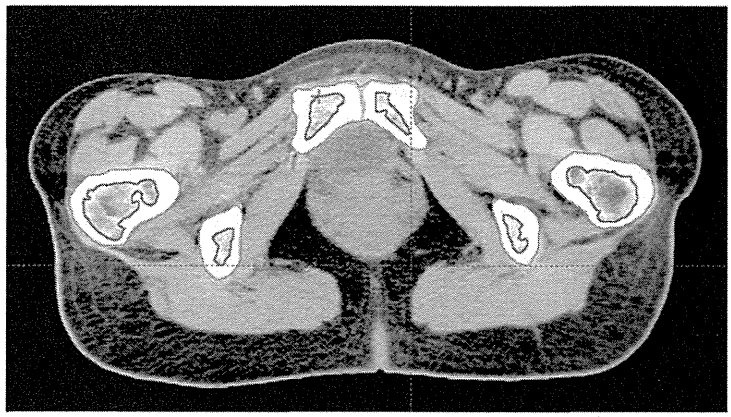

Figure 1. *An axial figure showing contour for inner cavity of pelvic bone (green line) and whole pelvic bone (pink line).*

Table I. *Patients' characteristics (n=10).*

Median age (range)	39 (25-66)
Clinical stage	
IB1	6
IB2	2
IIA	0
IIB	2
Histology	
Scc	5
Adeno	3
Adenosquamous	2
Parametrium invasion	
Yes	7
No	3
Pelvic LN metastasis	
Yes	9
No	1
Median Number of pelvic LN metastasis (range)	2 (0-8)
Surgical margin	
Positive	0
Negative	10
Median tumor size (cm, range)	4.1 (1-5.4)

Table II. *The mean value of DVH parameters for PTV.*

	Normal-IMRT		BMS-IMRT		
	mean (%)	SD†	mean (%)	SD†	*p*-Value
PTV D_{max}	109.7	3.7	110.8	1.4	0.258
PTV $D_{95\%}$	94.6	2.3	92.5	1.9	*<0.01
PTV $D_{97\%}$	93.4	2.5	90.8	2.6	*0.05
PTV median	100.4	0.4	100.8	0.5	*0.05

†SD: standard deviation, PTV: planning target volume, DVH: dose volume histogram.

between both plans. Therefore it was supposed that these small differences would not bring about any clinical relevant differences. For comparison, another plan was created in which the PTV coverage was prioritized to be of the same degree as normal-IMRT. In these plans, favorable sparing of bone marrow did not occur (data not shown).

Figure 2b shows boxplots of DVH parameters for OARs. There were no statistical difference between normal-IMRT and BMS-IMRMT in the mean value of rectum V_{30Gy}, V_{40Gy}, V_{50Gy}, bladder V_{45Gy}, V_{50Gy}, Bowel V_{35Gy}, V_{40Gy}, V_{45Gy}, and V_{50Gy}. There was a statistically significant difference between the mean value of bladder V_{35Gy} and femoral head V_{30Gy}. Although a statistical significance was found between normal- and BMS-IMRT in bladder V_{35Gy}, as shown in the Figure 2 the difference was quite small; therefore it was not known whether this small difference would be connected to clinically evident difference. The dose of femoral head was effectively lowered in BMS-IMRT than that of normal-IMRT. It was supposed that this difference was caused because the femoral head was included in the structure of pelvic bone.

Figure 3a and 3b show boxplots of DVH parameters for WPB and ICPB. Both in WPB and ICPB, the mean value of V_{10Gy}, V_{20Gy}, V_{30Gy}, and V_{40Gy} of BMS-IMRT were statistically lower than that of normal-IMRT. Figure 4 shows an example of dose distribution of BMS-IMRT and normal-IMRT. In this Figure, the area receiving a dose of 40 Gy or higher was colored. Visually, it was clear that the spinal body was effectively avoided in BMS-IMRT compared with normal-IMRT. Because the 15 MV photon beam is not used in the majority of institutions in the United States, another BMS-IMRT plan was created in 3 representative patients using a 6 MV photon beam and the DVH was compared.

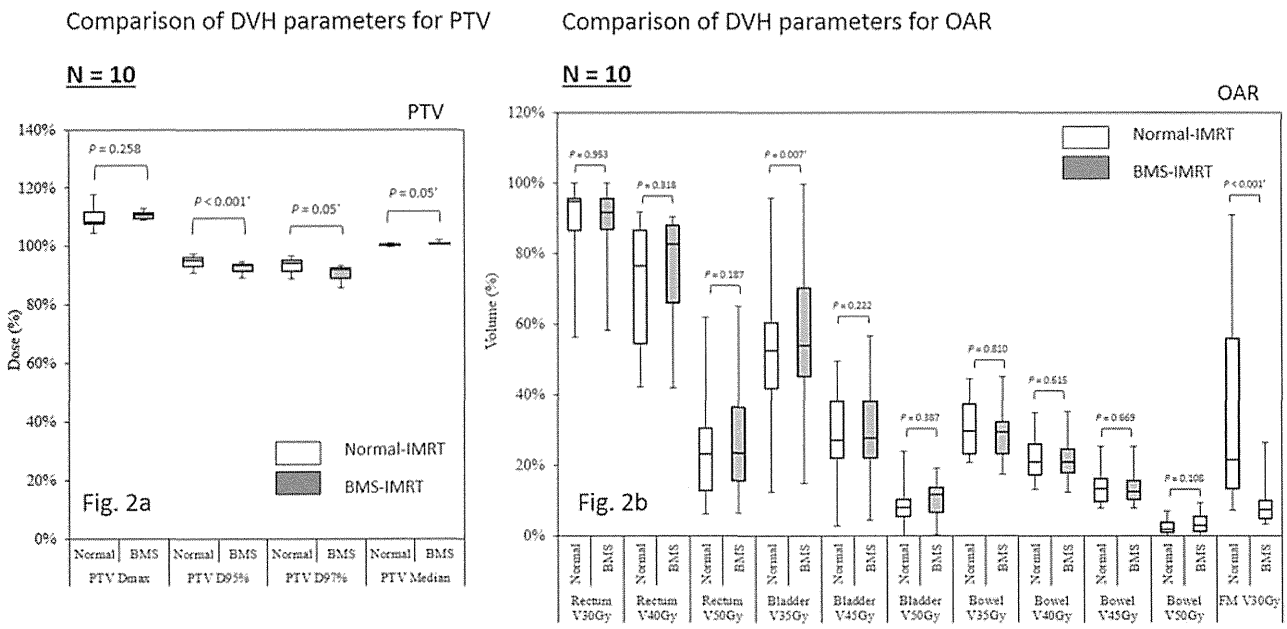

Figure 2. *Boxplots of dose volume histogram (DVH) parameters for planning target volume (PTV) (a) and organ at risks (OARs) (b).Note: spell out Femoral head (FM).*

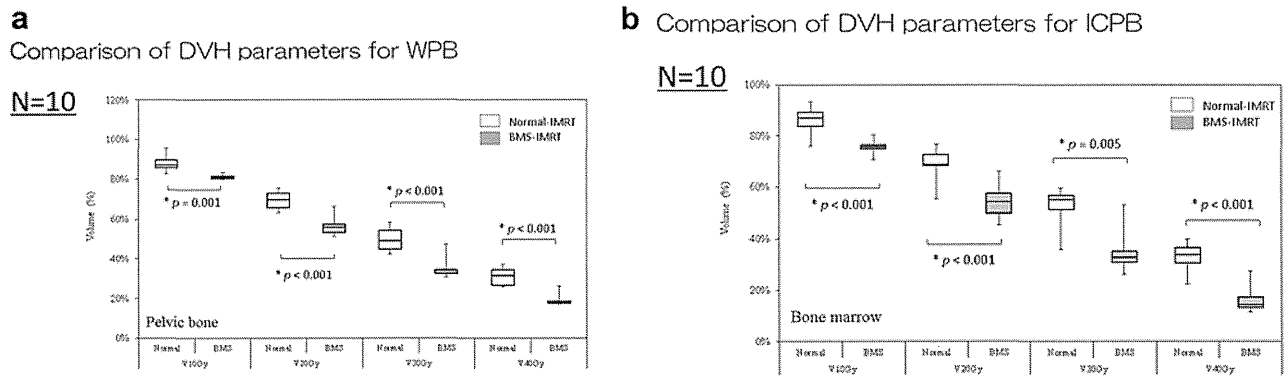

Figure 3. *Boxplots of dose volume histogram (DVH) parameters for whole pelvic bone (WPB) (a) and inner cavity of pelvic bone (ICPB) (b).*

Table III summarizes the difference of DVH parameters between the 6 MV and 15 MV photon beams. The same degree of BMS and OAR sparing was achieved by using the 6 MV photon beam, while the monitor unit of this beam was higher because of its lower photon energy.

Discussion

It was demonstrated that early-stage cervical cancer patients who underwent radical hysterectomy and exhibited high-risk feature(s) could benefit postoperative concurrent chemoradiotherapy by a multicenter prospective phase III randomized clinical trial (2). It was also shown in this study that it was important to deliver as many chemotherapy cycles as possible. In the RTOG 0418 trial (12), postoperative endometrial and cervical cancer patients were entered and treated by postoperative radiation therapy by IMRT. In the present study, cervical cancer patients were treated by concurrent chemoradiotherapy using weekly

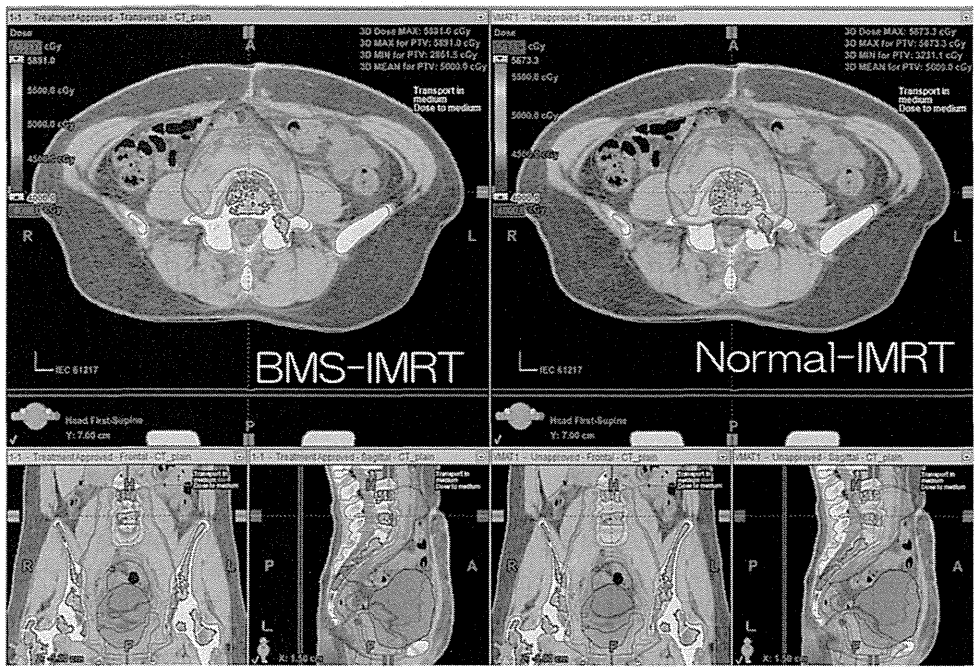

Figure 4. *An example of dose distribution of bone marrow sparing (BMS)–IMRT and normal-IMRT. The area which received 40 Gy or more was colored.*

cisplatin 40 mg/m^2. Although bone marrow sparing was not intended in the protocol of RTOG 0418, favorable hematological profiles were shown; 83% received 5 or more cycles of cisplatin and 90% received at least 4 cycles of cisplatin (13). The RTOG 0418 data demonstrated that pelvic bone V_{40Gy} >37% was associated with grade 2 or higher hematologic toxicity (13). On the other hand, Rose *et al*. reported that patients with V10Gy ≥95% as well as V_{20Gy} ≥76% were more likely to experience grade ≥3 leukopenia (21). Mell *et al*. also showed from a small sized retrospective study that bone marrow V_{10Gy} was a strong predictor for grade 2 or worse leukopenia (22). Albuquerque *et al*. reported the importance of the volume of bone receiving 20 Gy (23). Therefore V_{10Gy}, V_{20Gy}, V_{30Gy} and V_{40Gy} were extracted and compared in the current study since controversies still remain over whether lower or higher dose on bone marrow affects its function. Mahantshetty *et al*. demonstrated that ICPB was a better surrogate of active bone marrow than whole pelvic bone WPB (20); therefore, both WPB and ICPB were examined in this study.

In the current study, it was clearly demonstrated that both lower and higher dose for WPB as well as ICPB were significantly lower in the BMS-IMRT plan compared with the normal-IMRT plan without changing the coverage of target volume and other organs at risk. Therefore, it is important to include bone marrow structures into dose constraint structures for post-hysterectomy radiation therapy by IMRT especially with concurrent use of chemotherapy. Our institution uses a 15 MV photon beam for irradiating the pelvic region because a high energy photon beam has an advantage of delivering photons to deep seated organs with

Table III. *Comparison of dose volume histogram parameters of bone marrow sparing intensity-modified radiation therapy plans between 6 MV and 15 MV photon beams.*

6X-15X (%)		6X-15X (%)	
PTV D_{max}	0.80	Rectum V_{30Gy}	–2.07
PTV $D_{95\%}$	0.07	Rectum V_{40Gy}	–0.33
PTV $D_{97\%}$	0.10	Rectum V_{50Gy}	2.17
PTV Median	–0.01	Bladder V_{35Gy}	0.97
PB V_{10Gy}	0.53	Bladder V_{45Gy}	0.53
PB V_{20Gy}	0.60	Bladder V_{50Gy}	1.67
PB V_{30Gy}	0.10	Bowel V_{35Gy}	0.73
PB V_{40Gy}	0.23	Bowel V_{40Gy}	0.50
BM V_{10Gy}	0.37	Bowel V_{45Gy}	0.70
BM V_{20Gy}	–0.57	Bowel V_{50Gy}	0.83
BM V_{30Gy}	–0.03	FM V_{30Gy}	0.80
BM V_{40Gy}	0.50	MU	93 (MU)

PTV: planning target volume, PB: pelvic bone, BM, bone marrow, FM: femoral head, MU: monitor unit.

less attenuation; however, the 15 MV photon beam has a concern about creating neutrons along with photons. Therefore, another BMS-IMRT plan was created using a 6 MV photon beam. Since the same trend was obtained by using the 6 MV photon, BMS-IMRT may protect the patient's bone marrow function in institutions where such a beam is used for cervical cancer.

Among the limitations of this work it has to be noted that it was a single-institution retrospective study with small number of patients that analyzed only DVH parameters virtually based on CTs taken before radiotherapy. Also, it is important to maintain the patient's anatomical relationship as planning CT, namely monitoring the filling of bladder and the emptiness of rectum because otherwise intended OAR sparing will not be achieved. Since this study was represents a "plan-to-be" approach, we have to verify the efficacy of BMS-IMRT in a clinical setting. Recently RTOG 1203 was launched in order to validate whether IMRT could decrease acute gastrointestinal toxicity compared with conventional radiotherapy with bone marrow sparing included in the protocol. Therefore, important information about the influence of IMRT over bone marrow will be available soon.

Whether WPB or ICPB should be more appropriate surrogate structures for bone marrow function(s) will be confirmed by future prospective studies. Also, the appropriate doses for bone marrow's protection need to be further investigated in the near future.

Conclusion

Both lower and higher dose for WPB as well as ICPB were effectively decreased by IMRT with an intention of avoiding damage of bone marrow structure without compromising the coverage of target volume and other organs at risk.

Conflicts of Interest

There is no conflict of interest to be declared.

Acknowledgements

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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 1×10^6 cervical lymphocytes were isolated from each patient's cervix. 5×10^4 cervical lymphocytes (CxLs) or peripheral blood mononuclear cells (PBMCs) were incubated for 24 h at 37 °C with antigen presenting cells or 5×10^4 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.

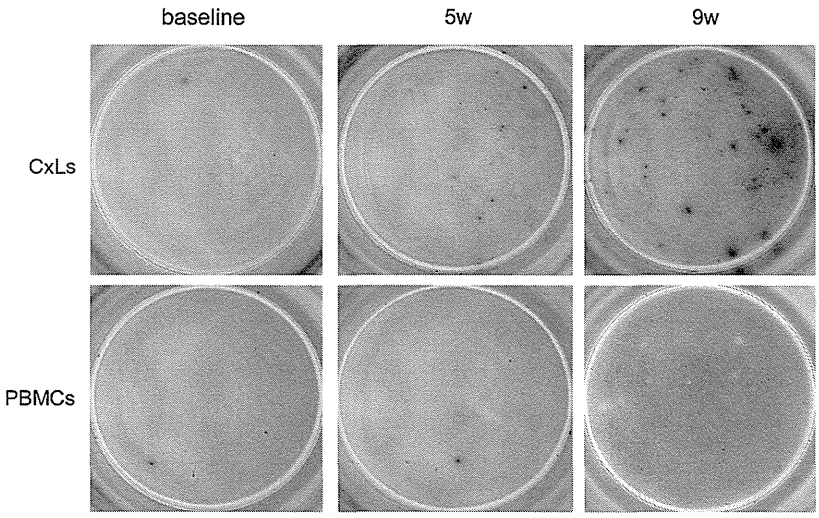

Fig. 1. Immunological response to vaccination (4 capsules/day) in a representative patient. (ELISpot assay images). CxLs (upper) and PBMCs (lower) were collected from patients 2–7 at baseline and at weeks 5 and 9. Purple dots indicated E7-specific IFNγ-producing lymphocytes. CxLs (cervical lymphocytes); 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

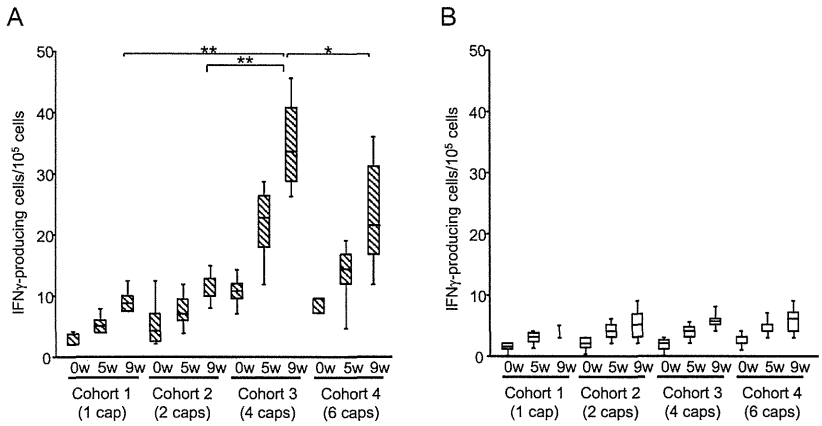

Fig. 2. Immunological responses to different dose of vaccination. (A) Stripe boxes indicate E7-CMI (E7-specific IFNγ-producing cells) in cervical lymphocytes (CxLs) for each cohort. (B) White boxes indicate E7-CMI in PBMCs for each cohort. For each cohort, E7-CMI at pre-vaccination (0 wk), weeks 5 (5 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.

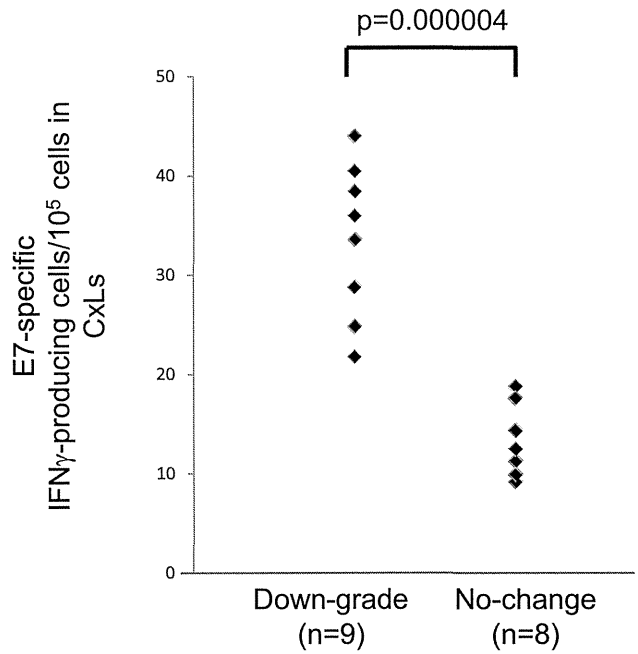

Fig. 3. Correlation of E7-CMI in CxLs with pathological responses. E7-CMI (E7-specific IFNγ-producing cells) at week 9 was compared between pathological down-grade ($n=9$) and no change ($n=8$) groups. The cervical E7-CMI of down-graded patients increased significantly more than that of patients who 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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Treatment results for Stage Ib cervical cancer after stage subdivision by MRI evaluation

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Summary

Purpose of investigation: The authors analyzed treatment results for cervical cancer after subdividing Stage Ib into Stages Ib1 and Ib2 according to magnetic resonance imaging (MRI) information. *Materials and Methods:* The subjects comprised 40 cases of Stage Ib cervical cancer treated by definitive radiotherapy in Kitasato University hospital and Tokyo University hospital from January 2000 to December 2008. The patients' ages ranged from 28 to 85 years (median: 68 years). The maximum tumor diameter measured with MRI ranged from undetectable to 60 mm (median: 25 mm). The authors classified tumors with the greatest dimension less than 40 mm as Stage Ib1 (29 cases) and those with the greatest dimension more than 40 mm as Ib2 (11 cases). All cases were treated with a combination of external beam irradiation and high-dose-rate intra-cavitary brachytherapy. Chemotherapy was combined with radiotherapy in 11 cases. *Results:* The follow-up time was from four to 109 months (median: 53 months). At the time of last observation, 37 cases survived, local recurrence was seen in none, and two cases showed distant metastasis. The two- and five-year overall survival rates of all cases were 97.5% and 89.5%, respectively. When a stage was subdivided and examined, the five-year overall survival rate of Stage Ib1 was 100% and that of Stage Ib2 was 50.5% ($p = 0.001$). *Conclusion:* The authors suggest that the subdivision of stages using image information reflects the prognosis of Stage Ib cervical cancer.

Key words: Stage Ib; Cervical cancer; Stage subdivision; MRI.

Introduction

According to the International Federation of Gynecology and Obstetrics (FIGO) staging of cervical carcinoma, Stage Ib is a clinical lesion confined to the cervix or a preclinical lesion greater than Stage Ia, and all gross lesions, even those with superficial invasion, are Stage Ib cancers. In 1995, the FIGO staging system was revised and, using 40 mm as a cut-off value, the Stage Ib tumor was re-classified as Stage Ib1 (≤ 40 mm) and Ib2 (> 40 mm) [1]. Even if there is no stromal invasion, few reports have been published on radiation therapy of Stage Ib1 and Ib2 tumors in order to evaluate how the size influences prognosis.

Diagnostic work-up for carcinoma of the uterine cervix includes physical examination (including bimanual pelvic and rectal examinations), inspection, colposcopy, conization, dilatation and curettage, punch biopsies, cystoscopy, rectosigmoidoscopy, intravenous pyelography (last three examinations are not mandatory), chest and bone radiography. Computed tomography (CT) and magnetic resonance imaging (MRI) evaluations were increased from the revision of April 2012, when "the use of diagnostic imaging techniques to assess the size of the primary tumor is encouraged but is not mandatory". Conventionally, it was decided, "the test results by CT or MRI may be used for treatment plan decision, but staging must not be influenced by these results."

MRI is not officially incorporated in the FIGO staging system, but is already widely accepted as the most reliable imaging technique for the diagnosis, staging, treatment planning, and follow-up of both endometrial and cervical cancer. The role of MRI in gynecologic oncology has evolved during the past two decades. There is now a substantial body of evidence that MRI is useful in evaluating malignant conditions of the pelvis [2]. MRI has been shown to be superior to CT in staging of cervical carcinoma and has been shown to be superior to CT in measuring tumor volume. The advantages of MRI are (a) space resolving power is high in the MRI, (b) the correlation between the high signal region of the T2-weighted MRI image and the tumor diameter is high [3], (c) MRI can also be used in a treatment plan for the image-guided intra-cavitary irradiation. Wagenaar *et al.* [4] demonstrated that tumor volume measurement with MRI was correlated with clinical stage and that inter-observer consistency was high.

The authors analyzed treatment results for Stage Ib cervical cancer after using MRI information to subdivide it into Stages Ib1 and Ib2.

Materials and Methods

This investigation examined retrospectively MRI images that were obtained before treatment as of April 2012. The images were correlated with stages of the disease based on a new evaluation standard introduced from April 2012.

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Table 1. — *Patient and tumor characteristics.*

		No.	Median (range)
FIGO Stage	Ib1	29	
	Ib2	11	
Age Histopathology	SqCC	36	
	AC	4	
Tumor greatest dimension by MRI			25 mm (undetectable - 60 mm)
PLN swelling	With	1	
	Without	39	
Dose fraction of EBRT			1.8 Gy (1.8 - 2.0)
Total dose EBRT			50 Gy (45 - 50.4)
Dose without MB			20 Gy (0 - 37.8)
Dose fraction of ICRT			6 Gy (5 - 6)
Total dose of ICRT			24.5 Gy (24 - 35)
Combined chemotherapy	With	11	
	Without	29	

FIGO: the International Federation of Gynecology and Obstetrics;
SqCC: squamos cell carcinoma; AC: adenocarcinoma; PLN: pelvic lymph node;
EBRT: external beam radiation therapy; MB: midline block;
ICRT: intra-cavitary radiation therapy; MRI: magnetic resonance imaging.

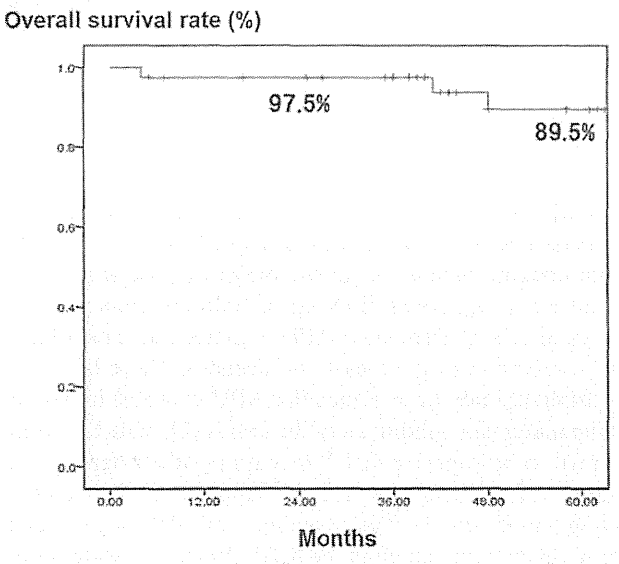

Figure 1. — Overall survival curve.

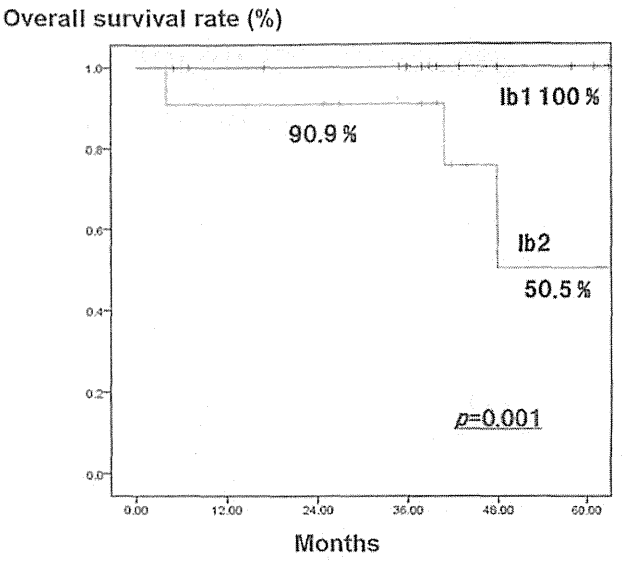

Figure 2. — Overall survival curves by stages subdivided with MRI.

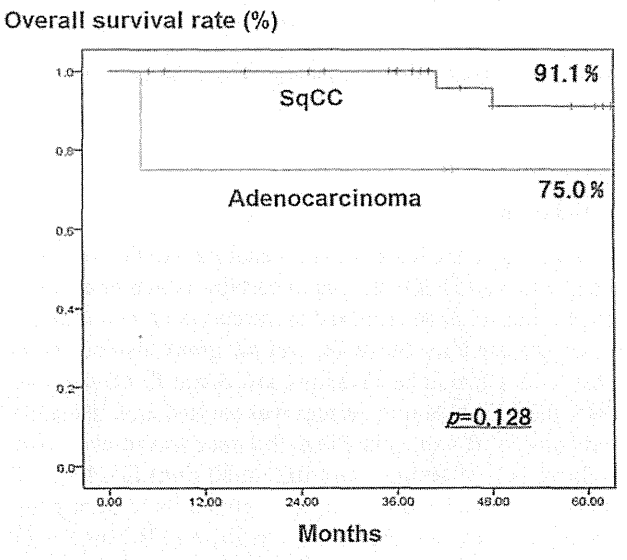

Figure 3. — Overall survival curves by histopathological types.

The study comprised 40 cases with Stage Ib cervical cancer treated by definitive radiotherapy in Kitasato University Hospital and Tokyo University Hospital from January 2000 to December 2008. The ages ranged from 28 to 85 years (median: 68) (Table 1). The maximal tumor diameter measured with MRI ranged from undetectable to 60 mm (median: 25 mm). The authors classified tumors with the greatest diameter dimension less than 40 mm as Stage Ib1 (29 cases) and those with the largest dimension more than 40 mm as Ib2 (11 cases) (Table 1). All cases were treated with a combination of external beam irradiation and high-dose-rate intra-cavitary brachytherapy. The chemotherapy was combined with radiotherapy in 11 cases (Table 1). Most of Stage Ib2 cases were combined with chemotherapy.

Results

Patients

The follow-up time ranged from four to 109 months (median: 53). At the time of last observation, 37 cases survived, local recurrence was seen in none, and distant metastasis was seen in two cases. The two-and five-year overall survival rates of all patients were 97.5% and 89.5%, respectively (Figure 1). When the authors subdivided the stage and examined the survival, the five-year overall survival rate of Stage Ib1 was 100% and that of Stage Ib2 was 50.5% (p = 0.001, Figure 2). The survival curves by histopathological

types showed that the five-year overall survival rate of squamous cell carcinoma (SqCC) was 91.1% and that of adenocarcinoma was 75.0% ($p = 0.13$, Figure 3). The prognosis of Stage Ib1 was significantly better than that of Stage Ib2.

The local control rate was 100%. Only two patients had disease recurrence outside the pelvis; one had lung metastases and the other pleural dissemination.

According to Common Terminology Criteria for Adverse Events (CTCAE) ver. 4.0, all non-blood toxicity was below Grade 3. Grade 3 hematological toxicity occurred in three cases, but none of them needed discontinuation of the treatment.

According to European Organization for Research and Treatment of Cancer (EORTC) / Radiation Therapy Oncology Group (RTOG) Late Radiation Morbidity Scoring Schema, late adverse events higher than Grade 3, small intestine toxicity (ileus) was recognized in only one case.

Discussion

In this study, the tumor diameter (Ib1 vs. Ib2) affected prognosis more significantly than previously thought. The relationship between tumor size and survival is not clear. Some studies have demonstrated a statistically significant relationship between tumor size and survival in univariate analysis [5-6], while others did not [7]. However, the most valuable analysis is a multivariate analysis, which determines independent prognostic factors. Some studies have demonstrated that tumor size is an independent prognostic factor [8], while others have not [9].

Rutledge *et al.* [5] (Ib1 vs. Ib2, median follow-up: 35 months) found that survival rate decreased from 92.5% in Stage Ib1 to 74.3% in Stage Ib2 ($p = 0.004$), but in a multivariate analysis, this decrease was not statistically significant. Horn *et al.* [8] (Stages IIa-IIb, median follow-up: 54 months) found that tumors larger than 40 mm affected the five-year survival rate significantly in an invariant analysis (49.5% vs. 67.4%, $p = 0.0015$), and tumor size was an independent prognostic factor. Kamelle *et al.* [6] (Stage Ib2, median follow-up: 25 months) found that two-year DFS was 86% when tumor size was 40-49 mm, while it was 72% when tumor size was 50 mm ($p = 0.29$). Lee *et al.* [7] (Stage Ib, median follow-up: 51 months), showed that 40 mm did not have a significant effect on disease-free survival and overall survival ($p = 0.06$, $p = 0.29$ respectively). The present study, which showed that the five-year overall survival rate of Stage Ib1 and Stage Ib2 were 100% and 50.5%, respectively, did not have inferiority to these previous reports.

In this study, Stage Ib2 comprised many adenocarcinoma groups. Adenocarcinoma has a very poor prognosis in cervical cancer. According to Niibe *et al.* [10], the five-year overall survival rate of Stage IIIb adenocarcinoma of the uterine cervix treated with high-dose-rate intracavitary brachytherapy combined with external beam radiation therapy in Japan was 20.2%, and adenocarcinoma has a generally poor prognosis.

In this study, all the recurrences were distant metastasis. The method of local radiation therapy will not have to be improved because there were few side effects. For Stage Ib2 cases, more intensive systemic therapy such as consolidation chemotherapy after concurrent chemoradiation may be necessary [11-12]. Abe *et al.* [11] concluded that patients with para-aortic lymphadenopathy who received concurrent chemoradiation therapy (CCRT) and adjuvant chemotherapy had a more favorable overall and disease-free survival than did those treated with CCRT alone. The same thing may be said of patients in Stage Ib2.

Conclusion

The authors suggest that the subdivision of stages using MRI information reflects prognosis of Stage Ib cervical cancer. It may be necessary to consider other regimens of chemoradiation for Stage Ib2.

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ORIGINAL ARTICLE

Outcomes of abdominal radical trachelectomy: results of a multicenter prospective cohort study in a Tohoku Gynecologic Cancer Unit

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Abstract
Background This study aimed to evaluate surgical, pregnancy, and prognostic outcomes of radical abdominal trachelectomy (RAT) for Japanese patients with early-stage cervical cancer.
Methods This was a multicenter prospective cohort study conducted in member facilities of Tohoku Gynecologic Cancer Unit. Patients with FIGO 1A–1B1 squamous cell carcinoma were included.
Results A total of 42 patients were registered in this study, and all patients underwent planned RAT. The median stromal invasion and median horizontal spread of resected specimens were 4.6 (range 1.0–10.0) and 12.4 mm (range 3.0–28.0), respectively. The median surgical time and median blood loss were 304 min (range 233–611) and 848 mL (range 250–3984), respectively. Five patients (11.9 %) received blood transfusion. Five of 18 (27.8 %) patients who attempted to conceive achieved pregnancy, and 3 patients had healthy babies. However, all pregnancies required assisted reproductive technology with in-vitro fertilization and embryo transfer. Four patients (9.5 %) received postoperative adjuvant therapy, and 3 patients (7.1 %) developed disease recurrence.
Conclusions RAT may be safely performed for Japanese patients with FIGO 1A–1B1 squamous cell carcinoma of the cervix, even in educational medical facilities. However, less-invasive surgery should be considered more often to improve pregnancy outcomes.

Keywords Abdominal trachelectomy · Fertility-sparing surgery · Cervical cancer · Prospective cohort study · Outcome

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Introduction

Since first reported as a fertility-sparing surgery in 1994 [1], radical trachelectomy has now become widely accepted as a standard treatment modality for patients with early-stage cervical cancer. The National Comprehensive Cancer Network Guidelines version 1.2014 [2] currently recommends performing radical trachelectomy with pelvic lymph node dissection +/− para-aortic lymph node sampling when patients wish to preserve fertility and have

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stage IA2–IB1 disease, tumor diameter ≤2 cm, and squamous histology. However, treatment outcomes of radical trachelectomy, including the laparoscopic approach, in Japanese patients with early stage cervical cancer have been reported only in retrospective studies at single institutions [3–5]. Therefore, to evaluate the surgical, prognostic, and pregnancy outcomes of radical abdominal trachelectomy (RAT), we conducted a prospective cohort study in Tohoku Gynecologic Cancer Unit.

Materials and methods

Study design

This prospective cohort study was conducted from August 2002 to May 2013 in the following member institutions of Tohoku Gynecology Cancer Unit: Tohoku University Graduate School of Medicine; Hirosaki University School of Medicine; Iwate Medical University; Akita University Graduate School of Medicine; Yamagata University Faculty of Medicine; and Fukushima Medical University. Patient eligibility criteria were as follows: (1) histologically confirmed cervical cancer; (2) FIGO stage IA2–IB1; (3) squamous cell carcinoma; (4) tumors less than 2 cm in diameter determined by preoperative magnetic resonance imaging; (5) patients desired fertility-sparing surgery; (6) and age ≤40 years. This study was conducted to evaluate the safety of surgery, pregnancy outcomes, and prognostic outcomes. All patients were registered pre-operatively, and we prospectively collected patient data, including surgical records, clinicopathological characteristics, pregnancy and fertility outcomes, and prognostic outcomes. This study was approved by the ethics committee at each participating facility.

Surgical procedures

All radical trachelectomies were performed via open laparotomy with a low abdominal incision, and all surgeons had received RAT education so that those performing the procedure in each facility were not fixed. The uterine cervix was resected by a nerve-sparing procedure with the bilateral cardinal ligament and vaginal wall ≥2 cm. In addition, pelvic lymphadenectomy was performed, including removal of the common iliac, external iliac, internal iliac, obturator, supra-inguinal, sacral, and cardinal lymph nodes.

Adjuvant therapy

If there was any risk factor, such as lymph vascular space involvement (LVSI) or pelvic lymph node metastasis (PLNM) was observed in resected specimens, postoperative adjuvant therapy was recommended. Radiotherapy or concurrent chemoradiotherapy (CCRT) were adopted when patients had intermediate risk (only LVSI or deep stromal invasion) or high risk (PLNM and/or cardinal ligament invasion) of recurrence. Moreover, combination chemotherapy of taxan and platinum was permitted as an optional therapy for patients who had a strong wish to preserve ovarian function.

Results

Clinicopathological characteristics

A total of 42 patients were registered for this study. The clinicopathological characteristics of patients are shown in Table 1. Thirty-seven (88.1 %) patients received diagnostic cervical conization before undergoing RAT. One patient who was diagnosed with stage 1A2 disease by colposcopy-directed biopsy was ultimately diagnosed with stage 1A1 disease based on the surgically resected specimens after RAT. The median stromal invasion and median horizontal spread of resected specimens were 4.6 (range 1.0–10.0) and 12.4 mm (range 3.0–28.0), respectively. Four patients received postoperative adjuvant therapy. Two patients with PLNM received CCRT with paclitaxel and carboplatin chemotherapy; 1 patient with vaginal invasion received whole pelvic radiation therapy; and 1 patient with LVSI received paclitaxel and cisplatin chemotherapy.

Treatment outcomes

All patients underwent the planned surgery. Surgical outcomes are shown in Table 2. The following surgical complications were observed: uretheral injury in 1 patient, postoperative ileus in 1 patient, and pelvic lymphocyst in

Table 1 Clinicopathologic characteristics of patients

Total number of patients	42
Median age, years (range)	32 (22–39)
FIGO (2008) stage (%)	
1A1	1 (2.3)
1A2	4 (9.6)
1B1	37 (88.1)
Histological subtype	Squamous cell carcinoma
Median follow-up period, months (range)	29.9 (1–122)
Median stromal invasion, mm (range)	4.6 (1.0–10.0)
Median horizontal spread, mm (range)	12.4 (3.0–28.0)
Lymph vascular space involvement (%)	5 (11.9)
Pelvic lymph-node metastasis (%)	2 (4.8)

FIGO International Federation of Gynecology and Obstetrics

Table 2 Surgical outcomes

Median surgical time, min (range)	304 (233–611)
Median blood loss, ml (range)	848 (250–3984)
Median number of PLNs resected (range)	35 (7–68)
Blood transfusion (%)	5 (11.9)
Surgical complications (%)	
Ureteral injury	1 (2.4)
Ileus	1 (2.4)
Pelvic lymph cyst	4 (9.5)

PLN pelvic lymph-node

Table 3 Pregnancy outcomes

Attempted to conceive (%)	18/42 (42.9)
Fertility treatment (%)	9/18 (50.0)
AIH	1/18 (5.6)
IVF-ET	8/18 (44.4)
Pregnancy (%)	5/18 (27.8)
Spontaneous abortion	1/5 (20.0)
Artificial abortion	1/5 (20.0)
Spontaneous delivery[a]	1/5 (20.0)
Cesarean section[b]	2/2 (40.0)

AIH artificial insemination by husband, *IVF-ET* in-vitro fertilization and embryo transfer

[a] Delivery at 38 weeks' pregnancy

[b] Delivery at 33 and 36 weeks' pregnancy

Table 4 Clinical outcomes

Number of recurrences (%)	3 (7.1)
Recurrence sites (%)	
Vaginal stump	1 (2.4)
Retroperitoneal cavity	2 (4.8)
Survival	
Disease-free alive	39 (92.8)
Alive with disease	1 (2.4)
Died of disease	2 (4.8)

4 patients. Pregnancy outcomes are shown in Table 3. A total of 18 patients attempted to conceive, resulting in 5 pregnancies and 3 healthy babies. However, all pregnancies were achieved by in-vitro fertilization and embryo transfer (IVF-ET), and none of the women who achieved pregnancy received adjuvant treatment after RAT. Although all patients met the eligibility criteria, 3 patients (7.1 %) developed disease recurrence, and 2 patients died because of disease progression. Recurrences were observed in the vaginal stump in 1 patient and in the retroperitoneal cavity in 2 patients (Table 4). Among 3 patients with recurrence, 1 patient had LSVI and refused to undergo postoperative adjuvant chemotherapy, but another 2 patients showed neither LSVI nor PLNM even after re-diagnosis of surgically resected specimens. However, considering the recurrence sites, the existence of an undetectable skip lesion in the preserved uterus cannot be ruled out.

Discussion

A systematic review of radical trachelectomy for cervical cancer [6] reported that among patients who were treated via a vaginal approach, 146 of 480 (30.4 %) patients achieved pregnancy, and 23 of 618 (3.7 %) patients developed disease recurrence. However, among those who underwent RAT, 30 of 147 (20.4 %) patients achieved pregnancy, and 7 (4.8 %) patients developed recurrence. Moreover, a more recent review of 485 RAT patients (stage 1A1 in 33, 1A2 in 90, 1B1 in 330, and ≥1B2 in 11) [7] reported that 47 (9.7 %) patients were converted to radical hysterectomy, 25 of 438 (5.7 %) patients received adjuvant treatment including hysterectomy, 75 of 413 (18.2 %) achieved pregnancy, and 16 of 438 (3.7 %) patients developed disease recurrence. With regard to surgical outcomes, operative times ranged from 110 to 586 min, and blood loss ranged from 50 to 5568 mL. The actual status of RAT in Japan was first revealed by a survey conducted between 2000 and 2008 [8]. This survey reported that among 26 institutions that responded to the survey, a total of 269 patients underwent radical trachelectomy, and 74.7 % of patients were treated via an abdominal approach. Moreover, although 46 % of institutions did not consider histological subtype, tumor size ≤2 cm and stage 1B1 disease were the indication criteria for RAT. Furthermore, 20 pregnancies and 13 deliveries were achieved, and, among the pregnant patients, 8 delivered later than the 29th gestational week. Compared with recent studies [4, 9–12] of RAT (Table 5), our present study showed lower median blood loss and a longer median operative time. However, because the median number of resected pelvic lymph nodes was greater in this study compared with previous studies, and because the present study was performed only in academic educational facilities, we believe RAT is a safe surgical procedure for fertility sparing in patients with early-stage cervical cancer. However, the 3 recurrent cases in the present study suggest that strict determination of surgical margin and LSVI by examination of a sufficient number of pathological specimens is necessary to prevent disease recurrence. Furthermore, our present study raised a problem regarding fertility treatment after RAT. Although 5 of 18 patients managed to achieve pregnancy, no spontaneous pregnancies were observed; all pregnancies were achieved by IVF-ET. In a study by Nishio et al. [4], although 31 pregnancies were achieved in 25 patients, natural conception was observed only in 9 (29.0 %) patients, and 20 (66.7 %) patients achieved pregnancy by IVF-ET. Although

Table 5 Results of recent studies

Factors	Reference					Present study
	[9]	[10]	[11]	[4]	[12]	
No. of patients	64	30	101	114	73	42
Age, years (range)	29.5[a] (11–41)	32.5[b] (23–41)	31[b] (19–43)	33[a] (25–40)	31[a] (22–39)	32[a] (22–39)
FIGO stage						
1A1	16	0	3	9	5	1
1A2	7	2	8	12	10	4
1B1	36	25	88	93	58	37
≤2 cm	22	20	–	–	29	34
>2 cm	14	5	–	–	24	3[c]
≥1B2	0	3	2	0	0	0
Histology						
SCC	50	15	40	99	64	42
Non-SCC	12	15	61	15	9	0
Median follow-up, months	22.8	24	–	33	20.6	29.9
Recurrences	0	2	0	–	0	3
Pregnancy	2	3	28	31	0	5
Median operation time, min	148	170	–	–	177[b]	304
Median blood loss, ml	–	813	–	–	322[b]	848
Blood transfusion, %	6.25	20	–	–	–	11.9
Median no. of PLN retrieved	25	24	24	–	26	35

FIGO International Federation of Gynecology and Obstetrics, *SCC* squamous cell carcinoma, *PLN* pelvic lymph node

[a] Median value

[b] Mean value

[c] Postoperative evaluation

cervical stenosis has been reported as a major complication of trachelectomy, the frequency of postoperative cervical problems has been reported to range from 7.8 [9] to 21 % [13]. Moreover, recent results of total laparoscopic radical trachelectomy [14] reported that 52 % of patients also needed assisted reproductive technology, though intra-pelvic inflammation was correlated with fertility problems. Although the detailed mechanisms are still unknown, less-invasive surgical procedures should be performed to prevent surgical complications, including fertility problems. A more recent review reported that less-radical surgical options, such as simple trachelectomy and cervical conization with or without sentinel lymph node biopsy and pelvic lymph node dissection, may be considered for cases of low-risk early-stage cervical cancer, including squamous carcinoma, adenocarcinoma, or adenosquamous carcinoma; tumor size <2 cm; stromal invasion <10 mm [15]. Previously, we have reported the usefulness of sentinel lymph node detection in early-stage cervical cancer [16–18]. As the results of robot-assisted laparoscopic radical trachelectomy have been recently reported [19], radical trachelectomy using a robot-assisted laproscopic approach with sentinel lymph node sampling may be implemented in the near future.

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Conflict of interest The authors declare that they have no conflict of interest.

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