Expression of Bcl-2 Family in 4-Nitroquinoline 1-Oxide-Induced Tongue Carcinogenesis of the Rat

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The number of patients with tongue carcinoma is increasing rapidly among young individuals in many parts of the world. Oral carcinoma progresses from hyperplastic lesion through dysplasia to invasive carcinoma and the concept of "field cancerization" with molecular alteration has been suggested for oral cavity carcinogenesis. Significant improvement in treatment and prognosis will depend on more detailed understanding of the multi-step process leading to cancer development.

To induce tongue carcinoma in rat by 4-NQO, each drinking water was made to 10 ppm, 25 ppm, 50 ppm and control (only D.W. without 4-NQO). Specimens were classified into 4 groups such as control, I (mild & moderate dysplasia), II (severe dysplasia and carcinoma in situ), III (carcinoma). The mRNA expressions of Bcl-2 family were evaluated by RT-PCR technique.

For anti-apoptotic Bcl-2 family, mRNA expression of Bcl-w was down-regulated in all stages of tongue carcinogenesis model. However, mRNA expression of Bcl-2 was up-regulated. For pro-apoptotic Bcl-2 family, all members were down-regulated in all stages of tongue carcinogenesis model except for Bad mRNA in group III. In terms of BH3 only protein, mRNA expressions of Bok and Mcl-1 were down regulated in all stages of specimen, but Bmf in group II and BBC3 in group III were up-regulated.

Our current findings demonstrated the involvements of mRNA expression of Bcl-2 family in multi-step tongue carcinogensis. This highlights the necessity for continued efforts to discover suitable biomakers (Bcl-2 family) for early diagnosis of the disease, and to understand its pathogenesis as a first step in improving methods of treatment. The discovery of these potential biomarkers and molecular targets for cancer diagnostics and therapeutics has the potential to significantly change the clinical approach and outcome of the disease.

Key words : Oral cavity carcinogenesis, 4-nitroquinoline 1-oxide, mRNA expressions of Bcl-2 family, RT-PCR technique

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Ⅰ. INTRODUCTION

Oral cancer progresses from hyperplastic epithelial lesions and epithelial dysplasia to invasive carcinoma. The concept of "field cancerization" with molecular alterations can be applied to oral cavity carcinogenesis[1-3]. Also, significant improvement in

treatment and prognosis will depend on more detailed understanding of the multi-step process leading to cancer development[4].

The most frequently used animal models in oral cancer research studies have been the hamster buccal pouch model, rat and less frequently, mouse[4-6].

A fat-soluble 7,12-dimethylbenz[a]anthracene (DMBA) and a water-soluble 4-nitroqinoline 1-oxide (4-NQO) are the most frequently used carcinogens in the studies. Since 4-NQO is water-soluble, it is well suited in examining the role of xenobiotics in experimental oral carcinogenesis. Topical application of 4-NQO and administration of 4-NQO in drinking water both induce premalignant and malignant transformation in the rat oral cavity. This results in papilloma and invasive squamous cell carcinoma, resembling the clinical and histologic changes observed in these neoplasms in humans[6-10].

Cell proliferation, apoptosis, and tumor cell implants were used to monitor malignant properties. Cell proliferation plays an important role in multi-step carcinogenesis with multiple genetic changes[11]. Therefore, control of cell proliferations is important for cancer prevention[12]. Furthermore, apoptosis induction is one of the important characteristics of candidate cancer chemopreventive agents[13].

Bcl-2 family serve as critical regulators of pathway involved in apoptosis, acting to either inhibit or promote cell death. Bcl-2 family members can be grouped into three categories, the anti-apoptotic member including Bcl-2, Bcl-xL, and Mcl-1, the multidomain pro-apoptotic members such as Bax and Bak, and the BH3 domain only proteins such as Bim, Bid, Bad, and Bik[14]. This family of Bcl-2 converges on the mitochondrial membrane depolarization that ultimately determine cell fate.

The Bax subfamily has been implicated as the "gateway" to apoptosis[15]. These Bcl-2 family are required to initiate most forms of apoptosis[15,16]. During apoptosis , Bax translocates to the mitochondria and oligomerizes, causing cytochrome *c* release from the mitochondria[17,18]. Translocation and oligomerization of Bax are preceded by a conformational change[19].

Bcl-2 plays an important role in cancer and resistance of cancer to conventional therapies. Bcl-2 can contribute to neoplastic cell expansion by preventing normal cell turnover caused by physiological cell death mechanisms[20,21]. High expression of Bcl-2 is found in a wide variety of human cancers and mediates the resistance of cancers to a wide spectrum of chemotherapeutic drugs and irradiation which act by inducing apoptosis in tumor cells[22].

Anti-apoptostic Bcl-2 family members function at least in part by inhibiting cytochrome *c* release from the mitochondria. They perform this task by preventing translocation and/or activation of Bax-like proteins on the mitochondria[23,24], however, the mechanism of this inhibition is not entirely clear. In addition, it is not certain that the role of anti-apoptotic Bcl-2 family members is limited to the mitochondria. Much emphasis has been placed on Bcl-2 function on the mitochondria, although it has been reported that wild type Bcl-2 localizes to the mitochondria, endoplamic reticulum (ER), and nuclear membranes[25]. And there is growing evidence that the ER is important in apoptosis[26-28].

Our current findings demonstrated the involvements of mRNA expression of Bcl-2 family in multi-step tongue carcinogensis.

Ⅱ. MATERIALS AND METHODS

1. Chemicals and Animals

Animal experiment care and use of all animal laboratory accompanied system and morals experiment that Chonnam Dental college animal laboratory provision of “Guidelines and Regulations for Use and Care of Animals” based on “Guide for the care and use of laboratory animals (National Research Council, USA)” and “Policy on humane care and use of laboratory animals (United States

Table 1. Experimental Criteria

Histologic grade		Experimental group
Normal		Control
Dysplasia	mild	I
	moderate	
	severe	II
Carcinoma in situ		
Cancer		III

Public Health service, USA)".

Male Sprague-Dawley rats weighing about 30 g and 4 weeks old were used and 4-NQO was obtained from TCI Co., Ltd. (Tokyo, Japan). 4-NQO solution was given to rats to induce multi-step tongue carcinogenesis. 4-NQO was given in drinking water for 4 to 8 months and each drinking water made 10 ppm, 25 ppm, 50 ppm and control (only D.W. without 4-NQO). The rats were sacrificed every week from 4 to 8 months after 4-NQO drinking. All animals were housed in wire

Table 2. Bcl-2 family mRNA primer.

Bcl-2	NM_016933	Forword	CCTGTGGATGACTGAGTACC
		Reverse	GAGACAGCCAGGAGAAATCA
Bax	NM_017059	Forword	GTTTCATCCAGGATCGAGCAG
		Reverse	CATCTTCTTCCAGATGGTGA
Mcl	NM_021846	Forword	GGCGTGCAGCGCAACCAC
		Reverse	TCCTGCCCCAGTTTGTTACGCC
Bmf	NM_139258	Forword	GAGCTTGCTCTCTGCTGACCT
		Reverse	GTCACCCCACAAGGCAGC
Bad	NM_022698	Forword	AAGGGACTTCCTCGCCC
		Reverse	GATCCCACCAGGACTGGA
BBC3	NM_173837	Forword	GAGTCGCCCGTGCCCAG
		Reverse	TGAGGTCGTCCGCCATCC
Bok	NM_017312	Forword	TGGAGGTGCTGCGGCGC
		Reverse	CTCCAGGAGAGGCCGGCG
Bcl-W	NM_021850	Forword	GTGACCCCAGGCTCAGCCCA
		Reverse	CACTGCTGTGGATCCAGTCAGCC
Bcl-xL	NM_031535	Forword	GGACAATGGACTGGTTGAGCC
		Reverse	GGTTGCCATTGATGGCACTG
Bak1	NM_053812	Forword	GGGCCGTGTGGTGGCTCT
		Reverse	TGGCCCAACAGAACCACACC
GAPDH	NM_017008	Forword	TGCATCCTGCACCACCAACT
		Reverse	CGCCTGCTTCACCACCTTC

cages with free access to drinking and under controlled conditions of humidity (50 ± 5%), lighting (12hr light/dark cycle) and temperature (22 ± 2℃). A half of tongue was kept frozen in liquid nitrogen and another half prepared for H&E staining. Experimental group was classified according to histomorphological findings (Table 1).

2. Histologic study

Rats were sacrificed and the tongue were longitudinally cut, followed by fixation in 10% phosphate-buffered formalin for 48 h, routinely embedded in paraffin, and serially sectioned at 3-4 ㎛. The sections were used for H&E staining. And the remaining samples were frozen at deep freezer for RNA study.

3. RNA isolation and RT-PCR

Total RNA was isolated from control and 4-NQO-induced tongue by means of Tri® Reagent (Invitrogen, USA.), according to the manufacturer's instructions. 3 ㎍ of total RNA was reverse-transcribed in a total volume of 50 ㎕, using *Accupower*® RT premix (Bioneer, seoul, korea). Oligonucleotide primers (5→3) were generated consulting NCBI (Genebank). The primers used in present study are listed in table 2. 2 ㎕ of cDNA was mixed PCR premix (Bioneer, korea) a final total volume of 20 ㎕, performed using Mygenic 96 Thermal Block (Bioneer, korea). Amplofocation profiles included denaturation for 40 s at 94℃, annealing for 40 s at 56℃: Bcl-2, Bax and Bad ; annealing for 40 s at 66℃:Mcl, Bmf, Bak1, BBC3, Bok, Bcl-w, Bcl-xL and primer extension for 90 s at 72℃ and the final step extended to 10 min at 72 ℃. The PCR precedure was performed at least three times for each sample.

Ⅲ. RESULTS

1. 4-NQO induced oral carcinogenesis

4-NQO was treated to induce multi-step carcinogenesis by time and dose dependent manner. Observing the histologic findings, histologic degrees (dysplasia, carcinoma in situ and SCC) were different to time and dose dependent manner (Table 3). With 25ppm, 8month needed to induce SCC, while 6month at 50ppm. And all specimen was followed the multi-step carcinogenesis. Fig. 1 to 5 showed the gross and histologic findings.

2. RT-PCR

RT-PCR were performed to investigate a variety of Bcl-2 mRNA expression in 4-NQO induced tongue carcinogenesis of rat. Bcl-2 family. covariance anaylsis has been used to normalize data using values obtained for mRNA expression of the house keeping gene GAPDH.

1) Bcl-2
mRNA expression of Bcl-2 was increased in multistep carcinogenesis than in control (Fig. 6).

Table 3. Time intervals of lesion induced by 4-NQO.

4-NQO concentration	Gross appearances	Histologic degrees		
	detected by naked eye	mild & moderate dysplasia	severe dyspalsia & carcinoma in situ	cancer
10 ppm	8 month	6 month	8 month	*
25 ppm	6 month	5 month	6 month	8 month
50 ppm	4 month	3 month	4 month	6 month

using values of mRNA expression shows 1 in control and other using values of mild or/ and moderate dysplasia shows 1.25 (SD=±0.04) compare with control. using values of severe dysplasia shows 1.53 (SD=±0.17) and squamous cell carcionoma shows 1.21 (SD=±0.05) compare with control (Fig. 6). Image data show a little different band.

2) Bax

mRNA expression of Bax decreased in multistep carcinogenesis than in control (Fig. 7). using values of mRNA expression shows 1 in control and using value of the resr mRNA expression of Bax show each 0.5 (SD=±0.12), 0.71 (SD=±0.08), 0.39 (SD=±0.02) compare with control. mRNA expression of squamous cell carcinoma were significant difference and decrease 2.5 folds compare with control (Fig. 7). Image data show that normal be seen band but other band be seen a glimmer.

3) Bcl - W

Bcl-W result shows oppsition result of Bcl-2. Bcl-W gradually decreases mRNA expression than in control (Fig. 8). using values of mRNA expression shows 1 in control and mild or/and moderate dysplasia shows 0.79 (SD=±0.18) as using value of mRNA expression compare with control. severe dysplasia shows that mRNA expression decread than in other dysplasia and using values of mRNA expression shows 0.49 (SD=±0.11) compare with control. mRNA expression of squamous cell carcinoma decrease than in control and uing values of mRNA expression shows decrease 0.25 folds compare with control (Fig. 8). Image data show that normal be seen band but other band be seen a glimmer.

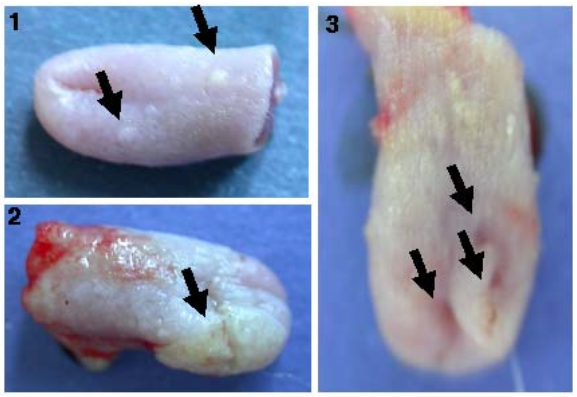


Fig. 1-3. Various gross appearances after treating 4-NQO in rat tongue. Papules (Fig. 1), patches (Fig. 2) and ulcers (Fig. 3) were examined.

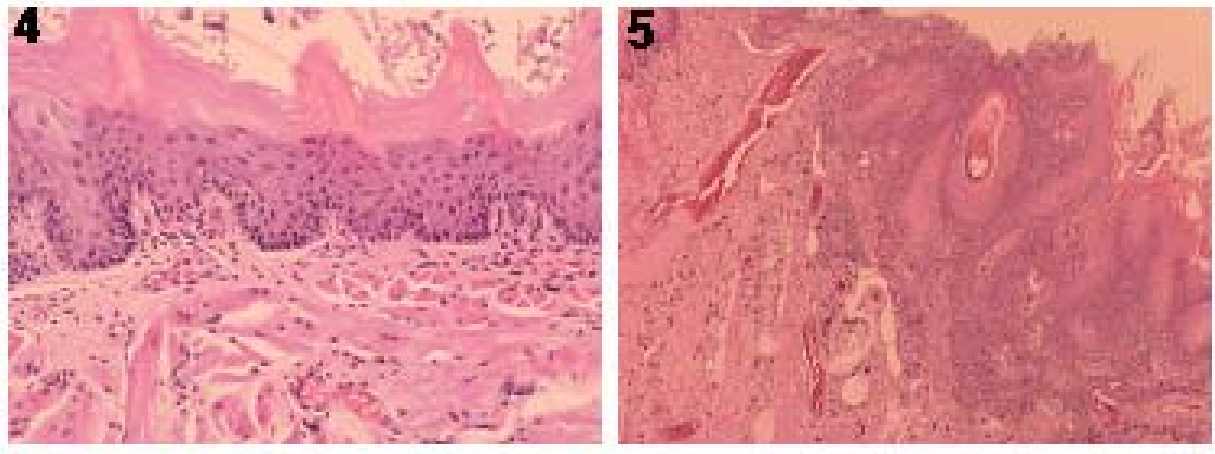
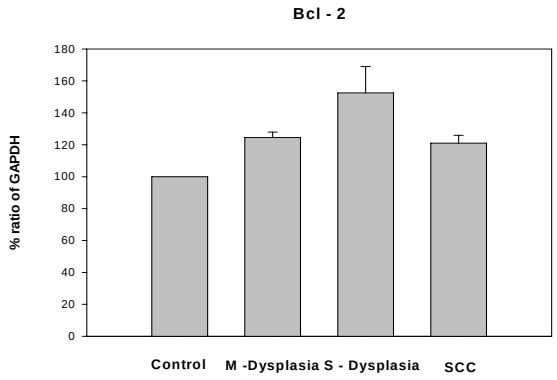
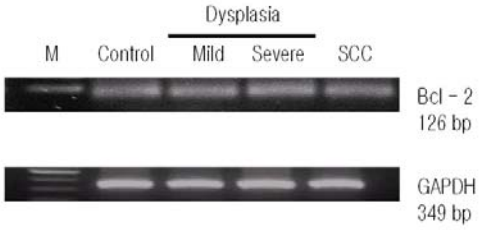


Fig. 4-5. Histologic appearances after treating 4-NQO in rat tongue. Moderate dysplasia (Fig. 4) and well-differentiated squamous cell carcinoma (Fig. 5) were observed.

A . B.



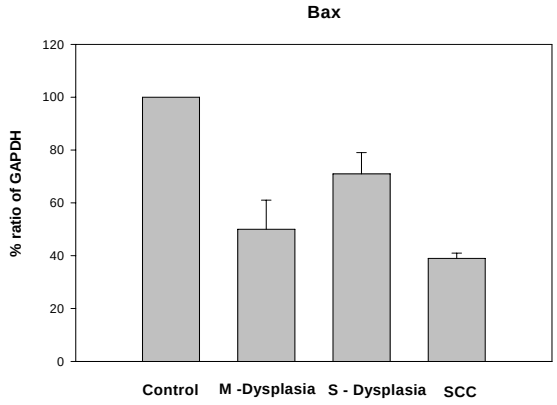


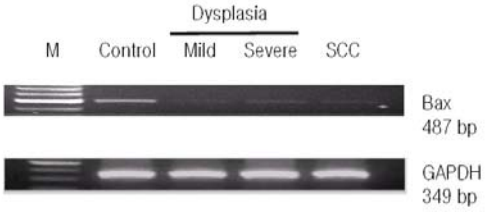
C.

Bcl - 2	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	1.25 (SD=±0.04)	1.53 (SD=±0.17)	1.21 (SD=±0.05)

Fig. 6. mRNA expression of Bcl-2 in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

A. B.



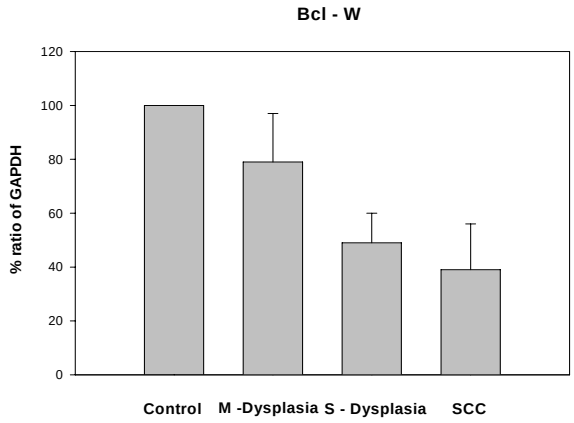


C.

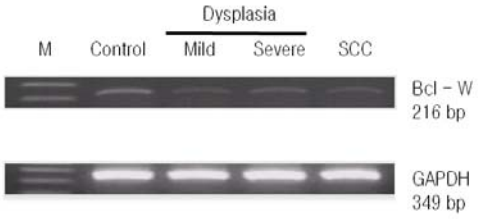
Bax	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	0.5 (SD=±0.12)	0.71 (SD=±0.08)	0.39 (SD=±0.02)

Fig. 7. mRNA expression of Bax in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

A.



B.

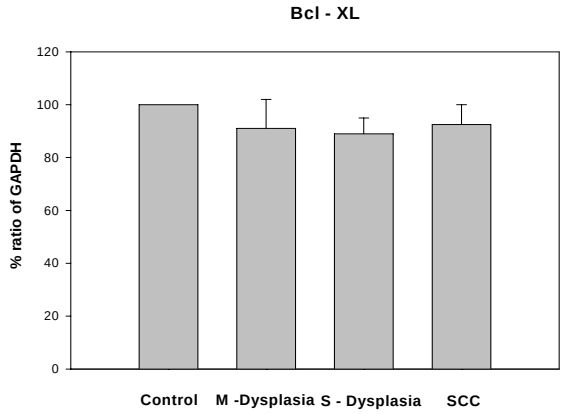


C.

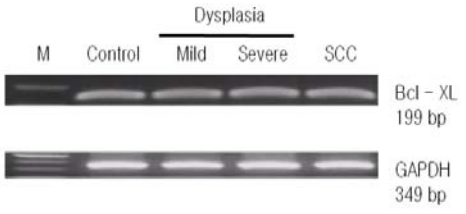
Bcl - W	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	0.79 (SD=±0.18)	0.49 (SD=±0.11)	0.39 (SD=±0.17)

Fig. 8. mRNA expression of Bcl-w in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

A.



B.



C.

Bcl - xL	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	0.91 (SD=±0.11)	0.89 (SD=±0.06)	0.93 (SD=±0.08)

Fig. 9. mRNA expression of Bcl-xL in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

4) Bcl - XL

mRNA expression of Bcl-XL shows reveals almost same mRNA expression in multistep carcinogenesis including the control (Fig. 9). using calues of mRNA expression shows 2 in control and using value of the rest mRNA expression of Bcl-XL shows each 0.91 (SD=±0.11), 0.89 (SD=±0.06) and 0.93 (SD=±0.08) compare with control. in other words, each dysplasia and squamous cell carcinoma shows a little difference mRNA expression and almost it is smilar. Image shows almost similar (Fig. 9).

5) Bak

mRNA expression of Bak decrease in dyplasia and mRNA expressin in sqmous cell carcinoma is more increased than in dysplasia (Fig. 10). Image data show upward band in normal, squamous cell carcinoma. Using values of mRNA expression shows 1 in control and mild or/and moderate using values shows 0.29 (SD=±0.04) compare with control. using values of mRNA expression in severe dysplasia show 0.22 (SD=±0.12) and squamous cell carcinoma shows 0.62 (SD=±0.02) (Fig. 10).

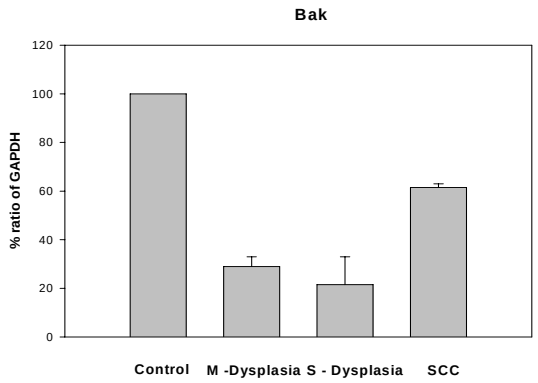
6) Bad

mRNA expression of Bad shows reveals almost same mRNA expression in multistep carcinomage-nesis including the control and alone mRNA expression of squsmous cell carcinoma slight increase (Fig. 11). using values of mRNA expression shows 1 in control and using value of the reat mRNA expression of Bad shows each 1.02 (SD=±0.13), 0.94 (SD=±0.03), 1.24 (SD=±0.03) compare with control. also Image data show similar (Fig. 11).

7) Bok

mRNA expression of Bok decrese in mild or/and moderate dysplasia and grdually increase in severe dysplaisa, in squamous cell carcinoma than in mild or/and moderate dysplasia (Fig. 12). Using values of mRNA expressoin of mRNA expression shows 1 in control and mild or/and moderate dysplasia shows 0.37 (SD=±0.03) as using values of mRNA expression compare with control. severe dysplasia shows that mRNA expression decrease than in control also increase than in mild or/and moderate dysplasia. using values of mRNA expression shows

A.



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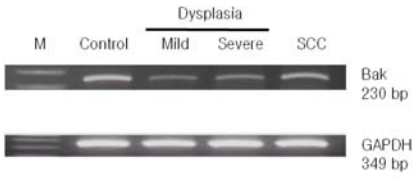


C.

Bak	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	0.29 (SD=±0.04)	0.22 (SD=±0.12)	0.62 (SD=±0.02)

Fig. 10. mRNA expression of Bak in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

0.57 (SD=±0.07) compare with control. mRNA expression of squamous cell carconoma increase than in control and using values of mRNA expression shows increased 1.23 folds compare with control. Image data show decreased in mild dysplasia (Fig. 12).

8) Bmf

mRNA expression of Bmf shows a smilar tendency in Bcl-2 (Fig. 13). mRNA expression of Bmf was increased in multistep carcinogenesis than in control. using values of mRNA expression shows 1 in control and other using values of mild or/ and moderate dysplasia shows 1.12 (SD=±0.03) compare with control. using values of severe dysplasia shows 1.49 (SD=±0.01) and squamous cell carcionoma shows 1.08 (SD=±0.04) compare with control (Fig. 13). Image data show a little different band.

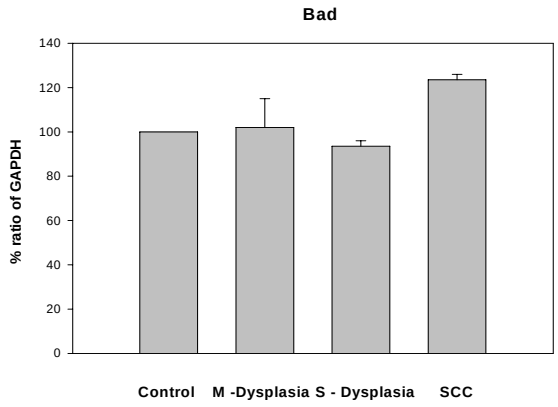
9) Mcl

Mcl result shows oppsition result of Bmf or BCl-2. Mcl gradually decreases mRNA expression than in control (Fig. 14). using values of mRNAexpression shows 1 in control and mild or/and moderate dysplasia shows 0.69 (SD=±0.17) as using value of mRNA expression compare with control. severe dysplasia shows that mRNA expression decread than in other dysplasia and using values of mRNA expression shows 0.65 (SD=±0.24) compare with control. mRNA expression of squamous cell carcinoma decrease than in control and using values of mRNA expression shows decrease 0.2 folds compare with control (Fig. 14). Image data show that normal be seen band but other band be seen a glimmer.

10) BBC3

mRNA expression of BBC3 shows some increased mRNA expression in multistep carcinomagenesis including the control (Fig. 15). Using values of mRNA expression shows 1 in control and using value of the rest mRNA expression of Bad shows each 1.08 (SD=±0.06), 1.11 (SD=±0.04), 1.43 (SD=±0.03) compare with control. Image data shows similar (Fig. 15).

A.



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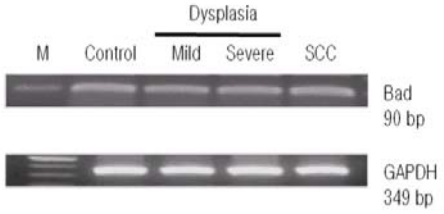
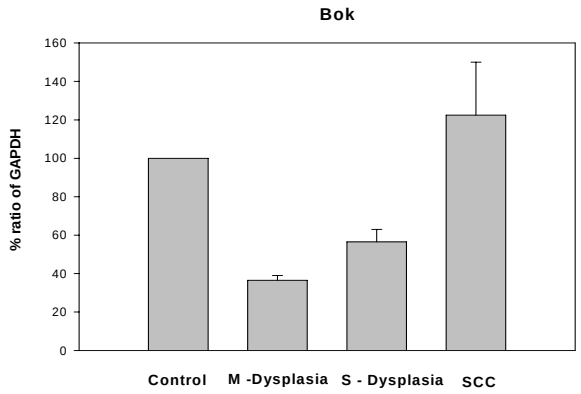


C.

Bad	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	1.02 (SD=±0.13)	0.94 (SD=±0.03)	1.24 (SD=±0.03)

Fig. 11. mRNA expression of Bad in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

A.



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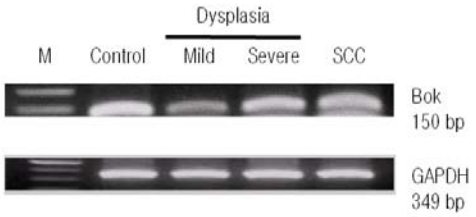
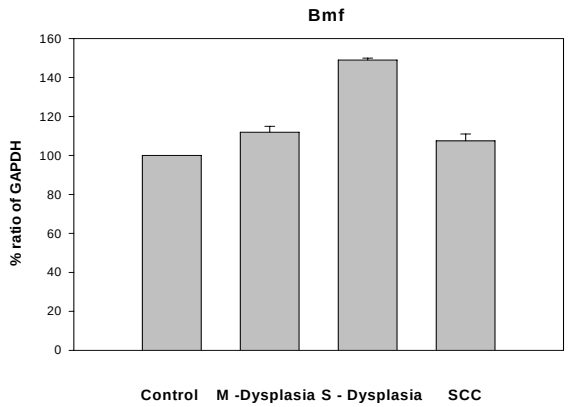


C.

Bok	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	0.37 (SD=±0.03)	0.57 (SD=±0.07)	1.23 (SD=±0.28)

Fig. 12. mRNA expression of Bok in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

A.



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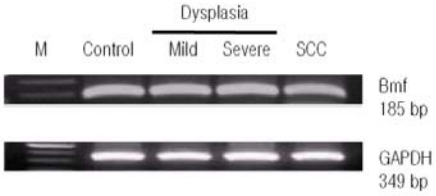
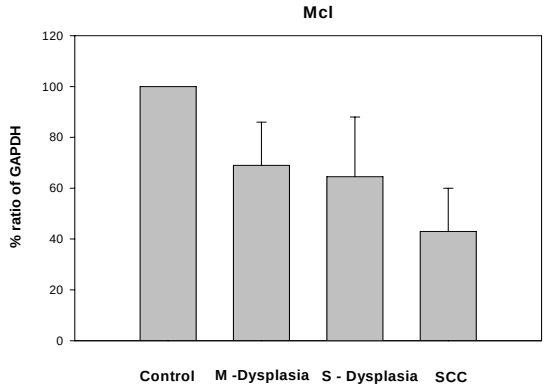


C.

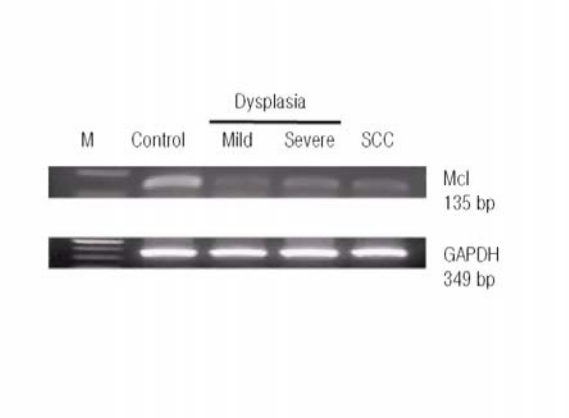
Bmf	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	1.12 (SD=±0.03)	1.49 (SD=±0.01)	1.08 (SD=±0.04)

Fig. 13. mRNA expression of Bmf in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

A.



B.

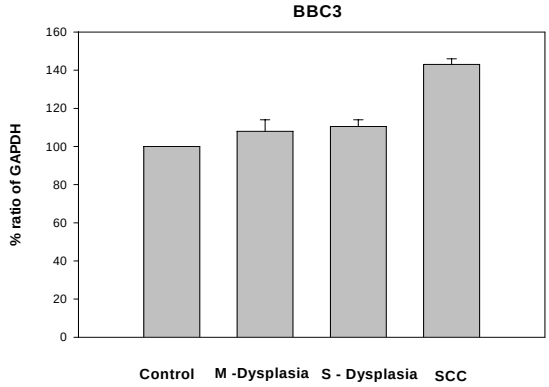


C.

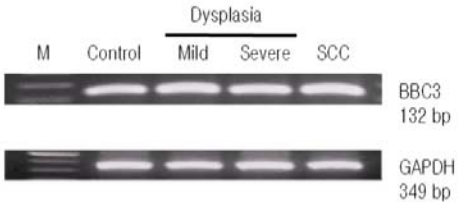
Mcl	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	0.69 (SD=±0.17)	0.65 (SD=±0.24)	0.43 (SD=±0.17)

Fig. 14. mRNA expression of Mcl in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

A.



B.



C.

BBC3	Control	M-Dysplasia	S-Dysplasia	SCC
Fold Increase (Bcl-2/GAPDH)	1	1.08 (SD=±0.06)	1.11 (SD=±0.04)	1.43 (SD=±0.03)

Fig. 15. mRNA expression of BBC3 in multistep carcinoma were decrease and the latter phase inceased mRNA expression (a). RT-PCR result shows image (b) and anaylysis has been used to data that using values obtained for mRNA expressin compare with control (c).

Ⅳ. DISCUSSION

In present study, bcl-2 mRNA expression level in multistep tongue carcinogenesis of rat was evaluated by RT-PCR. This is the first trial, as far as we've known, to examine the all bcl-2 family in multistep tongue carcinogenesis. The results for bcl-2 family mRNA expression could be largely categorized into 3 patterns such as 'increase', 'decrease' and 'fluctuant'. The Bcl-2 family showing 'increase' pattern are bcl-2, bmf and bbc3. bax, bcl-W, bcl-XL, bak and mcl showed 'decrease' pattern in present study. The 'fluctuant' pattern was showed in bad and bok. We could also observed the pattern of steadily 'increase' or 'decrease' as the histologic grade was advanced. bbc3 was the only one whose expression was increased and bcl-W and mcl showed decreased pattern as the histologic grade was advanced.

In present study, we performed delivery of 4-NQO in the drinking water at the doses, resulted in easily incidence of oral cavity carcinogenesis and the duration of cancer incidence came to be short at dose dependent manner.

In the literature, there are partly divergent results regarding the expression of bcl-2 in oral squamous cell carcinoma[41,42]. and in other epithelial tumors [43,44]. In present study, mRNA expression of bcl-2 was increased comparing with control. Considering the highest expression in severe dysplasia group, bcl-2 mRNA expression can be used as a bio-marker to differentiate severe dysplasia from other dysplastic grade and oral squamous cell carcinoma.

In protein level, Bax is also detected in many normal tissues[45], including normal oral epithelium [46], and in neoplastic tissues, including cancers[42]. The result of present study, mRNA expression for bax protein was down-regulated in all groups comparing with the expression of control. A milestone in understanding how Bcl-2 functions came when it was discovered that Bcl-2 was capable of heterodimerizing with its pro-apoptotic relative Bax[31,33]. Apoptosis of cancer emerged that anti-apoptotic proteins such as Bcl-2 and pro-apoptotic proteins such as Bax do battle with each other by hand-combat, with the Bcl-2:Bax ratio dictating the relative sensitivity or resistance of cells to a wide variety of apoptotic stimuli[31,32,33]. The result of their ratio (control : M-dysplasia : S-dysplasia : SCC = 1 : 2.5 : 2.2 : 3.1) in present study was supported by other's study[31,32,33], but was not consistent with Loro's et al[47]. The highest score was observed in SCC group, which indicated the anti-apoptotic tendency.

Bcl-w was expressed at relatively high levels in certain tumor cell lines of an epithelial origin, such as colonic, cervical, and breast cancer cells in protein level[48]. To our knowledge, there is no report regarding bcl-w mRNA expression in oral squamous cell carcinoma. In present study, its mRNA expression was decreased as the histologic grade was advanced, which was not consistent with the fact that expression of Bcl-w was increased in various cancer cell line and colorectal[49] and gastric adenocarcinoma[50]. This might be due to the different site involved in carcinoma. Also, bcl-w mRNA could be used as a bio-marker to trace the multistep carcinogenesis in tongue.

Bcl-XL has known to be an anti-apoptotic molecules in Bcl-2 family. Investigators have sensitized the cancer cells line and carcinoma by down-regualting Bcl-XL protein[51-54]. In present study, its expression was not significantly changed in all group. However, considering the Bcl-xL/Bax ratio in transcriptional level, mild, severe dysplasia and SCC were scored at 1.82, 1.25 and 2.38 in one another comparing with control. This meant that dysplasia and SCC were tended to be anti-apoptosis.

Bak immunoreactivity has been demonstrated in a wide variety of human tissues, including nasopharyngeal and esophageal epithelium, and epidermal keratinocytes[55,56]. Xie et al. suggest it shows a different pattern of expression in oral dysplasias and carcinomas than other sites and its expression , particularly in combination with Bax expression has prognostic value in tongue SCC[59].

Bak immunoreactivity in diseased oral mucosal lesions appeared uniform from basal to surface layer, as opposed to upper layer staining, as reported for normal nasopharynx, esophagus, and epidermis[56]. In contrast, Bak levels were found to be reduced in primary colorectal adenocarcinomas and gastric adenocarcinomas, as compared to normal gut mucosa[57,58], suggesting that Bak expression may be site dependent. In transcriptional level, in present study, its expression was decreased in all group comparing with the expression of control. Especially, Bak mRNA expression in severe dysplasia was the lowest in all Bcl-2 family, which was the opposite to bcl-2, the highest one in Bcl-2 family. Considering the fold increase of bak mRNA expression in severe dysplasia, it can be used as a bio-marker to discriminate severe dysplasia and SCC. Lindsten et al. reported Bax/Bak have been shown to be essential for programmed cell death. Considering the ratio of Bax/Bak, their ratio was contrast to Bcl-xL/Bax in SCC group. On our basic knowledge, the ratio of Bax/Bak was not favorable to predict the apoptosis.

Bad shares homology with Bcl-2 family only in the BH3 region, also plays an essential role in the regulation of cell death[60]. Scarcity of literature regarding Bad, it is hard to discuss. The result for Bad mRNA in present study showed its expression was not changed in dysplasia group, but slightly increased in SCC.

There was no report regarding Bok, Bmf, Mcl and BBC3 in oral squamous cell carcinoma, as far as we've known. Bok is a pro-apoptotic Bcl-2 family protein identified in the ovary based on its dimerization with the anti-apoptotic Bcl-2 proteins and cell killing[34]. In present study, Bok mRNA expression was decreased in dysplasia group comparing with the expression of control. But, in SCC group, its expression is not significant. Bok mRNA can be useful to discriminate dysplasia group from normal and SCC.

The Bcl-2 modifying factor, Bmf, is a pro-apoptotic member of the Bcl-2 family of apoptosis-related protein that has been shown to initiate apoptosis in response to the loss of attachment of cells from their basal lamina[35]. In present study, mRNA expression of bmf, like bcl-2, increased comparing with its expression in control. And a trend of expression level was similar to bcl-2.

Mantle cell lymphoma (MCL) is a mature B-cell proliferation characterized by the presence of translocation t(11;14)(q13;q32), an aggressive clinical course, and poor response to chemotherapy [61]. In present study, Mcl mRNA expression, like bcl-W, was decreased as histologic grade advanced. It can be used as a bio-marker together with bcl-w to discriminate all group of mulistep oral carcinogenesis.

BBC3 encodes a BH3-only protein that is induced by the p53 tumor suppressor and other apoptotic stimuli[62,63]. In contrast to decreasing pattern of mcl mRNA, BBC3 mRNA expression was increased as the histologic grade was advanced in present study, which suggest that it could be used as a bio-marker to discriminate the all group of multistep carcinogenesis together with mcl and bcl-2.

Taken together, Bcl-2 family mRNA expressions were complicated in mulitstep tongue carcinogenesis. And several Bcl-2 family in transcriptional level can be used as a biomarker to proper diagnosis for normal to malignant process through dysplasia stages.

V. CONCLUSIONS

The number of patients with tongue carcinoma is increasing rapidly among young individuals in many parts of the world. Oral carcinoma progresses from hyperplastic lesion through dysplasia to invasive carcinoma and the concept of "field cancerization" with molecular alteration has been suggested for oral cavity carcinogenesis. Significant improvement in treatment and prognosis will depend on more detailed understanding of the multi-step process leading to cancer development.

To induce tongue carcinoma in rat by 4-NQO,

each drinking water made 10 ppm, 25 ppm, 50 ppm and control (only D.W. without 4-NQO). Specimens were classified into 4 groups such as control, I (mild & moderate dysplasia), II (severe dysplasia and carcinoma in situ), III (carcinoma). mRNA expressions of Bcl-2 family were evaluated by RT-PCR.

For anti-apoptotic Bcl-2 family, mRNA expression of Bcl-w was down-regulated in all stages of tongue carcinogenesis model, however, up-regulation of Bcl-2 mRNA was evaluated. For pro-apoptotic Bcl-2 family, all members were down-regualted in all stages of tongue carcinogenesis model except Bad mRNA in group III. In terms of BH3 only protein, mRNA expressions of Bok and Mcl 1 were down regulated in all stages of specimen, but Bmf in group II and BBC3 in group III were up-regualted.

Our current findings demonstrated the involvements of mRNA expression of Bcl-2 family in multi-step tongue carcinogensis. This highlights the necessity for continued efforts to discover suitable biomakers (Bcl-2 family) for early diagnosis of the disease, and to understand its pathogenesis as a first step in improving methods of treatment. The discovery of these potential biomarkers and molecular targets for cancer diagnostics and therapeutics has the potential to significantly change the clinical approach and outcome of the disease.

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국문요약

백서 혀에서의 4-nitroquinoline 1-oxide 유도 발암과정에서 Bcl-2 계 유전자의 발현

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국은별[3] · 장미선[3] · 고미경[3] · 정권[3] · 최홍란[3] · 김옥준[3]

전 세계적으로 구강암의 빈도는 점점 증가 추세이며, 특히 한국인의 있어 혀(tongue)는 구강암이 가장 호발하는 장소이다. 구강암은 발암 단계에서부터 과증식 병소(hyperplastic lesion), 이형성(dysplasia) 및 상피내암(carcinoma in situ) 을 거쳐 악성 암종으로 발전하는 다단계 발암과정을 보이며, 분자 생물학적 변이가 구강암을 진행시킴이 널리 알려져 있다. 또한, 구강암은 일반적으로 암세포의 증식 및 고사(apoptosis)의 억제가 중요한 역할을 하고 있다 알려져 있다. 그리고, Bcl-2 family 는 세포 고사에 주요한 역할을 하고 있음이 알려져 있다. 그러나, 이들과 관련한 구강암 발생과정의 변화에 대해서는 널리 연구된 바가 없다.

본 연구는 백서에서 발암 물질인 4-NQO로 구강암을 유도시키고, 구강암 발생 다단계별로 Bcl-2 family의 mRNA 변화를 RT-PCR을 이용해 살펴보았다.

Bcl-2 family는 크게 3군, 즉 1) anti-apoptotic, 2) pro-apoptotic, 그리고 3) BH3 only protein으로 분류할 수 있으며, 본 연구에서 anti-apoptotic molecules인 Bcl-w는 모든 군에서 발현이 감소되었으며, Bcl-2는 발현이 증가 되었다. pro-apoptotic molecules에서는 Bad가 제 3군 (편평세포암종)에서 발현이 증가 되었고, 나머지는 감소하였다. BH-3 only protein에서는 Bmf가 제 2군에서, BBC3가 제 3군에서 발현이 증가하였고, 나머지는 모든 군에서 감소하였다.

결론적으로, 4-NQO로 유도된 백서의 발암단계에서, Bcl-2 family의 mRNA 양상은 다양하게 관찰되었으나, Bad 및 BBC3 mRNA가 제 3군에서, Bmf mRNA가 제 2군에서의 발현이 특별함을 알 수 있어, 다단계 발암과정에서의 구강암을 진단하는데 유용하리라 사료된다.

주제어: 구강암, 4-nitroquinoline 1-oxide, mRNA expressions of Bcl-2 family, RT-PCR technique