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# A retrospective study of immunotherapy using the cell wall skeleton of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG-CWS) for cervical cancer

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**Abstract**

*Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) has the potential to promote adaptive immunity. We sought to examine the synergistic effect of BCG-CWS vaccination on cervical cancer patients undergoing standard treatments including surgery, chemotherapy, and/or radiation. We retrospectively analyzed 103 patients (13 cases administered with BCG-CWS vaccine and 90 controls without BCG-CWS) who underwent a standard treatment for cervical cancer from 2005 to 2021. The BCG-CWS group underwent repeated intradermal injections of the BCG-CWS vaccine before or immediately after the standard therapy start from 2011 to 2018. The vaccination was repeated weekly for 1 month, and then every 4 weeks thereafter. The effectiveness of the BCG-CWS vaccination on cervical cancer treatment was evaluated by determining the hazard ratios of overall survival between the BCG-CWS group and the control group with multivariate analysis using the Cox model. Hazard ratios between 2 groups were determined after adjustment by clinical parameters including surgery, chemotherapy, radiation, age, clinical stage, presence of human papillomavirus, and pathology. Long-term follow-up revealed a significantly better prognosis (hazard ratio: 0.2108, $P = .008$ by the Cox model) for patients with cervical cancer in the BCG-CWS group compared to patients in the control group. Among patients with advanced cancer worse than stage IB2, some completely cleared the disease, whereas the others showed long-term survival with recurrence. BCG-CWS therapy appears to be an effective immune adjuvant therapy for cervical cancer, although randomized control studies are needed to confirm this. We also need to clarify the underlying mechanisms slowing the progression of cervical cancer in those receiving this vaccination. This study sheds light on the potential of immunostimulatory drugs such as BCG-CWS and suggests the important role of immunity for cancer elimination in combination therapy.

**Abbreviations:** BCG-CWS = Cell wall skeleton of *Mycobacterium bovis* Bacillus Calmette-Guérin, CD = cluster of differentiation, CI = confidence interval, CTL = cytotoxic T lymphocytes, DC = dendritic cells, FOXP3 = Forkhead box P3, HPV = human papillomavirus, HR = hazard ratio, OS = overall survival, PD-1 = programmed cell death protein 1, QOL = quality of life, SCC = squamous cell carcinoma, WT1 = Wilms tumor 1.

**Keywords:** BCG-CWS, cervical cancer, HPV, immunomodulator, immunotherapy

## 1. Introduction

Cervical cancer is the fourth most prevalent neoplastic disease in women.[1] It afflicts 6.6% of females worldwide, with 310,000 deaths each year.[1] The latest treatments for cervical cancer, including surgery, chemotherapy, radiation, and their various combinations, are effective and have prolonged the survival of patients with cervical cancer. However, more than a few women diagnosed with cervical cancer at an early stage suffer from long-term morbidity after complicated surgery.[2] For patients with locally advanced cervical squamous cell carcinoma at stage IB to IIA, there are 2 primary treatment options, either surgery or radiotherapy, given the assumed equivalence of these therapies.[2,3] However, surgery for advanced cervical cancer can lead to increased urological complications and reduced quality of life (QOL).[3] Even now, outcomes for recurrent cervical cancer remain poor.[2] The 5-year survival rate of patients with stage IV cervical cancer is still very low, at approx. 17%.[4] Gynecologists should present treatment methods that take into consideration QOL issues for all patients.[2] Immune therapy as an adjuvant strategy might offer a way to improve patients' QOL while also increasing the cervical cancer survival rate.

Human papillomavirus (HPV) is a causative agent for cervical cancer, and prophylactic HPV vaccines targeting the viral capsid antigen are used worldwide to prevent infection.[5] It

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had been demonstrated that the prophylactic HPV vaccine covering types 16 and 18 in girls <17 years old lessens the incidence of precursor lesions, high-grade cervical intraepithelial lesions (precancer), and cervical cancer.[6] It is thought that neutralizing antibodies prevent HPV infections in girls who have undertaken this vaccine before the onset of sexual behaviors.

HPV-positive cervical cancer has been shown to have a better prognosis than HPV-negative cancer.[7] This suggests that cell-mediated immunity induced by cytotoxic T lymphocytes (CTLs) against HPV antigens may have important roles in the elimination of cervical premalignant lesions or cancer. Based on this concept, clinical trials of therapeutic HPV vaccines targeting HPV oncoprotein E6 or E7 were conducted and demonstrated the vaccines' capacity to clear HPV-related premalignant diseases.[8,9] However, such therapeutic HPV vaccines were not so effective against invasive cancer.[10] This limitation might occur because cancer cells are able to evade the immune responses in various ways and inhibit the activities of lymphocytes through programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 pathways. The effectiveness of a combination therapy using an HPV vaccine and an immune checkpoint inhibitor is currently under investigation.[11,12]

The use of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is not a new strategy for cancer treatment. It is known that the incidence of any cancer among patients with active tuberculosis lesions is low,[13] and live BCG has been widely used for more than 30 years as an immunomodulator to treat bladder cancer.[14] The cell wall skeleton of BCG (BCG-CWS) is known to function as a component of complete Freund's adjuvant, which has produced strong immune responses to tumor cells in animal model experiments.[15] The mechanism of the antitumor effect of BCG-CWS is speculated to involve the enhancement of immunogenicity by the activation of professional antigen-presenting cells including dendritic cells (DCs) and macrophages.[16–19] BCG-CWS was reported to be an agonist of toll-like receptors, leading to activation of the myeloid differentiation primary response and the maturation of DCs.[16,19] The emulsification of BCG-CWS with mineral oil was a critical step forward since this form is thought to be more readily engulfed by phagocytes. BCG-CWS binds to toll-like receptors-2 in antigen-presenting cells, which then migrate to the draining lymph nodes.[18] This action is an important step in the activation of myeloid differentiation primary response 88 and DC maturation. After administrating both BCG-CWS and selected antigens in mice, the antigen-specific CTLs increased.[18] Smaller-size emulsified BCG-CWS particles were demonstrated to have even stronger antitumor effects in an animal model with bladder cancer.[20,21] It has been reported that the lipid antigens that constitute components of BCG-CWS activate T cells through the CD1 molecule.[22]

Historically, BCG-CWS vaccine without any additional tumor antigens has been used to treat ovarian cancer and lung cancer, and this treatment exhibited safety and efficacy in several Japanese trials.[23,24] As an improvement to BCG-CWS treatment, a recent phase 1 study by Nishida et al[25] revealed the T cell activation effect of BCG-CWS in combination with Wilms tumor 1 (WT1) peptide for WT1-expressing advanced cancers including colorectal, hepatobiliary, ovarian, and lung cancers, and melanoma. BCG-CWS vaccine may enhance immunity to the HPV 16 E6 or E7 oncoprotein expressed on premalignant lesions or cervical cancer. The purpose of the present study was to observe whether BCG-CWS could be an effective adjuvant treatment for cervical cancer or not. We investigated the prognosis of patients with cervical cancer who underwent vaccination with BCG-CWS in a private clinic as a part of a clinical study of the use of BCG-CWS in various treatment-resistant cancers. Here, we describe the adjuvant effect of BCG-CWS vaccination on survival in cervical cancer patients who received standard treatment in a retrospective study with long-term follow-up.

## 2. Methods

### 2.1. Patients

This was a retrospective and observational study. All patients with cervical cancer treated at our facility were included in this observational study. We analyzed the prognosis of a total of 103 patients (13 patients administered the BCG-CWS vaccine and 90 consecutive control patients without BCG-CWS) who underwent standard treatment such as surgery and/or chemotherapy and/or radiation for cervical cancer at Kanazawa Medical University during the period from 2005 to 2021. The BCG-CWS vaccination was performed from 2011 to 2018 in a private clinic as described below.

### 2.2. Ethics approval

After receiving written informed consent, the patients agreed to receive the BCG-CWS vaccine according to the protocol of the clinical study of BCG-CWS cancer immune therapy for therapy-resistant cancers conducted by MBR Co. (Ibaraki, Osaka, Japan) at a private hospital, Kanazawa-Riga Clinic (Kanazawa, Japan). We retrospectively examined the clinical records of those patients with cervical cancer and analyzed them in comparison with our large group of control patients without vaccination to determine the effectiveness of the BCG-CWS vaccine on cervical cancer. This observational study was approved by an Institutional Board, Kanazawa Medical University (nos. I599 and I268).

### 2.3. Intradermal inoculation with BCG-CWS

The BCG-CWS injection schedule was determined in principle so that the injections were administered before or immediately after the start of the standard treatment such as surgery, chemotherapy, and/or radiation. BCG-CWS vaccination was continued until July 2018. Freeze-dried BCG-CWS (SMP-105, MBR Co.) was suspended with Montanide ISA51 (Seppic Inc., Paris, France) to make a solution of BCG-CWS. The emulsification was performed by pushing out the solution more than 20 times between 2 syringes connected with each other. After incubation of the solution at 60°C for 30 minutes, an aliquot of 100 μL of BCG-CWS emulsified with 6 mg/mL of Montanide ISA 51 was used as the vaccination. The 100 μL emulsion was injected intradermally at the shoulder 1×/wk for 1 month and then every 4 weeks. The injection site was alternated between the right and left shoulder.

### 2.4. HPV typing

HPV types were identified by the Uniplex E6/E7 PCR method[26] or GENOSEARCH™ HPV31 (Medical & Biological Laboratories, Co., Ltd., Minato-ku, Tokyo) using cytological sampling from the cervix or formalin-fixed, paraffin-embedded cervical tissue.[27]

### 2.5. Immune-histochemical analyses for determining subsets of the immune cells

In the case study, immunohistochemical staining with $CD4^+$, $CD8^+$, $CD20^+$, $CD56^+$, and $FOXP3^+$ markers was performed to evaluate the presence of each subset of immune cells in the cervical cancer tissue or metastatic lesions of patients receiving BCG-CWS immunizations.

### 2.6. Analyses

We did not perform matching or randomization but performed multivariate analysis with patient background factors to reduce patient selection bias. We investigated whether the BCG-CWS vaccination was effective by determining the hazard ratios (HRs) of death versus overall survival (OS), between

**Table 1**

**The background of patients with cervical cancer.**

| Clinical factor | | Control (n = 90) | BCG-CWS (n = 13) | *P* value |
|---|---|---|---|---|
| Age, yr, mean ± SD | | 58.4 ± 15.6 | 46.6 ± 10.2 | .001624†* |
| Stage, n (%) | I | 30 (33.3) | 6 (46.2) | .8059‡ |
| | II | 15 (16.7) | 2 (15.4) | |
| | III | 21 (23.3) | 3 (23.1) | |
| | IV or recurrence | 24 (26.7) | 2 (15.4) | |
| HPV,§ n (%) | Positive | 59 (65.6) | 13 (100) | .3507‡ |
| | Negative | 10 (11.1) | 0 (0) | |
| Pathology, n (%) | SCC | 56 (62.2) | 5 (38.5) | .1342‡ |
| | Other type | 34 (37.8) | 8 (61.5) | |
| Surgery, n (%) | Yes | 47 (52.2) | 12 (92.3) | .006327‡* |
| | No | 43 (47.8) | 1 (7.7) | |
| Chemotherapy, n (%) | Yes | 55 (61.1) | 6 (46.2) | .3708‡ |
| | No | 35 (38.9) | 7 (53.8) | |
| Radiation, n (%) | Yes | 55 (61.1) | 6 (46.2) | .3708‡ |
| | No | 35 (38.9) | 7 (53.8) | |

HPV = human papillomavirus, SCC = squamous cell carcinoma.

*$P < .05$.

†*t* test.

‡Fisher's exact test.

§The reason why the total does not reach 100% is that there were patients whose HPV was unknown because the samples were too old to be tested.

the BCG-CWS vaccine group and the control group (no BCG-CWS) using the Cox model for multivariate analysis. HRs were calculated using the following clinical parameters: BCG-CWS (used or not), surgery (performed or not), chemotherapy (performed or not), radiation (performed or not), age, stage (I, II, III, and IV or recurrence), HPV (positive or not), and pathology (squamous cell carcinoma [SCC] or not). The purpose of this study was to investigate whether the use of BCG-CWS could enhance the therapeutic effect of conventional treatment methods. Therefore, we used a COX model that incorporated the interaction effects of the following explanatory variables related to treatment strategy: BCG-CWS, surgery, chemotherapy, and radiation. As other explanatory variables related to the patient background (e.g., age, stage, HPV, and pathological results) need to be considered for survival analysis of cervical cancer, they were also included in the COX model. By incorporating patient background features at risk of confounding results (i.e., age, stage, HPV, and pathological results) into the COX model, an adjusted HR was calculated, and the relationship between BCG-CWS and OS was evaluated by these adjusted HRs. Staging of cancer was based on International Federation of Gynecology and Obstetrics staging 2008.

### 2.7. Statistical analyses

The *t* test and Fisher's exact probability test were used to compare clinical data between BCG-CWS use and BCG-CWS non-use. The Cox proportional hazards models were analyzed with the R packages survival and survminer.[28,29] HR was calculated by the following formula: res.cox <- coxph(Surv(time, status) ~ (BCG-CWS + Surgery + Chemotherapy + Radiation)^4 + Age + Stage + HPV + Pathology, data = metadata). The "time" means observation day. The "status" means dead or alive. The "^4" indicates a calculation of the interaction effects of the following explanatory variables: BCG-CWS, surgery, chemotherapy, and radiation. Probability (*P*) values < .05 for differences between 2 groups were considered significant.

## 3. Results

### 3.1. The patients' clinical information

The BCG-CWS vaccination was performed in 13 patients, and 90 consecutive patients without the vaccination were analyzed as controls. Of the 13 BCG recipients, ten patients were started on BCG-CWS before initial treatment including radical hysterectomy (9 patients) and concurrent chemoradiotherapy (CCRT, 1 patient). Three patients with possible residual disease were started on BCG-CWS during the postoperative follow-up period. The BCG-CWS group was significantly younger at 46.6 ± 10.2 years old (mean ± SD) than the control group (58.4 ± 15.6, $P = .002$, *t* test, Table 1). The proportion of staging of cervical cancer did not differ between the BCG-CWS group (stage I, 46.2% [n = 6]; stage II, 15.4% [n = 2]; stage III, 23.1% [n = 3]; and stage IV or recurrence, 15.4% [n = 2]) and the control group (stage I, 33.3% [n = 30]; stage II, 16.7% [n = 15]; stage III, 23.3% [n = 21]; and stage IV or recurrence, 26.7% [n = 24], $P = .806$, Fisher's test, Table 1). The prevalence of HPV was 100% (13/13 patients) in the BCG-CWS group and 65.6% (59/90 patients) in the control group, but this difference in prevalence was not significant ($P = .351$, Fisher's test, Table 1). One possible reason for many negative results for HPV in the control may be due to the use of older samples (those from 2005–2011) in the control group than in the vaccine one.[26] The prevalence of SCC was 38.5% (5/13 patients) in the BCG-CWS group and 62.2% (56/90 patients) in the control group ($P = .134$, Fisher's test, Table 1). The rate of surgery was significantly higher in the BCG-CWS group than in the control group (92.3% [n = 12] vs 52.2% [n = 47], $P = .006$, Fisher's test, Table 1). No significant differences were observed for rates of chemotherapy (BCG-CWS: 46.2% [n = 6] vs control: 61.1% [n = 55], $P = .371$, Fisher's test, Table 1) and radiation (BCG-CWS: 46.2% [n = 6] vs control: 61.1% [n = 55], $P = .371$, Fisher's test, Table 1), as shown in Table 1.

### 3.2. The efficacy of BCG-CWS

This observational study revealed that multimodal therapy using BCG-CWS had a positive effect on the apparent survival effect for patients with cervical cancer. The Cox proportional hazards model analysis demonstrated that BCG-CWS significantly improved the OS (HR: 0.1145, 95% confidence interval [CI]: 0.0293–0.4482, $P = .002$, Fig. 1A and B). The OS rate for the BCG-CWS group was 72.9% at day 1308, while it was only 63.2% at day 1413 in the control group. The OS rate of the control group eventually dropped to 51.9% at day 3724.

### *3.3. Other clinical factors affecting OS*

As expected, the standard treatments including surgery (HR: $2.4 \times 10^{-9}$, 95% CI: $9.2 \times 10^{-10}$–$6.1 \times 10^{-9}$, $P < .001$), chemotherapy (HR: $1.3 \times 10^{-9}$, 95% CI: $4.7 \times 10^{-10}$–$3.7 \times 10^{-9}$, $P < .001$), and radiation (HR: $1.7 \times 10^{-11}$, 95% CI: $5.0 \times 10^{-12}$–$5.6 \times 10^{-11}$, $P < .001$) had positive effects on OS (Fig. 1A). For age, although there were differences between the 2 groups and concerns that this might be a confounding factor, the effect of age on survival was limited (HR: 1.061, 95% CI: 1.029–1.094, $P < .001$, Fig. 1B). As expected, the HR increased as the stage progressed, resulting in a poor prognosis for advanced stages (stage I; reference, stage II; HR: 3.9, $P = .04$, stage III; HR: 1.6, $P = .43$, and stage IV or recurrence; HR: 8.5, $P < .001$, Fig. 1A). Consistent with the report that HPV-positive cervical cancer has a better

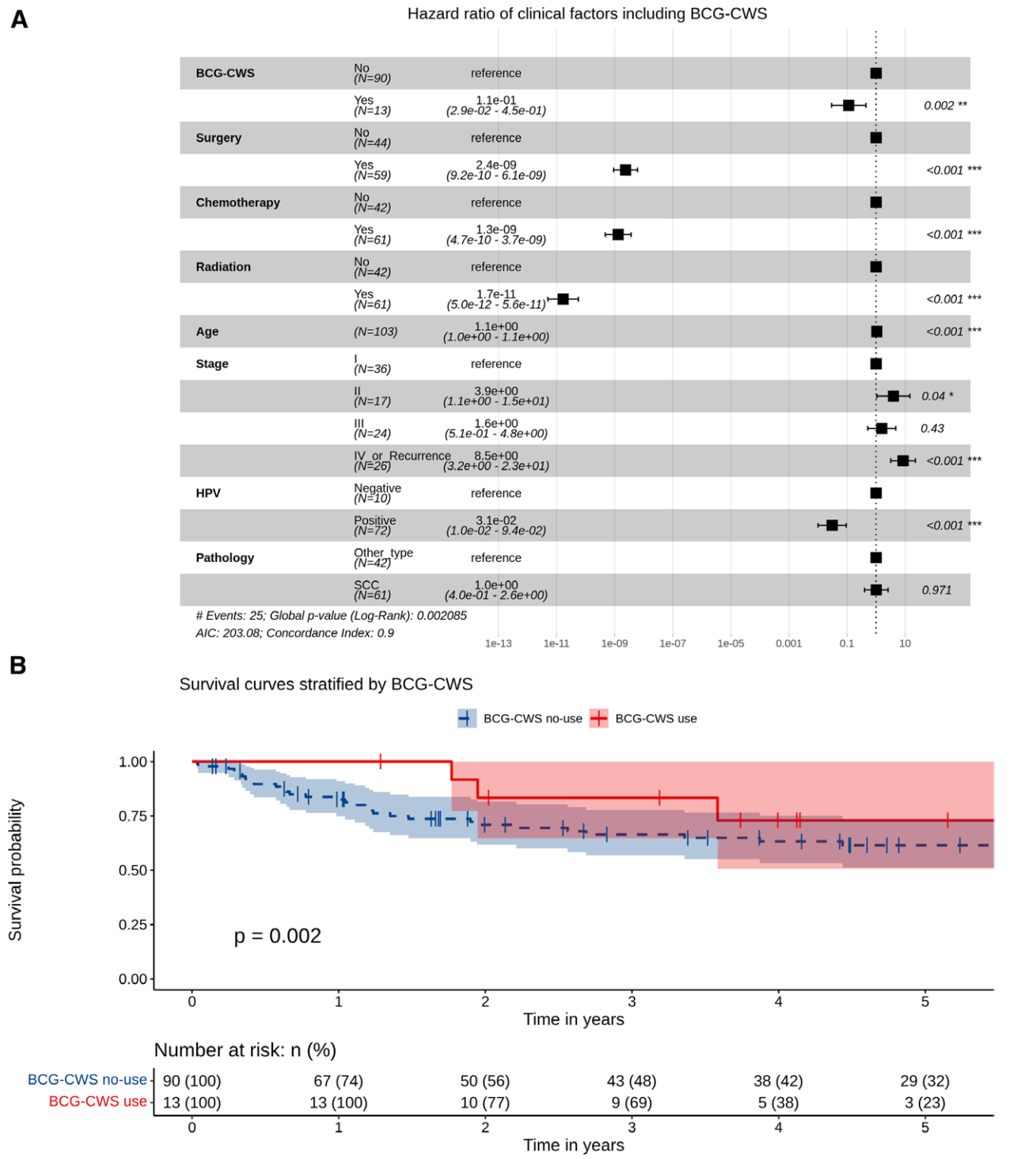


**Figure 1.** (A) The hazard ratio of BCG-CWS, surgery, chemotherapy, radiation, age, stage, HPV, and pathology for patients with cervical cancer. BCG-CWS significantly improved patient survival (hazard ratio: 0.1145, 95% CI: 0.0293–0.4482, $P = .002$). (B) Overall survival curves of cervical cancer patients with or without the BCG-CWS. The *red line* represents the survival curve with 95% CI of the cervical cancer patients receiving multimodal treatment with BCG-CWS. The *blue line* represents the survival curve with 95% CI of the patients receiving multimodal treatment without BCG-CWS. *Vertical bar*: censor. BCG-CWS = Cell wall skeleton of *Mycobacterium bovis* Bacillus Calmette-Guérin, CI = confidence interval, HPV = human papillomavirus.

prognosis than HPV-negative cancer,[7] this observational study demonstrated a clearly better prognosis for HPV-positive cervical cancer patients than for HPV-negative patients (HR: 0.031, 95% CI: 0.010–0.094, $P < .001$, Fig. 1A). Pathology (SCC or not) did not affect HR (HR: 1.018, 95% CI: 0.400–2.591, $P = .971$, Fig. 1A).

### 3.4. Adverse effect of the BCG-CWS

Transient fever and skin reactions were observed as adverse events. Almost all patients who received the BCG-CWS vaccine developed skin reactions such as swelling, erosion, and ulceration within a couple of days after the third or fourth vaccinations. The skin lesions disappeared within a couple of weeks in most cases, but the next inoculation was postponed if the lesions were not completely cured. Vaseline ointment with gentamycin was prescribed for treatment in some patients if the severity of ulceration required it. After ulcer formation, various levels of induration, pigmentation, or keloid formation were observed at injection sites in almost all patients. None of the patients required discontinuation of the vaccination due to a severe adverse event.

### 3.5. Representative cases demonstrating the effects of BCG-CWS

We report 4 representative cases (patient #1–#4) that demonstrate the effect of the BCG-CWS immunotherapy and 1 case (patient #5) with a poor outcome.

Patient #1 (in her 30s, stage IB2 cancer in the third trimester of pregnancy, HPV18-positive) had undergone a radical hysterectomy just after a cesarean section. The pathological diagnosis was SCC, stage IIB, N0M0, with the presence of cancer in lymphatic and vascular spaces. She did not wish further treatment, due to concerns about caring for her infant. She was free of disease for 2 years, but a metastatic lung tumor appeared 2 months after the cessation of BCG-CWS. After the surgical resection of a part of the lung containing a 3-cm tumor, she underwent 3 courses of chemotherapy. One year later, an approx. 4-cm tumor appeared on her scalp. Both the lung tumor and skin tumor were confirmed to have the same histology as the primary tumor. The patient was alive 3 years after the first recurrence with the re-start of BCG-CWS therapy.

Patient #2 (in her 50s, stage IV; brain metastasis, HPV18-positive) received focal irradiation to the brain tumor and systemic chemotherapy. BCG-CWS vaccination was started after simple hysterectomy. She had no recurrence in the next 6 years until it was noted that the level of the tumor marker had risen and para-aortic lymph node swelling had developed. She remains alive 7 years later, having metastatic tumorectomy, chemotherapy, and irradiation to the para-aortic lymph nodes.

Patient #3 (in her 30s, HPV16-positive) had a >5-cm cervical tumor with bleeding at the first visit and underwent focal radiation therapy to stop the massive bleeding. A radical hysterectomy and pelvic lymphadenectomy (stage IIB, N+, M0) were performed. BCG-CWS was administered before surgery and continued for 2 years. She refused chemoradiation therapy after the surgery but has been disease-free for 5 years.

Patient #4 (in her 40s, HPV52-positive) had a radical hysterectomy and pelvic lymphadenectomy (stage IIB, N+, M0). She declined adjuvant radiotherapy due to concerns about side effects and continued on BCG-CWS. She has been alive without recurrence for 5 years.

Patient #5 (in her 30s, HPV18-positive, adenosquamous cell carcinoma, stage IIB) continued habitual smoking. Patient #5 underwent hysterectomy after 6 administrations of BCG-CWS but died 3 years after the first operation.

### 3.6. Examination of subsets of immune cells infiltrating cancer tissue after BCG-CWS immunization

To evaluate the immune responses induced by BCG-CWS therapy, we analyzed the different subsets of immune cells infiltrating cancer tissue after immunizations with BCG-CWS in patients #1, #2, #3, and #4 as long-term survivors and #5 as a patient with a poor outcome. Among the long-term survivors (#1, #2, #3, and 4#), many cells stained positive for $CD4^+$ (helper T cells) and $CD8^+$ (cytotoxic T cells) in the cancer nests or stroma, but few for $CD20^+$ (B cells), $CD56^+$ (natural killer cells) or $FOXP3^+$ (immune-regulatory T cells; Fig. 2). On the other hand, although $CD4^+$ and $CD8^+$ were accumulated in the patient (#5) who continued to smoke and died, notable $FOXP3^+$ accumulation, especially in the cancer nest, emerged as a difference between her and the long-term survivors (Fig. 2).

## 4. Discussion

In the present study, the combination of BCG-CWS immunostimulatory therapy and conventional treatment was administered to patients with cervical cancer. The results revealed that the BCG-CWS adjuvant immune therapy provided improved survival compared to controls without BCG-CWS for cervical cancers at any stage. The role of host immuno-response against cancer is usually unrecognized in standard treatments such as surgery, radiation, and chemotherapy. Many clinicians do not believe in the effectiveness of immunotherapeutic agents other than checkpoint inhibitors. On the other hand, clinicians sometimes experience unexpected failure such as metastasis or recurrence in immunocompromised patients with early-stage cancer, suggesting the important role of immunity for cancer elimination regardless of treatment type.

As shown in the present case study and immunohistochemical staining, many $CD8^+$ T cells as well as $CD4^+$ T cells infiltrate cancer or stroma tissue after BCG-CWS administration (Fig. 2). Especially, the high $CD8^+$ T cell populations suggest that the induction of an adaptive immune response is predominant, indicating that the BCG-CWS therapy may have induced an antigen-specific immune response. In patient #5 whose smoking habit likely affected her immune system, although BCG-CWS did induce the accumulation of $CD8^+$ T cells in the cancer, the disease course may have been affected by the accumulation of $FOXP3^+$ in the cancer nest. Smoking is an important issue in terms of management of cancer patients since smoking has been suggested to inhibit the T cell response to *M tuberculosis*.[30] $CD20^+$ was also accumulated in patient #5. Regulatory B cells may release immune-suppressive cytokines that bring an anti-tumor response.[31] In a further study, we will confirm whether these immune responses, such as elevated levels of $CD4^+$ and $CD8^+$ and low levels of $FOXP3^+$ or $CD20^+$ are reliable signs of anti-cancer immunity in patients immunized with BCG-CWS.

The treatment success of immunostimulatory drugs such as BCG-CWS might be due to the sequencing of the regimen. CTLs kill cancer cells by targeting some peptides derived from oncoproteins exposed on cancer cell surfaces. This may mean that presence of oncoproteins is a prerequisite at the time of activation for CTL by BCG-CWS vaccination. In this study, BCG-CWS was administered before treatments such as surgery, chemotherapy, and radiation in 10 of 13 patients. The presence of target antigens at the time of the innate immune stimulation is critical, since DCs engulfing the antigens are matured by various inflammatory cytokines released in innate immunity,[32] and this is a critical step to induce the CTLs response. The CTL response is hardly induced without the presentation of some antigens by activated DCs. Conversely, removing all cancer cells either by surgery or radiation before administration of BCG-CWS might be certain to reduce if not eliminate the presence of cancer-specific antigens, diminishing the efficacy of cancer-specific CTL responses. Even so, we

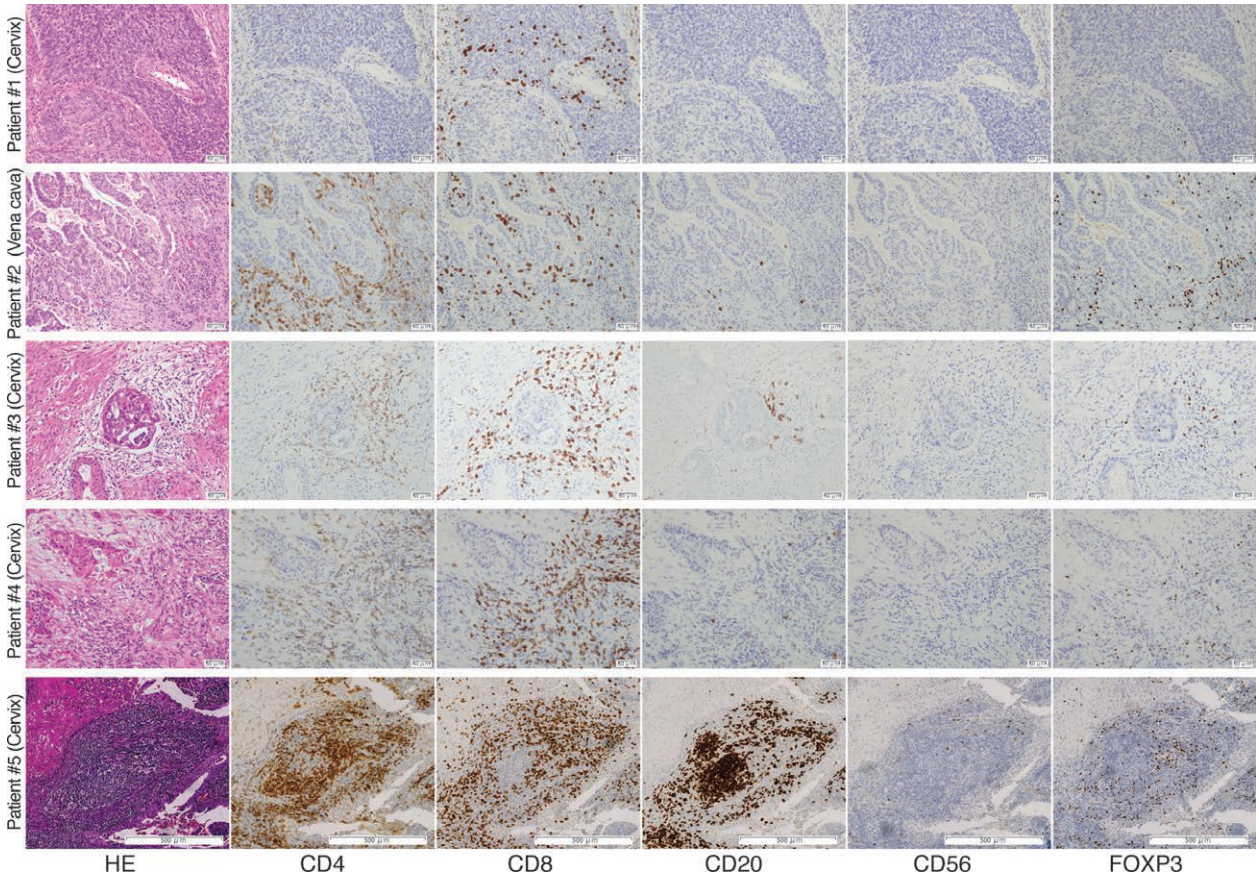


**Figure 2.** Five cases showing infiltration of immune cells into the tumor after BCG-CWS. Patients #1, #2, #3, and #4 are long-term survivors. Patient #5 had a poor outcome. After BCG-CWS, $CD8^+$ and $CD4^+$ accumulation in the cancer nests or stroma was observed in all patients regardless of prognosis. We found $FOXP3^+$ accumulation, especially within the cancer nest, was a key difference marking a poor prognosis. In patients #1, #3, #4, and #5, tissues of cervical cancer after BCG-CWS administration are presented. In patient #2, tissues of a metastatic inferior vena cava wall after BCG-CWS administration are presented. (A) Hematoxylin-eosin stain. (B) $CD4^+$. (C) $CD8^+$. (D) $CD20^+$. (E) $CD56^+$. (F) $FOXP3^+$. BCG-CWS = Cell wall skeleton of *Mycobacterium bovis* Bacillus Calmette-Guérin.

believe that BCG administration immediately after surgery or radiation may also be an acceptable strategy since exposure of cancer-specific antigens, neoantigens, and/or viral antigens to T cells is likely caused by the destruction of cancer tissue by standard treatments.

An important factor in the elimination of cervical cancer is the presence of HPV. Although HPV can transform cervical cells into malignant ones, HPV is a foreign antigen targeted by CTLs. Thorsson et al[33] investigated the immunogenicity of various carcinomas and reported that cervical cancer retains a strong CD8 signal and is characterized by a high degree of T cell receptor diversity. Although it is a new hypothesis based on the results of the present research, it is possible that some $CD8^+$ T cells without PD-1 receptors are present in cervical cancer tissue with high $CD8^+$ T cell infiltration, and that BCG-CWS might have the potential to activate those $CD8^+$ T cells without PD-1 receptors and thus help slow tumor growth. In the report of Nagasaki et al,[34] most tumor-specific T cell clones express PD-1, but some T cells do not. BCG-CWS may be able to activate $CD8^+$ T cells without PD-1. This hypothesis could be tested by tracking the number of $CD8^+$ T cell clonotypes before and after BCG-CWS administration in single-cell T cell receptor sequencing; indeed, we hope to conduct such a study in the future.

Co-administration of BCG-CWS and cancer antigens may be another promising strategy. Antigen-conjugated BCG-CWS may produce a Th1-skewed immune response more strongly than Freund's adjuvant alone.[15] Nishida et al[25] reported an increase in neutrophils, monocytes, and $CD4^+$ T cells in some patients with advanced cancer after the administration of a combination of BCG-CWS and WT1 peptide. WT-1-specific $CD8^+$ T cell responses were also exhibited.[25] Regarding cervical cancer, oncoproteins like E6 or E7 might be candidate antigens to use in combination immune therapy with BCG-CWS.

The primary limitations of this study derive from its nature as an observational, retrospective analysis. The relationship between OS and BCG-CWS has the possibility of confounding by age, surgery rate, and HPV infection rate, and it is difficult to completely eliminate confounding by matching when the number of subjects is limited in a retrospective study like this one. Therefore, we analyzed the effect of BCG-CWS on OS by calculating the adjusted hazard ratios after avoiding the disproportionate effects of age and surgical rates by multivariate Cox regression analysis. A randomized control study is needed to clarify the effect of BCG-CWS, as causal relationships cannot be inferred from observational data. A randomized study might eliminate some of the potentially confounding factors that we tried to eliminate statistically. Of course, randomized clinical studies require a great deal of coordination and ethical consideration.

Our results suggest that using BCG-CWS as an adjuvant to conventional treatment has a positive synergistic effect on tumor control and improves survival rates. BCG-CWS has great potential as a clinically applicable immune-adjuvant therapy for various cancers, with the clear advantages of low toxicity, easy administration, and cost-effectiveness. We emphasize that BCG-CWS does not compromise the patient's QOL, as demonstrated by the safety of its use, both historically and in our study. The combination of cancer immunotherapy

using cancer antigens and BCG-CWS offers promise for treatment of several types of cancer. Randomized prospective studies incorporating BCG-CWS should be supported generously by public institutions and foundations seeking to reduce the burden of cancer worldwide.

## Author contributions

T Sasagawa is responsible for the study's conception and design. T Shibata and T Sasagawa wrote and revised the manuscript and conducted the analyses. T Shibata, ET, JS, MT, and T Sasagawa interpreted the data and contributed to the acquisition of the data. AS and SY performed pathological diagnoses.
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