Original Research Article

Immunoexpression of tumor suppressor protein p53 and deubiquitinating enzymes in oral squamous cell carcinoma

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Abstract

Introduction: The ubiquitin-proteasome system is regulated by deubiquitinating enzymes (DUBs), which include the complex Ubiquitin-specific protease 1 (USP1) and WD40 repeat-containing protein 48 (WDR48). In normal conditions, these proteins contribute to genome integrity by regulating the DNA repair pathways. However, studies have associated abnormalities in this complex with the pathogenesis of cancer. Simultaneously, Tumor Suppressor Protein p53 is also regulated by ubiquitin-dependent degradation and its overexpression suggests that several DUBs are interacting and deubiquitinating this protein. **Objective:** To evaluate the immunoexpression of p53, USP1, and WDR48 in Oral Squamous Cell Carcinoma (OSCC). **Material and methods:** Thirty cases of OSCC and 40 non-neoplastic oral epithelium (NNOE, control group) were selected for immunohistochemical investigation. The histopathological classification was performed using H&E-stained sections. Values were statistically analyzed using the non-parametric test Kruskal Wallis. **Results:** Higher positivity of the markers was found in OSCC (p53:65%; USP1:96.4%; WDR48: 68.9%) than in NNOE (p53:35.1%; USP1:85%; WDR48: 65.2%). Poorly-differentiated cases of OSCC exhibited higher nuclear immunostaining of all proteins when compared with well-differentiated samples. Conclusion: This is a pioneer study and suggests that p53 and deubiquitinating enzymes (USP1 and WDR48) can affect the biological behavior of OSCC as they are related to the tumor development and histological malignancy grading.

Introduction

The ubiquitin-proteasome system (UPS) is a highly regulated key intracellular protein degradation pathway, which consists of an enzymatic cascade controlling protein ubiquitination and has critical functions in almost any cellular process, such as cell survival, proliferation, development, and DNA damage response (DDR) [31, 33]. This is antagonized by Deubiquitinating Enzymes (DUBs), an important class of regulators of the UPS. By cleaving covalently attached ubiquitin molecules from target substrates or polyubiquitinated chains, DUBs are carefully regulated and act as balancers of the ubiquitination-proteasome system in different ways and at different sites [9].

There are approximately 90-95 DUBs encoded in the human genome, divided into 5 different families [31]. The most abundant family is the ubiquitin-specific proteases (USPs) with 60 members [32]. USPs are regulated by changes in the catalytic domain, where the catalytic triad may be misarranged, via additional domains within the protein itself, via post-translational modifications, or by subcellular localization [28]. The primary responsibility of this enzyme is the degradation and clearance of misfolded or damaged proteins as well as of dysfunctional organelles, which compromise cellular homeostasis [33].

The Ubiquitin-specific protease 1 (USP1), a member of the USP family, is one of the best-known DUBs [5, 38], responsible for mediating the deubiquitination of inhibitors of DNA binding (IDs) [38], which are HLH transcription factors that inhibit differentiation and senescence. USP1 also enhances stem cell maintenance by regulating CKN1A expression [38], deubiquitinates Fanconi Anemia pathway proteins (FANCD2 and FANCI) [6, 29], a step required for the completion of this DNA repair pathway [17], and deubiquitinates the Proliferating Cell Nuclear Antigen (PCNA), an important safeguard against error-prone translesion synthesis (TLS) of DNA [17, 29, 38].

The deubiquitinase activity of USP1 is activated by complex formation with WD40 repeat-containing protein 48 (WDR48) (also called USP1-associated factor 1 [UAF1] or p80) [4, 5, 34], which leads to increased catalytic turnover for these enzymes [5]. Several studies have tried to uncover the detailed mechanism of USP1 activation by WDR48 and also the interfaces involved in the formation of this complex. It was suggested that WDR48 binding modulates the active site conformation of USP1, resulting in a productive catalytic triad [5, 36, 37]. Together, USP1 and WDR48 are important contributors to genome integrity at least in part by regulating the homologous recombination and translesion synthesis DNA repair pathways [5, 36, 37].

Extensive subsequent studies identified abnormalities related to USP1/WDR48 machinery, which has been linked to the pathogenesis of cancer [14, 30, 33]. Increased levels of USP1 are detected in certain types of human neoplasia, but little is known about the significance of this overexpression in cancer development [15]. Simultaneously, the stability of p53 also is regulated by ubiquitin-dependent degradation [20], and this regulation is essential for functions such as control of the degradation, localization, and activity of this protein [2]. Consequently, overexpression of mutated p53 suggests that several DUBs are interacting and deubiquitinating this protein [16].

Targeting the USP1/WDR48 complex in neoplastic cells with increased expression of mutated p53 may be predicted to achieve therapeutic success with chemotherapeutic agents [7]. The main advantage of inhibiting USPs, in cases of therapeutic intervention, is to improve treatment efficacy and decrease non-specific collateral effects. Therefore, human USPs are arising as a magnificent target for pharmacologic intervention [4].

In the scientific literature, there are studies about the association between p53, USP1, and WDR48 during the progression and establishment of several diseases [29, 38]. However, there are no reports about the expression of these proteins in Oral Squamous Cell Carcinoma (OSCC) [22], which represents more than 90% of malignant neoplasms in the oral cavity and oropharynx [1]. Based on this information, the objective of this study was to evaluate the immunoexpression of p53, USP1, and WDR48 in OSCC and compare these expressions with histopathological characteristics.

Material and methods

Sample selection

This study was approved by the Ethical Committee (protocol number 1.049.451) and performed following the Declaration of Helsinki. Thirty cases of OSCC were obtained from the Oral

Pathology Laboratory at the Federal University of Santa Catarina after sample calculation using Pearson's Chi-square. Forty non-neoplastic oral epithelium (NNOE) samples were used as the control group.

Histological malignancy grading

An expert observer (pathologist with more than 10 years of experience) analyzed all sections stained with hematoxylin and eosin (H&E) to confirm the diagnosis of OSCC and perform the histological malignancy grading. The professional was calibrated and an intra-examiner Kappa value higher than 0.7 was obtained. OSCC cases were classified as well-differentiated (WD), moderately-differentiated (MD) and poorly-differentiated (PD) according to Bryne *et al.* [3].

All cases were from different patients and none of them had received preoperative treatments, such as radiotherapy or chemotherapy. Information such as sex, age, ethnicity, deleterious habits and site of the lesion were collected from the records.

Immunohistochemistry

Immunohistochemical reactions were performed using a standard streptavidin-biotin-peroxidase protocol. After endogenous peroxidase activity blocking, antigen retrieval, and blocking of non-specific binding sites, the specimens were incubated overnight with anti-phospho-p53 antibody (AB76242, Abcam, Cambridge, United Kingdom, 1:400 dilution), anti-USP1 (AB84772, Abcam, Cambridge, United Kingdom, 1:200 dilution) and anti-WDR48 (HPA038421, Sigma Aldrich, St. Louis, Missouri, United States of America, 1:300 dilution). The reactions were revealed with streptavidin-biotin complex (Kit LSAB Peroxidase K0690 - DAKO Corporation, Carpinteria, CA, USA) followed by diaminobenzidine DAKO Liquid plus (DAB, Dako Corporation, Carpinteria, CA, USA) and stained with Harris hematoxylin. Brain, Placenta and Colon specimens were used to detected positive immunoreactivity for p53, USP1, and WDR48, respectively. Negative control for each reaction was treated in the same way, but omitting the primary antibody.

Immunohistochemical analyses

Although some cases showed cytoplasmic and nuclear expression, p53 immunoreactivity was evaluated only in the nucleus, since this is the site where its main functions are performed. USP1 and WDR48 expressions were evaluated in both sites, cytoplasm, and nucleus. Immunoreactivity was quantified by a previously calibrated single investigator, with an intraclass correlation coefficient of 0.88 (calibration performed with 2 examinations of 50 fields, at an interval of 1 week).

For each sample, a minimum of 500 cells were counted with ImageJ version 1.41 (National Institute of Health, Bethesda, Maryland, USA) in five fields, at 400X magnification, equidistantly captured with a camera (Canon A620, Beijing, China) attached to a light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany).

Positive and negative cells (unstained cells) were counted for each antibody. Then, immunopositivity to each sample was expressed as the percentage of positive cells over the total number of counted cells. OSCC cases were considered overexpressed when the positive percentage of each protein exceeded the mean of expression obtained in NNOE.

Statistical analysis

Data were analyzed by IBM SPSS Statistics 21.0 software (International Business Machines Corporation, United States of America). Nonparametric statistical tests were used because of the non-Gaussian distribution of data. Immunopositivity for each protein was compared between each group and associated with histopathological features using the Kruskal-Wallis test. Statistical differences were defined as $P<0.05$.

Results

Clinical information

OSCC samples were obtained from 30 patients, 90% male and 10% female, with an overall mean age of 59.8 ± 11.32 (mean $\pm$ standard deviation) years at diagnosis. Regarding NNOE (n=40), 57.5% were female and 42.5% were male with a mean age of 51.23 ± 15.25 years (mean $\pm$ standard deviation).

Smoking and alcoholism were habits frequently related in OSCC (n=26 and n=20, respectively) However, these characteristics were uncommon in NNOE (n=5 and n=1 respectively). The most prevalent sites reported were the floor of the mouth and tongue to malignant neoplasia and inner cheek and lip mucosa for NNOE samples.

Immunohistochemical features

In most specimens, there was stronger immunopositivity in OSCC than in NNOE (figure 1).

P53 was overexpressed in 65% of the OSCC cases; this percentage of positive nuclei was statistically higher than in NNOE (35.1%). In opposition, 35% and 64.9% of the cells were unmarked in OSCC and NNOE, respectively. In NNOE, this expression was commonly found in the middle third of the lining epithelium. Few cells were stained in the basal layer and upper third (table I).

In general, 96.4% of all OSCC cells presented positive staining of USP1, compared to 85.0% of NNOE cells. Specifically, in the cytoplasm of OSCC cells (46.3%), the expression of USP1 was significantly higher than in non-malignant cells (32.0%) and, consequently, the percentage of negative cells was lower in OSCC (3.3%) than in NNOE (14.8%). This expression was diffuse in all cells from OSCC (nucleus and cytoplasm) and commonly found in the middle third and basal layer in NNOE (table I).

Regarding WDR48, 68.9% of all OSCC cells were stained, compared to 65.2% of all NNOE cells. This protein exhibited predominant cytoplasmic staining, for this reason; the number of positive nuclei was low. In OSCC, WDR48 immunostaining occurred in all neoplastic epithelial islands, while in NNOE this expression was predominant in the epithelial lower third (table I).

Moreover, it is important to highlight that USP1 has its activity potentiated when bound to WDR48, by the formation of a complex. Based on this information, the levels of immunostaining of USP1 and WDR48 were compared with each other in the same group (OSCC or NNOE). However, there was no similarity (p=0.0000). In general, WDR48 protein presented cytoplasmic expression.

On the other hand, the USP1 protein presented a significant expression when the two sites (nucleus and cytoplasm) were evaluated together.

A comparison of immunoreactivity between these histological gradings showed that PD cases exhibited higher nuclear immunostaining of all proteins when compared with WD samples (table II); however, there was no statistically significant difference.
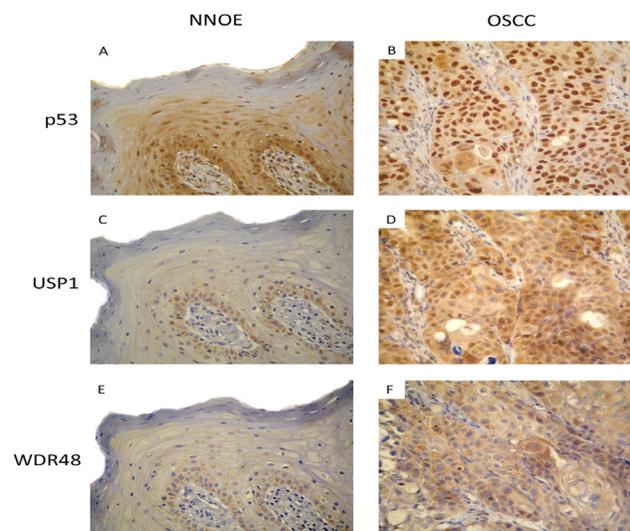
Figure 1 – Expression pattern of the biomarkers (p53, USP1, and WDR48) in oral squamous cell carcinoma (OSCC) and non-neoplastic oral epithelium (NNOE): A) NNOE showing positive nucleus to p53 at the middle and basal third of the epithelium; B) Strong nuclear p53 immunostaining in OSCC; C) Expression of USP1 in the middle third and basal layers of NNOE; D) Nuclear and cytoplasmic staining of USP1 in OSCC; E) Expression of WDR48 at the lower and middle third of NNOE; F) Nuclear and cytoplasmic expression of WDR48 in OSCC (LSAB, 400x)

Table I – Average and standard deviation for all antibodies in OSCC and NNOE

	P53		USP1				WDR48			
	N	**UC**	**N**	**C**	**N+C**	**UC**	**N**	**C**	**N+C**	**UC**
OSCC	65.0 ±21.8	35.0 ±21.8	0.8 ±2.9	46.3 ±21.0	49.3 ±19.3	3.3 ±5.9	2.6 ±8.7	64.5 ±33.8	1.8 ±5.7	30.9 ±30.3
NNOE	35.1 ±23.1	64.9 ±23.1	0.9 ±2.33	32.0 ±20.4	52.1 ±23.72	14.8 ±16.62	0.01 ±0.07	64.6 ±28.7	0.66 ±1.74	34.6 ±28.9
P-value	0.0000*	0.0000*	0.4440	0.0028*	0.4405	0.0000*	0.2596	0.6350	0.2216	0.3734

OSCC: oral squamous cell carcinoma; NNOE: non-neoplastic oral epithelium; N: positive nucleus; C: positive cytoplasm; N+C: positive nucleus and cytoplasm; UC: unmarked cells

Values are expressed as means ± standard deviation (%)

* Statistically significant data: P<0.05

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Table II – Average and standard deviation according to histological malignancy grading in OSCC

		wd OSCC (n=7)	md OSCC (n=19)	pd OSCC (n=4)	P-value
P53	N	65.8 ±29.4	62.5±20.7	75.1 ±11.6	0.4531
USP1	N	0.02±0.06	0.77± 3.34	2.5±3.0	0.4375
	C	56.2±14.4	46.7±22.0	27.5 ±14.8	0.0731
	N+C	41.5±12.7	49.5±20.6	62.4 ±18.9	0.2063
WDR48	N	5.60±14.83	0.60±1.55	7.3 ±14.6	0.5832
	C	54.7±41.0	68.5±32.5	62.4 ±31.6	0.6812
	N+C	4.69±11.0	1.0±2.9	0.70 ±0.81	0.8185

OSCC: oral squamous cell carcinoma; WD: well-differentiated OSCC; MD: moderately differentiated OSCC; PD: poorly differentiated OSCC

Values are expressed as means ± standard deviation (%).

* Statistically significant data: $P<0.05$

Discussion

P53, in normal conditions, is a tumor suppressor and prevents cancer formation. The stability of p53 is regulated by ubiquitin-dependent degradation. This regulation is critical for the function, degradation control, localization and activity of this protein [2]. Overexpression of mutated p53 suggests that several DUBs are interacting and deubiquitinating this protein [16]. For example, USP1 and WDR48 form a complex with p53 and control the level of ubiquitination during the response to stress signals [13]. Consequently, high levels of USP1/WDR48 expression can be connected to high levels of p53 [38].

In this study, the connection among p53, USP1, and WDR48 proteins is reinforced when the levels of expression are observed according to OSCC histological grading. Poorly differentiated OSCC presented higher expression of p53 and the USP1/WDR48 complex than well-differentiated samples. Corroborating with the literature, studies have shown that high-grade carcinomas presented more mutations on the TP53 gene than low-grade ones [18, 35]. In the same way, Dave *et al.* [10], using immunohistochemistry, found a significant association between poor differentiation and p53 overexpression. However, there are no reports in the literature on the association between WDR48/USP1 complex and tumor histological grading. This is the first study to describe this connection.

When neoplastic and non-neoplastic tissues are compared in this study, there was overexpression of p53 in OSCC. Confirming this result, Dave *et al.* [10] evaluated p53 staining in OSCC samples, and the results showed positive expression in 62% of the carcinomas. Lee *et al.* [18] found that 34 OSCC specimens were positive for mutated p53, whereas 23 specimens were negative. Also, Yang *et al.* [39] found positive p53 expression only in 3 of 16 normal oral epithelium samples, compared to 42 of 72 OSCC samples. It should be noted that only the nuclear expression of this protein was considered in the present study because its activity is directly related to the nucleus [16]. P53 is activated in response to cellular stresses, inducing the expression of genes involved in cell-cycle arrest, DNA repair or apoptosis [16].

Several methods described in the literature are able to measure the expression levels of USP1 and WDR48 proteins. The most relevant ones are immunohistochemical and other antibody-based assays that determine the levels of this complex in a cell or patient sample. Alternatively, molecular methods such as PCR, qPCR, Southern analysis, and Northern analysis also can be used to define the levels of the nucleic acid encoding these proteins [8].

D'Andrea [8] published a patent reporting the possible methods to identify the USP1 deubiquitinating enzyme. The author suggests that, in patients with a cancer diagnosis or increased susceptibility to develop malignant neoplasia, the level of USP1/WDR48 expression may be increased by at least 10% compared to control. Based on this information, it can be inferred that the present study found overexpression of USP1 since the amount of stained neoplastic cells was greater than 10% when compared to the control group (non-malignant cells). On the other hand, although the expression of WDR48 in OSCC had been higher than in the

control group, it was not expressive enough (only 3.7% higher) to be considered overexpression.

Furthermore, Williams *et al.* [38], using the immunohistochemical technique, found that osteosarcomas overexpressed USP1 when compared to healthy bone tissues. Liu *et al.* [23] also examined the expression of USP1 in 30 samples from patients with osteosarcoma. From the 30 samples, 26 were classified as a positive expression of this protein, while rare visible USP1 staining was detected in cartilage tumor tissue and normal bone tissues.

Liu *et al.* [24] detected high levels of USP1 in Non-Small Cell Lung Cancer by immunohistochemical analysis and found that suppression of USP1 downregulated the expression of many proteins associated with tumor genesis and development [23]. Also, Ma *et al.* [25] measured the presence of USP1 with RT-PCR in two cell lines (Saos2 and hFOB); consistently, Saos2 cells showed increased expression of USP1. In addition, using mRNA data and immunoblot analysis, Lee *et al.* [19] found that USP1 proteins were highly expressed in patients' glioma specimens compared to non-tumor brain tissues.

Regarding WDR48, the present study found only higher expression of this protein, but McClurg *et al.* [26] found overexpression in prostate cancer patients because staining was significantly higher when compared to benign controls from the prostate. Moreover, Park *et al.* [30] found that knockdown of WDR48 inhibits Ras-induced proliferation, tumorigenesis, control multiple cellular functions, including homologous-recombination repair.

Concerning the site of WDR48 expression, predominant cytoplasmic expression was found in this study, although this protein executes nuclear functions. On the other hand, the USP1 protein presented high expression in both sites (nucleus and cytoplasm). However, although different localizations had been observed, USP1 and WDR48 act together in the nucleus. According to Garcia-Santisteban *et al.* [14], USP1 and WDR48 form a complex in the cytoplasm that is subsequently translocated to the nucleus, where it exerts its function, through two nuclear localization signals (NLSs). This information justifies the results found in this study and corroborates the findings of other authors such as McClurg *et al.* [26]; who found significant cytoplasmic staining for WDR48 in prostate carcinoma, and Liu *et al.* [23]; who found predominance of USP1 in the nuclei and cytoplasm of osteosarcoma tissues.

The presence of this complex in the nucleus, in abnormal conditions, can be related to the cancer stem cell (CSC) hypothesis [38]. The neoplastic state of a stem cell initiates when the binding between the Basic-Helix-Loop-Helix transcription factor (bHLH) and DNA is restricted by heterodimerization with Inhibitor of DNA-binding (ID). IDs prevent cellular differentiation, promote growth and sustain tumor development. Nevertheless, USP1 is reported by the deubiquitination and stabilization of IDs. USP1 builds a complex with WDR48 and its activity is increased. For this reason, this complex plays an important role during the oncogenic process and is related to the CSC hypothesis, mainly by inhibition of differentiation and maintenance of a stemness state [15]. Consequently, the high expression of USP1/WDR48 complex, as identified in this study, can suggest a proliferative and undifferentiated cell pattern, compatible with the profile of a CSC.

Based on this perspective, this complex has been identified as a therapeutic possibility against neoplastic cells [15, 40]. Several studies have found that selective molecule inhibitors act on deubiquitinase complex USP1/WDR48, as well as Pimozide [4], ML323 [11, 12, 21], C527 [27] and GW7647 [4]. Alone, USP1 has low catalytic potential. Pharmacological inhibition of the complex promotes the degradation of the ID protein by ubiquitination. Without the blockade promoted by the ID protein, the proliferation of immature cells cannot occur and the cells continue the differentiation process [30].

As reported in this discussion, over the years, studies have identified the overexpression of this complex and its potential to be a histological biomarker in cancer diagnosis and prognosis [24]. Especially, scientific studies have been reporting that the USP1/WDR48 complex is a potential anti-cancer therapeutic target [15]. Despite the inherent limitations of immunohistochemistry, this is the first study that confirms the high immunoexpression of these proteins in OSCC and the association with histological malignancy grading.

Conclusion

In conclusion, this work allowed a better understanding of the expression and behavior of these proteins in OSCC, suggesting the involvement of Tumor Suppressor Protein p53 and Deubiquitinating Enzymes in the initial phases of carcinogenesis and correlation with histological malignancy grading.

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