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Correlation between cell cycle proteins and hMSH2 in actinic cheilitis and lip cancer

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Abstract This study aims to evaluate and verify the relationship between the immunoexpression of hMSH2, p53 and p21 in actinic cheilitis (AC) and lower lip squamous cell carcinoma (SCC) cases. Forty AC and 40 SCC cases were submitted to immunoperoxidase method and quantitatively analyzed. Expression was compared by Mann–Whitney test, Student *t* test or one-way ANOVA. To correlate the variables, Pearson's correlation coefficient was calculated. The expression of p53 and p21 showed no significant differences between histopathological grades of AC or lower lip SCC ($p > 0.05$). Immunoexpression of p53 was higher in SCC than in AC ($p < 0.001$), while p21 expression was more observed in AC when compared to SCC group ($p = 0.006$). The AC group revealed an inverse correlation between p53 and hMSH2 expression ($r = -0.30$, $p = 0.006$). Alterations in p53 and p21 expression suggest that these proteins are involved in lower lip carcinogenesis. Moreover, p53 and hMSH2 seem to be interrelated in early events of this process.

Keywords Actinic cheilitis · Squamous cell carcinoma · Lip carcinogenesis · Oral cancer

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Introduction

Actinic cheilitis (AC) is a potentially malignant disease that can display varied degrees of epithelial dysplasia and may progress to squamous cell carcinoma (SCC) of the lower lip [39, 42]. Both lesions have similar characteristics with skin lesions and also share the same main etiological agent, ultraviolet (UV) radiation, particularly the UVB [33]. The carcinogenesis process induced by UV, is still not fully elucidated, but it is known that exposure to endogenous and exogenous genotoxic agents, such as UV, can lead to oncogenic mutations resulting from perturbation of the cell cycle and damage to DNA repair systems [15, 30, 31].

Loss of genomic stability is orchestrated by an interaction between the repair system of badly paired bases genes (MMR—mismatch repair), tumor suppressor genes and oncogenes [14, 36]. The hMSH2 gene, the main component of the MMR repair system, has been extensively studied [12]. Inactivation of hMSH2 induces a decrease in the function of the mismatch repair and, therefore, leads to microsatellite instability (MSI). MSI induces the activation of oncogenes or inactivation of tumor suppressor genes, which can initiate cellular carcinogenesis [12, 26, 32]. Our group have described that low immunoexpression of hMSH2 is an early event in the carcinogenesis of lower lip cancer in a previous study [33]. We found a significantly higher expression of hMSH2 in ACs with absent/mild dysplasia than in the group with moderate or severe dysplasia. In the lower lip SCC cases, the comparison of positive-hMSH2 cells showed a slightly higher expression in low grade of malignancy than in the high-grade ones, but this result was not statistically significant. The comparison of hMSH2 immunoreactivity among different lesions revealed that the mean positive epithelial cells significantly

decreased, as the lesion was morphologically more aggressive. Therefore, the ACs classified as absent/mild dysplasia showed the highest mean of positive cells, the moderate/severe dysplasia group showed intermediate values and the SCCs exhibited the lowest means.

The relationship between MMR genes and function of the p53 tumor suppressor gene has been of interest since that p53 is essential in the protection of cell DNA damage induced by UVB radiation, by promoting cell cycle arrest or apoptosis of damaged cells [1, 12, 31, 37]. Activation of p53 induces the expression of several genes, including cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ gene (p21) [18, 19]. The DNA alkylating agents induce the phosphorylation and activation of p53, leading to an increase in p21 expression [6]. Thus, p21 may suppress tumors by its function in mediated p53-dependent G1 growth arrest [20]. Despite this, paradoxical oncogenic effects have been linked to p21, especially by its anti-apoptotic activity [27, 41].

The aim of the present study was to evaluate the epithelial immunoexpression of p53 and p21 proteins in AC and SCC of the lower lip and then analyze the relationships between these proteins and the previously reported hMSH2 expression in the same sample [33], to determine the participation of these proteins in different morphological stages of carcinogenesis lip.

Materials and methods

Tissue specimens

The study was approved by the Research Ethics Committee of UFRN (CEP/UFRN-028/2010). This retrospective study analyzed 40 cases of AC (male-to-female ratio = 3:1; mean age = 53.5 ± 17.04 years) and 40 cases of lower lip SCC (male-to-female ratio = 3:1; mean age = 61.28 ± 20.18 years), obtained from stored tissue blocks of the Laboratory of Oral Pathology, Department of Dentistry, Federal University of Rio Grande do Norte (UFRN), Natal, Rio Grande do Norte, and from the Scientific Diagnostic Center (SDC), Campina Grande, Paraíba, Brazil. The cases were intentionally selected on basis of the histopathological diagnosis. The diagnosis of AC or SCC of the lower lip was confirmed by reviewing the clinical and histopathological data in the patient records, as performed by Sarmento et al. [33].

Morphological study

For morphological analysis, 5-μm-thick sections were embedded in paraffin blocks, stained with hematoxylin/eosin, and examined by light microscopy. The cases of lower lip SCCs were classified as high and low grade of malignancy according to the histological grading system proposed by Bryne [4]. The 2005 morphological criteria of the World Health Organization (WHO) [3] were used to determine the degree of epithelial dysplasia in cases of AC. Twenty-two (55.0 %) of the 40 cases of lower lip SCC were classified as low grade of malignancy and 18 (45.0 %) as high grade. With respect to the degree of epithelial dysplasia in the 40 cases of AC, no dysplasia was observed in 16 (40.0 %), mild dysplasia in 10 (25.0 %), moderate dysplasia in 11 (27.5 %), and severe dysplasia in 3 (7.5 %).

Immunohistochemistry

Primary mouse monoclonal antibodies against p53 and p21 were used. For our previous study [33], the specimens of this sample were stained with monoclonal antibody against hMSH2. The specifications of the primary antibodies (clone, manufacturer, dilution, antigen retrieval and incubation) are shown in Table 1. After antigen retrieval, endogenous peroxidase was blocked with a 1:1 solution of methanol and 3 % hydrogen peroxide. Antibodies were detected by immunoperoxidase staining using a dextran polymer-based signal enhancement technique (ADVANCE™, Dako, Carpinteria, CA, USA). The reaction was developed with diaminobenzidine as chromogen. The sections were counterstained with Mayer's hematoxylin and mounted in Permount® (Fisher Scientific, Fair Lawn, NJ, USA). Negative controls consisted of the replacement of the primary antibodies with bovine serum albumin; and samples of normal oral mucosa and colon carcinoma with known positive reactivity were included as positive controls. A colon carcinoma specimen previously shown to be strongly positive for the antibodies analyzed was used as positive control. Only epithelial cells that exhibited brown nuclear immunoreactivity were considered to be positive, irrespective of the intensity of staining.

Quantification of immunostained cells

For the quantification of immunostained cells in AC, no distinction between the basal and suprabasal layers was made. According to Caldeira et al. [5], it is more relevant to consider the epithelium as a whole than to evaluate epithelial layers separately. In the cases of lower lip SCC, immunostaining was evaluated at the invasion front. Areas showing the highest immunostaining were analyzed in the two groups.

All specimens were examined under a light microscope (Olympus CH30, Olympus Co., Tokyo, Japan). First, the area with the largest number of immunostained cells was identified in the slide at 200× magnification. Next, an Infinity 1-3C camera (Lumenera Co., Ottawa, Canada)

Table 1 Primary antibody, clone, manufacturer, dilution, antigen retrieval and incubation

Primary antibody	Clone	Manufacturer	Dilution	Antigen retrieval	Incubation
Anti-hMSH2 [2]	G219-1129	Pharmingen (San Diego, CA, USA)	1:200	No antigen retrieval	Overnight (18 h)
Anti-p53	DO-7	Dako (Carpinteria, CA, USA)	1:400	Citrate, pH6.0, Pascal	30′
Anti-p21	4D10	Dako (Carpinteria, CA, USA)	1:50	Tris–EDTA, pH9.0, Pascal	60′

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coupled to a light microscope (Nikon Eclipse E200MV, Nikon, Tokyo, Japan) was used to photograph 10 consecutive fields of each specimen at 400× magnification. The captured images were processed using the Lumenera Infinity Analyze software, version 5.0.3. Epithelial cells exhibiting brown nuclear staining were considered to be positive. Positive and negative cells were counted in each field using the ImageJ program (ImageJ for Mac OS X, version 1.44, 64 bit Java) until a number of 1000 cells was reached. This approach permitted a more reliable comparison of the data since the number of cells was the same, irrespective of the lesion analyzed.

Statistical analysis

The data were analyzed using the SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA) and submitted to specific tests, adopting a level of significance of 5 %. Since p53 data showed a normal distribution, the Student *t* test was used to compare the mean immunoexpression of this protein. The p21 data showed a nonparametric distribution; thereby the Mann–Whitney test was used to compare the immunoexpression of this protein. Correlation between the levels of hMSH2, p53 and p21 expression was calculated using Pearson's and Spearman's correlation coefficient.

Results

p53 immunohistochemistry

All cases of AC and SCCs of the lower lip revealed positivity to p53. Comparison of the two types of lesion showed a significant higher p53 expression in the lower lip SCCs (561.33 ± 228.70), than in the AC cases (208.48 + 147.27) ($p < 0.001$; Table 2).

There was no significant difference in p53 expression between ACs with moderate/severe dysplasia (249.14 + 146.53) when compared with the absent/mild dysplasia group (186.58 + 145.76; Fig. 1a) ($p > 0.05$; Table 3). With respect to lower lip SCCs, no significant difference was observed between the mean number of p53-positive neoplastic epithelial cells between low-grade cases (Fig. 1b) when compared to high-grade SCCs ($p > 0.05$; Table 3).

Table 2 p53 immunoexpression and comparison between means of AC and SCC

Lesion	p53 immunoexpression		
	n	Mean ± SD	p^a
AC	40	216.85 ± 143.47	<0.001*
SCC	40	561.33 ± 228.70	

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AC actinic cheilitis, *SCC* squamous cell carcinoma, *SD* standard deviation

* Results are statistically significant

[a] Student *t* test

p21 immunohistochemistry

P21 staining was present in 29 (72.5 %) of the 40 AC specimens, with a pattern of nuclear expression mainly in the basal and suprabasal layers. When different lesions were compared, the AC cases showed a significant higher median immunoexpression of p21 than lower lip SCCs ($p < 0.05$) (Table 4).

Comparison of p21 expression between AC cases with different degrees of epithelial dysplasia showed higher expression of this protein in mild lesions (absent/mild dysplasia) (Fig. 1c) but this difference was not significant ($p > 0.05$) (Table 5). Analysis of lower lip SCC revealed 24 (60 %) positive cases, showing expression restricted to the nucleus of neoplastic cells. A larger median number of p21-positive neoplastic epithelial cells in low-grade cases (Fig. 1d) was observed when compared to high-grade SCCs ($p > 0.05$) (Table 5).

Correlation between the levels of hMSH2, p53 and p21 expression

Spearman's correlation coefficient was calculated to assess the possible correlations between the hMSH2 and p21, and between p53 and p21 expression. No correlation could be noted between hMSH2 and p21 ($r = 0.273$, $p = 0.08$) or p53 and p21 ($r = 0.015$, $p = 0.926$) in AC cases. In lower lip SCC, no significant correlation was found between hMSH2 and p21 ($r = -0.105$, $p = 0.517$) or p53 and p21 proteins ($r = 0.123$, $p = 0.450$).

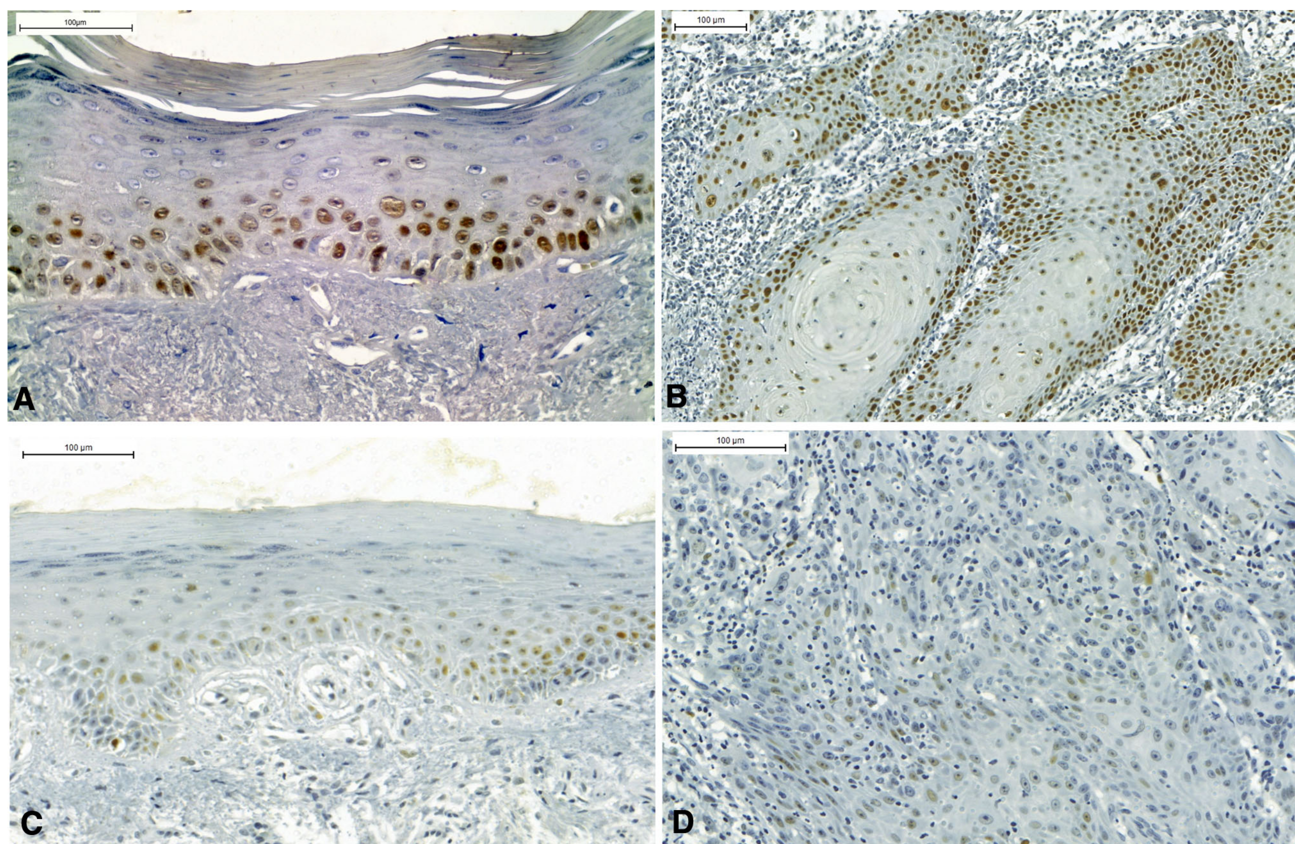

Fig. 1 Immunohistochemical expression of p53 and p21 in AC and SCC of the lower lip. **a** Immunoexpression of p53 in AC with mild dysplasia and **b** in SCC of the lower lip low with grade of malignancy; **c** immunoexpression of p21 in AC with mild dysplasia and **d** in SCC of the lower lip with low grade of malignancy (ADVANCE; *scale bar* 100 μm)

Table 3 p53 immunoexpression in AC and SCC and comparison of means between groups of each lesion

Lesion	Degree	p53 immunoexpression		p^a
		n	Mean ± SD	
AC	WD/MD	26	199.12 ± 141.38	0.293
	MOD/SED	14	249.79 ± 146.69	
SCC	Low grade	22	570.45 ± 239.15	0.784
	High grade	18	550.17 ± 221.59	

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AC actinic cheilitis, *SCC* squamous cell carcinoma, *WD* without dysplasia, *MD* mild dysplasia, *MOD* moderate dysplasia, *SED* severe dysplasia, *SD* standard deviation

[a] Student *t* test

Possible correlations between the expression of hMSH2 and p53 were investigated by the Pearson's correlation analysis. In AC cases, the level of hMSH2 expression was inversely correlated to the expression of p53 protein ($r = -0.30$, $p = 0.006$) (Fig. 2). In lower lip SCCs, p53 and hMSH2 showed no correlation ($r = -0.04$, $p = 0.777$).

Table 4 Sample, median, quartiles 25 and 75 of p21 immunoexpression between AC and SCC

Lesion	*n*	Median	Q_{25}–Q_{75}	Mean rank	p^a
AC	40	34.5	0–104.75	47.53	0.006*
SCC	40	4	0–26.5	33.48	

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AC actinic cheilitis, *SCC* squamous cell carcinoma

* Results are statistically significant

[a] Mann–Whitney test

Discussion

Among of the disorders that can undergo a process of malignant transformation there is the AC, which in some situations can turn into SCC of the lower lip [9, 21]. The rate for malignant transformation of these lesions ranges from 10 to 30 % [28] and solar radiation is the main etiological factor in its development and consequent progression to SCC [9, 28].

Genetic damage caused by ultraviolet radiation is usually repaired, especially via DNA repair [29]. Defective MMR

Table 5 Sample, median, quartiles 25 and 75 of p21 immunoexpression in AC and SCC and comparison of mean rank between groups of each lesion

Lesion	*n*	Median	Q_{25}–Q_{75}	Mean Rank	p^a
AC					
WD/MD	26	54.0	7–100.25	21.65	0.390
MOD/SED	14	7.0	0–138.25	18.36	
SCC					
Low grade	22	6.0	0–29.75	22.82	0.152
High grade	18	0	0–17.25	17.67	

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WD without dysplasia, *MD* mild dysplasia, *MOD* moderate dysplasia, *SED* severe dysplasia

[a] Mann–Whitney test

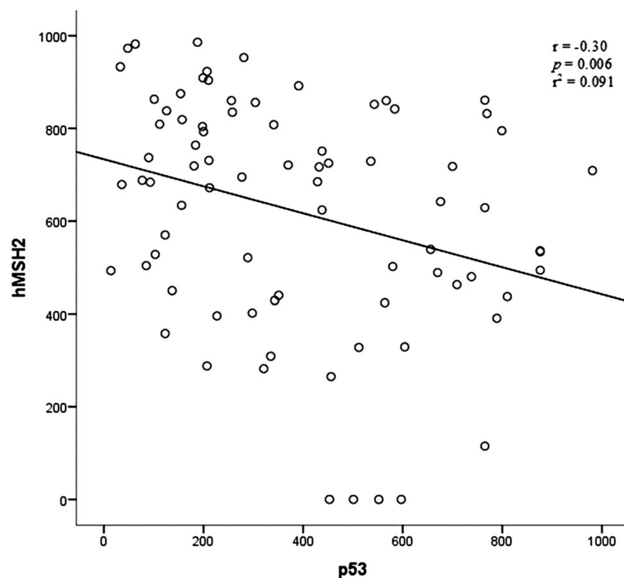

Fig. 2 Inverse correlation between p53 and hMSH2 expression in actinic cheilitis. The best linear fit and statistically significant correlation are identified in the *graph*

system is a very frequent genetic alteration in many malignancies, including squamous cell carcinoma, and recent studies have demonstrated these alterations in potentially malignant disorders, such as AC [33, 35]. In addition, changes in system MMR genes can cause disorders in cell cycle proteins such as p53 [22]. Based on this, we performed the immunohistochemical analysis of cell cycle proteins p53 and p21 in the same sample used in our previous study [33] to verify if there is any relationship between the alterations in hMSH2 expression and the aforementioned cell cycle proteins in different stages of lip carcinogenesis.

The p53 protein is essential in protecting the cells against DNA damage induced directly and indirectly by UV radiation [1, 12, 18]. Immunohistochemical expression of p53 is largely accepted as a marker of malignant transformation, and its overexpression has been associated with malignant potential in potentially malignant lesions of the head and neck [23, 24]. In the current study, p53 was present in AC, being slightly higher in AC with moderate/severe dysplasia group than in the no/mild dysplasia one. This finding confirms previous reports that demonstrated p53 expression in precancerous skin lesions and in chronically sun-exposed normal skin, suggesting that p53 mutation is an early event in skin carcinogenesis [7, 25, 43]. Therefore, according to Bäckvall et al. [2] and Mercuţ et al. [23] the expression of p53 oncoprotein in lesions localized on facial regions that were typically exposed to sunlight and these epidermal p53 clones seems to develop in skin areas chronically exposed to sunlight.

In SCC of the lower lip cases, the present results revealed that p53 expression was significantly higher in tumors presenting low grade of malignancy than in high-grade tumors, similar to other studies [12, 39]. Comparing the two studied lesions, we found a significantly greater expression of p53 in cases of SCC of the lower lip than in the AC ones. Therefore, our data showed that immunoreactivity for p53 protein was observed in both premalignant and malignant lesions of the lower lip. These data along with previous studies [13, 23] corroborate and emphasize that the overexpression of p53 protein is mainly present in the early stages of oral carcinogenesis process.

The p21 expression is induced through both p53-dependent and independent mechanisms [8, 10, 27]. Its importance in cell cycle arrest by inhibiting cyclin-dependent kinase, in apoptosis process and terminal differentiation of squamous epithelium has been described [12, 41]. Moreover, as one of its major functions, p21 is known to regulate differentiation in many different cell lineages [38]. The present results showed a lower immunostaining for p21 in AC with moderate/severe dysplasia than in the group with milder dysplasias; and the lower lip SCC group exhibited less positive cases than the AC group, the expression being more evident in SCCs low grade of malignancy.

The controversial prognostic potential of p21 may be associated with paradoxical functions performed by this protein. Even though p21 is best known for its cyclin-dependent kinase inhibitory function, this protein also can inhibit apoptosis, which might account for its oncogenic activities [20, 41]. Most of the studies suggested that p21 is present at the nucleus when considered as a tumor suppressor, and at the cytoplasm when considered as an oncogene [20]. The significant low nuclear expression of p21 in lip SCC cases when comparing to AC cases demonstrated here may reflect the tumor suppression effect of p21 protein. However, according to Tron et al. [38], p21

overexpression is associated with differentiation in proliferating cutaneous SCC but is not sufficient to suppress cancer development.

Even though the p21 deficiency reflects p53 wild-type inactivation, in AC and lower lip SCC studied, no correlation could be detected between p21 and p53 or hMSH2 expression. As p53 is abnormal or nonfunctional in most cases of cutaneous SCC, we hypothesized that expression of the downstream effector of p53 would be lost in these tumors [38]. Martínez et al. [21] did not find significant correlation between p21 and p53 in their AC sample and suggested that in earlier stages of lip carcinogenesis p53 could be inefficient to transactivate p21 expression.

There seems to be a close interaction between p53 and hMSH2 in carcinogenesis. Mutation in the hMSH2 gene reportedly could lead to activation of p53 mutation, without previous MSI, in sporadic digestive tract tumors [16]. Therefore, the relationship between p53 and hMSH2 has been investigated in several cancers [11, 12, 14, 17, 32, 36, 37, 40]. Helal et al. [12] found an inverse correlation between the level of expression of p53 and hMSH2 in oral SCC. Our data showed no correlation between these proteins in the lower lip SCC; however, an inverse correlation was observed in the AC cases. To our knowledge, this is the first report of such relationship in AC. Staibano et al. [37] emphasized the importance of this correlation in the UV-related carcinogenic process, as they found similar results in basal cell carcinomas. In fact, DNA damage promoted by UV irradiation has been shown to cause p53-dependent activation of human mismatch repair hMSH2 gene transcription in vitro, through functional interaction with c-Jun [34].

In summary, the present study supports the hypothesis that alterations in the immunoexpression of cell cycle proteins p53 and p21 are related to lip carcinogenesis. Additionally, hMSH2 and p53 proteins seem to be interrelated in early events of this process, but changes in p21 expression seem to be an independent event.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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