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Modification in CLIC4 Expression is Associated with P53, TGF-β, TNF-α and Myofibroblasts in Lip Carcinogenesis

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Chloride intracellular channel-4 (CLIC4) is regulated by p53 and tumor necrosis factor-α (TNF-α), it is linked to the increase of transforming growth factor-β (TGF-β), and myofibroblastic differentiation in skin carcinogenesis. This study analyzed the immunoexpression of CLIC4, p53, TGF-β, TNF-α, and α-SMA in 50 actinic cheilitis (AC) and 50 lower lip squamous cell carcinoma (LLSCC). AC and LLSCC immunoexpression were categorized as score 1 (<5% positive cells), 2 (5-50%) or 3 (>50%). For CLIC4, nuclear and cytoplasmic immunostaining of epithelial cells was considered individually. For morphologic analysis, the World Health Organization criteria were used to epithelial dysplasia grade of ACs, and Bryne grading of malignancy system was applied for LLSCC. Higher nuclear CLIC4 (CLIC4n) and TGF-β were observed in ACs with low-risk of transformation, while cytoplasmic CLIC4 (CLIC4c), p53 and TNF-α were higher in the high-risk cases ($p<0.05$). In LLSCCs, CLIC4c was higher in cases with lymph node metastasis, advanced clinical stages, and histological high-grade malignancy. p53 expression was higher in high-grade LLSCCs, whereas TGF-β decreased as the clinical stage and morphological grade progressed ($p<0.05$). ACs showed an increased expression of CLIC4n and TGF-β, while CLIC4c and α-SMA were higher in LLSCCs ($p<0.0001$). Both lesions showed negative correlation between CLIC4n and CLIC4c, while in LLSCCs, negative correlation was also verified between CLIC4c and p53, as well as CLIC4c and TGF-β ($p<0.05$). Change of CLIC4 from the nucleus to cytoplasm and alterations in p53, TGF-β, TNF-α, and α-SMA expression are involved in lip carcinogenesis.

Key Words: squamous cell carcinoma, carcinogenesis, uv radiation.

Introduction

Intracellular ion channels are involved in the pathogenesis of several malignancies (1,2). The chloride intracellular channel 4 (CLIC4) is one of the seven members of the chloride intracellular channel (CLIC) family of proteins (CLIC1-CLIC5, p64, and parchorina), which are expressed in diverse tissue types and participate in multiple biological process critical for cellular homeostasis (2,3).

CLIC4 (p64H1, RS43 or mtCLIC) is regulated by p53 and tumor necrosis factor-α (TNF-α) and is abundantly observed in the nuclei of normal keratinocytes, specially in the skin (4). In cells undergoing stress conditions, cytoplasmic CLIC4 translocates to the nucleus, where it acts as a tumor suppressor protein by induction of cell growth arrest via enhancement of the transforming growth factor (TGF-β) signaling, and by participation in p53-mediated apoptosis (4-6). In contrast, upregulation of CLIC4 in the mitochondria and the presence of its soluble form in the keratinocyte cytoplasm, has been associated to the tumor necrosis factor-alpha (TNF-α), which lead to cell apoptosis, a process mediated by p53 and eMyc (4).

In human malignant cells, CLIC4 is nearly or totally absent in the nucleus (7), with reports of direct relationship between the decreasing nuclear expression and the progression of cancer (6,7). Decrease or absence of CLIC4 protein in the tumor cells nucleus is thought to be associated with loss of beta-transforming growth factor (TGF-β) response and the occurrence of p53 gene mutations (5). On the other hand, CLIC4 is upregulated in tumors stroma, where it usually co-localizes with alpha smooth muscle actin (α-SMA) in a positive association with TGF-β-mediated myofibroblasts differentiation and epithelial-mesenchymal transition (8), both phenomena related to cancer progression (1,8).

Current knowledge indicates that changes in expression and subcellular localization of CLIC4 in early steps of cutaneous squamous cells carcinoma (SCC) tumorigenesis (6). Similar to cutaneous SCC, lower lip squamous cell carcinoma (LLSCC) follows a photo carcinogenesis model, in which chronic exposure to ultraviolet (UV) radiation is the main risk factor (9,10). Most LLSCC is preceded by a potentially malignant disorder known as actinic cheilitis (AC) (11); however, the precise mechanisms associated with progression from AC to LLSCC are not fully understood. In view of the CLIC4 functions in tumorigenesis, the present study aimed to investigate the participation of this protein

in lower lip carcinogenesis and its relationship with the expression of the p53, TNF-α, TGF-β and α-SMA.

Material and Methods

Tissue Specimens

The Ethics Committee of the Federal University of Rio Grande do Norte approved this study (Protocol No. 721.541). This retrospective study analyzed stored tissue blocks of AC (n=50; male to-female ratio=4:1; mean age=48.8+14.6 years) and LLSCCs (n=50; male to-female ratio=2.3:1; mean age=64.7+12.5 years) diagnosed between January 2009 and December 2012 at the at the Oral Pathology Service of the Federal University of Rio Grande do Norte (UFRN). Were included cases of LLSCC treated by surgical excision that allow the evaluation of the invasive tumor front and with the clinical files of the patients contain information regarding the clinical staging (TNM) of the lesions, outcome, and survival and lymph node metastasis. Cases of LLSCC that were previously submitted to radio or chemotherapy were excluded. The cases of AC with incomplete data on sex, age, occupation and clinical characteristics of the lesion were also excluded. The diagnosis of AC or LLSCC was confirmed by reviewing the clinical data of the patient records and histological evaluation. Clinical features were retrieved from patients' medical records.

Morphological Study

For morphologic evaluation, paraffin sections (5μm) were routinely prepared and stained with Hematoxylin & Eosin and evaluated under light microscopy. AC specimens were histologically graded as non-dysplastic, or with mild, moderate or severe epithelial dysplasia according to the World Health Organization (WHO) criteria (12) and adapted by Warnakulasuriya et al. (13). Then, AC cases with no dysplasia and mild dysplasia were grouped as low-risk lesions, and the remaining cases were grouped as high-risk lesions as suggested by Caldeira et al. (14). Histopathological grading of malignancy of LLSCC was performed as proposed by Bryne (15), and then classified as low-grade (4 to 8 total points) or high-grade of malignancy (>8 total points), as performed by Silveira et al. (16).

Immunohistochemistry

For immunohistochemical analysis, 3-μm thick tissue sections were mounted on organosilane-coated slides (3-aminopropyltriethoxysilane; Sigma Chemical Co., St. Louis, MO, USA). Deparaffinization, rehydration and antigen retrieval were performed using Trilogy (Cell Marque, CA, USA) diluted in distilled water (1:100) and heated in a Pascal pressure cooker. Endogenous peroxidase and nonspecific antibody reaction were blocked with 3% hydrogen peroxide and Protein Block (Thermo Scientific, Runcorn, UK), respectively. The tissues were incubated with primary antibodies anti-CLIC4 (EPR14253, Abcam, 1:4000, 60'), anti-p53 (DO7, DAKO, 1:400, 60'), anti-TGF-β (3C11, Santa Cruz, 1:500, 60'), anti-TNF-α (52B83, Santa Cruz, 1:400, overnight) and anti-α-SMA (1A4, DAKO, 1:800, 60'). Antibodies were detected using the HiDef DetectionTM HRP Polymer system (Cell Marque) and reaction was developed with diaminobenzidine as chromogen (DAB, Sigma Chemical, St Louis, MO, USA). The sections were counterstained with Mayer's hematoxylin and mounted in Permount® (Fisher Scientific, Fair Lawn, NJ, USA). Replacement of the primary antibodies with bovine serum albumin was used as negative control and human melanoma tissue served as positive control.

Analysis of Immunostained Cells

The slides were analyzed under light microscopy (Nikon Eclipse E20MV, Niko Co., Tokyo, Japan) by two previously calibrated examiners. Immunoexpression of CLIC4, p53, TGF-β, TNF-α and α-SMA in AC and at the invasive front of LLSCC was verified by light microscopy (100x and 400x). For all proteins, the expression was assessed at the epithelial component, with exception to α-SMA, which was evaluated in the connective tissue/stroma. Positive cells were regarded as cells showing brown staining in nucleus and/or cytoplasm. Kappa coefficient demonstrated a moderate to strong concordance among the examiners for the immunostaining evaluation of CLIC4 (0.758), TGF-β (0.705), TNF-α (0.629) and α-SMA (0.573). Semiquantitative analysis was performed for CLIC4, TGF-β and TNF-α, in which the following scores adapted from Piva et al. (17) were attributed: score 1 (low expression/negative; <5% positive cells), score 2 (moderate expression, 5-50% positive cells), or score 3 (high expression, >50% positive cells). For CLIC4 expression in AC and LLSCC, the subcellular localization of the protein was evaluated separately (nucleus or cytoplasm). In AC, the epithelial layer (basal and/or suprabasal) expressing CLIC4 was also evaluated. For p53 protein, cell counting of positive and negative cells was performed in 10 fields to obtain the labelling index by the quantitative method suggested by Rocha et al. (18) and for final analysis the values were divided into the scores applied to the other proteins.

Statistical Analysis

Data were analyzed in the Statistical Package for the Social Sciences (SPSS 24.0; Inc., Chicago, IL, USA) for descriptive analysis and statistical tests. To compare the proteins expression scores between the lesions, and between clinical and histological features, the non-parametric test U of Mann-Whitney was performed. Spearman's correlation coefficient was calculated to verify possible correlations

between the protein expression in the two lesions. The p-values of less than 0.05 were considered statistically significant.

Results

CLIC4 and Associated Proteins Expression in Actinic Cheilitis

Among the 50 AC, most cases (n=31; 62%) were classified as low-risk AC. Most ACs with low risk of transformation (n=25; 92.6%) presented with nuclear CLIC4 (CLIC4n) expression restricted to the epithelial basal layer, while in the high-risk cases (n=17; 73.9%), this protein was predominantly observed in all layers (p<0.0001). P53 was positive in the epithelial basal and suprabasal layers of most ACs (n=41; 82%). A moderate TGF-β expression was observed in the cytoplasm of keratinocytes of 28 (56%) cases, whereas TNF-α protein showed a high expression in 30 (60%) cases. Regarding α-SMA, it was poorly reactive in myofibroblastic cells and demonstrated a discontinuous expression at the lamina propria of 34 (68%) cases.

Comparing the scores of the proteins studied between low-risk and high-risk ACs, significant differences were observed for the expression of CLIC4n, cytoplasmic CLIC4 (CLIC4c), p53, TGF-β, TNF-α and α-SMA. In low-risk ACs, scores for CLIC4n and TGF-β were revealed to be significantly higher than in high-risk cases (p<0.0001; p<0.001, respectively). CLIC4c, p53, TNF-α and α-SMA expression enhanced significantly as the morphological risk of malignant transformation of the ACs increased (p<0.05; Table 1, Fig. 1).

CLIC4 and Associated Proteins Expression in Lower Lip Squamous Cells Carcinoma

The majority of the LLSCC patients (n=39; 78%) did not exhibit metastasis to the lymph nodes at the time of diagnosis. Clinical stage I or II (n=37;74%) were more frequent, while only 13 cases (26%) presented with advanced stages (III or IV). Morphological examination revealed that most LLSCC (n=28;56%) were low grade of malignancy tumors. The LLSCCs with lymph node metastasis, advanced clinical stages and a high degree of malignancy presented a significantly higher expression of CLIC4c (p=0.005, p=0.029, p=<0.0001, respectively). p53 revealed significantly higher ranks in the LLSCCs with high grade of malignancy than in the low-grade group (p=0.001). TGF-β expression significantly decreased at tumor parenchyma in tumors that presented more advanced clinical stage (p=0.042) or with a higher histological grade of malignancy (p=0.023). TNF-α and α-SMA were observed in most cases, but no significant differences were observed between the clinical and histological variables (Table 2, Fig. 2).

Comparison of CLIC4 and Associated Proteins Between Lesions

The expression of CLIC4n and TGF-β were significantly higher in ACs than LLSCCs (p<0.0001). LLSCCs showed a higher CLIC4c and α-SMA expression compared to ACs (p<0.0001). No significant differences were observed for p53 and TNF-α expression (Table 3).

Correlation between Proteins in AC and LLSCC

Analysis of AC cases revealed a weak negative correlation between CLIC4n and CLIC4c (r=0.554; p<0.0001). In LLSCC, the expression of CLIC4n was negatively correlated with CLIC4c (r=-0.378; p=0.007) and p53 (r=-0.317; p=0.025), while CLIC4c exhibited a negative correlation with TGF-β (r=-0.310; p=0.029). No significant correlation was observed between the other proteins in LLSCCs (Table 4).

Discussion

There is ample evidence demonstrating that solar UV induces human lip cancer and AC, an oral potentially malignant disorder. However, despite advances in the study of the complex photocarcinogenesis pathways, the mechanisms underlying the malignant transformation of AC remain obscure. To our knowledge, this is the first study to investigate the expression of CLIC4 in LLSCC and AC. Our findings reveal marked changes in the expression of CLIC4, and its subcellular localization, suggesting dynamic participation of this protein on lip carcinogenic microenvironment.

CLIC4 are one of the most active ion channels and participate in diverse biological functions, such as trans-

Table 1. Sample size, median, quartiles 25 and 75, m0ean rank and statistical significance for the proteins' expression in actinic cheilitis

Protein	Histological grade	n	Median	Q25-Q75	Mean rank	p†
CLIC4n	Low-risk High-risk	31 19	2 1	2-3 1-2	31.40 15.87	<0.0001*
CLIC4c	Low-risk High-risk	31 19	1 2	1-2 1-2	22.21 30.87	0.020*
p53	Low-risk High-risk	31 19	2 3	2-3 3-3	19.56 35.18	<0.0001*
TGF-β	Low-risk High-risk	31 19	2 1	2-3 1-2	30.29 17.68	0.001*
TNF-α	Low-risk High-risk	31 19	1 2	1-2 2-3	21.33 32.47	0.005*
α-SMA	Low-risk High-risk	31 19	1 2	1-1 1-2	19.92 34.61	0.0001*

CLIC4n, CLIC4 in the nucleus; CLIC4c, CLIC4 in the cytoplasm; CLIC4nc, CLIC4 in the nucleus and cytoplasm; † Mann-Whitney U test; *Result is statistically significant.

epithelial fluid transport, ion homeostasis, and cell pH and volume balance (2,7,19). Accumulating evidence has indicated that an abnormal CLIC activity occurs in the development of some human malignant neoplasms, particularly due to their importance in the cell proliferation, migration and apoptosis (7,19). In the present study, CLIC4 expression change pattern from the nucleus to the cytoplasm highlight its activity in lip carcinogenesis.

Several studies have investigated the expression of CLIC4 in cancer cells, highlighting the frequent loss or decline of CLIC4 in tumor cells of epithelial cancers, while its expression enhance in the tumor stroma (7,19). Moreover, changes in the subcellular localization of CLIC4 are thought to contribute to the malignant phenotype (7). In the present study, a pattern of expression emerged, revealing that CLIC4 in the nucleus of the keratinocytes decreased with increasing of the AC morphologic grade, while there was a higher expression in the cell's cytoplasm

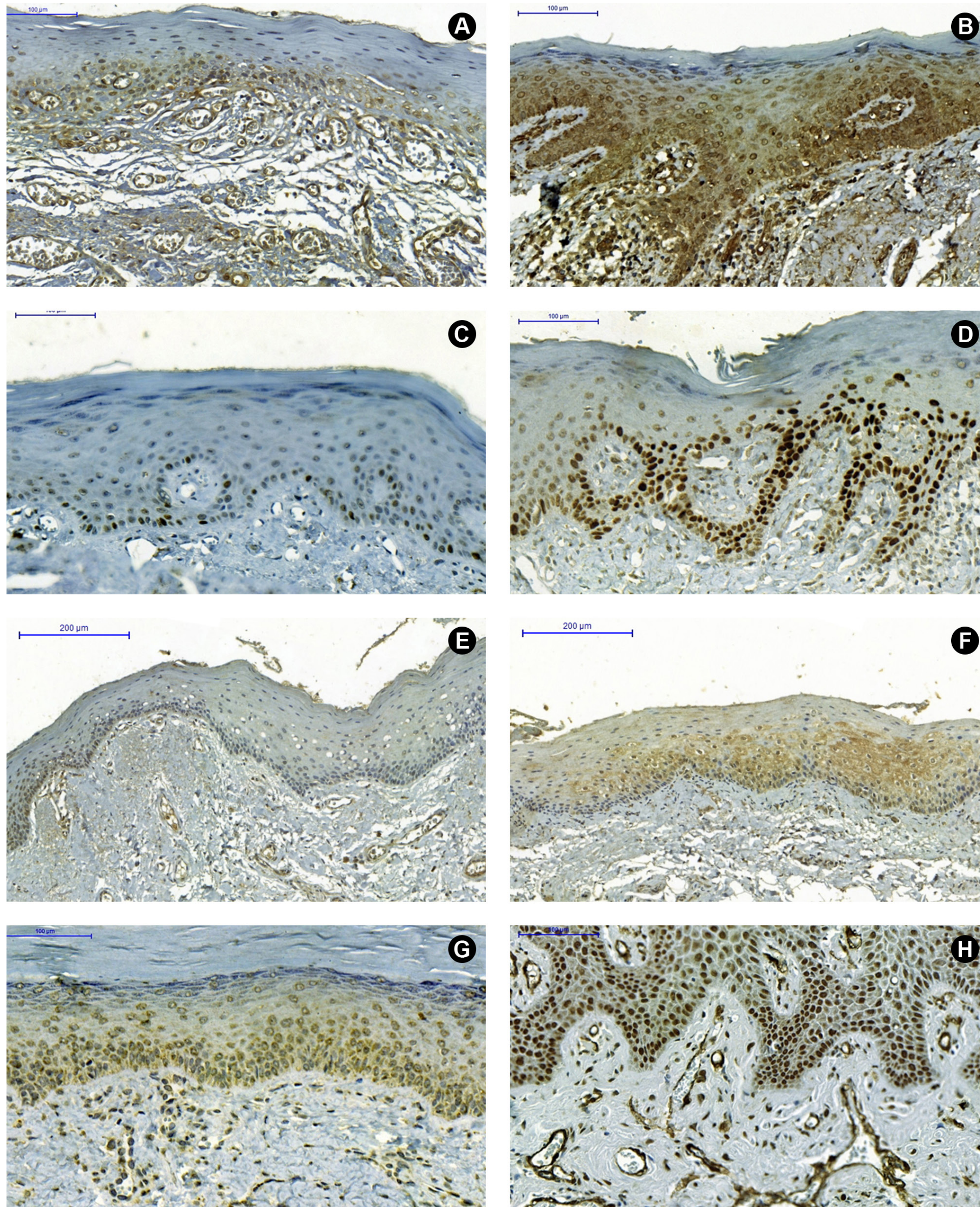

Figure 1. Immunoexpression of the proteins in AC – Low expression of CLIC4n in (A) low-risk AC compared to (B) high-risk AC. Low and basal expression of p53 in (C) low-risk AC, while the (D) high-risk AC shows high expression up to intermediate layer. Low TNF-α expression in (E) low-risk compared to (F) high-risk AC. Moderate TGF-β expression in the cytoplasm of AC's keratinocytes (G). Discontinuous and low α-SMA expression in the fibroblasts of AC's lamina propria (H). Scale bars=100 µm (A, B, C, D, G and H) and 200 µm (E and F)

of high-risk AC and high-grade LLSCC. The same pattern of nuclear CLIC4 translocation to the cytoplasm was observed when AC was compared to LLSCC and reinforced by the correlations results. We also observed an increase in the cytoplasmic CLIC4 in the LSCC with more aggressive clinical behavior. According to Shukla and Yuspa (5), this nuclear to cytoplasmic translocation appears to be related to the loss of growth arrest and proapoptotic functions in tumor cells. These authors showed that a reduction in nuclear CLIC4 is observed with progression of skin squamous tumors from benign to malignant phenotype.

Table 2. Sample size, median, quartiles 25 and 75, mean rank and statistical significance for the proteins' expression in lower lip squamous cells carcinoma

	Variable	Categories	n	Median	Q25-Q75	Mean rank	p†
CLIC4n	Lymph node metastasis	No	39	1	1-2	26.09	0.493
		Yes	11	1	1-1	25.86	
	Clinical stage	I or II	37	1	1-2	25.86	0.704
		III or IV	13	1	1-1,5	24.46	
	Histological grade	Low grade	28	1	1-2	28.23	0.057
		High grade	22	1	1-1	22.02	
CLIC4c	Lymph node metastasis	No	39	2	1-3	22.62	0.005*
		Yes	11	3	2-3	35.73	
	Clinical stage	I or II	37	2	1-3	23.00	0.029*
		III or IV	13	3	2-3	32.62	
	Histological grade	Low grade	28	1,5	1-2	19.25	<0.0001*
		High grade	22	3	3-3	33.45	
p53	Lymph node metastasis	No	39	3	2-3	24.17	0.117
		Yes	11	3	3-3	30.23	
	Clinical stage	I or II	37	3	2-3	23.72	0.061
		III or IV	13	3	3-3	30.58	
	Histological grade	Low grade	28	3	2-3	20.89	0.001*
		High grade	22	3	3-3	31.36	
TGF-β	Lymph node metastasis	No	39	1	1-2	27.00	0.087
		Yes	11	1	1-1	20.18	
	Clinical stage	I or II	37	1	1-2	27.49	0.042*
		III or IV	13	1	1-1	19.85	
	Histological grade	Low grade	28	1	1-2	28.82	0.023*
		High grade	22	1	1-1	21.27	
TNF-α	Lymph node metastasis	No	39	1	1-2	24.40	0.272
		Yes	11	1	1-3	29.41	
	Clinical stage	I or II	37	1	1-2	23.91	0.154
		III or IV	13	2	1-2.5	30.04	
	Histological grade	Low grade	28	1	1-2	23.36	0.200
		High grade	22	2	1-2	28.23	
α-SMA	Lymph node metastasis	No	39	1	1-2	24.03	0.142
		Yes	11	2	1-3	30.73	
	Clinical stage	I or II	37	2	1-3	24.35	0.305
		III or IV	13	3	2-3	28.77	
	Histological grade	Low grade	28	2	1-3	22.55	0.079
		High grade	22	3	2-3	29.25	

CLIC4n, CLIC4 in the nucleus; CLIC4c, CLIC4 in the cytoplasm; † Mann-Whitney U test; *Result is statistically significant.

Previous studies have suggested that when in the nucleus, CLIC4 is directly related to the P53 and c-Myc, two key genes of cancer development (1,20). CLIC4 appears to be a direct transcriptional target for p53, described as a downstream effector of the p53-mediated apoptosis, and therefore, a promising target for cancer therapy (4). It is well known that p53 protein protects the cells against DNA damage promoted by UV radiation, and that overexpression of mutant p53 is common on UV-inducted cancer and precancer, as observed in our results for AC and LLSCC (9,11). In this situation, p53 loses its ability to regulate protective mechanisms, while the mutant cells proliferate leading to chromosome translocations and deletions in potentially malignant lesions (11). In the present study, we found that the decrease of CLIC4 in the nucleus was significantly correlated to the increase of the mutant p53 protein in LLSCC, reinforcing the hypothesis that CLIC4 participates in p53-mediated growth arrest mechanisms in the nucleus.

P53 acts as a master regulator of TGF-β signaling, which involves the induction of inhibitory Smads and Smad dephosphorylation (6,21). TGF-β pathway critically regulates cellular mechanisms related to growth, differentiation, apoptosis, adhesion and motility (21). According to Shukla and Yuspa (5), the regulation of CLIC4 expression by p53 may be mediated through its effect on TGF-β-dependent gene transcription. In fact, in the nucleus, CLIC4 is thought to enhance TGF-β signaling through binding to phosphorylated Smadson DNA and prolonging their activated state (8). Interestingly, CLIC4 is reported to mirror the dual roles described for TGF-β in the carcinogenesis, which is considered as

tumor suppressor in early carcinogenesis by inhibiting cell proliferation, and as tumor-promoter in cancers at advanced stage by favoring tumor invasion (21,22).

We verified a progressive decreasing in the TGF-β

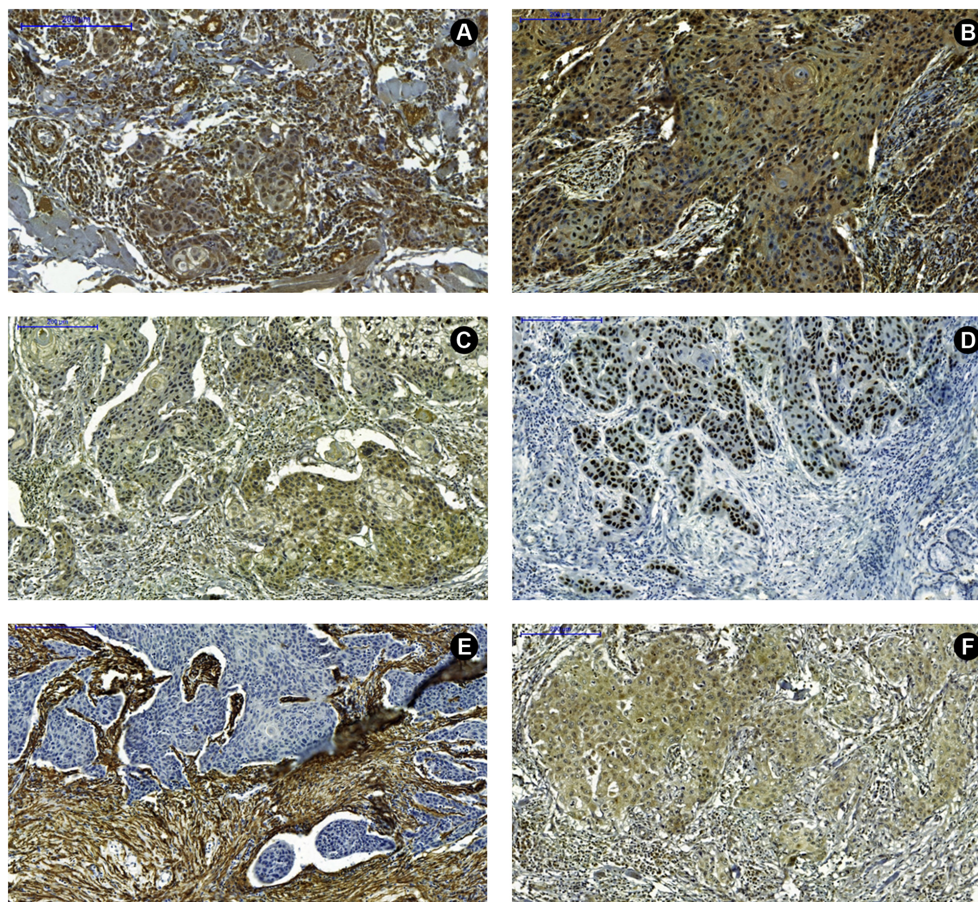

Figure 2. Immunoexpression of the proteins in LLSCC – High grade of malignancy tumors showing abundant nuclear and cytoplasmic CLIC4 (A, B), nuclear p53 (D) and cytoplasmic TNF-α (F) expression. Low-grade LLSCC exhibiting a low TGF-β expression (C). Continuous and high α-SMA expression in the stroma of a high-grade LLSCC (E). Scale bars=200 µm.

Table 3. Sample size, median, quartiles 25 and 75, mean rank and statistical significance for the protein expression according to the lesion type

Marker	Lesion	Median	Q25-Q75	Mean rank	p†
CLIC4n	AC LLSCC	2 1	1-2 1-2	60.74 40.26	<0.0001*
CLIC4c	AC LLSCC	1 2	1-2 1-3	41.05 59.95	<0.0001*
p53	AC LLSCC	3 3	2-3 2-3	46.50 54.50	0.097
TGF-β	AC LLSCC	2 1	1-2 1-2	62.11 38.89	<0.0001*
TNF-α	AC LLSCC	2 2	1-3 1-2	54 47	0.195
α-SMA	AC LLSCC	1 2,5	1-2 2-3	34.5 66.5	<0.0001*

AC, Actinic cheilitis; LLSCC, Lower lip squamous cells carcinoma; CLIC4n, CLIC4 in the nucleus; CLIC4c, CLIC4 in the cytoplasm; †Mann-Whitney non-parametric test; *Results are statistically significant.

Table 4. Spearman's correlation between the expression of the proteins according to the type of lesion

Proteins expression	AC		LLSCC	
	r	p	r	p
CLIC4n x CLIC4c	-0.554	<0.0001*	-0.378	0.007*
CLIC4n x p53	-0.244	0.087	-0.317	0.025*
CLIC4n x TGF-β	0.243	0.089	0.169	0.241
CLIC4n x TNF-α	-0.012	0.936	0.237	0.097
CLIC4n x α-SMA	-0.252	0.078	-0.073	0.615
CLIC4c x p53	-0.010	0.948	0.230	0.108
CLIC4c x TGF-β	-0.057	0.695	-0.310	0.029*
CLIC4c x TNF-α	0.135	0.350	0.015	0.916
CLIC4c x α-SMA	0.112	0.440	0.252	0.077

AC, Actinic cheilitis; LLSCC, Lower lip squamous cells carcinoma; CLIC4n, CLIC4 in the nucleus; CLIC4c, CLIC4 in the cytoplasm; *Results are statistically significant.

epithelial expression from low-risk to high-risk AC, and from low-grade to high-grade LLSCC. Lower TGF-β expression was also observed in LLSCC with advanced clinical stage. These results corroborate the observations of Salvadori et al. (23) in lip carcinogenesis. Our results also highlighted that an increase of CLIC4 in the cytoplasm of malignant keratinocytes was correlated to loss of TGF-β epithelial expression. Accumulation of CLIC4 in the cytoplasm, may indicate that this protein is not translocating to the nucleus, where its main antitumor functions take place. In the cancer context, the absence of CLIC4 in the keratinocytes nucleus has been linked to loss of TGF-β response (5), while elevating CLIC4 in tumor stroma occurs in conjunction to TGF-β in this tumor component and is associated to tumor growth and tumor cell invasion (8). In this last scenario, CLIC4 is often co-localized with α-SMA in fibroblastic cells, indicating its participation on the fibroblast's differentiation to myofibroblasts, an important TGF-β-mediated event during tumor epithelial-mesenchymal transition (1,8,24).

In the current study, a higher α-SMA expression was evident in the subepithelial region of high-risk AC, compared to low-risk cases. We also observed a highly significant increase in α-SMA expression in LLSCC stroma compared to AC. Acquisition of α-SMA-positive fibroblasts, known as myofibroblasts or carcinoma-associated fibroblasts (CAF) is associated to the establishment of desmoplasia in the tumor stroma and invasion of the tumor cells (25). However, the few studies that investigated myofibroblasts in the lip malignant transformation showed an increase in myofibroblastic cells in AC and lip cancer compared to control tissues but failed to reach statistical significance (26,27).

Shukla et al. (8) observed that human breast cancer cell lines induce expression of both CLIC4 and α-SMA in stromal fibroblasts via TGF-β signaling. They have also demonstrated that fibroblasts without CLIC4, even under the induction of TGF-β, markedly reduce the activation of p38MAPK. Non-activation of p38MAPK substantially inhibited the ability of TGF-β to regulate α-SMA expression, thus, TGF-β is thought to regulate the conversion of myofibroblasts through p38MAPK signaling. However, we found no correlation between CLIC4 and α-SMA in the lesions studied.

Nuclear translocation of CLIC4 may also be related to the TNF-α signaling, in a process that lead the cell to apoptosis (4,28). Fernández-Salas et al. (28) demonstrated that upregulation of CLIC4 can be induced by TNF-α in p53-null keratinocytes. These authors highlighted the potential implication of this protein in UV-mediated skin carcinogenesis, as induction of both p53 and TNF-α after UV exposure could superinduce CLIC4, resulting in apoptosis of altered cells. There was no significant correlation between CLIC4 and TNF-α, but we observed a progressive increase in the expression of TNF-α comparing low-risk to high-risk AC. This result highlights the paradoxal effect of TNF-α as an inflammatory cytokine inducing pro-tumoral changes at early stages of carcinogenesis, including skin carcinogenesis (29,30).

In summary, the results of this study suggest that the change in CLIC4 expression pattern from the nucleus to the cytoplasm, along with changes in p53, TGF-β and TNF-α expression, and increase of myofibroblasts is involved in lip carcinogenesis. These changes in intracellular localization and expression in malignant transformation and cancer progression, may offer a promising target for cancer prevention and therapy.

Resumo

O canal intracelular de cloreto 4 (CLIC4) é regulado pela p53 e fator de necrose tumoral α (TNF-α) e está relacionado ao aumento do fator de crescimento transformador β (TGF-β) e na diferenciação miofibroblástica na carcinogênese cutânea. Este estudo analisou a imunoexpressão de CLIC4, p53, TGF-β, TNF-α e α-SMA em 50 queilites actínicas (QA) e 50 carcinomas de células escamosas de lábio inferior (CCELI). A imunoexpressão da QA e CCELI foram categorizadas em escore 1 (<5% de células positivas), 2 (5-50%) ou 3 (>50%). Para CLIC4, a imunomarcação nuclear e citoplasmática das células epiteliais foi considerada separadamente. Para análise morfológica, foram utilizados os critérios da Organização Mundial da Saúde para a gradação das displasias epiteliais nas QAs, e o sistema de gradação de malignidade de Bryne foi utilizado para os casos de CCELIs. Alta imunoexpressão de CLIC4 nuclear (CLIC4n) e TGF-β foi observada em QA de baixo risco de transformação, enquanto CLIC4 citoplasmática (CLIC4c), p53 e TNF-α foram elevadas nos casos de alto risco ($p<0.05$). No CCELI, a imunoexpressão de CLIC4c foi maior em caos com metástase linfonodal, estágio clínico avançado e alto grau histológico de malignidade. A expressão de p53 foi elevada em CCELI de alto grau, enquanto o TGF-β diminuiu à medida que o estádio clínico e o grau morfológico progrediram ($p<0.05$). QAs exibiram uma elevada expressão de CLIC4n e TGF-β, enquanto o CLIC4c e α-SMA foram elevados em CCELIs ($p<0.0001$). Ambas as lesões mostraram correlação negativa entre CLIC4n e CLIC4c, enquanto nos CCELIs, também se verificou correlação negativa entre CLIC4c e p53, assim como entre CLIC4c e TGF-β ($p<0.05$). Alteração do CLIC4 do núcleo para o citoplasma e alterações na expressão de p53, TGF-β, TNF-α, e α-SMA estão envolvidas na carcinogênese labial.

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