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Original Article

E3 ligase carboxyl-terminus of Hsp70-interacting protein (CHIP) suppresses fibrotic properties in oral mucosa

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Background/purpose: Oral submucous fibrosis (OSF) represents a precancerous lesion of oral mucosa that may progress into oral cancer and its major etiological factor is areca nut chewing. Carboxyl-terminus of Hsp70-interacting protein (CHIP) functions as an ubiquitin E3 ligase and is associated with fibrosis diseases. In the current study, we sought to investigate whether CHIP participated in the areca nut-mediated OSF development.
Methods: The mRNA expression of CHIP in arecoline-stimulated buccal mucosal fibroblasts (BMFs) and OSF tissues was determined by qRT-PCR. Collagen gel contraction, migration and invasion assays were carried out to evaluate the myofibroblast activation. The protein expression levels of α-SMA and transglutaminase 2 (TGM2) were assessed by Western blot.
Results: The expression level of CHIP was reduced in BMFs following arecoline treatment in a dose-dependent manner, which was consistent with the observation of lower CHIP expression in OSF specimen compared to the normal counterparts. Ectopic expression of CHIP mitigated the myofibroblast activities, including elevated collagen gel contractility and cell motility. In addition, we showed that overexpression of CHIP downregulated the α-SMA and TGM-2

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expression, which may lead to less fibrosis alteration.
Conclusion: CHIP may not only function as a key regulator of protein quality control but also a critical deciding factor to oral fibrogenesis. Our findings suggested that CHIP possesses the anti-fibrotic effect, which may be mediated by TGM2 regulation. Restoration of CHIP could be a therapeutic direction to help OSF patients.

Introduction

Oral submucous fibrosis is one type of oral potentially malignant disorders, which is featured by chronic inflammation, epithelial atrophy and palpable fibrosis bands in the oral cavity. OSF patients often suffered from burning sensation and difficulty of mouth opening due to fibrosis, which has a negative impact on their quality of life in terms of social- and health-related aspects. To date, the detailed pathological mechanism of OSF development has not been fully revealed. It is known that the habit of areca nut chewing is a major contributing factor for OSF progression, and other factors like mechanical trauma, excessive alcohol intake, smoking, or ageing may further increase the risk of malignant transformation in OSF.[1] As such, it is important to identify the differentially expressed molecules that contribute to the oral fibrogenesis and prevent it from malignant transformation.

Carboxyl-terminus of Hsp70-interacting protein (CHIP) was first identified as a co-chaperone protein as it interacts with Hsp70 and negatively regulates chaperone functions.[2] CHIP also functions as an ubiquitin E3 ligase to promote the ubiquitination and the subsequent degradation of many oral cancer-associated factors, including p53[3] and HIF-1α,[4] via proteasomal machinery. In fibrosis diseases, CHIP has been revealed to target immature cystic-fibrosis transmembrane-conductance regulator for proteasomal degradation.[5] Besides, the Hsp90-CHIP axis has been shown to regulate integrin-linked kinase (ILK), an adaptor required to maintain the connection between integrins and the actin cytoskeleton.[6] They demonstrated the impaired fibroblast migration and a dramatic reduction in fibrosis of bleomycin-treated mice when ILK was ubiquitinated by CHIP and degraded by proteasome. Another study revealed that proteasomal degradation of NOX4 through CHIP regulation was responsible for inhibiting TGF-β-induced myofibroblast differentiation in lung fibroblasts.[7] These findings suggested that CHIP may possess the anti-cancer or anti-fibrosis potential. Nevertheless, researches regarding its expression level or functional role in the progression of precancerous OSF are still lacking.

In this present study, we examined the relative expression of CHIP in arecoline-treated buccal mucosal fibroblasts (BMFs) and OSF tissues to see if the expression of CHIP was altered in areca nut-associated OSF. Subsequently, we tested the effect of upregulation of CHIP on myofibroblast activities and myofibroblast marker, α-smooth muscle actin (α-SMA). We also assessed the expression of another fibrosis-related factor, transglutaminase 2 (TGM2), following overexpression of CHIP in fibrotic BMFs (fBMFs).

Materials and methods

Chemical, tissues acquisition and cell culture

Arecoline was purchased from Sigma—Aldrich (St. Louis, MO, USA). OSF patients were recruited at Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan. All procedures followed the approved guidelines from the Institutional Review Board of Chung Shan Medical University Hospital and informed written consent was obtained from each individual prior to commencing the study. Specimens from the histologically normal or fibrotic mucosa were excised, minced and washed with PBS supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml of amphotericin). Primary normal buccal mucosal fibroblasts (BMFs) and human fibrotic buccal mucosal fibroblasts (fBMFs) from OSF tissue were established and cultivated as previously described.[8] Cell cultures between the third and eighth passages were used in this study.

Overexpression of CHIP

CHIP cDNA will be cloned into pLV-EF1a-MCS-IRES-Puro (BioSettia, Cat. No: cDNA-pLV01; San Diego, CA, USA). Lentivirus production will be performed by co-transfection of plasmid DNA mixture with vector plus helper plasmids (VSVG and Gag-Pol) into 293T cells (American Type Culture Collection, Manassas, VA) using Lipofectamine 2000 (LF2000, Invitrogen, Calsbad, CA, USA).

Western blot analysis

Western blot was conducted as previously described.[9] The sample was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL, USA). The primary antibody against CHIP, α-SMA and TGM2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The corresponding secondary antibody was added to the membrane subsequent to the primary antibody incubation. GAPDH was used as protein loading control. The immunoreactive bands were developed using an ECL-plus chemiluminescence substrate (PerkinElmer, Waltham, MA, USA) and captured by LAS-1000

plus Luminescent Image Analyzer (GE Healthcare, Piscataway, NJ, USA).

Quantitative RT-PCR

Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extrac total RNA of cells according to the manufacturer's instruction. Superscript III first-strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) was utilized to reversely transcribe the total RNAs. qRT—PCR was performed using TaqMan miRNA assays with specific primer sets (Applied Biosystems, Carlsbad, Calif). qRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems).

Collagen contraction assay

fBMFs (2×10^5 cells) were suspended in 0.5 ml of 2 mg/ml collagen solution (Sigma—Aldrich, St. Louis, MO, USA) and added into one well of 24-well-plate followed by incubation at 37 °C for 2 h. After polymerization of collagen cell mixture, the gels were detached from wells and further incubated for 48 h. Contraction of the gels was photographed and measured using ImageJ software (NIH, Bethesda, MD, USA) to calculate their areas.

Migration and invasion assays

The 24-well Transwell system with a polycarbonate filter membrane of 8-μm pore size (Corning, United Kingdom) was employed. For the invasion capacity analysis, the membrane of upper chamber was coated with Matrigel. fBMFs were placed in the upper chamber (Corning, Acton, MA) with serum free medium, and medium containing 10% FBS was added to the lower chamber as chemoattractant. After 24 h of incubation, cells attached to the reverse side of the membrane were stained with crystal violet subsequent to the filter membrane fixation. These cells were counted from five different visual areas of 100-fold magnification under a microscope.

Statistical analysis

Three replicates of each experiment were performed. Data were expressed as the mean ± SD and analyzed by Student's *t*-test. The result was considered to be significant if *p*-value was <0.05.

Results

The main areca nut alkaloid, arecoline, has been demonstrated to induce myofibroblast transdifferentiation.[8] As shown in Fig. 1, we observed that the expression of CHIP was downregulated in arecoline-treated normal BMFs as the concentration increased from 0 to 20 μg/ml at mRNA (Fig. 1A) and protein (Fig. 1B) level, suggesting that the dose-dependent reduction of CHIP may be associated with the arecoline-induced myofibroblast activation. Meanwhile, the concordant increase of α-SMA and TGM2 expression following the CHIP downregulation with the treatment of arecoline (Fig. 1B). In accordance with this result, we also found that the relative expression of CHIP was aberrantly downregulated in OSF tissues ($n = 25$) and fBMFs compared to the normal counterparts (Fig. 2), indicating that CHIP may play an anti-fibrotic role in OSF progression.

To test this hypothesis, we overexpressed CHIP in two patient-derived OSF cell lines and examined the myofibroblast activities, including collagen gel contractility, migration and invasion abilities as myofibroblasts are the activated fibroblast migrated to the injured site for wound closure.[10] Our results demonstrated that upregulation of CHIP successfully suppressed the collagen gel contraction ability of two fBMFs (Fig. 3A). Also, the increased migration (Fig. 3B) and invasion (Fig. 4A) features of fBMFs were ameliorated in CHIP-overexpressed cells. Furthermore, we found that the expression of myofibroblast marker, α-SMA, was inhibited in CHIP-overexpressed fBMFs (Fig. 4B), which explained the finding of downregulated collagen gel contractility we observed in Fig. 3A as α-SMA is responsible to the contractile activity.[11] Additionally, the expression of TGM2 was abrogated in CHIP-overexpressed cells (Fig. 4B). In associated with the result that the increased expression of TGM2 mediated areca nut associated OSF progression,[12] our finding showed that upregulated the expression of CHIP resulted in blocked TGM2 expression and lower myofibroblast activities.

Discussion

Over the past decades, the concept that CHIP is involved in the progression of cystic fibrosis has been presented in a number of studies.[5,13] Nevertheless, its function in other fibrosis diseases remains obscure. In this study, we showed that areca nut consumption may lead to downregulation of the anti-fibrotic CHIP and the lower expression of CHIP in OSF tissues supported our *in vitro* finding. Secondly, ectopic expression of CHIP in fBMFs successfully repressed the elevated myofibroblast activation, including cell contractility and motility, as well as the expression of myofibroblast marker, α-SMA. These results provided a fundamental insight into the development of effective therapies as to relief of limited mouth opening for OSF patients.

TGM2, also known as tissue transglutaminase, is a calcium-dependent enzyme that catalyzes post-translation modification of proteins and the most commonly expressed member of the transglutaminase family.[14] Among its diverse functions, TGM2 has been shown to participate in various types of pathological fibrosis.[15,16] In OSF tissues, TGM2 also has been found to be aberrantly elevated in fibrosis specimen.[12] This phenomenon may be associated with the higher TGF-β expression during OSF progression. TGF-β activation has been proven to play a causative role in the areca nut-associated OSF[17] and fibroblast-derived TGM2 has been known as a downstream effector of TGF-β-induced fibrogenesis.[18] Moreover, the elevation of TGM2 in OSF has been shown to be implicated in fibrogenesis via upregulation of ROS.[12] These findings all suggested that TGM2 exhibited the pro-fibrogenic effect in OSF, while

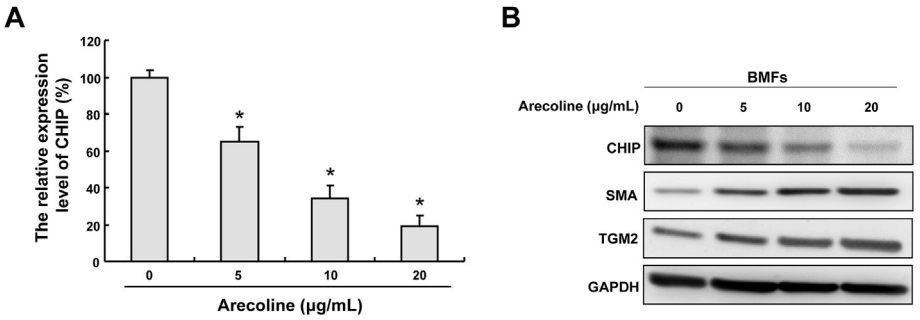

Fig. 1 CHIP is down-regulated in arecoline stimulated buccal fibroblasts (BMFs) in a dose-dependent fashion. The transcripts and protein expression levels of CHIP in normal BMFs treated with various concentration of arecoline (0—20 μg/ml) was determined by qRT-PCR (A) and western blot analysis (B).

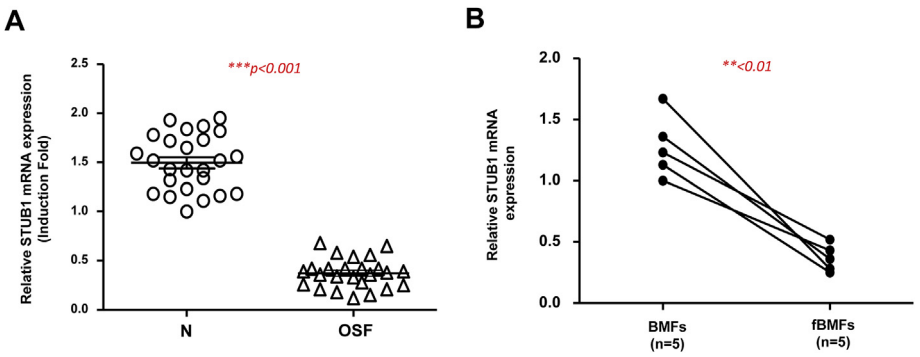

Fig. 2 Repressed expression of CHIP is OSF tissues. (A) The expression levels of CHIP in OSF specimen (n = 25) and its normal counterparts were presented. $^{**}p < 0.01$ compared with normal counterparts. (B) The relative expression level of CHIP was lower in human fibrotic buccal mucosal fibroblasts (fBMFs) relative to human buccal mucosal fibroblasts (BMFs).

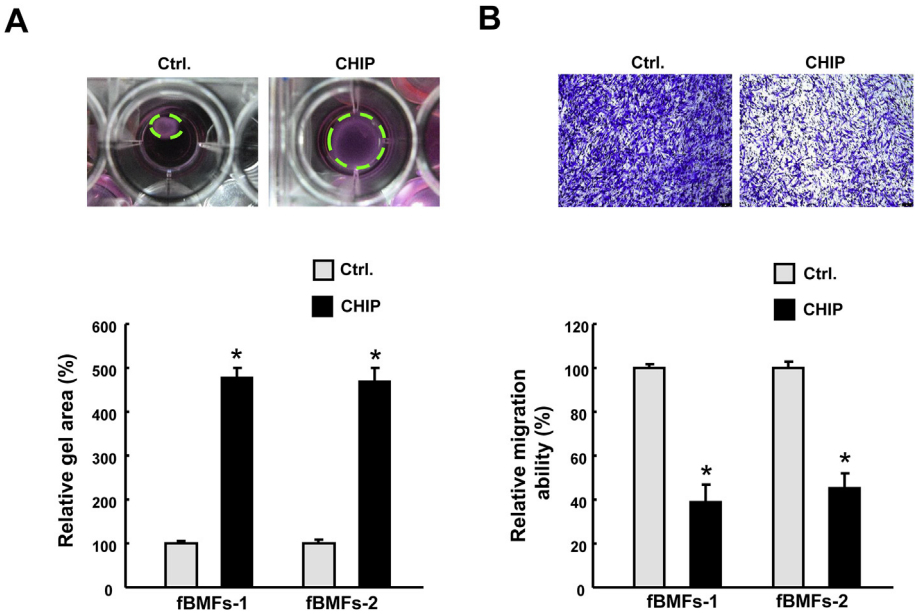

Fig. 3 Upregulation of CHIP alleviates the collagen gel contractility and migration ability in fBMFs. (A) The collagen gel contraction assay was used to evaluate their contractility in two fBMFs with or without CHIP overexpression; (B) Transwell migration assay was utilized to assess cell motility. $^{*}p < 0.05$ compared with control.

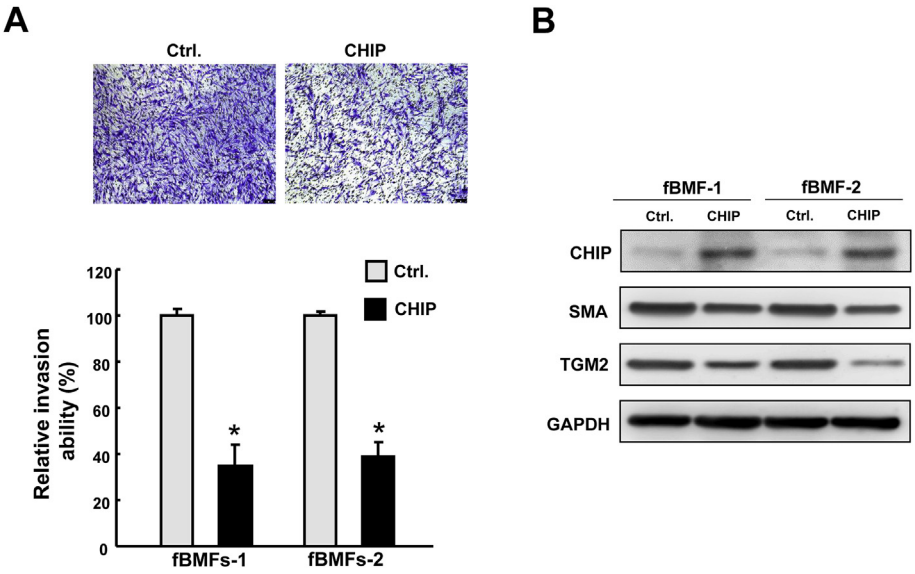

Fig. 4 Elevation of CHIP reduces the invasion capacity and fibrosis-associated factors in fBMFs. The invasion (A) ability of fBMFs was assessed by transwell invasion assay and the quantification result was presented as the relative value of control; (B) The protein expression levels of CHIP, α-SMA, and TGM2 following CHIP overexpression in two fBMFs. *$p < 0.05$ compared with scramble control.

results of the current study demonstrated the overexpression of CHIP revoked the elevated TGM2 expression. In agreement with it, one of the recent studies has shown that CHIP promoted the polyubiquitination and the subsequent proteasomal degradation of TGM2, leading to suppression of kidney tumor growth and angiogenesis.[19] In the present study, we found the inhibited expression of TGM2 and myofibroblast activities following overexpression of CHIP. The detailed mechanisms underlying the negative regulation of myofibroblast activation by CHIP-mediated degradation of TGM2 requires further experiments to confirm this hypothesis.

In conclusion, the present study suggested areca nut-associated OSF may be mediated by the reduced CHIP expression following the stimulation of areca nut constituents, such as arecoline. Downregulation of CHIP in normal cells may result in myofibroblast transdifferentiation and abnormal TGM2 accumulation, which likely causes further fibrosis changes. Therefore, restoration of CHIP expression may provide a viable strategy to counter the effect of areca nut chewing.

Declaration of competing interest

None declared.

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