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

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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. 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. There are no molecular markers that can predict malignant transformation in potentially malignant oral lesions.

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The BCL-2 family comprises cell death antagonists, such as BCL-2 and BCL-XL, 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. The proapoptotic protein BAX binds to the antiapoptotic protein BCL-2, leading to inactivation of the antiapoptotic function of BCL-2. BCL-2 inhibits the release of cytochrome C from the mitochondria through specific mitochondrial channels. The release of cytochrome C activates apoptotic pathways in cells by its ATP dependent binding to apoptosis protease activating factor 1. Mutations in the BH4 domain of BCL-2 convert it from an antiapoptotic to a proapoptotic protein. 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. Single nucleotide substitutions resulting in loss of the proapoptotic function of BAX have been reported within the BH1 and BH3 domains, and proximal to the C-terminus.

Aberrant expression of BCL-2 has been reported in several human cancers, including oral cancer. 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. 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
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 = 6), 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. 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. Samples were incubated overnight (18 hours) at 4°C in a humidified 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. 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; Qiagen DNA mini kit and Qiagen DNA blood mini kit handbook; Hilden; Qiagen, 2001) or an automatic DNA extractor (BioRobot M48 workstation; Qiagen; Sisleyangel 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, 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, 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 µlM 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 (1.5M; 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).
RESULTS

BCL-2 and BAX protein and mRNA expression

BCL-2 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 mantel 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 mantel 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-2a and BCL-2b 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 epithelium13 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

| Table 1 Primers used for molecular analysis of the BCL-2 and BAX genes |
|-----------------|----------------------|-----------------|--------|
| Forward sequence | Reverse sequence | Product | T_a (°C) | MgCl2 |
| Exon 2 | P1 | ctcgctggtcgtctgc | ggtggggtgggtctgc | 291 bp | 56 °C | 2.0mM |
| Exon 2 | P2 | cacccgggcatcttctc | gttggcaggcatgttgac | 284 bp | 55 °C | 0.5mM |
| Exon 2 | P3 | cccggcgacgacttct | aagcccacccgcactc | 367 bp | 61.7 °C | 1.2mM |
| Exon 2 | P4 | ggtggggtgggtctgc | gttggcaggcatgttgac | 224 bp | 54 °C | 1.0mM |
| Exon 1 | P1 | ctcgctggtcgtctgc | ggtggggtgggtctgc | 131 bp | 64 °C | 1.5mM |
| Exon 1 | P2 | cacccgggcatcttctc | gttggcaggcatgttgac | 400 bp | 58 °C | 1.5mM |
| Exon 1 | P3 | cccggcgacgacttct | aagcccacccgcactc | 94 bp | 58 °C | 1.5mM |
| Exon 1 | P4 | ggtggggtgggtctgc | gttggcaggcatgttgac | 209 bp | 58 °C | 1.5mM |
| Exon 1 | P5 | ggtggggtgggtctgc | gttggcaggcatgttgac | 129 bp | 60 °C | 1.5mM |
| Exon 1 | P6 | ggtggggtgggtctgc | gttggcaggcatgttgac | 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 previously.†Primer sets for amplification and sequencing of BAX were published previously except for the laboratory designed primers for exon 1 of BAX.

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-amin0-9-ethylcarbazole) and 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 \textit{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 \textit{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.\textsuperscript{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.\textsuperscript{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 \textit{BCL-2} and \textit{BAX} genes indicate that the aberrant expression of \textit{BCL-2} in OED and OSCC, and of \textit{BAX} in poorly differentiated OSCC, may not be the result of mutations in these genes. The lack of mutations in these genes suggests that \textit{BCL-2} in oral premalignant and malignant lesions and \textit{BAX} in poorly differentiated OSCC may be under transcriptional regulation. There is only one other study on oral cancer that has investigated \textit{BCL-2} at both the protein and mRNA levels. Our findings differ from that study, which reported that \textit{BCL-2} protein and mRNA are upregulated in OSCC, particularly in poorly differentiated OSCC, and suggested that \textit{BCL-2} may be under post-transcriptional regulation because the concentration of \textit{BCL-2} protein was much lower than that of the mRNA.\textsuperscript{20}
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 \( \beta \), 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\(_i\) 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-2.33 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-227 (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 \( \text{BAX} \) 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 \( \alpha \), 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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