

ORIGINAL ARTICLE

Loss of *BCL-2* in the progression of oral cancer is not attributable to mutations

L L Loro, A C Johannessen, O K Vintermyr

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Correspondence to:
Dr L L Loro, Department of
Odontology-Oral
Pathology and Forensic
Odontology, The Gade
Institute, Haukeland
University Hospital,
N5021 Bergen, Norway;
lado.loro@gades.uib.no

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Background: *BCL-2* and *BAX* are important in the regulation of apoptosis. There have been reports of loss of *BCL-2* in basal cells of oral epithelial dysplasia (OED) and in oral squamous cell carcinoma (OSCC), and suppression of *BAX* in poorly differentiated OSCC.

Aim: To investigate whether loss of *BCL-2* in OED and OSCC, and of *BAX* in poorly differentiated OSCC could be attributed to *BCL-2* and *BAX* mutations.

Methods: Immunohistochemistry and in situ hybridisation were used to confirm *BCL-2* and *BAX* expression. DNA was extracted from archival samples of OED (n = 22) and OSCC (n = 28). The connective tissue part from each section was collected separately and used as the normal reference.

Results: No mutations were detected in *BCL-2* or *BAX* that could explain their aberrant expression at the mRNA and protein levels in OED and OSCC. The reported A/G polymorphism at codon 7 of *BCL-2* was detected in 18 of 50 samples and a novel C/T polymorphism at codon 100 was detected in three of 50 samples.

Conclusions: No mutations were found that could explain loss of *BCL-2* in oral dysplasia and carcinoma. An unreported C/T polymorphism in *BCL-2* was detected. Downregulation of *BCL-2* in OED and OSCC may be the result of transcriptional regulation.

Oral cancer is the 11th most common cancer globally in terms of numbers.¹ Oral squamous cell carcinoma (OSCC), the most prevalent type of oral cancer, is often preceded by a premalignant lesion. Histologically, oral premalignant lesions frequently show features of epithelial dysplasia, with moderate and severe epithelial dysplasia carrying the highest risk for malignant transformation.² There are no molecular markers that can predict malignant transformation in potentially malignant oral lesions.

“Aberrant expression of *BCL-2* has been reported in several human cancers, including oral cancer”

The *BCL-2* family comprises cell death antagonists, such as *BCL-2* and *BCL-X_L*, and death agonists, such as *BAX* and *BAD*. Members of the *BCL-2* family have several conserved domains named *BCL-2* homology (BH) domains, which participate in the formation of dimers and in the regulation of cell death.³ The proapoptotic protein *BAX* binds to the antiapoptotic protein *BCL-2*, leading to inactivation of the antiapoptotic function of *BCL-2*.³ *BCL-2* inhibits the release of cytochrome C from the mitochondria through specific mitochondrial channels.⁴ The release of cytochrome C activates apoptotic pathways in cells by its ATP dependent binding to apoptosis protease activating factor 1.⁵ Mutations in the BH4 domain of *BCL-2* convert it from an antiapoptotic to a proapoptotic protein.⁶ Frameshift *BAX* mutations confined to a tract of eight deoxyguanosines (G₈) (ATG GGG GGG GAG) in the third exon have been reported in over 50% of human microsatellite mutator phenotype positive colon cancers.⁷ Single nucleotide substitutions resulting in loss of the proapoptotic function of *BAX* have been reported within the BH1 and BH3 domains,⁸ and proximal to the C-terminus.⁹

Aberrant expression of *BCL-2* has been reported in several human cancers, including oral cancer.¹⁰ Previously, we reported the loss of *BCL-2* in basal cells of oral epithelial

dysplasia (OED) and OSCC, and greatly reduced expression of *BAX* in poorly differentiated OSCC.^{11 12} In our present study, we report that loss of *BCL-2* in basal cells of OED and OSCC is not attributable to a mutated *BCL-2* gene. In addition, loss of *BAX* expression in poorly differentiated OSCC was not related to mutations in *BAX*. The A/G single

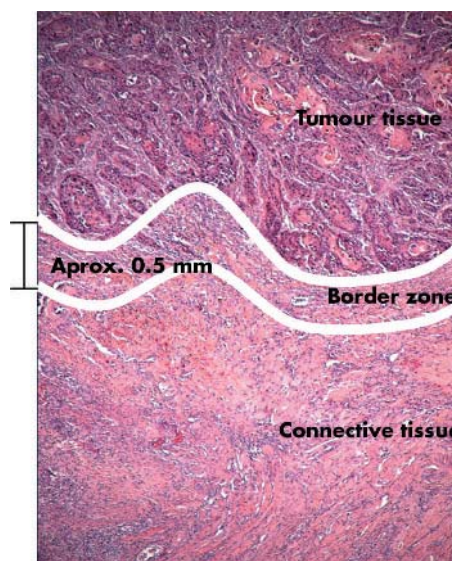


Figure 1 Haematoxylin and eosin section of a well differentiated oral squamous cell carcinoma illustrating the method used for the collection of samples for DNA extraction from the cancerous and connective tissue parts, with an intervening border zone that was not collected.

Abbreviations: BH, *BCL-2* homology; ISH, in situ hybridisation; OED, oral epithelial dysplasia; OSCC, oral squamous cell carcinoma; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

nucleotide polymorphism (SNP) at codon 7 of BCL-2 was frequently seen (18 of 50 samples), whereas a novel C/T polymorphism at codon 100 was less frequently seen (three of 50 samples) in premalignant and malignant oral epithelia.

MATERIAL AND METHODS

Tissue samples

Formalin fixed, paraffin wax embedded tissue sections of human OED (n = 22) and OSCC (n = 28) were used. The specimens comprised mild (n = 6), moderate (n = 7), and severe (n = 9) OED, and well differentiated (n = 19), moderately differentiated (n = 6), and poorly differentiated (n = 3) OSCC. All the dysplasia and OSCC samples were examined by an experienced pathologist (ACJ) and diagnosed according to the World Health Organisation criteria for the classification of cancer and precancer of the oral mucosa.¹³ Tonsillar tissues were used as controls for BCL-2 and BAX mRNA and protein expression. The connective tissue parts of the dysplasia and OSCC samples were used as non-cancerous control tissues for mutational analysis.

Immunohistochemistry

Sections (5 µm thick) were dewaxed in xylene and rehydrated in graded alcohol before pretreatment with 0.1% protease type xxiv (Sigma, St Louis, Missouri, USA) and microwave treatment, as described previously.¹² Samples were incubated overnight (18 hours) at 4°C in a humidified chamber with polyclonal antihuman BCL-2 (1/100 dilution; Pharmingen, San Diego, California, USA) or polyclonal antihuman BAX (1/800 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA) antibody. The specimens were further processed and the specificity of the anti-BCL-2 antibody tested with recombinant human BCL-2 protein (Pharmingen), as described previously.¹¹ Human tonsillar tissues were used as positive controls.

In situ mRNA hybridisation

Sections (5 µm thick) of OSCC were processed for the detection of BCL-2 and BAX mRNA by the catalysed signal amplification¹⁴ method developed for in situ hybridisation (ISH) (Genpoint kit; DakoCytomation, Glostrup, Denmark). The BCL-2 and BAX oligonucleotide probes, the relevant controls, and the ISH protocol have been described previously.¹² After dewaxing and rehydration, endogenous peroxidase activity was quenched in 0.3% H₂O₂ in 100% methanol for 20 minutes, followed by microwave treatment. Biotinylated "poly dT", sense or antisense BCL-2 and BAX probes were applied to samples at a concentration of 3 ng/µl in mRNA hybridisation solution (DakoCytomation) and hybridised overnight (18 hours) in a humidified chamber.

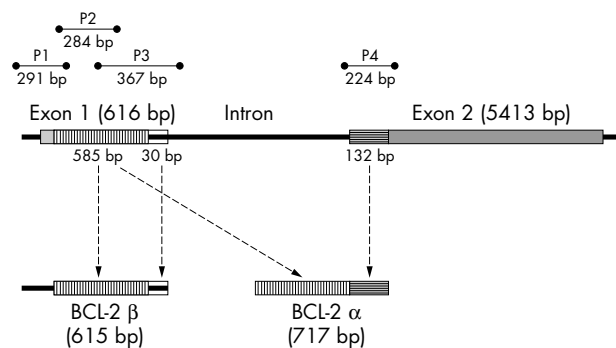


Figure 2 Schematic diagram of the BCL-2 gene, its isoforms, and the polymerase chain reaction oligonucleotide primers used. The primer sets (P1, P2, P3, and P4) were designed to span the coding regions for the α and β isoforms of BCL-2.

The hybridisation reaction was amplified twice using catalysed signal amplification for ISH (DakoCytomation), visualised with diaminobenzidine chromogen, and counterstained with Mayer's haematoxylin. Positive controls for BCL-2 and BAX mRNA comprised sections of human tonsillar tissue.

DNA isolation

Paraffin wax embedded OED or OSCC samples placed on uncoated microscopic glass slides were used for the extraction of genomic DNA. Three 10 µm sections were macrodissected from each sample. Before DNA extraction, neoplastic or dysplastic epithelium was carefully macrodissected from the surrounding normal stroma (fig 1). The stroma immediately adjacent to the dysplastic or neoplastic epithelium was left as a border zone, whereas the rest of the underlying stroma was collected and used as an internal normal reference. To avoid potential errors caused by the field cancerisation effect, superficial oral epithelium adjacent to OED or OSCC was never included as normal control tissue. For all samples, a haematoxylin and eosin stained section was used to guide tissue macrodissection. Tissues were then digested with proteinase K (0.2 mg/ml) in 200 µl digestion buffer (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.5% Tween 20) for 48 hours and DNA isolated using either silica spin columns (Qiagen, Hilden, Germany; *QIAamp DNA mini kit and QIAamp DNA blood mini kit handbook*. Hilden: Qiagen, 2001) or an automatic DNA extractor (BioRobot M48 workstation; Qiagen; *Supplementary protocol: isolation of genomic DNA from paraffin-embedded sections using MAgAttract DNA mini M48 kit*. Hilden: Qiagen, 2003), as described by the manufacturer. The quality and quantity of isolated genomic DNA was evaluated by electrophoresis and spectrophotometry (UV-1601; Shimadzu Corp, Kyoto, Japan).

Amplification and sequencing of BCL-2 and BAX

Four BCL-2 oligonucleotide DNA primers, three of which overlapped (table 1; fig 2), were used for amplification of the coding regions of exons one and two of BCL-2. Other than the reverse second primer,¹⁵ new polymerase chain reaction (PCR) primer pairs (P1, P3, and P4) were designed using the Oligo Primer Analysis Software (Molecular Biology Insights, Cascade, Colorado, USA). Primers used for amplification and sequencing of the six exons of BAX were as described previously,¹⁶ except for a new set of laboratory designed primers for amplification and sequencing of exon one (table 1).

For both BCL-2 and BAX the PCR amplification reaction mix (50 µl) contained 100 ng genomic DNA, 1.875 U Amplitaq Gold polymerase (Applied Biosystems, Warrington, UK), 0.25 mM dNTP, and 0.125 µM of each primer in PCR buffer (Applied Biosystems) supplemented with 0.5–2.0 mM MgCl₂ (table 1). The PCR reaction was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) for 40 cycles at the appropriate annealing temperature and MgCl₂ concentration for each primer set (table 1). Betaine (1.5 M; Sigma) was added to the PCR mix for the second set of BCL-2 primers (P2). PCR efficiency was evaluated by electrophoresis in a 3% agarose gel and visualised by ethidium bromide staining.

PCR products were purified using ExoSAP-IT (USB Corp, Cleveland, Ohio, USA), as described by the manufacturer. The purified PCR products (2.0–3.5 µl) were supplemented with 1 µl BigDye version 1.1 (Applied Biosystems), 1.5 µl 5× BigDye buffer (Applied Biosystems), and 0.2 µM primer and adjusted to 10 µl with deionised H₂O. Direct sequencing of PCR products in the forward and reverse directions was carried out using a capillary automatic sequencer (ABI PRISM 3100 genetic analyser; Applied Biosystems).

Table 1 Primers used for molecular analysis of the *BCL-2* and *BAX* genes

	Forward sequence (5'–3')	Reverse sequence (5'–3')	Product	T _a (°C)	MgCl ₂
<i>BCL-2</i>					
Exon 1 P1	cctcgccaagaatgcaa	gctgggaggagaagat	291 bp	56°C	2.0mM
P2	cgcaaccgggcatctctcccagc	gagagctctccaccaccg*	284 bp	55°C	0.5mM
P3	cccggcgacgactct	agagcccaccggcactc	367 bp	61.7°C	1.2mM
Exon 2 P4	cgfggggfggcattctc	gggcaggcatgttgac	224 bp	54°C	1.0mM
<i>BAX</i>					
Exon 1	cggagcggcgggtgatt†	aagccccaggccggtag†	131 bp	64°C	1.5mM
Exon 2/3	accctagaacccaagagtc	gctgagagtcctgtgtcc	400 bp	58°C	1.5mM
Exon 3 (G ₈) tract	atccaggatcgagcaggggcg	atcgctcagcttctgggtg	94 bp	58°C	1.5mM
Exon 4	tctctgcaggatgattgc	tcccaggctctcacagat	209 bp	58°C	1.5mM
Exon 5	caggcagfggggacaagg††	gggfgfggggfagggag	129 bp	60°C	1.5mM
Exon 6	ccctggccgagtcactgaa	agcccatgtccccaatc	237 bp	60°C	1.5mM

T_a, annealing temperature for each primer set.

*All *BCL-2* primers were laboratory designed for amplification and sequencing of the *BCL-2* gene in paraffin wax embedded samples except for the second reverse primer (P2), which was published previously¹⁵; †Primers for amplification and sequencing of *BAX* were published previously,¹⁶ except for the laboratory designed primers for exon 1 of *BAX*.

RESULTS

***BCL-2* and *BAX* protein and mRNA expression**

BCL2 expression was low in OSCC samples (fig 3A, B), as we have reported previously.¹¹ *BAX* was widely detected in tumour cells of well differentiated OSCC but in only a few cells in poorly differentiated OSCC (fig 3C, D). In control tonsillar tissue, *BCL-2* was predominantly detected in the mantle zones of tonsillar follicles and *BAX* mainly in the central zones of tonsillar follicles (data not shown).

BCL-2 mRNA was detected in a few cells in the tumour islands of well differentiated OSCC (fig 4A). Strong *BCL-2* mRNA reactivity was seen in tumour associated inflammatory cells (fig 4A). Only a few cells were positive for *BCL-2* mRNA in poorly differentiated OSCC (fig 4B). *BAX* mRNA was widely expressed in tumour islands of poorly differentiated OSCC and in tumour related inflammatory cells (fig 4C), but sparse in samples of poorly differentiated OSCC (fig 4D).

In control experiments, the demonstration of mRNA using a “polydT” oligonucleotide probe resulted in positive staining in all cells (data not shown). In positive control tonsillar tissues, *BCL-2* was detected mainly in follicular mantle zones

(fig 4E) and *BAX* in the follicular centres (fig 4F). Both *BCL-2* and *BAX* mRNA could also be detected in interfollicular lymphocytes. Incubation with *BCL-2* or *BAX* sense probes or elimination of probes resulted in very low or no reactivity, respectively.

Mutation analyses of *BCL-2* and *BAX*

The two exons of *BCL-2* and six exons of *BAX* were amplified and sequenced in our study samples. No mutations were detected in sequences encoding both the *BCL-2*α and *BCL-2*β isoforms. The reported A/G SNP at codon 7¹⁷ (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1801018) was detected in 18 of 50 and an unreported C/T SNP at codon 100 of *BCL-2* was detected in three of 50 samples in both dysplastic and neoplastic tissues, and corresponding reference samples from underlying stroma (fig 5). Tumour associated genetic alterations have been reported in apparently normal oral epithelium¹⁸ and in tumour associated stroma of breast carcinomas.¹⁹ Therefore, to test the level of detection of genetic alterations in tumour associated stroma or contamination of the connective tissue by tumour cells, tumour DNA containing the C/T SNP and DNA from stroma

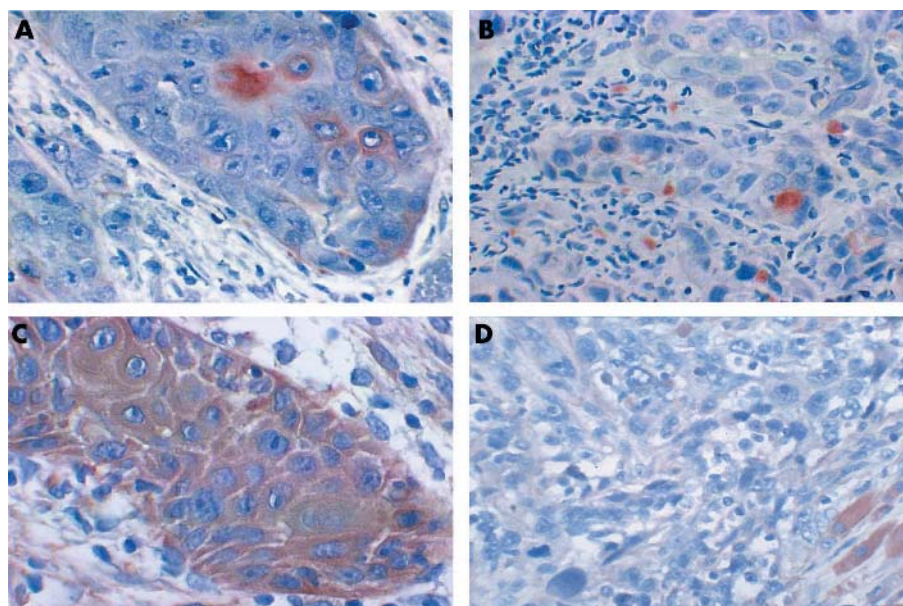


Figure 3 Immunohistochemical staining for *BCL-2* in (A) well differentiated and (B) poorly differentiated oral squamous cell carcinoma (OSCC). *BAX* immunoreactivity in (C) well differentiated and (D) poorly differentiated OSCC. Sections stained with AEC (3-amino-9-ethylcarbazole) and counterstained with Mayer's haematoxylin.

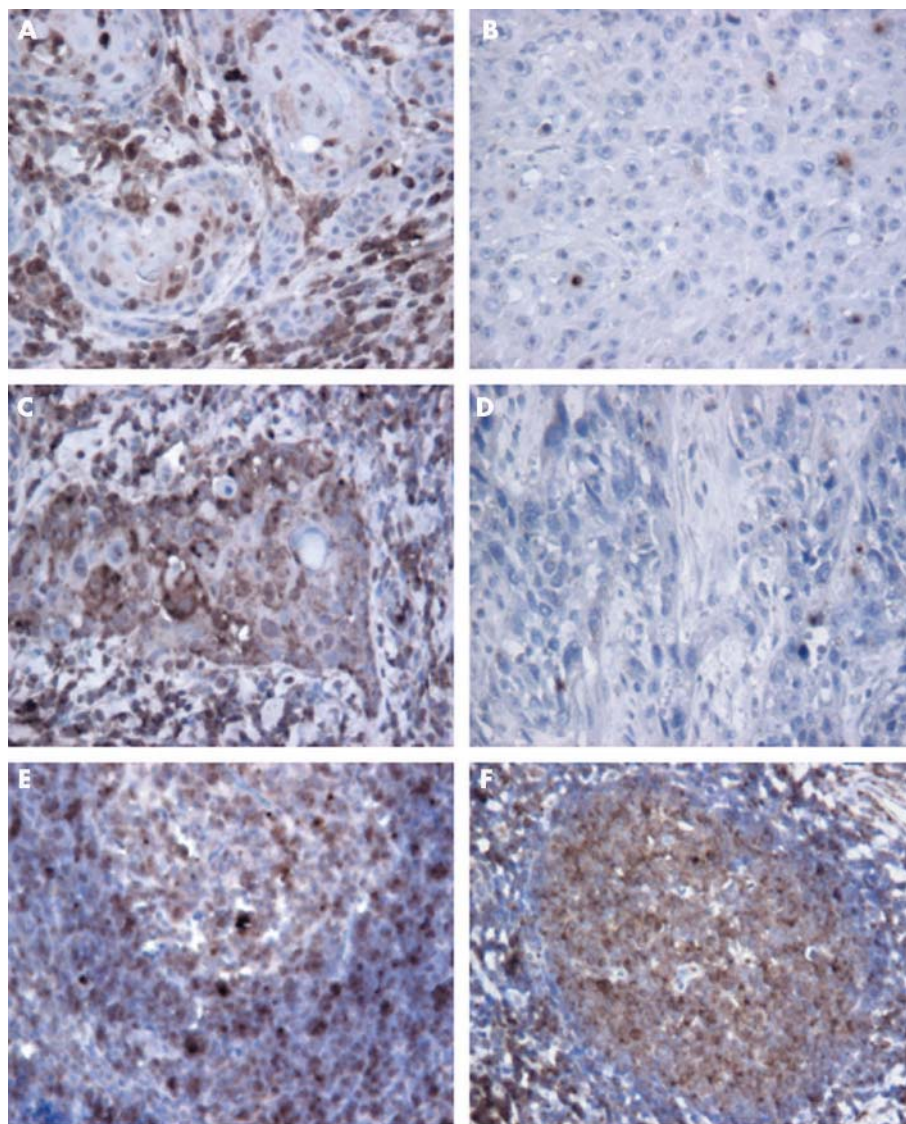


Figure 4 BCL-2 mRNA localisation in (A) well differentiated and (B) poorly differentiated oral squamous cell carcinoma (OSCC). BAX mRNA staining in (C) well differentiated and (D) poorly differentiated OSCC. Reactivity for (E) BCL-2 mRNA and (F) BAX mRNA in control tonsillar tissue. BCL-2 and BAX mRNA signals (A–F) were visualised with diaminobenzidine and the sections were counterstained with Mayer's haematoxylin.

without the C/T SNP were serially mixed to produce 50% (wt/wt), 12.5%, and 5% DNA containing the C/T SNP (fig 5A–F). In our hands, the level of detection of the C/T SNP in the mixed DNA was in the order of 5%. In addition, DNA extracted from samples from other sites (skin and pharynx) from patients with the C/T SNP also showed the C/T polymorphism (fig 5G), verifying that this was a germline polymorphism. No mutations in the hot spot eight deoxyguanosine (G₈) tract in the third exon of *BAX* were detected in OSCC samples. In a limited number of OSCC samples (n = 9), including three poorly differentiated OSCCs, exons one to six of the *BAX* gene were amplified and sequenced. However, no mutations were detected in these samples. The reported intronic G/A polymorphism, 13 bases downstream of the 3' end of exon three, was detected in one of nine OSCC samples.

DISCUSSION

Previously, we showed that BCL-2 mRNA and protein were progressively lost with increasing degree of dysplasia.¹² In addition, BCL-2 protein was lost in basal cells of well

differentiated and poorly differentiated OSCC, whereas BAX was maintained in dysplasia and in well differentiated OSCC, but much reduced in poorly differentiated OSCC.^{11, 12} Therefore, we wanted to analyse samples of OED and OSCC for mutations that may underlie the loss of BCL-2 in OED and OSCC, and of BAX in poorly differentiated OSCC.

Our mutational analyses based on sequencing of the entire coding regions of the *BCL-2* and *BAX* genes indicate that the aberrant expression of *BCL-2* in OED and OSCC, and of *BAX* in poorly differentiated OSCC, may not be the result of mutations in these genes. The lack of mutations in these genes suggests that *BCL-2* in oral premalignant and malignant lesions and *BAX* in poorly differentiated OSCC may be under transcriptional regulation. There is only one other study on oral cancer that has investigated BCL-2 at both the protein and mRNA levels. Our findings differ from that study, which reported that BCL-2 protein and mRNA are upregulated in OSCC, particularly in poorly differentiated OSCC, and suggested that *BCL-2* may be under post-transcriptional regulation because the concentration of BCL-2 protein was much lower than that of the mRNA.²⁰

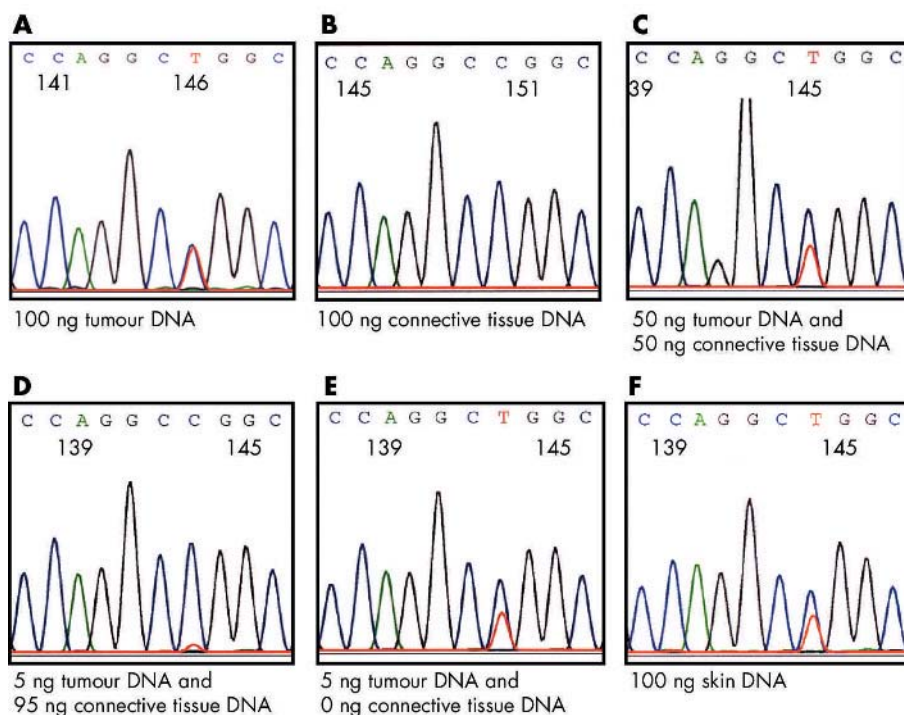


Figure 5 (A) The novel C/T single nucleotide polymorphism (SNP; arrow) at codon 100 of the *BCL-2* gene detected in a tumour sample. (B) DNA from the connective tissue of a tumour without the C/T SNP. (C, D) Mixture of tumour DNA with the C/T and connective tissue from the tumour without the C/T SNP. (E) Detection of the C/T SNP in 5 ng of DNA from the sample shown in (A). (F) DNA from the skin of the same patient as in (A).

The agreement between our immunohistochemical and in situ mRNA hybridisation findings suggests that both *BCL-2* and *BAX* may be under transcriptional regulation in dysplastic and neoplastic oral epithelia.

Several studies have shown that *BCL-2* expression is regulated transcriptionally and post-transcriptionally. A wide variety of molecules can downregulate or upregulate *BCL-2* mRNA or *BCL-2* protein including lymphokines, transforming growth factor β , Epstein Barr virus latent membrane protein 1, p53, retinoids, and phorbol esters.²¹⁻²⁶ Transcriptional suppression of *BCL-2* may also result from modulation of the negative response element located at the 5' untranslated region.²⁶⁻²⁸

“The agreement between our immunohistochemical and in situ mRNA hybridisation findings suggests that both *BCL-2* and *BAX* may be under transcriptional regulation in dysplastic and neoplastic oral epithelia”

BCL-2 and *BCL-X_L* are known to have antiproliferative effects, by delaying progression to S phase from quiescence.²⁹⁻³⁰ In addition, the antiproliferative effect of *BCL-2* has been shown to inhibit tumour progression in animal tumours.³¹⁻³² Therefore, loss of the suppressive effects of *BCL-2* may be advantageous for potentially malignant and malignant oral keratinocytes. A suppressive effect of *BCL-2* on proliferation is consistent with our earlier study reporting increased proliferative activity (as assessed by Ki-67 labelling) in basal cells of OED and OSCC, which had lost *BCL-2*.¹²⁻³³ Therefore, loss of *BCL-2* in oral keratinocytes may be involved in dysplastic and malignant progression of oral epithelium by making oral keratinocytes more responsive to mitotic stimuli.

The known A/G SNP at codon 7 of *BCL-2*¹⁷ (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1801018) was

seen frequently (18 of 50 samples) in our study. A less frequent (three of 50) and unreported C/T polymorphism was detected in oral dysplastic and neoplastic samples. The occurrence of SNPs in the human genome has been associated with susceptibility to many diseases, including cancers.³⁴ The observed C/T polymorphism could be a new SNP; however, its low frequency and absence from the SNP databases warrants further validation.

Downregulation of *BAX* in poorly differentiated OSCC suggests a role for *BAX* in the development of a more aggressive oral cancer phenotype. Molecular analysis of the conserved eight deoxyguanosines (G₈) tract in exon three and of the other five exons revealed no mutations. Because deregulated *BAX* expression in poorly differentiated OSCC could not be attributed to mutations in the *BAX* gene, and because of the concordance of *BAX* mRNA and protein expression, we suggest that *BAX* dysregulation in poorly differentiated OSCC may be the result of transcriptional regulation. Transcriptional induction of *BAX* by p53 requires a cofactor, the regulation of which is thought to be responsible for the tissue specific expression of *BAX* and induction of apoptosis.³⁵ Myc can also transcriptionally activate *BAX* expression by binding to a region in the *BAX* promoter.³⁶ Regulation of *BAX* expression and the interplay between modulating factors is still not well understood.

The molecular biology of oral carcinogenesis is characterised by the activation of oncogenes such as those encoding transforming growth factor α , the epidermal growth factor receptor, c-myc/N-myc, cyclin D1, and STAT-3, and inactivation of the tumour suppressor genes p53 and p16.³⁷ Our findings suggest that loss of *BCL-2* is an early event during oral carcinogenesis and decreased *BAX* expression seems to be associated with poorly differentiated OSCC. These alterations in *BCL-2* and *BAX* may be involved in dysregulation of apoptosis and contribute to the molecular carcinogenesis of oral cancers.

Take home messages

- Loss of BCL-2 in basal cells of potentially malignant and malignant oral epithelia and loss of BAX in poorly differentiated oral squamous cell carcinoma (OSCC) is not associated with mutations in the coding regions of these genes, but may be the result of transcriptional regulation
- These alterations in BCL-2 and BAX may be involved in dysregulation of apoptosis and contribute to the molecular carcinogenesis of oral cancers
- The aberrant expression of BCL-2 and BAX suggests a role, in cooperation with other molecular changes, in the progression of oral dysplasia and oral squamous cell carcinoma

In conclusion, our findings suggest that loss of BCL-2 in basal cells of potentially malignant and malignant oral epithelia and loss of BAX in poorly differentiated OSCC is not associated with mutations in the coding regions of these genes. The aberrant expression of BCL-2 and BAX suggests a role, in cooperation with other molecular changes, in the progression of oral dysplasia and oral squamous cell carcinoma.

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Authors' affiliations

L L Loro, A C Johannessen, Department of Odontology-Oral Pathology and Forensic Odontology, Haukeland University Hospital, N5021 Bergen, Norway

O K Vintermyr, Department of Pathology, The Gade Institute, University of Bergen, Haukeland University Hospital

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L L Loro, A C Johannessen and O K Vintermyr

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