Acta Odontologica Scandinavica, 2013; 71: 1513–1519

informa
healthcare

ORIGINAL ARTICLE

Oestrogens and androgen receptors in oral squamous cell carcinoma

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Abstract

Objective. To investigate the gender-related expressions of androgen (AR), estrogen alpha (ERα) and beta (ERβ) receptors and aromatase enzyme in oral squamous cell carcinomas (OSCC). ***Materials and methods.*** A total of 60 cases of OSCC (30 from males and 30 from females) were retrieved and submitted to immunohistochemistry. Also, steroid expression was studied in two OSCC cell lines using Western blotting and immunofluorescence. ***Results.*** Immunohistochemistry demonstrated that ERβ was expressed in almost 40% of the cases and AR in 26%. Aromatase enzyme and ERα were less commonly expressed. Only AR presented statistically significant differences between genders. Western blotting and immunofluorescence analysis demonstrated that ERβ was abundantly expressed in the nuclei of both cell lines and aromatase enzyme presented a cytoplasmic expression. ***Conclusion.*** The detection of steroid hormones, especially ERβ, can indicate a role of these proteins in the process of carcinogenesis of some OSCC. Further studies of the mechanisms involved may provide important biological information regarding therapeutic approaches.

Key Words: *oral squamous cell carcinoma, androgen, estrogen, aromatase*

Introduction

Oral squamous cell carcinoma (OSCC), the most common form of oral cancer, stands amongst the eight most common cancers in the world [1] and presents a great variability in incidence among countries [2,3]. In spite of all the research that has been done, OSCC mortality and morbidity rates have not improved consistently over all the years, and the overall survival rate remains largely unchanged [4]. The optimal treatment or therapy for OSCC remains a controversial issue, and surgery continues to be the treatment of choice. However, wide resections frequently cause significant esthetic and functional problems for the patient [5]. Therefore, the identification of proteins involved in the mechanisms of carcinogenesis can contribute to the development of new treatments.

The role of androgens and estrogens in several endocrine-related malignancies—such as breast, prostate, colon and ovaries—is well known [6–11]. The successful use of hormones antagonists in the treatment of these neoplasms has prompted researchers to investigate the presence of hormones receptors in other tissues and tumor types. In oral epithelium investigations have shown the expression of androgen, estrogens receptors and aromatase—the enzyme responsible for estrogen biosynthesis, suggesting a physiologic role of sex steroids hormones in oral keratinocytes [12–16].

In OSCC, neoplastic keratinocytes have shown the same expression of normal keratinocytes regarding estrogen beta receptors and aromatase [14–22]. Estrogen alpha receptors were not expressed in normal keratinocytes [12], but in OSCC it exhibits conflicting results among different cell lineages [17,18,22]. Regardless of the type of estrogen receptor, ER antagonist (tamoxifen) is shown to be effective in inhibiting the proliferation of OSCC cells and in inducing cells to apoptosis. This suggests that, in spite of the ER sub-type in different cell lines, the response to chemotherapeutic agents may be the same [17,18,22].

Towards AR there are few studies about its expression in OSCC [15,20], but the occurrence of OSCC, predominantly in men [1,3,23] besides noxious habits, could also be related to hormonal [15]. Based on this, in the present study, the gender-related immunohistochemical expressions of androgen receptor (AR),

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(Received 27 February 2012; accepted 1 February 2013)

ISSN 0001-6357 print/ISSN 1502-3850 online © 2013 Informa Healthcare
DOI: 10.3109/00016357.2013.775335

estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and aromatase enzyme were assessed in cases of OSSC. In addition, the presence of these proteins was investigated in two OSCC cell lines.

Materials and methods

The study was performed in accordance with the ethical principles of the World Medical Association Declaration of Helsinki and was independently reviewed and approved by an institutional ethical board (IRB).

Sixty cases from incisional biopsies of tongue OSSC (30 from males and 30 from females) were retrieved from the archives of the Oral Pathology Department of the University of São Paulo. Patients ages ranged from 45–65 years and none of them was under any kind of hormonal or anti-hormonal therapy, such anti-estrogen therapy. All original slides were reviewed in order to properly select representative tissue for immunohistochemical reactions. Fisher's exact test was used to evaluate the association between gender and the immunoexpression of AR, ERα, ERβ and aromatase enzyme. The chi-square test was used to compare significant differences of positivity between antibodies. A p-value under 0.05 was considered to be significant in all statistical analyses. The software used for statistical analysis was Microsoft Excel and Bio-Estat 3.0.

Immunohistochemical technique

Three-micrometre sections were obtained from the formalin-fixed, paraffin-embedded tissue and then mounted on silanized slides, deparaffinized and rehydrated. Antigen retrieval was performed using TRIS EDTA (pH 9.0) for 30 min at 95°C (for all antibodies). After that, the slides were incubated in a 6% hydrogen peroxide and methanol solution v/v to quench endogenous peroxidase activity (30 min at room temperature). After rinsing with water, sections were incubated in TBS, pH 7.4, for 15 min and then submitted to the primary monoclonal antibody: anti-AR (1:25, Dako, AR 441), anti-ERα (1:50, Dako, 1D5), anti-ERβ (1:50, Dako, EMRO2) and anti-aromatase enzyme (1:25, AbD Serotec, H4) for 120 min, followed by incubation with a polymeric-labelling two-step method (ADVANCE, DakoCytomation) for 30 min. Antibody complexes were revealed by a buffered diaminobenzidine substrate (Liquid DAB + Substrate chromogen system K3468, DakoCytomation) for 10 min. Sections were then counterstained with Mayer's haematoxylin for 10 min, de-hydrated in ethanol, cleared with xylene and mounted in xylene-based Permount (Fisher Scientific, Fair Lawn, NJ). The steps from primary antibody to counterstaining were performed in an autostainer (DakoCytomation), in which Tris-buffered saline Tween-20 (TBST) was used as buffer.

As positive controls, prostate adenocarcinoma was used for AR, placental tissue for aromatase enzyme and mammary adenocarcinoma (previously known as positive for each antibody) for ERα and ERβ. Negative controls were included in all reactions.

Immunohistochemical analysis

All sections were analyzed in a blind manner by two pathologists (LSM and SOMS) and were scored on the basis of visual analysis as positive and negative. Neoplastic cells were considered positive to anti-AR and anti-ER (alpha and beta) when they showed nuclear reactivity. For aromatase enzyme, immunoexpression was observed in the cytoplasm.

Cell culture

Two OSCC cell lines were used: SCC-9 and SCC-25 (ATCC, Manassas, VA) originated from the tongue. The cells were maintained in DMEM (Dulbelco's Modified Eagle's Medium/Sigma-Aldrich, St. Louis, MO) and F-12 (Nutrient Mixture Ham/Invitrogen Gibco Grand Island, NY) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C in a humidified atmosphere and 5% CO_2.

As controls, for AR, cell lysates of LNCap were kindly provided by Professor Hernandes Faustino de Carvalho (ICB – UNICAMP, Brazil); for ERα, cell lysates of MCF-7 and, for ERβ, cell lysates of MDA were kindly provided by Professor Ruy G. Jaeger (ICB-USP, Brazil). For aromatase enzyme, placental cells were extracted from fresh tissue.

Western-blotting

Cells were lysed with lysis buffer (50 mmol/L Tris–HCl, 1 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 1% DOC, 0.1% SDS) containing freshly added protease inhibitor cocktail (Sigma, St. Louis, MO) at 4°C for 20 min. The cells were scraped and the lysate was collected and cleared by centrifugation at 14 000 rpm for 20 min at 4°C. Protein concentrations of all samples were determined using the Pierce BCA method (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. Twenty-four micrograms of protein were loaded on polyacrylamide gels—10% for aromatase and beta estrogen; 12% for androgen receptor and 7.5% for alpha estrogen receptor—and then transferred to a PVDF membrane. After blocking in 5% non-fat dry milk or BSA, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-androgen receptor at 1:100 (Dako, AR 441), anti-alpha estrogen receptor at 1:100 (Dako; 1D5), anti-beta estrogen receptor at 1:100 (Dako, EMRO2), anti-aromatase at 1:100 (AbD Serotec, H4) and anti-

Table I. Expression of hormone receptors and aromatase in OSCC.

	AR	Aromatase	ERα	ERβ	*p*
Positive %	16 (26.6)	8 (13.3)	5 (8.3)	23 (38.3)	< 0.001
Negative %	44 (73.4)	52 (86.4)	55 (91.4)	37 (61.4)	
Total	60 (100.0)	60 (100.0)	60 (100.0)	60 (100.0)	

beta-actin at 1:6000 (Sigma-Aldrich, St. Louis, MO); and for 1 h at room temperature with a peroxidase-conjugated secondary antibody (IgG-HRP, Santa Cruz Biotechnology, Santa Cruz, CA). A bound antibody was detected by a colorimetric method using the Opti 4CN kit (Bio-rad Laboratories, Hercules, CA). Beta-actin was used to control the total volume of each sample.

Immunofluorescence

The cells were seeded over coverslips, grew until subconfluence, and were fixed in cooled absolute methanol at −20°C for 6 min. Briefly, cells were incubated with blocking solution (1% bovine serum albumin) for 30 min and followed by incubation with antibodies to AR (1:25, Dako, AR 441), ERα (1:50, Dako, 1D5), ERβ (1:50, Dako, EMRO2) and aromatase enzyme (1:50, Santa. Cruz Biotechnology, H4) diluted in PBS/1% BSA for 90 min at room temperature in a humidified chamber. Next, the cells were washed in PBS (Phosphate Buffered Saline) and incubated with a FITC conjugated antibody (Vector Laboratories, Ind., Burlingame, CA) for 45 min in the dark. After PBS washing, the coverslips were mounted using mounting media containing DAPI (Vectashield: DAPI, Vector Laboratories, Ind.) and photographed with a Zeiss Axio Imager A1 microscope (Carl Zeiss, Germany).

Results

Immunohistochemical staining demonstrated that AR and ERβ were more frequently expressed in OSCC than aromatase and ERα receptors (Table I).

ERβ was the most expressed in both genders among the studied steroids: 11 cases from men (36.6%) and 12 from women (40%). No statistical difference was seen between genders (Table II, Figure 1D). AR was expressed in four (13.3%) cases of women and 12 (40%) cases of men, showing statistically significant differences ($p = 0.023$) (Table II, Figure 1A). A comparison through the chi-squared test between the two more commonly expressed receptors (AR and ERβ) presented no statistically significant difference ($p = 0.256$).

Anti-aromatase enzyme was immunoexpressed in the cytoplasm of eight cases of OSCC, six (20%) from women and two from men (6.7%) (Table I, Figure 1B). Between the three steroid hormones receptors studied, ERα had the lowest expression, with three (10%) in women and two (6.7%) in men (Tables I and II, Figure 1C). In both, a comparison between sexes showed no statistically significant difference (Table II).

Regarding AR, results of Western blotting were quite similar to the immunohistochemical findings (Figure 2). Moreover, immunofluorescence showed nuclear localization of this protein only in the SCC9 cell line (Figure 3). Anti-aromatase enzyme was observed in the cytoplasm and nuclei of both cell lines in the immunofluorescence and also expressed in Western blotting (Figures 2 and 3). On the other hand, ERα was not observed either in Western blotting analysis or in the immunofluorescence technique (Figures 2 and 3). In agreement with the immunohistochemical results, Western blot analysis demonstrated that ERβ was abundantly present in SCC9 and SCC25 cell lines, and immunofluorescence confirmed nuclear localization of this receptor (Figures 2 and 3).

Discussion

Although oral mucosa is a non-classical target tissue for sex steroid hormones, they appear to play a significant role in its physiology, mainly estrogen [12–16,22].

Table II. Hormonal expression and gender.

	Female	%	Male	%	*p*
AR					
Positive	4	13.3	12	40	
Negative	26	86.7	18	60	0.023
Total	30	100	30	100	
Aromatase					
Positive	6	20	2	6.7	
Negative	24	80	28	93.3	0.153
Total	30	100	30	100	
ERα					
Positive	3	10	2	6.7	
Negative	27	90	28	93.3	0.676
Total	30	100	30	100	
ERβ					
Positive	12	40	11	36.7	
Negative	18	60	19	63.3	0.798
Total	30	100	30	100	

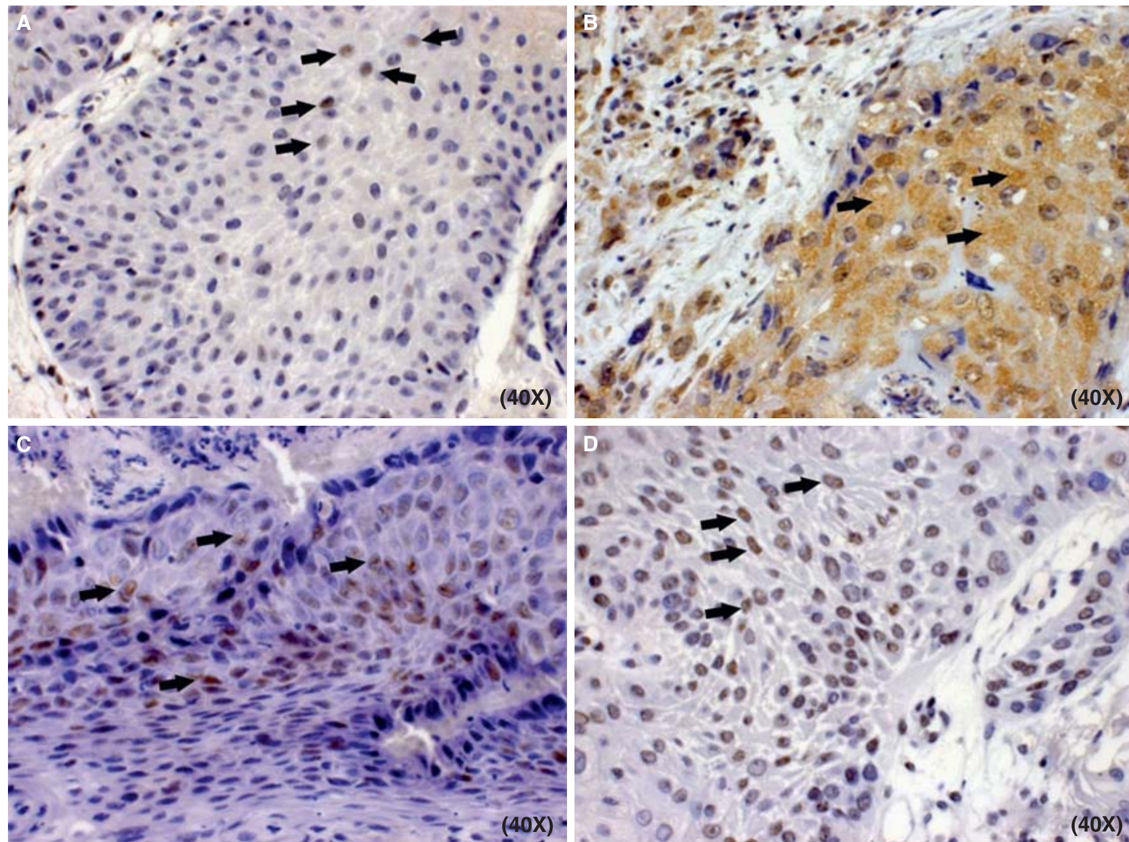

Figure 1. Immunohistochemical staining. (A) Nuclear expression of AR in neoplastic cells, (B) Cytoplasmic expression of aromatase enzyme, (C) ERα in a nuclear localization, and (D) Nuclear expression of ERβ. Streptavidin-biotin.

So, previous studies have studied the expression of steroid hormones in OSCC, presenting variable results [14–22]. This fact can be explained by the use of different clones of antibodies, as well as by the different mechanisms of antigen retrieval in immunohistochemistry [20,24,25].

In the present study, the expressions of some steroid hormones (estrogens and androgen) and also aromatase enzyme were evaluated in OSCC from men and women and in OSCC cell lines. A possible relation between hormonal expression and patient gender was investigated, making this the first investigation of gender-related steroid expressions in OSCC.

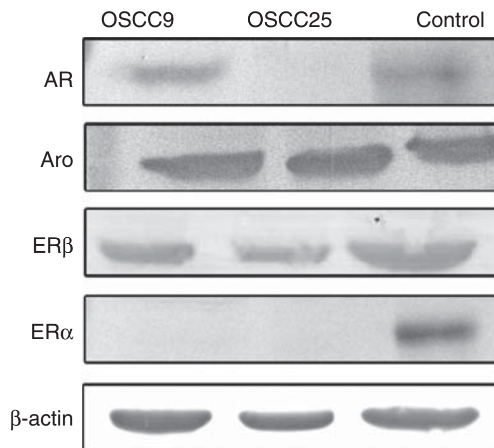

Figure 2. Western blotting results AR in SSC 9 and lack of AR expression in SCC 25. Aromatase enzyme in both cell lines (Aro). Presence of ERβ in SCC9 and SCC 25. Negative reaction to ERα, in both cell lines. Beta actin control.

Independent of gender, among the studied proteins, ERβ was the most commonly expressed, being present in 23/60 cases (40% of the cases in females and 36.7% of the cases in males). Both cell lines also expressed ERβ, as confirmed by immunofluorescence and by Western blotting. AR was also expressed by a considerable number of cases (40% of the men and 13.3% of the women), with a statistically significant difference between men and women.

The expression of estrogens in OSCC has been studied by immunohistochemistry and also in cell lines [17–19,21,22]. Through different techniques, other studies have shown the presence of ERβ in OSCC cell lines [17,18,22]. Although assessing only ERα receptors, Ku and Crowe [20] observed a light expression of this receptor in three cell lines: OSCC4, OSCC 9 and OSCC 25, two of which are the same lineages used in the present study. However, the total amount of protein lysates used in their research was almost 3-times greater than that used in this study. In other types of OSCC cells, Lukits et al. [23] demonstrated that ERα was expressed more often than ERβ. This finding reinforces the idea

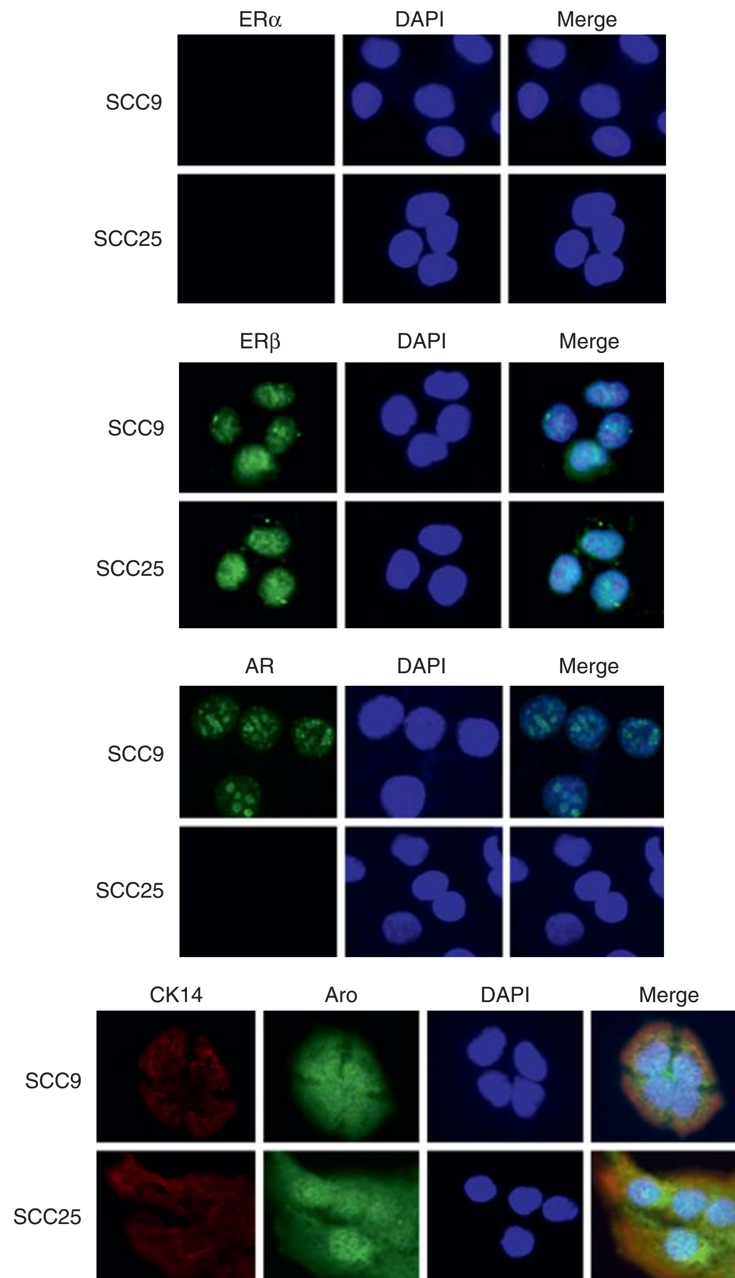

Figure 3. Negativity of both cell lines to ERα. Notice nuclear expression of ERβ in both cell lines. AR is positive only in SCC-9. Cytoplasmic expression of aromatase in both cell lines.

that different cell lineages could have different levels of ER isoforms. Ultimately, as the present study shows, a different expression of ER (β or α) both in the tissue and in OSCC cell lines when compared with that found in normal oral keratinocytes [12,14,18,22], suggests that this receptor is likely to play a role in tumor development or progression.

In some studies already mentioned above [17–19], regardless of the type of estrogen receptor and the OSCC cell line, ER antagonist (tamoxifen) was shown to be effective in inhibiting the proliferation of OSCC cells and in inducing cells to apoptosis. This suggests that, in spite of the ER sub-type in different cell lines, the response to chemotherapeutic agents may be the same. On the other side, recent studies have shown that, in breast and prostate cancer, while ERα promotes cell proliferation, ERβ is protective against carcinoma progression into an invasive state [11,25–29]. A recent study of laryngeal carcinomas showed that the expression of ERβ correlated positively with the maintenance of cell junctions and negatively with increased TNM stage. The authors suggest that ERβ could protect tumor cells from acquiring aggressive epithelial-mesenchymal transition features [30]. Future studies relating ER status to the clinical behavior of OSCC can clarify these points.

A statistically significant expression of AR in men (40%) in comparison to women (13%) was found in

the present study. In cell lines, only SCC9 expressed AR in the nuclei (Figures 2, 3A and B). Nehse and Tunn [21] using a different laboratorial method, found positivity to AR in 13 out of 18 OSCC cases. An interesting observation of these authors was that the concentration of these receptors was significantly lower in the tumor when compared to normal oral mucosa, suggesting that malignant transformation could be associated with a decrease in concentration of AR. Nonetheless, the possible effect of hormonal therapy in the positive cases remains to be explored. Although studying only cases from males, Dos Santos et al. [15] suggested that more than 12 polymorphisms found in the CAG sequence in the first exon of the AR gene could be associated with an increased risk to manifest an OSCC.

The determination of steroids expression in breast and prostate cancer is currently a requirement for the treatment of these tumors [11]. However, some controversies are still present regarding scoring systems for the evaluation of staining. Some systems consider, besides the amount of positive cells, the intensity of expression as well. ER status is usually considered positive in a range that varies from 1–10% of positive cells [31,32]. According to the recommendations of Hammond et al. [32] and considering the aim of this study—to demonstrate the presence or absence of these proteins in initial OSCC lesion—tumors presenting 1% or more positive cells were considered positive, regardless of staining intensity.

Aromatase is the enzyme that catalyzes the conversion of androgen to estrogen [34]. Cheng et al. [16] reported aromatase positivity in non-neoplastic keratinocytes and 6/14 cases of OSCC, all of which were well-differentiated positive cases. They found no aromatase expression in the poorly differentiated tumors, suggesting that aromatase expression in oral SCC may be related to the differentiation of the tumor. In our study, it was expressed in 13% of the cases studied, with an insignificant statistical difference between male and female cases. Although we did not classify the cases studied by degree of differentiation, most of them were poorly differentiated—at least in the areas analyzed. This could be a reason for the few cases positive to aromatase. No relation between aromatase and estrogen (alpha or beta) positive cases was found. However, as the expression of aromatase enzyme indicates local estrogen synthesis, the cases studied expressed more estrogen than aromatase. The cytoplasmic and nuclear expression, as seen in both immunohistochemistry and immunofluorescence, is explained by its localization in the endoplasmic reticulum [33].

In conclusion, the expressive number of cases expressing ERβ and AR, aromatase expression in poorly differentiated cases and the variable expression of ERα, justifies further studies that could reveal the importance of these hormones in the positive cases, either as a drug target or as an indicator of prognosis to OSCC.

Acknowledgments

We are thankful to Drs Fernando Augusto Soares, Ruy G. Jaeger, Vanessa M. Freitas and Hernandes F. Carvalho for providing the positive controls and CAPES for financial support.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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