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# Ⅳ．研究成果の刊行物・別刷

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# Oral vaccination against HPV E7 for treatment of cervical intraepithelial neoplasia grade 3 (CIN3) elicits E7-specific mucosal immunity in the cervix of CIN3 patients

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ABSTRACT

*Background:* Cervical intraepithelial neoplasia grade 3 (CIN3) is a mucosal precancerous lesion caused by high-risk human papillomavirus (HPV). Induction of immunological clearance of CIN3 by targeting HPV antigens is a promising strategy for CIN3 therapy. No successful HPV therapeutic vaccine has been developed.

*Methods:* We evaluated the safety and clinical efficacy of an attenuated *Lactobacillus casei* expressing modified full-length HPV16 E7 protein in patients with HPV16-associated CIN3. Ten patients were vaccinated orally during dose optimization studies (1, 2, 4, or 6 capsules/day) at weeks 1, 2, 4, and 8 (Step 1). Seven additional participants were only tested using the optimized vaccine formulation (Step 2), giving a total of 10 patients who received optimized vaccination. Cervical lymphocytes (CxLs) and peripheral blood mononuclear cells (PBMCs) were collected and E7 specific interferon-γ-producing cells were counted (E7 cell-mediated immune responses: E7-CMI) by ELISPOT assay. All patients were re-evaluated 9 weeks after initial vaccine exposure using cytology and biopsy to assess pathological efficacy.

*Results:* No patient experienced an adverse event. E7-CMI in both CxLs and PBMCs was negligible at baseline. All patients using 4–6 capsules/day showed increased E7-CMI in CxLs, whereas patients using 1–2 capsules/day did not. No patient demonstrated an increase in E7-CMI in their PBMCs. In comparison between patients of cohorts, E7-CMI at week 9 (9 wk) in patients on 4 capsules/day was significantly higher than those in patients on 1, 2, or 6 capsules/day. Most patients (70%) taking the optimized dose experienced a pathological down-grade to CIN2 at week 9 of treatment. E7-CMI in CxLs correlated directly with the pathological down-grade.

*Conclusions:* Oral administration of an E7-expressing Lactobacillus-based vaccine can elicit E7-specific mucosal immunity in the uterine cervical lesions. We are the first to report a correlation between mucosal E7-CMI in the cervix and clinical response after immunotherapy in human mucosal neoplasia.



## 1. Introduction

Human papillomavirus (HPV) is a major risk factor for the development of cervical cancer, the second most common cancer among women [1]. Some 99% of cervical cancer cases are associated with genital infection with oncogenic HPVs. Among them, HPV type 16 (HPV16) infection is most commonly associated with cervical cancer [2–5]. Recent prophylactic HPV vaccines have been shown to prevent genital infection with HPV types 16 and 18 (HPV16/18) and reduce the incidence of HPV16/18-related high-grade CIN [6–10]. However, little effect will likely be noted among

*Abbreviations:* CIN3, cervical intraepithelial neoplasia grade 3; HPV, human papillomavirus; CxLs, cervical lymphocytes; PBMCs, peripheral blood mononuclear cells; E7-CMI, E7cell-mediated immune responses; CTL, cytotoxic T lymphocytes.

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patients who were already infected by HPV prior to vaccination. The limitations of prophylactic HPV vaccines demonstrate a pressing need for novel approaches, possibly immune-mediated, to eradicate HPV-associated neoplasia and suggest that the development of therapeutic HPV vaccines for the treatment of HPV-associated lesions should remain an important goal [11]. The combined actions of the high-risk HPV E6 and E7 oncoproteins are essential for the maintenance of the neoplastic phenotype. Since E6 and E7 are the only HPV proteins expressed in precursor lesions, they represent reliable antigenic targets for immunotherapy of CIN3. Immunization with E6 and/or E7 of HPV16, with the resultant generation of antigen-specific CTL (cytotoxic T lymphocytes), has been a frequent immunotherapeutic approach for HPV-associated neoplasia and has utilized a wide array of potential vaccine delivery systems [12–23]. Previous clinical trials of HPV therapeutic vaccines have been able to elicit systemic E7-specific type 1 cell-mediated immune responses (systemic E7-CMI) using subcutaneous or intramuscular delivery, but few have studied mucosal E7-specific type 1 cell-mediated immune responses (mucosal E7-CMI). Because CIN lesions develop in the cervical mucosa, local mucosal lymphocytes possessing E7-CMI in the cervix are likely to play a direct role in immunological clearance of CIN lesions.

The lymphocytes involved in mucosal immunity are found in the inductive sites of organized mucosa-associated lymphoid tissues and in a variety of effector sites such as the mucosa of the intestine, respiratory tract, and genital tract. Integrin β7 is the most common homing receptor expressed on gut-derived mucosal lymphocytes [24]. We have demonstrated that cervical brushing methodology enables us to preferentially collect integrin β7+ mucosal cervical lymphocytes (CxLs) from CIN lesions [25]. Using murine models, several studies on immunization with Lactobacillus-based vaccines have demonstrated an induction of systemic E7-CMI and regression of subcutaneous HPV16 E7-positive tumors [26–28]. However, they have neither provided an insight into mucosal T cell responses to oral vaccination nor into the anti-tumor effects on mucosal intraepithelial neoplasms. We have observed an induction of mucosal E7-CMI within intestinal mucosa after oral administration of *Lactobaccilus casei* expressing HPV16 E7 in mice [29]. These studies suggested that oral vaccination may surmount some of the deficiencies seen with systemic immunization in previous CIN therapeutic vaccine clinical trials and encouraged us to embark on a clinical trial using GLBL101c. To assess the safety, immunogenicity, and clinical efficacy of GLBL101c, we designed a Phase I/IIa study involving patients with HPV16-positive CIN3. This is the first clinical trial to use oral vaccination for the treatment of HPV-associated neoplasia.

## 2. Materials and methods

### 2.1. Patients

Enrolled patients had (1) histologically confirmed ectocervical CIN3 lesions and were (2) infected only with HPV16 (exclusion of other high-risk HPV types) as documented by in-house PGMY-CHU HPV genotyping methods which can detect 34 HPV subtypes [30]. Other eligibility criteria included: (3) age 18–45, (4) colposcopic evidence of a persistent high-grade lesion 4 weeks after biopsy, (5) normal pretreatment laboratory blood values, and (6) signed informed consent. Exclusion criteria included: (1) any signs of invasive disease, (2) endocervical involvement, (3) pregnancy/lactation, and (4) HIV positivity, immunosuppressive disease or use of immunosuppressive medications.

### 2.2. Study design

Since the spontaneous regression rate of CIN3 at a 9-week time point is thought to be less than 10% [13,31,32], this study was designed as a single-center, single-arm (non-controlled), observational Phase I/IIa study. The primary end points were to evaluate the safety and the pathological efficacy of vaccination and the secondary endpoints were mucosal and systemic HPV16 E7-CMI and local cytological efficacy. For initial safety assessments, the minimal formulated amount (250 mg) of GLBL101c (one capsule) was administered daily. Next, four small scale dose-escalation cohorts (one or three patients per cohort) were treated with 1, 2, 4, or 6 capsules/day for four total rounds (Step 1). If no adverse effects were observed in cohorts given lower doses, the dose was escalated by one capsule per day in the next cohort. If no clinical response was observed at a given dose, the trial was discontinued for that dose level. Once a safe and effective dose was identified, seven more patients were enrolled at that dose level for a total of ten patients using the optimized dose of GLBL101c (Step 2). All patients received four rounds of oral vaccination at week 1, 2, 4, and 8. Each dose of GLBL101c was administered orally once each morning after fasting for five days, each treatment week. We followed our subjects for only 9 weeks prior to reassessment and possible treatment to ensure optimal patient safety. All vaccinations were performed from February 2009 to November 2012. The study was sponsored by the Ministry of Health, Labour and Welfare of Japan for the Third-Term Comprehensive Strategy for Cancer Control, and for Comprehensive Strategy for Practical Medical Technology, Japan, and approved by the medical ethics committee of the University of Tokyo, Faculty of Medicine. All patients gave written informed consent. All data underwent independent third-party management and analysis and were evaluated by a third-party committee for efficacy and safety.

### 2.3. Composition of the vaccine

GLBL101c was provided by GENOLAC BL Corp (Japan), generated from a recombinant *L. casei* expressing mutated HPV16 E7 as previously described and attenuated using heat. Briefly, the HPV16 E7 gene was modified by inserting point mutations into the Rb-binding site (the D, C, and E in E7 aa21, aa24, and aa26, respectively, were all replaced by a G) [29]. Through these mutations, the carcinogenicity of E7 was abrogated, but its immunogenicity remained intact [33]. The attenuated *L. casei* were purified by washing several times with distilled water then dried to powder. 250 mg of GLBL101c powder was enclosed in a capsule designed to degrade in the bowel.

### 2.4. Collection and processing of cervical specimens

Cervical cells were collected using a Digene cytobrush as described previously [34]. The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was then placed into a 15-mL tube containing R10 media (RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B) and an anticoagulant (0.1 IU/mL of heparin and 8 nM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37 °C for 15 min with shaking, the cytobrush was removed. The tube was centrifuged at 330 × g for 4 min. The pellet was resuspended in 10 mL of 40% Percoll, layered onto 70% Percoll, and centrifuged at 480 × g for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was >95%, as confirmed by trypan blue exclusion, and it is noted that all samples were frozen until use for further immunological assay [25].

### 2.5. Assessment of clinical efficacy

Visual examinations and histological and cytologoical specimen collections were performed under colposcopy. Biopsies of the same site were obtained at baseline and one week after the final vaccination round (at week 9). As a primary end point, grading of the lesions was performed by several experienced independent blinded pathologists according to strict criteria [35]. If CIN3 was downgraded to CIN2 or less, further surgical intervention was averted. Otherwise, patients with CIN3 underwent cervical conization or laser ablation. We used pathological response criteria that were modified based on the previous study [13]. Patients who did not receive surgical treatment had repeat cytologic evaluation at 6 and 12 months.

### 2.6. Safety and tolerability

Clinical assessments, laboratory testing and adverse events monitoring were conducted after each round of vaccination. Adverse events were graded according to version 3.0 of the Common Terminology Criteria for Adverse Events (CTCAE), which grades events on a scale of 1–5, with higher grades indicating greater severity.

### 2.7. Immunological responses to HPV16 E7 (E7-CMI)

All lymphocyte samples were frozen immediately after isolation and stocked in −40 °C freezer until use for immunological assay. Approximately $1 \times 10^6$ cervical lymphocytes were isolated from each patient's cervix. $5 \times 10^4$ cervical lymphocytes (CxLs) or peripheral blood mononuclear cells (PBMCs) were incubated for 24 h at 37 °C with antigen presenting cells or $5 \times 10^4$ PBMCs were treated with mitomycin C (75 μg/mL, Nakarai, Japan) and washed four times with PBS [25]. Ten microliters of synthesized peptides (working concentration 1 μg/mL) covering the entire 98 aa HPV16 E7 amino acid sequence with 18 HPV16 E7 15-mer overlapping peptides (overlapped by 10 amino acids) [36], mitogen (PMA 40 ng/mL + ionomycine 4 μg/mL) or medium alone (negative control) were added to a 96 well ELIIP plate (Millipore, USA) coated with anti-human interferon-γ (IFNγ) monoclonal antibodies (15 μg/mL) according to the manufacturer's protocols for ELISpot for IFNγ (MABTECH AB, Sweden). Spot numbers representing IFNγ-producing lymphocytes were analyzed as E7-CMI with a computer assisted video imaging analysis system, KS ELISPOT (Carl Zeiss Vision, Germany) [29]. The experiment was performed on three to six wells each to allow statistical analysis.

### 2.8. Statistical analysis

ELISpot data are presented as mean ± standard deviation. ELISpot numbers were compared between immunization groups using Mann–Whitney *U*-test. A *p*-value of <0.05 was considered significant.

## 3. Results

### 3.1. Study population and adverse events

Participant characteristics are summarized in Table 1. All enrolled patients were Japanese women. Ten patients were enrolled in safety and dose escalation studies (Step 1) and seven additional patients were studied only at the optimized vaccination dose (4 capsules/day; Step 2). The distribution of participant HLA haplotypes was similar to that in the Japanese population [37,38]. No patient experienced serious side effects induced by GLBL101c according to CTCAE. No patient was withdrawn because of adverse events or progression of their disease.

**Table 1**
Baseline characteristics of the patients.

| Pt. ID | Age | Pre Tx[a] | Dose[b] | Cytology[c] | Histology | HLA-A allele |
|---|---|---|---|---|---|---|
| Step 1 | | | | | | |
| 1–1 | 40 | Untreated | 1 | HSIL, S | CIN3 | 02:06/31:01 |
| 1–2 | 42 | Laser | 2 | HSIL, S | CIN3 | 24:02/33:03 |
| 1–3 | 34 | Untreated | 2 | HSIL, S | CIN3 | 24:02/24:20 |
| 1–4 | 42 | Untreated | 2 | HSIL, S | CIN3 | 11:01/24:02 |
| 1–5 | 39 | Untreated | 4 | HSIL, M | CIN3 | 02:06/26:01 |
| 1–6 | 43 | Laser | 4 | HSIL, S | CIN3 | 02:06/26:01 |
| 1–7 | 35 | Laser | 4 | HSIL, S | CIN3 | 24:02/24:02 |
| 1–8 | 33 | Untreated | 6 | HSIL, S | CIN3 | 11:01/26:01 |
| 1–9 | 41 | Laser | 6 | HSIL, S | CIN3 | 31:01/31:01 |
| 1–10 | 37 | Untreated | 6 | HSIL, S | CIN3 | 02:01/02:01 |
| Step 2 | | | | | | |
| 2–1 | 42 | Laser, conization | 4 | HSIL, S | CIN3 | 01:01/24:02 |
| 2–2 | 36 | Laser | 4 | HSIL, S | CIN3 | 24:02/31:01 |
| 2–3 | 35 | Laser | 4 | HSIL, S | CIN3 | 24:02/24:02 |
| 2–4 | 42 | Untreated | 4 | HSIL, S | CIN3 | 24:02/26:01 |
| 2–5 | 38 | Untreated | 4 | HSIL, S | CIN3 | 11:01/26:01 |
| 2–6 | 29 | Untreated | 4 | HSIL, S | CIN3 | 02:01/31:01 |
| 2–7 | 30 | Untreated | 4 | HSIL, M | CIN3 | 02:01/24:02 |

HSIL,S: high-grade squamous intraepithelial lesion, severe dysplasia (CIN3).
HSIL,M: high-grade squamous intraepithelial lesion, moderate dysplasia (CIN2).
[a] Previous treatment before the enrollment.
[b] Number of capsules of GLBL101c (250 mg/capsule) administered daily.
[c] Both Bethesda system classification and expected diagnoses were described.

### 3.2. E7 specific cell-mediated immune responses (E7-CMI)

Numbers of E7-specific IFNγ-producing cells in CxLs and PBMCs were separately examined for E7-CMI [25]. Fig. 1 depicts representative pictures of our ELISpot assays. In this patient (patient 2–7), oral administration of GLBL101c elicited a time-dependent increase in E7-specific IFNγ-producing CxLs but had no effect on PBMCs. A summary of E7-CMI results at baseline and week 9 is shown in Table 2 and Fig. 2. At baseline, all patients lacked E7-CMI in PBMCs while three patients had barely detectable levels of E7-CMI in CxLs. At week 9, all patients had increases in cervical E7-CMI

**Table 2**
E7-CMI in the cervix and peripheral blood before and after administration.

| Pt. ID | Dose | E7-CMI[a] | | | |
|---|---|---|---|---|---|
| | | Cervical lymphocyte[b] | | PBMC | |
| | | Baseline | 9 wk | Baseline | 9 wk |
| Step 1 | | | | | |
| 1–1 | 1 | 2.8 ± 0.4 | 9.2 ± 0.5 | 3.1 ± 0.3 | 8.0 ± 0.5 |
| 1–2 | 2 | 7.5 ± 1.0 | 12.3 ± 3.0 | 4.9 ± 1.0 | 6.2 ± 1.5 |
| 1–3 | 2 | 4.8 ± 1.0 | 9.9 ± 1.5 | 6.8 ± 1.5 | 7.2 ± 1.0 |
| 1–4 | 2 | 2.8 ± 0.5 | 11.3 ± 2.0 | 4.8 ± 0.6 | 6.4 ± 2.0 |
| 1–5 | 4 | 9.6 ± 0.4 | 28.8 ± 0.8 | 3.1 ± 0.7 | 7.0 ± 0.3 |
| 1–6 | 4 | 12.0 ± 0.4 | 38.4 ± 0.4 | 2.7 ± 0.3 | 5.3 ± 0.5 |
| 1–7 | 4 | 12.0 ± 0.4 | 44.0 ± 0.5 | 2.5 ± 0.4 | 5.7 ± 0.3 |
| 1–8 | 6 | 8.0 ± 0.2 | 33.6 ± 0.4 | 5.6 ± 0.2 | 19.2 ± 0.4 |
| 1–9 | 6 | 8.8 ± 0.2 | 17.6 ± 0.2 | 5.6 ± 0.2 | 12.8 ± 0.5 |
| 1–10 | 6 | 8.0 ± 0.2 | 14.4 ± 0.4 | 7.2 ± 0.4 | 10.4 ± 0.6 |
| Step 2 | | | | | |
| 2–1 | 4 | 8.3 ± 1.6 | 18.8 ± 1.2 | 2.3 ± 0.4 | 6.0 ± 0.9 |
| 2–2 | 4 | 6.8 ± 0.7 | 33.0 ± 1.8 | 2.3 ± 0.4 | 6.5 ± 0.3 |
| 2–3 | 4 | 14.3 ± 2.5 | 40.5 ± 1.7 | 2.5 ± 0.5 | 6.3 ± 0.2 |
| 2–4 | 4 | 6.0 ± 1.1 | 21.8 ± 2.0 | 2.0 ± 0.3 | 6.8 ± 0.4 |
| 2–5 | 4 | 6.8 ± 0.7 | 24.8 ± 2.2 | 1.8 ± 0.4 | 6.3 ± 0.5 |
| 2–6 | 4 | 8.3 ± 2.0 | 14.3 ± 1.2 | 2.3 ± 0.5 | 6.3 ± 0.4 |
| 2–7 | 4 | 9.0 ± 1.1 | 36.0 ± 3.8 | 2.0 ± 0.4 | 5.3 ± 0.2 |

[a] Numbers of E7-specific IFNγ-producing cell (/$10^5$ cells).
[b] Lymphocytes obtained from cervical lesion using the cytobrush method.

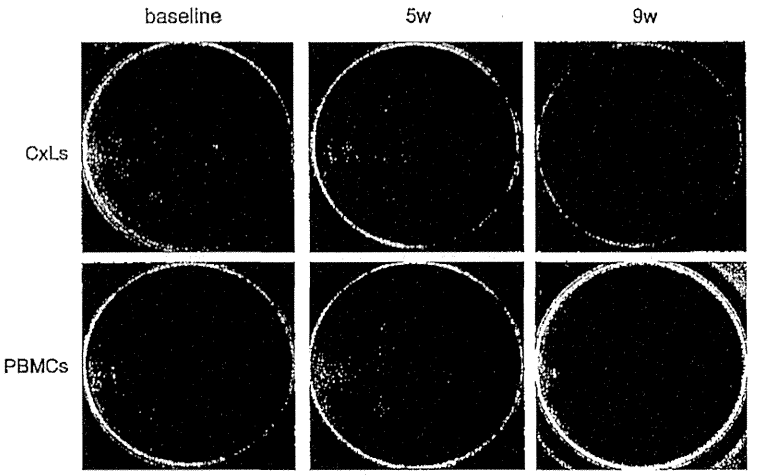


Fig. 1. Immunological response to vaccination (4 capsules/day) in a representative patient. (ELISpot assay images). CxLs (upper) and PBMCs (lower) were collected from patients 2–7 at baseline and at weeks 5 and 9. Purple dots indicated E7-specific IFNγ-producing lymphocytes. CxLs (cervical lymphocytes); PBMCs (peripheral blood mononuclear cells). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

significantly. In particular all patients taking 4 or 6 capsules/day had marked increases in cervical E7-CMI than in PBMCs. In comparison between patients of cohorts, E7-CMI at week 9 (9 wk) in patients on 4 capsules/day significantly higher than those in patients on 1, 2, or 6 capsules/day (Fig. 2). Only two of the 13 patients receiving four or six capsule doses had significant increases in E7-CMI in PBMCs. These data indicate that oral administration of GLBL101c induces predominantly mucosal E7-CMI homing to the cervical epithelium.

### 3.3. Clinical responses to administration

Supplementary Fig. 1 displays the clinical response of a representative patient experiencing a pathological down-grade to CIN1-2. Clinical responses at week 9 after vaccination and follow-up, cytological evaluations for all subjects are summarized in Table 3. In Step 1, the four patients taking 1–2 capsules/day had no pathological response. Two of three patients using 4 capsules/day experienced a pathological down-grade to CIN2. One of three patients on 6 capsules/day experienced a pathological down-grade to CIN2 while two had no pathological changes noted. Taken together these results with immunological responses (Fig. 2), 4 capsules/day was chosen as the optimal dose of GLBL101c and seven additional patients were enrolled at this dosage in Step 2. Combining Step 1 and 2 patients receiving 4 capsules/day, 7 of 10 patients (70%) using this regimen had a pathological down-grade to CIN2 at week 9 and one other patient (Patient ID: Step 1–5) had a pathological down-grade to CIN2 at week 12. Of the 13 patients receiving 4–6 capsules/day, nine patients (69%) with pathological down-grade to CIN2 did not require additional surgical treatment and were followed cytologically. Among the patients without additional treatment, five patients (56%) showed further cytological regression to LSIL or normal cytology by 12 months after administration. All nine patients continued to have no evidence of CIN3 when followed without intervention between 14 and 33 months. There was no correlation between clinical response and patient background characteristics (pre-treatment, baseline cytology, or HLA-A allele types).

### 3.4. Correlation between E7-CMI in CxLs and clinical efficacy

The number of E7-specific IFNγ-producing cells in CxLs for each patient was plotted along the y-axis as shown in Fig. 3 and divided

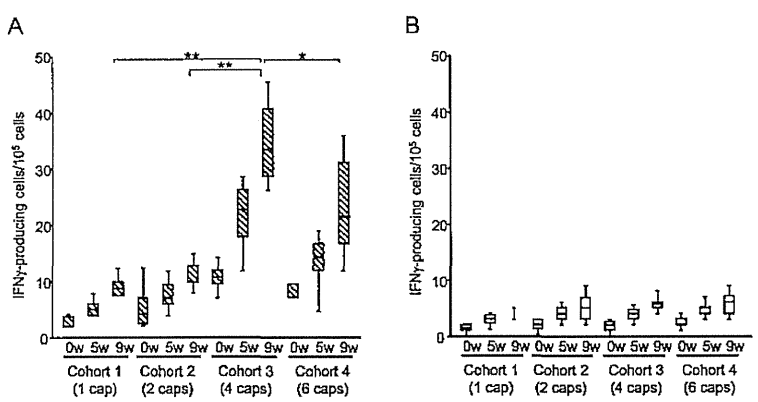


Fig. 2. Immunological responses to different dose of vaccination. (A) Stripe boxes indicate E7-CMI (E7-specific IFNγ-producing cells) in cervical lymphocytes (CxLs) for each cohort. (B) White boxes indicate E7-CMI in PBMCs for each cohort. For each cohort, E7-CMI at pre-vaccination (0 wk), weeks 5 (5 wk) and 9 (9 wk) was plotted. Each median, Inter Quartile Ranges (IQR), and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively. Asterisks indicate those comparisons with statistical significance (*: $p < 0.01$, **: $p < 0.001$).

**Table 3**
Clinical efficacy of GLBL101c oral administration.

| Pt. ID | Dose | Histology(9 wk) | Cytology[a](9 wk) | Tx[b](10–12 wk) | Follow-up | |
|---|---|---|---|---|---|---|
| | | | | | 6 months | 12 months |
| Step 1 | | | | | | |
| 1–1 | 1 | CIN3 | HSIL, S | Conization | | |
| 1–2 | 2 | CIN3 | HSIL, S | Conization | | |
| 1–3 | 2 | CIN3 | HSIL, S | Conization | | |
| 1–4 | 2 | CIN3 | HSIL, S | Conization | | |
| 1–5 | 4 | CIN3 | HSIL, M | (–) | HSIL, M | LSIL |
| 1–6 | 4 | CIN2 | HSIL, S | (–) | HSIL, M | HSIL, M |
| 1–7 | 4 | CIN2 | HSIL, M | (–) | LSIL | NILM |
| 1–8 | 6 | CIN2 | HSIL, M | (–) | HSIL, M | NILM |
| 1–9 | 6 | CIN3 | HSIL, S | Laser | | |
| 1–10 | 6 | CIN3 | HSIL, S | Laser | | |
| Step 2 | | | | | | |
| 2–1 | 4 | CIN3 | HSIL, M | Laser | | |
| 2–2 | 4 | CIN2 | HSIL, M | (–) | HSIL, M | HSIL, M |
| 2–3 | 4 | CIN2 | HSIL, S | (–) | HSIL, M | HSIL, M |
| 2–4 | 4 | CIN2 | HSIL, M | (–) | HSIL, M | HSIL, M |
| 2–5 | 4 | CIN2 | HSIL, S | (–) | LSIL | NILM |
| 2–6 | 4 | CIN3 | HSIL, S | Laser | | |
| 2–7 | 4 | CIN2 | HSIL, M | (–) | LSIL | LSIL |

HSIL,S: high-grade squamous intraepithelial lesion, severe dysplasia (CIN3).
HSIL,M: high-grade squamous intraepithelial lesion, moderate dysplasia (CIN2).
LSIL: low-grade squamous intraepithelial lesion.
NILM: negative for intraepithelial lesion or malignancy.
[a] Both Bethesda system classification and expected diagnoses are presented.
[b] Surgical treatment methods at week 10–12. (–): The patients with no surgical treatment were evaluated with standard cervical cytology.

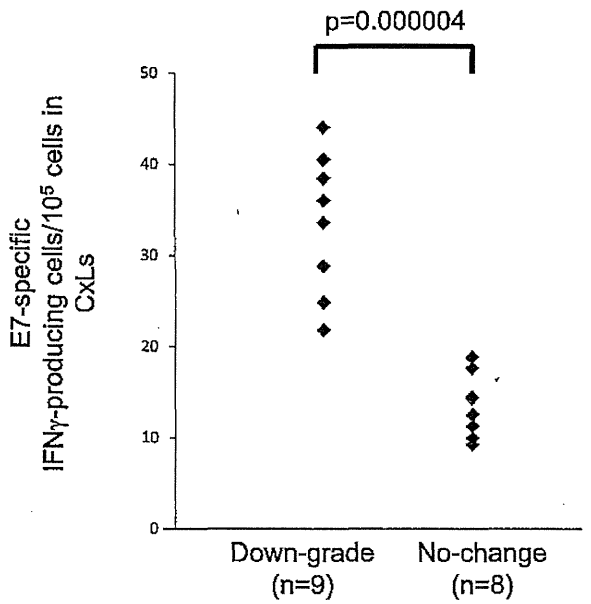


Fig. 3. Correlation of E7-CMI in CxLs with pathological responses. E7-CMI (E7-specific IFNγ-producing cells) at week 9 was compared between pathological down-grade ($n=9$) and no change ($n=8$) groups. The cervical E7-CMI of down-graded patients increased significantly more than that of patients who had no change (Mann–Whitney $U$-test: $p=0.000004$). The ROC curve indicated a cut-off value = 21.8 cells/$10^5$ cells and AUC = 0.994.

into groups, patients experiencing a pathological down-grade ($n=9$) and those who had no pathological change ($n=8$). E7-CMI in the CxLs of patients experiencing a pathological down-grade was clearly higher than that among patients with no pathological change ($p=0.000004$). ROC analysis of E7-CMI in CxLs indicated a cut-off value of 21.8 IFNγ-producing cells/$10^5$ cells (AUC = 0.994) for pathological regression and a sensitivity and specificity using this cut-off value of 94.5% and 99.2%, respectively (data not shown).

## 4. Discussion

Our study is the first report demonstrating that oral vaccination promotes clinical response in a mucosal intraepithelial neoplasm by inducing vaccine antigen-specific mucosal CMI. Interestingly, the clinical responses to GLBL101c correlated directly with mucosal E7-CMI within the uterine cervix but systemic E7-CMI was not generally elicited. This suggests strongly that mucosal effector T cells are induced by oral administration of the Lactobacillus-based HPV vaccine at intestinal mucosal inductive sites, eg Peyer's patches, and that these cells home to the cervical mucosa to direct the immunological microenvironment to type1 immune responses to HPV-related intraepithelial neoplasia. Mucosal T cells possessing E7-CMI and educated in the gut are thought to enter the peripheral circulation to home to the cervix. However, E7-CMI in the peripheral blood was negligible in our ELISpot data regardless of GLBL101c dose. The concentration of E7-specific mucosal T cells in the peripheral blood may have been too low to be detected by ELISpot assay due to dilution of the lymphocytes in the circulation. In contrast, the integrin β7+ mucosal T cells possessing E7-CMI accumulate and are retained in the mucosal epithelium via interactions of integrin αEβ7 with E-cadherin expressed in the epithelium [39].

In this trial, the pathological down-grade to CIN2 in response to a 4 capsules/day GLBL101c regimen was 80%. In Japan, CIN3 is treated surgically whereas CIN2 is generally monitored without surgical intervention by gynecologic oncologist. Therefore Japanese pathologists are routinely required to discriminate CIN2 from CIN3 in an effort to direct clinical management. Pathological down-grading continues to have important clinical implications in Japan. Data from several previous clinical trials of HPV therapeutic vaccines estimate a spontaneous regression rate from CIN3 to CIN2 of 10% based on data of non-intervention cohort studies or the placebo-arm of randomized clinical trials [13,31]. We purposely delayed repeat specimen collection until week 9, because the biopsy procedure can itself promote spontaneous regression [40]. Although

the best clinical response during 9 weeks of this study was a down-grade to CIN2, the observed clinical response rate was significantly higher than either the estimated or described rate of spontaneous down-grading to CIN2. Notably, 9 of 13 (69%) patients with pathological responses to 4–6 capsules/day remained free of CIN3 for 14–33 months after administration. Moreover, five of them had further cytological regression to LSIL or normal cytology (NILM) at 12 months after the last administration.

*L. casei* is one of the most commonly consumed bacterial species worldwide and its safety is well-demonstrated. No adverse event greater than grade 2 has been reported in any exposed patient in prior studies. Several studies on immunization with Lactobacillus-based vaccines have demonstrated an induction of systemic E7–CMI and regression of subcutaneous TC-1-induced tumors [26–28]. However, they have neither provided an insight into mucosal T cell responses to oral vaccination nor into the anti-tumor effects on mucosal intraepithelial neoplasms. While this could represent a difference between humans and mice, the mucosal specificity of the response in this human trial remains a useful attribute for further vaccine development. We previously reported a marked induction of mucosal T cells possessing E7–CMI within intestinal mucosae after oral administration of *L. casei* expressing HPV16 E7 in mice [29] and developed this clinical trial in response.

In this study, one of three patients on 6 capsules/day had clinical response while all patients using 4 capsules/day had a down-grade to CIN2 by week 12. Furthermore, E7-CMI in patients on 4 capsules/day was significantly higher than that in patients on 6 capsules/day. This may be the result of small sample size and will need to be studied further. Although we cannot conclusively state that 4 capsules/day is the optimal dose, we can state that this dose is both safe and effective.

Our GLBL101c regimen was associated with pathological down-grading from CIN3 to CIN2. Although this changes clinical management in Japan, changes in worldwide diagnostic guidelines mask the therapeutic clinical benefit of our regimen because CIN2 and CIN3 are grouped and both are treated surgically. Nevertheless, in this study, nine patients who experienced a down-grade to CIN2 were followed without surgery and remained free of CIN3. These patients may benefit from oral GLBL101c administration. Future randomized placebo-controlled studies to evaluate clinical efficacy of oral vaccination with GLBL101c should therefore include follow-up time points of at least 4–6 months after completion of the regimen.

## 5. Conclusion

Oral administration of a Lactobacillus-based HPV therapeutic vaccine succeeded in inducing mucosal but not systemic E7-CMI. This study is the first to report a correlation between mucosal CMI and clinical response of an immunotherapy in human mucosal neoplasia. This vaccine strategy may be a novel HPV-targeting immunotherapy for cervical cancer involving the induction of E7-specific mucosal immunity. Furthermore, the oral administration of Lactobacillus-based vaccine may extend to other diseases that develop at mucosal sites including bowel, bronchial, and oropharyngeal epitheliae.

This clinical trial is registered to UMIN-CTR which is accepted by ICMJE.

Clinical registration ID: UMIN000001686 (2009/02/06).

IRB approval No.: P9002144-11X.

The director of this study is K.K. This study was designed by K.K, Y.O, and T.F. K.K and K.A wrote the main manuscript text and prepared all figures. K.K, K.A, S.K, A.T, K.T, A.Y, H.N, K.N, T.A, O.W-H, and K.O collected samples from patients. K.A, S.K, A.T, K.T, K.N, T.Y, and H.N performed these experiments. T.Y and T.S provided GLBL101c. All authors reviewed the manuscript.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.09.020.

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# Characterization of Novel Transcripts of Human Papillomavirus Type 16 Using Cap Analysis Gene Expression Technology

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**We have performed cap-analysis gene expression (CAGE) sequencing to identify the regulatory networks that orchestrate genome-wide transcription in human papillomavirus type 16 (HPV16)-positive cervical cell lines of different grades: W12E, SiHa, and CaSki. Additionally, a cervical intraepithelial neoplasia grade 1 (CIN1) lesion was assessed for identifying the transcriptome expression profile. Here we have precisely identified a novel antisense noncoding viral transcript in HPV16. In conclusion, CAGE sequencing should pave the way for understanding a diversity of viral transcript expression.**



Cervical cancer is the third most common female cancer worldwide, with 530,000 new cases and 275,000 deaths annually (1), and infection by one of a subset of high-risk human papillomaviruses (HPVs) is a prerequisite for its development (2).

A number of elegant studies on the HPV type 16 (HPV16) transcriptome (3–10) have shown that transcription in all HPVs occurs unidirectionally from at least two promoters and involves multiple splice sites. The major promoters in HPV16 are the early promoter (p97), located in the locus control region (LCR), and the differentiation-inducible promoter (p670), with additional promoters in the LCR and within E4 and E5, potentially generating late transcripts. HPV transcription is polycistronic, undergoing differential splicing to produce numerous viral mRNAs (at least 13 transcripts from 8 HPV16 genes in W12E cells [11]). Previous analyses considered only coding RNA, and it is possible that new transcription start sites (TSSs) are obscured by prominent promoters. Therefore, we have used cap analysis gene expression (CAGE) for high-throughput transcriptome analysis (12–16) CAGE specifically determines transcriptional start sites (TSS) genome wide, by capping 5′ ends of RNA transcripts, reverse transcribing them, and mapping cDNAs to the reference genome to identify TSSs (17–20).

To assess all potential TSSs in HPV16-infected cell lines, we performed CAGE analysis using cervical carcinoma-derived CaSki and SiHa cells (21, 22), cervical intraepithelial neoplasia grade 1 (CIN1)-derived W12E (20863) cells, containing episomal copies of HPV16 (3, 23) (3T3 feeder cells removed by trypsinization), and a biopsy specimen from a CIN1 cervical lesion (approved by the Institutional Review Board, University of Tokyo Hospital). From each sample, 5 μg RNA was extracted using a miRNeasy kit (Qiagen). RNA quality was assessed by the use of a Bioanalyzer (Agilent) and standardized at an RNA integrity number (RIN) of >7.0. Quantitation by NanoDrop analysis confirmed that the $A_{260}/A_{290}$ and $A_{260}/A_{230}$ ratios were >1.7.

First-strand cDNAs were transcribed to the 5′ end of capped RNAs and attached to CAGE "bar code" tags (Table 1), and the sequenced CAGE tags were mapped to the HPV16 genome and the human hg19 genomes using BWA software (v0.5.9), discarding ribosomal or non-A/C/G/T base-containing RNAs (24). CAGE tags were normalized by the total number of tags per sample mapped to the human genome and are indicated as tags per million (TPM) (25). For HPV16 genes, CAGE tag 5′ coordinates were input for Paraclu clustering, with these parameters: (i) a minimum of 5 tags/cluster, (ii) (maximum density/baseline density) ≧ 2, and (iii) 100-bp maximal cluster length (18).

**HPV16 transcriptomes differ between carcinoma- and CIN1-derived cells.** Viral gene expression was high in W12E cells and CIN1 clinical samples (Fig. 1C) but was low in CaSki and SiHa cells (Table 1). To quantitatively visualize CAGE-tagged HPV16 genes from each cell line, we compared the data to the HPV16 reference genome (NCBI GI no. 333031). We found 25 positive-strand and 4 negative-strand tag clusters (TCs [regions with >0.5 TPM/sample]) in W12E cells (Fig. 1A), and only 1 positive-strand TC in CaSki and SiHa cells (Fig. 1B), corresponding to the p97 promoters. In W12E cells and a CIN1 clinical sample, numerous additional TSSs span the HPV16 genome, suggesting that precancerous cells show diversity and abundance of viral gene expression that are lost upon viral genome integration and disease progression, although investigation of W12 derivatives with integrated HPV16 and of additional clinical samples is needed to confirm this.

The six most highly expressed TCs in each cell line are compared in Fig. 2. The six TCs most frequently found in W12E cells originate from nucleotide (nt) 90 to 97, nt 1125 to 1149, nt 2017 to

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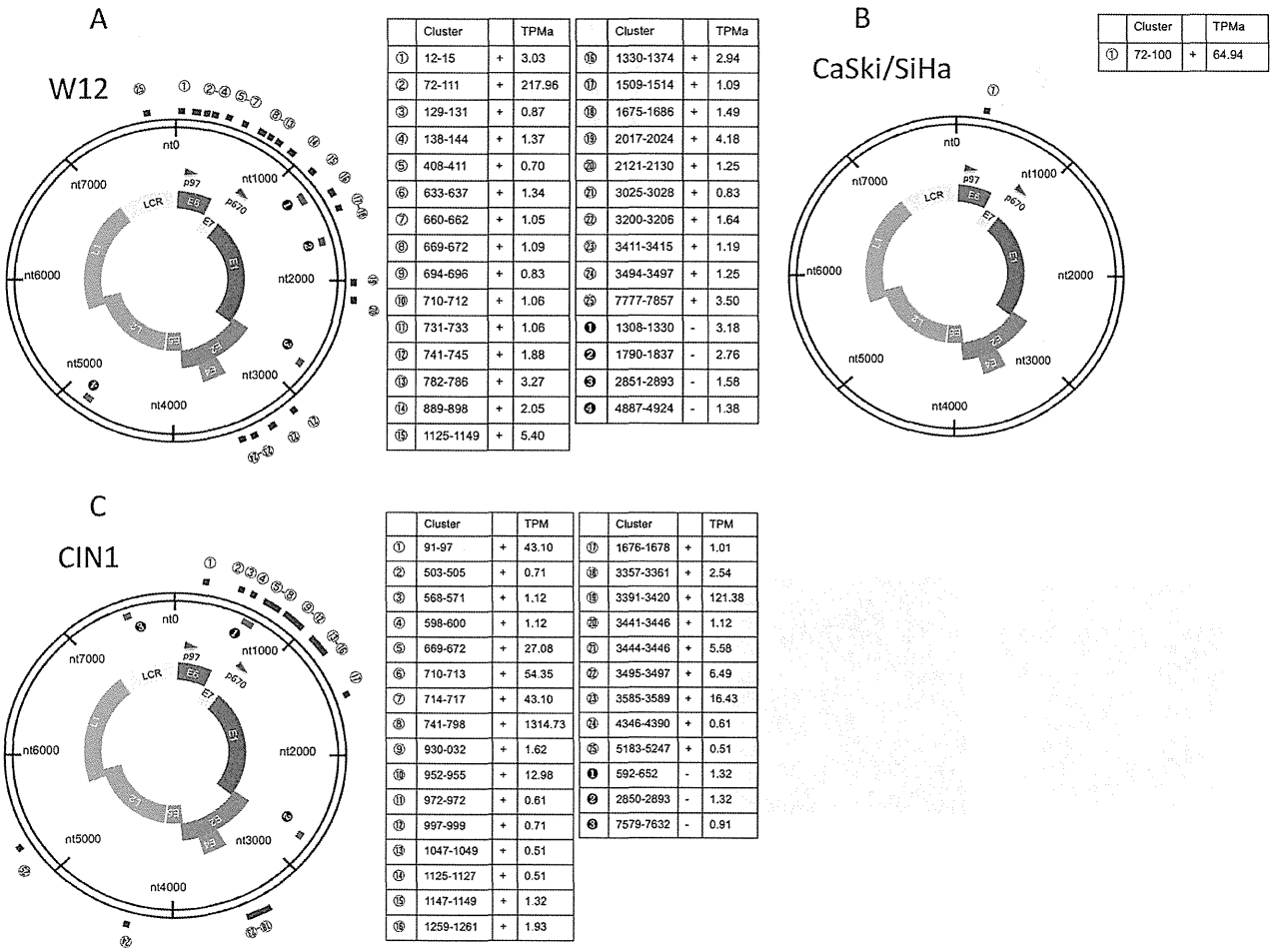
TABLE 1 Number of tags mapped to HPV16 genome

| Cell line | Bar code | No. of tags mapped to HPV16 genome | No. of tags mapped to hg19 genome | Tags per million (TPM) |
|---|---|---|---|---|
| CaSki-1 | GTA | 548 | 9,632,333 | 56.89 |
| CaSki-2 | ACC | 664 | 13,139,414 | 50.53 |
| CaSki-3 | CAC | 494 | 11,535,883 | 42.79 |
| SiHa-1 | AGT | 1,504 | 12,064,850 | 124.66 |
| SiHa-2 | GCG | 991 | 9,050,850 | 109.49 |
| W12-1 | TAC | 5,750 | 15,955,328 | 350.95 |
| W12-2 | GCT | 4,019 | 11,517,132 | 348.96 |
| W12-3 | ATG | 3,928 | 12,018,142 | 326.84 |
| CIN1 | ATG | 20,181 | 9,861,351 | 2,046.47 |

2024, nt 12 to 15, nt 1330 to 1322, and nt 741 to 745, some of which were previously identified (26). The TC at nt 90 to 97 (Fig. 2A) corresponds to the p97 promoter, while those at nt 129 to 131 and nt 138 to 144 had probably leaked from the P97 promoter (21). The TC at nt 1125 to 1149, detected only in W12 cells (Fig. 2B), is probably a TSS of E8-E2 (27, 28), and the TC at nt 12 to 15 corresponds to a promoter upstream of p97, indicating limited E1 transcription (Fig. 2D) (27). The TCs at nt 741 to 745 would be a TSS of E1Ê4, corresponding to the p670 promoter (Fig. 2F) (3). Most interesting, however, was the previously unreported TC at nt 1330 to 1322, which is a potential antisense cluster, highly expressed in W12E cells (Fig. 2E). In addition, we identified another new TC at nt 2017 to 2024 (Fig. 2C).

To verify these novel transcripts, W12 RNA with or without poly(A) was reverse transcribed using a ReverTra Ace qPCR RT kit (Toyobo), and 3′ rapid amplification of cDNA ends (3′ RACE)






| | Cluster | | TPMa |
|---|---|---|---|
| ① | 12-15 | + | 3.03 |
| ② | 72-111 | + | 217.96 |
| ③ | 129-131 | + | 0.87 |
| ④ | 138-144 | + | 1.37 |
| ⑤ | 408-411 | + | 0.70 |
| ⑥ | 633-637 | + | 1.34 |
| ⑦ | 660-662 | + | 1.05 |
| ⑧ | 669-672 | + | 1.09 |
| ⑨ | 694-696 | + | 0.83 |
| ⑩ | 710-712 | + | 1.06 |
| ⑪ | 731-733 | + | 1.06 |
| ⑫ | 741-745 | + | 1.88 |
| ⑬ | 782-786 | + | 3.27 |
| ⑭ | 889-898 | + | 2.05 |
| ⑮ | 1125-1149 | + | 5.40 |

| | Cluster | | TPMa |
|---|---|---|---|
| ⑯ | 1330-1374 | + | 2.94 |
| ⑰ | 1509-1514 | + | 1.09 |
| ⑱ | 1675-1686 | + | 1.49 |
| ⑲ | 2017-2024 | + | 4.18 |
| ⑳ | 2121-2130 | + | 1.25 |
| ㉑ | 3025-3028 | + | 0.83 |
| ㉒ | 3200-3206 | + | 1.64 |
| ㉓ | 3411-3415 | + | 1.19 |
| ㉔ | 3494-3497 | + | 1.25 |
| ㉕ | 7777-7857 | + | 3.50 |
| ❶ | 1308-1330 | - | 3.18 |
| ❷ | 1790-1837 | - | 2.76 |
| ❸ | 2851-2893 | - | 1.58 |
| ❹ | 4887-4924 | - | 1.38 |

| | Cluster | | TPMa |
|---|---|---|---|
| ① | 72-100 | + | 64.94 |

| | Cluster | | TPM |
|---|---|---|---|
| ① | 91-97 | + | 43.10 |
| ② | 503-505 | + | 0.71 |
| ③ | 568-571 | + | 1.12 |
| ④ | 598-600 | + | 1.12 |
| ⑤ | 669-672 | + | 27.08 |
| ⑥ | 710-713 | + | 54.35 |
| ⑦ | 714-717 | + | 43.10 |
| ⑧ | 741-798 | + | 1314.73 |
| ⑨ | 930-032 | + | 1.62 |
| ⑩ | 952-955 | + | 12.98 |
| ⑪ | 972-972 | + | 0.61 |
| ⑫ | 997-999 | + | 0.71 |
| ⑬ | 1047-1049 | + | 0.51 |
| ⑭ | 1125-1127 | + | 0.51 |
| ⑮ | 1147-1149 | + | 1.32 |
| ⑯ | 1259-1261 | + | 1.93 |

| | Cluster | | TPM |
|---|---|---|---|
| ⑰ | 1676-1678 | + | 1.01 |
| ⑱ | 3357-3361 | + | 2.54 |
| ⑲ | 3391-3420 | + | 121.38 |
| ⑳ | 3441-3446 | + | 1.12 |
| ㉑ | 3444-3446 | + | 5.58 |
| ㉒ | 3495-3497 | + | 6.49 |
| ㉓ | 3585-3589 | + | 16.43 |
| ㉔ | 4346-4390 | + | 0.61 |
| ㉕ | 5183-5247 | + | 0.51 |
| ❶ | 592-652 | - | 1.32 |
| ❷ | 2850-2893 | - | 1.32 |
| ❸ | 7579-7632 | - | 0.91 |

FIG 1 Clustered TSSs of the HPV16 genomes of cervical cell lines of different grades. CAGE tags of HPV16 genes from each cell lines are quantitatively visualized. The data are representative of the results determined with the respective cell lines. The CaSki and SiHa cultured cells, representing a transcriptional program characteristic of the conditions in a tumor, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. W12E (20863) is an HPV16-positive cell line derived from a CIN1 lesion (23). W12E (20863) cells were cocultured with mitomycin C-treated 3T3 fibroblast feeder cells. (A) Circular map depicting viral transcripts identified by Paraclu clustering. CAGE tags were clustered for HPV16 genes from W12E cells. Regions containing over 0.5 tags per million (TPM) in all samples were selected as tag clusters (TCs) and mapped to the circular HPV16 genome. (B) We defined regions containing over 0.5 TPM in the sample as TCs. Then, CAGE tags were clustered for HPV16 genes from the CaSki and SiHa cells. (C) CAGE tags were clustered for HPV16 genes from a CIN1 clinical sample. Notably, we found 25 positive-strand TCs and 4 negative-strand TCs in W12E cells (A), whereas only 1 positive-strand TC was found in CaSki or SiHa cells (B). TPMa, the average number of TPMs.

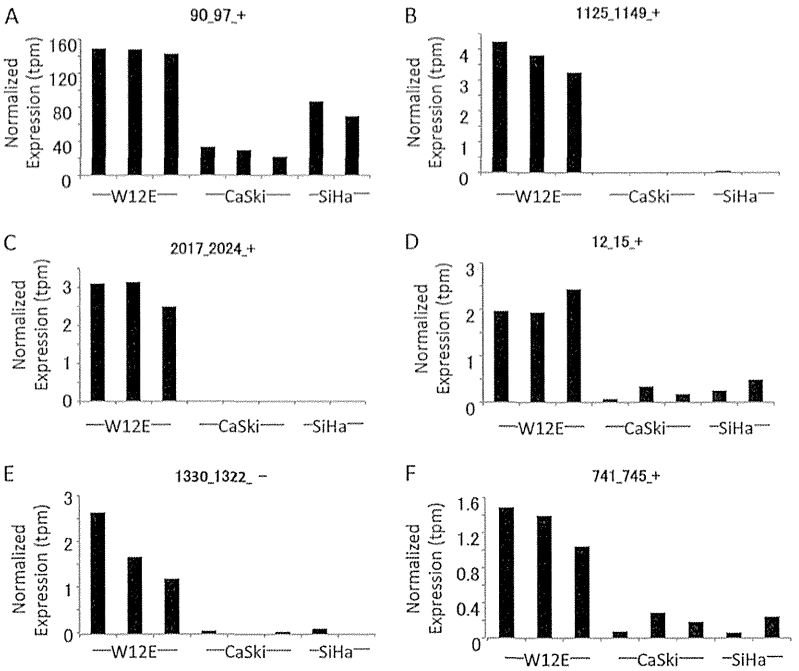
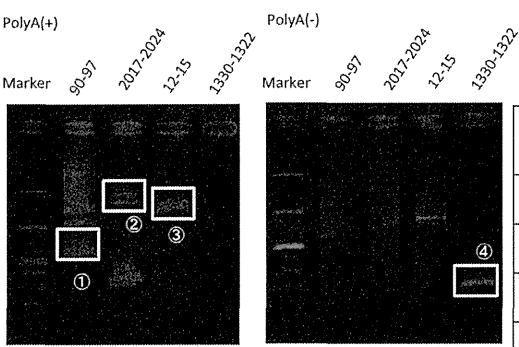



FIG 2 Expression levels of the top 6 tag clusters (TCs) in W12E, CaSki, and SiHa cells. The top 6 expressed TCs were selected, and expression levels are indicated as tags per million (TPM) (25). Six TSS clusters were frequently found to be prominent in W12E cells, originating from nt 90 to 97 (90_97_+), nt 1125 to 1149 (1125_1149_+), nt 2017 to 2024 (2017_2024_+), nt 12 to 15 (12_15_+), nt 1322 to 1330 (1330_1322_−), and nt 741 to 745 (741_745_+).

was carried out using *Ex Taq* (TaKaRa) on the detected clusters found in clustering analysis of HPV16 genome expression, using the nt-97 cluster as a positive control.

To verify RNA without poly(A), a 3′ preadenylated DNA adaptor sequence was ligated at the 3′ end of RNA. To increase specificity, we carried out first RACE and second RACE experiments. Primer pairs for each peak [oligo(dT)] adaptor are shown in Fig. 3. The PCR conditions for the first RACE and second RACE exper-




| Bands | Coordinate |
|---|---|
| 1 | 97-226^3391-3761 polyA(+) |
| 2 | 2017-3761 polyA(+) (lower band)<br>2017-4230 polyA(+) (upper band) |
| 3 | 12-226^3358-4230 polyA(+) |
| 4 | 1330-997 polyA(-) |

**Primer sequence of 3′ RACE and polyA(-) RT**

| Peaks | 1st/ 2nd RACE | Primers |
|---|---|---|
| nt90-97 | 1st<br>2nd | 5′-GCGACCCAGAAAGTTACCAC-3′<br>5′-ATGCACAGAGCTGCAAACAAC-3′ |
| nt2017-2024 | 1st<br>2nd | 5′-GGATTGTGCAACAATGTGTAG-3′<br>5′-CAAATGAGTATGAGTCAATGG-3′ |
| nt12-15 | 1st<br>2nd | 5′-CGGTTGAACCGAAACCGGTTAG-3′<br>5′-TATGCACCAAAAGAGAACTGC-3′ |
| nt1330-1322 | 1st<br>2nd | 5′-TTGCCATACCCGCTGTCTTCGC-3′<br>5′-ATTATTGTCTACACATCCAC-3′ |
| polyA(+) | 1st<br>2nd | 5′-CAAGCAGAAGACGGCATACGATT TTTTTTTTTVN-3′<br>5′-CAAGCAGAAGACGGCATACGA-3′ |
| polyA(-) | 1st<br>2nd | 5′-CATCATTGATGGTGCCTACAG -3'<br>5′-CATCATTGATGGTGCCTACAG -3' |
| polyA(-) RT-primer | | 5'- CCGATCTCACCTCCTGCATCATTGATG GTGCCTACAG -3' |
| 3′adaptor | | 5'- rApp-CTGTAGGCACCATCAAT-ddC -3' |

FIG 3 Validation of novel transcripts from 3′ RACE analysis. Agarose gel electrophoresis of transcripts from W12 cell eluted RNA. The results of the experiment indicate that the transcript originating at nt 1330 to 1322 could correspond to a newly identified viral antisense noncoding RNA without poly(A). Further, we could encode a newly identified transcript of nt 2017 to 2024 starting in the middle of E1 coding gene. ddC, dideoxycytosine; rApp, adenylation-5′; RT, reverse transcriptase.

iments were as follows: for the first RACE experiment, 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; for second RACE experiment, 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The results are shown in Fig. 3. PCR products were extracted (Qiagen) and sequenced by Applied Biosystems 3130xl (Fasmac). Importantly, we identified, for the first time, the full coordinates of a novel antisense transcript starting from nt 1330 as a noncoding RNA (ncRNA) without poly(A) and also a novel transcript, nt 2017 to 2024, starting in the middle of the E1 gene. Although most viral RNAs are considered to be polyadenylated at the 3′ end, our finding confirms that the noncoding viral RNA starting from nt 1330 lacks poly(A) signals.

In this study, we performed CAGE analysis and investigated TSSs from the perspective of both the HPV16 and human genomes, using cell lines containing episomal and integrated HPV sequences. All the TSSs used in the HPV16 transcriptome, in the HPV16-containing cervical keratinocyte W12E cell line, and in the cervical cancer-derived SiHa and CaSki cell lines were investigated. Intriguingly, we also show that a diversity of viral transcripts, seen in W12E cells, shared some identity with the CIN1 biopsy specimen subjected to a precise colposcopic examination. Recently, as shown previously in the mammalian transcriptome (29), abundant virally encoded noncoding RNAs (ncRNAs) were identified (30), but this is the first full confirmation of a preliminary observation of antisense transcripts in HPV.

Using the CAGE method, we have successfully established unbiased analyses of reproducible transcriptional start sites across the HPV16 genome, potentially identifying novel transcripts, including ncRNAs, for future RNA therapies (31). Further comprehensive studies will aim to identify possible markers to predict the outcome of infections with HPV.

**Nucleotide sequence accession number.** The sequence data reported are available in the DDBJ BioProject under the accession number PRJDB3385.

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# Positive peritoneal cytology at interval surgery is a poor prognostic factor in patients with stage T3c advanced ovarian carcinoma: A retrospective study

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## Abstract

***Aim:*** The purpose of our study is to investigate clinically significant prognostic factors at the time of interval surgery (IS), comprising interval look surgery and interval debulking surgery, for T3c (International Federation of Gynecology and Obstetrics stage IIIc to IV) advanced ovarian cancer (AOC) patients during primary treatment.

***Methods:*** We reviewed records of patients with T3c AOC who underwent IS following neoadjuvant chemotherapy or up-front primary debulking surgery with adjuvant chemotherapy at our institution between January 1996 and December 2010. For analysis of prognostic factors, cytology of peritoneal exfoliative cells at IS was added to clinicopathological variables.

***Results:*** A retrospective analysis was performed on 50 cases. The median age was 61.1 years (range, 38–78), with median follow-up of 45.9 months (range, 12–122). Macroscopic tumors were completely resected in 32 cases (64%) at IS. Univariate analyses of clinicopathological factors for IS identified preoperative serum cancer antigen-125 levels (≥20 IU/mL; $P = 0.0539$), number of residual lesions at IS (≥20; $P = 0.0554$), incomplete surgery at IS ($P = 0.0171$) and positive peritoneal cytology at IS ($P = 0.0015$) as significant factors for prognosis regarding progression-free survival (PFS). Multivariate analysis identified positive peritoneal cytology ($P = 0.0303$) as a unique independent predictor of poor prognosis in PFS.

***Conclusion:*** Positive peritoneal cytology at IS appears to be a significant factor for poor prognosis in PFS, which may provide useful information for post-IS chemotherapy planning. IS in the treatment of AOC may be useful for not only complete resection, but also for identification of patients with poor prognosis.

**Key words:** interval surgery, ovarian cancer, peritoneal cytology, prognostic factor.

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## Introduction

Ovarian cancer is the fifth most frequent cause of death among women.[1] The estimated annual incidence of ovarian cancer is approximately 225 000 women, resulting in 140 200 deaths per year.[2,3] In Japan, ovarian cancer is diagnosed in approximately 7000 women annually and continues to increase.[4] Approximately 70% of patients are diagnosed at an advanced stage (stage III to IV) as there are little or no warning symptoms during early stages and screening has not proven to be effective. Aiming for complete (no macroscopic residual tumors) or optimal (residual tumors of <1 cm) cytoreduction, primary debulking surgery (PDS), followed by platinum-based combination chemotherapy is the mainstay of treatment for patients with advanced ovarian cancer (AOC).[5–8] However, PDS is often not feasible, especially in patients with advanced disease in the upper abdomen or for those with poor functional status.[9,10]

To improve survival after incomplete primary treatment, interval surgery (IS) is often pursued, allowing a less aggressive surgery to be performed. However, whether neoadjuvant chemotherapy (NAC) or PDS is a more effective starting point in primary treatment, followed by secondary surgery, remains controversial.[11–15] Thus, two treatment options remain available for treating patients with AOC. Recently, a large phase III trial by the European Organization for Research and Treatment of Cancer (EORTC) Gynecologic group has suggested that NAC followed by interval debulking surgery (IDS) had the same survival rate as PDS followed by adjuvant chemotherapy, among those with International Federation of Gynecology and Obstetrics (FIGO) stage IIIC and IV ovarian cancer.[16] In addition, ongoing work of a second phase III randomized controlled trial (CHORUS), investigating timing of initial surgery, has been consistent with results from EORTC55971, strengthening evidence for NAC as a suitable alternative to PDS (Kehoe *et al.* J Clin Oncol (Meeting Abstracts)). In total, a number of reports have recommended NAC followed by IDS. In contrast, a multicentered prospective randomized trial conducted by the Gynecologic Cancer Intergroup demonstrated that it was unnecessary to perform secondary cytoreductive surgery in cases in which PDS was performed by a specialized gynecologic oncologist.[12] They concluded that NAC plus IDS improved neither progression-free survival (PFS) nor overall survival (OS) compared with appropriately aggressive PDS. However, even among specialized gynecologists, surgical treatment remains widely variable by country and individual, depending heavily on both surgical skill and patient performance status.[17–19]

The data supporting IS for AOC has remained compelling and, consequently, most patients with stage IIIc to IV primary ovarian cancer are treated with IS for residual diseases following incomplete primary treatments. Previous reports have demonstrated that PFS was significantly improved after complete resection at IDS.[20] Furthermore, Onda *et al.* have recently shown that the optimal goal of IS is limited to removal of as many tumor deposits as completely as possible, which leads to relatively good OS compared with those with microscopic residuals.[21] These data suggest that complete surgical resection at IS confers a survival advantage. While many studies have demonstrated the goal of surgical procedure, clinicopathological factors at IS which may influence prognosis have neither been well defined nor thoroughly investigated. Therefore, importantly, we retrospectively assessed factors at IS associated with poor prognosis, including those on preoperative evaluation.

## Methods

### Patients

All patients with stage IIIc to IV (T3c) primary ovarian carcinoma presenting to the University of Tokyo Hospital from 1994 and 2010 were eligible for this study. All cases were diagnosed as T3c per FIGO criteria, defined as unilateral or bilateral ovarian cancer with macroscopic peritoneal metastasis beyond the pelvis, with tumor size of more than 2 cm in greatest dimension. Diagnosis of adenocarcinoma was proven by surgical histology for PDS cases. NAC cases were staged using imaging and primary investigation of peritoneal cytology obtained by paracentesis or culdocentesis. Staging and histologic data of study participants are shown in Table 1. The study was performed with written informed consent from patients in accordance with ethical guidelines at the University of Tokyo Hospital.

### Treatments

All cases received one of the following first-line platinum-based combination chemotherapy regimens: CAP (cyclophosphamide, 400–600 mg/m$^2$, and doxorubicin, 30–40 mg/m$^2$, every 3 weeks with cisplatin, 50–75 mg/m$^2$); conventional TC (paclitaxel 175 mg/m$^2$, carboplatin area under the curve [AUC] 6.0); weekly TC (paclitaxel 60 mg/m$^2$ and carboplatin AUC 2.0, on

**Table 1** Patient and treatment characteristics before IS ($n = 50$)

| | No. of patients ($n = 50$) |
|---|---|
| Age, median (range), years | 61.1 (38–78) |
| Follow-up period (months) | 45.9 (12–122) |
| FIGO | |
| Stage IIIc | 36 (72%) |
| Stage IV | 14 (28%) |
| Histology | |
| High-grade serous, endometrioid | 37 (74%) |
| Clear cell, mucinous, low-grade serous | 4 (8%) |
| †Others | 9 (12%) |
| Duration of primary chemotherapy (months) | 3.9 (2–6) |
| Pre-IS treatment | |
| PDS + chemotherapy | 33 (66%) |
| NAC | 17 (34%) |
| Response of pre-IS chemotherapy | |
| ‡cCR | 26 (52%) |
| Non-cCR | 24 (48%) |

†Unclassifiable adenocarcinomas were included in this group. ‡cCR was evaluated in all cases by Response Evaluation Criteria in Solid Tumors, but one non-measurable case was included in this group. cCR, clinical complete response; FIGO, International Federation of Gynecology and Obstetrics; IS, interval surgery; NAC, neoadjuvant chemotherapy; PDS, primary debulking surgery.

days 1, 8 and 15); or dose dense weekly TC (paclitaxel at 80 mg/m$^2$ on days 1, 8, and 15 and carboplatin AUC 6.0). PDS cases received bilateral/unilateral salpingo-oophorectomy plus infragastric/radical omentectomy with or without hysterectomy following three to four cycles of chemotherapy prior to IS. After surgery, pathological tumor–node–metastasis characterization was performed. NAC cases received three to four cycles of chemotherapy before IDS. After IS, all cases received three to four courses of postoperative adjuvant therapy to complete a total of eight courses of the chemotherapy. Within 4 weeks of IS, all cases were treated with postoperative chemotherapy. All clinical records are kept as prospective computerized records at the University of Tokyo Hospital.

## Clinical evaluation

Patients were routinely evaluated at the end of treatment, including blood testing with preoperative elevated tumor markers (e.g. cancer antigen [CA]-125, carcinoembryonic antigen, CA19-9), chest and abdominal X-ray, transvaginal and transabdominal ultrasound examination, computed tomography (CT), magnetic resonance imaging and positron emission tomography, for evidence of disease recurrence. The following data were recorded for statistical analysis: host characteristics (age, gravidity, performance status), pathologic findings (histological diagnosis, cell type, ascites cytology, touch smear in peritoneal cavity), duration of primary/subsequent perioperative chemotherapy, extent of disease (FIGO stage, clinically measurable diseases detected by preoperative CT scanning or at surgery, preoperative serum CA-125 levels, size of residual disease, cytological examination and ascites volume) and success of surgical cytoreduction (optimal/complete or incomplete). Clinical complete response (cCR) was defined per Response Evaluation Criteria in Solid Tumors 1.1 criteria.

## IS

The IS, comprising the interval look surgery (ILS) and the IDS, have been performed in our institute as previously described.[21] The surgical procedures at interval surgery were all performed by specialized gynecologic oncologists. Our standard interval surgery is comprised of total abdominal hysterectomy, bilateral salpingo-oophorectomy and infragastric/radical omentectomy if these organs had not been removed at initial management. When appropriate, complete intraperitoneal debulking with the above standard surgery plus pelvic and aortic lymphadenectomy were performed, except for patients with low performance status (PS) or with any severe complications. Further surgical attempts at cytoreduction following procedures were performed, for example, rectosigmoid colectomy, bladder resection, splenectomy, diaphragmatic peritonectomy, hepatic resection and pancreas resection. Evaluation of residual lesions was determined by specialized gynecologic oncologists for all visualized abnormalities, including the size or number of residuals at visceral and peritoneal surface.

## Collection of peritoneal cytology at IS

Immediately after direct visualization of pelvic organs, ascites was aspirated from the pelvic cavity. For insufficient volume for floating cell cytology, peritoneal washing was performed by instilling approximately 50 mL of 37°C normal saline into the peritoneal cavity. The fluid was allowed to immerse the peritoneal surfaces and then aspirated from the pelvic region. In addition, exfoliative peritoneal cells were intensively wiped by cotton swab from five representative sites on the abdominal peritoneum: (i) vesicouterine pouch; (ii) cul-de-sac; (iii) right paracolic gutter; (iv) left paracolic gutter; and (v) right subphrenic space. For staining of

cytology, the conventional staining, consisting of Papanicolaou, periodic acid-Schiff, May-Grunwald and Giemsa were used before fixation. Peritoneal cytology was considered positive if cancer cells were detected microscopically in either peritoneal fluid or wiped swabs.

### End-points and statistical analysis

Standard statistical analyses were utilized. We analyzed the duration of response, PFS and OS in 50 patients who received secondary surgical cytoreduction, including IS, with residual disease after primary treatment (NAC or PDS). PFS was calculated from the first day of the secondary surgery. Patients whose disease progressed at secondary surgery and subsequently post-treatment, and who were therefore never progression-free, were considered to have a time to progression of zero. OS was calculated from the first day of chemotherapy to death; patients who were still alive at the last follow-up appointment were censored. Life-tables were constructed to assess PFS and OS using the Kaplan–Meier method and were compared between groups using the log–rank test. Univariate and multivariate analyses assessing prognostic factors on PFS were performed using Cox proportional hazards regression. All variables associated with $P < 0.10$ on univariate analysis were included in multivariate analysis. All statistical tests were two-tailed at a level of 0.05, and differences were considered statistically significant at $P < 0.05$. JMP-Pro version 10.0.2 software (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

## Results

### Pre-IS clinical characteristics of the patients

A total of 50 T3c cases were analyzed in this study. The median observation period in the group was 45.9 months (range, 12–122). The median age was 61.1 years (range, 38–78). The majority of patients were FIGO stage IIIc (72%); the remaining were stage IV (28%). The median preoperative serum CA-125 level was 2902 U/mL. Characteristics of all cases are summarized in Table 1. Of 50 cases, 33 (66%) were post-PDS, while the remaining (34%) underwent NAC. Regarding histological findings, 33 cases evidenced serous adenocarcinoma (66%). Both PDS and NAC cases received IS followed by post-IS platinum-based adjuvant chemotherapy. Combination paclitaxel–carboplatin therapy (TC) was introduced as first-line standard therapy in our hospital in the year 2000. Clinical response of pre-IS chemotherapy was evaluated by preoperative CT scanning. Twenty-six cases (52%) demonstrated cCR after primary treatment, while 24 (48%) did not (non-cCR). Within these 26 cases, one patient with non-measurable lesions (longest diameter, <20 mm) confirmed by normalized tumor markers with sufficient shrinkage of lesions was included in this cCR group. Twenty-four cases (48%) did not demonstrate any response (non-cCR) during follow-up.

### Macroscopic and cytological findings at IS

Macroscopic findings at IS and impact on tumor resection by IS are shown in Table 2. The number of residual tumors was 19 or fewer in 37 cases (74%) at IS, while 20 or more in 13 cases (26%). The diameter of the largest residual tumors was smaller than 2 cm in 25 cases (50%). Complete cytoreduction was achieved in 32 cases (64%).

Both peritoneal floating and exfoliative cells were collected from all cases by washing and touch swab, respectively, at IS laparotomy. Of 50 cases, 22 (44%) were cytology-positive, while the remaining (56%) were negative. Complete cytoreduction by IS was achieved in 82% (23/28) and 41% (9/22) of cytology-negative and -positive cases, respectively (Table 3). Cytology-negative cases were significantly more likely to achieve complete cytoreduction compared with cytology-positive cases ($P = 0.0035$). Interestingly, nine of 32 cases with complete cytoreduction were cytology-positive, suggesting that residual cancer cells remained microscopically present in the peritoneum.

**Table 2** Findings at IS and impact on tumor resection by IS ($n = 50$)

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|---|---|
| Diameter of largest residual tumor nodule found at IS | |
| <1 cm | 21 (42%) |
| 1–2 cm | 4 (8%) |
| ≥2 cm | 25 (50%) |
| No. of residual lesions found at IS | |
| ≤5 | 25 (50%) |
| 6–19 | 12 (24%) |
| ≥20 | 13 (26%) |
| Impact on IS | |
| Complete | 32 (64%) |
| Incomplete | 18 (36%) |
| ≤1 cm | 14 (28%) |
| >1 cm | 4 (8%) |

IS, interval surgery.