Prognostic Value of p53 Protein Accumulation in Cancer Cell Nuclei in Adenocarcinoma of the Uterine Cervix

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Adenocarcinoma of the uterine cervix (CxAd) is one of the most distressing malignancies of the female reproductive system because of its tendency to spread aggressively and to be resistant to radiation and systemic therapies. To clarify the prognostic significance of p53 alteration in CxAd, we immunohistochemically examined the incidence of p53 nuclear accumulation, which is considered to be mostly parallel with p53 gene mutation, and its association with clinicopathological parameters in 26 patients with CxAd. The overall incidence of p53 nuclear accumulation was 46% (12 of 26), being higher in groups with clinically advanced disease, higher degrees of cellular atypia, and deeper myometrial invasion, but significantly lower in patients with integration of human papillomavirus (HPV) type 16 or 18 DNA. Nuclear p53 immunoreactivity as well as lymph node status, depth of invasion and the absence of HPV-DNA integration were significant indicators of a poor prognosis. Examination of p53 nuclear accumulation could be applied to biopsy material, and would be of practical assistance in predicting the prognosis of CxAd both preoperatively and postoperatively.

Key words: Adenocarcinoma — Uterine cervix — p53 — Prognosis — Immunohistochemistry

Although adenocarcinoma accounts for only 10–20% of all cancers of the uterine cervix, its occurrence relative to squamous cell carcinoma is increasing. Adenocarcinoma of the uterine cervix (CxAd) is generally of a more aggressive clinical nature than squamous cell carcinoma.^{1,2} The treatment of CxAd is difficult because of its tendency for remote spread, poor prognosis and resistance to radiation and systemic therapies. In CxAd, certain abnormalities in tumor-associated genes have been shown to correlate with the clinical behavior of the tumor. For example, the incidence of amplification of the *c-erbB-2* proto-oncogene and overexpression of its product have been shown to be higher in patients at advanced clinical stages and to indicate a poor prognosis.³ p53 gene mutation at the DNA level, which has been shown to correlate with nuclear accumulation of the p53 protein in cancer cells in various types of gynecological cancer, is also more frequent in patients at advanced clinical stages and with higher histological grades.^{4–6} Immunohistochemical examination for nuclear accumulation of p53 protein should be able to detect the majority of CxAd patients with p53 gene mutations and might be able to predict clinically aggressive tumor behavior. This method is simple and can solve the problem of normal cell contamination, which always causes difficulty in the interpretation of the results of DNA analyses. In the pres-

ent study, p53 protein accumulation was immunohistochemically examined in 26 patients with CxAd, and its association with clinicopathological parameters and prognosis was analyzed.

MATERIALS AND METHODS

Cases Among the patients with uterine adenocarcinomas postoperatively diagnosed as CxAd at the National Cancer Center Hospital between June 1989 and March 1993, we performed immunohistochemical study for 26 cases in which p53 gene mutation and the integration of human papillomavirus (HPV) DNA had already been examined.⁶ Among the 26 specimens, 14 were of the endocervical subtype and 12 were endometrioid. The grade of cellular atypia was expressed as a combination of structural and nuclear atypia. When the degree of structural or nuclear atypia of tumor cells was low, moderate, and high, the cases were scored as 1, 2, and 3, respectively. When total scores combining nuclear and structural atypia were 2–3, 4, and 5–6, the cases were judged as low-grade, moderate-grade and high-grade atypia, respectively.

Staging of the cases was performed according to the system of the International Federation of Gynecology and Obstetrics.⁷ In the 22 patients at stages I to III, radical hysterectomy with pelvic lymph node dissection was performed, whereas total or partial pelvic exentera-

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tion was performed in the 4 patients with stage IV disease. From histopathological data, the depth of myometrial invasion and the presence of lymph node metastasis were documented. In a previous study, we examined the presence of mutation of the *p53* tumor-suppressor gene at the DNA level by polymerase chain reaction (PCR) — single-strand conformation polymorphism analysis and the integration of HPV types 16 and 18 DNA into the tumor DNA by Southern blot analysis in these 26 CxAd patients.⁶⁾ In brief, 0.1 μ g aliquots of DNA isolated from cancer and non-cancer tissue were enzymatically amplified by PCR using [γ -³²P]ATP-labeled synthetic oligonucleotide primers encompassing the exons 4, 5, 6, 7, 8 and 9 of the *p53* gene. The PCR products were denatured and electrophoresed in non-denaturing gel, and their mobility shifts in the gel, which represent conformational changes due to gene mutations, were detected on the autoradiogram. To detect the clonal integration of HPV DNA in cancer cells, 10 μ g of DNA was cut completely with a restriction enzyme, electrophoresed in agarose gel, denatured, and blotted onto a nitrocellulose filter. Single-stranded DNA on the filter was hybridized to [α -³²P]dCTP-labeled HPV type 16 or 18 DNA probe, and the hybridized signals of tumor DNA were detected on the autoradiogram.

Immunohistochemistry Routinely processed formalin-fixed, paraffin-embedded tissue blocks from 26 tumor specimens were cut into 5 μ m sections on lysine-coated glass slides (Muto Pure Chemicals, Tokyo). The sections were deparaffinized in xylene, and rehydrated in a descending ethanol series, ending in water. They were then incubated for 30 min in 0.3% hydrogen peroxide in methanol. Sections were placed in 5% zinc sulfate heptahydrate and exposed to microwave radiation for 6 min at 90°C using a BioRad Microwave Processor H2500 (BioRad, Richmond, CA). After having been cooled for 30 min to room temperature, slides were washed with water, and preincubated in 2% normal swine serum in phosphate-buffered saline. Immunohistochemical examination was performed by the avidin-biotin-complex method as described by Iwaya *et al.*⁸⁾ using anti-rabbit

anti-p53 polyclonal antibody, RSP53 (Nichirei Inc., Tokyo), which was raised against a synthetic peptide corresponding to amino acid residues 54 to 69 (Phe-Thr-Glu-Asp-Pro-Gly-Pro-Asp-Glu-Ala-Pro-Arg-Met-Pro-Glu-Ala) of human p53 protein. The immunoreaction spectrum of the RSP53 is identical with that of PAb1801 (Oncogene Science Inc., Manhasset, NY). When $\geq 20\%$ of cancer cells exhibited a nuclear immunoreaction for p53 protein, the specimen was judged as having positive nuclear p53 immunoreactivity.

Statistical analyses Thirteen patients had already died due to spread of the CxAd between 2 and 44 months after initial surgical therapy. In the other 13 patients, the mean follow-up period was 35.6 months (23–47 months). Two patients are alive with recurrent CxAd. The association of p53 immunoreactivity with clinical and pathological parameters was statistically analyzed by Fisher's exact test. Survival curves for patient groups were drawn by using the Kaplan-Meier method.⁹⁾ The difference between the survival curves was examined by means of the log-rank test.¹⁰⁾

Table II. Association of Clinicopathological Parameters with Nuclear p53 Immunoreactivity in Cervical Adenocarcinoma

Parameters	Number of cases (%)		
	Total	Nuclear p53 positive	P-value
A. Clinical stage			
I	12	3 (25)	0.02
II	7	3 (43)	
III	3	2 (67)	
IV	4	4 (100)	
B. Depth of myometrial invasion			
≤ 1/2	4	0 (0)	0.07
> 1/2 and ≤ 2/2	10	4 (40)	
> 2/2	12	8 (67)	
C. Lymph node status			
Negative	11	3 (27)	NS
Positive	15	9 (60)	
D. Histological type			
Endocervical	14	7 (50)	NS
Endometrioid	12	5 (42)	
E. Histological grade			
Low	9	2 (22)	0.08
Intermediate	11	6 (55)	
High	6	4 (67)	
F. Integration of HPV DNA			
Positive	7	0 (0)	0.005
Negative	19	12 (63)	

NS, not significant.

Table I. Association of Nuclear Accumulation of p53 Protein with *p53* Gene Mutations

Nuclear p53 immunoreaction	No. of cases (%)		
	Total	<i>p53</i> gene mutation	
		Detected	Not detected
Positive	12	8 (67)	4 (33)
Negative	14	1 (7)	13 (93)
Total	26	9	17 (100)

$P < 0.005$.

RESULTS

Association between p53 immunoreaction and p53 gene mutation in CxAd A nuclear immunoreaction in $>20\%$ of cancer cells was observed in 12 specimens (46%). Of these 12 specimens, eight (67%) also had p53 gene mutations (Table I). On the other hand, only one of the 14 specimens with a negative p53 immunoreaction revealed a p53 gene mutation. In this specimen, a single-base deletion (CCC to CC at codon 219) in exon 6 generated a downstream frameshift.⁶ The gene mutation lay outside the highly conserved region. In the eight specimens with both p53 gene mutations and p53 nuclear accumulation, the p53 gene mutation was a single-base substitution in the highly conserved region containing exons 5, 7 and 8.⁶

Association between p53 nuclear accumulation and clinicopathological parameters Nuclear p53 accumulation was detected in six of seven cases (86%) at stage III or IV, but only in six (32%) of the 19 stage I or II cases

($P=0.02$) (Table II). The incidence of p53 immunoreactivity was higher in patient groups with a myometrial invasion depth ratio $>1/2$ than in those with a depth ratio of $\leq 1/2$ ($P=0.07$). Among 15 patients with lymph node metastasis, p53 immunoreaction was positive in nine (60%), whereas the reaction was positive in only three (27%) of 11 patients without lymph node metastasis.

Among the 14 endocervical types, p53 nuclear accumulation was detected in 7 (50%) specimens, including 6 of 9 (67%) with high-grade or intermediate-grade atypia, but only one (20%) of five with low-grade atypia. Among the 12 cases of endometrioid type, p53 nuclear immunoreactivity was detected in 5 (42%) specimens, i.e., two of five (40%) with intermediate atypia and two of three (67%) with high-grade atypia, but only one of four (25%) with low-grade atypia. All specimens exhibiting a positive p53 immunoreaction had no integration of HPV DNA (Table II).

Association of p53 nuclear accumulation with poor prognosis Conventional prognostic factors, i.e., lymph node

Fig. 1. Survival curves of patients with adenocarcinoma of the uterine cervix. A. Survival curves for lymph node metastasis-negative (a) and lymph node metastasis-positive (b) groups. The two curves are significantly different ($P<0.001$). B. Curves for groups with different depths of myometrial invasion. a, $\leq 1/2$; b, $>1/2$ and $\leq 2/2$; c, $>2/2$ (curves a and b vs. curve c, $P<0.01$). C. Curves for the group with HPV-DNA integration (a) and the group without HPV-DNA integration (b). The two curves are significantly different ($P<0.005$). D. Survival curves for p53-immunoreaction-negative (a) and p53-immunoreaction-positive (b) groups of patients with CxAd. The two curves are significantly different ($P=0.02$).

status and the depth ratio of myometrial invasion were significantly associated with the prognosis of CxAd (Fig. 1A–1B). HPV DNA type 16 or 18 integration was also significantly associated with prognosis ($P < 0.005$): all seven patients with HPV-DNA integration remain alive with no recurrence, whereas 13 of the 19 patients without HPV-DNA integration died due to spread of the CxAd, and the remaining two are alive but have experienced recurrence (Fig. 1C). In the 19 patients with no HPV DNA integration, 10 of 11 patients with p53-positive tumors died of cancer, whereas only three of eight patients with p53-negative tumors died of cancer, and their survival rates at three years after surgery were 36% and 55%, respectively.

Among the 12 patients with positive p53 nuclear immunoreactions, 10 died of metastasis of CxAd within 44 mo after surgery, and one of the remaining two is suffering from recurrence eight months after initial surgery. On the other hand, among the 14 patients with no p53 nuclear immunoreactivity, three died of spread of CxAd within 29 mo after surgery, and one of the remaining 12 suffered a recurrence at 32 mo after surgery. The group with p53 immunoreactivity exhibited a significantly poorer prognosis than the group with no reactivity ($P < 0.025$) (Fig. 1D). Of the four patients in whom p53 mutation was negative but p53 immunoreactivity was positive, two died of cancer recurrence and one is alive with recurrence. One patient with mutation of the p53 gene but with no p53 immunoreactivity is still alive without recurrence 35 mo after surgery.

Patients were subdivided into lymph-node metastasis-positive and negative groups. All the nine p53-positive cases with lymph-node metastasis died of cancer spread within 40 mo after surgery. Among the six with lymph-node metastasis but without p53 nuclear accumulation, three died of cancer spread within 29 mo after surgery and one of the remaining three is suffering a recurrence. Among the three p53-positive patients without lymph node metastasis, one died of cancer spread 44 mo after surgery and one of the remaining two is suffering from recurrence at 42 mo after surgery. All eight patients with neither lymph node metastasis nor p53 nuclear accumulation are alive without recurrence from 16 to 46 mo after surgery.

DISCUSSION

Although there is a significant association between nuclear p53 immunoreactivity and mutation of the p53 gene, such an association is not always the case.^{11, 12} In four cases which exhibited immunoreactivity for p53 protein in cancer cell nuclei, no p53 gene mutation was detected, whereas one patient with a p53 mutation produced no nuclear p53 immunoreaction. In the former

four patients, the prognosis was poor, as observed in patients positive for both mutation and immunoreaction. Thus, positive nuclear immunoreactivity for the p53 protein appears to be an indicator of aggressive CxAd regardless of the detection of p53 gene mutation. In the latter one case, the absence of a nuclear p53 immunoreaction was explained by the fact that the non-sense mutations or small deletions which cause frameshifts downstream of the mutation site are usually linked with the absence of a p53 immunoreaction.¹²

It has been shown that the prognosis of CxAd is largely influenced by the clinical stage, lymph node status, and/or the degree of cancer cell differentiation.¹³ In the present study, p53 immunoreactivity was significantly associated with the extent of tumor spread and the absence of HPV-DNA integration. Both the presence of a p53 immunoreaction and the absence of HPV DNA type 16 or 18 integration were shown to correlate with a poor prognosis of CxAd. The presence of HPV-DNA integration in CxAd could be an indicator of an excellent prognosis. This inverse association between HPV-DNA integration and prognosis is identical to that reported in carcinoma of the uterine cervix.¹⁴ In the present study, Cox's life-table regression analysis was considered inappropriate because survival rate at 47 mo after surgery was 0% in patient groups with $>2/2$ depth ratio and with no HPV-DNA integration. Thus, we could not determine the independent prognostic value of each factor by Cox's analysis.

Our results suggested that p53 immunoreactivity is an indicator of poor prognosis in both groups with and without lymph node metastasis, although the number of patients was limited. When the survival curves presented in Fig. 1 are compared, p53 immunoreactivity appeared not to have such a strong association with prognosis as lymph node metastasis and HPV DNA type 16 or 18 integration. Nonetheless, p53 immunoreactivity would be of practical help as an objective indicator of poor prognosis and spread in preoperative biopsy specimens. Furthermore, the combination of postoperative examinations of p53 and lymph node status would give more accurate information with regard to the prognosis of patients with CxAd. p53 immunostaining seemed to be superior to other parameters including p53 gene and HPV DNA analyses in technical simplicity, reliability and cost benefits.

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