ONCOPROTEIN EXPRESSION PREDICTS RESPONSE TO RADIOTHERAPY OR THERMORADIOTHERAPY IN HUMAN CERVICAL CANCER

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УРОВЕНЬ ЭКСПРЕССИИ ОНКОБЕЛКОВ КАК ПРОГНОСТИЧЕСКИЙ МАРКЕР ОТВЕТА НА ЛУЧЕВУЮ ТЕРАПИЮ ИЛИ ЛУЧЕВУЮ ТЕРАПИЮ В СОЧЕТАНИИ С ГИПЕРТЕРМИЕЙ ПРИ ЛЕЧЕНИИ РАКА ШЕЙКИ МАТКИ

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The levels of Bax and Bcl-2 expression after 10.8 Gy radiotherapy are useful prognostic markers in patients with human cervical carcinoma. Thermoradiotherapy was found to result in better treatment responses than radiotherapy for patients with stage IIIB cervical carcinoma. An additive or synergistic antitumor effect of thermoradiotherapy is likely to occur through induction of apoptosis involving one of the *bax* pathways.
Kew Words: **cervical carcinoma, radiotherapy, thermoradiotherapy, Bax, Bcl-2, prognostic markers, apoptosis.**

Уровень экспрессии белков Bax и Bcl-2 после проведенной лучевой терапии (доза 10,8 Гр) у больных раком шейки матки может служить прогностическим маркером. Было показано, что лучевая терапия в сочетании с гипертермией при лечении больных раком шейки матки в стадии IIIB дает более выраженный эффект по сравнению с одной лишь лучевой терапией. Аддитивный или синергический противоопухолевый эффект при сочетанном применении лучевой терапии и гипертермии может быть связан с индукцией апоптоза по пути, опосредуемому продуктами гена *bax*.
Ключевые слова: **рак шейки матки, лучевая терапия, гипертермия, Bax, Bcl-2, прогностические маркеры, апоптоз.**

Cervical cancer is the second leading cause of death from cancer in women worldwide [1]. Radiotherapy (RT) is the most important non–surgical treatment for cervical carcinoma. Several potential markers of response to RT in cervical carcinoma, such as stage and bulk, histological type and grade, the presence of lymphovascular invasion, and lymph node status, have been identified [2]. The tumor stage is still believed to be the most important determinant of prognosis, and tumor size has also been used as a marker of prognosis for many years [3].

The molecular mechanisms of carcinogenesis have recently become clearer. In particular, the role of the p53 protein in radiation–induced apoptosis has been documented in a large number of studies [4]. The activation of the p53–dependent signal transduction pathway is one of the responses of cancer cells to DNA damage caused by ionizing radiation [5] or chemotherapeutic agents [6]. In cancer therapy, the appearance of apoptotic cells is a useful marker of better survival [7].

Apoptosis may be blocked by the product of the proto–oncogene *bcl–2*. This protein resides primarily in the outer mitochondrial membrane, in the nuclear envelope, and in parts of the endoplasmic reliculum [8].

Overexpression of Bcl–2 and Bax has been reported in different human cancers, although the correlations with clinical outcome are conflicting, and depend on the tumor type and site [9]. In addition, the clinical significance of abnormal Bcl–2 expression in cervical carcinoma remains unknown.

Falkvoll and Real [10] showed that, for human melanoma xenografts given a single radiation dose (7.5 Gy, 15 Gy, or 25 Gy), there is a dose–dependent decrease in the fraction of apoptotic cells, with a corresponding increase in the number of necrotic cells. We found the same tendency in cervical tumors irradiated with a fractionated regimen. That is, although apoptotic cells were detectable in hematoxylin and eosin–stained tissue speciments after 10.8 Gy (6 fractions of 1.8 Gy over 8 days), an increase in the dose to 19.8 Gy (11 fractions of 1.8 Gy over 15 days) caused necrosis, and apoptotic cells could hardly be identified (data not shown). On the basis of this evidence, we chose 10.8 Gy (6 fractions of 1.8 Gy over 8 days) as the dose appropriate for the purposes of the present investigation.

Thermoradiotherapy (TRT) has been reported to yield higher complete and durable responses compared with RT alone in both superficial and deepseated tumors [11], and it is believed to be another promising treatment modality for the management of advanced cervical carcinoma [12].

In this study, we evaluated Bax and Bcl–2 expression both before and during RT or TRT, and assessed their impact on the outcome of treatment.

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Abbreviations used: RT – radiotherapy; TRT – thermoradiotherapy.

(A) BAX AND BCL–2 EXPRESSION PREDICTS RESPONSE TO RADIOTHERAPY IN HUMAN CERVICAL CANCER

MATERIALS AND METHODS

Patient characteristics. Between October 1994 and July 1997, 44 patients with histologically proven carcinoma of the uterine cervix (2 stage Ib, 3 stage IIb, 1 stage IIIa, 29 stage IIIb, 4 stage IVa, 3 stage IVb, 3 cervical stump recurrences after hysterectomy) were treated with definitive RT at Kansai Medical University. The follow–up for the surviving patients ranged from 1.7 to 40 months (mean 19.4 months). The median patient age was 64.1 years (range, 35–87 years). The primary tumors ranged in diameter from 2 cm to 10.5 cm (mean 6.1 cm), measured by computed tomography (CT) and/or magnetic resonance imaging (MRI). The tumors consisted of 39 squamous cell carcinomas, 4 adenocarcinomas, and 1 adenosquamous carcinoma (Table1). No patient received chemotherapy prior to the RT.

Table 1. Clinical characteristics of 44 patients with cervical carcinoma

Total number of Patients	44
Mean age (years)	64.1
SD	11.1
FIGO stage	
Ib	2
IIb	3
III	29
IV	7
Reccurrence	3
Histology	
Squamous cell ca.	39
Adeno ca.	4
Adenosquamous ca.	1
Tumor size (cm)	
Mean	6.1
SD	2
p53 status	
Wild–type	40
Mutant–type	4
HPV infection	
Positive	17
Negative	27

FIGO — the International Federation of Gynecology and Obstetrics classification; ca. — carcinoma; HPV — human papilloma virus

Irradiation techniques and dose. All patients entered in the protocol were treated with external pelvic radiation therapy using 6–MV high–energy linear accelerator. A total of 30.6 Gy was provided to the whole pelvis, plus an additional dose to the parametria with central shielding to complete 52.2 Gy, along with ^{192}Ir high–dose–rate intracavitary brachytherapy. Radiation was delivered to the tumor in fractions of 1.8 Gy daily, 5 days per week. The dose of ^{192}Ir brachytherapy, administered at high dose rate, was 30 Gy to a point, given as 7.5 Gy per session once per week.

Tissue specimens and immunohistochemistry. Tumor samples obtained by punch biopsy prior to and after RT were formalin–fixed and paraffin–embedded, and consecutive 3–μm–thick sections were cut from the respective tumor blocks. An immunoperoxidase method employing an avidin–biotinylated horseradish peroxidase complex was used to detect Bcl–2 and Bax proteins in deparaffinized tissue sections, as previously described [12]. The primary anti–Bcl–2 monoclonal antibody (mAb) was a murine antihuman mAb, subclass IgG1, that recognized a cytoplasmic epitope of Bcl–2 [13]. The anti–Bax mAb was a murine anti–(human Bax) mAb, subclass IgG, that recognizes an amino–terminal epitope of the human Bax molecule. For both mAb, staining was developed by immersing the slides in 3,3'–diamino–benzidine tetrahydrochloride, followed by counterstaining with hematoxylin and mounting using an aqueous medium. Staining without antibodies was performed routinely as a negative control. As positive controls, infiltrating lymphocytes were stained for Bcl–2 and Bax. On the basis of the percentages of Bcl–2 and Bax–immunopositive cells in the tumors, the lesions were subdivided into three categories as follows: negative, no or fewer than 5% positive cells; 1+, more than 5% but fewer than 30% positive cells; 2+, more than 30% positive cells. Positive immunoreactivity required more than 30% positive cells.

Detection and typing of HPV. Tumor samples were obtained from the 44 patients by punch biopsy prior to RT. Punch biopsies 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. Tumor DNA was amplified by the polymerase chain reaction (PCR) with primers specific for human papilloma virus (HPV) 16, 18, and 33 E6, as previously described [14]. The 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.

RESULTS

Overall, 86.4% of the patients (38 of 44) had advanced or recurrent disease. At present, 19 patients (43.2%) are alive with no evidence of disease (NED), 15 patients had a local failure, 3 patients had distant metastases (multiple metastases 2, lung 1), and 7 had both local failure and distant metastases (multiple metastases 3, lung 2, para–aortic lymph nodes 2). 7 patients are alive with cancer disease (AWD); 18 patients (40.9%) have died from recurrent disease (CD, cancer–caused death). 40 patients had wild–type *p53* in their tumors, and the remaining four had mutant *p53*.

Relationships among stage, tumor size, response and outcome. As shown in Table 2, there was a correlation between stage of disease and response. All 5 patients with stage I or II showed NED. In contrast, 25 of 39 patients with advanced–stage or recurrent cancer were AWD or had died (CD) ($P = 0.01$). The tumor sizes were 4.5 ± 1.9 cm in the stage I–II patients, 6.3 ± 1.8 cm in stage III, 6.8 ± 1.6 cm in stage IV, and 5.4 ± 2.7 cm in the group with recurrent cancer. The statistical analysis using a two–tailed Student's *t*–test revealed significant differences between stage I–II and stage III ($P = 0.049$), and between stage I–II and stage IV ($P = 0.046$). The tumor size in–

Table 2. Relationship between tumor stage and response to radiotherapy

Stage	NED	AWD/CD	*P*
I + II	5	0	0.001
III + IV + recurrence	14	25	

NED — no evidence of disease; AWD — alive with disease; CD — cancer–caused death; *P* — value calculated by Fisher's exact test

creased with the stage of disease. At the time at which the tumor size of the NED patients was 5.2 ± 1.6 cm, it had increased to 6.6 ± 2.6 in the AWD patients, and to 6.9 ± 1.6 cm in the patients who subsequently died (CD). The difference in size was significant between the NED and CD groups (P = 0.003, two–tailed Student's t–test). The median tumor diameter was 6.1 ± 2.0 cm. We divided the patients into those with tumors less than 6.1 cm in diameter (n = 23) and those with tumors 6.1 cm in diameter or larger (n = 21). However, there was no significant difference in survival between these groups (P = 0.07, Breslow—Gehan—Wilcoxon–test).

The presence of HPV infection, stage, tumor size, and outcome. HPV 16, 18, or 33 genomes were detected by PCR in 17 patients (38.6%): 1 patients with stage I, 3 stage II, 9 stage III, 2 stage IV, and 2 patients with cervical stump recurrence. The tumor size was 5.6 ± 2.2 cm in the HPV–positive tumors compared to 6.5 ± 1.8 cm in the HPV–negative tumors; there was no correlation between tumor size and HPV status (two–tailed Student's t–test). Of the HPV–positive patients, 8 have NED, 3 are AWD, and 6 suffered CD. Of the HPV–negative patients, 11 have NED, 4 are AWD, and 12 suffered CD. Thus, there was no significant correlation between the response and HPV status (Fisher's exact test), nor was there any significant difference in survival between the HPV–positive and HPV–negative patients (P = 0.53, Breslow—Gehan—Wilcoxon–test).

Relationships between Bax and Bcl–2 expressions before treatment and stage, tumor size, and outcome. Positive Bax expression before RT was seen in 10 of 44 tumors (22.7%): in 1 case of stage I, 1 stage II, 4 stage III, 2 stage IV, and 2 cervical stump recurrences. The tumor size was 6.0 ± 2.6 cm in the Bax^+ tumors and 6.2 ± 1.8 cm in the Bax^- tumors. Therefore, there was no correlation between tumor size and Bax status (two–tailed Student's t–test). Of the Bax–positive patients, 4 have NED, 3 are AWD, and 3 have suffered CD. Of the Bax–negative patients, 15 have NED, 4 are AWD, and 15 have suffered CD. When we divided the patients into an NED group and an AWD/CD group, there was no significant relationship between these groups and Bax positivity (Fisher's exact test), nor was there any significant difference in survival between the Bax^+ and Bax^- patients (P = 0.55, Breslow—Gehan—Wilcoxon–test).

Positive Bcl–2 expression before RT was seen in 27 of 44 tumors (61.4%) — in 2 cases of stage I, 3 stage II, 15 stage III, 4 stage IV, and 3 cervical stump recurrences. The tumor size was 6.0 ± 2.1 cm in the $Bcl–2^+$ tumors and 6.3 ± 1.6 cm in the $Bcl–2^-$ tumors. There was no correlation between tumor size and Bcl–2 status (two–tailed Student's t–test). Of the $Bcl–2^+$ patients, 9 have NED, 6 are AWD, and 12 have suffered CD. Of the $Bcl–2^-$ patients 10 have NED, 1 is AWD and 6 have suffered CD. There was no significant relationship between the NED group and ADW/CD group and Bcl–2 positivity (Fisher's exact test). There was no significant difference in survival between the Bcl–2–positive and negative patients (P = 0.9, Breslow—Gehan—Wilcoxon–test).

Relationships between Bax and Bcl–2 expression after RT and stage, tumor size, and outcome. Positive Bax expression after 10.8 Gy radiation was seen in 16 of 44 tumors (36.4%), with distinct dense nuclear staining: in 2 cases of stage I, 3 stage II, 8 stage III, 2 stage IV, and 1 cervical stump recurrences. The tumor size was 5.8 ± 1.9 cm in the Bax^+ and 6.3 ± 2.0 cm in the Bax^- tumors after RT; there was no correlation between tumor size and post–radiation Bax status (two–tailed Student's t–test). Of the Bax^+ patients, 13 have NED, 2 are AWD, and 1 has suffered CD. Of the Bax^- patients, 6 have NED, 5 are AWD, and 17 have suffered CD. There was a significant relationship between the NED group and the AWD/CD group and Bax positivity (P = 0.0002, Fisher's exact test).

Positive Bcl–2 expression after RT was seen in 28 of 44 tumors (63.6%), with cytoplasmic and perinuclear staining. Tumor size was larger in $Bcl–2^+$ patients than in $Bcl–2^-$ ones after RT (6.6 ± 1.8 cm compared to 5.2 ± 1.9 cm, P = 0.02, two–tailed Student's t–test). Of the $Bcl–2^+$ patients, 7 have NED, 5 are AWD and 16 have suffered CD. Of the $Bcl–2^-$ patients, 12 have NED, 2 are AWD, and 2 have suffered CD. There was significant relationship between the NED group and the AWD/CD group and Bcl–2 positivity (P = 0.002, Fisher's exact test).

The survival of the patients who were Bax^+ after radiation was significantly better than that of the Bax^- patients (P = 0.04, Breslow—Gehan—Wilcoxon–test, Fig. 1, a). The survival of the patients with $Bcl–2^-$ tu–

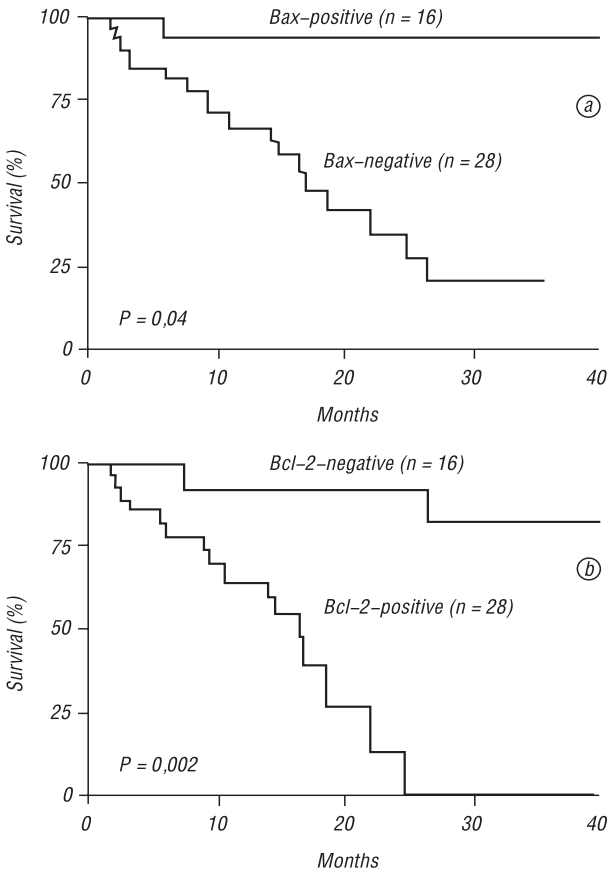

Fig. 1. Survival of patients with respect to Bax (a) and Bcl–2 (b) expression after 10.8 Gy radiation, plotted using the Kaplan—Meier method and analyzed by the Breslow—Gehan—Wilcoxon–test

mors after radiation was significantly better than that of the patients with Bcl–2$^+$ tumors (P = 0.002, Breslow—Gehan—Wilcoxon–test, Fig. 1, b).

Changes of Bax and Bcl–2 expression before and after radiation. As summarized in Table 3, 9 tumors changed from being Bax$^-$ before treatment to being Bax$^+$ after RT, and all of the patients in this group now have NED. The pre–radiation tumor size in this group was 5.9 ± 1.4 cm. A total of 25 initially Bax$^-$ tumors did not change their Bax expression after treatment; six of these patients have NED, and 19 patients are AWD or have suffered CD. The tumor size in this group was 6.2 ± 1.9 cm. The group that was Bax$^-$ before treatment and Bax$^+$ after radiation showed significantly better responses than the group that was Bax$^-$ before and after RT (P = 0.0001, Fisher's exact test). However there was no correlation with tumor size in either group (two–tailed Student's t–test). Seven tumors did not change their initially Bax–positivity after treatment; 4 of these patients have NED, and 3 are AWD or have suffered CD. The tumor size in this group was 5.6 ± 2.6 cm. Three tumors changed from being Bax$^+$ before treatment to being Bax$^-$ after radiation, and all of the patients in this group are AWD or have suffered CD. The tumor size in this group was 7.0 ± 3.1 cm. There was no correlation between radioresponse (Fisher's exact test) and tumor size (two–tailed Student's t–test) in these groups (Table 3).

Table 3. Bax expression before and after RT

Expression	NED	AWD/CD	P^a	Tumor size (cm)	P^b
Bax$^-$→Bax$^+$	9	0	0.0001	5.9 ± 1.4	NS
Bax$^-$→Bax$^-$	6	19		6.2 ± 1.9	
Bax$^+$→Bax$^+$	4	3	0.17	5.6 ± 2.6	NS
Bax$^+$→Bax$^-$	0	3		7.0 ± 3.1	

[a] — Fisher's exact test
[b] — two–tailed Student's t–test

7 tumors changed from being Bcl–2$^-$ before treatment to being Bcl–2$^+$ after RT; 1 patient in this group now has NED and 6 patients are AWD or have suffered CD. The pre–radiation tumor size in this group was 7.3 ± 0.9 cm. Ten tumors did not change their Bcl–2–negative expression after treatment; 9 patients have NED, and 1 patient died from cancer. The tumor size in this group was 5.6 ± 1.7 cm. The group that was Bcl–2$^-$ before treatment and Bcl–2$^+$ after radiation showed significantly poorer responses than the group that was Bcl–2$^-$ both before and after treatment (P = 0.004, Fisher's exact test). In addition, the mean tumor size of the group that was Bcl–2$^-$ before treatment and Bcl–2$^+$ after radiation was larger than that of the group that was Bcl–2$^-$ before treatment and Bcl–2$^-$ after RT (P = 0.02, two–tailed Student's t–test) in these groups. Altogether 21 tumors did not change their Bcl–2$^+$ expression after treatment; 6 of these patients have NED, and 15 patients are AWD or have suffered CD. The tumor size in this group was 6.4 ± 2.4 cm. 6 tumors changed from being Bcl–2$^+$ before treatment to being Bcl–2$^-$ after RT; 3 of these patients have NED and 3 patients are AWD or have suffered CD. The tumor size in this group was 4.7 ± 2.4 cm. There was no correlation between radioresponse (Fisher's exact test) and tumor size (two tailed Student's t–test) in these groups (Table 4).

Table 4. Bcl–2 expression before and after RT

Expression	NED	AWD/CD	P^a	Tumor size (cm)	P^b
Bcl–2$^-$→ Bcl–2$^+$	1	6	0.004	7.3 ± 0.9	0.02
Bcl–2$^-$→ Bcl–2$^-$	9	1		5.6 ± 1.7	
Bcl–2$^+$→ Bcl–2$^+$	6	15	0.3	6.4 ± 2.4	NS
Bcl–2$^+$→ Bcl–2$^-$	3	3		4.7 ± 2.4	

[a] — Fisher's exact test
[b] — two–tailed Student's t–test

(B) BAX AND BCL–2 EXPRESSION FOLLOWING RADIATION THERAPY VERSUS RADIATION PLUS THERMORADIOTHERAPY IN STAGE IIIB CERVICAL CARCINOMA

The relative amounts of Bcl–2 and Bax proteins determine cell survival or death following an apoptotic stimulus. To clarify the molecular mechanism of cell death after radiotherapy of thermoradiotherapy and its relation to the response of AJCC/UICC Stage IIIB cervical carcinomas, the expression of Bax and Bcl–2 proteins was investigated both before and in the course of treatment given during this study.

MATERIALS AND METHODS

Patient characteristics. Between October 1994 and June 1998, 37 patients with Stage IIIB carcinoma of the uterine cervix were enrolled in this study at Kansai Medical University. The patient eligibility criteria for entry into the study were as follows: 1) histologically proven cervical carcinoma; 2) International Federation of Gynecology and Obstetrics (FIGO) Stage IIIB disease; 3) Estern Cooperative Oncology Group (ECOG) performance status of 0–2; 4) no prior chemotherapy, RT, or surgery; 5) adequate bone marrow, liver, and renal function; 6) no concomitant malignancies; and 7) provision of informed consent. The protocol of this trial was approved by the Kansai Medical University Review Board. Randomization to treatment groups was performed by a computer–generated random number list. The patients were divided into the following two groups: the RT group of 19 patients who received RT alone, and the TRT group of 18 patients treated with RT and hyperthermia once a week for a total of 3 sessions, for up to 30.6 Gy of radiation. All randomized patients were evaluated for clinical response as well as *p53* status, HPV infection, and Bax and Bcl–2 immunostaining.

As shown in Table 5, there were no significant differences in patients' demographics, tumor characteristics, or follow–up between the two groups. Meanwhile, the frequency of mutation in *p53* was significantly higher in the TRT group than that in the RT group (P = 0.046, Fisher exact test).

Hyperthermia. Hyperthermia was delivered via a radiofrequency (RF) capacitive heating device (Thermotron RF–8, Yamamoto Vinita Co., Osaka, Japan) that uses 8 MHz RF electromagnetic waves as a source of heat. The output power ranged from 800 to 1500 W. The heating was performed as previously described

Table 5. Clinical characteristics of patients with cervical carcinoma stage IIIB

Characteristic	RT (n = 19)	TRT (n = 18)	*P* value
Mean age (years)	61.6	63.8	NS*
SD	11.2	9.5	
Follow–up time (month)			
Mean	22.2	24.2	NS*
Range	3.5–44.5	5.9–48.8	
Histology			
Squamous cell ca.	18	15	NS**
Adeno ca.	1	3	
Tumor size (cm)			
Mean	6.3	6	NS*
SD	1.7	2.3	
P53 status			
Wild–type	19	14	0.046
Mutant–type	0	4	
HPV infection			
Positive	6	4	NS**
Negative	13	14	

ca. — carcinoma; HPV — human papilloma virus
* — two tailed Students' *t*–test
** — Fischer's exact test

[15]. The electromagnetic power was applied between 2 external disk electrodes 25 or 30 cm in greatest dimension placed on opposite sides of the pelvic region. The temperature of the tumor was measured in all patients using a 4–point microthermo–couple sensor, which was inserted into the tumor through a 21–gauge catheter with the aid of ultrasonography (US). Hyperthermia was applied 10–20 min after external RT once a week for a total of 3 sessions. The first heating was usually performed after the third of fifth fraction of external RT.

Clinical response. The response of the tumor to the treatment was defined as follows: complete response (CR) when no tumor was detected by physical examination or magnetic resonance imaging (MRI), and cytologic or biopsy studies were negative for malignant cells for at least 1 month after treatment; partial response (PR) when the tumor mass was reduced by ≥ 50%; and no change (NC) when the reduction in the tumor mass was <50%.

RESULTS

Treatment response in the RT and TRT groups. The number of patients with CR was significantly greater in the TRT (15 of 18 patients, 83.3%) than in the RT group (10 of 19 patients, 52.6%) ($P = 0.049$, Fisher's exact test). Clinical outcome was also better for the TRT group (Table 6).

Pretreatment and posttreatment Bax expression in the RT and TRT groups. Pretreatment Bax was positive in 2 of 19 (10.5%) in the RT group and 3 of 18 tumors (16.7%) in the TRT group. Therefore, there was no correlation between the two groups and Bax status either before or after treatment (Fisher's exact test). In the course of the treatment, the total numbers of Bax–positive tumors became 15.8% (3 of 19) and 44.4% (8 of 18) in the RT and TRT groups respectively. A relative increase in Bax expression was observed in 10.5% of tumors (2 of 19) in the RT group versus 44.4% of tumors (8 of 18) in the TRT group. Thus, the absolute number of tumors that showed a relative increase in Bax expression was higher in the TRT group than in the RT group ($P = 0.02$, Fisher's exact test). Changes in Bax expression scores before and after 10.8 Gy of radiation are shown for the RT and TRT groups in Fig. 2, *a, b*.

Table 6. Treatment response and clinical outcome

	RT (n = 19)	TRT (n = 18)	*P* value
Treatment response			
CR	10	15	0.049
PR	5	2	NS
NC	4	1	NS
Clinical outcome			
Disease–free	9	12	NS
Local failure	6	2	NS
Local failure and distant metastases	3	1	NS
Distant metastases	1	3	NS

P value calculated by Fisher's exact test

Pretreatment and posttreatment Bcl–2 expression in the RT and TRT groups. Pretreatment Bcl–2 was positive in 7 of 19 tumors (36.8%) in RT group and 6 of 18 tumors (33.3%) in the TRT group. Therefore, there was no correlation between the two groups and Bcl–2 status either before or after treatment (Fisher's exact test). In response to treatment, an increase in Bcl–2 expression was seen in 21.1% of tumors (4 of 19) in the RT group and 27.8% of tumors (5 of 18) in the TRT group. Therefore, there was no significant difference between the two groups and the changes of Bcl–2 expression in response to treatment.

DISCUSSION

Apoptosis plays an important role in the cytotoxic effects of radiation therapy [7] and chemotherapy [16]. The activation of *p53* is associated with induction of apoptosis [4]. When the cellular DNA is damaged by the stress such as ultraviolet, radiation and hyperthermia, the level of wild type p53 will be increased, then the cell cycle is arrested at G_1 stage. In this period, DNA replication is carried out, cell cycle again will be going on, DNA synthesis begins. But if the replication is not possible, mutant cell will be eliminated through apoptosis. These functions of the wild *p53* as the G_1–checkpoint are observed not only in normal cell but in cancer cell having wild type *p53* gene. As stated above, when the DNA is damaged, various kind of gene will be expressed by the signal transduction pathway around *p53* and cell cycle and apoptosis are controlled as shown in Fig 3.

In the case of having mutant *p53* gene, the cell cycle is not arrested by even the stress raising DNA damage, and apoptosis is not seen. Activation of the p53 pathway under genotoxic stress leads to the down–regulation of *bcl–2* and the up–regulation of *bax* [17]. Bax homodimerizes and forms heterodimers with Bcl–2 *in vivo* [18]. Oltvai *et al.* reported that the ratio of Bcl–2 to Bax determines survival or death following an apoptotic stimulus [18]. They described that when Bcl–2 is in excess, cells are protected; however, when Bax is in excess and Bax homodimers dominate, cells are susceptible to apoptosis.

The findings regarding Bcl–2 are controversial. The prognostic importance of Bcl–2 overexpression has been reported for various solid carcinomas. Bcl–2 pro–

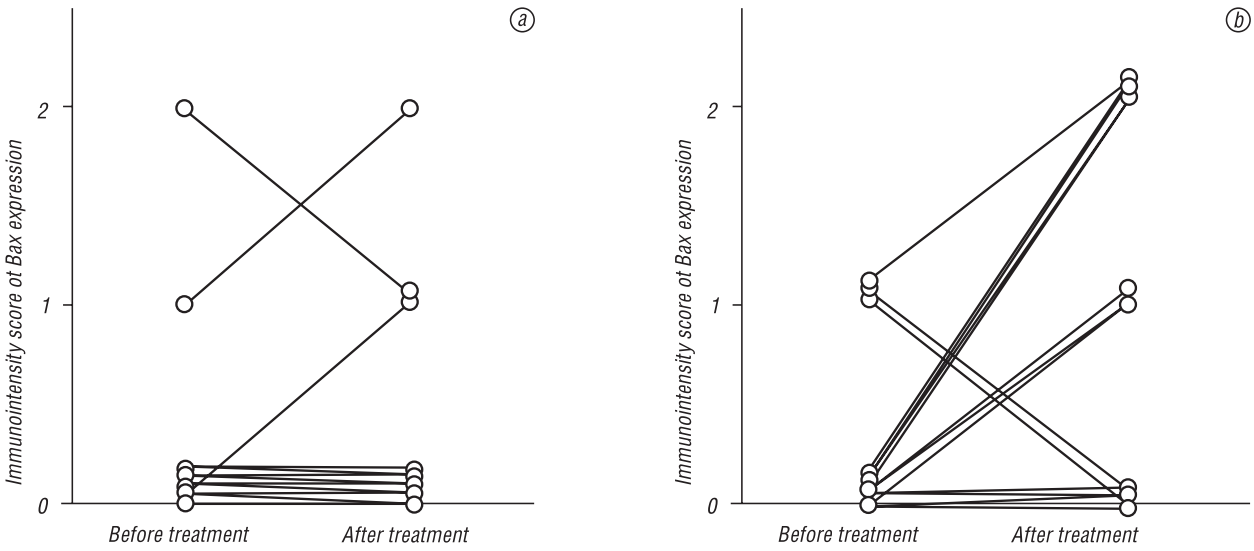

Fig. 2. Changes in the score of BAX expression before and after 10.8 Gy radiation in the RT group (n=19) (*a*) and in the TRT group (n=18) (*b*)

tein expression has been linked to a poor prognosis in bladder cancer [19] and prostate cancer [20]; a better prognosis in well–differentiated breast carcinoma [21], and no correlation was found in laryngeal squamous cell carcinoma [22]. In addition, Bax^+ $Bcl–2^-$ tumor expression had a significant negative influence on survival in patients with non–small–cell lung carcinoma [9]. In contrast, neither Bax nor Bcl–2 expression has been linked to prognosis in gastric carcinoma [23]. In the present study, we observed no significant difference in survival between the patients with pre–treatment Bax^+ or $Bcl–2^-$ tumors and the patients with Bax^- or $Bcl–2^+$ tumors.

Since there is controversy surrounding the prognostic value of Bax and Bcl–2 expression in pre–treatment specimens of cervical carcinoma, we investigated the correlation between Bax and Bcl–2 expression before and after radiotherapy and outcome. West *et al*.

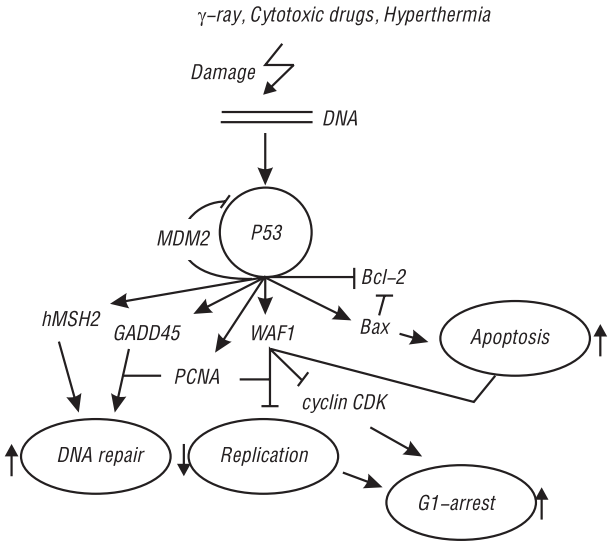

Fig. 3. Signal transduction pathway around p53

[24] found a correlation between intrinstic radiosensitivity and outcome in cervical cancer, using the Courtenay–Mills clogenic assay. In that trial of 88 patients with carcinoma of the cervix treated with radiation therapy, tumor cell survival *in vitro* following a dose of 2 Gy (SF2) was significantly predictive of outcome.

Using as an end–point the accumulation of Bax and Bcl–2 in tissue specimens taken after the administration of 10.8 Gy, we found that there was a significantly increased survival rate among the patients with Bax^+ tumors, compared with Bax^- tumors ($P = 0.04$). Thus, Bax expression may reflect the susceptibility to apoptosis, revealing a prominent early radiation response in radiosensitive tumors. Our data are in agreement with earlier *in vitro* [25] and *in vivo* observations [26]. In contrast to Bax, there was a significantly decreased survival rate among patients with $Bcl–2^-$ tumors ($P = 0.002$).

The importance of Bax and Bcl–2 expression in the response of cervical carcinoma to TRT remains unknown. In the current study, we found a positive correlation between the relative increase of Bax in the course of the treatment and patients' treatment responses. The TRT treatment caused more efficient activation of *bax* apoptotic pathway (44.4% of patients (8 of 18) in the TRT group vs. 10.5% (2 of 19) in the RT group, $P = 0.02$) and superior local tumor control (83.3% of patients (15 of 18) in the TRT group vs. 52.6% (10 of 19) in the RT group, $P = 0.0049$). In contrast, there was no correlation between the increase of Bcl–2 expression and treatment response in this study.

In contrast to the situation in many other human tumors, *p53* mutations are only rarely detected in cervical cancers [27]. In the present study also, only 4 patients (9.1%) were found to have mutations in the *p53* gene, as evaluated by an SSCP analysis of genomic DNA. 4 tumors with mutant *p53* had G C to A T muta–

tions. Our data are in agreement with a report on the high frequency of G C to A T mutations in cervical carcinoma [28]. In one patient with a mutant–*p53*–positive tumor, Bax expression with no Bcl–2 expression was seen, which resulted in a good prognosis. There are two explanations for the presence of Bax expression in patients bearing mutant *p53*. Either the mutated *p53* gene does not lose the ability to activate the *bax* gene, or, more probably, *bax* is activated through a *p53*–independent pathway.

Activation of one of the *bax* pathways is a proposed mechanism of synergistic antitumor effects of RT and hyperthermia.

REFERENCES

1. **Ponten J, Adami HO, Bergstrom R, Dillner J, Friberg LG, Gustafsson L, Miller AB, Parkin DM, Sparen P, Trichopoulos D.** Strategies for global control of cervical cancer. Int J Cancer 1995; **60**: 1–26.

2. **Toita T, Nakano M, Higashi M, Sakumoto K, Kanazawa K.** Prognotic value of cervical size and pelvic lymph node status assessed by computed tomography for patients with uterine cervical cancer treated by radical radiation therapy. Int J Radiat Oncol Biol Phys 1995; **15**: 843–9.

3. **Virostek LJ, Kim RY, Spencer SA, Meredith RF, Jennelle RL, Soong SJ, Salter MM.** Postsurgical recurrent carcinoma of the cervix: reassessment and results of radiation therapy options. Radiology 1996; **201**: 559–63.

4. **Lowe SW, Schmitt EM, Smith SW, Osborne BA.** P53 is required for radiation – induced apoptosis in mouse thymocytes. Nature 1993; **362**: 847–9.

5. **Kasten M, Onyekwere O, Sidransky D, Vogelstein B, Craig RW.** Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991; **51**: 6304–11

6. **Lowe SW, Bodis S, McClatchey A, Remington L, Ruley HE, Fisher DE, Housman DE, Jacks T.** P53 status and the efficacy of cancer therapy in vivo. Science 1994; **266**: 807–10.

7. **Chyle V, Pollack A, Czerniak B, Stephens LC, Zagars GK, Terry NHA, Meyn RE.** Apoptosis and downstaging after pre-operative radiotherapy for muscle-invasive bladder cancer. Int J Radiat Oncol Biol Phys 1996; **35**: 281–7.

8. **Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC.** Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrances. Cancer Res 1993; **53**: 4701–14.

9. **Apolinario RM, van der Valk P, de Jong JS, Deville W, van Ark-Otte J, Digemans AM, van Mourik JC, Postmus PE, Pinedo HM, Giaccone G.** Prognostic value of the expression of p53, bcl-2, and bax oncoproteins, and neovascularization in patients with radically resected non-small-cell lung cancer. J Clin Oncol 1997; **15**: 2456–66.

10. **Falkvoll KH, Real C.** Quantitative histological changes in a human melanoma xenograft following exposure to single dose irradiation and hyperthermia. Int J Radiat Oncol Biol Phys 1991; **21**: 989–94.

11. **Nagata Y, Hiraoka M, Nishimura Y, Masunaga S, Mitumori M, Okuno Y, Fujishiro M, Kanamori S, Horii N, Akuta K, Sasai K, Abe M, Fukuda Y.** Clinical results of radiofrequency hyperthermia for malignant liver tumors. Int J Radiat Oncol Biol Phys 1997; **38:** 359–65.

12. **Hornback NB, Shupe RE, Shidnia H, Marshall CU, Lauer T.** Advanced stage IIIB cancer of the cervix treatment by hyperthermia and radiation. Gynecol Oncol 1986; **23**:160–7.

13. **Pezzella F, Tso AGD, Cordell JL, Pulford KAF, Gatter KC, Mason DY.** Expression of the bcl-2 oncoprotein is not specific for the 14;18 chromosomal translocation. Am J Pathol 1990; **137**: 225–32.

14. **Yoshikawa H, Kawana T, Kitagawa K, Mizuno M, Yoshikura H, Iwamoto A.** Amplification and typing of multiple cervical cancer-associated human papillomavirus DNAs using a single pair of primers. Int J Cancer 1990; **45**: 990–2.

15. **Tanaka Y, Yamamoto K, Murata T, Nagata K.** Effects of multimodal treatment and hyperthermia on hepatic tumors. Cancer Chemother Pharmacol 1992; **31**: S111–S114.

16. **Harima Y, Harima K, Hasegawa T, Shikata N, Tanaka Y.** Transcatheter arterial embolization with Cisplatin: Apoptosis in VX2 tumor uterus transplants. Anticancer Res 1996; **16**: 193–9.

17. **Haldar S, Negrini M, Monne M, Sabbioni S, Croce CM.** Down-regulation of bcl-2 by p53 in breast cancer cells. Cancer Res 1994; **54**: 2095-7.

18. **Oltvai ZN, Milliman CL, Korsmeyer SJ.** Bcl-2 heterodimerizes in vivo with a conserved homolog, bax, that accelerates programmed cell deth. Cell 1993; **74**: 609–19.

19. **Lipponen PK, Aaltomaa S, Eskelinen M.** Expression of the apoptosis suppressing bcl-2 protein in transitional cell bladder tumors. Histpathology 1996; **28:**135–40.

20. **Bubendorf L, Sauter G, Moch H, Jordan P, Blochlinger A, Gasser TC, Mihatsch MJ.** Prognostic significance of Bcl-2 in clinically localized prostate cancer. Am J Pathol 1996; **148**: 1557–65.

21. **Lipponen P, Pierilainen T, Kosma VM, Aaltomaa S, Eskelinen M, Syjanen K**. The apoptosis suppressing protein bcl-2 is expressed in well-differentiated breast carcinomas with a favourable prognosis. J Pathol 1995; **177**: 49–55.

22. **Spafford MF, Koeppe J, Pan Z, Archer PG, Meyers AD, Franklin WA.** Correlation of tumor markers p53, bcl-2, CD34, CD44H, CD44v6 and Ki-67 with survival and metastasis in laryngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 1996; **122**: 627–32.

23. **Koshida Y, Saegusa M, Okayasu I.** Apoptosis, cell proliferation and expression of Bcl-2 and Bax in gastric carcinomas: immunohistochemical and clinicopathological study. Br J Cancer 1997; **75**: 367–73.

24. **West CML, Davidson SE, Roberts SA, Hunter RD**. Intrinsic radiosensitivity and prediction of patient response to radiotherapy for carcinoma of the cervix. Br J Cancer 1993; **68**: 819–23.

25. **Macklis RM, Lin JY, Beresford B, Atcher RW, Hines JJ, Humm JL**. Cellular kinetics, dosimetry, and radiobiology of α-particle radioimmunotherapy: induction of apoptosis. Radiat Res 1992; **130:** 220–6.

26. **Stephen LC, Ang KK, Schultheiss TE, Milas L, Meyn R**. Apoptosis in irradiated murine tumors. Radiat Res 1991; **127**: 308–16.

27. **Chang F, Syrjanen S, Syrjanen K.** Implications of the p53 tumor-suppressor gene in clinical oncology. J Clin Oncol 1995; **13**: 1009–22.

28. **Park DJ, Wilczynski SP, Paquette RL, Miller CW, Koeffler HP.** P53 mutations in HPV-negative cervical carcinoma. Oncogene 1994; **9**: 205–10.