antibody-independent protective immunity against *Salmonella enterica* serovar Enteritidis. Vaccine 2007;25(18):3599–605.
[27] Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res 1996;56(1):21–6.
[28] Sakaue G, Hiroi T, Nakagawa Y, Someya K, Iwatani K, Sawa Y, et al. HIV mucosal vaccine: nasal immunization with gp160-Encapsulated hemagglutinating virus of Japan-liposome induces antigen-specific CTLs and neutralizing antibody Responses1. J Immunol 2003;170(1):495–502.
[29] Ishikawa H, Li Y, Abeliovich A, Yamamoto S, Kaufmann SH, Tonegawa S. Cytotoxic and interferon g-producing activities of gdT cells in the mouse intestinal epithelium are strain dependent. Proc Natl Acad Sci USA 1993;90(17):8204–8.
[30] Dupuy C, Buzoni-Gatel D, Touzé A, Bout D, Coursaget P. Nasal immunization of mice with human papillomavirus type 16 (HPV-16) virus-like particles or with the HPV-16 L1 gene elicits specific cytotoxic T lymphocytes in vaginal draining lymph nodes. J Virol 1999;73(11):9063–71.
[31] Klavinskis LS, Barnfield C, Gao L, Parker S. Intranasal immunization with plasmid DNA–lipid complexes elicits mucosal immunity in the female genital and rectal tracts. J Immunol 1999;162(1):254–62.
[32] Stagg AJ, Kamm MA, Knight SC. Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. Eur J Immunol 2002;32(5):1445–54.
[33] Rank RG, Bowlin AK, Kelly KA. Characterization of lymphocyte response in the female genital tract during ascending Chlamydial genital infection in the guinea pig model. Infect Immun 2000;68(9):5293–8.
[34] Cohen MS, Anderson DJ. Genitourinary mucosal defenses. In: Holmes KK, Mardh PA, Sparling PF, Wiesner PJ, Cates Jr W, Lemon SM, Stamm WE, editors. Sexually transmitted diseases. 3rd ed. New York: McGraw-Hill; 1999. p. 173–90.
[35] Lund JM, Linehan MM, Iijima N, Iwasaki A. Plasmacytoid dendritic cells provide innate immune protection against mucosal viral infection. In Situ J Immunol 2006;177(11):7510–4.
[36] Soderberg KA, Linehan MM, Ruddle NH, Iwasaki A. MAdCAM-1 expressing sacral lymph node in the lymphotoxin beta-deficient mouse provides a site for immune generation following vaginal herpes simplex virus-2 infection. J Immunol 2004;173(3):1908–13.
[37] Stoddard E, Cannon G, Ni H, Karikó K, Capodici J, Malamud D, et al. Vaginal submucosal dendritic cells, but not langerhans cells, induce protective Th1 responses to herpes simplex virus-2. J Immunol 2007;179(5):3126–32.
[38] Kraus I, Molden T, Ernø LE, Skomedal H, Karlsen F, Hagmar B. Human papillomavirus oncogenic expression in the dysplastic portio: an investigation of biopsies from 190 cervical cones. Br J Cancer 2004;90(7):1407–13.
[39] Levine MM, Dougan G. Optimism over vaccines administered via mucosal surfaces. Lancet 1998;351(9113):1375–6.
[40] Mohamadzadeh M, Olson S, Kalina WV, Ruthel G, Demmin GL, Warfield KL, et al. Lactobacilli activate human dendritic cells that skew T cells toward T helper 1 polarization. Proc Natl Acad Sci USA 2005;102(8):2880–5.
[41] Koizumi S, Wakita D, Sato T, Mitamura R, Izumo T, Shibata H, et al. Essential role of Toll-like receptors for dendritic cell and NK1.1(+) cell-dependent activation of type 1 immunity by *Lactobacillus pentosus* strain S-PT84. Immunol Lett 2008;120(1–2):14–9.
[42] Liu B, Ye D, Song X, Zhao X, Yi L, Song J, et al. A novel therapeutic fusion protein vaccine by two different families of heat shock proteins linked with HPV16 E7 generates potent antitumor immunity and antiangiogenesis. Vaccine 2008;26(10):1387–96.
[43] Bian T, Wang Y, Lu Z, Ye Z, Zhao L, Ren J, et al. Human papillomavirus type 16 L1E7 chimeric capsomeres have prophylactic and therapeutic efficacy against papillomavirus in mice. Mol Cancer Ther 2008;7(5):1329–35.
[44] Boursnell ME, Rutherford E, Hickling JK, Rollinson EA, Munro AJ, Rolley N, et al. Construction and characterisation of a recombinant vaccinia virus expressing human papillomavirus proteins for immunotherapy of cervical cancer. Vaccine 1996;14(16):1485–94.

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ORIGINAL ARTICLE

Comparison Between Conventional Surgery Plus Postoperative Adjuvant Radiotherapy and Concurrent Chemoradiation for FIGO Stage IIB Cervical Carcinoma

A Retrospective Study

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Objective: To compare treatment outcome of conventional surgery followed by adjuvant postoperative radiotherapy (PORT) versus concurrent chemoradiation therapy (cCRT) for stage IIB cervical carcinoma.

Methods: A retrospective analysis was conducted of 59 patients with stage IIB uterine cervical cancer treated with radical surgery plus PORT (N = 34) or cCRT-alone (N = 25) from April 1996 to June 2008. The median follow-up time was 27 months (range, 3–150 months) in the cCRT group and 44 months (range, 4–134 months) in the PORT group. The median age was 59 years (range, 37–85 years) in the cCRT group and 49 years (range, 32–74 years) in the PORT group. All 34 patients in the surgery group underwent hysterectomy with pelvic lymph node dissection and received PORT. Twenty-five patients (42%) were assigned to the cCRT group.

Results: The 3-year overall survival rates for surgery plus PORT and cCRT-alone were 80.0% and 75.1%, respectively. The difference between these 2 treatments was not statistically significant (log-rank $P = 0.5871$). The late complication rate of grade 3–4 was 12% in the cCRT group and 16% in the surgery group.

Conclusion: This retrospective study suggests that survival results with cCRT and with conventional surgery plus PORT for patients with stage IIB cervical carcinoma are comparable.

Key Words: cervical carcinoma, surgery, chemoradiotherapy, high-dose-rate brachytherapy, stage IIB

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Cervical cancer is the most common gynecologic malignancy in Japan, with an estimated 5 new cases per 100,000 females every year. High dose-rate intracavitary brachytherapy (HDR-ICBT) in combination with external beam irradiation (EBRT) has become an acceptable treatment for carcinoma of the cervix.[1] HDR-ICBT has been widely used in treatment of uterine cervical cancer in Asia and Europe. Although some controversy exists in the United States over the use of HDR-ICBT,[2] an increasing frequency of its adoption has been noted.[3,4]

Recently published randomized clinical trials demonstrated a significant improvement in pelvic disease control and survival when concurrent chemotherapy consisting of cisplatin-containing regimens was added to radiotherapy (RT) in patients with locally advanced cervical cancer.[5–7] These results led to significant changes in the standard treatment of cervical cancer.

Radiotherapy has long been recognized as a successful treatment modality for all stages of carcinoma of the uterine cervix. In Japan, however, because patients present first at gynecologic clinics, gynecologists usually determine the treatment modality without additional inputs from radiotherapists. In general, Japanese gynecologists consider surgical treatment to be superior to RT, and, as a result, the majority of patients with stage IIB are subjected to radical hysterectomy plus pelvic, and with or without para-aortic lymphadenectomy followed by preventive postoperative RT (PORT). Consequently, other than this preventive postoperative RT, radiation oncologists in Japan have treated only stage IIB patients who refused surgery or who were not indicated for surgery because of other coexisting disease.

Although RT has been widely used in Western countries, there are only a few reports on definitive RT for early stages (stages I–II) of cervical carcinoma. Some studies indicated that RT for early stage patients was a feasible definitive treatment.[8–11] In Japan, no prior report has compared surgery and RT. We now report the results of a retrospective study in which the survival outcomes of surgery and RT were compared for stage IIB cervical cancer. The hypothesis was to be certified that definitive cCRT is not inferior to survival and less frequency about severe complications than radical hysterectomy plus PORT for stage IIB cervical cancer in this single institution.

PATIENTS AND METHODS

Patients

Between April 1996 and June 2008, a total of 59 consecutive patients were treated for FIGO (International Federation of Gynaecology and Obstetrics classification) stage IIB carcinoma of the cervix with conventional surgery plus adjuvant PORT or concurrent CRT at our institution. All patients with stage IIB treated during the 13-year period (1996–2008) were included in the study. Patients included were those previously untreated and who had a histologic diagnosis of squamous cell carcinoma, or adenocarcinoma in FIGO stage IIB. Patients with adenocarcinoma (n = 13) were also included in this study. Median age was 53 years (range, 32–85 years). Table 1 shows the patients' characteristics. Surgically treated patients comprised 58% (34/59) and cCRT-alone patients 42% (25/59) (Table 1). The patients submitting to definitive CRT were those with comorbidities or who refused surgery in our institution. T1

Patients were evaluated with a physical and pelvic examination without anesthesia, routine blood counts, blood chemistry profile, chest radiograph, intravenous urogram, and barium enema. Computed tomography (CT) scan and magnetic resonance imaging (MRI) were used only for detecting lymphadenopathy. Pelvic and para-aortic lymph nodes greater than 10 mm in minimum diameter

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TABLE 1. Comparison of Patients' Characteristics

	Surgery Plus PORT	cCRT	*P*
Total no. patients	34	25	
Age			
Median (range) (yr)	49 (32–74)	59 (37–85)	0.0001
Histopathology			
Squamous cell carcinoma	24 (71%)	22 (88%)	0.2026
Adenocarcinoma	10 (29%)	3 (12%)	
Pelvic nodal status			
Positive	17 (50%)	5 (20%)	0.0185
Negative	17 (50%)	20 (80%)	
Paraaortic nodal status			
Positive	3 (8%)	2 (8%)	0.9106
Negative	31 (92%)	23 (92%)	
Maximum tumor diameter (mm)			
>40	19 (56%)	14 (56%)	0.9928
≦40	15 (44%)	11 (44%)	
Median (range)	49.5 (18–100)	45.5 (30–80)	0.6153

detected by CT and MRI were considered to be positive for metastases. Neither lymphangiography nor surgical evaluation of lymph nodes was performed.

Concurrent CRT

EBRT

All patients received EBRT using a linear accelerator with a photon-beam-energy of 10 MV to the whole pelvis with the 4-field box technique for a total dose of 30.6 Gy in 17 fractions (3.4 weeks, 1.8 Gy fractions from Monday to Friday). The irradiated volume was to include the whole uterus, the paracervical, parametrium and uterosacral regions, as well as the external iliac, hypogastric and obturator lymph node. Minimum margins were the upper margin of L-5 (superiorly), the lower margin of the obturator foramen or the lowest extension of the disease (inferiorly), and 2.0 cm beyond the lateral margins of the bony pelvis and its widest plane (laterally). For the lateral fields, the anterior margin was the anterior edge of the symphysis or 3 cm in front of the sacral promontory. The posterior margin was the S2–S3 interspace or the posterior border of the uterine cervix assessed by CT for treatment planning plus a 2 cm margin. After that, a midline block, 4 cm in width at midplane, was inserted with the anteroposterior parallel 2-field technique for a dose of 19.8 Gy in the last 11 fractions (ie, parametrial boost). This block extended to the top of the uterine.

Two patients who had para-aortal lymph node involvement received whole pelvic irradiation plus para-aortal irradiation using the 4-field box or conformal technique. Total dose to para-aortal lymph nodes was 50.4 Gy in 28 fractions.

ICBT

In our department, EBRT preceded ICBT. A midline block was inserted at the same time as the first application of ICBT. Four intracavitary iridium-192 (^{192}Ir) insertions were performed weekly, starting 3.4 weeks after starting EBRT. High-dose-rate intracavitary therapy was used. Brachytherapy was delivered using after-loading applicators placed in the uterine cavity and vagina. A Manchester system applicator (Nucleotron microSelectron HDR source) was used. The dose distribution was calculated for each individual patient and placement. Patients were treated in the dorsal lithotomy position. Point A was defined on radiographs as being 2 cm superior (along the tandem) to the flange abutting the external cervical os and 2 cm lateral from the axis of the tandem. Source loading corresponded to the Manchester System for uterine cervical cancer.[12] HDR-ICBT was performed once a week with a daily dose of 6 Gy at point A. The details of the method for ICBT were shown in our previous report.[13,14]

Total dose to the central area was 54.6 Gy and to the parametrium area was 50.4 Gy. When using biologically effective dose with α/β = 10 Gy, total dose to the central area was 74.5 Gy and to the parametrium was 59.5 Gy.

Chemotherapy

Concurrent CDDP-based chemotherapy combined with RT for stage IIB has been routinely performed in our department. All patients received platinum series-based chemotherapy combined with RT. All patients received CDDP (75 mg/m^2 in a bolus infusion on days 1, 22, and 43).

Surgery Plus Adjuvant RT

Radical hysterectomy with pelvic lymphadenectomy was performed on 34 patients. Radical hysterectomy at our institution includes resection of the uterus along with its attached parametrial soft tissue and a margin of the upper vagina, as in the world standard. Even when positive nodes were found, radical hysterectomy was continued without stopping. No radical hysterectomy was aborted. Para-aortic lymphadenectomy up to the level of the inferior mesenteric artery was performed for patients with adenocarcinoma or enlarged pelvic lymph nodes assessed by preoperative CT or MRI (N = 13). Because it is well-known that cases with positive pelvic lymph nodes are indicative of a very poor prognosis, lymphadenectomy up to the level of the inferior mesenteric artery was added for these high-risk cases in our institution. The median operation time was 390 minutes (range, 150–550 minutes) and the median quantity of operative blood loss was 1450 mL (range, 400–5600 mL).

All patients in the surgery group received postoperative adjuvant RT because of invasion to the parametrium. Adjuvant RT consisted of external pelvic irradiation (10 MV x-rays) with the 4-portal technique, one fraction of 1.8 Gy daily, with a total dose of 50.4 Gy over 5.6 weeks. The para-aortic region was irradiated with a dose of 50.4 Gy over 5.6 weeks with the conformal technique when metastases were detected in the surgical specimens of para-aortic nodes.

Follow-Up

Both radiation and gynecologic oncologists were involved in the follow-up the treated patients. The patients were seen every month for the first year, every 2 to 3 months for the next 2 years, and at least every 6 months thereafter. No patients were lost to follow-up. Follow-up procedures included pelvic examination, palpation of supraclavicular nodes, cervical Papanicolaou smear, and review of serum squamous cell carcinoma related antigen and cytokeratin 19 fragment antigen values. When central and/or parametrial recurrence was suspected by pelvic examination and/or Papanicolaou smear, a biopsy was taken for confirmation. Intravenously enhanced chest, abdominal, and pelvic CTs were performed annually. Other imaging studies, such as MRI, ultrasound and bone scintigraphy, were not routinely performed. Both acute and late complications were graded in accordance with the National Cancer Institute Common Toxicity Criteria Version 2.0.

Statistical Analysis

Statistical analyses were performed using StatView Dataset File version 5.0 J for Windows computers (Cary, NC). OS, progression-free survival (PFS), and local (ie, within pelvic) recurrence-free survival (LRFS) were calculated from the first date of curative treatment. Survival time was plotted using the Kaplan–Meier

method. Differences in patients' characteristics were analyzed by the χ^2 test or Fisher exact test for 2 × 2 columns and unpaired t test for a succession of numbers. Differences in survival by treatment were evaluated using the log-rank test.

RESULTS

Patients and Tumor

The age and pelvic nodal status distributions were significantly different between the 2 groups of patients (Student t test or χ^2 test). The median age was 59 years (range, 37–85 years) in the cCRT group and 49 years (range, 32–74 years) in the PORT group (Table 1). The patient FIGO stage, follow-up time, para-aortic nodal status, and histopathology distributions showed no significant differences (Table 1). The Karnofsky Performance Status for all patients was more than 80%. The proportion of patients with tumors less than 4 cm in diameter was 41% (14/34) for the surgery group and 44% (11/25) for the CRT. The median size was 50 mm (range, 18–100 mm) for the surgery group and 45.5 mm (range, 30–80 mm) for the cCRT group. The positive rate of surgical margins for the surgery group was 21% (7 cases). In the surgery group, bilateral parametrial involvement was seen in only one patient, although it was unilateral in the other 33 patients. Of those with unilateral involvement, only one patient had more than half extension of parametrial involvement and the other 32 patients had less than half.

In the surgery group, the positive rate of pelvic nodal metastasis was 32% (11 cases) assessed by clinical method and 50% (17 cases) by histopathological method. The pelvic nodal status showed no significant difference between surgery and definitive CRT groups if assessed by same method (clinically) (χ^2 test, $P = 0.2916$).

Median follow-up time was 27 months (range, 3–150 months) in the cCRT group and 44 months (range, 4–134 months) in the PORT group. The proportion of the surviving patients was 74% (25/34) for the surgery and 76% (19/25) for the cCRT groups.

The first site of progression was local-alone (ie, within pelvis) in 8% (2 cases) for the definitive CRT group and in 12% (4 cases) for the surgery group. Additionally, it was distant-alone progression in 4% (1 case) for CRT group and in 18% (6 cases) for surgery group, and both local and distant in 8% (2 cases) for CRT group and 0% for the surgery group. In other words, the recurrent rate within the pelvis was 14% (8/59 cases).

Survival

When this analysis was closed, 44 of 59 patients were alive. Deaths resulted in all 15 patients with cervical cancer (6 patients for definitive CRT group and 9 patients for surgery group). No treatment-related deaths were encountered. Figure 1 shows a comparison of the OS curves for the definitive CRT and surgery groups. The 3-year OS rates were 75% for the cCRT group and 80% for the surgery, respectively; the difference between these 2 rates was not significant (log-rank $P = 0.5871$). The 3-year PFS rates were 79% for the cCRT group and 70% for the surgery, respectively ($P = 0.7247$, HR = 0.825, 95% CI = 0.281–2.422). The 3-year LRFS rate was 83% for the cCRT group and 86% for the surgery, respectively ($P = 0.4908$, HR = 1.622, 95% CI = 0.404–6.513). Clearly, none of these analyses was of any significance.

Complications

In the cCRT group, nonhematological acute toxicity and all late toxicity (complications that persisted or occurred for more than 60 days after treatment) of grade 3+ were noted in 3 patients (12%). Small bowel perforation without tumor recurrence (grade 4) and melena from radiation proctitis in 2 cases (both grade 3) were seen at 5, 8, and 11 months after the completion of CRT. Moreover,

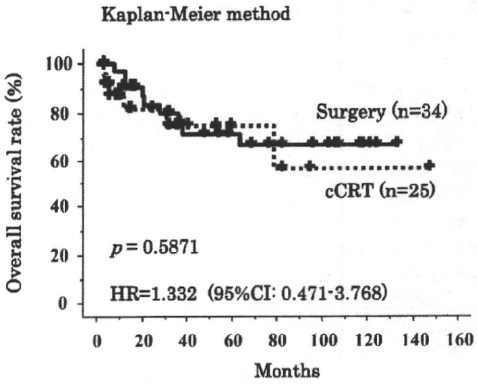

FIGURE 1. Overall survival curves in stage IIB patients comparing surgery plus postoperative radiotherapy (surgery) and concurrent chemoradiation (cCRT).

grades 1 or 2 melena and lymphoedema of the lower limbs were seen in each of 4 patients (16%).

In the surgery group, the bladder damage was seen in one example for a complication during operation. Nonhematological acute toxicity of grade 3+ was seen in 9 patients (16%). Postoperative ileus (grade 4 in 1 patient and grade 3 in 3 patients), grade 3 pelvic lymphocyst in one patient, grade 3 pulmonary infarction in 2 patients, grade 3 bilateral hydronephrosis in one patient, and lymphangitis of lower extremities (grade 3) in one patient were seen. Moreover, grade 2 hypertension, urinary tract infection, unilateral functionless kidney, and pelvic lymphocyst were seen in each of a single patient (total was 16%). No ureteral stricture was seen in the neither PORT or surgery groups.

DISCUSSION

This is a retrospective analysis of 59 patients with FIGO stage IIB cervical cancer treated with surgery plus PORT (n = 34) or concomitant CRT-alone (n = 25). Some centers especially in Japan still consider both treatment modalities as a standard for FIGO stage IIB cervical cancer. In this study, the 3-year OS, PFS, and LRFS were the same for both groups, although the surgery cases had at least a debulking of lymph nodes when positive as compared with the definitive CRT group. Also, CRT patients received a slight low dose for tumor control: only 50.4 Gy with EBRT plus 4 × 6 Gy with HDR-ICRT. These techniques are quite different from those used to treat the same category of patients in the United States and Europe. Additionally, surgery plus PORT presented 16% of grade 3+ complications. Patients who received definitive CRT were at higher risk, when age and probable comorbidities were considered. This suggests that there is no treatment of choice with respect to local control of disease. Conceivably, the patient population might be too small to draw any conclusions about the superiority of either one of the 2 treatment modalities.

The type of radical hysterectomy performed was III and ureteral resection was performed, although we had 21% of positive margins. The median and mean number of excised nodes was 61 and 63.4 (range, 25–133).

Many previously published results[8–11,15,16] suggest that radical hysterectomy or definitive RT is standard treatments for IB–IIA. In the United States and Europe, definitive RT has been selected in many cases. Radical hysterectomy is not a world standard for stage IIB patients. In contrast, surgery is preferentially used over RT in Japan even for stage IIB cervical cancer, although those patients with stage IIB ideally should have been treated with concomitant

CRT. RT is usually selected only for the elderly or inoperable cases because of coexisting disease in Japan.

The age of the patients and pelvic nodal status were significantly different between those patients going to surgery and cCRT patients (Table 1). The mean age of the cCRT patients was significantly greater than that of patients in the surgery group (P = 0.0001). There was a tendency for cCRT to be performed for elderly patients who were fearful of surgery or general anesthesia. Regardless of these fears, the cCRT patients had survival rates comparable with the surgery patients.

For the surgery group, pelvic nodal status was pathologically assessed in the surgical specimen, whereas clinically assessed by CT or MRI in the cCRT group. The positive rate of pelvic nodal status for the surgery group was significantly higher than in the cCRT group (50% vs. 20%, P = 0.0185). In the surgery group, the positive rate of pelvic nodal metastasis was 32% (11 cases) assessed clinically. The pelvic nodal status showed no significant difference between surgery and definitive CRT groups if assessed by the same method (clinically) (χ^2 test, P = 0.2916).

Horn et al[17] concluded that tumor size, when bulky disease was defined as tumors larger than 4 cm, was also of prognostic importance in FIGO stage II cervical carcinomas. In this study, there was no significant difference in the maximum tumor diameter between the 2 groups (P = 0.6153), although stage IIB varied from minimal to medial and even to lateral parametrial invasions, just short of pelvic wall fixation. To determine the size of the tumor, pathologic evaluation was used in the PORT group and pretreatment MRI in the CRT group.

Rotman et al[18] concluded that pelvic RT after radical surgery significantly reduced the risk of recurrence and prolonged PFS in women with stage IB cervical cancer whereas PORT appeared to be particularly beneficial for patients with tumors comprised of adenocarcinoma or adenosquamous histologies. In this study, there was no significant difference in the number of adenocarcinomas between the 2 groups (P = 0.2026).

It was shown in a previous publication[19] that for the stage IIB patients with lateral parametrial involvement had significantly higher rates of pelvic failure and of survival in comparison with those patients with medial parametrial involvement.

In our experience, the low rate of major complications after cCRT suggests that this approach is well tolerated in most patients. Treatment-related toxicity of grade 3+ developed in 16% of the surgery patients and in 12% of the definitive CRT group. This difference was not significant, probably because of the small numbers of patients in both groups.

Our results confirm earlier findings that have suggested that cCRT is not inferior to surgery plus PORT for FIGO stage IIB cervical carcinoma regardless of age bias, although the 2 treatment groups were not similar (ie, the cCRT group was older and had more comorbidities) and the pelvic node status was different as well. The 3-year outcomes in both groups were also been shown to be compatible with previous reports.[2–8] Because the t test based on a sample of 59 is underpowered, clinical trails with more patients should be needed to further confirm the efficacy of cCRT or surgery plus PORT on FIGO stage IIB cervical carcinoma. By the power analysis, to detect the difference between 2 independent groups, when the input is assumed that tail = one, effect size r = 0.5 (large), 0.3 (medium), or 0.1 (small), α err probability = 0.05, power (1-β err probability) = 0.8, and allocation ratio N2/N1 = 1, total sample size is calculated as 102, 278, or 2476, respectively. This study is too underpowered to conclude whether such both techniques as radical hysterectomy plus PORT and definitive CRT are feasible.

In this study, it was to be certified that definitive cCRT is not inferior with regards to survival and less frequency about severe complications than radical hysterectomy plus PORT for stage IIB cervical cancer in this single institution. The limitations of our study included the retrospective nature of the study, heterogeneity of the patient population in the 2 treatment arms, shorter follow-up and physician's bias in the selection of the patients. A matched-pair analysis would be better to compare the 2 groups but could not be done in our study because of the small number of patients. Nevertheless, we hope that our experience will initiate further prospective studies especially in Japan.

REFERENCES

1. Morita K. Cancer of the cervix. In: Vahrson HW, ed. *Radiation Oncology of Gynecological Cancers*. Berlin, Germany: Springer; 1997:143–239.
2. Eifel PJ. High-dose-rate brachytherapy for carcinoma of the cervix: high tech or high risk? *Int J Radiat Oncol Biol Phys*. 1992;24:383–386.
3. Eifel PJ, Moughan J, Owen J, et al. Patterns of radiotherapy practice for patients with squamous carcinoma of the uterine cervix: patterns of care study. *Int J Radiat Oncol Biol Phys*. 1999;43:351–348.
4. Nag S, Orton C, Young D, et al. The American brachytherapy society survey of brachytherapy practice for carcinoma of the cervix in the United States. *Gynecol Oncol*. 1999;73:111–118.
5. Rose PG, Bundy BN, Watkins EB, et al. Concurrent cisplatin-based chemotherapy and radiotherapy for locally advanced cervical cancer. *N Engl J Med*. 1999;340:1144–1153.
6. Whitney CW, Sause W, Bundy BN, et al. A randomized comparison of fluorouracil plus cisplatin versus hydroxyurea as an adjunct to radiation therapy in stages IIB–IVA carcinoma of the cervix with negative para-aortic lymph nodes: a Gynecologic Oncology Group and Southwest Oncology Group study. *J Clin Oncol*. 1999;17:1339–1348.
7. Morris M, Eifel PJ, Lu J, et al. Pelvic radiation with concurrent chemotherapy compared with pelvic and paraaortic radiation for high-risk cervical cancer. *N Engl J Med*. 1999;340:1137–1143.
8. Eifel PJ, Morris M, Wharton JT, et al. The influence of tumor size and morphology on the outcome of patients with FIGO stage IB squamous cell carcinoma of the uterine cervix. *Int J Radiat Oncol Biol Phys*. 1994;29:9–16.
9. Horiot JC, Pigneux J, Pourquier H, et al. Radiotherapy alone in carcinoma of the intact uterine cervix according to G. H. Fletcher guidelines: a French cooperative study of 1383 cases. *Int J Radiat Oncol Biol Phys*. 1988;14:605–611.
10. Liu W, Meigs JV. Radical hysterectomy and pelvic lymphadenectomy. A review of 473 cases including 244 for primary invasive carcinoma of the cervix. *Am J Obstet Gynecol*. 1955;69:1–32.
11. Newton M. Radical hysterectomy or radiotherapy for stage I cervical cancer. *Am J Obstet Gynecol*. 1975;123:535–542.
12. Tod MC, Meredith WJ. A dosage system for use in the treatment of cancer of the uterine cervix. *Br J Radiol*. 1938;11:809–824.
13. Yamashita H, Nakagawa K, Tago M, et al. Treatment results and prognostic analysis of radical radiotherapy for locally advanced cancer of the uterine cervix. *Br J Radiol*. 2005;78:821–826.
14. Yamashita H, Nakagawa K, Tago M, et al. Comparison between conventional surgery and radiotherapy for FIGO stage I–II cervical carcinoma: a retrospective Japanese study. *Gynecol Oncol*. 2005;97:834–839.
15. Morley GW, Seski JC. Radical pelvic surgery versus radiation therapy for stage I carcinoma of the cervix (exclusive of microinvasion). *Am J Obstet Gynecol*. 1976;126:785–798.
16. Landoni F, Maneo A, Colombo A, et al. Randomized study of radical surgery versus radiotherapy for stage Ib–IIa cervical cancer. *Lancet*. 1997;350:535–540.
17. Horn LC, Fischer U, Raptis G, et al. Tumor size is of prognostic value in surgically treated FIGO stage II cervical cancer. *Gynecol Oncol*. 2007;107:310–315.
18. Rotman M, Sedlis A, Piedmonte MR, et al. A phase III randomized trial of postoperative pelvic irradiation in Stage IB cervical carcinoma with poor prognostic features: follow-up of a gynecologic oncology group study. *Int J Radiat Oncol Biol Phys*. 2006;65:169–176.
19. Perez CA, Grigsby PW, Chao KS, et al. Tumor size, irradiation dose, and long-term outcome of carcinoma of uterine cervix. *Int J Radiat Oncol Biol Phys*. 1998;41:307–317.

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Advanced age is a significant determinant of poor prognosis in patients treated with surgery plus postoperative radiotherapy for endometrial cancer

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Abstract

Aim: A review was conducted in which the effect of age on survival was assessed in a population of endometrial cancer patients treated with surgery and adjuvant radiation therapy in our institution.

Methods: From 1988 to 2008, 111 endometrial cancer patients underwent total abdominal hysterectomy and adjuvant whole pelvic radiation therapy (RT). After surgery, for patients with low or intermediate risk without lymph node metastasis, no postoperative adjuvant therapy was performed. For patients with high risk or positive cytology from the abdominal cavity, postoperative radiation therapy was performed. A total dose of 50–50.4 Gy of RT was delivered sequentially. Forty-four patients (44%) were given chemotherapy consisting of epirubicin/cisplatin/carboplatin or paclitaxel/carboplatin. Univariate and multivariate analyses were performed to identify significant prognostic clinicopathological factors.

Results: With a median follow-up time of 59.2 months, the 5-year overall survival was 74% for those 60 years or older versus 90% for those younger than 60 years ($P = 0.044$). For disease-free survival, it was 65% for those 60 years or older, versus 85% for those younger than 60 years ($P = 0.013$). On multivariate analysis, poor disease-free survival was associated with age $\geq$ 60 years ($P = 0.035$).

Conclusions: Older patients (age $\geq$ 60 years) with endometrial cancer had significantly lower overall survival and disease-free survival following postoperative RT independent of other prognostic factors and/or treatment technique.

Key words: age, endometrial carcinoma, prognostic factors, radiation therapy, treatment.

Introduction

The preponderance of data in the literature indicates that advanced age is a predictor of poor outcome in patients with endometrial carcinoma.[1–4] Whether the poor outcome among elderly patients can be accounted for entirely by a more advanced stage at the time of diagnosis, staging, treatment or that endometrial carcinoma among the elderly is intrinsically more aggressive than in younger patients remains to be determined.[5,6] In general, older patients with endometrial carcinoma tend to have deep myometrial invasion, poorly differentiated histology, or extra-uterine spread.[7,8] Consequently, the perception of a negative influence of advanced age on outcome was prevalent even in patients who underwent full surgical staging or those with well- to moderately differentiated tumors.[2,9]

Poor outcome in some of the published reports may be attributed to the less aggressive adjuvant therapy (i.e. radiation therapy), offered to elderly patients.[6] This

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is conceivably a valid argument, especially when dealing with elderly patients who are less likely than younger patients to consent to and tolerate recommended adjuvant therapy in general.[10,11] According to Alekitar *et al.*,[12] even when treated in a similar fashion, endometrial carcinoma patients aged ≥ 70 years appeared to fare worse than younger patients independent of other prognostic factors, thus mandating further improvement in their treatment strategies. Therefore, to determine whether advanced age is an intrinsically poor prognostic factor or whether it is due to less aggressive adjuvant therapy, a comparison was made of the outcomes according to age in a group of patients who all received adjuvant radiation therapy.

Methods

Patients

A total of 111 consecutive endometrial cancer patients were treated with postoperative radiation therapy in our institution between October 1988 and January 2008. All patients were followed in detail and evaluated. This was a retrospective study in a single institution.

In this study, several categories of risk were defined as follows:

1 Intermediate–low risk ($n = 2$, 2%): Stage IA + histological International Federation of Gynecology and Obstetrics (FIGO) grade 3, Stage IB + grade 2, and Stage IIA + grade 1–2 + <50% myometrial invasion (MI)
2 Intermediate–high risk ($n = 30$, 28%): Stage IB + grade 3, Stage IIA + grade 3 + <50% MI, Stage IC + grade 1–2, Stage IIA + grade 1–2 + ≥50% MI, or lymph vascular space invasion or 1/3 above + age ≥70, 2/3 above + age <50–69, or 3/3 above + age <50
3 High risk ($n = 76$, 70%): Stage IC + grade 3, Stage IIA + grade 3 + ≤50% MI, Stage IIB + any grade, or uterine papillary serous carcinoma or clear cell carcinoma, or Stage III–IV.

Basically, simple total abdominal hysterectomy (TAH), bilateral salpingo-oophorectomy (BSO), pelvic lymphadenectomy (PLA), para-aortic lymphadenectomy (PALA), and peritoneal washing cytology (PWC) were applied for the endometrial cancer patients with stage I or II where the disease was confined to the uterine body. Abdominal radical hysterectomy (ARH) or modified radical hysterectomy (mRH) was carried out in place of TAH for the patients with clinically obvious interstitial infiltration. TAH was performed in 12 cases (11%) and RH in the other cases (89%) including mRH for eight cases (7%). PLA was performed in 98 cases (88%), PALA in 92 cases (83%), and bilateral and ipsilateral SO in 60 (54%) and six cases (5%) in this study.

After surgery, for patients with low or intermediate risk without lymph node metastasis, no postoperative adjuvant therapy was performed. For patients with high risk or positive cytology from the abdominal cavity, postoperative radiation therapy (PORT) was performed.

Postoperative chemotherapy

For patients with lymph node metastasis, those with only one lymph node metastasis were given PORT alone and those with two or more lymph node metastases were given chemotherapy followed by external beam radiation therapy. From 2003, the cyclophosphamide, doxorubicin, and cisplatin (CAP) regimen was administered to patients with histological FIGO grade 1 and without vascular invasion and the paclitaxel and carboplatin (TC) regimen was administered for patients with histological FIGO grade 2 or 3 and/or with vascular invasion. Before 2003, only the CAP regimen had been used for postoperative chemotherapy. The CAP regimen consisted of three cycles of 70 mg/m^2 of cisplatin, 500 mg/m^2 of cyclophosphamide, and 50 mg/m^2 of doxorubicin. The TC regimen consisted of three cycles of paclitaxel at 175 mg/m^2 and carboplatin with an area under the curve (AUC) of 6, tri-weekly or monthly.

PORT

The whole pelvis was irradiated in all cases. For para-aortic lymph node metastasis, the para-aortic area and the whole pelvis were irradiated. Two parallel ports, the anterior–posterior and posterior–anterior, were used for whole pelvis irradiation until the year 2000. Thereafter, four ports (box field) were used. The upper edge included the bifurcation of the common iliac artery (around L4–5). The lower edge was between the obturator foramen and the ischial tuberosity, and the lateral edge was 1.5–2 cm outside the small pelvic cavity. The energy was basically 10 MV. The prescribed irradiation dose was 50–50.4 Gy/25–28 fractions. On the irradiation to the para-aortic area, the upper edge was between the 11th and 12th thoracic vertebrae, and the lateral edge included the transverse process.

Evaluation and follow up

Response to radiotherapy was evaluated using pelvic examination, computed tomography, and cytology. Follow up after PORT was usually conducted every

month for the first 2 years and every 3 months thereafter. Follow-up computed tomography was performed every 6 months and cytology every month. Swab samples were obtained from the vaginal stump. For patients suspected of recurrent disease, the follow up was conducted at more frequent intervals in consideration of alternative salvage treatment. Follow-up examination included physical and pelvic examinations and cytology.

In addition, toxicity was scored using the Common Terminology Criteria for Adverse Events (CTCAE) v3.0.

Statistical analysis

Associations between variables were assessed using the χ^2-test, Fisher's exact test, and linear-by-linear exact tests. The Kaplan–Meier product–limit method was used to estimate the probability of overall survival (OS) and disease-free survival (DFS); the log–rank test was used to estimate any differences. Multivariate analyses were performed using the Cox proportional hazards regression model. OS was calculated in months from the date of surgery to the date of death from any cause or to January 2009. Patients who were still alive in June 2008 were treated as censored. *P*-values < 0.05 were regarded as statistically significant. Statistical analyses were carried out using StatView Dataset File version 5.0 J for Windows.

Results

Patients

The patient characteristics are shown in Table 1. Of the 111 patients receiving PORT, the median age was 57 years (range, 28–78). One of these 111 patients was not followed up after PORT, and was therefore excluded from the analysis. Thirty-six patients had positive pelvic lymph nodes, 71 patients were negative, and others

Table 1 Univariate analysis of OS and DFS

Factor	*n*	%	5-y OS	*P*-value	5-y DFS	*P*-value
Age						
<60 y	67	61	90%	0.044	85%	0.013
≥60 y	43	39	74%		65%	
PLN						
(+)	36	34	81%	0.36	71%	0.150
(−)	71	66	87%		81%	
PALN						
(+)	24	22	72%	0.12	52%	0.001
(−)	86	78	88%		85%	
FIGO stage						
I	36	34	93%	0.11	87%	0.040
II–III	69	66	79%		71%	
Histological type						
EA	84	78	83%	0.51	76%	0.32
Not EA	24	22	87%		80%	
FIGO grade						
1	41	48	97%	0.35	81%	0.440
2	25	29	82%		76%	
3	18	23	77%		70%	
Risk group						
High	76	70	82%	0.61	72%	0.180
Intermediate–high	30	28	88%		86%	
Intermediate–low	2	2	100%		100%	
Chemotherapy						
With	46	44	82%	0.75	66%	0.13
Without	58	56	84%		84%	
Depth						
a–b†	18	18	100%	0.19	75%	0.67
c–d‡	80	82	83%		77%	

†<50% myometrial invasion. ‡>50% myometrial invasion. DFS, disease-free survival; EA, endometrioid adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; OS, overall survival; PALN, para-aortic lymph node; PLN, pelvic lymph node.

($n = 3$) could not be clearly determined. For the para-aortic lymph nodes, 24 patients were positive and 86 patients were negative. There were 36 patients (32%) in stage I, 15 patients (14%) in stage II, and 54 patients (50%) in stage III according to the FIGO staging. Five patients (4%) could not be staged because of insufficient FIGO staging information. Endometrioid adenocarcinoma, with 84 patients (76%), was the most frequent histological type encountered. Six other types were detected with considerably fewer frequencies. The histological types for two patients were not described in their medical records.

Among the cases not receiving postoperative chemotherapy, just the whole pelvis was irradiated in 86 cases (78%) and the para-aortic lymph node area plus the whole pelvis were irradiated in 24 cases (22%). As for the cases with postoperative chemotherapy, the CAP regimen was used in 32 cases (29%) and the TC regimen in 14 cases (13%).

To account for the high risk of local recurrence after surgery, a strong primary possibility was deep invasion in over 50% in 80 patients (82%) (Table 1). Secondarily, there were positive lymph node metastases in 36 patients (34%) (Table 1). Other reasons were ovarian invasion in five patients and cervical invasion in one patient. PORT was performed even for four patients with FIGO stage IA or IB with high risk of local recurrence. The reasons were histological FIGO grade 3 in three patients, and adenosquamous carcinoma in one patient. Although the intermediate low-risk group was not a target of PORT, two patients with intermediate low risk were given PORT because the residual lesions were strongly doubted by the surgeon in spite of negative histopathology.

Survival

The median follow-up time for all patients was 59.2 months (range; 6.5–235.2 months). The number of survivors at the end of the observation period was 93 (84%), and the number of disease-free survivors was 81 (73%). The OS rate at 5 years was 84%, and the DFS rate at 5 years was 77% for all patients. Both OS and DFS reached a plateau at approximately 3 years. Patients younger than 60 years had significantly better OS (log–rank $P = 0.044$, odds ratio [OR] = 0.383, and 95% confidence interval [CI] = 0.145–1.008) and DFS (log–rank $P = 0.013$, OR = 0.389, and 95%CI = 0.180–0.840) than patients 60 years or older (Fig. 1). Moreover, when limited to the histological type of endometrioid adenocarcinoma ($n = 84$), there was a significant difference in DFS between patients 60 years or older, and those

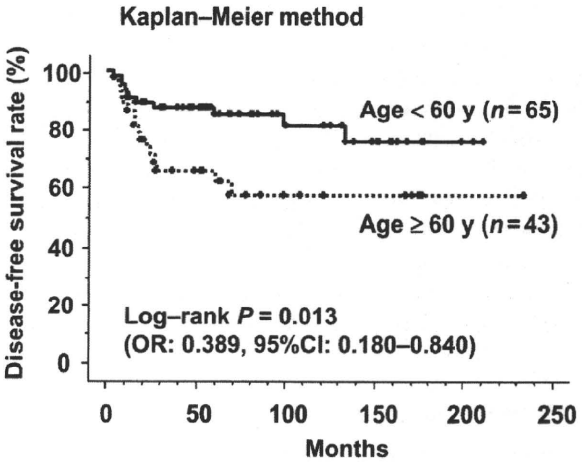

Figure 1 Disease-free survival curves by age (less than 60 years old vs 60 years or older). CI, confidence interval; OR, odds ratio.

under 60 years old (log–rank $P = 0.004$, OR = 0.289, and 95%CI = 0.119–0.703). However, when the age was raised to 65 years, the earlier significant difference in DFS shown for those younger than 60 years vanished ($P = 0.067$). When the age was raised to 70 years, there was a significant difference in DFS ($P = 0.0030$, OR = 0.274, and 95%CI = 0.109–0.685). The numbers of patients aged ≥65 and 70 years old were 25 (23%) and 10 (9%), respectively.

Certain prognostic factors (as determined by univariate analysis, Table 1) for both OS and DFS were examined. Pathological stage, with or without lymph node metastasis, and with or without chemotherapy, and age were studied. The 5-year OS and DFS rates were 93% and 87% in FIGO pathological stage I cases, 85% and 79% in stage II cases, and 83% and 74% in stage III cases, respectively. A significantly higher DFS rate in stage I ($n = 36$) emerged when compared with stages II–III ($n = 69$) (log–rank $P = 0.040$). Significant differences for DFS were not shown for those with and without lymph node metastasis (log–rank $P = 0.15$) and with and without postoperative chemotherapy (log–rank $P = 0.13$). However, for those with and without para-aortic lymph node metastasis the difference was significant (log–rank $P = 0.0006$, OR = 0.285, 95%CI = 0.133–0.612) (Table 1).

On multivariate analysis (Table 2), poor DFS correlated only with age ≥ 60 years ($P = 0.035$).

The distribution of variables according to age (age <60 vs age ≥60) is summarized in Table 3. There were significantly more cases of endometrioid

Table 2 Multivariate analysis of disease-free survival

Factor	*P*-value	OR	95%CI
Age			
<60 y	0.035	0.427	0.193–0.944
≥60 y			
PALN			
(−)	0.067	0.451	0.192–1.058
(+)			
FIGO stage			
I	0.18	0.484	0.167–1.405
II–III			

CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; OR, odds ratio; PALN, para-aortic lymph node.

adenocarcinoma on histological type ($P = 0.019$) and fewer high-risk cases ($P = 0.017$) in patients ≥60 years old than in patients <60 years old.

Complications

According to the CTCAE v3.0, lower limb edema, intestinal obstruction, and diarrhea were adverse events of grade 3 or more. Regarding complications of grade 3 or more, lower limb edema was seen in 16 patients (14%), intestinal obstruction was seen in nine patients (8%), and diarrhea was seen in three patients (3%). In this study, lower limb edema was recorded in 15 patients with grade 1 complications and 13 patients with grade 2 complications. There was no grade 3 or 4 myelosuppression in any of the cases.

Discussion

In this retrospective study of endometrial cancer in our institution, prognostic factors were evaluated in endometrial cancer patients and were focused particularly on the effect of advanced age on the outcome of surgery and PORT. The limitations of our study included the retrospective nature of the study and the heterogeneity of the patient population in the two arms (<60 years vs ≥60 years).

In a prospective randomized trial of postoperative radiation therapy in endometrial carcinoma (PORTEC) for stage I disease, Creutzberg *et al.*[13] reported that patient age ≥60 years was an independent predictor of death from endometrial carcinoma (hazard ratio of 3.1 and 95%CI , 1.2–8; $P = 0.02$). The data in the literature also suggest that there is an incremental increase in the risk of dying from endometrial carcinoma with increasing age. In a review of 819 patients with stage I–II endometrial carcinoma from the Gynecologic Oncology Group database, Zaino *et al.* demonstrated that the relative risk (RR) increased from 1.0 for patients who were aged ≤45 years (reference) at the time of diagnosis to 2.0 for patients aged 55 years, to 3.4 for patients aged 65 years, and to 4.7 for patients aged ≥75 years.[2] According to Alektiar *et al.*,[12] patient age ≥70 years was found to be an independent predictor of poor locoregional control (RR: 3 and 95%CI , 1–10; $P = 0.019$), DFS (RR: 2 and 95%CI , 1–13; $P = 0.03$), and OS (RR: 4 and 95%CI , 2–7; $P = 0.001$). Jolly *et al.*[14] concluded from a retrospective study that older endometrial cancer (age >63 years) patients had a significantly decreased OS, cause-specific survival, and greater risk of recurrence following PORT that were independent of other prognostic factors and/or treatment technique. According to Lee *et al.*,[15] their study ($n = 51\,471$) of a large population of uterine cancer patients demonstrated that those 40 years or younger have an OS advantage compared with women older than 40 years, independent of other clinicopathological prognosticators. Farley *et al.*[16] concluded that age (older than 50) is a specific and significant predictor of outcome in endometrioid adenocarcinoma of the uterus ($n = 328$). The frequent association between older age in endometrial carcinoma patients on the one hand and deep myometrial invasion and aggressive histologies always raises the possibility that the poor outcome in older patients is entirely the result of such an association. Why older patients with early-stage endometrial carcinoma tend to fare worse independent of other factors is not clear. Nevertheless, clinical efforts should be directed toward maximizing the therapeutic ratio in those patients. The notion of limited life expectancy should not hinder that effort because survival to the age of 80 years and beyond has been reported to have increased in many developed countries.[17] The remaining life expectancy of a white US woman aged 75 years is estimated to be 11.7 years.[18]

The treatment methods were changed for postoperative adjuvant therapy in our institution due to a pathological result after operation. Seeing the treatment outcome, the 5-year DFS rate of each FIGO stage was 82% in stage I, 79% in stage II, and 74% in stage III. These outcomes are comparable to other institutions. In the gynecology tumor committee report of 1993 in Japan,[19] the OS rate for five years was 84.0% in stage I, 73.5% in stage II, and 54.8% in stage III. As for our outcome results, only those cases receiving PORT in our department were evaluated, and it is likely that the results would show further improvement for stage I if the patients in the low-risk group were included. There

Table 3 Distribution of variables according to age

Variables	<60 years	($n = 67$)	≥60 years	($n = 43$)	*P*-value (×2)
PLN					
(+)	21	(41%)	15	(27%)	0.96
(−)	30	(59%)	41	(73%)	
PALN					
(+)	15	(23%)	9	(20%)	0.67
(−)	50	(77%)	36	(80%)	
FIGO stage					
I	20	(33%)	16	(36%)	0.65
II–III	41	(67%)	28	(64%)	
Histological type					
EA	43	(69%)	41	(89%)	0.019
not EA	19	(31%)	5	(11%)	
FIGO grade					
1	25	(50%)	16	(47%)	0.81
2	14	(28%)	11	(32%)	
3	11	(22%)	7	(21%)	
Risk group					
High	51	(80%)	25	(57%)	0.017
Intermediate–high	12	(19%)	18	(41%)	
Intermediate–low	1	(1%)	1	(2%)	
Chemotherapy					
With	30	(49%)	16	(37%)	0.21
Without	31	(51%)	27	(63%)	
Depth					
a–b†	10	(19%)	8	(18%)	0.27
c–d‡	44	(81%)	36	(82%)	

†<50% myometrial invasion. ‡>50% myometrial invasion. DFS, disease-free survival; EA, endometrioid adenocarcinoma; FIGO, International Federation of Gynecology and Obstetrics; OS, overall survival; PALN, para-aortic lymph node; PLN, pelvic lymph node.

were only 15 examples for stage II, and the number of cases might not be sufficient to analyze treatment results. On the other hand, there were 55 examples of stage III, which constitutes an excellent OS rate. A phase III randomized trial showed improved survival with the use of chemotherapy for stage III and IV endometrial cancer.[20,21] However, pelvic and abdominal failure rates were alarmingly high, which appears to be persuasive for the integration of radiation and chemotherapy as performed in our institution.

According to our multivariate analysis of DFS, being a senior citizen is in itself an independent risk factor. More intensive treatment may be necessary for senior citizens than for young people. A total dose of approximately 50 Gy in PORT has already been prescribed, and because any further dose increase is difficult, the inclusion of postoperative chemotherapy can be expected. According to a recent Japanese Gynecologic Oncology Group study,[22] adjuvant CAP chemotherapy may be a useful alternative to PORT for intermediate-risk endometrial cancer. Moreover, adjuvant vaginal high-dose-rate brachytherapy alone may be a safe and effective alternative to pelvic external beam PORT for surgical early stage endometrial cancer.[23,24]

References

1. Kosary CL. FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: An analysis of 1973–1987 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. *Semin Surg Oncol* 1994; **10**: 31–46.
2. Zaino RJ, Kurman RJ, Diana KL, Morrow CP. Pathologic models to predict outcome for women with endometrioid adenocarcinoma: The importance of the distinction between surgical stage and clinical stage – a Gynecologic Oncology Group study. *Cancer* 1996; **77**: 1115–1121.
3. Abeler VM, Kjørstad KE. Endometrial adenocarcinoma in Norway. A study of a total population. *Cancer* 1991; **67**: 3093–3103.
4. Irwin C, Levin W, Fyles A, Pintilie M, Manchul L, Kirkbride P. The role of adjuvant radiotherapy in carcinoma of the endometrium: results in 550 patients with pathologic stage I disease. *Gynecol Oncol* 1998; **70**: 247–254.

5. Poulsen MG, Roberts SJ. Prognostic variables in endometrial carcinoma. *Int J Radiat Oncol Biol Phys* 1987; **13**: 1043–1052.
6. Mundt AJ, Waggoner S, Yamada D, Rotmensch J, Connell PP. Age as a prognostic factor for recurrence in patients with endometrial carcinoma. *Gynecol Oncol* 2000; **79**: 79–85.
7. Grigsby PW, Perez CA, Kuten A *et al.* Clinical stage I endometrial cancer: Prognostic factors for local control and distant metastasis and implications of the new FIGO surgical staging system. *Int J Radiat Oncol Biol Phys* 1992; **22**: 905–911.
8. Lindahl B, Ranstam J, Willén R. Five year survival rate in endometrial carcinoma stages I-II: Influence of degree of tumour differentiation, age, myometrial invasion and DNA content. *Br J Obstet Gynaecol* 1994; **101**: 621–625.
9. Alektiar KM, McKee A, Venkatraman E *et al.* Intravaginal high-dose-rate brachytherapy for Stage IB (FIGO Grade 1, 2) endometrial cancer. *Int J Radiat Oncol Biol Phys* 2002; **53**: 707–713.
10. Samet J, Hunt WC, Key C, Humble CG, Goodwin JS. Choice of cancer therapy varies with age of patient. *JAMA* 1986; **255**: 3385–3390.
11. Schrag D, Cramer LD, Bach PB, Begg CB. Age and adjuvant chemotherapy use after surgery for stage III colon cancer. *J Natl Cancer Inst* 2001; **93**: 850–857.
12. Alektiar KM, Venkatraman E, Abu-Rustum N, Barakat RR. Is endometrial carcinoma intrinsically more aggressive in elderly patients? *Cancer* 2003; **98**: 2368–2377.
13. Creutzberg CL, Van Putten WL, Koper PC *et al.* Surgery and postoperative radiotherapy versus surgery alone for patients with stage-1 endometrial carcinoma: Multicentre randomized trial. PORTEC Study Group. Post Operative Radiation Therapy in Endometrial Carcinoma. *Lancet* 2000; **355**: 1404–1411.
14. Jolly S, Vargas CE, Kumar T *et al.* The impact of age on long-term outcome in patients with endometrial cancer treated with postoperative radiation. *Gynecol Oncol* 2006; **103**: 87–93.
15. Lee NK, Cheung MK, Shin JY *et al.* Prognostic factors for uterine cancer in reproductive-aged women. *Obstet Gynecol* 2007; **109**: 655–662.
16. Farley JH, Nycum LR, Birrer MJ, Park RC, Taylor RR. Age-specific survival of women with endometrioid adenocarcinoma of the uterus. *Gynecol Oncol* 2000; **79**: 86–89.
17. Manton KG, Vaupel JW. Survival after the age of 80 in the United States, Sweden, France, England, and Japan. *N Engl J Med* 1995; **333**: 1232–1235.
18. Taeuber CM. Sixty-five plus in America. Current Population Reports. Special Studies. Washington, DC: US Department of Commerce, Economics and Statistics Administration, Bureau of the Census, 1996; publication no. P23–173RV.
19. The Cancer Committee of the Japan Society of Gynecology. [The 41st treatment annual report. 5-year result of treatment started in 1993 for cervical and endometrial carcinoma.] *J Jpn Soc Obstet Gynecol* 2008; **60**: 1876–1901. (In Japanese.)
20. Randall ME, Brunetto VL, Muss H. Whole abdominal radiotherapy versus combination doxorubicin-cisplatin chemotherapy in advanced endometrial carcinoma: A randomized phase III trial of the Gynecologic Oncology Group. *Proc Am Soc Clin Oncol* 2003; **22**: 2.
21. Randall ME, Spirtos NM, Dvoretsky P. Whole abdominal radiotherapy versus combination chemotherapy with doxorubicin and cisplatin in advanced endometrial carcinoma (phase III): Gynecologic Oncology Group Study no. 122. *J Natl Cancer Inst Monogr* 1995; **19**: 13–15.
22. Susumu N, Sagae S, Udagawa Y *et al.* Japanese Gynecologic Oncology Group.Randomized phase III trial of pelvic radiotherapy versus cisplatin-based combined chemotherapy in patients with intermediate- and high-risk endometrial cancer: A Japanese Gynecologic Oncology Group study. *Gynecol Oncol* 2008; **108**: 226–233.
23. Jolly S, Vargas C, Kumar T *et al.* Vaginal brachytherapy alone: An alternative to adjuvant whole pelvis radiation for early stage endometrial cancer. *Gynecol Oncol* 2005; **97**: 887–892.
24. Solhjem MC, Petersen IA, Haddock MG. Vaginal brachytherapy alone is sufficient adjuvant treatment of surgical stage I endometrial cancer. *Int J Radiat Oncol Biol Phys* 2005; **62**: 1379–1384.

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ORIGINAL PAPER

Aromatase inhibitor anastrozole as a second-line hormonal treatment to a recurrent low-grade endometrial stromal sarcoma: a case report

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Abstract Low-grade endometrial stromal sarcoma (ESS) is a rare neoplasm and is generally an indolent tumor with estrogen and progesterone receptors. Objective responses by hormonal treatment with progestin or aromatase inhibitor have been reported, however, long-term management of this disease could be difficult if it becomes refractory to one of these hormonal therapies. A 34-year-old woman was diagnosed with stage I low-grade ESS at the time of hysterectomy for presumed uterine fibroma. Five years later, she recurred with multiple tumors in the lower abdomen. After an optimal surgery, she was free from progression for 6 years with progestin treatment (medroxyprogesterone acetate: MPA, 200–600 mg daily). Thereafter, she recurred twice during the MPA treatment and received debulking surgery each time. MPA was discontinued at age of 53, because another recurrent tumor grew up to 13 cm in diameter. Aromatase inhibitor anastrozole was then given at a daily dose of 1 mg with partial response (the tumor size decreased to 7 cm in diameter) for a duration of 9 months. After complete resection of the recurrent tumor, she remains progression-free for 16 months. Anastrozole was effective to recurrent low-grade ESS even after being refractory to progestin therapy. Aromatase inhibitor treatment may be a useful option as a second-line hormonal treatment to low-grade ESS.

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Keywords Low-grade endometrial stromal sarcoma · Uterine corpus · Recurrence · Aromatase inhibitor · Progestin therapy · Hormonal treatment

Introduction

Endometrial stromal sarcoma (ESS) is a rare neoplasm, accounting for 0.2% or less of gynecologic malignancies [1]. Low-grade ESS usually expresses estrogen receptors (ER) and progesterone receptors (PR), and estrogen acts as a growth stimulus [2, 3]. Objective responses have been obtained with progestin therapy, such as megestrol acetate and medroxyprogesterone acetate (MPA) [4, 5]. More recently, the efficacy of a non-steroid aromatase inhibitor has been also reported [6, 7], as it inhibits estrogen synthesis. Although either type of hormonal therapy might be useful as a first-line therapy, it is still uncertain whether a second-line hormonal treatment is effective to repetitively recurrent ESS with resistance to a first-line therapy.

We report a case of recurrent low-grade ESS with long-term survival, treated with MPA for 13 years as a first line and aromatase inhibitor anastrozole for 9 months as a second-line hormonal therapy.

Case report

A 34-year-old woman (gravida 4, para 2) underwent a total abdominal hysterectomy for presumed uterine fibroma at her local hospital in 1988. The histopathological result revealed stage I low-grade ESS of the corpus uteri. In December 1993, she was referred to our hospital, and a computed tomography (CT) scan revealed a 9-cm pelvic mass, bilateral ovarian masses (4 cm on the left and 7 cm

on the right), and para-aortic lymph node enlargement. She underwent secondary debulking surgery, including bilateral salpingo-oophorectomy, omentectomy, bowel resection, and biopsy of para-aortic lymph nodes. All the residual tumors were less than 1 cm in diameter. The final pathology revealed recurrence of the low-grade ESS (Fig. 1a), involving the bilateral adnexae, ileum, appendix, colon, omentum, and para-aortic lymph nodes. Immunohistochemical analysis showed a strong nuclear staining for both ER and PR (Fig. 1b, c), as well as CD10 (Fig. 1d) and vimentin, and a negative staining for HHF35, 1A4, Desmin, and CD34. Postoperatively, she was started on MPA at a daily dose of 600 mg. Three years after the MPA therapy, complete response was pathologically confirmed by second look laparoscopy. MPA was continued at a daily dose of 200–400 mg without any appreciable adverse effects.

In April 2000, surgical biopsy of a 2-cm mass around the liver confirmed the recurrence of the disease on peritoneum. Two years later, she received another debulking procedure with partial liver resection for a 5-cm tumor and resection of another 5-cm pelvic tumor. After the surgery, she was hospitalized four times within 2 years due to grade 2 ileus. In June 2006, a CT scan showed a 5-cm solid mass in the left upper quadrant. The patient did not choose a debulking surgery and was kept treated with MPA at a daily dose of 200–400 mg. Eight months later, she was found to have progression of disease, represented by enlargement of the mass up to 13 cm in diameter and appearance of 4 cm mesenteric mass in the pelvis (Fig. 2a). Then, MPA treatment was discontinued, and anastrozole at a daily dose of 1 mg was started with an informed consent. After 9 months of the treatment, the tumor in the left upper quadrant was decreased to 7 cm in diameter and the mesenteric tumor was undetected (Fig. 2b). Anastrozole was discontinued because of arthritis with grade 2 joint-function disorder. Then, she underwent complete resection of the recurrent tumor. Pathological findings also revealed the significant effect of anastrozole. As shown in Fig. 3, the majority of the tumor cells was necrotic and replaced by numerous foamy histiocytes. The viable cells remained partly in the marginal lesion with expression of ER and PR. She recovered from the joint-function disorder shortly after the surgery and remains asymptomatic and progression-free for 16 months.

Discussion

ESS is subdivided histopathologically into low-grade and undifferentiated (or high-grade) forms depending on the morphology, number of mitoses, cellularity, and necrosis. The primary treatment for low-grade ESS is mainly surgery, including an abdominal hysterectomy with bilateral salpingo-oophorectomy. Adjuvant treatment, such as radiotherapy or chemotherapy, is not routinely recommended

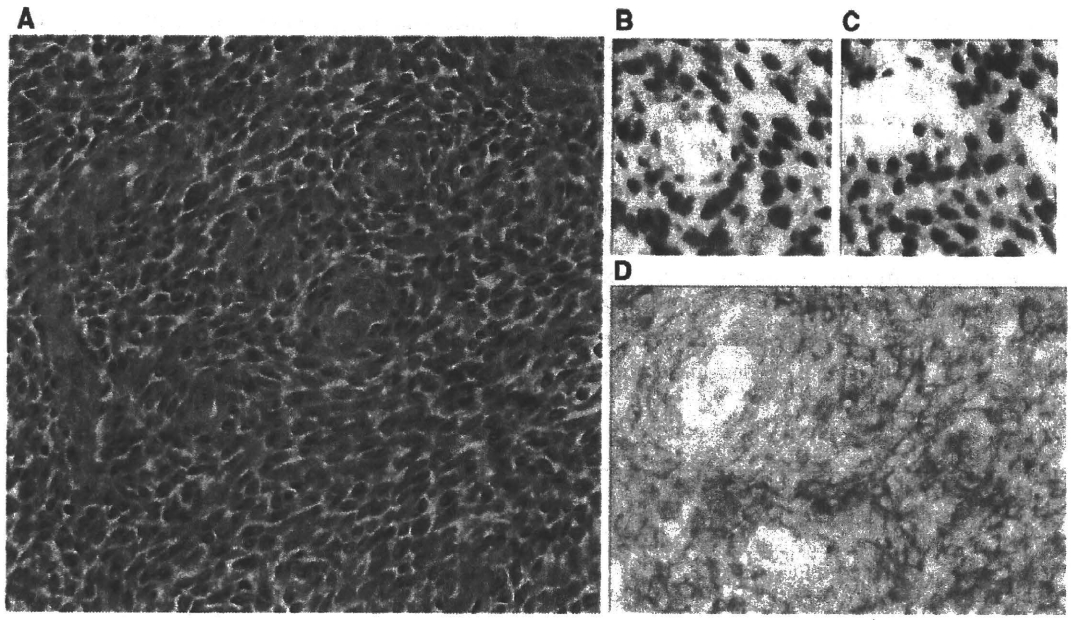

Fig. 1 Histological findings of the tumor, excised before MPA treatment. **a** High Power: Tumor cells in the pelvis, showing proliferation of endometrial stromal cells without significant atypia or pleomorphism, diagnosed as low-grade ESS. **b–d** High Power: Tumor cells are strongly positive for estrogen receptor (**b**) and progesterone receptor (**c**) and are diffusely positive for CD10 (**d**) by immunohistochemistry

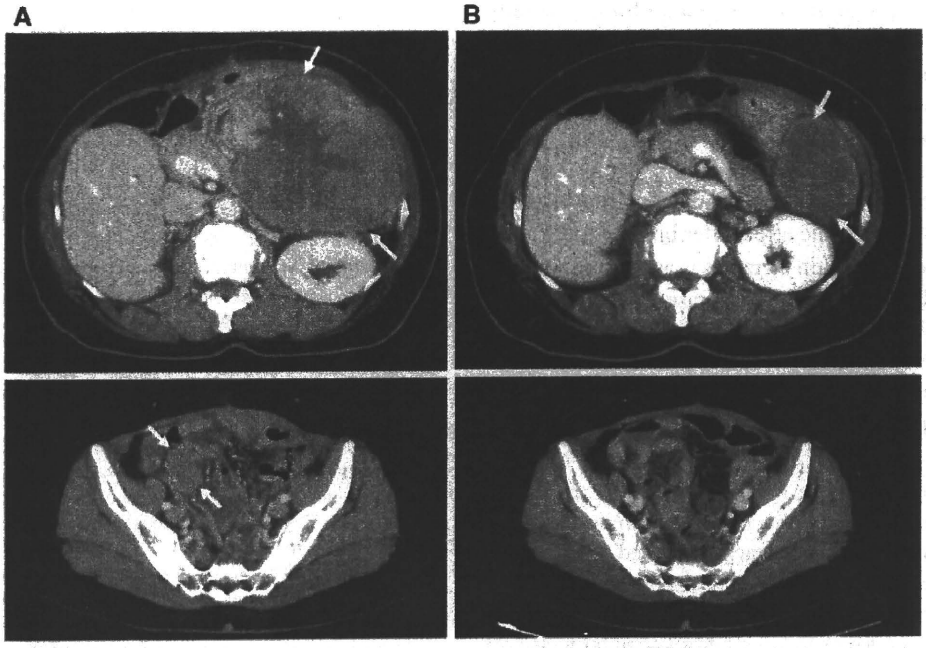

Fig. 2 Images of CT scan before and after anastrozole treatment. **a** Recurrent tumors with 13 cm in diameter in the left upper quadrant (*Upper*) and 4 cm in diameter in the pelvis (*Lower*). **b** The recurrent tumors were diminished to 7 cm in diameter (*Upper*) or became undetectable (*Lower*)

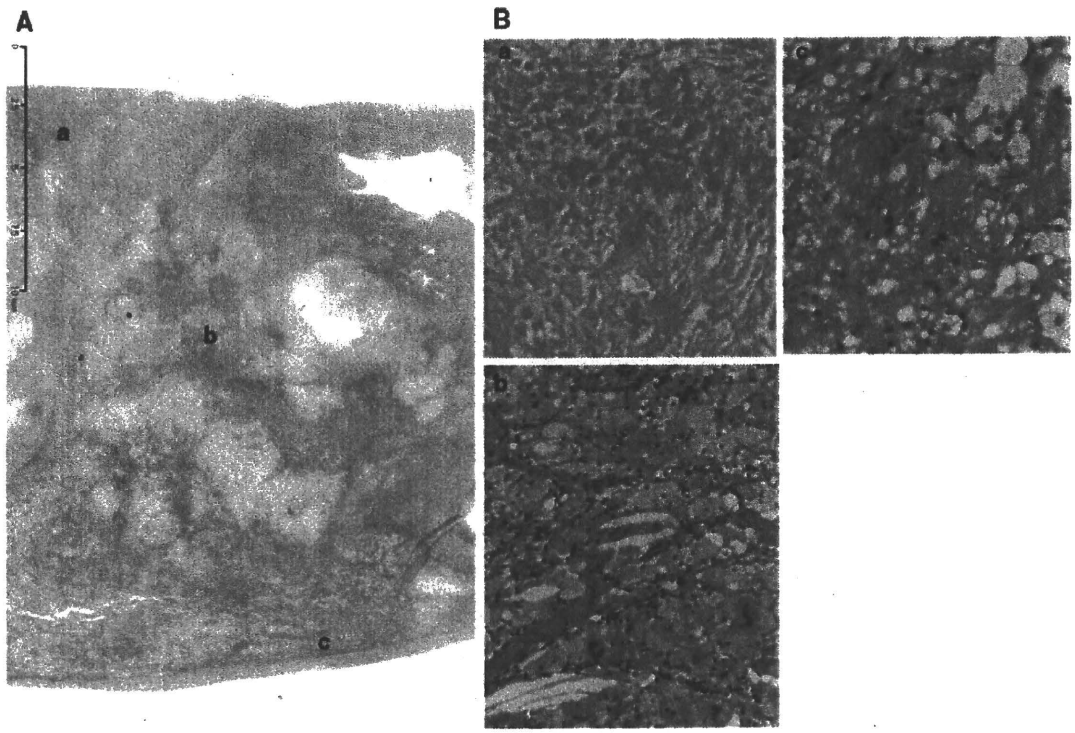

Fig. 3 Histological findings of the tumor, excised after anastrozole treatment. **a**. Low Power: Tumor cells with massive necrosis. **b** High Power: (*a*) Lesion with coagulative tumor cell necrosis, which occupies the majority of the tumor. (*b*) Center lesion with numerous foamy histiocytes. (*c*) Marginal lesion of the tumor with viable cells partly remaining

[8]. Although the prognosis of low-grade ESS is generally favorable with more than 90% of 5-year overall survival, the recurrence-free survival rate is reported to be about 50% [9, 10]. In addition to surgical resection, treatment option to recurrent low-grade ESS is hormonal therapy with progesterone derivative or aromatase inhibitor. MPA and megestrole acetate are synthetic derivatives of progesterone that exert an anti-estrogenic effect after binding to PR. The sensitivity to these progestin therapies is associated with the presence of ER and PR [11]. Aromatase inhibitors reduce estrogen levels by inhibiting its synthesis in peripheral sites. The distinct function suggests that suppressing aromatase might be still effective to recurrent ESS with resistance to progestin therapy.

The patient reported here suffered from repeated recurrences after becoming refractory to MPA treatment. Positive PR expression of the recurrent tumors suggests that the resistance to MPA therapy is caused by PR-independent manner. As a second-line hormonal therapy, anastrozole showed significant response to these recurrent tumors, suggesting that aromatase inhibitor might be useful for progestin-resistant low-grade ESS tumors. It is to be elucidated whether aromatase inhibitor is also effective to recurrent ESS tumors with negative PR expression.

Acknowledgments Conflict of interest statement All the authors declare no conflict of interest.

References

1. Koss LG, Spiro RH, Brunschwig A. Endometrial stromal sarcoma. Surg Gynecol Obstet. 1965;121:531–7.
2. Sabini G, Chumas JC, Mann WJ. Steroid hormone receptors in endometrial stromal sarcomas. A biochemical and immunohistochemical study. Am J Clin Pathol. 1992;97:381–6.
3. Wade K, Quinn MA, Hammond I, Williams K, Cauchi M. Uterine sarcoma: steroid receptors and response to hormonal therapy. Gynecol Oncol. 1990;39:364–7.
4. Gloor E, Schnyder P, Cikes M, Hofstetter J, Cordey R, Burnier F, et al. Endolymphatic stromal myosis. Surgical and hormonal treatment of extensive abdominal recurrence 20 years after hysterectomy. Cancer. 1982;50:1888–93.
5. Tsukamoto N, Kamura T, Matsukuma K, Imachi M, Uchino H, Saito T, et al. Endolymphatic stromal myosis: a case with positive estrogen and progesterone receptors and good response to progestins. Gynecol Oncol. 1985;20:120–8.
6. Maluf FC, Sabbatini P, Schwartz L, Xia J, Aghajanian C. Endometrial stromal sarcoma: objective response to letrozole. Gynecol Oncol. 2001;82:384–8.
7. Leunen M, Breugelmans M, De Sutter P, Bourgain C, Amy JJ. Low-grade endometrial stromal sarcoma treated with the aromatase inhibitor letrozole. Gynecol Oncol. 2004;95:769–71.
8. Reich O, Regauer S. Hormonal therapy of endometrial stromal sarcoma. Curr Opin Oncol. 2007;19:347–52.
9. Pink D, Lindner T, Mrozek A, Kretzschmar A, Thuss Patience PC, Dorken B, et al. Harm or benefit of hormonal treatment in metastatic low-grade endometrial stromal sarcoma: single center experience with 10 cases and review of the literature. Gynecol Oncol. 2006;101:464–9.
10. Kim WY, Lee JW, Choi CH, Kang H, Kim TJ, Kim BG, et al. Low-grade endometrial stromal sarcoma: a single center's experience with 22 cases. Int J Gynecol Cancer. 2008;18:1084–9.
11. Katz L, Merino MJ, Sakamoto H, Schwartz PE. Endometrial stromal sarcoma: a clinicopathologic study of 11 cases with determination of estrogen and progestin receptor levels in three tumors. Gynecol Oncol. 1987;26:87–97.

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Novel human papillomavirus type 18 replicon and its application in screening the antiviral effects of cytokines

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Human papillomaviruses (HPVs) infect the stratified epithelial organ. The infection induces benign tumors, which occasionally progress into malignant tumors. To elucidate the virus-induced tumorigenesis, an understanding of the lifecycle of HPV is crucial. In this report, we developed a new system for the analysis of the HPV lifecycle. The new system consists of a novel HPV replicon and an organotypic "raft" culture, by which the HPV-DNA is maintained stably in normal human keratinocytes for a long period and the viral vegetative replication is reproduced. This system will benefit biochemical and genetic studies on the lifecycle of HPV and tumorigenesis. This system is also valuable in screening for antiviral compounds. We confirmed its usefulness by evaluating the antivirus effect of cytokines. (*Cancer Sci* 2010; 101: 536–542)

The infection of high-risk type human papillomavirus (HPV) is a major risk factor for cervical cancer.[1–3] The World Health Organization has reported that the cases of HPV-associated cancers number about half a million, which corresponds to 10% of cancer cases in women.[4] This indicates the importance of the prevention of and urgently developing treatment for the cancer.

In order to control cervical cancer, it is essential to understand the regulatory mechanisms of the HPV infection. The primary target for HPV infection is the epithelial cells (keratinocytes) of the stratified squamous epithelium, and replication of HPV is strictly regulated by the differentiation program of the keratinocytes,[5] making it difficult to analyze the virus lifecycle in standard tissue culture systems. Several tissue culture conditions have been used for studying the differentiation-dependent lifecycle, such as a suspension culture of keratinocytes using methylcellulose "semisolid" medium,[6,7] a calcium-induced differentiation of keratinocytes,[8] and an organotypic raft culture.[9–11] Among them, the raft culture seems superior for studying the HPV lifecycle, because it is able to reproduce the stratified structure of epithelium, support the production of progeny virions in the differentiated layer, and capture the virus-induced hyperplasia as in the infected lesions. Although the raft culture has been successfully used for the analysis of the HPV lifecycle in combination with the genomic-type of HPV-DNA,[12] the drawbacks to using the culture system are the intricateness in the construction of it and the difficulty in obtaining the cell population maintaining the HPV-DNA.

In this manuscript, we tried to improve the suitability of the culture system for analysis of the HPV lifecycle. We constructed a new HPV replicon that could be maintained for a long period in comparison with the conventional method utilizing the genomic-type HPV-DNA. The raft culture system incorporating the new replicon could reproduce the physiological status of HPV-infected lesions: virus-induced hyperplasia accompanied by viral DNA amplification and late gene expression. This new system might accelerate the investigation of the HPV lifecycle. The usefulness of the system was verified by examining the effects of several cytokines on HPV replication and hyperplasia induction.

Materials and Methods

Construction of plasmid DNAs. HPV18 genomic DNA was isolated from a plasmid containing a full-length HPV18 DNA (GenBank accession no.: X05015). A new HPV18 replicon, p18FLneo, was constructed as illustrated in Figure 1. pEGFP1-ori(pBR) was constructed by replacing the origin of pEGFP1 (Clontech Laboratories, Mountain View, CA, USA) with pBR322-ori derived from pPUR (Clontech Laboratories). The long control region (LCR) of HPV18 (nucleotide number 7000 to 100; GenBank no.: X05015) was isolated by PCR, and then cloned into the vector pEGFP1-ori(pBR); the resultant plasmid was named 18LCR/pEGFP1-ori(pBR). Full-length HPV18 genome was cloned into the 18LCR/pEGFP1-ori(pBR) by using the *Afl*II recognition site. The genomic-type of HPV18 DNA was obtained by self-ligation of the full-length HPV18 DNA by following a method previously described.[13]

Cell culture and transfection. Human foreskin fibroblasts (HFFs) and human foreskin keratinocytes (HFKs) were commercially obtained (Kurabo Industries, Osaka, Japan), and maintained with 10% fetal bovine serum/DMEM and a serum-free keratinocyte growth medium (KGM) (EpiLife-KG2; Kurabo Industries), respectively. HFKs were transfected with 2 μg of p18FLneo or 1.5 μg of the genomic-type HPV18 DNA plus 0.5 μg of pEGFP1 by the nucleofection method (Nucleofector Kit; Amaxa, Cologne, Germany). The HFKs transfected with p18FLneo were cultured under the presence of G418 for more than 4 weeks, then used for the experiments.

Southern hybridization. Total DNA was extracted from the HFKs by following a standard protocol.[14] Five μg of the total DNA was digested with *Dpn*I and *Bgl*I, and then the DNA fragments were separated by 0.8% agarose gel electrophoresis, and transferred to a nylon membrane (Hybond N+; Amersham Biosciences UK, Little Chalfont, UK). For the detection of HPV18-specific DNA, a non-RI detection system was employed (Digoxigenin [DIG] Wash and Block Buffer Set and anti-DIG-alkaline phosphatase [AP]; Roche Diagnostics, Mannheim, Germany). The DIG-labeled probe for the LCR (7000–100 nt; GenBank no.: X05015) or L1 region (6137–7136 nt; GenBank no.: X05015) of HPV18 was obtained by a PCR-mediated method (PCR DIG Probe Synthesis Kit; Roche Applied Science, Manheim, Germany). The chemiluminescent signal

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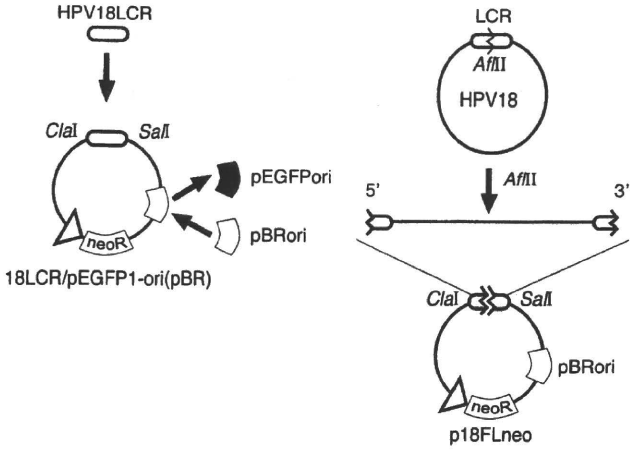

Fig. 1. Structure of a new human papillomavirus (HPV) replicon. A new replicon containing the full length of HPV18 genomic DNA constructed with a backbone plasmid, pEGFP1-ori(pBR), based on pEGFP1 plasmid. The replication origin (ori) element was replaced with that of pBR322. HPV18 long control region (LCR) was cloned into the pEGFP1-ori(pBR), and then the full-length HPV18 genome was inserted into the AflII site located in the LCR region. The obtained replicon was named p18FLneo.

was visualized with a chemiluminescent image analyzer (LAS-3000; Fuji Film, Tokyo, Japan).

Organotypic raft culture system. The construction of the organotypic raft culture and the preparation of frozen section have been described previously.[15–17] For BrdU incorporation, 50 g/mL BrdU (Sigma-Aldrich, St Louis, MO, USA) was added in the medium 6 h before harvest. The thickness of the epidermal layer was measured using image analysis software (AxioVision 3.1; Carl Zeiss Vision, Munich, Germany).

Immunoblot analysis. Total cell lysate of HFKs was obtained with a triple-detergent buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 0.5% sodium deoxycholate)[18] supplemented with a protease inhibitor cocktail (0.5 mM PMSF, 0.15 M aprotinin, 1 M E-64, 1 M leupeptin, 0.5 M EDTA) (Nakarai Tesuque, Kyoto, Japan) and 1 mM dithiothreitol. Equal amounts of cell lysate (5 μg protein) were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was blotted to a PVDF membrane (Hypond-P; Amersham Biosciences UK). The equalities of the loaded amounts were confirmed with the anti-actin immunoblot (1:50000) (clone AC-15; Sigma-Aldrich) (data not shown). Antibodies for p53 (1:1000) (Ab-6; Oncogene Research Products, San Diego, CA, USA) and pRb (1:1000; BD Biosciences Pharmingen, San Diego, CA, USA) were purchased commercially. Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000; Amersham Biosciences UK) and a luminal reagent (Western Blotting Luminol Reagent; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were purchased commercially. The chemiluminescent signal was visualized with a chemiluminescent image analyzer (LAS-3000; Fuji Film).

Immunohistochemistry (IHC). IHC for the tissue sections on slide glasses was performed as described previously.[15–17] Antibodies for BrdU (1:400) (clone 2B-1; MBL, Nagoya, Japan) and L1 (1:200) (MAB837; Millipore, Billerica, MA, USA), and p53 (Ab-6) (Oncogene Research Products), and pRb (BD Biosciences Pharmingen) were purchased commercially.

***In situ* hybridization (ISH).** Detection of HPV18 DNA signals in the tissue sections was performed with the TSA-biotin system (Perkin-Elmer, Boston, MA, USA) following the manufacturer's instructions. The DIG-labeled DNA for HPV18 LCR region (7000–100 nt; GenBank no.: X05015) (DIG high prime; Roche Diagnostics) was used as the probe. For the detection of DIG-labeled probe, HRP-labeled anti-DIG antibody (Dako, Glostrup, Denmark) was used. After hybridization, biotinyl tyramide working solution, SA-HRP (streptavidin–HRP), and metal-enhanced DAB solution (Roche Diagnostics) was used for detection of the signal. All slides were counterstained with hematoxylin.

Cytokine treatment. In monolayer culture, HFKs were incubated with cytokines (interferon [IFN]-β, 100 units/mL; transforming growth factor [TGF]-β, 1 ng/mL; tumor necrosis factor [TNF]-α, 5 ng/mL) for 3 days. IFNβ and TGFβ (Sigma-Aldrich) and TNFα (Merk Biosciences, San Diego, CA, USA) were purchased from the distributors. In the organotypic raft culture, cytokines were added in the culture medium for 7 days before the sample harvest.

Apoptosis induction by cytokine. 2×10^5 cells were cultured in the growth medium supplemented with the cytokines for 3 days. The treated cells were washed, trypsinized, and fixed with ice-cold methanol. Apoptotic cells were labeled by M30 antibody (CytoDEATH; Roche Diagnostics) and AlexaFLUOR488-antimouse antibody (Invitrogen, Carlsbad, CA, USA), and then counted by flow cytometry (BD Biosciences, San Jose, CA, USA).

Results

Construction of new HPV replicon. Genomic-type HPV-DNA was used as the replicon to obtain the keratinocytes maintaining HPV-DNA. The genomic-type DNA, which was constructed by re-circularization of full-length HPV-DNA, was transfected into the cells with the pSV2*neo* expressing a neomycin-resistance gene (neo^R).[13] The transfected cells were selected in the culture medium containing G418 for about 1 week, and then the surviving cells were expanded without the drug selection. The raft culture constructed with the cells could reproduce viral vegetative replication as reported previously.[12] By this method, there is no selectable pressure for the maintenance of HPV-DNA, causing a possible problem in that the cells lose HPV-DNA. It was also suggested that the transfection of the genomic-type DNA was inefficient because of its non-supercoiled structure.[19] To eliminate this potential problem, full-length HPV18 DNA was inserted into a plasmid containing the neo^R expression unit (Fig. 1). The replication of HPVs is regulated by the LCR as the promoter/enhancer for gene expressions at the 5′ region of the coding region and as the transcriptional terminator at the 3′ region. Therefore, the LCR was added at the both sides of the coding region. We named the new replicon p18FLneo (FL is an abbreviation for full length).

We verified the potential of p18FLneo as the replicon by examining whether it could be maintained stably in culture cells. HFKs were transfected with p18FLneo, then cultured under the presence of G418 for 4 weeks. Under the same condition, we found that the mock-transfected cells were dislodged within a week and that the cells transfected with pEGFP1, which contains the neo^R expression unit and is replication-defective in the HFKs, could not survive for more than 2 weeks. The total DNAs were collected from the cells, and then HPV-DNA was detected by Southern hybridization analysis. The result indicated that p18FLneo was stably maintained in the HFKs as a HPV18 replicon (Fig. 2A, p18FLneo). In order to validate its potential as the replicon, the genomic-type of HPV18 DNA was used as the control replicon (Fig. 2A, 18-ligation). The genomic-type HPV18 was introduced with the pEGFP1 into the HFKs. The transfected cells were selected under the presence of G418 for 1 week, and then the surviving cells were cultured without the drug-selection for 3 weeks. The amount of replicated HPV-DNA was analyzed by Southern blot analysis, and it appeared that the efficiency of the maintenance or the replication of the genomic-type HPV18

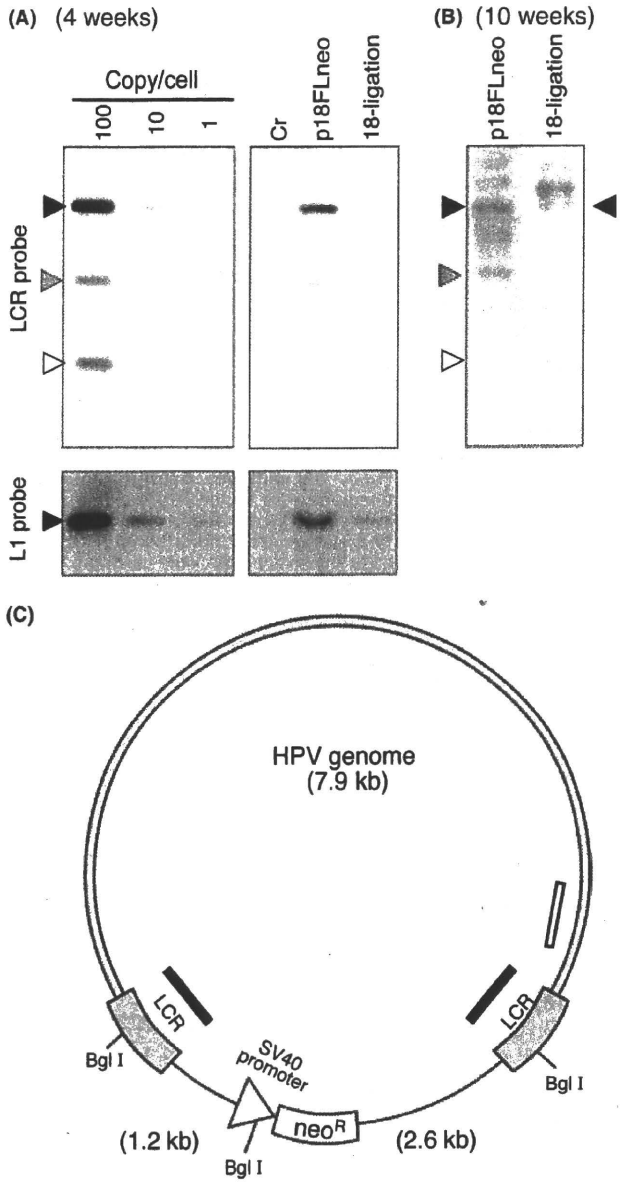

Fig. 2. Maintenance of the new human papillomavirus (HPV) replicon in primary keratinocytes. (A) Maintenance of the HPV replicon in the human foreskin keratinocytes (HFKs) 4 weeks after transfection was examined by Southern blot analysis. Total DNA was extracted from the normal HFKs (Cr), the HFKs harboring either the HPV replicon (p18FLneo) or the genomic-type HPV18 DNA (18-ligation) and subjected to *Dpn*I + *Bgl*I digestion. Each lane was loaded with 5 μg of the total DNA. p18FLneo DNA was used as the control for the copy number per cell. p18FLneo was digested with *Bgl*I and applied to the agarose gel at an amount equivalent to 1 copy, 10 copies, or 100 copies per cell. The probe used for the detection of HPV-DNA was DIG-labeled 18LCR or 18L1 DNA fragment. Closed triangle indicates the position of the full-length HPV18 genomic DNA. Gray and open triangles indicate the positions of the fragments containing a portion of long control region (LCR) and the backbone plasmid; those fragments could not be detected with L1 probe. (B) The maintenance of the HPV replicon in HFKs 10 weeks after transfection was examined as described in (A). Closed triangle indicates the position of the full-length HPV18 genomic DNA. Gray and open triangles indicate the positions of the fragments containing a portion of LCR and the backbone plasmid. The extra signals observed in each lane were considered the integrated form of HPV-DNA. (C) The scheme of pFL18neo. Gray region indicates HPV18 DNA. Black and white bars indicate the regions targeted by LCR and L1 probes, respectively. The recognition sites for BglI are indicated, and the digestion produced three DNA fragments, 7.9 kb, 2.6 kb, and 1.2 kb.

DNA was lower than that of p18FLneo. These results suggested the advantage of p18FLneo as the HPV replicon.

The HFKs containing p18FLneo could be maintained for more than 10 weeks under the presence of G418. The growth potential of normal HFKs apparently declined after 6 weeks of culturing and the cells acquired senescence status, indicating that the HFKs containing p18FLneo were immortalized by the functions of HPV E6 and E7. The existence of HPV-DNA in the cells was examined, and it was revealed that the majority of HPV-DNA was maintained as the episomal status and that some portion of the DNA might be integrated in the host chromosome (Fig. 2B, p18FLneo). In the accompanying experiment, the DNA status in the HFKs containing the genomic-type HPV-DNA was examined, and it found that most of the HPV-DNA was maintained as integrated form (Fig. 2B, 18-ligation).

Raft culture with HFKs harboring the new HPV replicon. The HFKs or the spontaneously immortalized keratinocytes (normal immortal keratinocytes, NIKS)[20] harboring HPV genomic DNA were used to organize the organotypic raft culture,[11,13,21,22] and they could support HPV replication in a differentiation-dependent manner. We examined whether the HFKs maintaining p18FLneo ($HFK_{p18FLneo}$) could also support the HPV lifecycle in the raft culture.

$HFK_{p18FLneo}$ could organize a stratified epithelial structure and it appeared that significant hyperplasia was induced (Fig. 3, H&E). In the epithelial layer of the $HFK_{p18FLneo}$ raft culture, BrdU-positive cells were detected in the parabasal layer. On the contrary, BrdU-positive cells were restricted at the basal layer in the raft culture with normal HFKs (Fig. 3, BrdU). This observation suggested that p18FLneo had the potential to disturbe the differentiation program of the epithelial cells and induced hyperproliferation.

We next examined whether the late-phase of the virus lifecycle was reproduced with $HFK_{p18FLneo}$. By ISH with the HPV18 probe, the cells positive for HPV18 DNA were detected in the suprasurface layer (Fig. 3, ISH), indicating that the copy number of p18FLneo was amplified in the differentiated layer of the epithelium. The expression of the late gene product, L1, was also detected in the differentiated layers by IHC (Fig. 3, IHC). These observations indicate that $HFK_{p18FLneo}$ in combination with the raft culture is an appropriate tool for the analysis of the HPV lifecycle. Although the $HFK_{p18FLneo}$ maintained for long period (10 weeks) was also used for the raft culture, it failed to organize a stratified epithelial layer, indicating it lost the property of normal cellular differentiation.

Application of the new HPV replicon: (1) Effects of cytokines on the latent phase of HPV infection. Cytokine production is a biological response *in vivo* to virus infection or inflammation. One of the cytokines, type I IFN, is used in chemotherapy for HPV-positive cervical neoplasia.[23] Other cytokines, TGFβ and TNFα, have been reported to be involved in the response to HPV infection.[24–30] We examined the effects of these cytokines on HPV replication using the new HPV replicon system.

The monolayer culture of $HFK_{p18FLneo}$ is supposed to represent the status of the latent infection of HPV in basal cells. We first examined the effects of the cytokines on the $HFK_{p18FLneo}$ monolayer culture. We chose doses of cytokines that had minimal effects on the growth of normal HFKs (IFNβ, 100 units/mL; TGFβ, 1 ng/mL; TNFα, 5 ng/mL) in order to observe the specific effects on HPV replication (Fig. 4). These doses of the cytokine treatments had also no significant effects on the growth of $HFK_{p18FLneo}$. The apoptosis induction by the cytokines was also examined by FACS analysis using an apoptosis-specific antibody (M30 CytoDEATH; Roche Diagnostics), and it appeared that these cytokine treatments did not induce any apoptotic response (data not shown). Note that the treatment of higher doses of the cytokines induced growth arrest or

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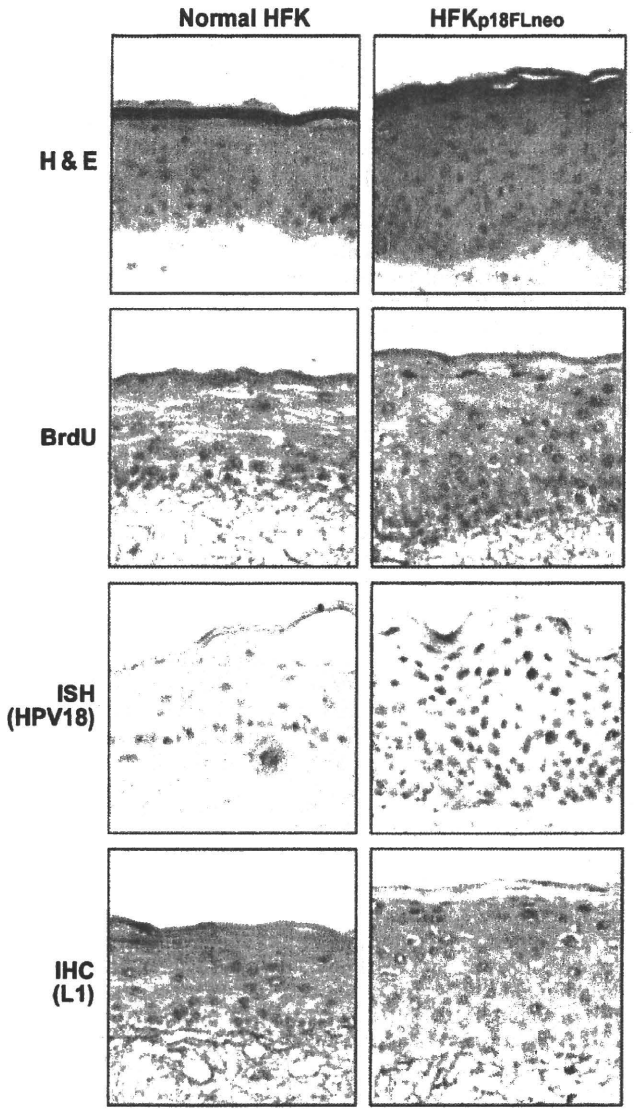

Fig. 3. Vegetative replication of the human papillomavirus (HPV) replicon in the raft culture. Thin sections (7 μm) were obtained from the raft culture organized with normal human foreskin keratinocytes (HFKs) and $HFK_{p18FLneo}$. The DNA synthesis of the cell was monitored by immunohistochemistry for incorporated BrdU. HPV18 DNA was detected by *in situ* hybridization (ISH) with DIG-labeled L1 probe. The expression of L1 protein was analyzed by IHC with anti-L1 antibody.

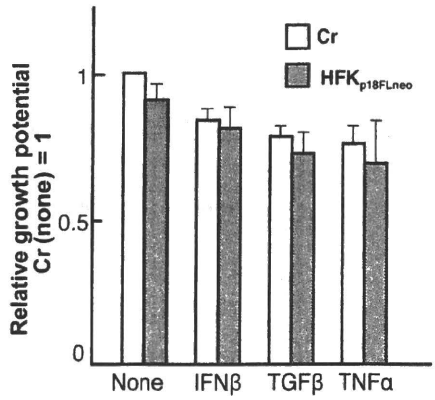

Fig. 4. Effects of cytokines on the proliferation of human foreskin keratinocytes (HFKs) harboring p18FLneo. The effects of cytokine treatments (interferon [IFN]-β, 100 units/mL; transforming growth factor [TGF]-β, 1 ng/mL; tumor necrosis factor [TNF]-α, 5 ng/mL) on the proliferation of HFKs were monitored. The growth rate in 72-h culture of the exponentially growing cells is indicated (growth rate of not treated normal HFKs, 1). The living cells were distinguished by Trypan blue exclusion. The value is the average of at least three independent experiments and the SD is indicated.

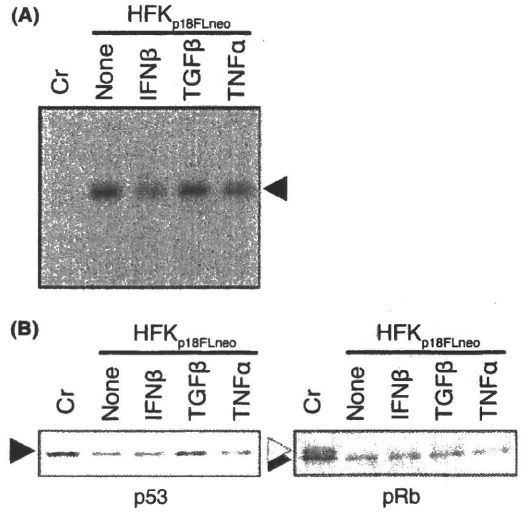

Fig. 5. Effects of cytokines on the maintenance of the human papillomavirus (HPV) replicon. (A) The $HFK_{p18FLneo}$ was treated with cytokines for 3 days and total DNA was extracted. The DNA was digested with both BglI and DpnI and 2 μg of it was subjected to Southern blot analysis with the DIG-labeled L1 probe. Normal human foreskin keratinocytes (HFKs) were used as the control (Cr). (B) Expressions of p53 and pRb in the same cells monitored by immunoblot analysis.

apoptotic cell death of both normal HFKs and $HFK_{p18FLneo}$ (data not shown).

Next, we examined the effects of the cytokines on the maintenance of HPV-DNA in $HFK_{p18FLneo}$, and the result indicated that IFNβ treatment moderately suppressed HPV-DNA replication (Fig. 5A). The TGFβ and TNFα treatments did not influence DNA maintenance significantly. In HPV-infected cells, the expressions of cellular tumor suppressors p53 and pRb are suppressed by the functions of viral oncoproteins E6 and E7, respectively. We analyzed the expressions of p53 and pRb in $HFK_{p18FLneo}$ by immunoblot analysis, and found that they slightly decreased as compared with those in the normal HFKs (Fig. 5B). This weak suppression indicated that the expression levels of the viral oncoproteins were kept low as found in the latent infection of HPV. The weak suppressions of p53 and pRb expressions were not modified significantly by the cytokine treatments.

The results indicated that the cytokine treatments used in this report did not affect the growth potential of $HFK_{p18FLneo}$ and the maintenance of HPV-DNA. Given that $HFK_{p18FLneo}$ is considered to represent basal cells latently infected with HPV, these results suggested that the cytokine treatments had no anti-HPV effect on the latently infected cells.

Application of the new HPV replicon: (2) Effects of cytokines on the late-stage of the HPV lifecycle. We next examined the effect of the same set of cytokines on the late-stage of the HPV lifecycle by using $HFK_{p18FLneo}$ and the raft culture. As described above, the raft culture with $HFK_{p18FLneo}$ showed a moderate hyperplasia at the epithelial layer (Fig. 3), and the hyperplasia