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Profile of apoptotic proteins in oral squamous cell carcinoma: A cluster analysis of 171 cases

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the eighth most prevalent cancer worldwide. In recent large-scale studies, by immunohistochemistry and cluster analysis, several markers were associated with patient survival in various tumors. The aim of this study was to analyze the expression profiles of 23 proteins that have been linked to the inhibition (Bcl-2, Bcl-x, Bcl-xL, Bcl-2-related protein A1, BAG-1, and survivin) and promotion (Bak, Bax, Bim/Bod, Bim-Long, Bad, Bid, PUMA, Apaf-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, caspase-10, Smac/DIABLO, and cytochrome c) of apoptosis in OSCC.

Methods: Two-hundred and twenty nine cases of OSCC, arranged in a tissue microarray, were immunohistochemically analyzed, and the results were quantified on an automated imaging system. The data were analyzed using a random forest clustering method.

Results: Overall protein expression patterns defined two chief clusters: an anti-apoptotic cluster (142 cases) and a pro-apoptotic cluster (29 cases). These groups could not be explained by any clinical or pathological characteristic, and overall and disease-free survival did not differ between them.

Conclusions: Although there was no association with survival, the cluster analysis demonstrated specific protein profiles that could be of interest for using targeted therapies: in one of the clusters, the expression of pro-apoptotic proteins was more prominent, demonstrating a pro-apoptotic profile and highlighting the importance of apoptosis during OSCC development.

Keywords: Oral squamous cell carcinoma, Apoptosis, Cluster analysis, Protein profile, Immunohistochemistry

Background

Oral squamous cell carcinoma (OSCC) is the eighth most prevalent cancer worldwide [1, 2]. In Brazil, the National Cancer Institute estimates that 11,140 males and 4350 females develop oral cancer annually [3].

Many studies have identified various changes in gene expression in head and neck squamous cell carcinomas compared with normal oral mucosa, identifying subgroups of head and neck squamous cell carcinomas with profiles that correlate with many aspects of prognosis [4–7].

Molecular profiling by gene array and its translation into surrogate immunohistochemistry profiles is affecting the classification and management of tumors, such as endometrial, breast, gastric cancer, and brain tumors [8–13]. It is important that similar approaches be taken to increase our understanding of OSCC.

The changes that have been observed in such molecular studies affect a wide range of processes that mediate the development of many cancers [4]. One of these processes is apoptosis. The primary function of apoptosis is to eliminate senescent or altered cells that are useless or harmful to a multicellular organism. Altered expression levels of apoptosis-related proteins have been reported in several cancers, including OSCC [14–16]. The development of resistance to apoptosis is a hallmark of malignant cells,

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enabling them to survive, despite apoptosis-inducing environmental signals and the loss of normal survival signals [17, 18].

Two pathways initiate apoptosis, both of which activate the executioner caspases 3, 6, and 7. The first pathway is the intrinsic, or mitochondrial, pathway, because mitochondria control the initiation of apoptosis. Apoptotic stimuli induce the release of cytochrome c and other apoptotic regulators from the intermembrane space of mitochondria. In the cytosol, cytochrome c, APAF-1, ATP, and the initiator procaspase 9 form the apoptosome, which effects the cleavage of effector caspases. The Bcl-2 family of proteins regulates mitochondrial permeability. The second pathway is known as the extrinsic pathway; it is mediated by various death receptors on the cell surface. These receptors, once activated by specific ligands, initiates the recruitment of FAS-associated death domain protein and procaspases 8 and 10 to the death domain, which forms the death inducing signaling complex and promotes the activation of caspase 8 [16, 19].

The aim of this study was to analyze the expression profiles of 23 proteins that are associated with both apoptosis pathways in a series of 229 cases of OSCC. Hierarchical cluster analysis was performed to identify apoptotic molecular subtypes of OSCC and determine if these different profiles are related to patient outcomes.

Methods

Tissue samples

Paraffin-embedded tissue samples from 229 oral squamous cell carcinoma cases and 10 non-neoplastic samples from oral mucosa tissue adjacent to tumor were obtained from the files of the Department of Pathology of the A.C. Camargo Cancer Center, São Paulo, Brazil. All retrieved cases had been untreated, underwent surgery as the initial treatment at the hospital and have been followed-up for at least 5 years. Clinical and histological details of the cases are provided in Table 1. This study was approved by the AC Camargo Cancer Center Ethics Committee (Protocol number 985/07) and complied with the Helsinki Declaration guidelines.

Tissue microarray (TMA)

To construct the TMA, H&E sections were analyzed, and a representative area of the deepest tumor sheet was marked on the slide. The tissues that corresponded to the selected areas were sampled from the donor block using a tissue microarrayer (Beecher Instruments, Silver Springs, USA). Each sample was arrayed twice with a 1.0-mm-diameter core that was spaced 0.2 mm apart, and two TMAs with different core regions were constructed. After the array was completed, the TMA blocks were sectioned at a thickness of 4 μm.

Table 1 Clinicopathological characteristics of the oral squamous cell carcinoma patients

Variables	Categories	Number of patients (%)
Age (years)	<= 56	117 (51.1)
	>56	112 (48.9)
Gender	Male	194 (84.7)
	Female	35 (15.3)
Tobacco smoking	No	19 (8.3)
	Yes	182 (79.5)
	n/a[a]	28 (12.2)
Alcohol consumption	No	45 (19.7)
	Yes	153 (66.8)
	n/a	31 (13.5)
T stage	T1/T2	131(57.2)
	T3/T4a	98 (42.8)
Clinical Stage	I/II	77 (33.6)
	III/IV	152 (66.4)
Tumor site	Oral tongue	122 (53.3)
	Floor of mouth	55 (24.0)
	Other	52 (22.7)
Lymph node metastasis (pN)	No	92 (40.2)
	Yes	112 (48.9)
	n/a	25 (10.9)
Perineural infiltration	No	129 (56.3)
	Yes	86 (37.6)
	n/a	14 (6.1)
Vascular invasion	No	72 (31.4)
	Yes	141 (61.6)
	n/a	16 (7.0)
Histological grade	Well differentiated	177 (77.3)
	Moderately/poorly differentiated	46 (20.1)
	n/a	6 (2.6)
Treatment	Surgery	118 (51.5)
	Surgery + Radiotherapy	111 (48.5)

[a]n/a, information not available

Immunohistochemistry

The expression of Bcl-2, Bcl-x, Bcl-xL, Bcl-2-related protein A1, BAG-1, Bak, Bax, Bim/Bod, Bim-Long, Bad, Bid, PUMA, Apaf-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, caspase-10, Smac/DIABLO, cytochrome c, and survivin was examined in OSCC tissue samples in a TMA [14,15]. Immunostaining was performed on two tissue slides from different cores of the same sample. The slides were deparaffinized, rehydrated, and subjected to antigen retrieval. Details on the antigen retrieval methods and primary antibodies clones, sources, and titers are listed in Table 2. The sections

Table 2 Primary serum, clones, source, working titers, and antigen retrieval

Primary serum	Clone	Source	Working titer	Antigen retrieval
Bcl-2	124	Dako	1:200	Citrate pH 6.0
Bcl-x	polyclonal	Dako	1:500	Citrate pH 6.0
Bcl-xL	7D9	Chemicon	1:2000	Citrate pH 6.0
Bcl-2 related protein A1	EP517Y	Epitomics	1:150	Citrate pH 6.0
BAG-1	3.10G3E2	Neomarkers	1:100	EDTA/Tris pH 9.0
Bak	polyclonal	Dako	1:300	Citrate pH 6.0
Bax	polyclonal	Dako	1:400	Citrate pH 6.0
Bim/Bod	polyclonal	Neomarkers	1:150	EDTA/Tris pH 9.0
Bim Long	5E5	Chemicon	1:100	Citrate pH 6.0
Bad	Y208	Epitomics	1:1500	EDTA/Tris pH 9.0
Bid	BH3	Epitomics	1:200	Citrate pH 6.0
PUMA	EP512Y	Epitomics	1:250	Citrate pH 6.0
APAF1	polyclonal	Novocastra	1:30	Citrate pH 6.0
Caspase 2	Y154	Epitomics	1:100	EDTA/Tris pH 9.0
Cleaved Caspase 3	polyclonal	Cell Signaling	1:600	Citrate pH 6.0
Caspase 6(c-term)	E180	Epitomics	1:150	EDTA/Tris pH 9.0
Caspase 7	7CSP01	Chemicon	1:150	EDTA/Tris pH 9.0
Caspase 8	11B6	Novocastra	1:250	Citrate pH 6.0
Caspase 9p10	H83	Santa Cruz	1:500	Citrate pH 6.0
Caspase 10 pro	E35	Epitomics	1:600	Citrate pH 6.0
Smac/Diablo	Y12	Epitomics	1:100	Citrate pH 6.0
Cytochrome c	CTC05	Chemicon	1:3000	Citrate pH 6.0
Survivin	polyclonal	Neomarkers	1:400	EDTA pH 8.0

were incubated in 3% aqueous hydrogen peroxide for 15 min to quench endogenous peroxidase activity and with Protein Block Serum-Free (Dako, CA, USA) for 20 min at room temperature to suppress nonspecific binding of subsequent reagents.

The sections were then incubated with primary antibody for 2 h at room temperature. The antigen-antibody complexes were visualized using the Advance Detection System (Dako, CA, USA) and incubated with 3’3 diaminobenzidine tetrachloride (DAB) (Dako, CA, USA) for 5 min, and the sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted with a glass coverslip and xylene-based mounting media. The results were quantified on an automated imaging system (ACIS III, Dako, CA, USA), which detects levels of hue, saturation, and luminosity, converting this signal into a numerical measurement of density (staining intensity) that ranges from 0 to 256. Qualitative analysis of the results considered the distribution of the protein (nucleus, cytoplasmic and membrane), the pattern of staining (diffuse or focal) and the intensity of staining (strong, moderate, weak). Negative controls were performed by replacing the primary serum with a non-immune reagent. Positive controls were used per the manufactures’ recommendations.

Cluster analysis

Hierarchical cluster analyses were performed using a random forest clustering method (TMEV, http://mev.tm4.org/), and the results were displayed using Tree-View. This method is an unsupervised learning method that creates molecular classifications, based on distinct global expression profiles, blinded to clinic and pathological covariates. Samples that did not presented interpretable protein expression in more than 80% of the 23 proteins analyzed were excluded from hierarchical cluster analysis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL assay was performed using Apoptag S7100 (Milipore) according to the manufacturer’s protocol.

The slides were deparaffinized, rehydrated, and subjected to proteinase K treatment. The sections were incubated in 3% aqueous hydrogen peroxide for 5 min to quench endogenous peroxidase activity. The sections were then

incubated with TdT enzyme working solution for 1 h at 37 °C, followed by incubation with anti-dig conjugate for 30 min at room temperature. The reaction was developed with TSA incubation for 8 min at room temperature. The sections were mounted with a glass coverslip and xylene-based mounting media.

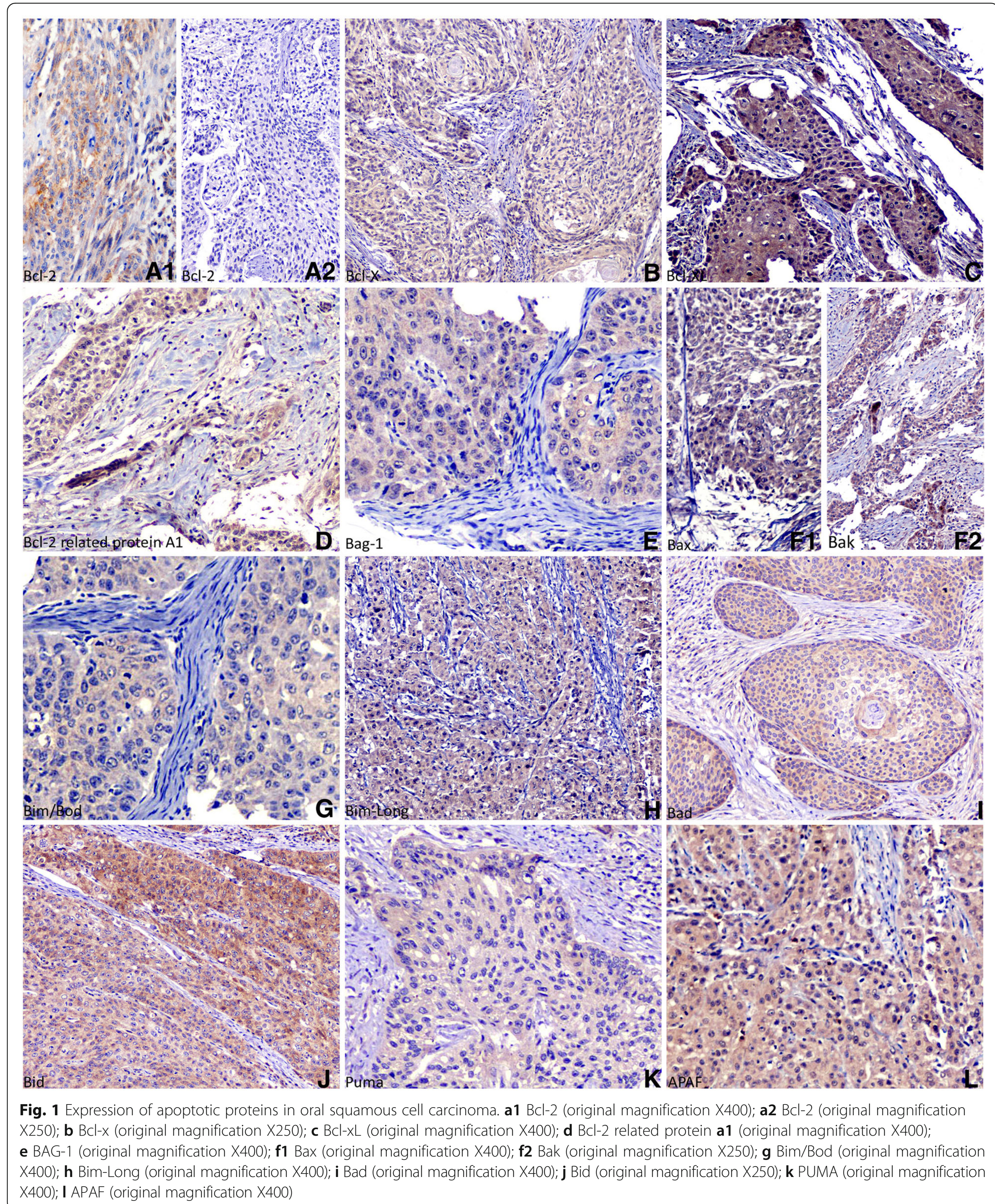

Fig. 1 Expression of apoptotic proteins in oral squamous cell carcinoma. **a1** Bcl-2 (original magnification X400); **a2** Bcl-2 (original magnification X250); **b** Bcl-x (original magnification X250); **c** Bcl-xL (original magnification X400); **d** Bcl-2 related protein **a1** (original magnification X400); **e** BAG-1 (original magnification X400); **f1** Bax (original magnification X400); **f2** Bak (original magnification X250); **g** Bim/Bod (original magnification X400); **h** Bim-Long (original magnification X400); **i** Bad (original magnification X400); **j** Bid (original magnification X250); **k** PUMA (original magnification X400); **l** APAF (original magnification X400)

Statistical analysis

The association of the demographic, clinical and pathological characteristics of the patients with protein expression profiles between clusters was analyzed by chi-square test. Overall and disease-free survival probabilities were calculated, based on the Kaplan-Meier method, and log-rank test was used to determine statistical significance. The significance level was 5% for all statistical tests. Statistical analyses were performed using R, version 2.13 (R Development Core Team (2010), Vienna, Austria, www.R-project.org).

Results

All antiapoptotic (Bcl-2, Bcl-x, Bcl-xL, Bcl-2-related protein A1, BAG1, survivin) and proapoptotic (Bak, Bax, Bim/Bod, Bim-Long, Bad, Bid, PUMA, Apaf-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, caspase-10, Smac/Diablo, cytochrome c) proteins were expressed in the oral squamous cell carcinoma (OSCC) samples (Figs. 1 and 2). The proteins varied in expression, and all were cytoplasmic in neoplastic cells and positive controls, except survivin that was expressed in a cytoplasmatic and/or nuclear pattern. Staining for Bad was robust in well-differentiated tumors. This pattern was also observed with Bcl-xL, which was strong in the fronts of invasive areas. Bcl-2-related protein A1 expression was strong in well-differentiated tumors, concentrated in the cytoplasm of keratinizing neoplastic cells that surrounded keratin pearls. There was no difference in the pattern of distribution of the staining of the other proteins in tumors. All proteins were expressed in non-neoplastic oral squamous mucosa in the middle and lower layers. These results are described in Table 3.

Hierarchical cluster analysis was performed after the exclusion of the samples with values that were missing in more than 20% of the 23 proteins. Therefore, 171 samples were allocated for the analysis. The combined protein expression patterns defined two clusters: cluster A (142 cases) and cluster B (29 cases; Fig. 3).

Nine proteins (BAG-1, Bcl-2, Bid, Bim/Bod, Bax, caspase-2, caspase-6, caspase-7, Smac/Diablo) were expressed equally between clusters. Apaf-1, caspase-3, caspase-9, cytochrome c, caspase-10, PUMA, survivin, Bad, Bak, Bcl-2-related protein A1, Bcl-x, Bcl-xL, and Bim-Long proteins were preferentially expressed in cluster B (p <0.0001, $p < 0.001$, $p < 0.001$, $p = 0.001$, $p = 0.001$, $p < 0.001$, $p = 0.007$, $p = 0.024$, $p = 0.028$, $p < 0.001$, $p <$ 0.001, $p < 0.001$ and $p = 0.006$, respectively), and caspase-8 was predominant in cluster A ($p < 0.001$) (Fig. 4). Based on the distribution of proteins in the clusters, we defined cluster A as the "anti-apoptotic

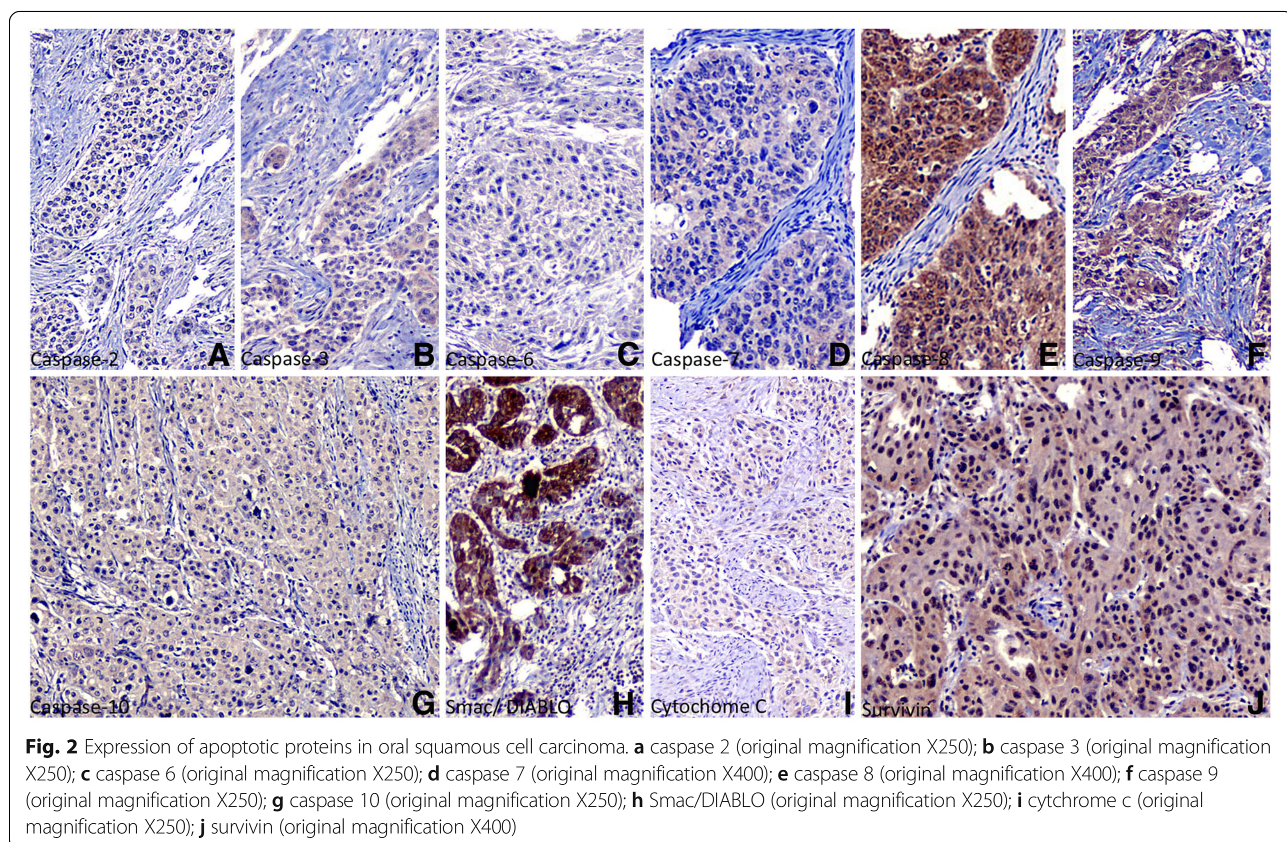

Fig. 2 Expression of apoptotic proteins in oral squamous cell carcinoma. **a** caspase 2 (original magnification X250); **b** caspase 3 (original magnification X250); **c** caspase 6 (original magnification X250); **d** caspase 7 (original magnification X400); **e** caspase 8 (original magnification X400); **f** caspase 9 (original magnification X250); **g** caspase 10 (original magnification X250); **h** Smac/DIABLO (original magnification X250); **i** cytchrome c (original magnification X250); **j** survivin (original magnification X400)

Table 3 Results of immunostaining quantitatively analyzed using an automated imaging system (ACIS III)

Protein	Intensity (range)	Median value of intensity	Intensity (range)	Median value of intensity
	Neoplastic cells		Normal cells	
Bcl2[a]	52.0–80.0	55.0	56.0–57.0	57.0
Bcl-x[a]	66.0–106.0	74.0	83.3–96.0	84.0
Bcl-xL[a]	109.5–156.0	136.9	80.7–95.0	83.0
Bcl-2 related protein A1[a]	78.5–128.0	93.8	74.7–87.0	79.2
BAG-1/RAP46[a]	69.0–102.0	77.0	68.0–76.0	74.6
Bak[a]	90.0–142.0	114.5	88.5–101.3	98.5
Bax[a]	99.0–138.3	114.5	74.0–75.0	74.7
Bim/Bod[a]	65.0–98.3	77.2	65.3–71.5	66.5
BIM-Long[a]	81.0–130.0	101.5	81.0–85.5	82.9
Bad (N-term)[a]	70.0–121.5	85.0	75.0–84.5	78.5
Bid[a]	65.5–98.5	76.5	62.3–65.0	63.5
Puma[a]	61.0–115.0	80.5	97.5–117.0	113.8
APAF1	102.0–150.0	124.0	83.0–93.0	87.0
Caspase 2	58.0–83.2	63.4	58.0–66.0	63.0
Cleaved Caspase 3[b]	68.8–107.0	81.0	61.0–73.0	62.5
Caspase 6 (c-term)[b]	63.0–90.0	74.0	62.0–82.0	66.5
Caspase 7[b]	69.5–107.5	80.3	65.0–72.0	66.0
Caspase 8[b]	85.0–151.0	115.4	78.0–115.0	82.0
Caspase 9p10[b]	90.5–138.0	107.0	55.0–68.0	59.0
Caspase 10 pro[b]	67.0–91.5	77.0	60.0–70.0	61.0
Smac/Diablo	72.0–135.8	90.0	71,0–83.0	74.0
Cytochrome c	63.0–87.0	73.0	63.0–71.0	70.5
Survivin	66.0–97.0	80.1	73.0–85.0	80.5

[a]Immunohistochemical data described in Coutinho-Camillo et al. [15]
[b]Immunohistochemical data described in Coutinho-Camillo et al. [16]

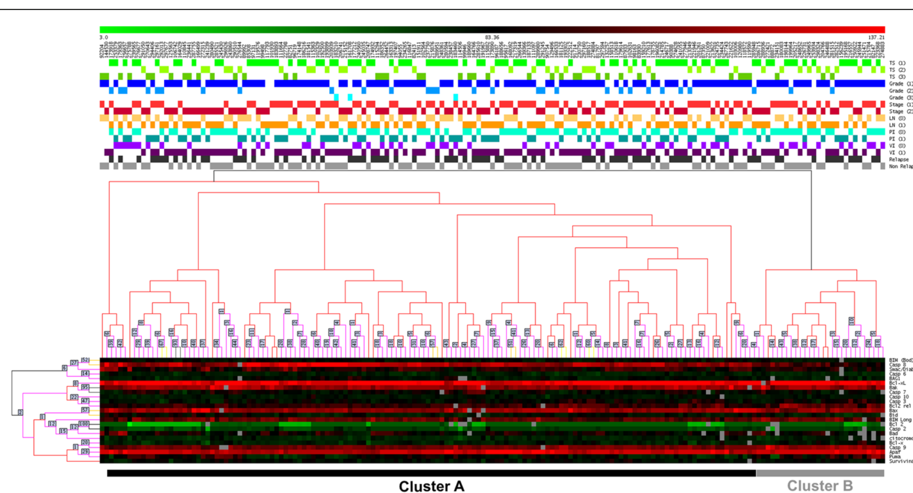

Fig. 3 Two-way hierarchical cluster analysis of 171 oral squamous cell carcinomas based on the expression of 23 proteins. Tumors were grouped into 2 clusters (A and B), based on the protein expression profile. Rows represent proteins, ordered according to their hierarchical distances. Colors in columns represent expression levels: red indicates positive staining, and green represents the absence of staining for each of the antibodies. Within each cluster, samples were ordered based on their correlation distances

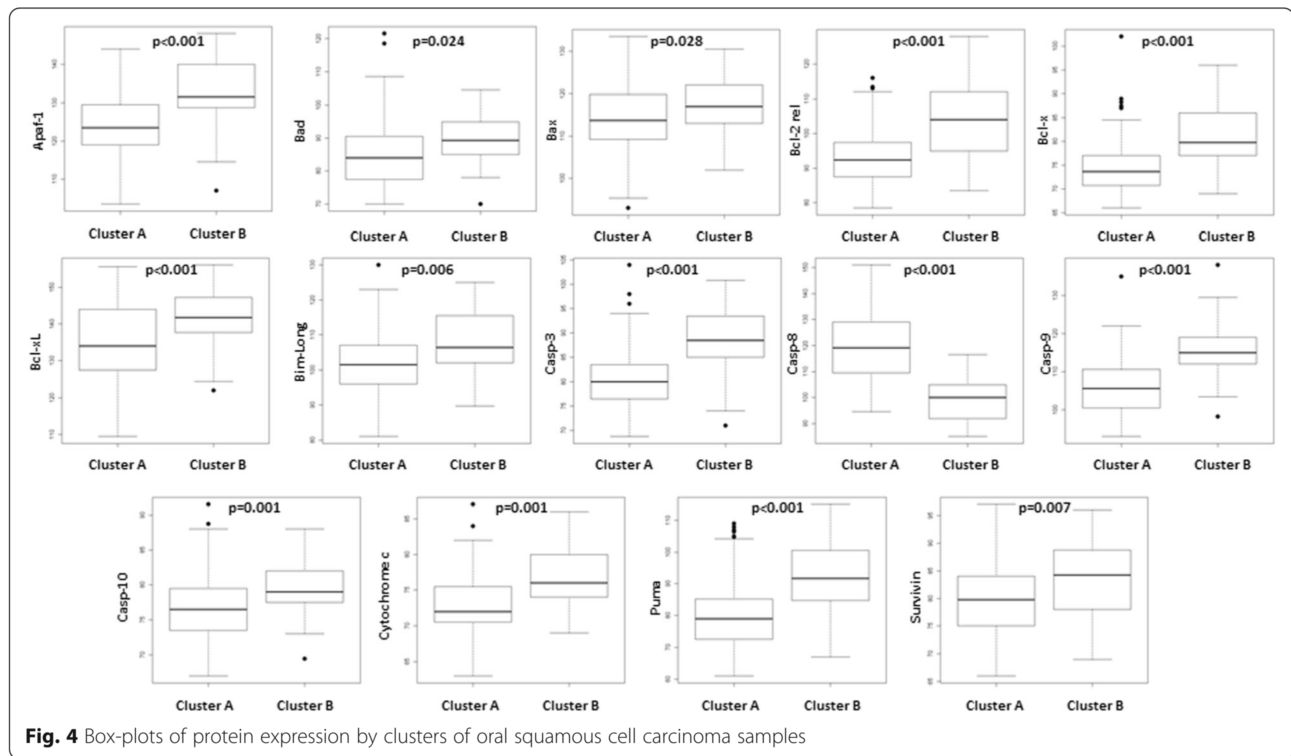

Fig. 4 Box-plots of protein expression by clusters of oral squamous cell carcinoma samples

cluster" and cluster B as the "pro-apoptotic cluster". Figure 5 shows the occurrence of apoptosis in OSCC samples using TUNEL assay. The apoptotic cells were mainly detected in areas with evidence of keratin pearl formation. The protein expression profile in cases that demonstrated apoptosis by TUNEL assay was similar to "pro-apoptotic cluster", however no statistical significance was observed.

To determine whether these two clusters represented clinically distinct subgroups of patients, univariate analysis was performed. Significant associations between clusters and clinical/pathological findings, such as location and histological grade, were not observed (Table 4). Five-year overall and disease-free survival rates did not differ between clusters A and B ($p = 0.69$ and $p = 0.68$, respectively).

Discussion

Alterations in the expression levels of apoptosis-related proteins have been reported in several cancers, including oral cancer [14–16]. In this study, by tissue microarray (TMA) analysis of 229 oral squamous cell carcinoma (OSCC) cases, the expression profiles of 23 apoptotic proteins were examined and a hierarchical cluster analysis was performed.

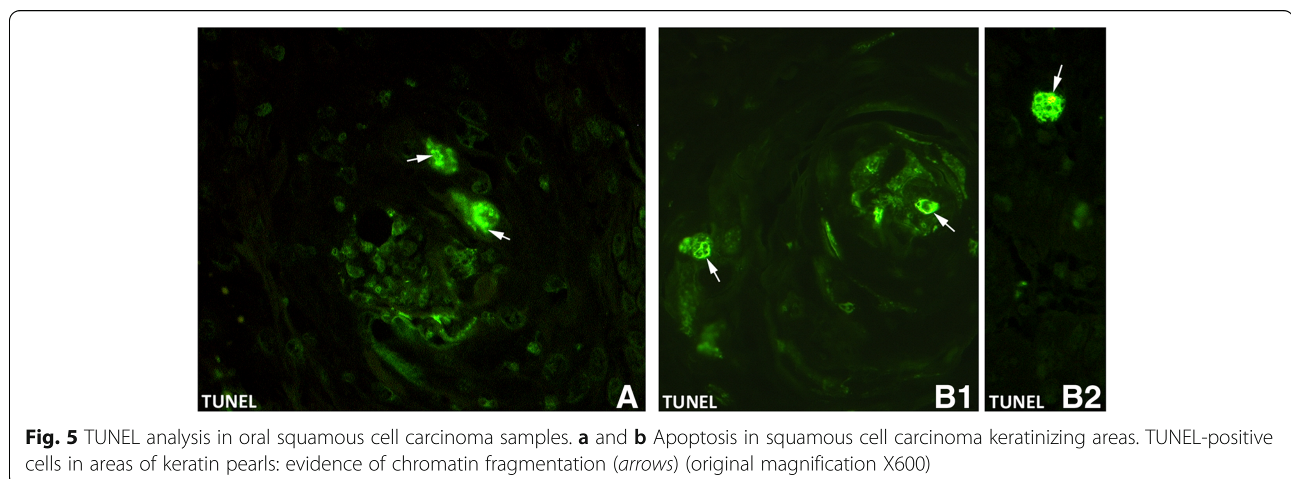

Fig. 5 TUNEL analysis in oral squamous cell carcinoma samples. **a** and **b** Apoptosis in squamous cell carcinoma keratinizing areas. TUNEL-positive cells in areas of keratin pearls: evidence of chromatin fragmentation (*arrows*) (original magnification X600)

Table 4 Association between clusters and clinical and pathological findings

Variable	Category	Cluster		Total	*p*-value
		A	B		
Tumor site	Oral tongue	72 (82.0)	16 (18.0)	88	0.1225
	Floor of mouth	32 (76.0)	10 (24.0)	42	
	Other	38 (93.0)	3 (7.0)	41	
T stage	T1/T2	73 (82.0)	16 (18.0)	89	0.7116
	T3/T4a	69 (84.0)	13 (16.0)	82	
Clinical stage	I/II	38 (79.0)	10 (21.0)	48	0.3990
	III/IV	104 (85.0)	19 (15.0)	123	
Histological grade	Well differentiated	108 (81.0)	25 (19.0)	133	0.4002
	Moderately differentiated	26 (90.0)	3 (10.0)	29	
	Poorly differentiated	3 (100.0)	0 (0.0)	3	
Vascular invasion	No	43 (81.0)	10 (19.0)	53	0.4956
	Yes	93 (85.0)	16 (15.0)	109	
Perineural infiltration	No	74 (80.0)	19 (20.0)	93	0.2045
	Yes	61 (87.0)	9 (13.0)	70	
Lymph node metastasis	No	59 (86.0)	10 (14.0)	69	0.7312
	Yes	76 (84.0)	15 (16.0)	91	

More than 60 gene expression-profiling studies of human clinical samples of head and neck squamous cell carcinoma (HNSCC) have been published. Unfortunately, these profiles have failed to effect any clinically relevant application in the management of HNSCC [20, 21]. Several studies have identified gene expression profiles that differ between normal mucosa, premalignant lesions, and invasive carcinoma. Other studies focused on tumor classification, prognostic or predictive value based on molecular characteristics [22–26].

Molecular profiling by gene array and its translation into surrogate immunohistochemistry profiles are being applied to classify and manage various tumors. Cluster analysis has been used, allowing one to make a more objective interpretation of immunoprofiles, based on staining with multiple antibodies, and holding great promise for the immunohistochemical classification of tumors [8–13]. Tissue microarrays have become a widely used tool to screen for protein expression patterns in a large number of tumors and these data could be used to classify tumors and determine whether these putative classes (clusters) are biologically and clinically meaningful [27].

Apoptosis has a significant function in OSCC. Based on the expression of 23 proteins, our group have demonstrated that decreased expression of caspase-3 correlates with lymph node metastasis, lower caspase-7 levels are associated with disease-free survival in oral cancer, high Bim-Long expression is linked to overall survival and that elevated PUMA levels are associated with disease-free survival in oral cancer patients [15, 16]. Elevated expression of Apaf-1 and survivin is associated with the absence of embolization, and the expression of Smac/DIABLO and cytochrome c is not significantly associated to any clinicopathological characteristic (unpublished results).

The contradictory results regarding the prognosis and expression of apoptotic proteins indicate that the induction of apoptosis is complex and that the influence of individual proteins varies between tumors. Further, the balance between anti- and proapoptotic proteins determines cell fate. In this study, a hierarchical cluster analysis was performed to identify protein profiles, which could distinguish different subtypes of OSCC, and how they relate to patient outcomes.

Overall protein expression patterns defined two chief clusters: an anti-apoptotic cluster (142 cases) and a pro-apoptotic cluster (29 cases). These groups could not be explained by any clinical or pathological characteristic, and overall and disease-free survival did not differ between them. Although there was no association with survival, the cluster analysis demonstrated specific protein profiles that could be of interest for using targeted therapies: in one of the clusters, the expression of pro-apoptotic proteins was more prominent, demonstrating a pro-apoptotic profile and highlighting the importance of apoptosis during OSCC development.

First studies in HNSCC focused on discriminatory profiles between normal and tumor samples [28–30]. Chung et al. [22] was the first group to classify HNSCC into prognostic groups based on gene expression profiles of 60 samples from different tumor sites. Zanaruddin et al. [31], using a panel of 11 proteins previously shown

to have a prognostic significance in HNSCC, described a 4-protein signature that predicts lymph node metastasis and survival in oral squamous cell carcinoma. Box et al. [32] identified a protein signature associated with EGFR-TKI resistance in head and neck squamous cell carcinoma cell lines.

Walter et al. [33] analyzed gene expression profile of 138 HNSCC samples from different sites and reported the same groups described by Chung et al. [22]. However, they did not observed an association between recurrence-free survival and tumor subtype. Méndez et al. [34] described 314 genes differentially expressed between tumor and normal samples. However, considering patient's outcome only one gene was differentially expressed between metastatic and non-metastatic tumors.

These apparent conflicting results regarding prognostic findings presented by different studies might be due to the use of different array platforms, analytical procedures, tumor sample selection, number of patients evaluated, and different endpoints evaluated [7, 20, 21].

One of the limitations of this study is the lack of data on other critical cellular pathways, besides apoptosis. Another possible limitation of this study is the use of Tissue microarrays, due to intra-tumor heterogeneity of protein expression. However, increasing the number of cores collected from the sample and/or increasing the core diameter can circumvent this limitation [35, 36]. Furthermore, the use of an automated scoring system enables a more objective and quantitative acquisition of staining result measurements due to its higher accuracy, sensitivity and better reproducibility of data [36, 37].

Despite these limitations, this study was able to demonstrate different biological behavior in OSCC. Further validation using another cohort and in vivo studies are necessary to evaluate if the cluster analysis could be predictive of response to therapeutic interventions.

In summary, the expression 23 apoptosis-related proteins was evaluated on 229 OSCC samples arranged on a tissue microarray. Although hierarchical cluster analysis identified two clusters, no significant associations between the clusters and clinical and pathological findings were observed. Based on the expression profiles of the clusters, a pro-apoptotic cluster and an anti-apoptotic cluster were defined, suggesting that apoptosis is linked to tumor behavior in OSCC.

Conclusions

Our results suggested that apoptosis is present in OSCC, but other mechanisms of cell growth outcome those of cell-death. This information may be of value in establishing new approaches to investigate the complex molecular roads that lead to uncontrolled cell proliferation in malignant neoplasms.

Abbreviations

DAB: 3'3 diaminobenzidine tetrachloride; OSCC: Oral squamous cell carcinoma; TMA: Tissue microarray; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Authors' contributions

CMCC designed the research study, performed the research, analyzed the data and wrote the paper; SVL designed the study and wrote the paper; RDP analyzed the data; ASD analyzed the data; THNT performed the research, analyzed the data; LPK designed the research study and revised the paper; FAS designed the research study and revised the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study was approved by the AC Camargo Cancer Center Ethics Committee (Protocol number 985/07) and complied with the Helsinki Declaration guidelines.

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