Detection of Apoptosis and Expression of Apoptosis-associated Proteins as Early Predictors of Prognosis after Irradiation Therapy in Stage IIIb Uterine Cervical Cancer

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We investigated the proportion of apoptotic cells and the expression of apoptosis-associated proteins after the delivery of the first week of irradiation for stage IIIb uterine cervical cancer. Thirty patients with stage IIIb squamous cell carcinoma of the uterine cervix who received only irradiation therapy were registered in this study. Specimens were obtained before irradiation therapy and at the end of the first week of irradiation. The apoptotic index (AI) of each tissue specimen was calculated by counting the apoptotic cells and expressed as a percentage. Immunohistochemical evaluation for apoptosis-related proteins, p53, Bcl-2, Bax, caspase-1 and caspase-3 was also performed. The AI was 0.8±0.9% (mean±SD) before irradiation and 1.7±1.3% at the end of the first week of irradiation. We observed that the patients who survived more than 5 years had AI levels of 2.1±1.3% at the end of their first week of therapy. This rate was significantly higher than the rate of 1.1±0.8% ($P=0.02$) of the patients who died within 5 years. When the cut-off value of the AI was set at 1.7%, the sensitivity, specificity, positive predictive value, and negative predictive value for the prediction of patients' prognosis after irradiation therapy were 73.4%, 72.4%, 82.4%, and 61.5%, respectively. In 17 of the AI-positive cases, expressions of Bax ($P=0.006$), caspase-1 ($P=0.045$), and caspase-3 ($P=0.013$) at the end of the first week were significantly higher than before irradiation. The proportion of apoptotic cells and the expression of apoptosis-associated proteins, Bax, caspase-1, and caspase-3, at the end of the first week of irradiation could be useful predictors of the prognosis in stage IIIb squamous cell carcinoma of the uterine cervix treated by irradiation therapy.

Key words: Uterine cervical cancer — Irradiation therapy — Apoptosis — Prediction of prognosis

Irradiation therapy has been the only effective therapy for stage IIIb uterine cervical cancer for a long time, although tumor response varies widely.[1] Formerly, the therapeutic effect of irradiation was thought to be the result of cellular necrotic changes. However, recently, the phenomenon of irradiation-induced apoptosis has generated interest because the therapeutic effect is now thought to be related to irradiation-induced apoptosis.[2, 3] The wide variation in tumor response to irradiation therapy might be explained by differences in tumor cell death-inducing effect. In experimental animal tumors, apoptosis has been shown to occur following treatment with irradiation.[4, 5] One such study reported that an acute apoptotic response after irradiation may be a feature of the radiosensitive tumor.[6] Spontaneous apoptosis is a mechanism of cell loss in untreated tumors,[3] so irradiation probably enhances the process of apoptosis that results in tumor cell death in irradiated patients.[7–9]

In human uterine cervical cancer, Ohno *et al*. recently reported the induction of apoptosis and an apoptosis-related protein, Bax, after irradiation therapy.[10] However, no data are available that indicate whether the induction of apoptosis in uterine cervical cancer tissue after the initial dose of irradiation therapy predicts the long-term prognosis of irradiated patients.

In this study, we investigated the relationship between the rate of apoptotic cells, the expression of apoptosis-associated proteins, i.e., p53, Bcl-2, BAX, caspase-1 and caspase-3, which play an important role in irradiation induced apoptosis,[11] at the end of the first week of irradiation, and the patients' outcome after the completion of irradiation therapy. Using these parameters, we attempted to determine whether the prognosis of patients with stage IIIb uterine cervical cancer treated with irradiation alone is predictable.

MATERIALS AND METHODS

Patients and radiation therapy   Thirty patients with stage IIIb squamous cell carcinoma of the uterine cervix who received only irradiation therapy at Toyama Medical and Pharmaceutical University Hospital, Japan, between 1981 and 1989 were studied. Clinical stages and histologi-

cal classification (29 cases were non-keratinizing type and one case was keratinizing type) were based on the criteria of the International Federation of Gynecology and Obstetrics (FIGO) and the World Health Organization. Patients were treated either with a combination of external and high dose intracavitary irradiation, or with external irradiation only. Initially, external whole pelvis irradiation was administered with anteroposterior and posteroanterior parallel opposed ports at a dose ranging from 1.8 to 2.0 Gy per fraction, 5 times per week, with the total doses ranging from 30 to 40 Gy. Central-shielding irradiation was delivered with a dose of 2.0 Gy per fraction, 5 times per week following the initial whole pelvis irradiation, with the total doses ranging from 10 to 20 Gy. Along with the central-shielding irradiation, these patients also received intracavitary irradiation by a remote after-loading system (RALS) using cobalt-60 sources. Patients received 3 insertions (1 per week) with fractional doses ranging from 6.0 to 7.0 Gy at point A, with the total doses ranging from 18 to 21 Gy. In those cases when only external whole pelvis irradiation was administered, anteroposterior and posteroanterior parallel opposed ports were delivered at a dose of 2.0 Gy per fraction, 5 times per week, to a total dose of 50 Gy.

All specimens were excised from the same site in the cervical tumors before irradiation therapy and after about a week of irradiation therapy (a total dose ranging from 9 to 14 Gy). Tissues were fixed in 10% formalin for one day and embedded in paraffin. Five-micrometer serial sections were cut from the paraffin blocks; one section was stained with hematoxylin and eosin (H&E), and the rest were subjected to immunohistochemical staining for apoptosis-related proteins.

Apoptotic index (AI) Apoptotic cells, which generally showed condensed and homogeneous chromatin and strongly eosinophilic cytoplasm with or without small nuclear fragments, were identified as cells with apoptosis according to the criteria of Kerr *et al.*[3] in H&E stained sections under high-power (×400) magnification. The AI of each tissue sample was calculated by counting apoptotic cells in at least 1000 nuclei in the specimens. AI was expressed as a percentage. Areas of severe inflammatory cell infiltration were excluded.

Immunohistochemistry Immunohistochemical studies were performed to detect expression of apoptosis-related gene products, p53, Bax, Bcl-2, caspase-1 (interleukin 1-β converting enzyme [ICE]), caspase-3 (cysteine protease p32 [CPP32]) by means of the streptavidin-biotin immunoperoxidase technique. Briefly, tissue sections were deparaffinized and rehydrated. A high-temperature unmasking procedure was performed using an autoclave at 121°C for 15 min in 0.01 M citrate buffer, pH 6. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min at room temperature, followed by washing twice for 5 min each in phosphate-buffered saline (PBS).

The sections were incubated for 10 min at room temperature with blocking agents (goat serum). After shaking off the blocking agents, the sections were incubated in a humidity chamber at 4°C overnight with the first antibodies. The first antibodies used were mouse monoclonal anti-p53 immunoglobulin (Ig) G (DO-7; DAKO, Copenhagen, Denmark) diluted at 1:100 in PBS, rabbit polyclonal anti-Bax IgG (P19; Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:800 in PBS, mouse monoclonal anti-Bcl-2 IgG (124, DAKO) diluted at 1:100 in PBS, rabbit polyclonal anti-caspase-1 IgG (C-20; Santa Cruz Biotechnology) diluted at 1:400 in PBS and goat polyclonal anti-caspase-3 IgG (N-19; Santa Cruz Biotechnology) diluted at 1:400 in PBS. These specimens were washed 3 times for 5 min each in PBS, then incubated in a humidity chamber at 37°C for 10 min with biotinylated secondary antibody, followed by washing 3 times for 5 min each in PBS. The specimens were incubated in a humidity chamber at 37°C for 5 min with streptavidin-biotinylated peroxidase complex, and each was washed 3 times for 5 min in PBS. Slides were then developed with 3,3-diaminobenzidine (DAB), lightly counter-stained with Mayer's hematoxylin solution, dehydrated, and mounted. At least 1000 cells were counted in the specimens, and immunohistochemically positive cells were also counted. Thereafter, the positive cell rate was calculated and expressed as a percentage for each sample. This procedure was done by one of the authors (H.Y.) who was blinded as to patient outcome.

Histological grading of the therapeutic effects after the first week of irradiation therapy Biopsy specimens taken after the first week of irradiation therapy were evaluated according to the modified Shimosato histological grading system.[12] Grade 0 was assigned to specimens that showed no effect after irradiation; G1—less than two-thirds of cancer tissue was eradicated or degenerated by irradiation therapy; G2—over two-thirds of the cancer tissue was eradicated or degenerated; and G3—no viable cancer cells were identified.

Evaluation of prognosis We divided the patients into two groups depending on the clinical outcome. The group of patients who survived more than 5 years without disease after irradiation therapy was defined as "the good survival group." The group of patients who died of disease within 5 years was defined as "the poor survival group." Mean age was 66.1 years (range, 49–83 years) in the good survival group, and 72.6 years (range, 59–84 years) in the poor survival group ($P=0.09$). The mean dose of irradiation within the first week was 12.4 Gy (range, 9–14 Gy) in the good survival group, and 11.3 Gy (range, 9–14 Gy) in the poor survival group ($P=0.19$). Mean and median follow-up periods were 115 and 112 months (range, 62–175 months) for the good survival group and 19 and 24 months (range, 2–36 months) for the poor survival group.
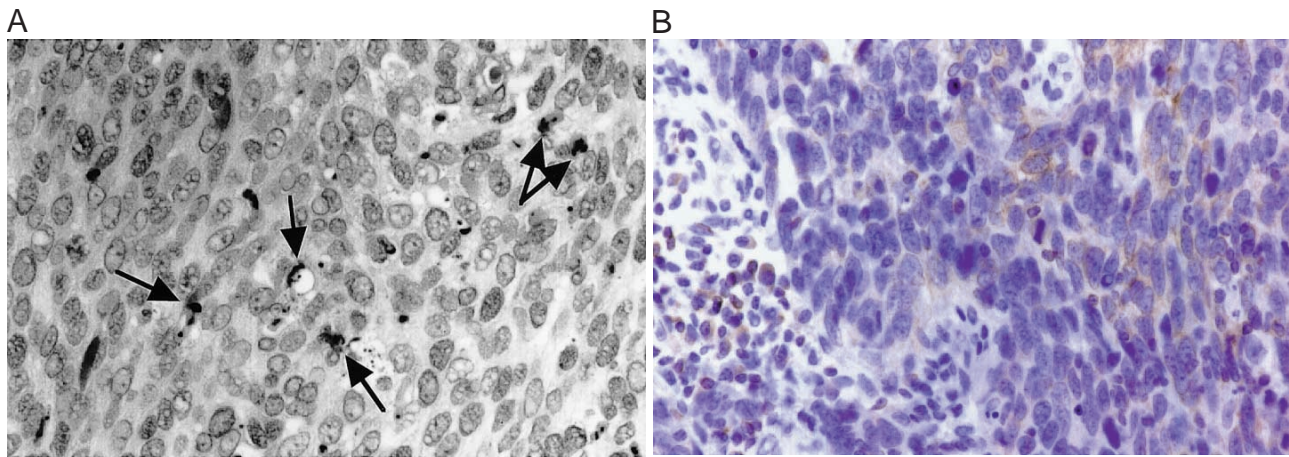
Fig. 1. A: Apoptotic cells, which showed condensed and homogeneous chromatin and strongly eosinophilic cytoplasm with or without small nuclear fragments (arrow). (H&E, original magnification ×400). B: Immunohistochemical staining for Bax. Positive staining was observed in the cytoplasm of tumor cells. (Original magnification ×400).

Statistical analysis The correlation between AI and the expression of each apoptosis-related protein in pre- and post-irradiation tissue were determined by the paired Student's t, the χ^2, and Fisher's exact tests at the significance level of $P<0.05$. Patients' survival was compared in terms of the Kaplan-Meier survival curve, and the log rank test was performed to evaluate statistical significance.

RESULTS

Apoptotic cells were identified as cells with condensed and homogeneous chromatin and strongly eosinophilic cytoplasm, with or without small nuclear fragments (Fig. 1A). The mean AI in all patients was 0.8% (range, 0–2.7%) before irradiation and 1.7% (range, 0–4.0%) at the end of the first week of irradiation. A significant increase of AI at the end of the first week of irradiation was observed ($P=0.002$, Fig. 2). The rate of AI of the good survival group and the poor survival group showed no significant difference before irradiation therapy. However, a significant increase in AI in the good survival group was observed at the end of the initial irradiation delivery compared with the AI of the poor survival group ($P=0.02$, Fig. 3). When the cut-off value of AI was set at 1.7%, which was the mean value plus one standard deviation (SD) of the AI before irradiation, a significant correlation between prognosis and AI at the end of the first week was found (Table I). Sensitivity, specificity, positive predictive value, and negative predictive value were 73.4%, 72.7%, 82.4% and 61.5%, respectively. The survival curves by the Kaplan-Meyer method for AI-positive and AI-negative patients are shown in Fig. 4. A significant difference
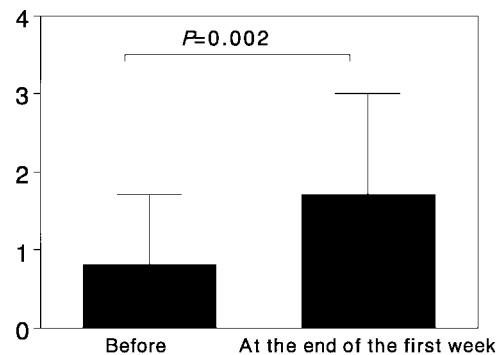

Fig. 2. Apoptotic index before and at the end of the first week of irradiation ($n=30$). Data are mean±standard deviation.

existed between the two groups (log rank; $P=0.018$). Looking at the pattern of relapses in AI-positive or negative poor survival patients, 2 out of 3 AI-positive patients died from distant metastasis. The other patient died from uremia without any evidence of local or distant relapse. Autopsy confirmed the absence of malignant disease in this case. Of the 8 AI-negative poor survival patients, 7 died from local relapse.

Histological grading of therapeutic effects after the first week of irradiation therapy could not distinguish between the poor survival group and the good survival group (Table II).

In the 17 AI-positive cases (AI≥1.7% at the end of the first week of irradiation), cells stained for Bax, caspase-1 and caspase-3 were significantly increased at the end of
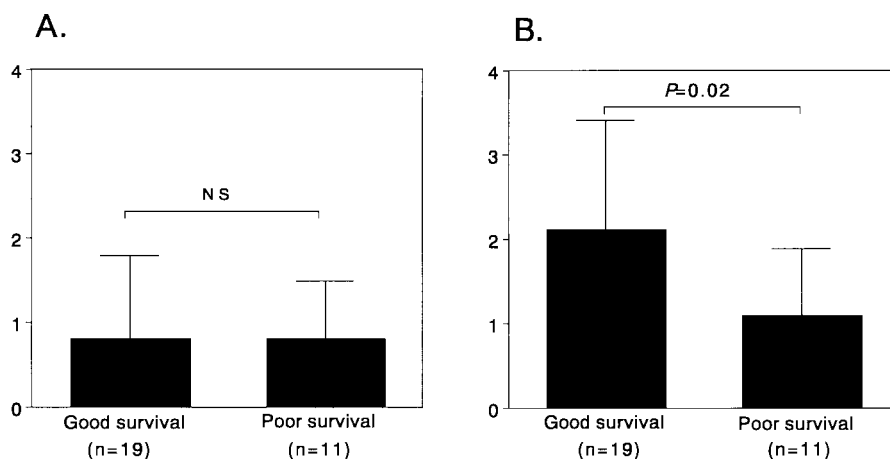
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A.

B.

Fig. 3. Apoptotic index in the good survival group and the poor survival group before and at the end of the first week of irradiation. A: AI before irradiation therapy. B: AI at the end of the first week of irradiation. Data are mean±standard deviation.

Table I. Correlation between AI at the End of the First Week of Irradiation and Long-term Prognosis

AI	Good survival[c]	Poor survival[d]	Total
Positive[a]	14	3	17
Negative[b]	5	8	13
Total	19	11	30

Fisher's exact test: $P=0.037$.
a) AI≥1.7% at the end of the first week of irradiation.
b) AI<1.7% at the end of the first week of irradiation.
c) The group of patients who survived more than 5 years after irradiation.
d) The group of patients who died within 5 years after irradiation.

Fig. 4. Kaplan-Meier survival curve of patients with AI-positive (—— AI≥1.7%, *n*=17) or AI-negative (------ AI<1.7%, *n*=13) tumors at the end of the first week of irradiation.

the first week compared with those before irradiation (Bax, $P=0.006$; caspase-1, $P=0.045$; caspase-3, $P=0.013$; Fig. 5). In several AI-positive areas, simultaneous positive staining of BAX and caspase-3 was observed in cells. We did not see this increase of positively stained cells in p53 and Bcl-2 investigation, although a tendency for increase of p53 and decrease of Bcl-2 was observed. Instead, in the 13 AI-negative cases (AI<1.7% at the end of the first week of irradiation), no significant difference was observed between the figures before and after the initial irradiation dose delivery. The mean positive rate of Bax was significantly higher in the poor survival group than in the good survival group after the initial irradiation dose delivery ($P=0.04$). However, no significant difference was observed in other apoptosis-associated proteins (data not shown). The cut-off value, which was set at 10% positivity
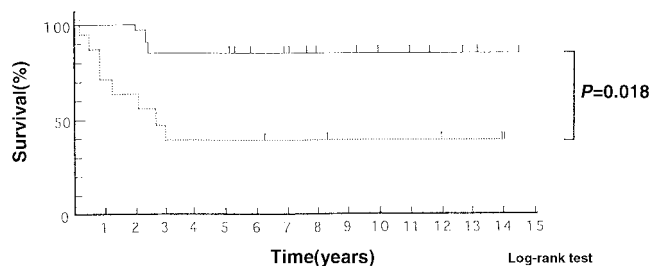
Table II. Correlation between Histological Effect at the End of the First Week of Irradiation and Prognosis

Histological effect	Good survival	Poor survival	Total
G0	9	6	15
G1−3	10	5	15
Total	19	11	30

Fisher's exact test: NS

in those immunohistochemical parameters, was set by previously published data.[13–16] Survival was significantly better in the Bax-positive group than in the Bax-negative group (Fig. 6; log rank, $P=0.014$).
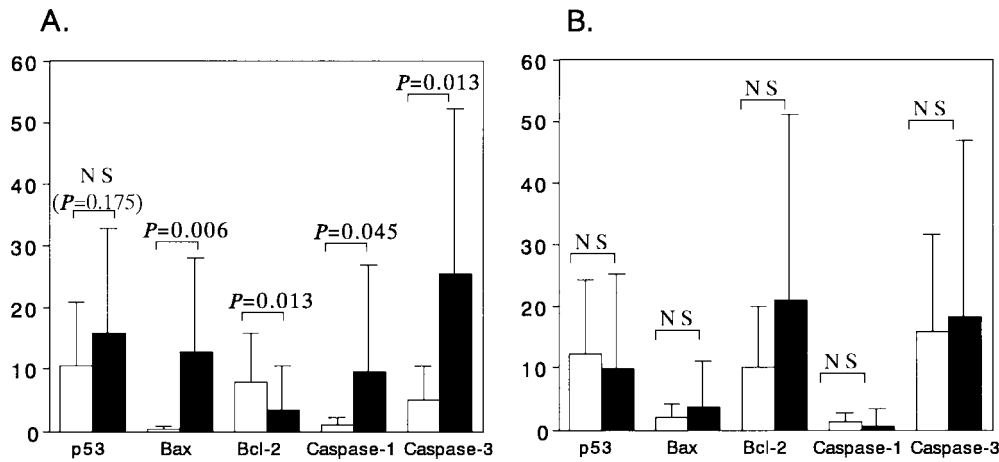
A.

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Fig. 5. Percentage of cells positive for apoptosis-associated proteins before and at the end of the first week of irradiation. A: AI-positive cases{AI≥1.7% at the end of the first week of irradiation (n=17)}. B: AI-negative cases{AI<1.7% at the end of the first week of irradiation (n=13)}. □ before, ■ at the end of the first week. Data are mean±standard deviation.

DISCUSSION

The main treatment for stage IIIb uterine cervical cancer, which is inoperable, has been irradiation therapy. However, the 5-year survival rate is 40 to 60%.[17–19] That rate indicates that some tumors respond to irradiation therapy effectively and some do not. If we could predict early which patients will not respond to conventional irradiation therapy, another treatment modality, possibly recently developed, effective, chemotherapeutic regimens, could be employed as an alternative. Trials have been conducted to determine the response of uterine cervical cancer in the early stage of irradiation therapy using histological therapeutic grading. However, this approach failed to predict prognosis.[12] A similar result was obtained in our study (Table II). Recently, the usefulness of induction of apoptosis after irradiation therapy as a predictor of short-term prognosis has been reported in patients with cancer other than uterine cancer.[8, 20–23] In uterine cervical cancer, similar observations were reported.[7, 24] However, it has not been established whether apoptosis after initial irradiation dose delivery can predict long-term prognosis. We investigated the correlation between the degree of appearance of apoptotic cells or the expression of apoptosis-associated proteins after initial irradiation dose delivery and long-term survival of patients with stage IIIb cervical cancer. In this study, the AI of the good survival group at the end of the first week of irradiation was increased significantly compared with the AI before irradiation. When the cut-off value of the AI was set at 1.7% at the end of the first week of irradiation, the AI appeared to be an effective predictor of prognosis. Several recent studies have used the terminal
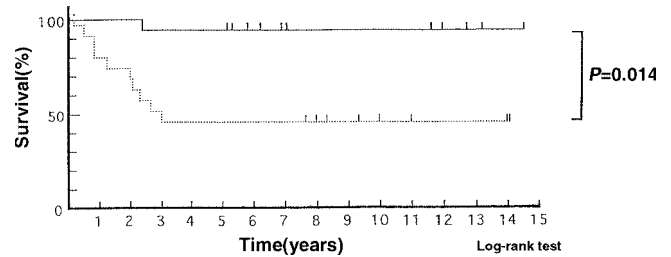
Fig. 6. Kaplan-Meier survival curve of patients with Bax-positive (—— BAX≥10%, n=12) or Bax-negative (------ BAX<10%, n=18) tumors at the end of the first week of irradiation.

deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method to detect apoptotic cells; however this method gave false positive findings in our study, probably because the specimens had been embedded in paraffin for a long time. The detection of apoptotic cells in H&E-stained specimens was reported to correlate closely with the results of the TUNEL method.[25] Therefore, we detected apoptotic cells according to the criteria of Kerr et al.[3] in H&E-stained sections. We also confirmed the presence of apoptotic cells by electron microscopy in the same specimens in which apoptosis was identified by H&E staining (data not shown).

In apoptosis induction by irradiation, many so-called apoptosis-associated proteins are known to be activated.[26, 27] We investigated immunohistochemically the expression of apoptosis-associated proteins, i.e., p53, Bax, the caspase family, especially caspase-1 and caspase-3,[11] and Bcl-2 as apoptosis-suppressing proteins in serial sec-

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tions. The expression of Bax in cervical cancer specimens after irradiation therapy was recently reported.[10] In their relatively short-term follow-up study, Harima *et al.* found a better short-term prognosis in a Bax-expressing uterine cervical cancer group given 10.8 Gy of irradiation.[28] Our results indicated a better long-term prognosis among the patients who expressed higher AI and Bax positivity at the time of delivery of the first week of irradiation. Our data indicate that induction of apoptosis through Bax activation has an important role in the eradication of cervical squamous cell carcinoma by irradiation therapy. The follow-up period in our study extended to 15 years with a mean follow-up period of 80 months and median of 83 months. This follow-up is long enough to establish the relationship of prognosis, tumor response to irradiation, and apoptosis induction in the early stage of irradiation therapy. We also found that in 3 cases of AI-positive poor survival patients, 2 cases died from distant metastasis. The other case died from other disease. Autopsy confirmed the absence of local and metastatic disease in this case. Conversely, of 8 AI-negative poor survival patients, 7 died from local relapse. These results indicate that positive AI at the end of the first week of irradiation therapy could predict good control of local disease after the irradiation therapy.

p53, which is one of the best-known tumor suppresser genes, down-regulates Bcl-2 and activates Bax.[29, 30] It is thought that tumor cells with wild-type p53 are sensitive to irradiation because wild-type p53 induces apoptosis through BAX and the caspase family, and tumor cells with mutant p53 are resistant to irradiation.[31] The anti p53 product antibody that we used in this study, i.e. OD-7, recognizes both wild and mutant types of p53 proteins,

though the detection rate of p53 mutation in cervical cancer is quite low.[32] We observed a tendency of p53 to increase in the AI-positive group and to decrease in the AI-negative group, which may imply a role of p53 in the upstream portion of the cascade of irradiation-induced apoptosis.

Bcl-2 is known to inhibit apoptosis and to promote cell death by forming heterodimers with Bax.[33, 34] There was a tendency for Bcl-2 to decrease in the AI-positive group and to increase in the AI-negative group after irradiation, but this was not statistically significant. Because the ratio of BAX/Bcl-2 influences the induction of apoptosis, a statistically significant increase in the expression of BAX and a tendency for a decrease in the expression of Bcl-2 in the good survival group indicate that apoptosis was progressively induced in this group.

In conclusion, induction of apoptosis measured in terms of AI and expression of apoptosis-associated proteins, especially Bax, at the end of the first week of irradiation, seem to be useful predictors of prognosis in stage IIIb squamous cell carcinoma of the uterine cervix treated by irradiation therapy. If AI and Bax expression are low at the end of the first week of irradiation therapy, the treatment plan should be changed to another treatment modality. For example, irradiation combined with cisplatin-based chemotherapy for cervical squamous cell carcinoma was recently confirmed as an effective treatment modality for cervical cancer.[35–38]

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