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# Enhanced Bax in oral SCC in relation to antitumor effects of chemotherapy

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**BACKGROUND: Antitumor effects of chemotherapeutic agents are commonly associated with the induction of apoptosis. Bax belongs to the Bcl-2 family and induces apoptosis. The present study was conducted to investigate the relationship between enhanced Bax expression in oral squamous cell carcinoma (SCC; cell lines and clinical cases) and the antitumor effects of chemotherapy.**

**METHODS: In three oral SCC cell lines, Bax expression before and after treatment with chemotherapeutic agents [docetaxel (TXT), cisplatin and 5-fluorouracil] was examined by reverse transcriptase-polymerase chain reaction and immunoblotting. The effects of treatment were assessed by counting the number of viable cells and determining sub-G1 cells. Tissue samples (both biopsy specimens before chemotherapy and surgically excised specimens after chemotherapy) from nine patients with oral SCC who underwent neoadjuvant chemotherapy were immunostained for Bax. The relationship between enhancement of Bax expression and chemotherapeutic effects was established.**

**RESULTS: Two of three cell lines did not express Bax mRNA or protein before treatment. After treatment, Bax expression was enhanced only by TXT in one cell line, but by all chemotherapeutic agents in the other two cell lines. In three of nine patients, Bax expression was not found before chemotherapy. Two of these three patients showed enhanced Bax expression after chemotherapy including TXT, but one still failed to express Bax. Both in cell lines and clinical cases, enhancement of Bax after chemotherapy was associated with antitumor effects.**

**CONCLUSION: Certain chemotherapeutic agents enhance Bax expression in oral SCC, and it is suggested that this contributes to the antitumor effects of chemotherapy.**
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**Keywords:** apoptosis; Bax; oral squamous cell carcinoma

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Chemotherapy has an important role in multidisciplinary treatment of head and neck cancer. There are now randomized data supporting the use of chemotherapy in the context of combined modalities treatment to cure patients with unresectable disease, as well as in a neoadjuvant approach to downstage disease in patients. Moreover, the use of concomitant chemotherapy and radiotherapy in advanced disease has been shown to provide a statistically significant survival benefit in selected studies (1).

Antitumour effects of chemotherapeutic agents are commonly associated with the induction of apoptosis, and there are numerous reports linking apoptosis with chemotherapeutic success (2–8). Apoptosis is an important phenomenon for determining sensitivity to chemotherapeutic agents in cancer chemotherapy. Gene products regulating chemotherapeutic agents often induce apoptosis, and genes of the p53 and Bcl-2 families are included in this category (9–11). These apoptotic control genes are known to be involved in regulating signal transduction pathways in chemotherapeutic drug-induced apoptosis. The p53 gene product regulates apoptosis by activating its downstream target genes such as Bax and Bcl-2 in the signal transduction pathways. In the p53-dependent pathway, when DNA damage occurs, Bax is induced by p53, whereas Bcl-2 is suppressed by p53 (12, 13). It has been noted that chemotherapeutic drug-induced apoptosis can be regulated by the activation of proapoptotic genes and the suppression of antiapoptotic genes.

Recently, Xie et al. (14) demonstrated that Bax expression is strongly associated with favorable clinical outcome in SCC of the head and neck. We have previously suggested that patients with oral SCC expressing Bax as detected by immunohistochemistry have good responses to neoadjuvant chemotherapy and better survival rates than patients without Bax expression (15).

In the present study, we investigated enhanced Bax expression, which would be expected to promote apoptosis, in relation to the antitumor effects of different chemotherapeutic agents using three oral SCC cell lines,

and specimens from nine patients treated with chemotherapy following surgery.

## Materials and methods

*Cell lines, culture conditions and chemotherapeutic treatment*

The three human oral SCC cell lines Ho-1-N-1, KOSC-3 (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) and SAS (obtained from the Institute of Development, Aging and Cancer, Tohoku University, Tohoku, Japan) were analysed. The Ho-1-N-1 line is a well-differentiated SCC derived from primary buccal mucosa cancer. KOSC-3 is also a well-differentiated SCC derived from primary lower gingival cancer. SAS is a poorly differentiated SCC derived from primary tongue cancer. All three of these cell lines carried mutated p53 (16, 17).

Cell lines were cultured in RPMI-1640 (Sigma Chemical Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (Cell Culture Technologies, Tokyo, Japan), 50 units/ml penicillin and 50 μg/ml streptomycin (GIBCO, Grand Island, NY, USA) at 37°C in humidified air containing 6% $CO_2$.

All cell lines were treated with docetaxel (TXT) (Aventis Pharma., Ltd, Tokyo, Japan), cisplatin (CDDP) (Nippon Kayaku Co., Ltd, Tokyo, Japan) and 5-fluorouracil (5-FU) (Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan). From each cell line, $5 \times 10^6$ cells were seeded into 10 cm culture dishes. Where indicated, cells were treated with 1 ng/ml TXT, 1 μg/ml CDDP or 1 μg/ml 5-FU and assayed at 12, 24 or 48 h after exposure to the chemotherapeutic agents.

*Reverse transcriptase-polymerase chain reaction*

Total RNA was isolated from each cell line before treatment as a control and after treatment with the chemotherapeutic agents, using ISOGEN (Nippon Gene). Thereafter, 10 μg of total RNA was reverse transcribed (RT) using the Superscript First-strand Synthesis System (Invitrogen, Tokyo, Japan), and then polymerase chain reaction (PCR) was performed. The forward and reverse primers were 5′-AGGGTTTCATC CAGGATCGAGCAG-3′ and 5′-ATCTTCTTCCAG ATGGTGAGCGAG-3′ for Bax, and 5′-TCCAC CACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCA TGCCATCAC-3′ for G3PDH (18). The cycling conditions for Bax cDNA amplification were 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, over 38 cycles. Conditions for G3PDH cDNA amplification were 94°C for 30 s, 58°C for 30 s, 72°C for 45 s over 28 cycles. PCR products were electrophoresed in 2% agarose gels and detected by staining with Ethidium Bromide Solution (GIBCO). Ready-load 100 bp DNA ladder (GIBCO BRL) was used as a size marker.

*Immunoblot analysis*

Cells from each cell line before treatment as a control and after treatment with chemotherapeutic agents were lysed with lysis buffer [40 mM HEPES (pH 7.5), 100 mM NaCl, 1% N-P40, 5% glycerol, 5 mM EDTA, 1 mM PMSF] and then sonicated. Equal amounts of protein (10 μg) from each of the Ho-1-N-1 or SAS or Kosc-3 cell lysates were loaded onto 15% sodium dodecyl sulphate-polyacrylamide gels and electrophoresed. The separated proteins were then transferred to nitrocellulose membranes (Trans-Blot Transfer Medium; Bio-Rad, Japan, Tokyo, Japan) and incubated with monoclonal antibody, antihuman Bax (clone 4F11, mouse IgG; Medical and Biological Laboratories Co. Ltd, Nagoya, Japan; 1:1000 dilution). The antibodies were visualized with the ECL Plus detection system (Amersham Biosciences, Piscataway, NJ, USA).

*Number of viable cells*

Each cell line before treatment as a control and after treatment with chemotherapeutic agents was stained with trypan blue and unstained (viable) cells were counted with a hemocytometer using a phase contrast microscope (magnification: 100×).

*Flow cytometric analysis*

Flow cytometric analysis of propidium iodide-stained nuclei was performed. The cells were trypsinized before treatment as a control and after chemotherapeutic treatment, rinsed twice with phosphate buffer solution (PBS) and fixed in 70% ethanol at 4°C for at least 5 h. The fixed cells were then rinsed twice with PBS, incubated with 1 μg/ml RNaseA (Sigma Chemical Co.) for 1 h at 37°C and stained with 50 μg/ml propidium iodide (Sigma Chemical Co.) for 1 h at room temperature. The stained cells ($5 \times 10^4$) were analyzed for relative DNA content using a FACS Calibur (Becton Dickinson Labware, Franklin Lakes, NJ, USA).

*Immunohistochemistry in SCC patients*

Paraffin-embedded specimens from nine patients with primary squamous cell caricinoma (both biopsy specimens before chemotherapy and surgically excised specimens after chemotherapy were employed).

The chemotherapy regimen was TXT (80 mg), CDDP (25–37.5 mg) or CDGP (75–120 mg), and/or 5-FU (1250 mg). Primary tumor sites were tongue ($n = 3$), floor of the mouth ($n = 1$), lower gingival ($n = 3$), central carcinoma of the jaw ($n = 1$), and buccal mucosa ($n = 1$). The following criteria were used to classify the nine patients: T and N categories, clinical stage, histopathological grading proposed by the UICC in 2002, DNA ploidy pattern given by Erhardt et al. (19). The clinical response of the tumor to chemotherapy was defined as follows: complete remission (CR), when no clinically detectable tumor has found after chemotherapy; partial remission (PR), when the measurable tumor mass was decreased by 50% after chemotherapy; minimal remission (MR), the same as partial remission, but the response did not meet the criteria of 50% reduction; progression, when the mass of one or more tumor sites increased by more than 25%; and stable disease, when the measurable tumor did not meet the criteria for CR, PR, MR or progression (20).

Both biopsied specimens before chemotherapy and resected specimens of the primary tumor after chemo-

therapy were fixed in 10% formalin, embedded in paraffin and 5-μm sections were made from each sample. Deparaffinized sections were autoclaved (121°C, 5 min) in 10 mM citrate buffer (pH 6.0) to unmask antigens. Monoclonal antibody, antihuman Bax (clone 4F11, mouse IgG; Medical and Biological Laboratories Co. Ltd., Nagoya, Japan; 1:200 dilution) was used (21). Immunohistochemical staining was performed using the CSA system (Dako Japan Co. Ltd, Kyoto, Japan). The sections were finally counterstained with hematoxylin and mounted.

A 5-μm paraffin section of cerebral cortex of an Alzheimer disease patient, which overexpressed Bax, was used as positive control (22). Mouse IgG in 0.05 M Tris buffer, pH 7.6, containing carrier protein and 15 mM sodium azide was used instead of primary antibody as a negative control.

We counted both the positively stained and total number of tumor cells in random fields of specimens under a 20× objective (magnification: 200×). In total, at least 1000 tumor cells were counted. Thereafter, the positive cell fraction was calculated and classified according to the method of Chen et al. (23), as follows: more than 50% of cells positive (+++); 25–50% positive (++); 5–24% positive (+); fewer than 5% positive or no staining (−). This procedure was carried out by one of the authors (K.T.) who was blinded as to patient outcome.

*Statistical analysis*

Differences between the number of cells in control lines and those treated with chemotherapeutic agents were tested by the multiple comparisons method of Sheffe. Values were considered significantly different when $P$ was less than 0.05.

## Results

*Bax mRNA and protein expression in oral SCC cell lines*

Bax mRNA and protein expression after treatment with the different chemotherapeutic drugs used in oral SCC cell lines was examined.

Untreated Ho-1-N-1 and SAS controls did not express Bax mRNA or protein, whereas KOSC-3 expressed both. TXT induced Bax mRNA and protein expression in all cell lines, but CDDP and 5-FU failed to induce Bax mRNA or protein expression in Ho-1-N-1. Bax mRNA and protein were inducible by all three chemotherapeutic drugs, although they were differently expressed in untreated SAS and KOSC-3 controls.

No Bax mRNA or protein was present before treatment with the chemotherapeutic drugs, but both Ho-1-N-1 and SAS cells were able to express Bax mRNA and protein following treatment. In particular, only TXT was able to induce Bax mRNA and protein expression in Ho-1-N-1 cells (Fig. 1).

*Inhibition of cell proliferation by chemotherapeutic agents*

The number of viable cells remaining after treating the

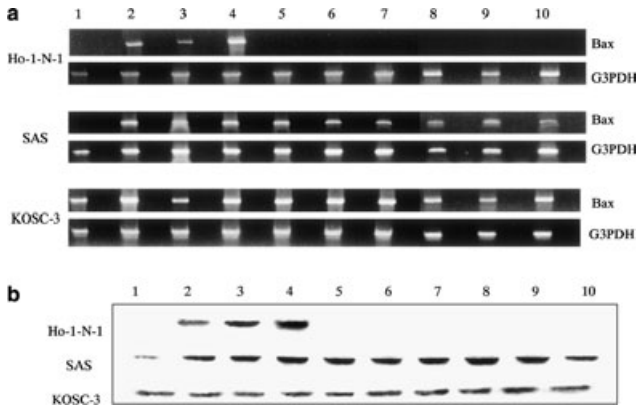


**Figure 1** Bax mRNA and protein in oral squamous cell carcinoma cell lines. The number of each lane shows the following: (1) control, (2) docetaxel (TXT) 1 ng/ml 12 h, (3) TXT 1 ng/ml 24 h, (4) TXT 1 ng/ml 48 h, (5) cisplatin (CDDP) 1 μg/ml 12 h, (6) CDDP 1 μg/ml 24 h, (7) CDDP 1 μg/ml 48 h, (8) 5-fluorouracil (5-FU) 1 μg/ml 12 h, (9) 5-FU 1 μg/ml 24 h, (10) 5-FU 1 μg/ml 48 h. (a) Detection of Bax mRNA by reverse transcriptase-polymerase chain reaction. (b) Detection of Bax protein by the immunoblot method.

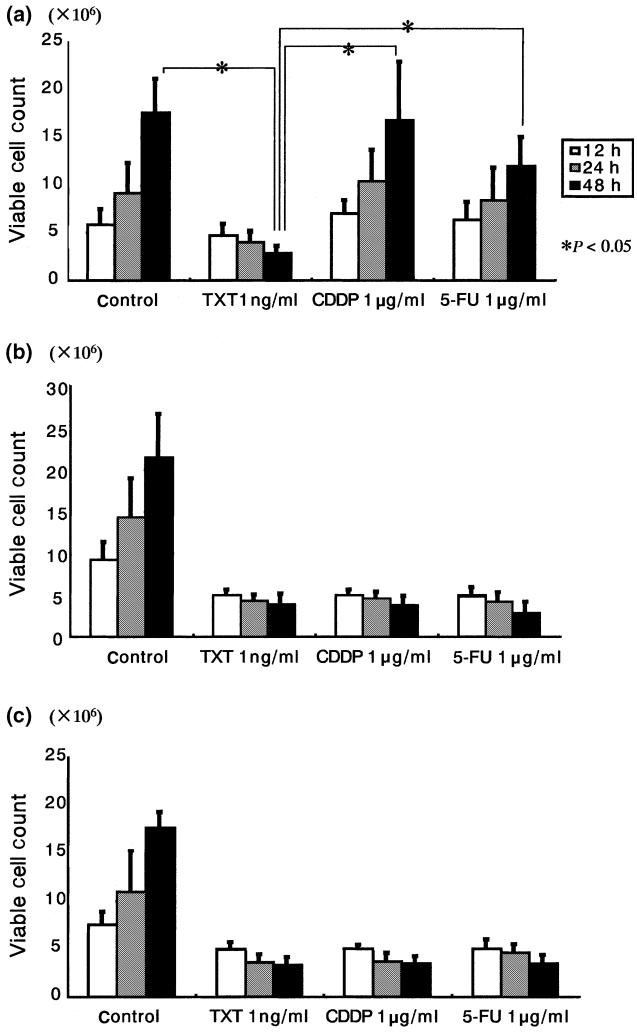


**Figure 2** Inhibition of cell proliferation by chemotherapeutic agents. (a) The number of viable cells in the Ho-1-N-1 line. The number of viable cells decreased significantly after 48 h, of treatment with docetaxel. (b) The number of viable cells SAS decreased after 48 h treatment with any of the chemotherapeutic agents. (c) The number of viable cells in KOSC-3 was likewise decreased by all agents after 48 h.

oral SCC cell lines with the different chemotherapeutic drugs was investigated.

In Ho-1-N-1, treatment with TXT caused a decrease in the number of viable cells and their proliferation was inhibited. In contrast, treatment with CDDP and 5-FU failed to prevent an increase in the number of viable cells and tumor cells proliferated. Therefore, the number of viable cells at 48 h after treatment with TXT was significantly decreased compared with CDDP- and 5-FU-treated cells ($P < 0.05$).

On the other hand, in SAS and KOSC-3, treatment with any of the three chemotherapeutic agents caused a tendential decrease in the number of viable cells in the cultures and their proliferation was inhibited. However, SAS and KOSC-3 did not show any significant reductions (Fig. 2).

In the cultured cell lines, together with Bax expression induced by chemotherapeutic treatment, there was a decrease in the number of viable cells. Inversely, where Bax was not induced, there was an increase in the number of viable cells.

*Peak accumulation of cells in sub-$G_1$*

Peak accumulation of cells in sub-$G_1$ in the Ho-1-N-1 line was examined. The proportion of sub-$G_1$ cells in untreated controls was 1.39%. In TXT-treated cells,

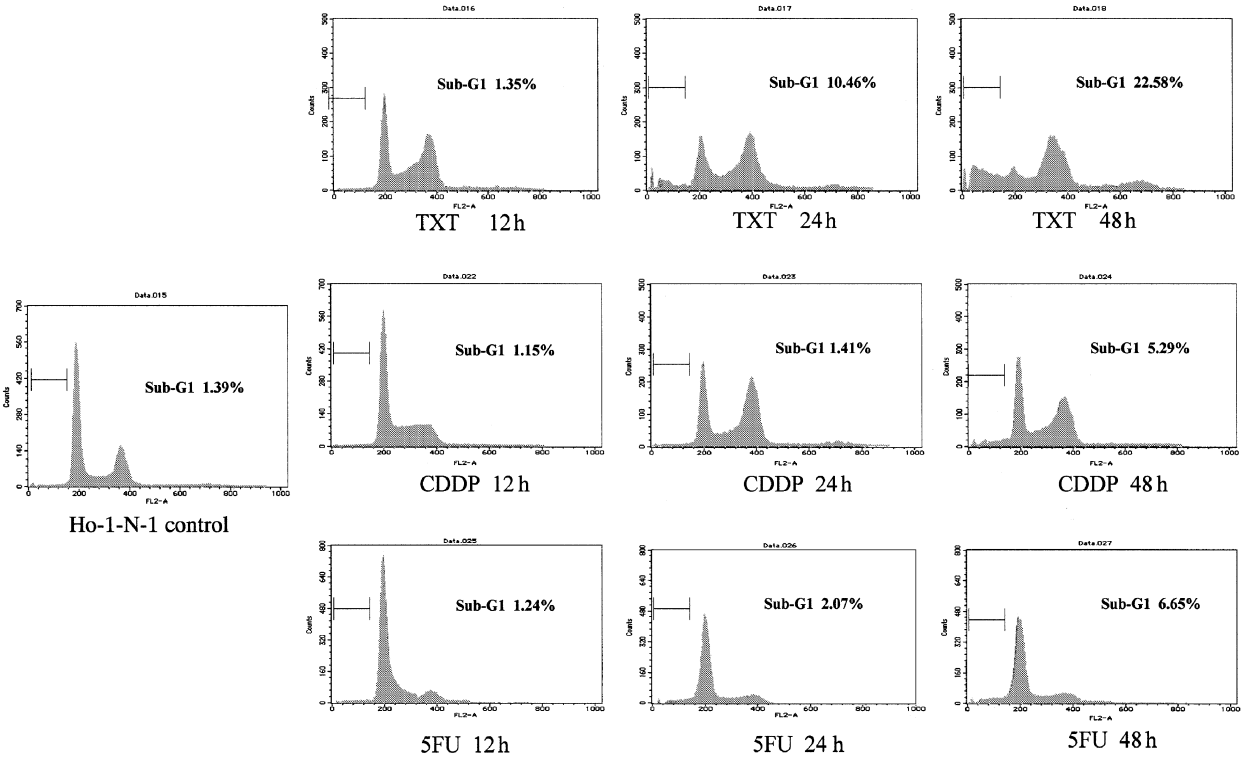


**Figure 3** Results of flow cytometric analysis. In Ho-1-N-1, only docetaxel treatment increased the fraction of cells in sub-G1. On the other hand, cisplatin and 5-fluorouracil treatment increased sub-G1 cells.

**Table 1** Patients with oral squamous cell carcinoma treated mainly with docetaxel

| *No.* | *T and N categories* | *Stage* | *Histopathological grading* | *Site* | *DNA ploidy* | *Chemotherapeutic agents*[a] | *Clinical response to chemotherapy*[b] | *Bax expression before chemotherapy → after chemotherapy* |
|---|---|---|---|---|---|---|---|---|
| 1 | T2N1 | III | G1 | Tongue | Aneulploidy | TXT, CDDP, 5-FU | PR | + + + → + + + |
| 2 | T2N1 | III | G2 | Oral floor | Aneulploidy | TXT, CDDP, 5-FU | MR | + + → + + + |
| 3 | T2N1 | III | G2 | Buccal mucosa | Diploidy | TXT, CDDP, 5-FU | PR | − → + + + |
| 4 | T2N2b | IV | G1 | Lower gingiva | Diploidy | TXT, CDDP, 5-FU | CR | + + + → + + + |
| 5 | T3N1 | III | G1 | Tongue | Aneulploidy | TXT, CDDP, 5-FU | MR | − → + + + |
| 6 | T3N1 | III | G2 | Tongue | Diploidy | TXT, 5-FU | MR | + + + → + + + |
| 7 | T4N1 | IV | G3 | Mandible | Aneulploidy | TXT, CDDP, 5-FU | NC | − → − |
| 8 | T4N2b | IV | G1 | Lower gingiva | Aneulploidy | TXT, CDGP | PR | + + + → + + + |
| 9 | T4N2c | IV | G2 | Lower gingiva | Aneulploidy | TXT, CDGP | PR | + + → + + + |

[a]CDDP, cisplatin; TXT, docetaxel; 5-FU, 5-fluorouracil.
[b]CR, complete remission; MR, minimal remission; PR, partial remission.

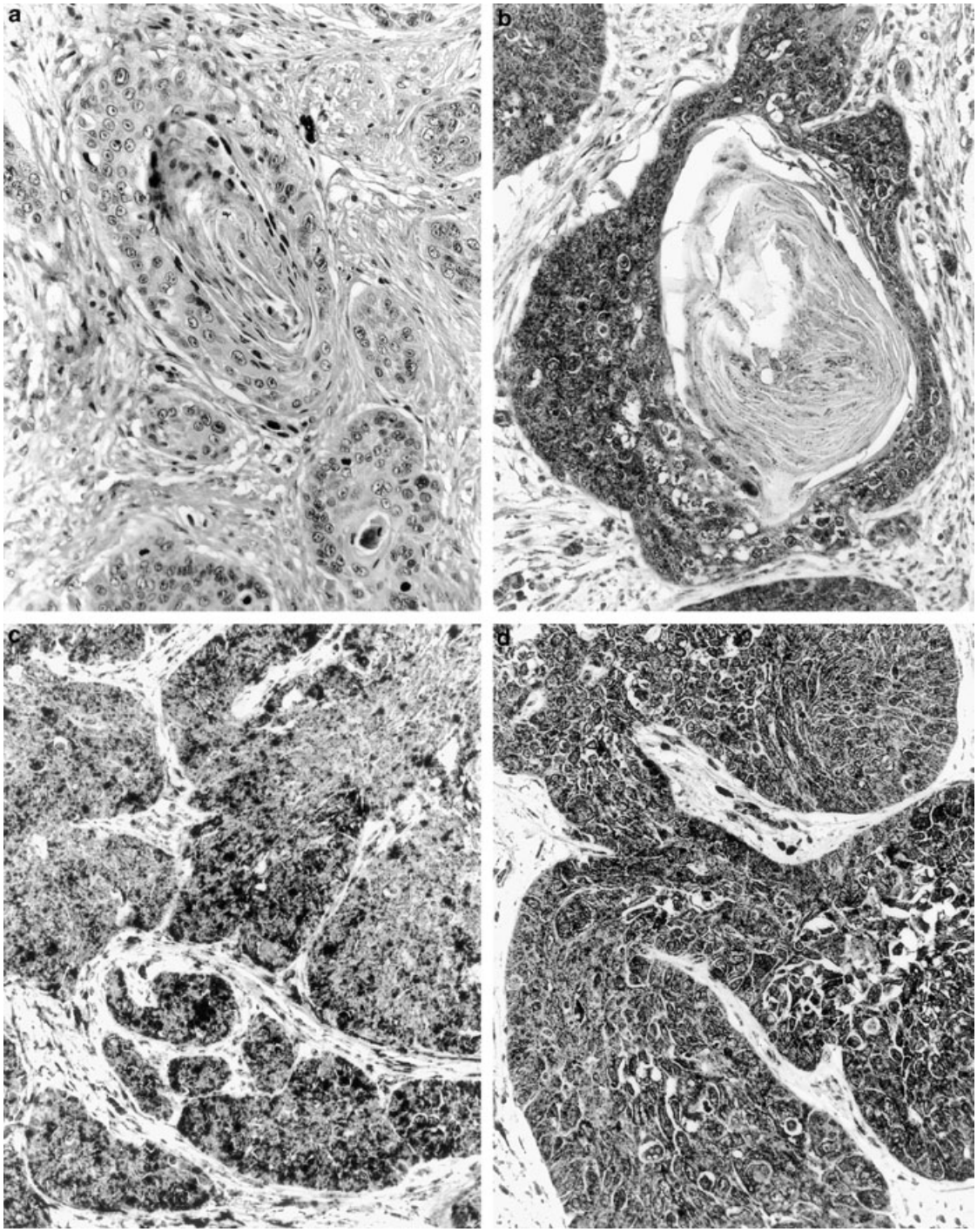


**Figure 4** Immunohistochemical detection of Bax protein in squamous cell carcinoma patients. (a) and (b) were case no. 5, (c) and (d) were case no. 8. (a) Biopsy specimen before preoperative chemotherapy consisting mainly of docetaxel (TXT). Bax protein was not expressed (Bax−) (200×). (b) Operated specimen of the primary tumor after chemotherapy. Bax protein is expressed by 100% of the cells (Bax + + +) (200×). (c) Biopsy specimen before chemotherapy preoperative chemotherapy also consisting mainly of TXT. Bax protein is expressed by 80% of the cells (Bax + + +) (200×). (d) Operated specimen of the primary tumor after chemotherapy. Expression of Bax protein is maintained (Bax + + +) (200×).

the fraction of cells in sub-$G_1$ increased in a time-dependent manner. After 48 h, the number of cells in sub-$G_1$ had reached 22.58%. In contrast, in CDDP- or 5-FU-treated cells, there was little increase in the sub-$G_1$ fraction with time, reaching 5.29 and 6.65%, respectively, after 48 h (Fig. 3). On the other hand, in the SAS and KOSC-3 cell lines, the fraction of cells in sub-G1 was increased to around 20% after 48-h treatment with any of the three chemotherapeutic agents (data not shown).

Thus, in the cultured cell lines, together with the Bax expression induced by treatment with chemotherapeutic

agents, there was an increase of in the fraction of cells in sub-G1.

*Immunohistochemical detection of Bax protein in SCC patients*

Immunohistochemical features for Bax protein expression in patients are summarized in Table 1 (Fig. 4). In nine clinical cases, three were classified as Bax(−), two cases as Bax(++) and four cases as Bax(+++) in biopsy specimens. Among the three cases which were Bax(−) at pre-treatment biopsy, two became Bax(+++) and only one remained Bax(−) after preoperative chemotherapy. Two cases which were Bax(++) at pre-treatment biopsy showed Bax expression enhanced to Bax(+++) after preoperative chemotherapy. Among the four cases which were Bax(+++), three remained Bax(+++) and one could not be evaluated for Bax expression because preoperative chemotherapy had resulted in a CR and no tumor cells could be identified. Regardless of the presence or absence of Bax in pre-treatment biopsy specimens, cases in which Bax expression was found after preoperative chemotherapy were characterized by a higher chemotherapy response level as indicated by the many CR and PR obtained in these patients.

## Discussion

In this study, we showed that various chemotherapeutic agents enhanced Bax expression in oral SCC cell lines, although there were differences between lines and chemotherapeutic agents in this respect. In cell lines in which Bax expression was enhanced or retained after treatment, chemotherapeutic effects characterized both by decreases in the number of viable cells and increases in the sub-G1 fraction were observed. Bax is a member of the Bcl-2 family and is a positive regulator of apoptosis. When DNA damage caused by chemotherapeutic agents, radiation, etc., occurs, Bax expression is induced by wild-type p53 (12). It was reported that TP53 status affects cell sensitivity to various chemotherapeutic agents including CDDP and antimetabolities, etc. in various tumor cell lines including oral cancer cell lines (24, 25). Each of the three cell lines which we used here carried p53 mutations. Accordingly, it was not expected neither that Bax expression would be induced with such DNA-damaging agents as CDDP and 5-FU, nor that apoptosis would be induced in Ho-1-N-1. However, Bax expression was enhanced after treatment of SAS with CDDP and 5-FU and maintained in KOSC-3 after treatment with these drugs. Although more detailed studies are still required, these data suggest that Bax expression and apoptosis might be induced by an as yet unidentified p53-independent pathway. On the other hand, Yoneda et al. (26) reported that 5-FU and radiation-induced apoptosis of SCC cells are not strongly regulated by Bcl-2/Bax, and 5-FU and γ-irradiation can induce apoptosis of SCC cells via a p53- and p21-independent pathway. Accordingly, apoptosis induction via other pathways should be considered even if enhancement of Bax expression is seen.

In contrast, TXT is a novel antimitotic inhibitor that promotes tubulin assembly in microtubules to inhibit their depolymerization (27, 28). In the present study, induction of Bax expression by TXT was detected in all three cell lines. It is reported that tumor cell lines have a high sensitivity to antimitotic agents such as paclitaxel and vincristine in spite of p53 aberrations (27). Moreover, it has been reported that Bax enhances paclitaxel-induced apoptosis through a p53-independent pathway, or at least that sensitivity to paclitaxel is not related to p53-dependent apoptosis (29, 30).

Representative Bcl-2 family members are Bcl-2 which suppresses and Bax which promotes apoptosis. Proteins of the Bcl-2 family interact with each other as homodimers and heterodimers, and the relative proportions of these pro- and anti-apoptotic factors regulate the process of apoptosis (10). There are some reports that have sought to correlate Bcl-2/Bax expression ratio with sensitivity to various chemotherapeutic agents and radiotherapy (31, 32). In different kinds of cancer, it has been suggested that Bax overexpression strongly associates with chemotherapeutic effects. It has been demonstrated that cancer cells transfected with Bax show increased sensitivity to chemotherapeutic agents (33–35).

Although it is still premature to draw a definitive conclusion from this study, Bax seems to serve as a predictive tool for chemotherapy in oral SCC. Considering the application of chemotherapeutic agents based on the results of the present study, Bax-positive cases may be predicted to experience more favorable outcomes using CDDP and 5-FU, as routinely employed. On the other hand, in Bax-negative cases, a regimen including TXT may be more effective (36–38).

Immunohistochemical examination in nine patients showed that Bax expression tended to be retained or enhanced following preoperative chemotherapy, except for one patient. It is interesting to note that the three patients whose tumors expressed Bax after chemotherapy had MR or noticeable chemotherapeutic effects, whereas the single Bax-negative patient had a poor response to chemotherapy. Thus, the desirability of further clinical studies to clarify the relationship between Bax expression in oral SCC and successful chemotherapy is suggested by these results.

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