

Loss of Heterozygosity on Chromosome 6p21.2 as a Potential Marker for Recurrence after Radiotherapy of Human Cervical Cancer¹

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ABSTRACT

Cervical carcinomas develop as a result of multiple genetic alterations, and specific alterations lead to specific clinical behavior. However, the effect of such alterations on the recurrence of cervical cancer after radiotherapy remains unknown. Chromosome arm 6p is one of those most frequently involved in a loss of heterozygosity (LOH) in patients with cervical carcinoma. The aim of this study was to identify the correlation between the LOH on chromosome 6p21.2 and the recurrence of cervical cancer after radiotherapy. A total of 62 patients with cervical cancer (stage I, 4 patients; stage II, 9 patients; stage III, 37 patients; and stage IV, 12 patients) were included in this study. All patients were treated with definitive radiotherapy. We analyzed specimens from the tumors and venous blood of all patients. Tumors and normal DNA were analyzed by PCR for genetic losses at three polymorphic microsatellite loci (*D6S276*, *D6S1624*, and *D6S1583*). Chromosome 6p21.2 is involved in the LOH in 46.8% (29 of 62) of the informative carcinomas. Ten patients had a local recurrence, 4 had distant metastases, and 13 had both local recurrence and distant metastases after radiotherapy. To evaluate the relationship between the recurrence after radiotherapy and LOH on chromosome 6p21.2, we divided the patients into those with cancer recurrence ($n = 27$) and those without recurrence ($n = 35$). LOH on chromosome 6p21.2 was correlated with recurrence after radiotherapy ($P = 0.006$). The tumors in patients with recurrence were significantly larger than those in patients without recurrence ($P = 0.003$). However, there was no correlation between the sizes and stages of tumors and the

LOH on chromosome 6p21.2. In addition, both overall survival and relapse-free survival were significantly worse for the patients with LOH as compared with those without LOH ($P = 0.02$ and $P = 0.002$, respectively). The results of this study suggest that LOH on 6p21.2 is correlated with recurrence of cervical carcinoma after radiotherapy.

INTRODUCTION

Cervical cancer is one of the most common tumors affecting women worldwide, both in incidence and mortality (1), and radiotherapy is the most important nonsurgical treatment for cervical carcinoma. The tumor's stage is still believed to be the most important determinant of prognosis in patients with cervical carcinoma, but tumor size has also been used as a marker for the response to radiotherapy and the patient's prognosis (2).

The involvement of the HPV³ in the development of cervical cancer has been firmly established. Because HPV infection does not always lead to cervical cancer, other genetic alterations must also play a role in tumor development. A LOH, which points to a role for TSGs, oncogene amplification, and point mutations, are all thought to be involved, but there is as yet no complete picture of the relative roles for each of these genetic changes in patients with cervical carcinomas. To play a role in tumorigenesis, both copies of a TSG must be inactivated. The loss of one allele in a chromosome region may point to the presence of a TSG in that region.

Several studies have shown that LOH at specific chromosomal sites is frequently associated with the recurrence of various cancers, *e.g.*, 13q14.3 in oral carcinoma (3), 10q in human lung cancer (4), and 11p15 in breast cancer (5). Although cytogenetic studies of cervical cancer are relatively few, they have revealed frequent, nonrandom chromosomal changes (6). Studies of LOH in patients with cervical carcinoma have also reported a high frequency of allelic deletions affecting 3p (7–9), 5p (10), 17p (11–14), and 18q (15). A LOH on chromosome 6p has also been reported in patients with cervical carcinoma (12, 13, 16, 17). However, the importance of LOH on chromosome 6p in the recurrence of cervical cancer after radiotherapy remains unknown.

In this study, we evaluated the incidence of LOH on 6p21.2 in the DNA of patients with cervical cancer and assessed the impact on the recurrence after radiotherapy.

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³ The abbreviations used are: HPV, human papillomavirus; LOH, loss of heterozygosity; TSG, tumor suppressor gene; MRI, magnetic resonance imaging; CR, complete response; PR, partial response; NC, no change; SSCP, single-strand conformation polymorphism.

Table 1 Clinical characteristics of 62 patients with cervical carcinoma

Characteristic	
Total number of patients	62
Mean age (yr)	66
SD	12.4
FIGO ^a stage	
I	4
II	9
III	37
IV	12
Histology	
Squamous cell carcinoma	55
Adeno carcinoma	7
Tumor size (cm)	
Mean	5.7
SD	2.3
<i>p53</i> status	
Wild-type	55
Mutant-type	7
HPV infection	
Positive	26
Negative	36

^a FIGO, the International Federation of Gynecology and Obstetrics classification.

MATERIALS AND METHODS

Patient Characteristics. Between January 1995 and April 1999, 62 patients with histologically proven carcinoma of the uterine cervix (4 stage Ib, 9 stage IIb, 1 stage IIIa, 36 stage IIIb, 6 stage IVa, and 6 stage IVb) were treated with definitive radiotherapy at Kansai Medical University (Table 1). The follow-up for the surviving patients ranged from 1 to 55.8 months, with a mean of 24.6 months. The mean patient age was 66 years (range, 27–90 years). The primary tumors ranged in diameter from 1 to 11.9 cm (mean, 5.7 cm), as measured with MRI. The typical examination included a medical history, a physical examination, routine blood counts, a blood chemistry profile, a chest radiograph, an i.v. urogram, colonoscopy, and MRI. Each patient's tumor was staged jointly by the radiation oncology and gynecological oncology staff according to the classification of the International Federation of Gynecology and Obstetrics (18), with modifications. The tumors consisted of 55 squamous cell carcinomas and 7 adenocarcinomas. No patient received chemotherapy prior to the radiotherapy. Each of the patients signed a consent form approved by the Kansai Medical University Review Board describing the experimental nature of the treatment.

Irradiation Techniques and Doses. All patients entered in the protocol were treated with external pelvic radiation therapy using 6-MV, high-energy linear accelerators. A total of 30.6 Gy was provided to the whole pelvis. An additional dose was given to the parametria with central shielding to complete 52.2 Gy, and patients also received ¹⁹²Ir high-dose-rate intracavitary brachytherapy. Radiation was delivered to the tumor in fractions of 1.8 Gy daily, 5 days/week. The dose of ¹⁹²Ir brachytherapy was 30 Gy to point A (2 cm lateral to the central canal of the uterus and 2 cm up from the mucous membrane of the lateral fornix in the axis of the uterus; Ref. 19), given at 7.5 Gy per session once a week.

Clinical Response. The response of the tumor to the treatment was defined as follows: CR when no tumor was

detected by physical examination or MRI and cytological or biopsy studies were negative for malignant cells for at least 1 month after treatment; PR when the tumor mass was reduced by $\geq 50\%$; and NC when the reduction in the tumor mass was $< 50\%$.

Detection and Typing of HPV. Tumor samples were obtained from the 62 patients by punch biopsy prior to radiotherapy. Samples were taken from two to four different parts of each tumor and frozen immediately at -80°C . Genomic DNA was extracted from each tumor according to standard protocols (20).

Tumor DNA was amplified by PCR with primers specific for HPV types 16, 18, 33, and 58 E6, as described previously (21). PCR was carried out for 40 cycles at 95°C for 1.5 min, 48°C for 1.5 min, and 70°C for 2 min, using a BioGene PHC-1 system (Techne, Cambridge, United Kingdom).

Investigation of *p53* Status. Mutations of the *p53* gene were identified by a SSCP analysis and DNA sequencing of tumor samples (22) with primers flanking the evolutionarily conserved regions of the gene from exon 5 to exon 8. Each exon was amplified separately using sense and antisense oligonucleotide primers flanking the exon as shown: exon 5, sense 5'-TCCTCTTCCTGCAGTACTC-3' and antisense 5'-GCCCCAGCTGCTCACCATCG-3'; exon 6, sense 5'-CACTGATTGCTCTTAGGTCTG-3' and antisense 5'-AGTTGCAAA-CCAGACCTCAG-3'; exon 7, sense 5'-CCAAGGCGCACT-bpGGCCTC-3' and antisense 5'-GCGGCAAGCAGAGGC TGG-3'; and exon 8, sense 5'-CCTATCCTGAGTAGTGGTAA-TC-3' and antisense 5'-GTCCTGCTTGCTTACCTCGC-3'. All mutations were confirmed by sequencing or an SSCP analysis of a second independent PCR reaction. The products of this reaction were subcloned into a TA vector (TA Cloning kit; Invitrogen). A mixture of at least 62 subclones was used as the template for DNA sequencing using a T7 sequencing kit (Boehringer Mannheim), and the results were visualized by exposure to Kodak XAR film with an intensifying screen at -80°C .

Investigation of LOH on 6p21.2. Regions of the genomic DNA extracted from the blood and tumor of each patient were amplified. PCR was used to detect the presence of LOH on chromosome 6p with the three microsatellite markers *D6S276*, *D6S1624*, and *D6S1583* (6p21.2). The PCR of each region was cycled 35 times at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min using each fluorescent set as described previously (23). The PCR products were loaded on a 6% polyacrylamide gel and were analyzed with automatic sequencing (ALFred; Pharmacia, Uppsala, Sweden). Allelic losses were scored as decreases in the intensity of one allele relative to the other, as determined from a comparison of tumor and normal DNA. The shift was indicated by either an addition or a deletion of one or more repeat units, resulting in the generation of novel microsatellite alleles. The analysis in patients was repeated at least twice, and the results were highly reproducible.

Statistical Methods. Survival was measured as the time (days) from the start of radiotherapy. The relationship between the presence or absence of genetic alterations on chromosome 6p21.2 and the radioresponse was analyzed with Fisher's exact test. The tumor size data were analyzed with a Wilcoxon rank sum test. Actuarial survival was estimated by the Kaplan-Meier (24) method, and differences in survival were analyzed with the

log-rank test (25). The statistical analyses were performed with Stata 4.0 Software (Stata Statistical Software, Release 4.0; Stata Corp., College Station, TX). $P < 0.05$ was considered significant.

RESULTS

In this series, 79% of patients (49 of 62) had stage III or stage IV disease. The number of patients with CR was 40 (64.5%), with PR was 10 (16.1%), and with NC was 12 (19.4%). At present, 32 patients (51.6%) are alive and well, 10 have had a local recurrence, 4 have had distant metastases (2 with multiple metastases, 1 with lung, and 1 with para-aortic lymph nodes), and 13 have had both local recurrence and distant metastases (9 with multiple metastases, 1 with lung, and 3 with para-aortic lymph nodes). Six patients are alive with cancer. Twenty-one patients (33.9%) have died from recurrent disease.

Relationships among Stage, Tumor Size, Clinical Response, and Outcome. There was a correlation between the stage of disease and the patients' response. Twelve of 13 patients with stages I and II showed CR. In contrast, 21 of the 49 patients with advanced stage cancer (*i.e.*, stage III or IV) showed PR or NC ($P = 0.02$, Fisher's exact test). The tumor sizes were 3.9 ± 1.8 cm in the stage I-II patients and 6.1 ± 2.2 cm in stage III-IV patients. Statistical analysis using the Wilcoxon rank sum test revealed significant differences in tumor sizes between stage I-II *versus* stage III-IV ($P = 0.002$). The tumor size increased with the stage of disease. The tumor sizes of patients with CR was 4.8 ± 1.9 cm; it increased to 7.2 ± 2.2 cm in patients with PR or NC. The difference in size was significant between the CR and PR/NC groups ($P = 0.0002$, Wilcoxon rank sum test). The tumor size of the patients without recurrence was 4.9 ± 2 cm; it increased to 6.7 ± 2.3 cm in patients with recurrence. The difference in size was significant between the presence and absence of recurrence ($P = 0.003$, Wilcoxon rank sum test; Table 2). The mean tumor diameter of all patients was 5.7 ± 2.3 cm. We divided the patients into two groups, those with tumors <5.7 cm in diameter ($n = 35$) and those with tumors ≥ 5.7 cm in diameter ($n = 27$). There was a significant difference in overall survival ($P = 0.0004$, log-rank test) and disease-free survival ($P = 0.01$, log-rank test) between these groups; the former group survived significantly longer than the latter group.

The Presence of HPV Infection, Stage, Tumor Size, Clinical Response, and Outcome. HPV types 16, 18, 33, or 58 genomes were detected with PCR in 26 patients (41.9%): in 1 stage I, 7 stage II, 13 stage III, and 5 stage IV. The tumor size was 5.9 ± 2.3 cm in the HPV-positive tumors *versus* 5.5 ± 2.3 cm in the HPV-negative tumors; there was no correlation between tumor size and HPV status (Wilcoxon rank sum test). Of the HPV-positive patients, 18 had CR, 3 had PR, and 5 had NC. Of the HPV-negative patients, 22 had CR, 7 had PR, and 7 had NC. There was no correlation between the tumor response and HPV status (Fisher's exact test). Of the HPV-positive patients, 12 had recurrence and 14 had no recurrence. Of the HPV-negative patients, 15 had recurrence and 21 had no recurrence. Thus, there was no correlation between recurrence and HPV status (Fisher's exact test; Table 2). There was also no significant difference in overall survival (log-rank test) and disease-

Table 2 Relationship between presence/absence of recurrence and clinicopathological factors of 62 patients with cervical carcinomas

	Recurrence (+) (n = 27)	Recurrence (-) (n = 35)	P
Age (yr)			
Mean	63.2	68.2	NS ^a
SD	13.7	11	
Stage			
I-II	3	10	NS ^b
III-IV	24	25	
Histological type			
Squamous cell carcinoma	24	31	NS ^b
Adenocarcinoma	3	4	
Tumor size (cm)			
Mean	6.7	4.9	0.003 ^a
SD	2.3	2	
p53 status			
Wild-type	24	31	NS ^b
Mutant-type	3	4	
HPV infection			
Positive	12	14	NS ^b
Negative	15	21	
6p LOH			
Positive	18	11	0.006 ^b
Negative	9	24	

^a Wilcoxon rank sum test. NS, not significant.

^b Fisher's exact test.

free survival (log-rank test) between HPV-positive and HPV-negative patients.

Relationships between p53 Status and Stage, Tumor Size, Clinical Response, and Outcome. p53 mutations were detected in seven patients (11.3%) with SSCP analysis, in five patients with stage III, and two with IV. We observed p53 mutations in several codons: in codon 245, changing GGC to AGC and causing the substitution of serine for glycine; in codon 146, TGG to TAG, resulting in the substitution of a stop codon for tryptophane; in codon 189, GCC to GTC, causing the substitution of valine for alanine; in codon 286, GAA to CAA, resulting in the substitution of glutamine for glutamic acid; in codon 285, GAG to AAG, resulting in the substitution of lysine for glutamic acid; in codon 213, CGA to TGA, resulting in the substitution of a stop codon for arginine; in codon 146, TGG to TAG, resulting in the substitution of a stop codon for tryptophane; and in codon 193, CAT to CGT, resulting in the substitution of histidine for arginine.

The tumor size was 7.7 ± 3.2 cm in the mutant p53 tumors *versus* 5.4 ± 2.1 cm in the wild-type p53 tumors; there was a correlation between tumor size and p53 status ($P = 0.049$, Wilcoxon rank sum test). Of the patients with mutant p53, four had CR and three had NC. Of the patients with wild-type p53, 36 had CR, 10 had PR, and 9 had NC. Thus, there was no significant relationship between any group and the p53 status (Fisher's exact test). Of the patients with mutant p53, three had recurrence and four had no recurrence. Of the patients with wild-type p53, 24 had recurrence and 31 had no recurrence. Thus, there was no correlation between recurrence and the patient's p53 status (Fisher's exact test; Table 2). There was also no significant difference in overall survival (log-rank test) and disease-free survival (log-rank test) between patients with wild-type p53 and those with mutant p53.

Relationships between LOH on Chromosome 6p21.2 and Stage, Tumor Size, Clinical Response, and Outcome.

Chromosome arm 6p21.2 was involved in LOH in 46.8% (29 of 62) of the informative carcinomas. The tumor size was 5.9 ± 2.1 cm in patients with LOH versus 5.5 ± 2.5 cm in the 33 patients without LOH; there was no correlation between tumor size and LOH status (Wilcoxon rank sum test). LOH on 6p21.2 was seen in 1 patient with stage I disease, 7 stage II, 14 stage III, and 7 stage IV. There was no correlation between the patient's LOH status and the tumor stage (stage I-II versus stage III-IV; Fisher's exact test).

Of the patients with LOH, 15 had CR, 6 had PR, and 8 had NC. Of the patients without LOH, 25 had CR, 4 had PR, and 4 had NC. The difference in LOH status was significant between the CR and PR/NC groups ($P = 0.04$; Fisher's exact test). Of the patients with LOH, 18 had recurrence and 11 had no recurrence. Of the patients without LOH, 9 had recurrence and 24 had no recurrence. The difference was significant between the presence/absence of recurrence and the LOH status ($P = 0.006$, Fisher's exact test; Table 2). In addition, there was a significant difference in overall survival ($P = 0.02$, log-rank test; Fig. 1A) and disease-free survival between patients with LOH and those without ($P = 0.002$, log-rank test; Fig. 1B). The latter group survived significantly longer than the former.

DISCUSSION

Several studies have shown that for intracavitary brachytherapy, local control and survival rates for high-dose rate and low-dose rate are quite similar (26, 27). In this series, patients with cervical carcinoma have received ^{192}Ir high-dose-rate intracavitary brachytherapy.

In recent studies of cytogenetics and allelotypes, many observations of allelic losses at specific chromosomal loci in a variety of human cancers have implicated the presence of putative TSGs on some chromosomes. In particular, deletions and rearrangements of the short arm of chromosome 6 (6p) are known to be frequent in cancers of the colon (28), lung (29), ovary (30), and kidney (31). Published allelotype analyses of cervical cancer identify LOH on chromosome 6p in 28% (12), 34% (16), 43% (13), and 47% (15) of specimens. However, the importance of LOH on chromosome 6p in the recurrence of cervical cancer after radiotherapy remains unknown. In this study, we investigated the correlation between recurrence after radiotherapy and the rate of LOH on chromosome 6p21.2 using three microsatellite markers (*D6S276*, *D6S1624*, and *D6S1583*) in both tumor tissue and blood of 62 patients with cervical carcinoma.

In this study, LOH on 6p21.2 occurred in 46.8% (29 of 62) of the patients. Patients with LOH had significantly poorer responses to radiotherapy ($P = 0.04$) and shorter disease-free survival ($P = 0.002$) compared with those without LOH. LOH on 6p21.2, where *WAF1* is situated, has been described in many cancers (28–31), suggesting that *WAF1* may be inactivated by a two-hit process in the corresponding tumors.

The *WAF1* gene is a cyclin-dependent kinase inhibitor that acts primarily as a negative regulator of cell proliferation at the G_1 cell cycle checkpoint, *i.e.*, to allow the replicating cell time to repair damaged DNA (32, 33). The inability of the cell to

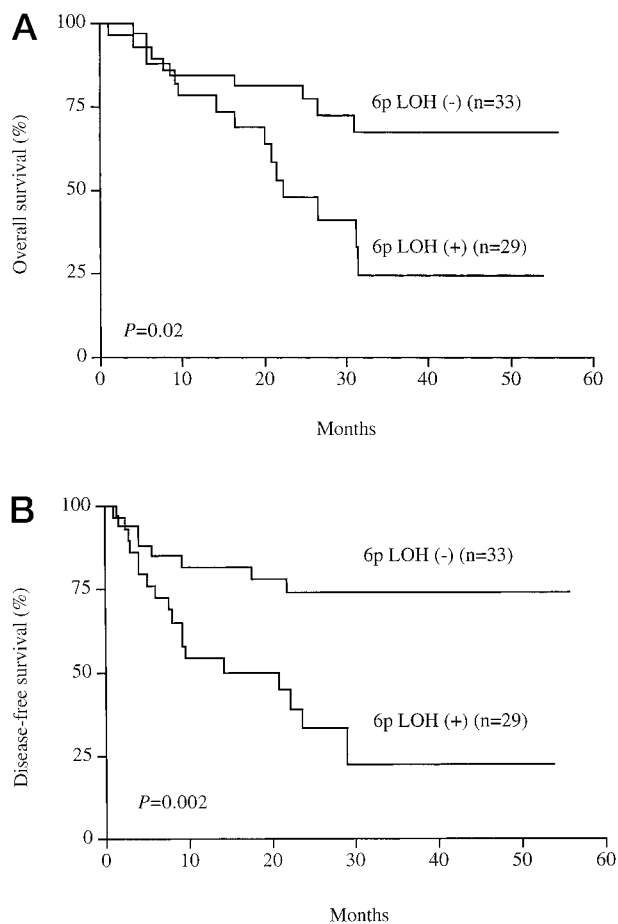


Fig. 1 A, overall survival of patients with respect to the presence or absence of LOH on chromosome 6p21.2 using the Kaplan-Meier method and analyzed with the log-rank test. The 33 patients without LOH survived significantly longer than the 29 patients with LOH ($P = 0.02$). B, disease-free survival of patients with respect to the presence or absence of LOH on chromosome 6p21.2 using the Kaplan-Meier method and analyzed with the log-rank test. The 33 patients without LOH survived significantly longer than the 29 patients with LOH ($P = 0.002$).

properly observe this interval increases the risk of incorporating DNA damage and could lead to the accumulation of gene mutations with resultant genomic instability and altered regulation of cellular proliferation (34, 35). Alterations in the expression or function of the *WAF1* gene can be expected to lead to aberrant control of cell proliferation and may predispose certain individuals to cancer. Tumor-associated mutation of the coding region of the *WAF1* gene is rare, although it has been reported in a small number of patients with Burkitt's lymphoma (36), prostate adenocarcinoma (37), and breast cancer (38). We also detected the somatic mutation of the *WAF1* gene in 9.1% (2 of 22) patients with cervical cancer.⁴ In addition, Shiohara *et al.* (39) have reported that the absence of *WAF1* alterations in a large series of 14 human malignancies, including cervical can-

⁴ Data in preparation.

cers, suggested that *WAF1* mutations may not play an important role in either the onset or the progression of these malignancies. Therefore, we hypothesized that other genes in this region might also be involved.

The smallest region of deletion is between 6p21 and 6p23. Kersemaekers *et al.* (17) observed a high percentage (54%) of LOH on chromosome 6p with primers at 6p22–23, which was also in the smallest region of overlap between *D6S105* and tumor necrosis factor. The HLA region is in this smallest area of deletion. HLA molecules are required for the immunological response to HPV infection, and tumors may thus evade the immune defense by a loss of HLA expression. The loss of HLA expression is observed in many tumor types, including cervical cancer (40). A genetic defect may explain this loss of expression, in view of the frequent LOH found in this region. Enlund *et al.* (41) reported that locus *D6S276* was associated with the HLA region. Although we have used the same microsatellite marker, we hypothesized that the *HLA* gene might be partially associated with the recurrence of advanced cervical cancers. Further mapping with more markers is needed to elucidate this issue.

The importance of the *p53* gene in clinical oncology has been reviewed (42). A poor response of human malignancies, such as breast cancer (43) and colon cancer (44), to different therapies is often associated with mutations of the *p53* gene. In contrast to many other tumors in humans, *p53* mutations are only rarely detected in cervical cancer (42). In our study, 7 of the 62 patients (11.3%) were found to have mutations in the *p53* gene, as evaluated by an SSCP analysis of genomic DNA. The seven tumors with mutant *p53* had G:C→A:T mutations. These data confirm the report of a high frequency of G:C→A:T mutations in patients with cervical carcinoma (45).

Most cervical carcinomas have been shown to contain HPV DNA sequences, including the high-risk HPV 16 and HPV 18 types (46). The binding of high-risk HPV type E6 viral protein to the *p53* protein has been shown to result in a rapid ubiquitin-dependent proteolytic degradation of *p53* (47). Therefore, the presence of high-risk forms of HPV is believed to result in a loss of the *p53*-mediated control of cell growth (48). In general, the presence of high-risk HPV in tumor cells is thought to be associated with a poor response of cervical cancer to treatment. It has been shown, however, that HPV-positive cancer cells may preserve the *p53* protein in its functional form (48). This observation may partially explain the discrepancies in the results of studies examining the correlation between the presence of various types of HPV DNA in tumors and the treatment outcome in patients with cervical cancer (49, 50). In the case of cervical carcinoma, there is an obvious need to study the effects of HPV infection on intrinsic tumor cell radiosensitivity (51). However, the literature detailing the differences in prognosis between patients with HPV-positive and HPV-negative tumors is incomplete (52, 53). In our study, HPV-positive tumors were found in 41.9% of the patients (26 of 62). This low rate is consistent with the report by Matsumoto *et al.* (54), who showed that HPV was detected in 49% of cervical cancers obtained from Japanese women as compared with 80% in Western countries (50). The expression of the *E6* or *E7* gene of high-risk HPVs (types 16, 18, and others) seems to be an essential but not a sufficient factor for the malignant conversion of the cervical epithelium in

Japanese patients (55). No correlation was found between HPV infection and treatment outcome in our study.

In this study, tumor size was found to be the important determinant of the response to radiotherapy and a poor overall survival ($P = 0.0004$, log-rank test) and disease-free survival ($P = 0.01$) after radiotherapy, which confirms data from previous reports (2). The difference in size was significant for the presence or absence of recurrence ($P = 0.003$). However, LOH on 6p21.2 was found to be the most significant determinant of the relapse-free survival time ($P = 0.002$). In addition, we found no correlation between LOH status and the size or stage of tumor. Thus, the results of this study suggest that LOH on 6p21.2 is an important predictor of recurrence after radiation treatment in patients with cervical carcinomas independent of tumor size. The higher rate of LOH on 6p21.2 in tumors may explain, at least in part, the poor prognosis of patients with cervical carcinoma after radiotherapy.

More recently, investigators have been looking for a way to improve local control by combining radiation therapy with chemotherapy (56, 57). The Gynecological Oncology Group (56) has explored the role of radiation therapy and concurrent chemotherapy with hydroxyurea, cisplatin, and 5-fluorouracil. In their study, the rate of local recurrences was significantly lower with cisplatin-based regimen than with hydroxyurea regimen, whereas the rate of distant recurrences was only reduced slightly. These results suggested that the principal effect of cisplatin is radiosensitization. In our series, the patients with local recurrence might be influenced by chemotherapy.

In this study, we evaluated the recurrence of cervical cancer, including both local recurrence and distant metastases after radiotherapy. In the future, we hope to investigate the correlation between LOH status and treatment failure according to local recurrence and distant metastasis.

The analysis of key gene-related LOH on 6p21.2 in patients with cervical carcinoma might help clarify the mechanisms of recurrence after radiotherapy in some individuals.

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