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# Photodynamic Therapy for Cervical Intraepithelial Neoplasia

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**Key Words**
Human papilloma virus · Excimer dye laser · YAG-OPO laser · Photofrin · Cervical cancer

**Abstract**
***Objectives:*** Photodynamic therapy (PDT) is a minimally invasive treatment for cervical intraepithelial neoplasia (CIN). We report the effectiveness of PDT in 105 cases of CIN. ***Methods:*** All patients received photofrin (PHE) 2 mg/kg intravenously and, 48–60 h later, phototherapy was performed using the Excimer dye laser or a YAG-OPO laser with an irradiation dose of 100 J/cm$^2$ using 630 nm wavelength. ***Results:*** Mild photosensitivity occurred in 48% (50/105) of patients. The complete response (CR) rate was 90% (94/105) at 3 months following treatment. In the remaining 11 patients, 5 patients had CIN1, 2 patients had CIN2, and 4 patients had mild cytologic findings. However, in 9 of these 11 patients, CR was achieved 6 months after PDT. In 69 patients, human papilloma virus (HPV) typing was performed before and after PDT therapy. Pre-treatment, 64 of 69 patients (93%), were HPV-positive including 30 cases of high-risk HPV (43%). Testing performed 3, 6 and 12 months following PDT revealed no HPV-DNA in 75% (52/69), 74% (48/65) and 72% (41/57) of patients. At present, the median follow-up period is 636 days (90–2,232 days). In 3 patients, recurrence requiring surgical treatment was identified at 646, 717 and 895 days after PDT. ***Conclusions:*** PDT is an effective and minimally invasive treatment for CIN, which also appears to eradicate HPV infection.



## Introduction

Cancer of the uterine cervix is one of the most common malignant neoplasms among women, and remains the leading female malignancy in developing countries [1]. In 1999, about 6,500 women were diagnosed with cervical cancer in Japan [2]. In the USA, approximately 13,000 women developed cervical cancer in the year 2000 [3]. Cervical intraepithelial neoplasm (CIN) is often the precursor to cervical cancer. In 70% of CIN, evidence of the human papilloma virus (HPV) is detected [4]. In cervical carcinogenesis, HPV is thought to inactivate the cell cycle regulators by inhibiting p53 and pRb proteins by E6 and E7 proteins [5–7]. HPV is divided into two types: high-risk types and low-risk types. Only the high-risk HPV can efficiently inactivate p53 and pRb.

The current treatment of CIN is primarily based on the surgical excision using laser, loop electrosurgical procedure or cold knife conization technique. Unfortunately, these treatments often lead to obstetric problems such

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as cervical incompetence in young women who go on to become pregnant [8]. There is a novel alternative for the treatment of neoplasia called photodynamic therapy (PDT). PDT involves the systemic administration of a tumor-localizing photosensitizer, followed by the laser irradiation of the affected area [9–11]. Since PDT is minimally invasive with no surgical excision, it should be a cervix-sparing treatment, which may be particularly attractive to women desiring to preserve fertility. This paper presents a large series of patients with CIN treated with PDT.

In this study we expand on our preliminary report of the therapeutic effect of PDT in 31 CIN cases which suggested that PDT is effective for treating cervical dysplasia, and for the eradication of cervical HPV [12]. We now describe the effectiveness PDT in 105 cases, of CIN with a median follow-up period of 636 days.

## Material and Methods

*Patients*

Between December 1996 and April 2004, 105 nonpregnant women with a histological diagnosis of CIN (CIN1: 4; CIN2: 6; CIN3: 95) were enrolled in this study. All patients hoped to retain their fertility and chose PDT for its potential as a cervix-preserving therapy. The nature and purpose of the study were fully explained to each patient, and all patients gave written informed consent. The study was approved by the institutional review board of Hyogo Medical Center for Adults and Osaka City General Hospital.

*PDT*

All patients received intravenous PHE, 2 mg/kg to photosensitize the lesions (Photofrin, Japan Wyeth Lederly , Tokyo, Japan). Phototherapy was performed using an Excimer dye laser (EDL) or a YAG-OPO laser (Ishikawajima-Harima, Heavy Industry, Tokyo, Japan) with an irradiation dose of 100 J/cm$^2$ using a 630-nm wavelength. The laser instruments were mounted on a colposcope with an optical path for the laser. For the endocervix, a specially designed intracervical probe was used. After treatment, all patients were hospitalized in a dark room with protection from light for 3 weeks. For the first week post-treatment, light was limited to 5–20 Lx, for the second week light was limited to 30–50 Lx, and for the third week light was limited to 50–100 Lx. The light was measured using a luxmeter. The patients were examined every 3 months after PDT treatment. The clinical effect was judged using cytology and directed biopsy. The primary responses were determined 3 months later after PDT. When these examinations showed no abnormal findings, the case was considered a complete remission (CR). Minor response (MR) indicates mild histological change indicating low or high grade squamous intraepithelial lesion (LSIL, HSIL) less severe than the primary disease, and partial response (PR) indicates mild cytologic findings indicating LSIL or HSIL without histologic change. Toxicity was determined using NCI-CTC ver2.

*Detection of HPV*

The cervical smears were collected with a cotton-tipped swab and preserved in the phosphate buffer at –80°C until analyzed. DNA was analyzed following the PCR-based methods previously described by Yoshikawa et al. [13] and Nagano et al.[14]. Briefly, samples were analyzed by L1 consensus primers for amplification and detection of HPV-DNA, and digested with *Rsa*I, *Dde*I, *Hae*III, *Hin*fI, *Xba*I, *Acc*I, *Pst*I and *Kpn*I for HPV typing by restriction fragment length polymorphism (RFLP). HPV-DNA types 16, 18, 31, 33, 51, 52, 56, 58, 61, 70 and 82 were determined with the sensitivity of 0.01–0.1 copy/cell.

## Results

*Clinical Response*

A total of 105 women enrolled in this study. The clinical characteristics of the patients are shown in table 1. Median age is 30 years (range: 19–49). Forty-eight patients were single, 50 patients were married and 7 patients were divorced. Seventy-four patients were nulliparous, and 31 patients were multigravida. Four patients had CIN1, 6 patients had CIN2 and 95 patients had CIN3. Toxicity was predominantly mild cutaneous photosensitivity (grade 1–2 in 49 patients, and grade 3–4 in 1 patient). Grades 1 and 2 cutaneous photosensitivity were cured within 2 weeks without any treatment. One patient suffered from grade 3 photosensitivity, because she worked in the sunshine during the summer season just after being discharged. She was cured with topical steroid treatment. Minimal vaginal discomfort and discharge was also described by some patients. Cervical stenosis occurred in 11 patients. PDT was performed safely for all the patients.

The CR rate was 90% (94/105) 3 months following PDT. In the remaining 11 patients, 5 patients had CIN1, 2 patients had CIN2, and 4 patients had mild cytological findings. However, in 9 of these 11 patients, CR was achieved 6 months following PDT. In contrast, 5 patients had newly detected disease after 6 months including 3 CIN1 lesions and 2 cases of mild cytological findings. For 76 patients, the 1-year follow-up results were as follows: 2 patients had CIN1, 2 patients had mild cytological findings and 72 patients achieved CR (95%: 72/76). In 15 CR cases, cervical cytology and biopsy were performed every 3 days after PDT for 2 weeks, and within 3 days of laser treatment, necrosis of the CIN region occurred and atypical cells disappeared in all cases.

At present, the median follow-up period is 636 days (range: 90–2,232 days). In 3 patients, recurrence occurred at 646, 717 and 895 days after treatment. Two of these

**Table 1.** Clinical course in all cases

| Patient | Age | MA | PRG | DEL | HIS | PS | HPV PRE | HPV 3M | HPV 6M | HPV 12M | HPV 24M | Effect 3M | Final prognosis | Duration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 27 | 1 | 0 | 0 | CIN3 | G1 | NEG | NEG | NEG | | | CR | NED | 1,253 |
| 2 | 35 | 1 | 4 | 1 | CIN3 | G1 | 16+OT | NEG | 52 | 52+OT | NEG | CR | NED | 1,361 |
| 3 | 29 | 1 | 0 | 0 | CIN3 | G1 | 16 | NEG | OT | OT | OT | CR | NED | 1,409 |
| 4 | 33 | 1 | 4 | 0 | CIN3 | G1 | 16 | NEG | NEG | NEG | NEG | CR | NED | 1,244 |
| 5 | 37 | 1 | 2 | 1 | CIN3 | G0 | 16 | NEG | NEG | NEG | NEG | CR | NED | 1,395 |
| 6 | 28 | 1 | 4 | 1 | CIN3 | G1 | 16 | NEG | NEG | NEG | | CR | NED | 567 |
| 7 | 29 | 1 | 1 | 1 | CIN3 | G1 | 16 | NEG | NEG | NEG | NEG | CR | NED | 1,255 |
| 8 | 26 | 1 | 1 | 1 | CIN3 | G1 | 16 | OT | 61 | 51 | NEG | CR | NED | 1,293 |
| 9 | 20 | 0 | 1 | 0 | CIN3 | G0 | 51 | NEG | NEG | NEG | NEG | CR | NED | 721 |
| 10 | 32 | 0 | 0 | 0 | CIN2 | G1 | 16 | NEG | NEG | NEG | NEG | CR | NED | 1,099 |
| 11 | 28 | 0 | 4 | 2 | CIN3 | G1 | 18 | NEG | NEG | NEG | NEG | CR | NED | 1,104 |
| 12 | 29 | 1 | 1 | 0 | CIN3 | G1 | 16 | NEG | NEG | NEG | NEG | CR | NED | 1,103 |
| 13 | 30 | 1 | 0 | 0 | CIN3 | G0 | 61 | NEG | NEG | NEG | NEG | CR | NED | 1,140 |
| 14 | 32 | 1 | 0 | 0 | CIN3 | G1 | | | | | | CR | NED | 1,140 |
| 15 | 25 | 0 | 4 | 0 | CIN3 | G0 | 53+OT | NEG | NEG | NEG | NEG | CR | NED | 1,116 |
| 16 | 33 | 1 | 6 | 2 | CIN3 | G0 | 52 | NEG | NEG | | NEG | CR | NED | 1,011 |
| 17 | 38 | 2 | 1 | 0 | CIN3 | G1 | 58 | NEG | NEG | NEG | NEG | CR | NED | 1,060 |
| 18 | 30 | 1 | 2 | 1 | CIN3 | G1 | 58 | NEG | NEG | NEG | OT | CR | NED | 810 |
| 19 | 36 | 0 | 0 | 0 | CIN3 | G0 | 35 | NEG | 16 | OT | NEG | CR | NED | 1,024 |
| 20 | 30 | 1 | 1 | 1 | CIN3 | G0 | 52 | NEG | NEG | NEG | NEG | CR | NED | 917 |
| 21 | 30 | 1 | 1 | 1 | CIN3 | G1 | 16 | NEG | NEG | NEG | | CR | RE | 717 |
| 22 | 25 | 0 | 0 | 0 | CIN3 | G1 | 16 | NEG | OT | NEG | | CR | NED | 372 |
| 23 | 33 | 0 | 0 | 0 | CIN3 | G0 | 52+OT | OT | NEG | | 52 | CR | NED | 919 |
| 24 | 19 | 0 | 1 | 0 | CIN3 | G0 | 70+OT | 70 | NEG | NEG | OT | MR | NED | 970 |
| 25 | 33 | 1 | 0 | 0 | CIN3 | G0 | 31 | 31 | OT | NEG | OT | PR | RE | 895 |
| 26 | 40 | 1 | 0 | 0 | CIN3 | G1 | 16+58 | OT | OT | OT | OT | CR | NED | 913 |
| 27 | 35 | 2 | 3 | 1 | CIN3 | G1 | 16 | 51 | 51 | NEG | NEG | CR | NED | 902 |
| 28 | 37 | 1 | 4 | 0 | CIN3 | G1 | NEG | NEG | NEG | NEG | NEG | CR | NED | 902 |
| 29 | 35 | 2 | 3 | 1 | CIN3 | G0 | 52+OT | NEG | NEG | NEG | 58 | MR | NED | 810 |
| 30 | 42 | 0 | 1 | 0 | CIN3 | G0 | 82 | 16 | NEG | 16 | 16 | CR | NED | 893 |
| 31 | 29 | 1 | 2 | 1 | CIN2 | G1 | 52 | NEG | NEG | NEG | | CR | NED | 803 |
| 32 | 28 | 0 | 0 | 0 | CIN3 | G1 | OT | NEG | NEG | NEG | NEG | CR | NED | 886 |
| 33 | 28 | 1 | 0 | 0 | CIN3 | G0 | 58 | NEG | NEG | NEG | | CR | NED | 733 |
| 34 | 39 | 1 | 1 | 0 | CIN3 | G0 | 16 | OT | NEG | NEG | OT | CR | NED | 756 |
| 35 | 29 | 1 | 1 | 1 | CIN3 | G0 | OT | NEG | NEG | NEG | OT | MR | NED | 620 |
| 36 | 30 | 1 | 1 | 1 | CIN3 | G0 | 82 | NEG | NEG | NEG | | CR | NED | 727 |
| 37 | 31 | 1 | 0 | 0 | CIN3 | G0 | 51 | NEG | NEG | NEG | | MR | NED | 557 |
| 38 | 33 | 1 | 3 | 2 | CIN3 | G0 | 51 | NEG | NEG | 51 | | CR | NED | 594 |
| 39 | 26 | 1 | 1 | 0 | CIN3 | G0 | NEG | NEG | OT | NEG | | CR | NED | 371 |
| 40 | 23 | 0 | 0 | 0 | CIN3 | G0 | 51 | NEG | NEG | | 16+53 | CR | NED | 636 |
| 41 | 36 | 1 | 0 | 0 | CIN3 | G1 | 16+35 | NEG | NEG | NEG | | CR | NED | 698 |
| 42 | 26 | 0 | 3 | 0 | CIN3 | G0 | 58 | NEG | NEG | NEG | | CR | NED | 558 |
| 43 | 23 | 0 | 0 | 0 | CIN2 | G0 | OT | 52 | 52+OT | 54 | | CR | NED | 547 |
| 44 | 33 | 0 | 0 | 0 | CIN3 | G0 | 51 | NEG | NEG | NEG | | CR | NED | 568 |
| 45 | 25 | 0 | 0 | 0 | CIN3 | G0 | 16 | 52 | 52 | 52 | | CR | NED | 529 |
| 46 | 28 | 0 | 0 | 0 | CIN3 | G0 | 58 | NEG | NEG | NEG | | CR | NED | 529 |
| 47 | 30 | 2 | 2 | 1 | CIN3 | G1 | OT | 34 | NEG | 16 | | CR | NED | 529 |
| 48 | 29 | 0 | 4 | 0 | CIN3 | G1 | 31 | NEG | OT | NEG | | CR | NED | 374 |
| 49 | 27 | 0 | 0 | 0 | CIN3 | G0 | 16 | NEG | NEG | OT | | CR | NED | 286 |
| 50 | 34 | 0 | 0 | 0 | CIN2 | G1 | 16 | NEG | | 68 | | CR | NED | 395 |
| 51 | 37 | 1 | 1 | 0 | CIN3 | G1 | 16 | NEG | NEG | NEG | | CR | NED | 381 |
| 52 | 31 | 0 | 3 | 0 | CIN3 | G0 | 18 | 18 | 18 | 18 | | CR | NED | 381 |
| 53 | 37 | 1 | 1 | 1 | CIN3 | G1 | 16 | NEG | NEG | NEG | | CR | NED | 371 |
| 54 | 32 | 1 | 2 | 0 | CIN3 | G0 | 16 | NEG | NEG | NEG | | CR | NED | 371 |
| 55 | 30 | 0 | 0 | 0 | CIN3 | G0 | 52 | OT | 51 | 68+OT | | CR | NED | 359 |

**Table 1** (continued)

| Patient | Age | MA | PRG | DEL | HIS | PS | HPV PRE | HPV 3M | HPV 6M | HPV 12M | HPV 24M | Effect 3M | Final prognosis | Duration |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 56 | 24 | 1 | 4 | 1 | CIN3 | G1 | NEG | NEG | NEG | | | CR | NED | 366 |
| 57 | 35 | 0 | 0 | 0 | CIN3 | G1 | 16+70 | 16+70 | NEG | NEG | | CR | NED | 356 |
| 58 | 33 | 1 | 1 | 1 | CIN3 | G0 | 16 | NEG | NEG | | | CR | NED | 480 |
| 59 | 31 | 1 | 4 | 2 | CIN3 | G1 | 16 | NEG | NEG | NEG | | CR | NED | 276 |
| 60 | 21 | 1 | 3 | 2 | CIN3 | G1 | 59 | 59 | NEG | | | CR | NED | 194 |
| 61 | 35 | 2 | 5 | 4 | CIN3 | G1 | OT | NEG | NEG | OT | | CR | NED | 283 |
| 62 | 22 | 0 | 0 | 0 | CIN1 | G0 | 52 | OT | 52+OT | | | CR | NED | 276 |
| 63 | 27 | 0 | 0 | 0 | CIN3 | G0 | 59+OT | OT | 33+OT | OT | | MR | NED | 324 |
| 64 | 29 | 1 | 0 | 0 | CIN1 | G0 | 58 | NEG | NEG | | | CR | NED | 352 |
| 65 | 26 | 0 | 0 | 0 | CIN3 | G0 | NEG | NEG | NEG | | | CR | NED | 269 |
| 66 | 21 | 0 | 1 | 0 | CIN3 | G1 | 16 | NEG | OT | | | CR | NED | 273 |
| 67 | 33 | 1 | 0 | 0 | CIN3 | G0 | 16 | NEG | NEG | | | CR | NED | 269 |
| 68 | 24 | 0 | 0 | 0 | CIN3 | G1 | | | | | | CR | MC | 269 |
| 69 | 29 | 0 | 3 | 1 | CIN3 | G1 | | | | | | MR | NED | 269 |
| 70 | 21 | 0 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 269 |
| 71 | 31 | 1 | 2 | 1 | CIN3 | G1 | | | | | | CR | NED | 266 |
| 72 | 41 | 2 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 276 |
| 73 | 24 | 0 | 3 | 0 | CIN3 | G1 | | | | | | CR | NED | 273 |
| 74 | 34 | 0 | 4 | 0 | CIN3 | G1 | | | | | | MR | MC | 153 |
| 75 | 40 | 0 | 0 | 0 | CIN3 | G1 | | | | | | CR | NED | 184 |
| 76 | 27 | 0 | 1 | 0 | CIN3 | G0 | | | | | | CR | NED | 90 |
| 77 | 38 | 0 | 4 | 0 | CIN3 | G1 | | | | | | CR | NED | 118 |
| 78 | 32 | 0 | 0 | 0 | CIN1 | G1 | | | | | | CR | NED | 94 |
| 79 | 33 | 1 | 1 | 1 | CIN3 | G0 | 16 | NEG | | NEG | | PR | RE | 646 |
| 80 | 25 | 0 | 0 | 0 | CIN3 | G1 | | | | | | CR | NED | 648 |
| 81 | 36 | 0 | 0 | 0 | CIN3 | G1 | | | | | | CR | NED | 101 |
| 82 | 35 | 2 | 1 | 1 | CIN3 | G3 | | | | | | CR | NED | 643 |
| 83 | 21 | 0 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 616 |
| 84 | 32 | 1 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 901 |
| 85 | 22 | 0 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 749 |
| 86 | 34 | 0 | 1 | 0 | CIN3 | G0 | | | | | | CR | NED | 845 |
| 87 | 24 | 1 | 0 | 0 | CIN3 | G1 | | | | | | CR | NED | 1,015 |
| 88 | 29 | 0 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 1,114 |
| 89 | 20 | 0 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 1,298 |
| 90 | 20 | 0 | 0 | 0 | CIN3 | G1 | | | | | | CR | NED | 1,112 |
| 91 | 27 | 1 | 1 | 0 | CIN3 | G1 | | | | | | CR | NED | 1,850 |
| 92 | 49 | 0 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 2,116 |
| 93 | 25 | 1 | 1 | 1 | CIN3 | G1 | | | | | | CR | NED | 1,410 |
| 94 | 26 | 1 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 2,232 |
| 95 | 29 | 0 | 0 | 0 | CIN3 | G0 | 58 | NEG | | NEG | NEG | PR | NED | 2,065 |
| 96 | 28 | 1 | 4 | 1 | CIN2 | G0 | 16 | NEG | | NEG | NEG | CR | NED | 2,035 |
| 97 | 25 | 1 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 1,653 |
| 98 | 34 | 0 | 1 | 0 | CIN2 | G0 | | | | | | CR | NED | 820 |
| 99 | 40 | 1 | 2 | 2 | CIN3 | G1 | | | | | | PR | NED | 481 |
| 100 | 35 | 1 | 2 | 1 | CIN3 | G0 | | | | | | CR | NED | 365 |
| 101 | 35 | 1 | 0 | 0 | CIN3 | G1 | | | | | | CR | NED | 360 |
| 102 | 39 | 0 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 354 |
| 103 | 33 | 1 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 180 |
| 104 | 30 | 0 | 0 | 0 | CIN1 | G1 | | | | | | CR | NED | 95 |
| 105 | 32 | 1 | 0 | 0 | CIN3 | G0 | | | | | | CR | NED | 93 |

MA = Marriage (0: single, 1: married, 2: divorced); PRG: pregnancy times; DEL = delivery times; HIS = histology; PS = photosensitivity; HPV PRE: HPV type before treatmenrt; HPV 3M = HPV type 3 months after PDT; NEG = negative; OT = Other; Effect 3M = effect of PDT after 3 months; PR = partial response; MR = minor response; NED = No evidence of disease; MC = minor change; RE = recurrence; Duration = duration after treatment (days).

patients had CIN3, and 1 patient (case 25) developed stage IB1 cervical cancer. All 3 patients required surgical intervention. Two additional patients had mild cytological changes. Of all 105 patients, 14 patients have become pregnant following PDT including 6 women who have delivered term babies without complications. The outcomes of the other 8 pregnancies include: 1 preterm delivery, 1 spontaneous miscarriage, 2 therapeutic abortions, 1 molar pregnancy, and 3 ongoing pregnancies. All clinical histories are summarized in table 1.

*HPV*

HPV typing was performed before and after PDT therapy for 69 patients. Before treatment, HPV was detected in 64 of 69 patients (93%), including 30 patients with high-risk HPV (16, 18). Three months after PDT, HPV-DNA could not be detected in 47 of 64 patients (73%) who showed HPV-DNA positive cervical smears before treatment. Seventeen patients still had HPV-DNA positive cervical smears, and in 13 of these 17 cases, HPV typing changed. Six months after PDT, 17 of 65 (26%) examined patients still had HPV-DNA positive cervical smears; however, these 16 patients had no abnormal cytological or histological findings. Additionally, in 15 of these 16 HPV-DNA positive cases, HPV typing changed compared to pre-PDT testing. One year after PDT, 16 of 57 (28%) examined patients still had HPV-DNA in cervical smears. Of these 16 patients, only 1 patient had mild abnormal cytological findings indicating LSIL. Additionally, in 13 of 16 HPV-DNA-positive patients, HPV typing changed compared to pre-PDT testing. Two years after PDT, 11 of 31 (35%) examined patients still had HPV-DNA in cervical smears. In 10 of 11 HPV-DNA positive patients, HPV typing changed compared to pre-PDT testing. Finally, 3 patients had recurrence (2 cases: CIN3; 1 case: invasive cancer); however, these 3 patients had negative HPV-DNA in cervical smears 1 year after PDT treatment.

## Discussion

In this study, we examined the effect of PDT on CIN and HPV in over 100 women. We found that over 90% of patients achieved CR after 3 months. Only three patients ultimately developed recurrent disease. There are several studies reporting disappointing results using PDT for the treatment of CIN [15–20]; however, these investigators used 5-aminolevulinic acid for a sensitization agent which we believe is inferior to the agent used in our study. For example, Hillemanns et al. [16] performed PDT using 5-aminolevulinic (5-ALA) for sensitization and an argon-ion-pumped dye laser in 7 women with high grade CIN. However, PDT did not appear to be effective in all patients. Keefe et al. [18] performed PDT using 5-ALA and argon-pumped dye laser in 40 CIN2 or 3 patients, and reported success rates at 4, 8 and 12 months were 51, 46 and 31%. Barnett et al. [19] reported that the response rate of PDT using 5-ALA was 33% in CIN1/2 patients. These reports suggest that PDT using 5-ALA is not effective for CIN. We achieved much better response when treating CIN with PDT using PHE for photo sensitization, and an ELD or YAG-OPO laser. In addition, PHE is reported to be more effective for cascular endothelial cell than 5-ALA [21, 22]. Muroya et al. [23] performed PDT using PHE and EDL for 56 patients (39 CIS and 17 dysplasia), and achieved high complete response rate comparable to our current study (96.4%, 54/56). However, in the Muroya report, follow up duration was short and HPV typing was not evaluated. Di Saia and Creasman [24] reported that the surgical treatment including cold-knife excision, electrocautery, cryosurgery and laser ablation achieved high success rates between 90 and 98%. Recurrence rate of conization for CIN was reported to be 0.6% [25–27]. From these findings, PDT may be somewhat inferior to surgical treatment for CIN. A comparative study is needed to solve this problem.

It is well known that HPV is the most prevalent etiologic agent in neoplastic transformation of squamous epithelial cells. Cervical carcinogenesis is related to specific high risk types of HPV, most commonly HPV 16 and 18. In our series, HPV was detected in 64 out of 69 patients (93%) and high-risk HPV (16, 18) detected in 30 (43%) patients before treatment. Finally, HPV-DNA was not detected in 75, 74 and 72% at 3, 6 and 12 months after PDT. These data are consistent with Wierrani et al.'s [17] report of 19 patients undergoing PDT. One year after PDT, 16 of 57 (28%) patients in our study still had HPV-DNA in cervical smears. Of these 16 patients, only 1 patient had mild abnormal cytological findings. Additionally, in 13 of 16 HPV-DNA-positive patients, HPV typing changed compared with testing before PDT. This suggests that the 13 patients might have been re-infected with other types of HPV since treatment. Furthermore, the 3 patients with recurrent pre-invasive or invasive disease had no HPV-DNA detected in cervical smears 1 year after PDT. Persistent HPV infection did not predict the recurrence of CIN. In fact, 16 patients with HPV persistence or re-infection had no recurrence of CIN during the follow-up period.

The follow-up period in our study is too short to determine the long term effectiveness of PDT for CIN treatment. Ylitalo et al. [28] reported that among HPV 16-positive women, the median incubation period from infection to carcinoma in situ was 7–12 years. In our study, 3 patients experienced recurrence despite negative HPV testing 1 year after PDT. Possibly, our HPV detection system may have lacked sensitivity in these cases. Alternatively, not only HPV infection, but also the status of the immune system, abnormality of cell cycle regulators, and p53 polymorphisms may contribute to the development cervical neoplasia [4, 29–31]. However, given the known lengthy incubation period of neoplasia, we speculate that these recurrent cases might be due to small undetectable lesions of CIN that persisted following PDT. Further study is needed to better understand the cases that are not cured by PDT.

In this study, all patients were hospitalized to ensure light deprivation for 3 weeks. With this rigorous light-deprivation protocol, 50 of 105 patients (48%) developed mild cutaneous photosensitivity (grades 1 and 2). Only one patient suffered from grade 3 cutaneous photosensitivity. It may be because she worked in the sunshine during the summer season just after being discharged. In other reports of PDT using 5-ALA, cutaneous photosensitivity was not reported despite light exposure [15–20]. However, we believe the superior therapeutic profile of PHE justifies its use despite the increased phototoxicity. We recognize that our protocol may be prohibitively expensive and inconvenient in many settings. Since toxicity was minimal, more liberal protocols of light deprivation may be appropriate. It was reported that lower doses of PHE such as 1 mg/kg was effective for cutaneous cancer [32–33]. Decreasing the PHE dose may reduces cutaneous photosensitivity. Establishing an outpatient protocol is one of our goals for future study.

In conclusion, PDT is an effective treatment for CIN, and for HPV infection. PDT may be an attractive alternative for women desiring to preserve cervical function for pregnancy. Furthermore, in our study, the persistence of HPV following treatment did not correlate well with CIN recurrence.

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S. Yamaguchi and H. Tsuda have contributed equally to this study.

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# Is laser conization adequate for therapeutic excision of adenocarcinoma *in situ* of the uterine cervix?

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## Abstract

***Aims:*** To determine the safety of uterine-preserving operations for adenocarcinoma *in situ* of the cervix.

***Methods:*** Fifteen cases of adenocarcinoma *in situ* (AIS) were diagnosed using neodymium:yttrium aluminum garnet (Nd:YAG) laser conization. The accuracy of preconization histology or cytology was evaluated in 15 AIS cases. In these AIS cases, we investigated how far the tumor was located from the squamocolumnar junction (SCJ) and the endocervix. Fourteen cases of the 15 AIS-affected patients were treated using laser conization alone. These patients were closely followed up.

***Results:*** Precise agreement between preconization diagnosis and conization histology was seen in 46.7% (7/15) of the AIS cases. In 14 of the 15 cases of AIS (93.3%), the tumor was adjacent to the transitional zone, within 3 mm of the SCJ, and in the other case (6.7%), the tumor was between 0 and 5 mm away from the SCJ. In all subjects, cone height was 8–18 mm (mean 13.1 mm). None of the 15 patients showed any recurrence of AIS during follow up ranging from 15 to 75 months (43.1 months on average).

***Conclusions:*** Women with AIS who want to preserve their fecundity might be treated with laser conization alone.

**Key words:** adenocarcinoma *in situ*, laser conization, squamocolumnar junction, uterine cervix, uterine preservation.

## Introduction

The incidence of uterine cervical adenocarcinoma among cervical cancers has been reported to be 5–9%,[1–4] or 5–15%;[5] thus, uterine cervical adenocarcinoma is assumed to be relatively rare. Cervical adenocarcinomas occur between the squamocolumnar junction (SCJ) and the internal os and are thus difficult to observe directly. Consequently, cervical adenocarcinoma might be missed in its early stages.

Even when a lesion is discovered, although it is still considered a microinvasive adenocarcinoma, a radical operation, including lymphadenectomy is frequently carried out because cervical adenocarcinomas are more aggressive than squamous cell carcinomas, and the prognosis is usually poor.[6] Thus, although the uterus often can be preserved in the treatment of early squamous cell carcinoma, preservation of the uterus is more difficult in cases of cervical adenocarcinoma. Some authors have reported that young women with cervical adenocarcinoma *in situ* (AIS) who want to preserve their fertility might be treated with a conservative procedure, such as conization of the uterine cervix if the surgical margins are free of cancer.[7–10] However,

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there is no consensus as to whether conization is adequate treatment for AIS because residual foci of tumor might remain and present subsequently as invasive adenocarcinoma.[11–13]

Comparing the diagnosis of pre- with post-cone biopsy, the accuracy of diagnosing AIS using cytology and punch biopsy also was evaluated in the present study. Furthermore, AIS patients treated at our hospital were reviewed retrospectively to determine the advisability of preserving the uterus in cases of AIS.

## Patients and Methods

Data from 15 patients with AIS of the cervix, as the final pathologic diagnosis treated at our hospital between 1989 and 2002, were reviewed for this study. All patients underwent laser conization of the uterine cervix. The same contact Nd:YAG laser conization procedure was carried out in all 15 patients. In summary, the excision was carried out with an output of 25 watts and at the endocervical margin, the cone was transected with scissors. The endocervical curettage was carried out in postoperative registration, and the cervical canal was always examined. All conization specimens were cut longitudinally. After being fixed at room-temperature in 10% formalin for 16–40 h, the cone specimens were step-sectioned by radical cuts, and the blocks were paraffin-embedded with sections cut at 3 μm and stained with hematoxylin and eosin. The mean number of blocks was 12.7 (range 8–24). The hospital's pathologist diagnosed all cases. In eight cases, the preoperative diagnosis was CIN3 or microinvasive carcinoma. In seven cases, laser conization was carried out because atypical glandular cells were present in cervical smears or AIS was identified in punch biopsy specimens. All patients were informed the risk of cervical adenocarcinoma if they underwent only laser conization.

The diagnosis before conization was compared with the pathologic diagnosis of the conization specimens. In the 15 cases where the pathologic diagnosis of the conization specimen was AIS, we measured the vertical distance of the cervical region domain between the SCJ and the distal edge of the tumor. The mean age of the AIS patients was 36.3 years. In the one case of AIS that was diagnosed using laser conization, an abdominal hysterectomy with lymphadenectomy was carried out. Postoperatively, all patients were followed up with cytologic and colposcopic examination every 3–4 months for as long as possible.

## Results

Among the eight cases where the preoperative diagnosis was CIN3 or microinvasive carcinoma, all conization specimens contained AIS. Of the 15 cases of AIS diagnosed postoperatively (cases 1–15), only seven (46.7%) were diagnosed accurately. Thirteen of the 15 women with AIS strongly wanted to preserve their fecundity and one case (no. 5) did not want the sequential hysterectomy. These women underwent only conization with close follow up. The remaining

**Table 1** The outcome of conization for adenocarcinoma *in situ* (AIS) of the cervix

| Case no. | Age (years) | Punch biopsy | Final diagnosis | Margin status | Follow up (months) | Recurrence |
|---|---|---|---|---|---|---|
| 1 | 37 | AIS | AIS + CIS | Cancer is free | 75 | (–) |
| 2 | 29 | AIS + CIS | AIS + CIS | Cancer is free | 67 | (–) |
| 3 | 40 | SD | AIS + SD | Cancer is free | 70 | (–) |
| 4 | 35 | AIS + CIS | AIS + CIS | Cancer is free | 36 | (–) |
| 5 | 49 | CIS | AIS + CIS | Cancer is free | 68 | (–) |
| 6 | 33 | CIS | AIS + CIS | Cancer is free | 57 | (–) |
| 7 | 44 | CIS | AIS + CIS | Cancer is free | 52 | (–) |
| 8 | 38 | AIS + CIS | AIS + CIS | Cancer is free | 17 | (–) |
| 9 | 35 | AIS | AIS + SD | Cancer is free | 51 | (–) |
| 10 | 41 | AIS | AIS + SD | Cancer is free | 15 | (–) |
| 11[†] | 36 | CIS | MIC + AIS | Cancer is free | 48 | (–) |
| 12 | 39 | MIC | MIC + AIS | Cancer is free | 32 | (–) |
| 13 | 32 | CIS | AIS + CIS | Cancer is free | 21 | (–) |
| 14 | 31 | AIS + SD | AIS + CIS | Cancer is free | 19 | (–) |
| 15 | 26 | CIS | AIS + CIS | Cancer is free | 18 | (–) |

[†]Patient no. 11 underwent sequential radical hysterectomy. Neither residual tumor nor lymph node metastasis was found in the hysterectomy specimen. CIS, carcinoma *in situ*; MIC, microinvasive carcinoma; SD, severe dysplasia.

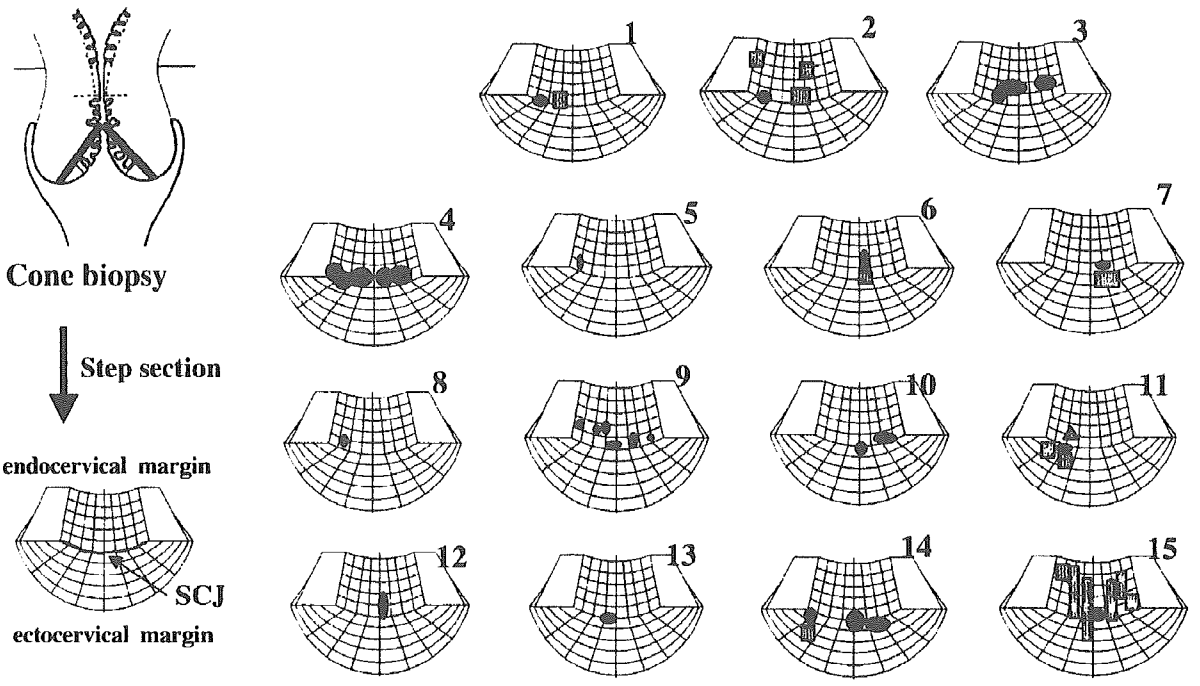


**Figure 1** Schematic representation of the location of (●) adenocarcinoma *in situ* (AIS), (▦) carcinoma *in situ* (CIS) and (▲) microinvasive carcinoma (MIC) in conization specimens. Conization specimens were divided into 8–24 blocks. Case numbers 3, 4, 5, 8, 9, 10, 12 and 13 contain neither CIS nor MIC in conization specimens. SCJ, squamocolumnar junction.

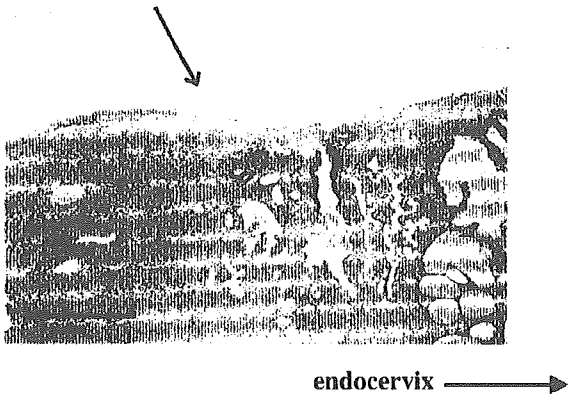


**Figure 2** Relation between adenocarcinoma *in situ* (AIS) and the squamocolumnar junction (SCJ; →). AIS exists in the surface of the uterine cervix. Normal cervical glands are seen in the deeper layers (hematoxylin and eosin, ×80).

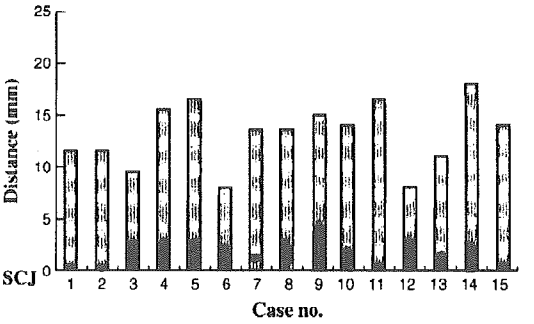


**Figure 3** Distance between the squamocolumnar junction (SCJ) and the surgical margin in patients with adenocarcinoma *in situ* (AIS) who underwent conization. AIS tumor was localized adjacent to SCJ. The surgical margins were free of cancer in all cases. (●) Distance between both tumor edges.

patient underwent hysterectomy and showed no cancerous lesions (Table 1). In all 15 patients, the locus in all cases except one was adjacent to the transitional zone, within 3 mm of the SCJ (Figs 1–3). In the remaining case, the tumor was 0–5 mm from the SCJ. Cone height of conization specimens in all patients was 8–18 mm (mean 13.1 mm). Four of the subjects became pregnant during the follow-up period. No patient suffered a recurrence. An abdominal operation was carried out in one subject.

## Discussion

The absence of recurrences among women with AIS after laser conization suggests that this modality is safe and effective for the treatment of these lesions.

If the surgical margin is always clear in conization specimens containing AIS, conization of the uterine cervix alone might be curative for AIS. However, AIS is difficult to diagnose using Papanicolaou smears, and the reported incidence is only 0.0002–0.004%.[5,14–18] Furthermore, AIS is difficult to diagnose in biopsy specimens because it does not always exhibit intraepithelial abnormalities during a colposcopy.[18,19] In this study, of 15 AIS cases, only seven were diagnosed as AIS preoperatively (46.7%; Table 1). In contrast, of 11 cases diagnosed or suspected as AIS preoperatively, four cases were diagnosed as having a microinvasive adenocarcinoma (MIAC) by laser conization procedure. Thus, conization of the cervix and histopathologic confirmation is essential for a definitive diagnosis of AIS. Some authors have stressed that conization of the cervix is not adequate treatment for AIS because the entire cervical gland area, where adenocarcinoma could occur, cannot be removed.[11,20,21] Azodi *et al.* reported that cervical adenocarcinomas are occasionally missed and that one of 16 cases of AIS (6.3%) thought to have been resected completely by conization had residual tumor tissue in subsequent conization or hysterectomy specimens.[20] Other authors have reported that when conization was carried out for AIS, with clear surgical margins, residual tumors have been found in 0–44% of surgical specimens when subsequent hysterectomies were carried out.[21–23] Thus, evidence exists that conservative treatment will cause incomplete resection in some cases.[11,12]

With regard to the possibility of incomplete resection, Lea *et al.* reported that endocervical curettage (ECC) is useful for detecting the presence of residual AIS.[24] In the present study, ECC was not carried out at the time of conization. Nevertheless, after conization, endocervical cytologic and colposcopic examination and ECC as needed were always carried out. We think doing so enabled early detection of residual lesions. However, the length of the cervical gland region of the conization specimens was not mentioned in those studies except one. Bertrand *et al.*[25] reported that the conization specimen should be at least 25 mm in depth and have negative margins to ensure that the patient has no residual disease and is therefore at a low risk of recurrence. In contrast, some authors state that AIS are adjacent to the SCJ. Andersen *et al.* maintained that because AIS develops in the transitional zone, conization with a clear surgical margin is adequate treatment.[18] Likewise, Teshima *et al.* reported that 90% of all AIS are located in the transitional zone or adjacent to the SCJ.[6] Of the 15 patients with AIS in this study, 14 tumors (93.3%) were within 3 mm of the SCJ. Our conization method excised at least 8 mm of the cervical gland region, so all 15 AIS surgical specimens had clear margins. Similarly, other authors have found that AIS might be treated using conization alone if the surgical margins are clear.[7–9]

It has been reported that adenocarcinomas often coexist with *in situ* squamous cell carcinomas.[6,11] All 15 of our confirmed cases of AIS coexisted with microinvasive carcinoma (stage Ia1), carcinoma *in situ* or severe dysplasia (CIN3).

The outcome of conservative treatments for AIS of the cervix is reportedly poor. Kuohung *et al.* found that AIS recurred in one of 12 patients (7%) treated with conization alone.[26] Widrich *et al.* described the outcome in 24 patients managed conservatively who had clear conization margins, two patients (8.3%) suffered a recurrence of AIS in that series.[27] In the present study, the follow-up period ranged from 15 to 75 months in patients treated with conization alone, and no patient developed recurrent disease.

In contrast, Östör *et al.* reported that the prognosis of microinvasive adenocarcinoma is similar to that of squamous cell carcinoma of the cervix and should be managed similarly.[28,29] However, other authors have emphasized that the risks of residual tumor and recurrent disease require more aggressive management.[10,30,31] We found that MIAC tended to occur relatively far from the SCJ compared with AIS.

Because AIS of the uterine cervix often occurs adjacent to the SCJ and is characterized by a low potential for lymph node metastasis,[32] women with AIS and clear surgical margins after conization might be followed without further treatment. However, radical surgery is probably advisable for patients with MIAC because MIAC often occurs at a relatively distant location from the SCJ, and conization might miss residual foci of tumor.

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# Comparison Between In Situ Hybridization and Real-time PCR Technique as a Means of Detecting the Integrated Form of Human Papillomavirus 16 in Cervical Neoplasia

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**Abstract:** Integration of the human papillomavirus (HPV) genome is thought to be one of the causes of cancer progression. However, there is controversy concerning the physical status of HPV 16 in premalignant cervical lesions, and there have been no reports on the concordance between detection of the integrated form of HPV16 by real-time PCR and by in situ hybridization. We investigated specimens of cervical intraepithelial neoplasia (CIN) and invasive carcinomas for the physical status of HPV 16 by real-time PCR and in situ hybridization. The presence of the integrated form was detected by both real-time PCR and in situ hybridization in zero of four cases of CIN1, three of six cases of CIN2, nine of 27 cases of CIN3, and two of six cases of invasive carcinomas. Integrated HPV 16 was present in some premalignant lesions but was not always present in carcinomas. The concordance rate between the two methods for the detection of the presence of the integrated form was 37 of 43 (86%) cases. Real-time PCR and in situ hybridization were found to be complementary and convenient techniques for determining the physical status of the HPV genome. We conclude that a combination of both methods is a more reliable means of assessing the physical status of the HPV genome in cervical neoplasia.

**Key Words:** human papillomavirus, real-time PCR, in situ hybridization, integration, cervical neoplasia

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Human papillomavirus (HPV) DNA is found in more than 90% of cervical carcinomas,[1] and HPV16 has been reported to be the dominant type of HPV in cervical carcinomas. Detection of HPV16 is proportional to the degree of cervical neoplasia: HPV16 has been detected in 15% of cervical intraepithelial neoplasia (CIN) 1, 30% of CIN2, and 50% of CIN3 and invasive carcinomas.[2,3] Integration of the HPV genome is thought to be one of the causes of cancer progression. Viral DNA that has been integrated into the host genome disrupts some early genes, such as the E1 and E2 open reading frames (ORFs), whereas the E6 and E7 ORFs remain intact. E6 protein binds to and degrades the tumor suppressor P53 protein via ubiquitin pathways.[4] E7 protein binds to the tumor suppressor Rb protein followed by E2F, transcriptional factor, compelled the cell-cycle rotation.[5] Münger reported that E7 protein is capable of causing aneuploidy in stratified squamous cells.[6] E7 protein plays an important role in the onset of dysplastic change, and the level of the expression of E7 protein may depend on the physical status of the HPV genome in cells. In an in vitro study, Lambert found that the integration of HPV16 results in an increase in E7 transcripts in cultured cells.[7] Thus, it is very important to determine the status of HPV16 genome in cervical neoplasia to elucidate the mechanisms of cervical cancer. In a study by real-time PCR, Peitsaro showed that integration had occurred in 23 of 24 premalignant lesions in patients with HPV16 infection.[8] By contrast, Southern blot analysis has shown that some cervical cancers contain the episomal form,[9,10] and thus there is still controversy concerning the physical status of the HPV16 genome in premalignant lesions and carcinomas of the cervix.[11–13] We realize that Southern blot analysis is the most reliable method of determining whether the integrated form is present in a specimen, but this analysis was not used in this study because only very small specimens were harvested from the premalignant lesions. By contrast, real-time PCR and in situ hybridization technique have been reported to be useful for investigating the physical status of the HPV genome in premalignant and cancerous specimens.[8,14–20] The question then arises as to the validity and concordance of real-time PCR and in situ hybridization. The purpose of this study was to assess the physical status of the HPV genome and to determine the degree of agreement between the results obtained by these two methods.

## MATERIALS AND METHODS

### Specimen and Sample Preparation

Patients diagnosed with cervical neoplasia on the basis of the results of biopsies performed during colposcopy at Keio University Hospital, in Tokyo, Japan, between October 2000 and April 2001 were enrolled in the study. Exfoliated cells into ThinPrep vials were collected with a broom device (Cervex Brush; Unimar, Wilton) just before colposcopy and then stored at ambient temperature, to be used for the HPV-DNA analysis

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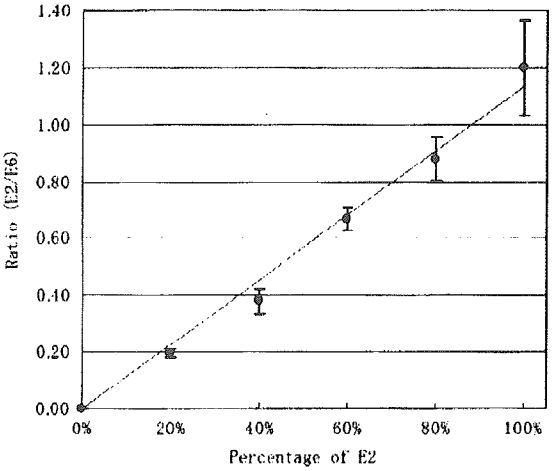


**FIGURE 1.** Control experiment using real-time quantitative PCR with the LightCycler system: the PCRE67 plasmid was mixed with a PK114/K plasmid to prepare a mixture of HPV 16 DNA. The PK114/K plasmid was replaced by an equivalent copy number of the PCRE67 plasmid in increments of 20%. DNA solutions containing episomal DNA (PK114/K exclusively), integrated DNA (pCRE67 exclusively), and a mixture form of HPV 16 DNA (20%-80% integrated) were prepared. The E2 and E6 copy number was quantitatively determined 6 times by real-time PCR assay, and the ratio of the E2 copy number to E6 copy number was calculated for each DNA solution. The cutoff value to distinguish the mixed form from the pure episomal form was set at 0.79. Details of calculation are described in the text.

within 12 months of collection. To perform the analysis, approximately 10 mL of preserved fluid from ThinPrep vials was centrifuged at 3,000 rpm for 30 minutes, and after washing the pellet once in phosphate-buffered saline, genomic DNA was extracted with proteinase K and phenol-chloroform. The quality and quantity of the extracted genomic DNA were monitored by ethidium-bromide– stained agarose gel electrophoresis. HPV DNA was identified by PCR analysis with consensus primer pairs designed to amplify an approximately 250-bp segment of HPV DNA.[21] These consensus primer pairs target the HPV L1 ORF and detect a broad range of genital HPVs. HPV typing and sequencing analysis were performed in all PCR-positive cases.[3]

## Real-Time PCR

To generate a pCR-TOPO E6/E7 plasmid (designated PCRE67 plasmid) which contains the E6 gene and E7 gene, a PK114/K plasmid containing a variant HPV16 genome, was amplified by PCR and introduced into the cloning site (Invitrogen, San Diego, CA). The primers for PCR amplification were as follows: E6/E7F, 5′-ATGTTTCAGGACCCACAGGAG-3′ (104-124) and E6/E7R, 5′-GGTAGATTATGGTTTCTGA-GA-3′ (844-864). The PK114/K plasmid contains one copy each of the E2 and E6 ORFs, whereas the PCRE67 plasmid carried the entire E6 ORF but lacked the E2 ORF. The PCRE67 plasmid was mixed with the PK114/K to prepare a mixture of the episomal form and the integrated form of HPV 16 DNA. The PK114/K was replaced by an equivalent copy number of the PCRE67 plasmid in increments of 20% to prepare DNA solutions containing the episomal form of HPV16 DNA (PK114/K exclusively), the integrated form of HPV16 DNA (PCRE67 exclusively), and both forms of HPV 16 DNA (20%-80% integrated form). The E2 and E6 copy numbers were quantitatively determined six times by real-time PCR, and the

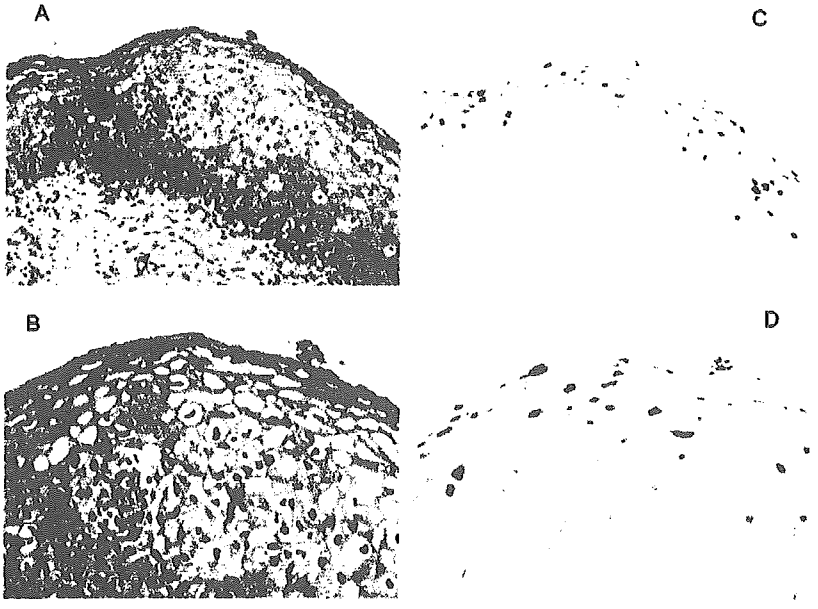


**FIGURE 2.** Case 3325, representative of the diffuse pattern: hematoxylin and eosin-stained section (A, ×20; B, ×40). In situ hybridization with the diffuse pattern (D) with the HPV16 probe (C, ×20; D, ×40).

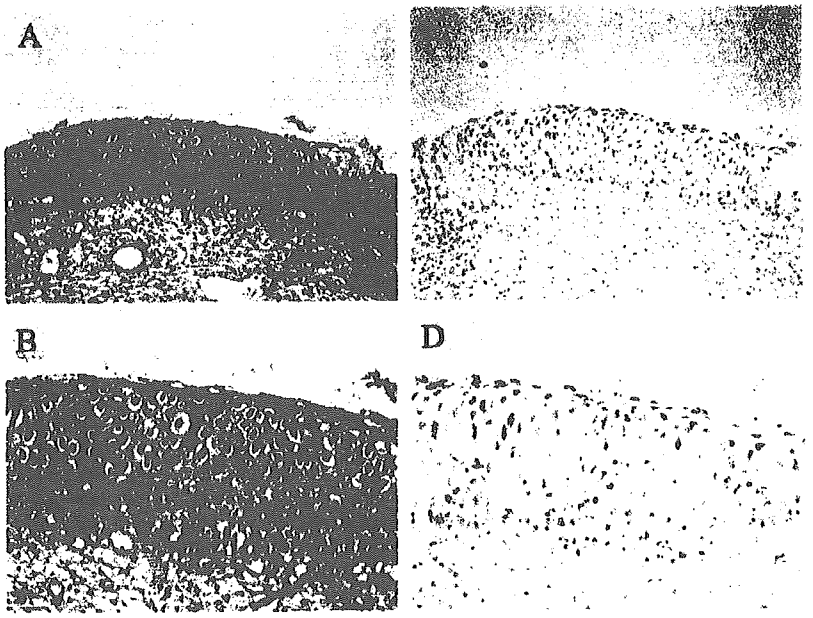


**FIGURE 3.** Case 613, representative of the spot pattern: hematoxylin and eosin-stained section (A, ×20; B, ×40). In situ hybridization with the spot pattern (S) with the HPV16 probe (C, ×20; D, ×40).

ratio of the E2 copy number to the E6 copy number was calculated for each DNA solution. Differences in ratio between the episomal form and other forms (pure integrated and mixed forms) were analyzed by the unpaired *t* test. Probability values less than 0.01 were considered statistically significant. Real-time PCR was performed with the LightCycler (Roche Diagnostics, Germany) system. PCR for the HPV-16 E2 gene was performed with 2 μL of master mix (LightCycler FastStart DNA Master Hybridization Probes, Roche Diagnostics) containing buffer, dATP, dCTP, dGTP, dUTP, Taq polymerase, 3 mmol/L $MgCl_2$, 0.2 μmol/L probe (Nihon Gene Research Laboratories, Japan), 0.6 μmol/L forward primer, 0.5 μmol/L reverse primer (Nihon Gene Research Laboratories Inc, Japan), a template DNA, and water added to a final volume of 20 μL. PCR for the HPV-16 E6 gene was performed with 2 μL of master mix (LightCycler FastStart DNA Master Hybridization Probes, Roche Diagnostics) containing buffer, dATP, dCTP, dGTP, dUTP, Taq polymerase, 4 mmol/L $MgCl_2$, 0.2 μmol/L probe (Nihon Gene Research Laboratories, Japan), 0.5 μmol/L of each primer (Nihon Gene Research Laboratories Inc, Japan), a template DNA, and water added to a final volume of 20 μL. The PCR cycle for the E2 and E6 gene included denaturation at 95°C for 10 seconds, annealing and extension 60°C for 25 seconds, and cooling at 40°C for 30 seconds, followed by 45 cycles of amplification. The primers used for PCR of the E2 gene were as follows: forward, 5′-TGTGTTTAGCAGCAACGAAG-3′ (3349-3368) and reverse, 5′-GCTGGATAGTCGTCTGTGTT-3′ (3454-3473). The probe was FAM-5′ CAAGGCGACGGCTTTGGTATGGGTC-3′-TAMRA (3418-3442). The primers used for PCR of the E6 gene were as follows: forward, 5′-CGACCCAGAAAGT-TACCA-3′ (125-142) and reverse, 5′-AGCAAAGTCATATA-CCTCACG-3′ (220-241). The probe used was FAM-5′ TTAT-GCACAGAGCTGCAAACAACTATACATGA-3′-TAMRA (146-177). Two standard curves were obtained by amplification of dilution series of $10^2$ to $10^7$ copies of the PK114/k plasmid.

**TABLE 1.** Concordance Between the Physical Status of the HPV Genome According to the Results of Real-time PCR and In Situ Hybridization

| | | ISH | | | | |
|---|---|---|---|---|---|---|
| | PCR | D | DS | S | Absence | Total |
| CIN I | Integration pattern | | | | | |
| | Presence | 0 | 0 | 0 | 0 | 0 |
| | Absence | 1 | 0 | 0 | 3 | 4 |
| CIN II | Integration pattern | | | | | |
| | Presence | 0 | 2 | 1 | 1* | 4 |
| | Absence | 2 | 0 | 0 | 0 | 2 |
| CIN III | Integration pattern | | | | | |
| | Presence | 3* | 3 | 6 | 0 | 12 |
| | Absence | 5 | 0 | 2* | 8 | 15 |
| MIC | Integration pattern | | | | | |
| | Presence | 0 | 0 | 1 | 0 | 1 |
| | Absence | 1 | 0 | 0 | 2 | 3 |
| SCC | Integration pattern | | | | | |
| | Presence | 0 | 0 | 1 | 0 | 1 |
| | Absence | 0 | 0 | 0 | 1 | 1 |
| Total | | | | | | 43 |

*Indicates discrepancies between the two methods.
MIC, microinvasive carcinomas; SCC, squamous cell carcinomas.
The shadow box represents the concordance between the results of the two methods for the presence of the integrated form.

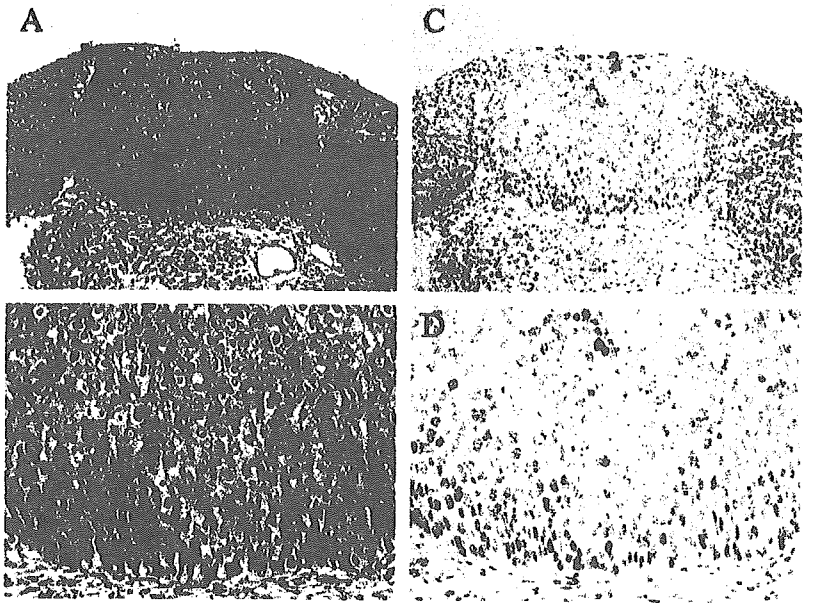


FIGURE 4. Case 91, representative of the diffuse and spot pattern: hematoxylin and eosin-stained section (A, ×20; B, ×40). In situ hybridization with the diffuse and spot pattern (DS) with the HPV16 probe (C, ×20; D, ×40).

### In Situ Hybridization

In situ hybridization was carried out with the GenPoint kit catalyzed signal amplification system (Dako Cytomation, Kyoto, Japan) as described in the instruction manual. Briefly, formalin-fixed, paraffin-embedded tissue slides were deparaffinized, pretreated with Target Retrieval Solution at 95°C for 40 minutes, and then exposed to proteinase K diluted 1:6,000 to 1:10,000 at room temperature for 10 minutes. The tissue slides were then soaked in 0.3% hydrogen peroxide in methanol at room temperature for 20 minutes, dehydrated through a graded alcohol series, and air dried. The biotin-labeled HPV16 probe was then applied to the tissue slides. After denaturing at 95°C for 5 minutes and hybridization at 37°C overnight, the tissue slides were washed in Stringent Wash Solution at 45°C or 55°C for 20 minutes and rinsed in Tris buffered saline with Tween 20 (TBST). The tissue slides were then incubated with 1:100 diluted horseradish peroxidase-conjugated streptavidin at room temperature for 15 minutes. After rinsing with TBST three times, the slides were incubated with biotinyl tyramine at room temperature for 15 minutes, and after washing the slides with TBST three times, they were incubated with horseradish peroxidase-conjugated streptavidin at room temperature for 15 minutes and washed three times in TBST. For signal development, 3, 3′-diaminobenzidine tetrahydrochloride was used as the substrate, and positive signals were detected in the form of a brown color under a light microscope. The sections were weakly counterstained with hematoxylin. The signal types were assessed as described previously.[22–24] Diffuse (D) signals, defined as diffuse positivity throughout the nuclei, were reported as the episomal form, Spot (S) signals localized in the nuclei, as the integrated form, and both diffuse and spot (DS) signals as a combination of the episomal form and the integrated form.

## RESULTS

### The Presence of the Integrated Form by Real-Time PCR

We examined 3,000 specimens of exfoliated cervical cells obtained from cervical cancer screening and investigated the 43 specimens in which HPV16 had already been identified by PCR followed by direct sequencing.[3] It is possible to investigate whether the integrated form is present or not by comparing the E2/E6 ratios by real-time PCR. In other words, if only the episomal form were present, the E2/E6 ratios would be almost the same, and if only the integrated form were present, the E2/E6 ratios would be very low. If the integrated form and episomal form were mixed, the E2/E6 ratios would be intermediate between the values for the pure episomal form and the integrated form. We therefore conducted the analysis in the assessment by real-time PCR in our study to determine whether the integrated form was present by examining the E2/E6 ratios. We set the cutoff value to distinguish the mixed episomal and integrated form from the pure episomal form was set at 0.79. The basis for setting the cutoff value at 0.79 is that we measured the E2/E6 ratio six times in a control experiment, and when we computed the mean and standard deviation and then calculated the 99% confidence interval, we obtained an interval of 0.79 to 1.61. Thus, if the E2/E6 ratio were less than 0.79, the chance of being the pure episomal form would be less than 1%, namely, integrated HPV16 DNA was therefore

considered to be present when the E2/E6 copy number ratio was below 0.79 (Fig. 1). Integrated DNA was found to be present in none of the four CIN1 cases, four of the six (66%) CIN2 cases, and 12 of the 27(44%) CIN3 cases. There were no significant differences between the CIN2 and CIN3 cases in percentage of positive cases, suggesting that integration may occur in the stage of CIN2. We also investigated six squamous cell carcinomas, consisting of microinvasive carcinomas and invasive squamous cell carcinomas, and two contained the integrated form (Table 1).

### Detection of the Integrated Form by In Situ Hybridization

One of four CIN1 cases yielded D signals, indicating the presence of the episomal form (Fig. 2). Five of the six CIN2 cases of yielded positive signals, and three (50%) yielded DS or S signals, indicating the presence of the integration. Twelve of 27 CIN3 cases yielded positive signals, and 9 (33%) yielded DS or S signals (Figs. 3 and 4). Investigation of six squamous cell carcinomas yielded S signals in two (33%), D signals in one, and DS signals in none (Table 1).

### Concordance Between the Results for the Detection of the Integrated Form by Real-Time PCR and by In Situ Hybridization

We compared the results of detection about the integrated form by both real-time PCR and in situ hybridization. The integrated form was not detected in CIN1 by either real-time PCR or in situ hybridization. In CIN2, both methods identified the integrated form in three (50%) of the six cases, and they identified the integrated form in nine (33%) of the 27 CIN3 cases. Although there was some difference between the results obtained by the two methods, the concordance rate for detection of the integrated form was 37 of 47 (86%) in Table 1. Thus, the results of the two methods for detection of the integrated form almost always agreed.

## DISCUSSION

HPV18 and HPV33 have been found in cervical carcinomas in their integrated forms, whereas HPV16 has been found in the pure integrated, the pure episomal form, or the mixed form. It is noteworthy that the physical status of HPV16 in cervical neoplasia is unique.[25,26] We therefore used real-time PCR and in situ hybridization to investigate whether cervical neoplasia contains the integrated form of the HPV16 genome. Real-time PCR has recently been used to determine the physical status of the HPV genome, and it has the advantage of being sensitive enough to use in small amount of samples. The template DNA can be obtained from exfoliated cervical cells preserved for liquid cytology, however, there has been a great deal of criticism of determination of the physical status of the HPV genome by real-time PCR. Since we selected the E2 and E6 regions for real-time PCR primers to compare differences in DNA amplification between the E2 and E6 ORFs, the physical status of the genome may have been misinterpreted based on the results. It is known to be limited discussion regarding the physical status only by real-time PCR. However, since there have been many reports that the E2 is most often disrupted when viral DNA is integrated into the host genome, we assessed the detection of the integrated form based on the E2/E6 ratio according to the results of real-time PCR. It is noteworthy that the incidence of integration was not as high as previously reported on the basis of real-time PCR.[8] We assume that the discrepancy is due to the cutoff values used in the control experiments.

Since the physical status of the HPV genome has also recently been investigated by in situ hybridization, we used in situ hybridization as an alternative assay for detection of the integrated form for comparison. The greatest advantage of in situ hybridization is that neoplastic area and nonneoplasite area of the same specimen can be examined by microscopy for positive signals. The in situ hybridization system used in our study was based on catalyzed amplification of positive hybridization signals with biotin tyramide complexes and does not require PCR amplification to improve sensitivity. Nevertheless, its sensitivity appeared to be poorer to that of the PCR technique, and that presumably explains the failure to detect positive signals in the CIN 1 (Table 1). Evans et al[15] reported detection of spot signals in 100% of CIN by in situ hybridization, but they observed spot signals even in low risk of HPVs genome such as HPV 6 or 11 under their conditions. There is controversy as to whether low-risk HPV is integrated into the host genome or not. Under the present circumstances, in which the low-risk type has not generally been recognized as being present as the integrated form, we should consider the sensitivity and specificity of their assay. Since the results obtained with our high-sensitivity in situ hybridization almost completely coincided with the results of real-time PCR, we concluded that our assay more closely reflects reality.

The discrepancy between the results obtained by two methods in our study may be due to the difference between the samples. Exfoliated cells were investigated by real-time PCR, whereas biopsy material was examined by in situ hybridization. Despite the difference in such an experimental condition, we found strong concordance between the results of real-time PCR and in situ hybridization. We conclude that a combination of real-time PCR and in situ hybridization is a more reliable method than using either method alone to determine the physical status of the HPV genome.

We also investigated the physical status of the HPV genome in our specimens and the clinical outcome of the patients. The group with having the integrated pattern tended to show persistent HPV infection; however, in these cases, laser vaporization was performed during the observation period at the patients' request (data not shown). Therefore, we could not conclude that integration is an indicator of a poor outcome. Although the lesions regressed spontaneously in most CIN2 cases, the integrated form was found in some CIN2 cases. It remains unresolved whether the presence of the integrated form is an indicator of progression of cervical neoplasia because the integrated form was not detected in all of the invasive carcinomas. From the standpoint of the gynecologic oncologist, the CIN classification still seems to be the ordinary international terminology for histologic diagnosis. Our data have demonstrated that the pattern of HPV gene presence is different in the CIN1 group and the CIN2 group. In other words, they clearly show that CIN1, ie, low-grade squamous

intraepithelial lesions (LSILs), differ from CIN2 or higher-grade lesions, ie, high-grade squamous intraepithelial lesions (HSILs), biologically as well. If it is found that SIL, which is generally used in cytologic terminology, is theoretically consistent from a molecular biology standpoint as well, it seems that the concept of SIL will be widely recognized in the future as histologic terminology. We feel that our study is important from this viewpoint as well. On the other hand, since clinical regression also occurs in many CIN2 cases, there is a problem in deciding on CIN2 and higher-grade cases as targets for treatment. Differentiating between CIN2 and CIN3 is important in terms of deciding on treatment policy. Thus, at the present time making a histologic diagnosis into just two groups, an LSIL group and an HSIL group, is difficult for clinicians to accept. That is why we used the biopsy samples and the CIN classification to describe them in the present study. The incidence of anti E7 antibody in cervical neoplasia patients has been reported to be 20% to 50%,[27,28] but the relationship between E7 expression in cells and the incidence of anti E7 antibody remains unresolved. If production of anti E7 antibody in cervical neoplasia patients is related to the physical status of the HPV genome, it might be useful to work out a strategy for therapeutic vaccination for the E7 protein in the future.

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# Transcriptional expression of the genes implicated in angiogenesis and tumor invasion in cervical carcinomas

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**Abstract**

*Objective.* Co-expression patterns of the genes implicated in angiogenesis and tumor invasion in cervical carcinoma cells were investigated together with invasive activity of tumor cells. Transcript levels of those genes were also compared between tumor cells and normal cervical tissues.

*Methods.* Real-time quantitative RT-PCR analysis was conducted on selected 11 genes (total VEGF-A, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, VEGF-B, C and D, bFGF, dThdPase, MMP-2 and uPA) using 11 cervical carcinoma cell lines and 14 normal cervical tissues. Protein expression of VEGF-C and MMP-2 and invasive activity of tumor cells were evaluated for each cell line by sandwich ELISA and haptoinvasion assay, respectively.

*Results.* Gene co-expression analysis revealed the significant correlation between angiogenic factors and proteinases in malignant but not in normal cervical samples. Gene or protein expression levels of VEGF-C and MMP-2 were well correlated with the number of invaded tumor cells. VEGF-A splicing variants were increased in malignant compared to normal cervical samples but not associated with the invasive activity of the cells.

*Conclusion.* VEGF-C and MMP-2 were closely related to invasive phenotype of tumor cells, whereas VEGF-A isoforms were considered to be involved in cervical carcinogenesis.


*Keywords:* Angiogenesis; Invasion; VEGF-C; MMP-2; Cervical carcinoma

## Introduction

Growth of solid tumors depends on angiogenesis, the process by which new blood vessels develop from the endothelium of a pre-existing vasculature [1]. Tumors promote angiogenesis by secreting various angiogenic factors, and newly formed blood vessels induce tumor cell proliferation and invasiveness. Various peptide growth factors, such as vascular endothelial growth factor (VEGF) [2,3], basic fibroblast growth factor (bFGF) [4,5] and thymidine phosphorylase (dThdPase) [6–8], have been found to stimulate the proliferation and motility of endothelial cells, thus inducing new blood vessel formation. Molecular cloning has revealed five different isoforms of VEGF-A which are generated from a single mRNA by alternative splicing and which have different biochemical features and biological effects [9,10]. Recently, three new members of the VEGF family, VEGF-B, C and D, have been also discovered and characterized [11–13]. It has been suggested that VEGF family members, bFGF and dThdPase, are expressed in a variety of human tumors in different ways.

Metastatic spread of the solid tumor depends on a critical cascade of events that includes tumor cell adhesion, migration, invasion, proliferation and ultimately neovascularization [14]. These biological processes require

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