First, a prostate cancer cell’s C4-2 was mixed with PBMCs prepared as illustrated in Fig. 1B, after which it was tested for adenoviral infection and expression of green fluorescence protein (GFP). As shown in Fig. 1B, 0.1 MOI of Ad5/35E1aPSESE4 mediated GFP expression in C4-2 cells at 48 h, suggesting that Ad5/35E1aPSESE4 detected PSA/PSMA-positive prostate cancer cells in the presence of as many as 3 × 10^6 PBMCs. To evaluate other prostate cancer cells, hormone-sensitive LNCaP and hormone-refractory prostate cancer cells, DU145 and PC3, were tested following the same protocol in Fig. 1C. One thousand cells were exposed to 1 MOI of Ad5/35E1aPSESE4 for 48 h and placed under fluorescence microscopy. All PSA/PSMA-positive cells tested enabled GFP expression at 48 h, but PSA/PSMA-negative PC3 and DU145 exhibited no GFP expression, as reported previously [14]. HT29 and Ku19-19 were included as non-prostate cancer cells. We used such a small number of virus particles that the GFP gene controlled by the universal CMV promoter in the Ad5/35E1aPSESE4 genome was not sufficiently expressed, with only transient viral transduction without viral replication.

Quantifying CTCs in human blood employing Ad5/35E1aPSESE4

Next, Ad5/35E1aPSESE4 was evaluated for its ability to determine how many CTCs existed in the presence of PBMCs. Quantitative analysis may be an important factor for exploiting adenovirus in detecting CTCs, as the number of CTCs in a certain amount of blood may provide correlative information regarding prognosis or treatment outcome in the clinic. To answer this question, the known number of C4-2 cells was mixed with PBMCs purified from a normal male blood donor and infected with 1 MOI of Ad5/35E1aPSESE4. The cells were observed and counted under fluorescence microscopy at 48 h and 72 h (Fig. 2A). When GFP-positive cells were counted at 48 h and 72 h post-infection, GFP-positive cell numbers were closely correlated with the C4-2 cells added to the culture dish. At 72 h, GFP-positive numbers decreased compared with input cells, most likely due to RCA-driven cell lysis. To confirm the virus replication in this experimental setting, two differing amounts of C4-2 cells were mixed and then infected with 1 MOI of Ad5/35E1aPSESE4. Total DNA was extracted and then used for counting the total number of virus particles by employing quantitative PCR (Fig. 2B). As shown in Fig. 2B, the number of virus particles at 48 h was much higher than that at 72 h, suggesting continuous virus replication up to 72 h, and the more effective time point was at 48 h for counting CTCs with fluorescence.

Detection of CTCs by Ad5/35E1aPSESE4 in an animal model

We investigated whether Ad5/35E1aPSESE4 had the ability to mark prostate cancer cells circulating in animal blood. C4-2 at a count of 1 × 10^6 cells was injected through the tail vein. Blood was drawn 6 h post-adenovirus injection and was treated with RBC-selective lysis solution. Subsequently, PBMC samples were infected with 1
MOI of Ad5/35E1aPSESE4 for 48 h. As shown in Fig. 3, different numbers of C4-2 cells in blood showed fluorescence upon infection with Ad5/35E1aPSESE4. Control mice, which were never exposed to Ad5/35E1aPSESE4, had no fluorescent cells. This experiment confirmed that prostate cancer cells in the blood maintained their viability throughout the RBC lysis procedure and mediated GFP expression upon adenovirus infection. Next, we explored whether CTCs could be observed in TRAMP mice that developed a prostate tumor when SV40-T antigens became highly abundant. Before the animal experiments, TRAMPc2 cells derived from a TRAMP mouse were infected with Ad5E1PSESE4, confirming that Ad5/35E1aPSESE4 enabled the expression of GFP in a TRAMP mouse-derived cell line (Fig. 4A). Tumor formation near the prostate in TRAMP mice was monitored using a [18F]FDG-PET scan at a 2 week interval. Interestingly, tumors in the prostate were not clear, as seen by [18F]FDG-PET during the entire period of the experiments (Fig. 4B), but CTCs started to be observed under fluorescence microscopy at week 16 (Fig. 4B), suggesting that adenovirus-mediated CTC detection is very informative for tumors near the prostate and their metastasis. As more weeks passed, more CTCs were counted. Our findings are not conclusive but suggest that Ad5/35E1aPSESE4-mediated CTC detection may be a useful diagnostic tool in the clinic for identifying CTCs in prostate cancer patients. To verify prostate tumors in TRAMP mice, a TRAMP mouse was sacrificed and specimens of the prostate were placed on a slide with H&E staining. Prostate tumors in the prostate and the seminal vesicle were histologically confirmed (Fig. 4C).

Validation of CTC detection in the blood of prostate cancer patients

PSA is a prostate-specific antigen, and its increase in blood is used to establish an abnormal status of the prostate such as hyperplasia and neoplasia. This biomarker is also a good indicator of cancer recurrence and disease progression. However, the PSA level has definitive limitations and requires additional information for the determination of cancer progression status. We explored whether CTCs in blood could be used for the determination of clinical status. Before Ad5/35E1aPSESE4 was applied to CTCs in the blood of prostate cancer patients, 20 non-cancerous people aged 20-40 years were enrolled in this study and their blood was tested. Ad5/35E1aPSESE4 mixed with PBMCs from the normal control group did not produce any detectable fluorescence, indicative of the very small possibility of false positivity. Then, we hypothesized that prostate cancer patients at the metastatic stage would have CTCs in their blood. Blood from patients at the metastatic stage was drawn, and leukocyte mixtures were infected with 0.01 MOI of Ad5/35E1aPSESE4. Patients determined to be at late stages based on pathological readings had a large number of CTCs (Supplementary Fig. S1). As summarized in Table 1, most of the patients harbored CTCs in which the fluorescence signal was amplified and that were cultivatable. Such results implied that detectable fluorescence and cultivatable CTCs in patient blood were strong biomarkers for cancer metastasis. Next, we investigated whether Ad5/35E1aPSESE4 could detect CTCs from patients bearing organ-confined prostate tumors. Blood obtained from patients after prostatectomy was infected with Ad5/35E1aPSESE4 and was observed under fluorescence microscopy. The majority of those patients (54 of 88 patients) did not exhibit fluorescence and had no cultivated cells upon Ad5/35E1aPSESE4 infection. Many patients (25 of 88 patients) had fluorescent cells but did not have cultivatable cells (Table 2). However, four patients had fluorescent and cultivatable CTCs marked by Ad5/35E1aPSESE4; interestingly, 3 patients had as low as 0.1 ng/mL PSA in the blood (Table 3). CTCs from one of 3 patients were further characterized in Fig. 5. Cultivated cells were exposed to various concentrations of docetaxel and 2 μM of docetaxel inhibited cell survival by 30% in cultivated CTCs (Fig. 5B). Furthermore, those cultivated CTCs were analyzed by RNA-seq and were shown to overexpress several metastasis-related genes (Fig. 5C). PSCA was included as a reference gene known to be expressed in prostate cancer cell. This finding suggests that cultured and fluorescent CTCs can be

Fig. 2. Quantitation of C4-2 cells in the presence of PBMCs by adenovirus Ad5/35E1aPSESE4. A. Different numbers of C4-2 cells from 0 to 100 were mixed with 1×105 PBMCs and counted at 48 and 72 h. B. C4-2 cells of 10 or 100 were infected with 1 MOI of Ad5/35E1aPSESE4. Forty-eight hours post-infection, cells were harvested and the total DNA was extracted. For counting virus particles inside cells, qPCR was performed using primers corresponding to adenovirus ITR as described in the Materials and Methods.

Fig. 3. Prostate CTCs in mice marked by Ad5/35E1aPSESE4. A total of 1×106 C4-2 cells were injected into the tail veins of BALB/c nude mice. After 6 h of injection, 200 µL of blood was withdrawn and lysed with RBC lysis buffer. PBMCs were seeded onto 12 well-plates and infected with 1 MOI of Ad5/35E1aPSESE4. Cells expressing GFP upon infection were observed and counted under a fluorescence microscope.

Fig. 4. Prostate CTCs in mice marked by Ad5/35E1aPSESE4. A. Different numbers of C4-2 cells from 0 to 100 were mixed with 1×105 PBMCs and counted at 48 and 72 h. B. C4-2 cells of 10 or 100 were infected with 1 MOI of Ad5/35E1aPSESE4. Forty-eight hours post-infection, cells were harvested and the total DNA was extracted. For counting virus particles inside cells, qPCR was performed using primers corresponding to adenovirus ITR as described in the Materials and Methods. C. A large number of CTCs (Supplementary Fig. S1). As summarized in Table 1, most of the patients harbored CTCs in which the fluorescence signal was amplified and that were cultivatable. Such results implied that detectable fluorescence and cultivatable CTCs in patient blood were strong biomarkers for cancer metastasis. Next, we investigated whether Ad5/35E1aPSESE4 could detect CTCs from patients bearing organ-confined prostate tumors. Blood obtained from patients after prostatectomy was infected with Ad5/35E1aPSESE4 and was observed under fluorescence microscopy. The majority of those patients (54 of 88 patients) did not exhibit fluorescence and had no cultivated cells upon Ad5/35E1aPSESE4 infection. Many patients (25 of 88 patients) had fluorescent cells but did not have cultivatable cells (Table 2). However, four patients had fluorescent and cultivatable CTCs marked by Ad5/35E1aPSESE4; interestingly, 3 patients had as low as 0.1 ng/mL PSA in the blood (Table 3). CTCs from one of 3 patients were further characterized in Fig. 5. Cultivated cells were exposed to various concentrations of docetaxel and 2 μM of docetaxel inhibited cell survival by 30% in cultivated CTCs (Fig. 5B). Furthermore, those cultivated CTCs were analyzed by RNA-seq and were shown to overexpress several metastasis-related genes (Fig. 5C). PSCA was included as a reference gene known to be expressed in prostate cancer cell. This finding suggests that cultured and fluorescent CTCs can be
Fig. 4. CTC detection in a TRAMP mouse by Ad5/35E1aPSESE4. A. A total of $1 \times 10^3$ TRAMPc2 cells were seeded into 12 well-plates and infected with 1 MOI of Ad5/35E1aPSESE4. Cells were monitored under fluorescence microscope at 48 h post-infection. B. On a regular basis from 8 weeks after birth, 200μL of blood was withdrawn from TRAMP mice developing orthotopic tumors inside the prostate and deprived of RBC. The remaining PBMCs were infected with Ad5E1PSESE4, and GFP was monitored and counted under fluorescence microscopy. Simultaneously, $[^{18}F]$FDG-PET/CT was employed to monitor tumor growth in the prostate. C. After the study was completed, the TRAMP mouse was sacrificed and tumors around the prostate were confirmed by H&E (hematoxylin and eosin) staining. SV, seminal vesicle; P, prostate; B, bladder.

Table 1
CTC detection in prostate cancer patients with tumor metastasis.

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>Number of detected CTCs</th>
<th>Number of patients</th>
<th>CTC culture or not</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cancer with metastasis</td>
<td>0</td>
<td>11</td>
<td>No</td>
</tr>
<tr>
<td>1–5</td>
<td>12</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>5–10</td>
<td>5</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>&gt;10</td>
<td>6</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Castration-resistant prostate cancer</td>
<td>0</td>
<td>6</td>
<td>No</td>
</tr>
<tr>
<td>1–5</td>
<td>5</td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>&gt;10</td>
<td>9</td>
<td></td>
<td>Yes</td>
</tr>
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Table 2
CTC detection in prostate cancer patients with no metastasis.

<table>
<thead>
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<th>Tumor stage</th>
<th>CTC culture or not</th>
<th>Number of detected CTCs</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk prostate cancer&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Localized prostate cancer&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1–5</td>
<td>23</td>
</tr>
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<td></td>
<td></td>
<td>5–10</td>
<td>1</td>
</tr>
<tr>
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<td>Yes</td>
<td>&gt;10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.1</td>
<td>3</td>
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<td></td>
<td></td>
<td>5–10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;10</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Patient received active surveillance of low risk prostate cancer.
<sup>b</sup> Patients underwent radical prostatectomy included localized or locally advanced prostate cancer.
detected from patient blood with serum PSA (\(<0.1\) ng/mL), indicating the potential of Ad5/35E1aPSESE4 to provide additional information that the PSA value alone cannot provide regarding disease status. Another interesting group of 5 patients with PSA > 0.1 ng/mL. No fluorescent cells but did have cultured cells. These cells may be non-prostate cancer cells or prostate CTCs that did not express PSA/PSMA, requiring further analysis.

**Discussion**

Radical prostatectomy is the standard treatment for localized prostate cancer. Although patients with prostate cancer undergo surgery, many patients still show PSA increasing within 10 years after surgery, which is called the biochemical recurrence of prostate cancer and is observed in more than half of patients [16]. To predict the risk of disease recurrence after treatment, physicians use clinical prognostic factors including baseline PSA level, stage (clinical/pathological), and Gleason scoring, which have been regarded as key predictive variables. However, the accuracy of those clinical variables is limited in predicting the prognosis of prostate cancer due to diverse PSA levels arising from heterogeneous tumor biology and the inconsistency of tumor grading based on the Gleason grading system. Many studies have aimed at developing candidate biomarkers in peripheral blood for prostate cancer with clinical use for the prognostication of disease recurrence and progression after treatment. In our previous studies, we found that some candidate tumor markers in peripheral blood have a potential role in predicting disease recurrence after surgery, such as PSCA and PSMA mRNA expression in cells in peripheral blood by the RT-PCR method [17,18]. Numerous reports have called into question the usefulness of RT-PCR in prostate cancer due to its limitations with respect to sensitivity and quantitation. When the results of CTC detection were analyzed in the present study, cases of advanced prostate cancer showed a higher rate of CTC detection in the peripheral blood of patients than those of early-localized prostate cancer. The CTCs were more frequently cultured in advanced disease, such as in cases with metastasis or castration-resistant diseases (Tables 1–3). Compared with CTC detection by RT-PCR amplification of PSA and PSMA genes using total RNA templates obtained from prostate cancer patients with metastasis (Supplementary Table S1) or CRPC patients (Supplementary Table S2), Ad5/35E1aPSESE4 was the more sensitive tool in judging the CTC existence as well as the total number in patient blood. These findings support the proposal of the potential role of CTC detection with adenovirus Ad5/35E1aPSESE4 as a molecular staging tool of prostate cancer. Furthermore, our method for Ad5/35E1aPSESE4 use detects only cells expressing PSA/PSMA proteins and is able to count fluorescent CTCs and to determine whether to culture CTCs. At least 3 months after surgery, we detected CTCs in 29 of 85 localized prostate cancer patients who underwent radical prostatectomy. Among 29 patients showing CTC in peripheral blood, 27 patients still showed no biochemical evidence of tumor recurrence, with an undetectable PSA level (PSA \(<0.1\) ng/mL). Furthermore, 3 of 27 patients harbored cultivatable CTCs (Table 3). Considering that the majority of CTCs originated from a primary tumor are eliminated by a host defense mechanism, the detection of CTCs that have not been eradicated in circulation is likely to indicate tumor recurrence or residual tumors in the primary site or a distant area. These discoveries demonstrate the potential role of CTC detection by Ad5/35E1aPSESE4 for the prognostication of prostate cancer after definite treatment including surgery. It would be interesting to conduct a longer follow-up in order to conclude whether clinically confirmed tumor recurrence occurred in those patients who presented with post-operative CTC detection. In addition, we succeeded in in vitro CTC culturing and performing Docetaxel-sensitivity tests (Fig. 5B), enabling our method to be

**Table 3**

<table>
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<tr>
<th>Tumor stage</th>
<th>CTC detection</th>
<th>CTC culture or not</th>
<th>Number of patients</th>
<th>PSA &lt;0.1</th>
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<td></td>
<td>Yes</td>
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applied to patient-specific therapy through drug-sensitivity tests or biologic phenotyping of cultured CTCs.

CTCs are present in the blood, and as a result, a so-called liquid biopsy by a simple procedure has a strong advantage over regular tissue biopsy, potentially providing a benefit to patients [19]. There are a number of clinical trials registered at www.clinicaltrials.gov investigating CTCs as biomarkers. To date, much effort has been exerted to develop a new detection method with better specificity and sensitivity. The most well-established methods utilize epithelial markers such as EpCAM [20] and CD45 [21]. These methods are efficient, but they have definite limitations in that large numbers of CTCs lose their epithelial phenotypes in the blood. Alternatively, CTCs of relatively large size are captured through small pores that can separate them from small PBMCs. This method is also likely to fail to capture cells as small as PBMCs, as some patients harbor as few of the relatively small CTCs as one per 5 mL of blood. In parallel with the need for a better method is the introduction of selectively labeling CTCs with a reporter gene delivered by RCA. Depending on the transmigration regulator used to control RCA replication, its sensitivity and specificity can be determined. The association of cancer cells with the hTERT promoter was employed to control the E1a gene of RCA, and hTERT-controlled RCA was applied to CTCs originating from breast cancer [12]. Another RCA for prostate CTC detection utilized a rat probasin promoter, and a luciferase was secreted into culture media upon infection of prostate cancer CTCs [13]. These two RCAs have distinct drawbacks in that they are unable to mark suspended CTCs in blood from late-stage prostate cancer patients. hTERT expression is not always high in the late-stage tumor [22,23], and the probasin is dependent on the androgen/androgen receptor. Probasin cannot be applied to CTCs in patients undergoing hormone therapy and in the hormone-deprivation stage. In our previous study, the PSES enhancer was introduced to produce an RCA with prostate cell specificity. PSES is a composite of two transcription regulatory elements controlling two common prostate specific proteins – PSA and PSMA [14] – and mediates high transcription activity in PSA/PSMA-expressing prostate cancer cells in the presence and absence of androgen. PSA and PSMA genes are the two most well-known genes expressed by the majority of androgen-dependent as well as androgen-independent prostate cancers. Therefore, PSA and PSMA, with strong androgen-independent promoter activity, make PSES superior to probasin for the detection of CTCs from patients undergoing androgen ablation therapy. Serum PSA has a limitation but is an effective biomarker in correlation with disease status prior to prostatectomy or hormone therapy.

The standard of care in first-line metastatic castration-resistant prostate cancer (mCRPC) is docetaxel-based chemotherapy. Docetaxel with estramustine or prednisone can prolong survival compared with mitoxantrone plus prednisone in mCRPC by a median of approximately 18 months [24,25]. Recently, patient-derived xenograft (PDX) has been employed to test the extent to which a certain tumor is responsive prior to chemotherapy. However, PDX is not well formed and does not reflect tumor heterogeneity. In this study, CTCs from many CRPC patients were cultured to enable further drug sensitivity tests, suggesting that some of the mCRPC patients could have benefited from CTC evaluation in determining the most effective chemotherapy regime. Furthermore, in cases of chemotherapy resistance, genome analysis of CTC culture would be useful to understand resistance mechanisms, as resistance depends on the genetic mutations of prostate tumors. Our data highlight a novel strategy to detect CTCs by a PSES-controlled adenosvirus, which effectively marks prostate cancer cells in patient blood. Because CTCs reflect the patient status for potential recurrence and disease progression, Ads535E1aPSES4-mediated CTC detection accompanied by the identification of CTCs is worthy of further investigation.

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Conflict of interest

This study has no financial and personal relationships with other people or organizations that could inappropriately influence this work.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.12.018.

References


