

# Preliminary Report of Expression of *bax* in Oral Cavity Pathologies

(*bax* / apoptosis / carcinoma / dysplasia / adamantinoma)

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**Abstract.** *Bax* is considered one of major effectors of apoptosis – programmed cell death. Immunohistochemical analysis of *in vitro* patterns of *bax* expression was mostly investigated in mammalian cell lines and tissues. The present study is the first *in vivo* molecular analysis of *bax* expression in oral cavity pathologies. The study population consisted of 45 patients with hyperplasia, neoplasm *in situ* malignancy, and carcinoma. Biopsies were taken from incision line, tumour section, and healthy tissue. *bax* expression was investigated depending on the site of biopsy material sampling and final histopathology result. No statistically significant difference was demonstrated in *bax* expression between four hyperplasia subgroups. However, statistically significant differences in *bax* expression were found between the three basic study groups ( $P = 0.001$ ). Statistically significant differences in *bax* expression were demonstrated depending on tissue collection site ( $P = 0.0002$ ). We conclude that differences in *bax* expression may play a role in the pathogenesis of neoplastic disease.

## Introduction

*Bax* is a proapoptotic molecule that effects genetically programmed cell death (Pirocanac et al., 2002). It belongs to the Bcl-2 family, and is one of the most thoroughly investigated genes (Gu et al, 2002); *bax* overexpression was found in various cell lines in prostate, colorectal, and uterine carcinoma (Kobayashi et al., 2000; Huh et al., 2001; Li et al., 2001; Lowe et al., 2001). Increased *bax* expression was also related to the histopathological type of thyroid cancer (Branet et al., 1996), and prostate cancer staging (Krajewska et al., 1996). According to Ogura et al. (1999), the prediction of patient outcome was more favourable in *Bax*-positive patients with rectal cancer when compared to those

who had been determined *Bax*-negative. Thus, *bax* has been recognized as a prognostic marker in the above-mentioned pathologies; Gu et al (2002) consider it as a potential therapeutic agent. As detectable in all epithelial layers, *Bax* may also, according to Drenning et al (2001), help differentiate squamous cells due to an increase in their susceptibility to those factors that induce apoptotic cell death. Expression of this proapoptotic protein is enhanced in early stages of head and neck squamous cell carcinoma, and diminishes as malignancy stage increases (Guan et al., 1997). However, in an analysis of tissue pathology, determination of both *bax* and *bcl-2* expression is definitely more informative than that of a single gene only (Hunter and Parslow, 1996). These two have similar amino-acid sequence, but opposite functions. The ratio of Bcl-2 and *Bax* lower than 1 is characteristic of uterine cancer; high Bcl-2 and *Bax* ratios suggest poor response to radio- and chemotherapy for prostatic cancer. The expression of *Bax*-*Bax*- homodimer and *Bax*-Bcl-2 heterodimers was investigated mainly immunohistochemically in mammalian cell lines and tissues. To our knowledge there is no literature on molecular analysis of *bax* expression in oral cavity pathologies.

## Material and Methods

Forty-five patients entered the study. Depending on final histopathology report, the patients were divided into three main groups (Groups I, II, and III). Four histopathology subgroups were determined within Group I: giant cell granuloma (Ia – 3 subjects), inflammatory and granulation hyperplasia (Ib – 17 subjects), fibroplasia and inflammatory infiltration (Ic – 12 subjects), and lichenoid lesions (Id – 1 subject). Group II was formed by 7 patients with neoplasm *in situ* malignancy (adamantinoma – 5 subjects, odontoma – 1 subject, and myxoma – 1 subject). Squamous cell carcinoma was found in 5 subjects – Group III. A digital photo of each pathology was taken prior to surgery. *bax* expression was assessed in tissue samples taken from incision line, resected tumour, and healthy gingival tissue – opposite to tumour side. The 135 biopsies

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(three from each patient) were secured in liquid nitrogen. Molecular analysis consisted of three stages: RNA extraction, amplification, and evaluation of amplification products specificity, which were carried out at the Medical Department of Molecular Biology and Genetics.

The investigations were approved by the Bioethic Committee of the Medical University of Silesia.

### RNA extraction

Tissue samples were homogenized in liquid nitrogen. Total RNA was extracted and purified by the acid guanidinium-phenol-chloroform (AGCP) method described by Chomczynski and Sacchi (1987). RNA concentration was determined by absorbance at 260 nm using a Gene Quant II spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ); its quality (degradation and purity) was evaluated by electrophoresis on 2% agarose gel stained with ethidium bromide.

### Real-time RT-PCR assay

Levels of mRNA of the studied gene were determined by real-time RT-PCR based on SYBR Green chemistry using an ABI PRISM 7000 Sequence Detector (Applied Biosystems, Foster City, CA). The PCR mix contains a fluorescent dye, SYBR Green, which, when binding to dsDNA, exhibits fluorescence enhancement. The latter is proportional to the initial concentration of cDNA template. The fluorescent signals of each sample were collected by a CCD camera. Following that, threshold cycles (CT) were calculated by the ABI PRISM 7000 software (Applied Biosystems), where the point was determined at which fluorescence intensity was 10 times greater than the standard deviation from baseline fluorescence.

RT was carried out in the same system immediately before PCR amplification. RT-PCR reaction tubes contained 25 µl QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Hilden, Germany) (HotStart Taq DNA Polymerase, QuantiTect SYBR Green RT-PCR Buffer, dNTP mix, SYBR Green I, passive reference dye ROX, and 5 mM MgCl<sub>2</sub>), 2.5 µl of forward and reverse primers (10 µM stock solution), 3 µg of unknown RNA template, 0.5 µl of OmniTect RT Mix (Qiagen) (containing Omniscript reverse transcriptase, and Sensi-script reverse transcriptase), and deionized water to a total volume of 25 µl. All RT-PCR reactions were conducted in 96-well microtitre plates covered with optical adhesive covers.

The thermal profile was 48°C for 30 min for reverse transcription, and 95°C for 15 min, 40 two-step cycles at 94°C for 15 s and 60°C for 30 s; followed by 72°C for 10 min for QPCR. Finally, in a 30-min dissociation protocol, RNA integrity was assessed by mRNA amplification of the house-keeping genes *GAPDH* and *β-actin*. For each studied gene and endogenous control the specificity of amplification was monitored with the

dissociation curve of the amplified product. All reactions were run in triplicate.

Sequence-specific PCR primers for Bax mRNA detection were designed using computer software Primer Express Version 1.0 ABI PRISM (Applied Biosystems). In order to rule out amplification of contaminating DNA, the primer sequences were designed to span exon-intron junctions. The following oligonucleotide 5' and 3' primer sequences were used: for Bax: sense: 'CCTGTGCACCAAGGTGCCGGAAGT3'; antisense: 'CCACCCTGGTCTTGGATCCAGCCC3' (amplification product 99 bp); for β-actin: sense: 5'TCACCC-ACACTGTGCCCATCTACGA; antisense: 'CAGCGGAACCGCTCATTGCCAATGG3' (amplification product 295 bp); for GAPDH: sense: 5'GAAG-GTGAAGGTCGGAGTCA3'; antisense: 5'GAAGATGGTGTATGGGATTTC3' (amplification product 226 bp).

PCR products were separated on 8% polyacrylamide gel and visualized using silver staining. The molecular weight marker was plasmid pBR 322/*Hae*III. The specificity of amplification products was analysed with Biotec-Fisher BAS-SYS 1D software. Specificity of PCR products was additionally confirmed by establishing their melting temperature in a dissociation curves assay and by enzymatic sequencing carried out with the use of ABI PRISM 377 DNA sequencer (Applied Biosystems).

### Statistical analysis

The values are expressed as SEM and standard deviation. Quantitative data were compared using ANOVA (the Tukey test). P values of < 0.05 were considered to indicate statistical significance. All calculations were performed with Statistica Version 6.0 Software.

### Results

*bax* expression was investigated in 135 biopsies collected from 45 patients. No statistically significant difference was demonstrated in *bax* expression between four hyperplasia subgroups. However, statistically significant differences in *bax* expression were found between the three basic study groups (P = 0.001). The difference was the least pronounced between carcinoma and hyperplasia (P = 0.02). Statistically significant differences in *bax* expression were demonstrated depending on the tissue collection site (P = 0.0002). In Group II and III patients, considerable differences were also found regarding the gene expression in tumour section and incision line (P = 0.0001), and incision line and healthy tissue (P = 0.0001) (Fig.1).

### Discussion

So far mainly *in vitro* evaluation of *bax* expression was carried out in different oral cavity pathologies by Loro et al. (2002), who did not find the gene in normal, dysplastic, and hyperplastic epithelium. However, Guan

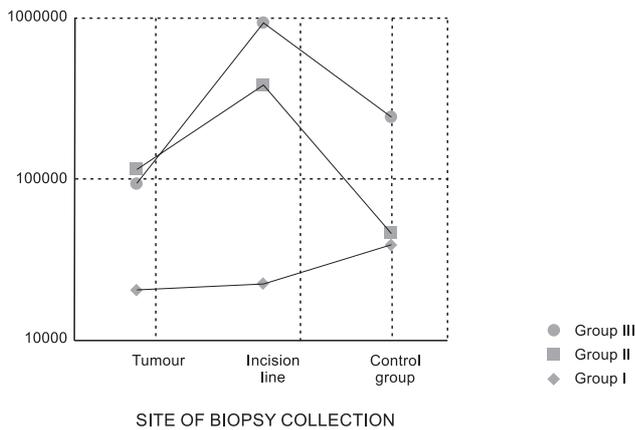


Fig. 1. Mean values of Bax mRNA levels depending on tissue collection site, and final histopathology result (Group I - hyperplasia, Group II - neoplasm *in situ* malignancy, Group III - invasive carcinoma).

et al. (1997) maintained that Bax was present in 60% of normal epithelium examined. All investigations into *bax* expression were performed using the immunohistochemical method. Our study is the first *in vivo* molecular analysis of *bax* expression in oral cavity pathologies. So far no opportunity has arisen to compare both methods.

In the present study *bax* expression is compared in different oral cavity pathologies than those already described in the literature. For the first time analysis has been performed for hyperplastic lesions, neoplasm *in situ* malignancies, and squamous cell carcinoma. Additionally, and also for the first time, *bax* expression has been investigated in three biopsies collected from each patient, i.e., incision line, tumour section, and healthy tissue from the side opposite to the tumour. Realizing well the discrepancy between the results of clinical and histopathological examinations of mucous membrane hyperplasias as well as unclear aetiology of these lesions, we decided to evaluate *bax* expression in hyperplasia subgroups – as suggested by histopathologists. Thus, separate analyses were undertaken for giant cell granulomas, inflammatory and granulation hypertrophy, fibroplasia and inflammatory infiltration, and lichenoid lesions. The lack of significant differences in *bax* expression between the above-mentioned four subgroups might suggest similar aetiopathogenesis thereof, thus indicating that similar therapy should be administered.

Three main study groups (i.e., Groups I, II, and III) showed different aetiology with statistically significant differences in *bax* expression found between the three basic study groups ( $P = 0.001$ ), which seemed to depend on biopsy collection site. Considerable differences in *bax* expression between *in situ* carcinoma (Group II), and invasive carcinoma (Group III) were found regarding the gene expression in tumour section and incision line ( $P = 0.0001/P < 0.0001$ ), and incision line and healthy tissue ( $P = 0.0001/P < 0.0001$ ). This might suggest pathology-associated, considerable

apoptotic activity at the border area between affected and healthy tissues. Drenning et al. (2001) confirmed the occurrence of *bax* protein in tissues adjacent to those affected by squamous cell carcinoma. According to the majority of researchers, *bax* expression is proportional to tumour malignancy (Guan et al., 1997; Loro et al., 2002; Jordan et al., 2003). Kumamoto and Ooya (1997) found higher *bax* expression in malignant vs benign adamantinomas.

In our investigations, differences in *bax* expression were less significant between cancer and neoplasm *in situ* malignancy, and quite pronounced between these two groups and hyperplasias. The highest gene activity was noted at perilesional areas of cancer and neoplasm *in situ* malignancies; it was only insignificant at corresponding areas of hyperplasias. A high level of *bax* expression is characteristic of healthy tissue, distant from cancer area; the level is though lower when compared to that observed in pericancerous area. It is the latter, and not inside-tumour gene expression, that reflects the malignancy stage of a neoplasm.

## References:

- Branet, F., Brousset, P., Krajewski, S., Schlaifer, D., Selves, J., Reed, J. C., Caron P. (1996) Expression of the cell death-inducing gene *bax* in carcinomas developed from the follicular cells of the thyroid gland. *J. Clin. Endocrinol. Metab.* **81**, 2726-2730.
- Chomczynski, P., Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.
- Drenning, S. D., Marcovitch, A. J., Johnson, D. E., Melhem, M. F., Tweardy, D. J., Grandis, J. R. (2001) Bcl-2 but not Bax expression is associated with apoptosis in normal and transformed squamous epithelium. *J. Oral Pathol. Med.* **30**, 309-315.
- Gu, J., Zhang, L., Huang, X., Lin, T., Yin, M., Xu, M., Ji, L., Roth, J. A., Fang, B. (2002) A novel single tetracycline-regulative adenoviral vector for tumor-specific Bax gene expression and cell killing *in vitro* and *in vivo*. *Oncogene* **21**, 4757-4764.
- Guan, W., Yu, S., Gao, Y. (1997) Expression of apoptosis-related protein in epithelial dysplasia and squamous cell carcinoma. *Oral Oncol.* **33**, 419-425.
- Huh, W. K., Gomez-Navarro, J., Arafat, W. O., Xiang, J., Mahasrehti, P. J., Alvarez, R. O., Barnes, M. N., Curiel, D. T. (2001) *Bax*-induced apoptosis as a novel gene therapy approach for carcinoma of the cervix. *Gyn. Oncol.* **83**, 370-377.
- Hunter, J. J., Parslow, T. G. (1996) A peptide sequence from *bax* that converts Bcl-2 into an activator of apoptosis. *J. Biol. Chem.* **271**, 8521-8524.
- Jordan, R. C., Catzavelos, G. C., Barrett, A. W., Speight, P. M. (2003) Differential expression of *bcl-2* and *bax* in squamous cell carcinomas of the oral cavity. *Pol. J. Pathol.* **54**, 49-52.
- Kobayashi, T., Sawa, H., Morikawa, J., Zhang, W., Shiku H. (2000) *Bax* induction activates apoptotic cascade via mitochondrial cytochrome c release and *bax* overexpression enhances apoptosis induced by chemotherapeutic agents in DLD-1 colon cancer cells. *Jpn. J. Cancer Res.* **91**, 1264.

- Krajewska, M., Krajewski, S., Epstein, J. I., Shabaik, A., Sauvageot, J., Song, K., Kitada, S., Reed, J. C. (1996) Immunohistochemical analysis of *bcl-2*, *bax*, *bcl-x* and *mcl-1* expression in prostate cancers. *Am. J. Pathol.* **148**, 1567-1576.
- Kumamoto, H., Ooya, K. (1997) Immunohistochemical analysis of *bcl-2* family proteins in benign and malignant ameloblastomas. *J. Oral Pathol. Med.* **26**, 419-425.
- Li, X., Marani, M., Yu, J., Nan, B., Roth, J. A., Kagawa, S., Fang, B., Denner, L., Marcelli, M. (2001) Adenovirus-mediated *Bax* overexpression for the induction of therapeutic apoptosis in prostate cancer. *Cancer Res.* **61**, 186-191.
- Loro, L. L., Vintermyr, O. K., Liavaag, P. G., Jonsson, R., Johannessen, A. C. (2002) Oral squamous cell carcinoma is associated with decreased *bcl-2/bax* expression ratio and increased apoptosis. *Oral Oncol.* **38**, 691-698.
- Lowe, S. L., Rubinchik, S., Honda, T., McDonnell, T. J., Dong, J. Y., Norris, J. S. (2001) Prostate-specific expression of *Bax* delivered by an adenoviral vector induces apoptosis in LNCaP prostate cancer cells. *Gene Ther.* **8**, 1363.
- Ogura, E., Senzaki, H., Yamamoto, D., Yoshida, R., Takada, H., Hioki, K., Tsubura, A. (1999) Prognostic significance of *Bcl-2*, *Bcl-xL/S*, *Bax*, *Bak* expressions in colorectal carcinomas. *Oncol. Rep.* **6**, 365-369.
- Pirocanac, E. C., Nassirpour, R., Yang, M., Wang, J., Nardin, S. R., Gu, J., Fang, B., Moossa, A. R., Hoffman, R. M., Bouvet, M. (2002) *Bax*-induction gene therapy of pancreatic cancer. *J. Surg. Res.* **106**, 346-351.