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# Adenovirus vector carrying *REIC/DKK-3* gene: neoadjuvant intraprostatic injection for high-risk localized prostate cancer undergoing radical prostatectomy

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As the First-In-Human study of *in situ* gene therapy using an adenovirus vector carrying the human *REIC* (reduced expression in immortalized cell)*/Dkk-3* gene (Ad-REIC), we conducted neoadjuvant intraprostatic injections in patients with high-risk localized prostate cancer undergoing radical prostatectomy (RP). Patients with recurrence probability of 35% or more within 5 years following RP, as calculated by Kattan's nomogram, were enrolled. Patients received two ultrasound-guided intratumoral injections at 2-week intervals, followed by RP 6 weeks after the second injection. After confirming the safety of the therapeutic interventions with initially planned three escalating doses of $1.0\times10^{10}$, $1.0\times10^{11}$ and $1.0\times10^{12}$ viral particles (vp) in 1.0–1.2 ml ($n=3$, 3 and 6), an additional higher dose of $3.0\times10^{12}$ vp in 3.6 ml ($n=6$) was further studied. All four DLs including the additional dose level-4 (DL-4) were feasible with no adverse events, except for grade 1 or 2 transient fever. Laboratory toxicities were grade 1 or 2 elevated aspartate transaminase/alanine transaminase ($n=4$). Regarding antitumor activities, cytopathic effects (tumor degeneration with cytolysis and pyknosis) and remarkable tumor-infiltrating lymphocytes in the targeted tumor areas were detected in a clear dose-dependent manner. Consequently, biochemical recurrence-free survival in DL-4 was significantly more favorable than in patient groups DL-1+2+3.

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## INTRODUCTION

The reduced expression in immortalized cell (*REIC*) gene was originally identified and cloned at the Okayama University (Okayama, Japan) and reported in 2000 as a gene whose expression is decreased through the immortalization of normal human fibroblasts.[1] The sequence of the *REIC* gene was found to be consistent with that of the human *Dkk-3* gene, a member of the *Dkk* family genes (*hDkk-1*, *-2*, *-3* and *-4*). The expression of the *REIC/Dkk-3* gene was found to be markedly downregulated in a broad range of human cancer cells as a tumor suppressor gene.[2–9] In our preclinical studies including *in vivo* study using immunocompetent orthotopic mouse tumor models, the therapeutic effects of Ad-REIC and its mechanisms of action have been clarified.[5,10–17] The simultaneous induction of cancer-selective apoptosis and augmentation of antitumor immunity is the most characteristic feature of Ad-REIC. Ad-REIC offers a new, sophisticated way to induce selective toxicity based on different sensitivities to Ad-REIC-induced unfolded protein responses in the endoplasmic reticulum (ER) between cancer and normal cells. ER stress-induced apoptosis triggered by the activation of c-Jun N-terminal kinase is mediated in Ad-REIC-infected cancer cells but not in infected normal cells. The overproduction of interleukin-7 (IL-7) by infected normal cells including cancer-associated fibroblasts is responsible for the activation of innate immunity involving natural killer (NK) cells.[18] Furthermore, secreted REIC protein with potent immunomodulatory function creates an optimal environment for the activation of host immune cells, inducing cytotoxic T lymphocytes (CTLs).[14,19] As reported previously, dendritic cells, induced by secreted REIC proteins, acquire possible cancer antigens from apoptotic cancer cells and induce tumor-associated antigen-specific $CD8^+$ CTLs.[20,21] The resulting CTLs are expected to have a major role in systemic antitumor immunity of *in situ* Ad-REIC as a personalized therapeutic cancer vaccine.

To develop Ad-REIC as a new class of therapeutic cancer vaccines, the First-In-Human clinical study, a phase I/IIa study of *in situ* Ad-REIC gene therapy for prostate cancer (PCa), was initiated at the Okayama University from January 2011. As reported previously,[22,23] in this phase I/IIa study, two groups of patients were treated: group A consisting of patients with castration-resistant PCa (CRPC) with or without metastasis, and group B consisting of patients with high-risk, localized PCa scheduled to undergo radical prostatectomy (neoadjuvant study). Ad-REIC was injected directly into the prostate or metastatic tumor using four escalating doses of viral particles (vp), starting from $1.0\times10^{10}$ to $3.0\times10^{12}$ vp. In group A, direct and indirect systemic effects induced by *in situ* gene therapy were clearly illustrated in a case of chemotherapy-resistant advanced CRPC with bulky lymph node metastases.[22] In the neoadjuvant study, patients treated with the initially planned three escalating dose levels (DLs) of $1.0\times10^{10}$, $1.0\times10^{11}$ and $1.0\times10^{12}$ vp in 1.0–1.2 ml ($n=3$, 3 and 6) showed remarkable safety profiles of Ad-REIC without reaching the maximum-tolerated dose. Then, an additional study with a higher DL-4 of $3.0\times10^{12}$ vp in 3.6 ml ($n=6$) was conducted. As of

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November 2014, therapeutic interventions of 18 cases in group B were completed and preliminary data on feasibility of neoadjuvant Ad-REIC gene therapy were reported in a separate brief note.[23] In this communication, precise clinical results with long-term follow-up in group B were presented.

## MATERIALS AND METHODS

### Patient eligibility

Patients with clinical stage T2a–T3a adenocarcinoma of the prostate and probable candidates for RP were recruited. Kattan's nomogram score of $\geqslant 115$ (calculated 5-year recurrence-free probability of $\leqslant 65\%$)[24,25] was used to select high-risk localized PCa. All participants were required to undergo needle biopsy of the prostate (at least 12 cores) to obtain tissue for pathologic analysis. A baseline chest X-ray, bone scan, computed tomography scan of the abdomen and pelvis and magnetic resonance imaging (MRI) of the pelvis and prostate were mandatory for determining the clinical stage. Patients reviewed the informed consent document and received individual counseling with a thorough discussion as to alternative treatments, including non-participation. Written informed consent was obtained from all patients.

### Study design and therapy administration

The present clinical protocol was approved by the Okayama University Institutional Review Board and the Japanese Government. This protocol was an open-label, dose escalation study without the inclusion of control subjects. After getting UMIN ID (UMIN000004929) for Japanese clinical trial registration, the clinical study was initiated at the Okayama University from January 2011. Initially, three escalating DLs of $1.0\times10^{10}$, $1.0\times10^{11}$ and $1.0\times10^{12}$ vp in 1.0–1.2 ml were planned. Patients received two ultrasound-guided intratumoral injections at 2-week intervals, followed by RP 6 weeks after the second injection. Based on MRI findings and biopsy mapping, one track injection to the most prominent cancer area was conducted. The injection was carried out by a newly developed injection machine driven by an air pressure (228AHBZ00005000; Nemoto Kyorindo, Tokyo, Japan) that can control injection speed and solution volume under ultrasound guidance. The safety of the therapeutic interventions with DL-1, -2 and -3 ($n=3$, 3 and 6) being confirmed, an additional higher dose, DL-4 of $3.0\times10^{12}$ vp in 3.6 ml ($n=6$), was further studied. As for DL-4, three track injections (three times injection of $1.0\times10^{12}$ vp in 1.2 ml) to multiple target cancer areas were conducted.

### Adenovirus vector

A full-length cDNA of human *REIC/Dkk-3* gene was integrated into a cosmid vector pAxCAwt and transferred into an E1/E2-deleted replication-deficient adenovirus type 5 vector with CAG (CMV early enhancer/chicken β-actin) promoter[26] by the cosmid cassettes and Ad DNA-terminal protein complex (COS/TPC) method (Takara Bio, Shiga, Japan). A cGMP product of Ad5-CAG-REIC, free of replication-competent adenoviruses, was developed and supplied by a startup biotech company, Momotaro-Gene Inc. (Okayama, Japan).

### Monitoring viral DNA detection and neutralizing antibody

Adenoviral DNA in blood and urine was determined by real-time PCR on day 0 (before viral injection) and on days 1, 3, 15, 17 and 56 after injection in a commercial-based laboratory (SRL, Tokyo, Japan). Neutralizing antibody titers against adenovirus were also determined on days 0, 14, 28, 56 and 84 (SRL, Tokyo, Japan).

### Therapeutic evaluations

Using standard sections of RP specimens with hematoxylin and eosin (H&E) staining, final pathological diagnosis and antitumor effects mediated by Ad-REIC were determined. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining for the detection of apoptosis of cancer cells and immunohistochemical staining for the analysis of tumor-infiltrating lymphocytes were conducted in selected cases. Peripheral blood lymphocyte subsets were analyzed by multicolor flow cytometry on days 0, 1, 3, 7, 14, 15, 17, 21, 28 and 56: $CD14^{-}CD45^{+}$, $CD3^{+}CD4^{+}CD8^{-}$, $CD3^{+}CD4^{-}CD8^{+}$, HLA-DR/CD3, HLA-DR/CD4, HLA-DR/CD8, $CD3^{+}CD19^{-}$, $CD3^{-}CD19^{+}$ and $CD3^{-}CD19^{-}CD16^{+}CD56^{+}$ (SRL). NK activity, interferon-γ, tumor necrosis factor-α, IL-6 and IL-7 were also measured (SRL). Serum prostate-specific antigen (PSA) levels were analyzed before and after Ad-REIC injections by measuring on days 0, 7, 14, 21, 28 and 56. The changing rate in PSA (PSA on day 56/PSA on day 0x100) was calculated as a therapeutic parameter. After RP, PSA was measured at months 1, 2, 3 and every 3 months thereafter or as clinically indicated. Biochemical recurrence was defined as an initial PSA value exceeding 0.2 ng $ml^{-1}$, followed by a subsequent confirmatory PSA value $>0.2$ ng $ml^{-1}$. If PSA levels did not decrease to $<0.2$ ng $ml^{-1}$ after surgery, the date of RP was defined as the date of disease recurrence.

### Statistical considerations

A conventional phase I/IIa clinical study was designed with a sample size of three to six patients for each of four DLs. The maximum-tolerated dose was the dose for which the incidence of dose-limiting toxicities was lesser than 33%. Although the trial was not expected to have sufficient power to detect small differences in biomarkers, the preliminary analyses were carried out using all patients and stratified by dose. Paired *t*-tests or Wilcoxon's signed-rank tests were used to evaluate the changes in biomarkers. The two-way analysis of variance test was used to compare changes in biological markers between the dose groups to assess differences. Log-rank test was applied to evaluate differences in Kaplan–Meier curves representing biochemical recurrence-free survival (BRFS) after RP among four DL groups.

## RESULTS

### Patient characteristics

Eighteen patients were enrolled with a median age of 65.5 (range 57–74) years. Clinical and pathological characteristics and follow-up duration after RP are shown in Table 1. As reported previously,[23] there were no significant differences in patient

**Table 1.** Baseline patient characteristics

| | *Vector dose level (vp)* | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *DL-1 ($1\times10^{10}$)* | | | *DL-2 ($1\times10^{11}$)* | | | *DL-3 ($1\times10^{12}$)* | | | | | | *DL-4 ($3\times10^{12}$)* | | | | | |
| | *Patient no.* | | | | | | | | | | | | | | | | | |
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* | *14* | *15* | *16* | *17* | *18* |
| Age (years) | 74 | 67 | 59 | 63 | 62 | 61 | 70 | 65 | 70 | 66 | 71 | 63 | 74 | 57 | 71 | 62 | 60 | 70 |
| PSA before Tx (day 0) | 25.2 | 9.44 | 15.75 | 33.41 | 11.84 | 5.34 | 16.18 | 9.82 | 13.41 | 14.23 | 10.81 | 13.36 | 13.32 | 25.51 | 33.36 | 17.18 | 10.87 | 21.82 |
| Clinical stage | T2c | T3a | T2a | T3a | T2c | T3a | T3a | T2c | T3a | T2c | T3a | T3a | T2c | T3a | T3a | T2c | T2a | T2a |
| Gleason score (biopsy) | 4+5 | 4+5 | 4+4 | 4+4 | 5+5 | 4+5 | 4+5 | 4+4 | 4+4 | 4+4 | 4+3 | 4+3 | 4+5 | 4+4 | 4+4 | 3+4 | 4+4 | 4+5 |
| Nomogram score | 148 | 141 | 124 | 176 | 140 | 133 | 167 | 127 | 161 | 137 | 143 | 118 | 137 | 172 | 173 | 137 | 122 | 123 |
| Follow-up (months) | 60.1 | 59.2 | 59.2 | 59 | 57.8 | 57.6 | 57.1 | 55.5 | 52 | 48.5 | 46.9 | 45.2 | 42.2 | 27.5 | 27.5 | 24.2 | 18.1 | 17.7 |

Abbreviations: DL, dose level; PSA, prostate-specific antigen; vp, viral particles.

characteristics, including Kattan's nomogram scores among groups of neoadjuvant Ad-REIC treatment (DL-1+2, DL-3 and DL-4). Most patients were regarded as a very high risk for recurrence; 83% (15/18) had a Gleason score of $\geqslant 8$ and 72% (13/18) had a Kattan's nomogram score of $>130$ (5-year recurrence-free probability of $<5\%$).

### Safety and feasibility

All DLs including the additional DL-4 were feasible without reaching maximum-tolerated dose. As for adverse events, only grade 1 or grade 2 fever was observed in DL-2, -3 and -4, but was transient and treatable with antipyretics. As for laboratory toxicities, only grade 1 or grade 2 elevated aspartate transaminase/alanine transaminase was observed in 4 out of 18 cases; grade 1 was in 2 of DL-3 and grade 2 was in 2 of DL-4 (Table 2). Neither intraoperative nor postoperative complications related to neoadjuvant Ad-REIC were observed, illustrating remarkable safety profiles of *in situ* Ad-REIC treatment. Changes in serum-neutralizing antibody titer against adenovirus are shown in Table 3. Dose-dependent response and boosting response to the second injection were not clearly observed. Neutralizing antibody response seemed to be in no relationship with clinical outcome including adverse events of *in situ* Ad-REIC treatment.

The risk of dissemination of a viral vector into the environment from the treated patient, a phenomenon called shedding, is a major safety concern, especially in Japan, where the Cartagena law is applied. Quantitative kinetic analysis of adenoviral DNA in blood and in urine by real-time PCR was conducted (Tables 4A and 4B). No adenoviral DNA in blood was detected in any of the samples studied, except for one sample on day 15, one day after the second injection, from no. 18 in DL-4. Adenoviral DNA in urine was detected on days 1 and 15 in 17 out of 36 samples, one day after the injection, in a dose-dependent manner, but not detected on days 3 and 17 in most samples (31/34). No. 7 of DL-3 showed enigmatic positive result before and after injections.

### Histopathological evaluation

Post-therapeutic findings and clinical outcome are summarized in Table 5. In terms of antitumor effects, the histopathological evaluation of RP specimens was found to be the most reliable form of measurement. Cytopathic effects illustrated by clear tumor degeneration with cytolysis and pyknosis and/or remarkable tumor-infiltrating lymphocytes (TILs) in significant areas of the targeted tumor regions were observed in a clear dose-dependent manner. Although no pathological effects were detected in DL-1, two out of three in DL-2 and three out of six in DL-3 showed significant cytopathic effects or TILs, evaluating as overall effects of grade 1. Three out of six in DL-3 and all six cases in DL-4 showed significant cytopathic effects together with remarkable TIL, evaluating as overall effects of grade 2 (see Figures 1–3). As demonstrated in Figure 2 from case 15 in DL-4, tumor degeneration with pyknosis and severe disturbance of the glandular structure were prominent without remarkable (Figure 4) TILs in the specimen from section no. 10 (2C), whereas remarkable TILs were detected without clear disturbance of the glandular structure in the specimen from section no. 12 (2D). Although these two different pathological responses might represent only different time course of the same antitumor effects of Ad-REIC, these two types of responses were detected distinctively in each Ad-REIC-targeted area. As demonstrated in Table 5, overall pathological effects were evaluated as grade 1 (+) or grade 2 (++), resulting in a clear dose-dependent clinical outcome.

### PSA and other biomarker response

Changes in PSA values related to two Ad-REIC treatments were quite different among DLs (see Supplementary Figure). In low DLs

**Table 2.** Adverse events

| | *Vector dose level (vp)* | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *DL-1 ($1\times10^{10}$)* | | | | *DL-2 ($1\times10^{11}$)* | | *DL-3 ($1\times10^{12}$)* | | | | | *DL-4 ($3\times10^{12}$)* | | | | | | |
| | *Patient no.* | | | | | | | | | | | | | | | | | |
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* | *10* | *11* | *12* | *13* | *14* | *15* | *16* | *17* | *18* |
| Fever | — | — | — | — | Grade 1 | Grade 1 | Grade 2 | Grade 1 | Grade 2 | — | Grade 1 | Grade 2 | Grade 2 | Grade 2 | Grade 2 | Grade 2 | Grade 2 | Grade 2 |
| TA elevation | — | — | — | — | — | — | — | Grade 1 | — | Grade 1 | — | — | Grade 2 | — | — | — | Grade 2 | — |
| Others (possibly related) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; DL, dose level; TA, transaminases (alanine transaminase and aspartate transaminase); vp, viral particles.

**Table 3.** Changes in serum-neutralizing antibody titer against adenovirus

| | *Day 0* | *Day 14* | *Day 28* | *Day 56* | *Day 84* |
|---|---|---|---|---|---|
| *DL-1* | | | | | |
| 1 | < 4 | < 4 | 32 | 8 | 32 |
| 2 | 16 | 16 | 8 | 4 | 16 |
| 3 | 16 | 512 | 512 | 1024 | 1024 |
| *DL-2* | | | | | |
| 4 | 4 | 16 | 64 | 128 | 128 |
| 5 | 16 | 64 | 128 | 32 | 128 |
| 6 | 32 | 512 | 256 | 1024 | 512 |
| *DL-3* | | | | | |
| 7 | < 4 | 64 | 128 | 128 | 32 |
| 8 | 128 | 256 | 512 | 256 | 256 |
| 9 | 32 | 256 | 256 | 256 | 512 |
| 10 | 4 | 4 | 4 | 8 | 16 |
| 11 | 64 | 32 | 32 | 32 | 64 |
| 12 | < 4 | 16 | 32 | 64 | 64 |
| *DL-4* | | | | | |
| 13 | < 4 | 128 | 128 | 128 | 128 |
| 14 | 4 | 32 | 64 | 32 | 64 |
| 15 | 16 | 32 | 128 | 128 | 64 |
| 16 | 16 | 32 | 64 | 1024 | 512 |
| 17 | 4 | 64 | 128 | 128 | 128 |
| 18 | 16 | 128 | 64 | 128 | 128 |

Abbreviation: DL, dose level.

**Table 4A.** Quantitative kinetic analysis of adenoviral DNA in blood by real-time PCR

| | *Day 0* | *Day 1* | *Day 3* | *Day 15* | *Day 17* | *Day 56* |
|---|---|---|---|---|---|---|
| *Adenoviral DNA quantity in blood (copies per ml)* | | | | | | |
| DL-1 | | | | | | |
| 1 | ND* | ND* | ND* | ND* | ND* | ND* |
| 2 | ND* | ND* | ND* | ND* | ND* | ND* |
| 3 | ND* | ND* | ND* | ND* | ND* | ND* |
| DL-2 | | | | | | |
| 4 | ND* | ND* | ND* | ND* | — | ND* |
| 5 | ND* | ND* | ND* | ND* | ND* | ND* |
| 6 | ND* | ND* | ND* | ND* | ND* | ND* |
| DL-3 | | | | | | |
| 7 | ND* | ND* | ND* | ND* | ND* | ND* |
| 8 | ND* | ND* | ND* | ND* | ND* | ND* |
| 9 | ND* | ND* | ND* | ND* | ND* | ND* |
| 10 | ND* | ND* | ND* | ND* | ND* | ND* |
| 11 | ND* | ND* | ND* | ND* | ND* | ND* |
| 12 | ND* | ND* | ND* | ND* | — | ND* |
| DL-4 | | | | | | |
| 13 | ND* | ND* | ND* | ND* | ND* | ND* |
| 14 | ND* | ND* | ND* | ND* | ND* | ND* |
| 15 | ND* | ND* | ND* | ND* | ND* | ND* |
| 16 | ND* | ND* | ND* | ND* | ND* | ND* |
| 17 | ND* | ND* | ND* | ND* | ND* | ND* |
| 18 | ND* | ND* | ND* | $2.6\times10^2$ | ND* | ND* |

Abbreviations: DL, dose level; ND, not detected; —, not measured. *ND: $< 1.0\times10^2$ copies per ml.

**Table 4B.** Quantitative kinetic analysis of adenoviral DNA in urine by real-time PCR

| | *Day 0* | *Day 1* | *Day 3* | *Day 15* | *Day 17* | *Day 56* |
|---|---|---|---|---|---|---|
| *Adenoviral DNA quantity in urine (copies per ml)* | | | | | | |
| DL-1 | | | | | | |
| 1 | ND* | ND* | ND* | ND* | ND* | ND* |
| 2 | ND* | ND* | ND* | ND* | ND* | ND* |
| 3 | ND* | ND* | ND* | ND* | ND* | ND* |
| DL-2 | | | | | | |
| 4 | ND* | ND* | ND* | ND* | — | ND* |
| 5 | ND* | ND* | ND* | ND* | ND* | ND* |
| 6 | ND* | ND* | ND* | ND* | ND* | ND* |
| DL-3 | | | | | | |
| 7 | $2.9\times10^3$ | $2.8\times10^3$ | $5.7\times10^2$ | $5.5\times10^3$ | $7.0\times10^3$ | ND* |
| 8 | ND* | $1.2\times10^2$ | ND* | ND* | ND* | ND* |
| 9 | ND* | ND* | ND* | $7.9\times10^2$ | ND* | ND* |
| 10 | ND* | ND* | ND* | ND* | ND* | ND* |
| 11 | ND* | $1.2\times10^2$ | ND* | ND* | ND* | ND* |
| 12 | ND* | $3.7\times10^2$ | ND* | $1.2\times10^2$ | — | ND* |
| DL-4 | | | | | | |
| 13 | ND* | ND* | ND* | $1.2\times10^3$ | ND* | ND* |
| 14 | ND* | $1.1\times10^2$ | ND* | $2.1\times10^2$ | ND* | ND* |
| 15 | ND* | $2.2\times10^3$ | ND* | $3.2\times10^2$ | ND* | ND* |
| 16 | ND* | $6.5\times10^3$ | $1.6\times10^3$ | $3.7\times10^4$ | ND* | ND* |
| 17 | ND* | ND* | ND* | $1.5\times10^4$ | ND* | ND* |
| 18 | ND* | $3.5\times10^3$ | $6.3\times10^3$ | $1.7\times10^2$ | ND* | ND* |

Abbreviations: DL, dose level; ND, not detected; —, not measured. *ND: $< 1.0\times10^2$ copies per ml.

of DL-1 and DL-2, PSA showed no remarkable changes before and after treatments, but minor reduction (PSA on day 56/PSA on day 0) was detected in two cases of DL-2 (Table 5). Similarly, minor reduction was detected in all six cases of DL-3. Although changes in PSA values in DL-4 showed declining tendencies following transient elevation because of injections, minor elevation (PSA on day 56/PSA on day 0) was detected in two cases of DL-4. Consequently, changes in PSA values were not regarded as a reliable parameter for the evaluation of clinical outcome in the present neoadjuvant study.

Peripheral blood lymphocyte subsets were analyzed by multicolor flow cytometry on days 0, 1, 3, 7, 14, 15, 17, 21, 28 and 56 in all cases. As reported previously,[23] changes in B cells, T cells, NK cells and $CD4^+$ lymphocytes showed no tendency to increase, whereas $CD8^+$ lymphocytes increased clearly in response to the Ad-REIC injection. The HLA-DR marker of activation was used to double label $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes as a relative measure of activated T cells. HLA-$DR^+CD8^+$ (activated CTL) and HLA-$DR^+CD3^+$ (activated T) lymphocytes showed increases after Ad-REIC treatment with a tendency of dose-dependent manner. As for cytokines, no detectable changes were observed in DL-1. Interferon-γ, tumor necrosis factor-α and IL-6 increased clearly on days 1 and 15, one day after Ad-REIC injection, in a dose-dependent manner. Changes in IL-7 were not constant as compared with other cytokines (Figure 4). In any case, cytokines were not regarded as a reliable parameter for the evaluation of clinical outcome.

Biochemical recurrence-free survival

BRFS of each dose group was compared using the Kaplan–Meier survival analysis, demonstrating BRFS in DL-4 was significantly more favorable than in DL-1+2 plus DL-3 group patients (Figure 5). In this phase I/IIa study, one track injection to the most prominent cancer area was conducted in the initially planned DL-1+2 plus DL-3 groups, as the primary end point was to assess the safety of the *in situ* Ad-REIC treatment. As demonstrated in Figure 1, overall pathological effects of grade 2 was detected in the histopathological sections from blocks no. 11 and 12 of targeted tumor areas, whereas large intact tumor areas were detected in the section from block no. 14. On the other hand, three track injections to multiple target cancer areas were conducted in the DL-4 group.

**Table 5.** Post-therapeutic findings and clinical outcome

| | *Patient no.* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *5* | *6* | *7* | *8* | *9* |
| Pathological stage | pT3bpN1 | pT3bpN0 | pT2cpN0 | pT3bpN0 | pT3bpN0 | pT3bpN1 | pT3bpN0 | pT2cpN0 | pT3apN0 |
| Gleason score (resected specimen) | 4+5 | 4+5 | 4+3 | 4+5 | 5+4 | 4+5 | 4+5 | 4+5 | 3+4 |
| PSA before RP (day 56) | 30.13 | 9.12 | 14.94 | 22.49 | 12.34 | 4.16 | 14.52 | 8.34 | 11.32 |
| PSA changing rate (%)[a] | 120 | 97 | 95 | 67 | 104 | 78 | 90 | 85 | 84 |
| *Evaluation of pathological effects* | | | | | | | | | |
| Cytopathic effects | – | – | – | – | + | – | + | + | + |
| Lymphocytic infiltration | – | – | – | – | – | + | + | + | – |
| Overall effects | – | – | – | – | + | + | ++ | ++ | + |
| Evaluation of MRI findings | SD | SD | SD | SD | SD | SD | SD | SD | PR |
| PSA recurrence after RP (months) | – | 9.3 | – | 0 | 0 | 6 | 0 | 9.2 | 16.1 |

| | *Patient no.* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *10* | *11* | *12* | *13* | *14* | *15* | *16* | *17* | *18* |
| Pathological stage | pT2cpN0 | pT3bpN0 | pT2cpN0 | pT2cpN0 | pT3bpN0 | pT3apN0 | pT2cpN0 | pT2cpN0 | pT2cpN0 |
| Gleason score (resected specimen) | 4+3 | 3+5 | 4+3 | 4+5 | 4+3 | 4+4 | 3+3 | 4+3 | 4+5 |
| PSA before RP (day 56) | 13 | 9.04 | 12.2 | 9.74 | 28.78 | 29.39 | 14.09 | 9.51 | 25.32 |
| PSA changing rate (%)[a] | 91% | 84% | 91% | 73% | 113% | 88% | 82% | 87% | 116% |
| *Evaluation of pathological effects* | | | | | | | | | |
| Cytopathic effects | + | + | – | + | + | + | + | + | + |
| Lymphocytic infiltration | + | – | + | + | + | + | + | + | + |
| Overall effects | ++ | + | + | ++ | ++ | ++ | ++ | ++ | ++ |
| Evaluation of MRI findings | SD | SD | SD | PR | SD | PR | SD | – | SD |
| PSA recurrence after RP (months) | 22.4 | – | 0 | – | – | – | – | – | 9.2 |

Abbreviations: MRI, magnetic resonance imaging; PR, partial response; PSA, prostate-specific antigen; RP, radical prostatectomy; SD, stable disease. [a]PSA changing rate (%): PAS on day 56/PSA on day 0 ×100.

As illustrated in Figure 2, clear pathological effects were obtained in the sections from three targeted tumor areas. As for the case no. 13 in DL-4, three track injections were carried out on only one cancer area detected by MRI, resulting in almost complete destruction of tumor tissues with remarkable TILs (Figure 3). These pathological changes were well accorded with MRI findings and a condensed exposure of Ad-REIC seemed to be more effective. Consequently, it was obvious that multitrack injections to multiple target cancer areas with a sufficient dose of Ad-REIC were clearly effective for successful neoadjuvant therapy.

## DISCUSSION

At present more than 1500 gene therapy protocols for cancer have been conducted around the world (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Interestingly, the second highest number of protocols is for PCa. The prostate is regarded as an ideal target organ for the development of cancer gene therapy. PCa is a leading internal malignancy and new therapies are needed, especially for metastatic CRPC. The prostate is easily accessible organ and biopsy and gene transfer can be easily conducted by ultrasound guidance. In addition, PSA, an extremely sensitive tumor marker, is available for clinical evaluation. More recently, a variety of clinical trials including *in situ* gene therapy[27–29] have been conducted as a form of neoadjuvant therapy, as this approach provides a paradigm for evaluating the activity and mechanism of action of new agents with histopathological analysis using tumor tissues before and after therapy.[30] In fact, histopathological analysis of RP specimens provided the most reliable parameters in the evaluation of clinical effects induced by *in situ* Ad-REIC treatment in the present study.

Neoadjuvant therapy is widely accepted in the treatment of patients with localized or locally advanced high-risk breast cancer and other solid cancers.[31–33] In PCa, however, the beneficial effects on pathological outcomes provided by neoadjuvant androgen deprivation therapy and chemotherapy with or without androgen deprivation therapy did not link to improved disease-free survival or overall survival.[34–39] Neoadjuvant therapy with androgen deprivation therapy and chemotherapy is not currently recommended in patients with high-risk localized PCa undergoing RP. Consequently, *in situ* immune gene therapy has a great potential to offer a new option for neoadjuvant therapy by generating indirect, systemic antitumor effects.[27–29]

The ultimate aim of *in situ* Ad-REIC cancer gene therapy is to develop a new generation of cancer vaccines based on the concept of simultaneous induction of selective killing of cancer cells and augmentation of antitumor immunity. This kind of cancer vaccine strategy will become a leading standard in the treatment of most solid cancers with gene therapy.[40] In October 2015, T-VEC (talimogene laherparepvec; an oncolytic herpes simplex type 1 virus armed with granulocyte–macrophage colony-stimulating factor)[41] was approved by the Food Drug and Administration for the treatment of melanoma in patients with inoperable tumors. Similarly, Pexa-Vec (pexastimogene devacirepvec; an oncolytic vaccinia poxvirus armed with granulocyte–macrophage colony-stimulating factor) has already been successfully developed.[42] In our preclinical studies,[5,11,12,14,18,20,21] Ad-REIC presented a new, sophisticated way to induce cancer-selective apoptosis because of ER stress, providing an ideal presentation of possible cancer antigens to the host immune system. Secreted REIC protein at the tumor site also creates an optimal environment, mediating tumor-associated, antigen-specific cytotoxic T cells. In addition,

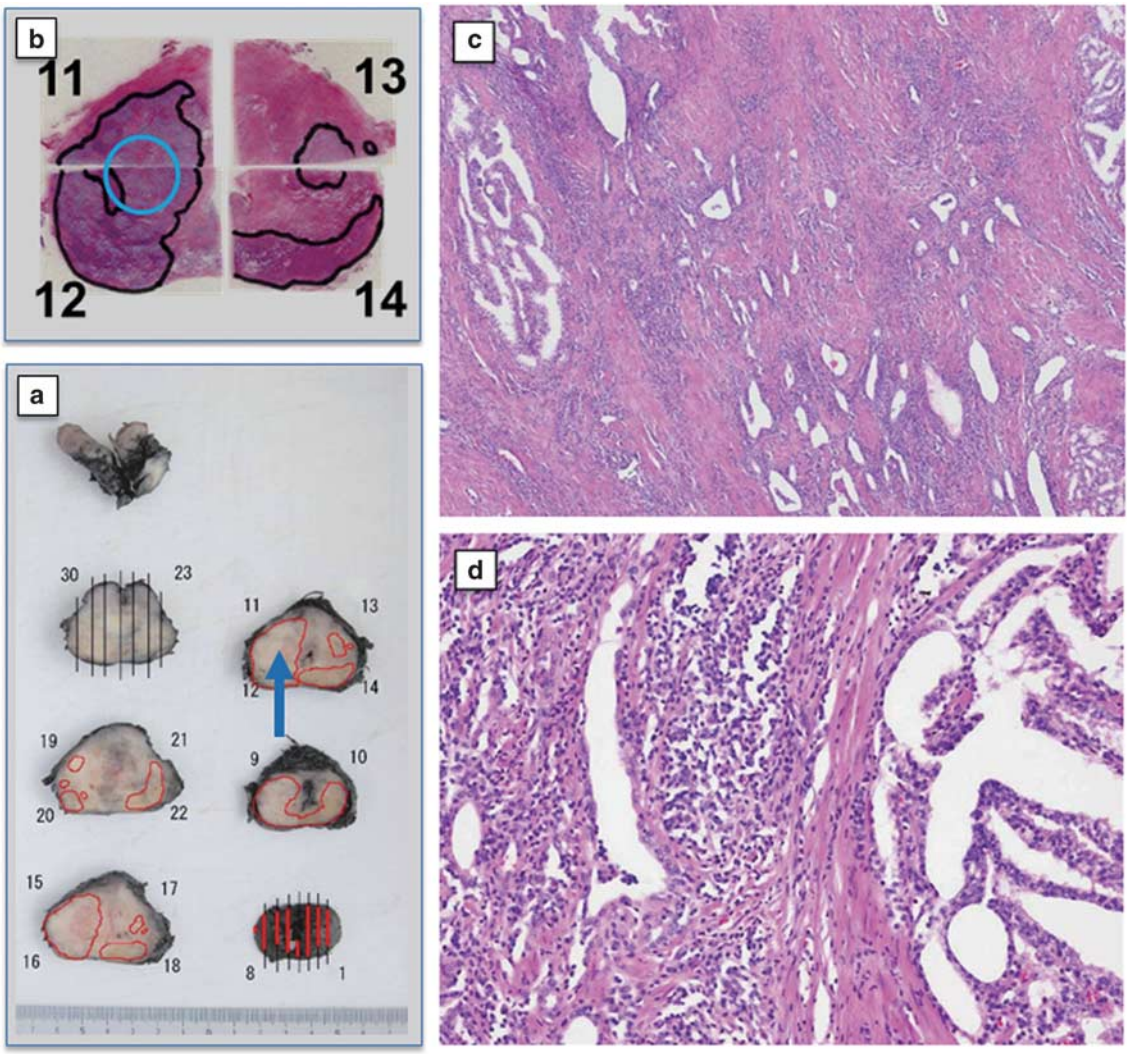


**Figure 1.** Surgical specimens from case 7 in dose level-3 (DL-3) treated by one track injection with $1x10^{12}$ vp of Ad-REIC. (**a**) Gross appearance of radical prostatectomy (RP) specimen sliced by a standard method. Cancer distribution areas are enclosed by the red line. A blue arrow indicates the Ad-REIC-targeted area. (**b**) Gross appearance of hematoxylin and eosin (H&E)-stained histopathological sections. Cancer distribution areas are enclosed by the black line. A blue circle indicates the targeted area. (**c**) A photomicrograph of the targeted area from section no. 12, demonstrating tumor degeneration with tumor-infiltrating lymphocytes (TILs) (H&E). (**d**) A photomicrograph of cancer distribution area from section no. 14, demonstrating a large intact tumor area (H&E).

the overproduction of IL-7 by cancer-associated fibroblasts activates innate immunity involving NK cells. As the induction of activated dendritic cells and antigen-specific CTLs is crucial for the development of therapeutic cancer vaccines by Ad-REIC, this process was carefully investigated in a mouse tumor model using E.G7-expressing OVA.[21] The intratumoral administration of Ad-REIC-mediated robust antitumor effects with the accumulation of OVA-specific CTLs in the tumor tissues and spleen and with the upregulation of the CD86-positive dendritic cells in the tumor draining lymph nodes. In a dual tumor-bearing mouse model in the left and right back, Ad-REIC injection in one side significantly suppressed the tumor growth on both sides and significant infiltration of OVA-specific CTLs into non-injected tumor was detected. These results clearly indicate that the ER stress-induced apoptosis by Ad-REIC mediates immunogenic cancer cell death.

Recently, the concept of immunogenic cell death has been introduced with the evidence that apoptotic cancer cells induced by anthracyclines[43] or ionizing irradiation[44] are able to induce a potent immune response. It is now clear that ER stress has a principal role in immunogenic cell death induced by several chemotherapeutic agents and oncolytic viruses and endogenous damage-associated molecular patterns releasing from apoptotic cancer cells are responsible for promising anticancer effects including the reinitiation of immune responses suppressed by the tumor microenvironment.[45–47] We are now complementing whole action mechanisms of Ad-REIC by considering roles of damage-associated molecular patterns associated with Ad-REIC treatment.

In the present neoadjuvant study, we believe that the proof of concept of simultaneous induction of selective killing of cancer cells and augmentation of antitumor immunity by Ad-REIC has been successfully established. Using RP specimens after two intratumoral Ad-REIC injections, tumor degeneration with cytolysis and pyknosis and remarkable TILs in significant areas of the targeted tumor regions were detected in a clear dose-dependent manner. As illustrated in the previous report,[23] terminal deoxynucleotidyl transferase dUTP nick-end labeling staining was helpful in the detection of apoptosis of cancer cells and immunohistochemical staining revealed concurrent infiltrations of $CD8^+$ lymphocytes and dendritic cells in the area of apoptotic cancer cells. In addition, peripheral blood $CD8^+$ T cells and

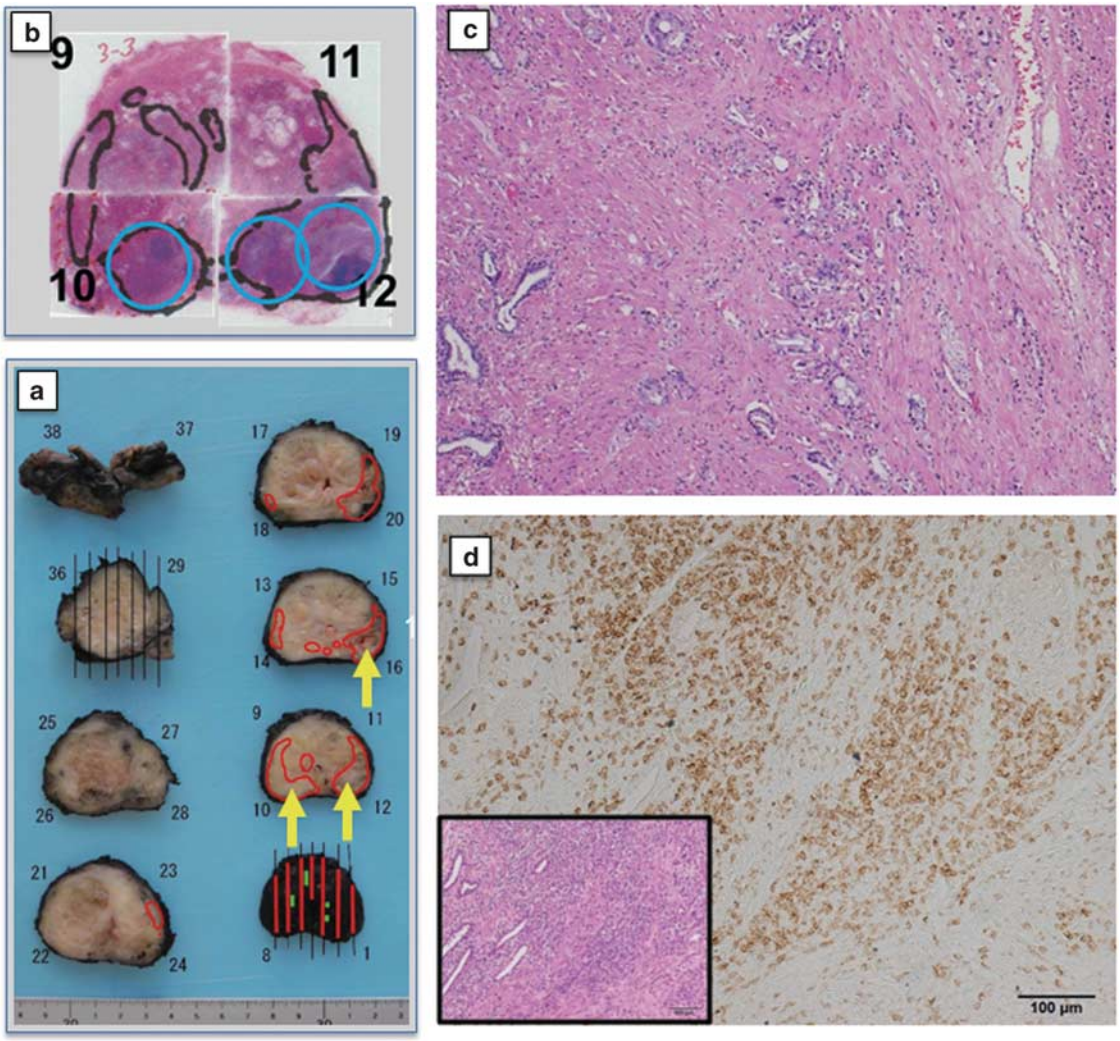


**Figure 2.** Surgical specimens from case 15 in dose level-4 (DL-4) treated by three track injections with $3x10^{12}$ vp of Ad-REIC. (**a**) Gross appearance of radical prostatectomy (RP) specimen sliced by a standard method. Cancer distribution areas are enclosed by the red line. Three yellow arrows indicate the Ad-REIC-targeted areas. (**b**) Gross appearance of hematoxylin and eosin (H&E)-stained histopathological sections. Cancer distribution areas are enclosed by the black line. Three blue circles indicate the targeted areas. (**c**) A photomicrograph of the targeted area from section no. 10, demonstrating tumor degeneration without significant tumor-infiltrating lymphocytes (TILs) (H&E). (**d**) Photomicrographs of the targeted area from the inner part of section no. 12. The inset shows low magnification view of pathological effects with remarkable TILs (H&E). Immunohistochemical staining, illustrating tumor-infiltrating $CD8^+$ lymphocytes.

$HLA\text{-}DR^+CD8^+$ (activated CTL) increased after Ad-REIC treatment in a dose-dependent manner. Although the direct detection of tumor-associated antigen-specific CTLs was not carried out mainly because of its technical difficulties, preliminary quantitative detection of autoantibodies to cancer antigens was conducted using stocked serum samples. Recently, Futami *et al*.[48] (Okayama University) have developed a sensitive multiplexed method for the analysis of autoantibodies to cancer antigens with chemically s-cationized full-length and water-soluble denatured proteins. Interestingly, this brand new technology using 50 cancer antigens including cancer/testis antigens revealed a clear elevation of auto-antibodies to multiple antigens after one and two intratumoral injections of Ad-REIC (unpublished data), illustrating a promising evidence of Ad-REIC as personalized therapeutic cancer vaccines.

In this phase I/IIa clinical study, we used one track injection to the most prominent cancer area in the initially planned DL-1+2 plus DL-3 groups and three track injections to multiple target cancer areas in the DL-4 group. BRFS in DL-4 was significantly more favorable than in DL-1+2 plus DL-3 group patients, demonstrating an expected outcome of Ad-REIC to be a promising neoadjuvant therapy for high-risk localized PCa. To evaluate the potential activities of Ad-REIC as therapeutic cancer vaccines, we re-examined histopathological effects of non-targeted tumor areas in DL-3 but we could not obtain a clearcut evidence for indirect antitumor effects mediated by Ad-REIC-induced CTLs. At this point, it is obvious that multitrack injections to multiple target cancer areas with a sufficient dose of Ad-REIC are essential for successful neoadjuvant therapy. Although further studies are needed to determine the optimal dose, dosing interval and number of doses, the present regimen using three track injections (three times injection of $1.0\times10^{12}$ vp in 1.2 ml) of Ad-REIC is regarded as one of the recommended options. To realize a more effective neoadjuvant therapy, a more potent, second generation of Ad-REIC using a super gene expression (SGE) system (Ad-SGE-REIC) has already been developed.[49–51] The SGE system is a newly developed plasmid vector, constructed by placing three enhancers in tandem after poly A to realize extremely high expression of the targeted REIC gene. A phase I/IIa study of *in situ* Ad-SGE-REIC

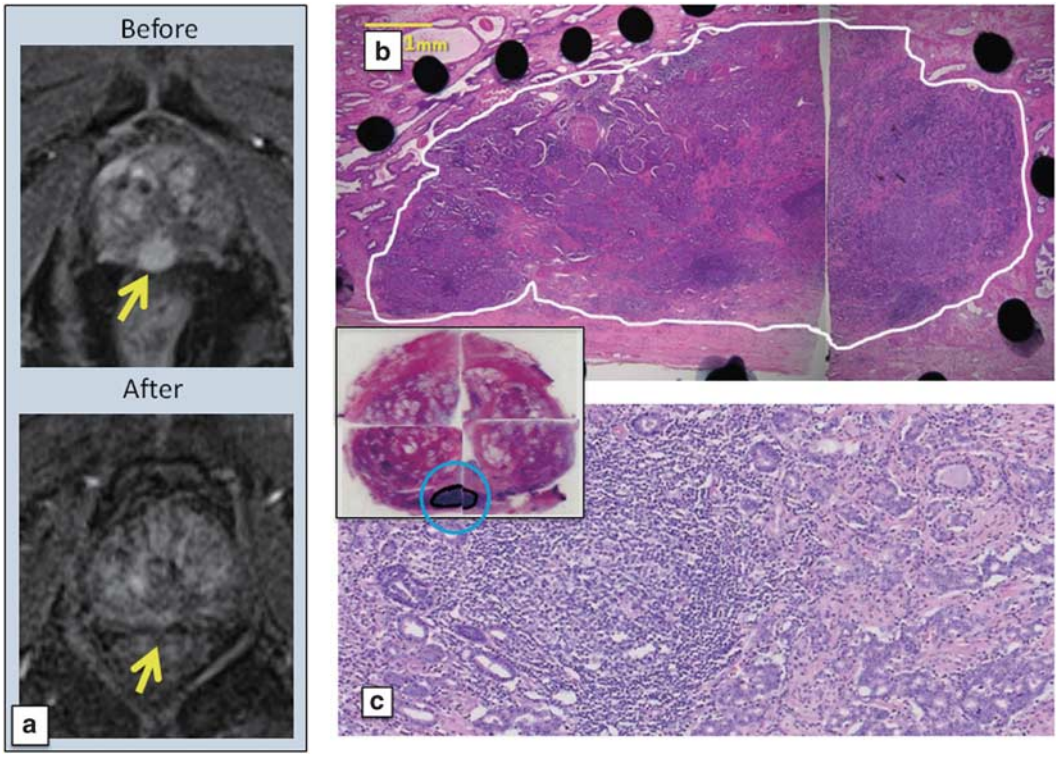


**Figure 3.** Magnetic resonance imaging (MRI) and histopathological findings of case 13 in dose level-4 (DL-4) treated by three track injections with $3x10^{12}$ vp of Ad-REIC. (**a**) Contrast enhancement MRIs. A strong enhancement area indicated by an arrow before Ad-REIC injections disappeared after injections. (**b** and **c**) Low and high magnification photomicrographs. Whole targeted cancer area is replaced by degenerated cancer cells with remarkable tumor-infiltrating lymphocytes (TILs). The inset shows gross appearance of hematoxylin and eosin (H&E)-stained histopathological sections.

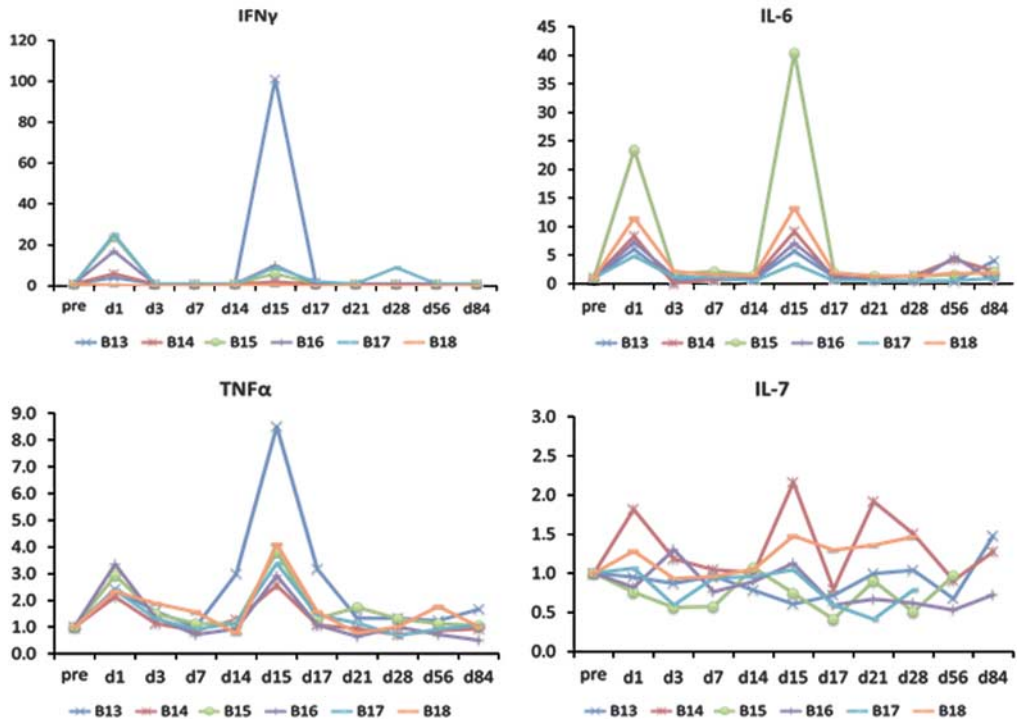


**Figure 4.** Changes in cytokines related to two Ad-REIC treatments in dose level-4 (DL-4), expressed by the ratio of the pre-treatment value. Clear increases in interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) on days 1 and 15 were noted, whereas changes in IL-7 were not constant.

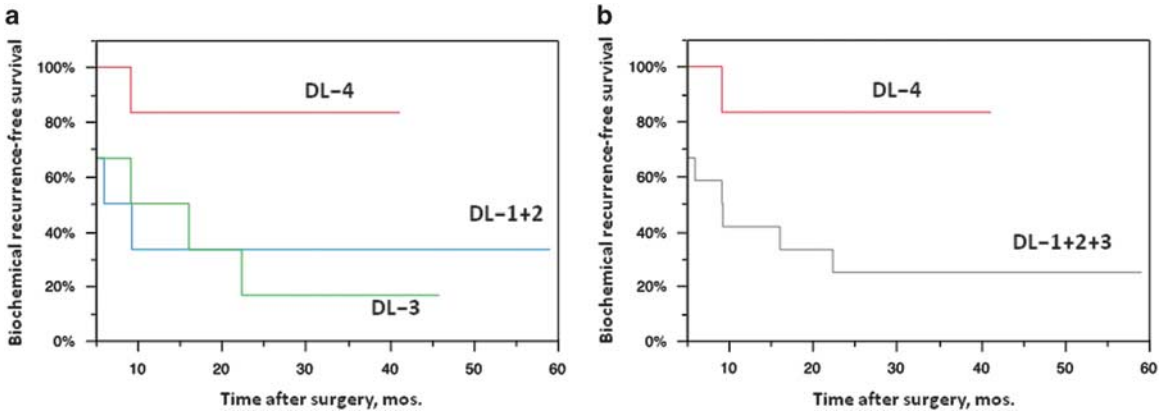


**Figure 5.** Kaplan–Meier curves representing biochemical recurrence-free survival (BRFS) in patients with high-risk prostate cancer treated with neoadjuvant Ad-REIC followed by radical prostatectomy. (**a**) BRFS curves of three dose level groups. The differences were not significant ($P = 0.101$, log-rank test). (**b**) BRFS curves of DL-1, -2 and -3 pooled group and DL-4 group. The difference was significant ($P = 0.034$, log-rank test).

therapy in patients with localized PCa sponsored by Momotaro-Gene Inc. is now ongoing successfully in the States.

Although historically, PCa was not regarded as an immunogenic cancer, recent clinical results including the efficacy of sipuleucel-T[52] for metastatic CRPC have led to a renewed interest in immunotherapy for PCa. More recently, the concept of cancer immunoediting[53] and the advent of immune checkpoint inhibitors[54,55] are changing the role of immunotherapy in cancer management. Ad-REIC, a new class of therapeutic cancer vaccines, combined with immune checkpoint inhibitors will offer a new option for immunotherapy in the treatment of patients with metastatic CRPC. As for neoadjuvant therapy for high-risk localized PCa undergoing radical prostatectomy, a definitive therapeutic regimen of Ad-REIC will be proposed through prospective randomized comparative studies.

## CONFLICT OF INTEREST

Okayama University and Momotaro-Gene Inc., a startup biotech company from the Okayama University, holds the patents of the Ad-REIC agent and develops the agent as a cancer therapeutic medicine. HK, MW and YN demonstrated the utility of the agent and also own stocks in Momotaro-Gene Inc. HK is the Chief Scientific Officer of Momotaro-Gene Inc. Okayama University and Momotaro-Gene Inc. are working together on the development of the Ad-REIC agent. Okayama University received cGMP-grade products of Ad5-REIC from Momotaro-Gene Inc. to perform clinical studies for the treatment of cancer patients.

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Supplementary Information accompanies the paper on Cancer Gene Therapy website (http://www.nature.com/cgt)