Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Evaluation of specific modified histones in lip carcinogenesis

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ARTICLE INFO

Keywords:
Actinic cheilitis
Squamous cell carcinoma
Lip cancer
Histone modifications
Epigenetics

ABSTRACT

Objective: Histones regulate chromatin density and therefore influence gene expression and cellular proliferation. These properties are modified by methylation, acetylation and phosphorylation of histones. The aim of this study was to investigate the variation of specific modified histones in actinic cheilitis (AC) and squamous cell carcinoma of the lip (SCCL).

Methods: Samples of non-neoplastic tissue of the lip (NNTL, n = 9), AC (n = 33), and SCCL (n = 27) were submitted to immunohistochemistry to detect the modified histones H3K36me3, H3K9ac, H4K12ac, and H3S10 ph.

Results: Reactivity for all of the modified histones was significantly decreased from NNTL to AC, but not from AC to SCCL. Dysplasia in AC or histological grade in SCCL were not related to the reactivity of any modified histones.

Conclusions: Histone modifications are related to initial actinic damage, but not to malignant transformation in the lip.

1. Introduction

Squamous cell carcinoma of the lip (SCCL) is one of the most common cancers of the head and neck [1]. It is a curable disease when diagnosed at initial stages, but reconstruction of lip defects ensued from oncologic treatment can be challenging. In addition, advanced SCCL is as aggressive as intraoral carcinomas, with poor survival rates [2]. Many SCCL are preceded by actinic cheilitis (AC) [3]. However, there is no accurate information about the prevalence of malignant transformation of actinic cheilitis, but it is known that the presence and intensity of epithelial dysplasia increases the chance of progression to lip cancer [4]. SCCL and AC are strongly related to chronic exposure to ultraviolet from sunlight, which triggers genetic and epigenetic changes in the keratinocytes [5].

Histone modifications are epigenetic changes since it does not alter the sequence of the DNA molecule, but interfere in the gene expression [6]. Histones are the main proteinaceous component of chromatin in the nucleus of eukaryotic cells, and constitute the nucleosomes altogether with DNA [7]. Acetylation, methylation and phosphorylation of histones interfere with chromatin packaging and accessibility of transcription factors to DNA, therefore exerting a critical epigenetic influence on gene expression [7]. Histone modifications have been associated with several biological events, with special mention to cellular proliferation [8–10]. In fact, methylation and acetylation in histone lysine amino acids have been extensively studied in different neoplasms [7,11,12]. Enzymes able to modify histones are overexpressed in AC compared to normal epithelium and in SCCL compared to AC [12,13]. However, the expression and influence of specific histone modifications are still unclear in lip carcinogenesis [14].

The present study aimed to evaluate the immunohistochemical reactivity for H3K36me3, H3K9ac, H4K12ac and H3S10ph in normal lip, AC and SCCL, looking for patterns related to lip carcinogenesis.

2. Material and methods

This study was previously reviewed and approved by the Institutional Ethical Reviewer Board of the Federal University of Uberlândia (CAAE #33410014.4.0000.5152)

Samples of AC (n = 33) and SCCL (n = 27), diagnosed and graded according to the criteria defined by the World Health Organization

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https://doi.org/10.1016/j.prp.2018.04.004
Received 13 February 2018; Received in revised form 12 April 2018; Accepted 12 April 2018

[15], were retrieved from the archives of the Oral Pathology Laboratory at the Federal University of Uberlândia (Uberlândia, Brazil). Samples of normal epithelial lining of the lip ($n = 9$ cases) were obtained from labial varices of without evidence of actinic aggression or inflammation. All of the lesions were located in the vermilion border of the lower lip.

Each case was then submitted to immunohistochemistry to detect H3K36me3, H3K9ac, H4K12ac and H3S10ph, with the streptavidin-peroxidase technique. Briefly, it was performed in formalin-fixed, paraffin-embedded 3 µm tissue sections. Antigen retrieval was performed with EDTA solution (1 mM, pH 8.0) in a pressurized chamber (Decloaking Chamber NxGen), for 15 min at 110 °C and 8PSI. Tissue sections were then incubated with the primary monoclonal antibodies against H3K36me3 (clone ab9050, titration 1:10000), H3K9ac (ab10812, 1:2000), H4K12ac (ab61238, 1:10000), or H3S10ph (ab5176, 1:500), all purchased from Abcam (Cambridge, UK), at room temperature for 2 h. Amplification was performed with streptavidin-biotin-peroxidase (Starr Trek, Biocare Medical, Concord, CA, USA) and staining was developed with diaminobenzidine (Biocare Medical, Concord, CA, USA), followed by hematoxylin counterstaining. Negative controls received the same treatment, but by omitting the primary antibody from the reaction. The epithelium of the oral mucosa was used as positive controls, given the information provided by the Protein Atlas database (www.proteinatlas.com).

Immunohistochemical staining was independently evaluated by two trained observers, looking for nuclear reactivity in the cells representative of each condition (normal, potentially malignant lesion and neoplastic keratinocytes). It was performed in five high-power microscopic fields at the most evident area of staining, by means of the Quickscore method [16]. This score corresponds to the product of values attributed to the staining intensity and proportion of positive cells, as follows: 0 (negative), 1 (weak intensity of staining), 2 (moderate) or 3 (strong); and 0 (negative), 1 (0–4% of reactive cells), 2 (5–19%), 3 (20–39%), 4 (40–59%), 5 (60–79%) or 6 (80–100%), respectively. The final score for each case was the highest value determined between the two observers except when the discrepancy was higher than one point—in this situation the case was evaluated again until consensus was reached.

Statistical analysis was performed with the GraphPad Prism software, version 6.03 (GraphPad Software, Inc. San Diego, USA). For each histone modification, the scores were compared among groups with the Kruskal-Wallis test. The median ranks of each histone were also used as cutoff values to compare the staining with clinicopathologic parameters with the Fisher exact test. In every instance, a significance level of 5% was considered to evaluate the difference between groups.

3. Results

Clinical and demographic information of the patients and histopathological classification of the samples are shown in Table 1.

Representative pictures of the immunohistochemical stainings are shown in Fig. 1. Staining was found in all of the epithelial layers, except for H3K9ac and H4K12ac in AC, in which staining was restricted to the basal and parabasal epithelial layers. As shown in Fig. 2, scores for all of the histones were significantly reduced from normal epithelium to AC, and from normal epithelium to SCCL for H3K36me3 and H2S10ph, but any significant variation was found from AC to SCCL. The grade of dysplasia in AC or grading of SCCL was not associated with the reactivity for any of the histones, as shown in Table 2. Reactivity for H3K9ac was associated with the sex of the patients with SCCL (Table 3).

4. Discussion

Epigenetic mechanisms can alter the expression of several genes related to the development of cancer [6,7]. In fact, histone modifications have been investigated in different types of cancer [11]. As in the present work, some of these previous studies compared neoplastic and normal tissues or precursor (potentially malignant) lesions by immunohistochemistry. For instance, modified histones are less common in malignant than benign renal tumors, without significant differences between benign and normal renal tissue [17]. On the contrary, the expression of specific modified histones was increased from normal to benign, and then to malignant colorectal tissues [18]. Modifications of histones are still poorly investigated in many human tumors, such as lip cancer.

UV radiation increases H3K9 acetylation rates [19] and H3K9ac is a modification associated with transcriptional activity [14]. Surprisingly, we observed that reactivity for H3K9ac was decreased from normal tissue to AC and SCCL. The histone acetylation process has been related to the carcinogenesis of different tumors [14] and correlated with prognostic factors [20–22]. The immunohistochemical expression of H3K9ac has been shown to be inversely correlated with recurrence and distant metastases in patients with non-small cell lung carcinoma [21]. The low level of H3K9ac was associated with oral squamous cell carcinoma malignization, lymph node progression, clinical stage, the degree of differentiation and poor prognosis [20,22]. We were unable to show evidence of similar data in our sample.

The study of histone modifications in oral cancer and potentially malignant oral lesions is still recent. Levels of H3K4ac, H3K9ac, H3K18ac and H3K27me3, H4K16ac have been associated with development, progression and prognosis of intraoral squamous cell carcinoma [20,22,23]. Chen et al. [23] included eight cases of SCCL in a study on histone modifications with a large sample of oral squamous cell carcinomas but did not provide specific data or comments about the patterns of reactivity in carcinomas of the lips. It should be noted that there are variations of genetic and epigenetic characteristics according to tumor location and etiology [12]. The level of HDAC2 (a histone deacetylase) is higher in AC compared with SCCL [12], but to the best of our knowledge, there is no previous study regarding the evaluation of specific histone modifications in AC.

The present study shows that the reduction of specific histone modifications is related to the transition from normal tissue of the lip to AC, which in turn is considered an initial stage of lip carcinogenesis [24]. On the other side, any variation of modified histones was found

Table 1
Characteristics of study subjects.

	AC n (%)	SCCL n (%)	NNTL n(%)
Number of cases	33 (100.0)	27 (100.0)	9 (100.0)
Sex			
Male	26 (78.8)	23 (85.2)	3 (33.3)
Female	7 (21.2)	4 (14.8)	6 (66.7)
Age			
Mean ± SD	53.2 ± 13.8	59.7 ± 16.0	42.8 ± 16.2
Range	23–82	38–100	23–69
≤60 years	25 (75.8)	14 (51.9)	7 (77.8)
> 60 years	8 (24.2)	9 (33.3)	2 (22.2)
Unknown	0	4 (14.8)	0
Race			
White	20 (60.6)	17 (63.0)	5 (55.5)
Brown or indigenous	6 (18.2)	3 (11.1)	0
Unknown	7 (21.2)	7 (25.9)	4 (44.5)
AC dysplasia grade (WHO)			
Mild	25 (75.8)	–	–
Moderate	5 (15.1)	–	–
Severe	3 (9.1)	–	–
Histopathological grading (WHO)			
Well	–	16 (59.3)	–
Moderate	–	9 (33.3)	–
Poor	–	2 (7.4)	–

SCCL: Squamous cell carcinoma of the lip; AC: actinic cheilits; NNTL: non-neoplastic tissue of lip; SD: standard deviation.