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Pansini, Paola Fernandes; do Valle, Isabella Bittencourt; Damasceno, Thabata Coeli Dias; de Abreu, Priscila Marinho; Có, Anna Clara Gregório; López, Rossana Verónica Mendoza; Lenzi, Jeferson; Rocha, Ricardo Mai; Souza, Evandro Duccini; Curado, Maria Paula; Mehanna, Hisham; Nankivell, Paul; de Podestá, José Roberto Vasconcelos; von Zeidler, Sandra Ventorin

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Paola Fernandes Pansini[1] (E-mail: paolafpansini@gmail.com)

Thabata Coeli Dias Damasceno[1] (E-mail: thabata.damasceno@gmail.com)

Priscila Marinho de Abreu[1] (E-mail: priscilamarinho84@gmail.com)

Isabella Bittencourt do Valle[1,2] (E-mail: isabellabdv@gmail.com)

Anna Clara Gregório Có[1] (E-mail: annaclaragc@gmail.com)

Rossana Verônica Mendoza López[3] (E-mail: rossana@alumni.usp.br)

Jeferson Lenzi[4] (E-mail: jefersonlenzi@hotmail.com)

Ricardo Mai Rocha[4] (E-mail: ricardomai@gmail.com)

Evandro Duccini Souza[4] (E-mail: evandroduccini@gmail.com)

José Roberto Vasconcelos de Podestá[4] (E-mail: podestajr@gmail.com)

Maria Paula Curado[5] (E-mail: mp.curado@accamargo.org.br)

Hisham Mehanna[6] (E-mail: h.mehanna@bham.ac.uk)

Sandra Ventorin von Zeidler[1] (E-mail: sandra.zeidler@ufes.br)

[1]Programa de Pós-Graduação em Biotecnologia, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil.

[2]Programa de Pós-Graduação em Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil.

[3]Instituto do Câncer do Estado de São Paulo (ICESP) - Center for Translational Research in Oncology, Universidade de São Paulo, São Paulo, Brazil.

[4]Programa de Detecção Precoce do Câncer Bucal, Setor de Cirurgia de Cabeça e Pescoço, Hospital Santa Rita de Cássia, Vitória, Espírito Santo, Brazil.

[5]Centro Internacional de Pesquisa, AC Camargo Cancer Center, São Paulo, São Paulo, Brasil.

[6]Institute for Head and Neck Studies and Education (InHANSE), University of Birmingham, Birmingham, United Kingdom.

Corresponding author: Sandra Ventorin von Zeidler D.D.S., MS, PhD. Departamento de Patologia, Programa de Pós-graduação em Biotecnologia, Centro de Ciências da Saúde - Universidade Federal do Espírito Santo, Av. Marechal Campos, 1468 Maruípe, Vitória, ES, Brazil. ZIP Code 29.043-900. Phone: + 55 27 3335-7299; Fax number: +55-27-3335-7290; E-mail: sandra.zeidler@ufes.br

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Statement of translational relevance

Oral cancer is the sixth most common malignancy worldwide, with approximately 300,000 new cases per year and with a high morbidity-mortality rate. Specifically, oral squamous cell carcinoma represents 95% of all oral cancers, with a delayed diagnosis in Brazil resulting in more aggressive therapies and low survival rates. From a clinical perspective, since there are no reliable biomarkers predicting the risk of malignant oral epithelium progression in oral dysplasia or oral squamous cell carcinoma, the aim of this study was to help identify potential biomarkers of the tumor development process. We thus analyzed eight proteins as biomarkers for oral cancer potentially applicable to the clinical routine for an early diagnosis, that could benefit the patient with less invasive treatment and a high survival rate compared to conventional diagnostic methods. Our findings indicate that the survivin, PLK1, p63 and p40 proteins are potential biomarkers capable of predicting malignant epithelial transformation in oral squamous cell carcinoma.

Abstract

Risk stratification of individuals regarding the development of oral squamous cell carcinoma (OSCC) continues to be based on clinical detection of potentially malignant oral disorders and histological assessment of oral epithelial dysplasia grade. To evaluate molecular epithelial changes, we investigated whether a profile of survivin, cyclin dependent kinase inhibitor 2A (CDKN2A), epidermal growth factor receptor (EGFR), polo like kinase 1 (PLK1), p63, p40 (Δnp63 isoform), cyclin D1 (CCND1) and BCL2 apoptosis regulator (BCL2) proteins could predict malignant transformation. Different tissue segments (tumor adjacent epithelium; dysplasia and tumor) from a total of 109 patients were analyzed by immunohistochemistry. The Kruskal-Wallis test confirmed increased expression of survivin ($p<0.001$), PLK1 ($p=0.001$), and p63 ($p<0.001$) in parallel to reduced immunostaining of p40 ($p<0.001$) and BCL2 ($p=0.029$) among the tissue segments analyzed. Our study revealed that survivin, PLK1, p63, p40 and BCL2 play a role in oral tumorigenesis and represent promising biomarkers able to recognize mesenchymal phenotype induction in the transition from nonmalignant cells to tumor cells. These results will provide further tools for predicting early progression in order to identify potential biomarkers of tumor development.

Key words: oral cancer, dysplasia, tumor, progression, immunohistochemistry

Introduction

During the development of oral squamous cell carcinoma (OSCC), transformed epithelial cells evade the mechanisms of cell cycle control and trigger the epithelial to mesenchymal transition (EMT), acquiring some specific changes such a loss of contact inhibition and reduction of cell polarity. These processes strengthen the migratory and invasive potential detected during tumor progression (1,2).

Some molecular epithelial changes progress to dysplasia or cancer, whereas others do not seem to need further changes in order to transform, mainly due to their biological behavior and molecular profile (3). Thus, although the World Health Organization 2017 three-tier grading system (4) is the gold standard for histological diagnosis of potentially malignant oral disorders, it has certain limitations. Therefore, the exclusive use of clinical and histopathological parameters for the clinical management of oral epithelial dysplasia could be inaccurate since this dysplasia can potentially undergo malignant transformation or can even regress, impacting on the identification of tumor progression (3,5,6). Thus, molecular biomarkers are required to overcome these limitations and to improve the prediction of the risk of further malignant transformation (7).

Several target molecules involved in cell growth, proliferation and survival mechanisms have been indicated as potential predictive biomarkers of tumor development; however, their diagnostic accuracy is still controversial (8,9). Different proteins, depending on their upregulated or downregulated status, can participate in the process of cancer development in addition to affecting tumor behavior and aggressiveness (10). Thus, based on the promising biomarkers published, we selected some outstanding targets such as: survivin and BCL2 apoptosis regulator (BCL2) that support cell division and anti-apoptotic function (11,12); cyclin dependent kinase inhibitor 2A (CDKN2A) that

regulates the progression from the G1 to the S phase in the cell cycle, the polo like kinase 1 (PLK1) that regulates the G2/M transition (13,14); epidermal growth factor receptor (EGFR) that promotes cell survival and proliferation (15); and nuclear cyclin D1 (CCND1) that, when accumulated, results in an aberrant increase in cell proliferation activity (16). The main function of p63 is to maintain the proliferative potential of progenitor epidermal cells, predominantly located in basal layers. This function is exerted primarily by the p40 isoform (Δnp63 isoform) (17,18).

On this basis, the objective of the present study was to investigate the expression profile of the target proteins survivin, CDKN2A, EGFR, PLK1, p63, p40, CCND1 and BCL2 as potential biomarkers of malignant transformation in OSCC to be used in clinical routine.

Methods

Patient samples

This was a prospective longitudinal analytical study of biological samples and clinical data from 109 patients treated at three Cancer Centers between 2010 and 2017. Fifty-seven patients were recruited from the Oral Cancer Early Detection and Prevention Program, Head and Neck Cancer Division, of Hospital Santa Rita de Cássia and Hospital Universitário Cassiano Antônio Moraes, both in Brazil, and 52 patients were selected from the University Hospitals Coventry and Warwickshire NHS Trust, United Kingdom. The study was approved by the Research Ethics Committee Integrated Center for Health Attention, Vitória, Espírito Santo, Brazil, (Protocol #318/2011), by the National Research Ethics Commission (Protocol #681/2011), and by the National Research Ethics Service (NRES) - Coventry & Warwickshire (EC.10.H1210.9).

Clinical and pathological data (e.g., age, sex, tumor subsite, TNM stage, alcohol consumption and tobacco exposure) were obtained by interview and from the medical records of the patients. The clinical stage of the tumor was reclassified according to the 7th edition of the AJCC Cancer Staging Manual (19).

According to the International Classification of Diseases for Oncology, 3rd edition (ICD-O-3) (20), only oral cavity squamous cell carcinomas were included (C00.3-C00.9; C01–C06) prior to any antineoplastic treatment. All tumors were confirmed by microscopy and were defined as squamous cell carcinoma (codes M8070, M8071, M8072, M8076, M8051 and M8083).

Tissue Microarray and immunohistochemistry

Tissue Mircroarrays (TMAs) were constructed in triplicate from representative formalin-fixed and paraffin-embedded tissues from the tumor adjacent epithelium, dysplasia and OSCC cores. Immunohistochemistry (IHC) was performed on 3 µm TMA slides for protein expression analysis. Following deparaffinization by immersion in xylol and rehydration in alcohol, the slides were immersed in citrate buffer, pH 6.0 for 3 minutes for antigen retrieval. The sections were then incubated with 3% hydrogen peroxide for 5 minutes and subsequently incubated at 4ºC overnight with the respective antibodies: monoclonal rabbit anti-survivin (Clone EP2880Y, Abcam) at 1:750 dilution; monoclonal human anti-CDKN2A (Clone E6H4, CINtec p16INK4a Histology Kit, Ventana Medical Systems, USA) at 1:5 dilution; EGFR monoclonal rabbit anti-EGFR (Clone D38B1, Cell Signaling Technology) at 1:100 dilution; monoclonal mouse rabbit anti-PLK1 (Clone 35-206, Abcam) at 1:1000 dilution; monoclonal human anti-p63 (Clone 4A4, DAKO) at 1:50 dilution; monoclonal mouse anti-p40 (Clone BC28, Biocare medical) at 1:100 dilution; monoclonal mouse anti-BCL2 (Clone 124, DAKO) at 1:50

dilution; and monoclonal rabbit anti-cyclin D1 (Clone SP4, Sigma-Aldrich) at 1:50 dilution. The slides were then incubated with the second antibody Novolink Polymer, Leica Novocastra™) according to the protocol suggested by the manufacturer. Finally, the sections were incubated with 3,3'- diaminobenzidine (K3468; DAKO, Glostrup, Denmark) for 2–5 min at room temperature, stained with Harry's hematoxylin (HHS80, Sigma-Aldrich®), and covered with Entellan® mounting medium (Merck Millipore). Negative controls were obtained by omitting the primary antibody. Human tonsil fragments (survivin, p63, BCL2 and CCND1), squamous cell carcinoma of the oropharynx (CDKN2A), basal layer of normal human oral mucosa (EGFR), human colon adenocarcinoma (PLK1), and human bladder (P40) of known positive reactivity were included as positive controls.

Cell counting and statistical analysis

A validation set was performed to assess the correspondence of whole section staining and TMA core staining. A whole section from 10 tumors was stained and scored, and then the scores of whole sections and their corresponding TMA cores were compared.

Protein immunostaining was analyzed in terms of H-score, considering a semi-quantitative and ordinal scale of 0 to 3 where 0 = absent immunostaining, 1 = low immunostaining, 2 = moderate immunostaining, and 3 = strong immunostaining intensity. The H-score was scaled based on the product of the intensity score (0 to 3) and the percentage of cell immunostaining in each core (0% to 100%), resulting in values of 0 to 300 (21). Epithelial cells were considered to be positive when showing brown staining. A cell count was performed by two blinded pathologists using a Zeiss light microscope (Carl Zeiss, Gottingen, Germany). Quadratic weighted kappa (κ) indices were calculated

to assess the TMA validation process and the level of interobserver agreement about TMA cores.

The statistical analyses were performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA). Patient profile data are reported as frequencies and percentages. IHC data were analyzed statistically using the non-parametric Kruskal–Wallis test and mean protein expression in the studied segments was compared by the Mann-Whitney *U* test and Bonferroni's test for multiple comparison adjustments. Correlations were evaluated using the Spearman test. The level of significance was set at 5% for all statistical hypotheses.

Results

Clinical and microscopic features

Table 1 lists the clinicopathological characteristics of all OSCC cases. The study population consisted predominantly of males (72.2%) and mean patient age was 60 years. There was a high percentage of tobacco smokers (43.2%) and alcohol drinkers (44.1%). Sixty-two (52.5%) cases were diagnosed with advanced stages (III and IV) and 52.2% had positive lymph node metastases.

Immunostaining of survivin, CDKN2A, EGFR, PLK1, p63, p40, CCND1 and BCL2

The positive immunostaining panel for the protein targets survivin, CDKN2A, EGFR, PLK1, p63, p40, CCND1 and BCL2 is illustrated in Figure 1. The immunoexpression profile of tumor adjacent epithelium, dysplasia and OSCC is shown in Table 2.

The largest mean number of p40-positive cells (222.09) was observed in the adjacent epithelium and the lowest mean number of CDKN2A-positive cells was

observed in tumor adjacent epithelium (15.09) (Table 2). The mean scores for survivin, PLK1, p63 and p40 differed significantly (p<0.001; Table 2) among segments.

A progressive linear trend was observed in mean survivin immunoexpression from tumor adjacent epithelium to OSCC (p<0.001), as shown in Figure 2 and Table 2. A highly significant positive correlation between survivin and PLK1 was observed in dysplasia (r=0.742, p=0.000), whereas CCND1 was moderately correlated with tumor adjacent epithelium (r=0.669, p=0.000) and dysplasia (r=0.627, p=0.000) (Table 3).

p63 immunoexpression differed significantly between tumor adjacent epithelium and OSCC and between dysplasia and OSCC (p<0.001), being approximately 2.27 times higher in OSCC than in dysplasia.

An inverse correlation of the reduction of p40 immunoexpression was detected between tumor adjacent epithelium, dysplasia and OSCC (p<0.001) and the difference between tumor adjacent epithelium/dysplasia and tumor adjacent epithelium/OSCC was confirmed by Bonferroni adjustment. BCL2 also showed decreased immunoexpression among the segments (p=0.029), although it was not confirmed by the Bonferroni post hoc test (Figure 2; Table 2).

Discussion

The carcinogenesis of OSCC is a multifunctional process involving deregulation between cell death and growth (22). It has been well-established in the literature that malignant transformation is related to mesenchymal phenotype acquisition during the EMT (2). Thus, the identification of molecular markers involved in EMT changes may indicate the risk of malignant transformation and tumor initiation (22). Alterations in cell proliferation in different steps of tumor progression can be detected by

immunohistochemical analysis, which can be easily performed in clinical practice, facilitating an early detection of potential malignant epithelial changes (23).

Overexpression of p63 has been demonstrated to be an essential tool for epithelial stem cell maintenance, cell proliferation and differentiation (24). Our investigations revealed that the role of p63 in carcinogenesis is supported by gradually increased expression in the segments analyzed, with high expression in OSCC, as also reported in previous investigations (25,26). Thus, oral carcinogenesis could be associated with the balance between p63 and its respective isoform, p40, due to their important influence on differentiation, survival, apoptosis and tumor suppression (27). Our study showed decreased intensity of p40 immunoexpression, in agreement with Goto et al. (28), with this change being related to the induction of a mesenchymal phenotype leading to malignant transformation and a more invasive profile of OSCC. Therefore, our results support previous studies that have pointed out p40 as a predictive marker of early events in tumorigenesis, involving cell cycle and cellular adhesion (18,23,29).

Altered protein expression linked to cell proliferation and apoptosis may be a strong baseline of potential epithelial malignant transformation to OSCC and ensures the identification of the early stages of oral carcinogenesis (30). Several studies have reported overexpression of BCL2 in OSCC, enabling carcinogenesis progression, while others have indicated reduced expression in oral dysplasia compared to normal epithelium. We report here a trend to a loss of BCL2 immunoexpression in the tumor, explained by the association of an anti-apoptotic role with increased proliferation of transformed cells. Similarly, Loro et al. (31) reported a remarkably reduced expression of BCL2 in oral tissues due to the expected anti-apoptotic function of transformed cells. This deregulation may represent one of the molecular alterations occurring in malignant transformation (32).

Survivin, another inhibitor of apoptosis, has been previously reported to be widely expressed in embryonic and fetal tissues, but barely expressed in terminally differentiated cells (33). In our study, survivin was overexpressed in OSCC, demonstrating an increased immunoexpression in the tumor, promoting progression of transformed cells ($p< 0.001$). The same condition was observed by Poomsawat et al. (34) during oral carcinogenesis and by Liu et al. (9) in normal oral tissues compared to tumor tissues.

Our investigation indicates that survivin is a remarkable factor during tumorigenesis due to its functional role in the control of cell division and apoptosis. The strong correlation between PLK1 (p=0.000) and survivin and the moderate correlation between CCND1 (p<0.000) and survivin observed here permitted us to elucidate how survivin may impact the transition in cell cycle phase arrangements, with PLK1 and CCND1 also sustaining the proliferative ability of cancer cells in many types of tumors (35–37). Previous reports have described a significant correlation coefficient for survivin and PLK1 in head and neck cancer (21) and a positive correlation between CCND1and survivin in mucinous ovarian neoplasms (35).

Several mechanisms have been investigated in order to explain the role of survivin in OSCC development, with data showing that cell growth and apoptosis regulation are important factors in tumorigenesis (38). Apoptosis deregulation by altered expression of survivin plays a critical role in tumor progression, as reported by Khan et al. (38) who showed that about 50% of premalignant lesions had survivin overexpression (39).

In summary, by studying different markers, we observed that protein immunostaining in tumors was not uniform in general and particularly for OSCC. We checked eight well-described markers as potential indicators of progression, and our findings revealed that survivin, PLK1, p63 and p40 stand out and play a central role in oral tumorigenesis. Specifically, survivin represented a promising biomarker of transition

from nonmalignant cells to OSCC, being involved in tumorigenesis due to a gradual increase in immunoexpression and to a significant correlation with PLK1 and CCND1 in tumor development.

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Tables

Table 1. The clinicopathological characteristics of OSCC patients (n=109).

Variables	Patient n (%)
Sex	
Male	85 (72.0)
Female	24 (20.3)
Age, years	
≤ 60	12 (10.2)
> 60	97 (82.2)
Smoke history	
Yes	51 (43.2)
No	35 (29.7)
Alcohol history	
Yes	52 (44.1)
No	24 (20.3)
T-primary tumor size	
T1-T2	56 (47.5)
T3-T4	48 (40.7)
N-regional lymph node metastasis	
Negative	37 (31.4)
Positive	62 (52.2)
TNM stage	
I-II	42 (35.6)
III-IV	62 (52.5)

[a] Summation lower than 109 due to the lack of data.

Table 2. Protein expression profile in tumor adjacent epithelium, dysplasia and OSCC.

Proteins/Segments	Mean	SD	P value
SURVIVIN			
Adjacent epithelium	36.88 [a,b]	30.66	< **0.001****
Dysplasia	53.73 [a]	40.87	
OSCC	62.65 [b]	35.38	
CDKN2A			
Adjacent epithelium	15.09	22.68	0.165
Dysplasia	35.60	71.07	
OSCC	33.71	81.10	
EGFR			
Adjacent epithelium	15.89	17.15	0.176
Dysplasia	23.88	60.14	
OSCC	34.14	64.70	
PLK1			
Adjacent epithelium	29.54 [a,b]	28.63	**0.001****
Dysplasia	47.14 [a]	35.49	
OSCC	69.05 [b]	62.68	
p63			
Adjacent epithelium	62.74 [a]	86.91	< **0.001****
Dysplasia	63.07 [b]	87.00	
OSCC	143.38 [a,b]	99.36	
p40			
Adjacent epithelium	222.09 [a,b]	64.78	< **0.001****
Dysplasia	141.86 [a]	94.17	
OSCC	112.25 [b]	78.24	
CCND1			
Adjacent epithelium	112.25	78.24	0.143
Dysplasia	101.34	74.41	
OSCC	126.80	74.60	
BCL2			
Adjacent epithelium	29.83	46.29	**0.029****
Dysplasia	22.67	35.49	
OSCC	15.42	21.97	

* N, total number of samples. Std Dev, standard deviation

** P value< 0.05

[a,b] Refers to the result of Bonferroni's post-hoc test. Different letters between groups mean indicates significant difference between them.

Table 3. Spearman's correlation coefficient for the expression profile of tumor adjacent epithelium, dysplasia and OSCC.

Segments		CDKN2A	EGFR	PLK1	p63	p40	CCND1	BCL2
AE	**SURVIVIN**	r= 0.400, p = 0.017	r= 0.432, p= 0.012	r= 0.384, p= 0.028	r= 0.412, p= 0.014	r= 0.118, p= 0.641	**r= 0.669, p= 0.000**	r= 0.109, p= 0.486
	CDKN2A		r= 0.068, p= 0.702	r= 0.302, p= 0.082	r= 0,230, p= 0.178	r= -0.408, p= 0.083	r= 0.373, p= 0.036	r= -0.149, p= 0.371
	EGFR			r= 0.253, p= 0.155	r= 0.168, p= 0.312	r= 0.100, p= 0.650	r= 0.062, p= 0.735	r= -0.080, p= 0.602
	PLK1				r= 0.111, p= 0.514	r= -0.037, p= 0.886	r = 0.148, p= 0.411	r = 0.077, p= 0.650
	p63					r= -0.293, p= 0.175	r= 0.239, p= 0.173	r= -0.072, p= 0.640
	p40						r= -0.148, p= 0.585	r= 0.309, p= 0.049
	CCND1							r= 0.137, p= 0.400
Dysplasia	**SURVIVIN**	r= -0.112, p=0.463	r= 0.370, p= 0.014	**r= 0.742, p= 0.000**	r= -0.006, p= 0.971	r= 0.100, p= 0.491	**r= 0.627, p= 0.000**	r= 0.418, p= 0.001
	CDKN2A		r= -0.173, p= 0.226	r= -0.081, p= 0.596	r= -0.031, p= 0.841	r= -0.036, p= 0.803	r= -0.017, p= 0.910	r= 0.165, p= 0.279
	EGFR			r = 0.171, p= 0.263	r= 0.131, p= 0.402	r= -0.090, p= 0.536	r= 0.027, p= 0.857	r= 0.258, p= 0.090
	PLK1				r= -0.216, p= 0.180	r= -0.077, p= 0.613	r= 0.393, p= 0.008	r= 0.098, p= 0.531
	p63					r= 0.086, p= 0.577	r= -0.063, p= 0.690	r= 0.037, p= 0.818
	p40						r= -0.07, p= 0.619	r= 0.007, p= 0.960
	CCND1							r= 0.454, p= 0.001
OSCC	**SURVIVIN**	r= 0.101, p= 0.337	r= 0.115, p= 0.266	r= 0.351, p= 0.001	r= 0.080, p= 0.446	r= 0.056, p= 0.732	r= 0.266, p= 0.007	r= 0.118, p= 0.450
	CDKN2A		r= -0.050, p= 0.628	r= -0.096, p= 0.365	r= 0.076, p= 0.468	r= 0.218, p= 0.223	r= -0.032, p= 0.761	r= -0.277, p= 0.103
	EGFR			r= 0.091, p= 0.387	r= -0.027, p= 0.793	r= 0.146, p= 0.403	r= 0.166, p= 0.100	r= 0.189, p= 0.257
	PLK1				r= 0.188, p= 0.074	r = 0.159, p= 0.361	r= 0.038, p= 0.718	r= -0.032, p= 0.847
	p63					r= 0.034, p= 0.850	r= -0,016, p= 0.875	r= -0,122, p= 0.467
	p40						r= 0.252, p= 0.113	r= 0.547, p = 0.000
	CCND1							r= 0.059, p= 0.708

TAE, tumor adjacent epithelium. r, Speraman's correlation coefficient.
P value< 0.001

Figure legends

Figure 1. Photomicrographs of tumor adjacent epithelium, dysplasia and OSCC showing survivin, CDKN2A, EGFR, PLK1, p63, p40, CCND1 and BCL2 positive immunostaining. Original magnifications x400. Representative cases showing survivin, CDKN2A, PLK1, CCND1 nuclear and cytoplasmic staining; EGFR membrane or membrane and cytoplasmic staining; p63, p40 nuclear staining; BCL2 cytoplasmic staining.

Figure 2. Representative standardized mean immunoexpression in tumor adjacent epithelium, dysplasia and OSCC. The plots indicate an increase or decrease in immunoexpression of survivin, CDKN2A, EGFR, PLK1, p63, p40, CCND1 and BCL2.