Regulation of Oral Squamous Cell Carcinoma Proliferation Through Crosstalk Between SMAD7 and CYLD

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Key Words
Oral squamous cell carcinoma (OSCC) • SMAD7 • CYLD • Cancer invasion

Abstract
Background/Aims: SMAD7 is a key inhibitor of transforming growth factor β (TGFβ) receptor signaling, which regulates the alteration of cancer cell invasiveness through epithelial-mesenchymal cell conversion. Dysfunction of protein ubiquitination plays a critical role in carcinogenesis, whereas the involvement of a deubiquitinating enzyme, cylindromatosis gene (CYLD), in the tumor invasion of oral squamous cell carcinoma (OSCC) is unknown. Methods: Here, we studied the role of CYLD in regulation of OSCC cell invasion, using clinic specimens and cell lines. We modified SMAD7 levels in OSCC cells, and examined its effects on CYLD mRNA and protein levels by RT-qPCR and by Western blot, respectively. We also modified CYLD levels in OSCC cells, and examined its effects on SMAD7 mRNA and protein levels by RT-qPCR and by Western blot, respectively. Then, we examined the cell invasiveness in CYLD and/or SMAD7-modified OSCC cells in a transwell cell invasion assay. Results: We found that the levels of CYLD and SMAD7 were significantly decreased in OSCC specimens, compared to the paired normal tissue. Metastatic OSCC appeared to contain lower levels of CYLD and SMAD7. Moreover, CYLD and SMAD7 levels strongly correlated in OSCC specimens. Low CYLD levels were associated with poor patients’ survival. Moreover, SMAD did not regulate CYLD, but CYLD regulated the levels of SMAD7 in OSCC cells. Furthermore, CYLD overexpression inhibited SMAD7-mediated cell invasion, while CYLD depletion increased SMAD7-mediated cell invasion in OSCC cells. Conclusion: Suppression of CYLD in OSCC cells may promote SMAD7-mediated cancer invasion. Thus, CYLD appears to be an intriguing therapeutic target to prevent OSCC metastases.
Introduction

Oral squamous cell carcinoma (OSCC) is a malignant cancer with poor prognosis, largely stems from its aggressive characteristics [1-5]. Hence, studies on the molecular regulation of OSCC invasion and targeting therapies are critical approaches in the area.

The epithelial-mesenchymal transition (EMT) is characterized by the feature of loss of cell polarity and cell-cell adhesion of epithelial cells to allow them to gain migratory and invasive properties [6-8]. EMT is essential for numerous biological processes during development, and in wound healing, organ fibrotic remodeling and in cancer initiation and progression [6-8]. Among all factors that induce EMT, transforming growth factor β1 (TGFβ1) has been shown to be the most potential one [6-14]. TGFβ receptor signaling initiates by the binding of a ligand to a type II TGFβ receptor, which catalyzes the phosphorylation of a type I TGFβ receptor, and subsequently the phosphorylation of two intracellular proteins SMAD2 and SMAD3 to form heteromeric complexes with SMAD4. The activated SMAD complexes are then translocated to the nucleus, where they modulate gene transcription [9, 15, 16]. SMAD7 is a general antagonist against TGFβ receptor signaling. Activation of TGFβ receptor signaling is essential for EMT to occur in cancer [17, 18]. Thus, SMAD7 appeared to be a tumor suppressor.

The posttranslational modification of different proteins via direct, covalent ubiquitin attachment may alter the stability, function, and localization of the modified protein. Ubiquitin is covalently bound to lysine residues or amino termini of substrate proteins to modify the substrates by monoubiquitination or polyubiquitination. Protein polyubiquitin chains trigger diverse cellular processes, including DNA repair, receptor trafficking, and endocytosis or modulate the activity of kinases and transcription factors, to allow cells to decode the regulatory signals. Ubiquitin modification is reversed by the action of enzymes known as deubiquitinating enzymes, such as cylindromatosis gene (CYLD). Recent studies have shown that CYLD can induce a variety of signaling pathways that suppress tumor cell proliferation and survival by cleavage of the ubiquitin chains from target proteins [19-24]. However, a role of CYLF in the OSCC has not been studied.

Here, we studied the role of CYLD in regulation of OSCC cell invasion. We found that the levels of CYLD and SMAD7 were significantly decreased in OSCC specimens, compared to the paired normal tissue. Metastatic OSCC appeared to contained lower levels of CYLD and SMAD7. Moreover, CYLD and SMAD7 levels strongly correlated in OSCC specimens. Low CYLD levels were associated with poor patients' survival. Moreover, SMAD did not regulate CYLD, but CYLD regulated the levels of SMAD7 in OSCC cells. Furthermore, CYLD overexpression inhibited SMAD7-mediated cell invasion, while CYLD depletion increased SMAD7-mediated cell invasion in OSCC cells. Thus, our data suggest that suppression of CYLD in OSCC cells may promote SMAD7-mediated cancer invasion. Thus, CYLD appears to be an intriguing therapeutic target to prevent OSCC metastases.

Materials and Methods

Patient tissue specimens

A total of 30 resected specimens from OSCC patients were collected for this study. OSCC specimens were compared with the paired normal hepatic tissue (NT) from the same patient. OSCC specimens were further divided into two groups based on presence of detectable distal metastases or not (metastasis; no metastases). All specimens had been histologically and clinically diagnosed at Hospital of Stomatology affiliated to Zhejiang University. The characteristics of the OSCC were summarized in Table 1. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Culture of a human OSCC cell line

SCC-15 is a human OSCC line purchased from ATCC, and was culturbed in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA), 100
U/ml penicillin and 100μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

**Plasmids transfection**

CYLD-modulating and SMAD7-modulating plasmids were prepared using a backbone plasmid containing a GFP reporter under CMV promoter (pIRES2-CMV-GFP, Clontech, Mountain View, CA, USA). The transgenes were cloned from human cDNA, and the short-hairpin interfering RNA for SMAD7 (shSMAD7) and CYLD (shCYLD), or a control scrambled sequence (scr) was all purchased from Sigma-Aldrich (St. Louis, MO, USA). Transfection was performed with Lipofectamine 2000 reagent (Invitrogen), according to the instructions of the manufacturer. One day after transfection, the transfected cells were purified by flow cytometry based on their expression of GFP (about 90% transfection efficiency), and then analyzed.

**Western blot**

The protein was extracted from the specimens, or from the cultured cells, in RIPA lysis buffer (Sigma-Aldrich) on ice. The SDS-polyacrylamide gels-separated proteins were transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were rabbit anti-SMAD7, anti-CYLD and anti-α-tubulin (Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Protein signals were enhanced by chemiluminescence detection kit. Blotting images were representatives from 5 repeats. α-tubulin was used as a protein loading control. The protein quantification was performed using ImageJ software (NIH, Bethesda, MA, USA).

**Transwell cell invasion assay**

Cell invasion was assessed using the Matrigel Invasion Chamber (Corning, NY, USA) in triplicate. The cells (1 × 10⁵) were harvested in serum-free medium containing 0.1% BSA and plated to the upper chamber precoated with Matrigel. Medium containing 10% fetal bovine serum in the lower chamber served as the chemoattractant. After the cells were incubated at 37°C in a humidified incubator with 5% CO₂ for 22 hours, the no invading cells were removed with cotton swabs. The invasive cells attached to the lower surface of the inserted membrane were fixed in 100% methanol at room temperature and stained with Wright-Giemsa. The number of invasive cells on the lower surface of the membrane was then counted under a microscope.

**Statistical analysis**

All statistical analyses were carried out using the GraphPad Prism 6.0 statistical software package (GraphPad Software, Inc. La Jolla, CA, USA). All values are depicted as mean ± standard deviation (SD) and are considered significant if p < 0.05. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher’s Exact Test for comparison of two groups. Kaplan-Meier curves were used to analyze the patient survival by CYLD levels. Bivariate correlations were calculated by Spearman’s rank correlation coefficients.
**Results**

*Decreased CYLD and increased SMAD7 are detected in OSCC specimens*

In the OSCC samples, we detected significantly lower levels of SMAD7, compared to paired non-OSCC tissue (NT; Fig. 1A). Moreover, the levels of SMAD7 in OSCC with distal metastases were even lower than those without metastasis (Fig. 1A). Also, we detected significantly lower levels of CYLD in OSCC, compared to NT (Fig. 1B). And the levels of SMAD7 in OSCC with distal metastases were even lower than those without metastasis (Fig. 1B).

*CYLD and SMAD7 levels correlate in OSCC specimens*

In order to examine the relationship between CYLD and SMAD7 in OSCC, we performed the correlation test using the 30 OSCC specimens. A strong correlation was detected between CYLD and SMAD7 (\(\gamma = 0.72, p < 0.0001, n = 30\)), suggesting the presence of a relationship between CYLD and SMAD7 in OSCC specimens.

*Low CYLD levels are associated with poor patients' survival*

Next, we investigated whether the levels of CYLD may correlate with overall survival of OSCC patients. After resection of the primary tumor, the 30 patients were followed-up for 5 years. The median value of all 30 cases was chosen as the cutoff point for separating CYLD-high cases (n = 15) from CYLD-low cases (n = 15). Kaplan-Meier curves were performed, *p < 0.05, **p < 0.01, N = 30.*
years. The median value of all 30 cases was chosen as the cutoff point for separating CYLD-high cases (n = 15) from CYLD-low cases (n = 15). Kaplan-Meier curves were performed, showing that CYLD-low OSCC patients had a worse 5-year survival, compared to CYLD-high OSCC patients (Fig. 1D). Thus, low CYLD levels seem to be associated with poor 5-year survival.

**SMAD7 does not regulate CYLD in OSCC cells**

In order to find out whether SMAD7 may regulate CYLD in OSCC cells, we modified SMAD7 levels in an OSCC cell line SCC-15 to evaluate its effects on the CYLD. Thus, we transfected SCC-15 cells with either a SMAD7-overexpressing plasmid, or a plasmid carrying shSMAD7, or a control plasmid carrying a scrambled sequence (scr). Modulation of SMAD7 in SCC-15 cells was confirmed by RT-qPCR (A), and by Western blot (B). Neither overexpression nor depletion of SMAD7 in SCC-15 cells significantly altered CYLD levels in SCC-15 cells, by RT-qPCR (C), by Western blot (D), * p < 0.05, NS: non-significant, N = 5.

**CYLD regulates SMAD7 in SCC-15 cells**

In order to find out whether CYLD may regulate SMAD7 in OSCC cells, we modified CYLD levels in SCC-15 cells to evaluate its effects on the SMAD7. Thus, we transfected SCC-15
cells with either a CYLD-overexpressing plasmid, or a plasmid carrying shCYLD, or a control plasmid carrying a scrambled sequence (scr). First, modulation of CYLD in SCC-15 cells was confirmed by RT-qPCR (Fig. 3A), and by Western blot (Fig. 3B). We found that overexpression of CYLD significantly increased SMAD7 levels, while CYLD depletion significantly decreased SMAD7 levels in SCC-15 cells, by RT-qPCR (C), by Western blot (D), * p < 0.05, NS: non-significant, N = 5.

**Fig. 3.** CYLD regulates SMAD7 in SCC-15 cells. In order to find out whether CYLD may regulate SMAD7 in OSCC cells, we modified CYLD levels in SCC-15 cells to evaluate its effects on the SMAD7. Thus, we transfected SCC-15 cells with either a CYLD-overexpressing plasmid, or a plasmid carrying shCYLD, or a control plasmid carrying a scrambled sequence (scr). (A-B) Modulation of CYLD in SCC-15 cells was confirmed by RT-qPCR (A), and by Western blot (B). (C-D) Overexpression of CYLD significantly increased SMAD7 levels, while CYLD depletion significantly decreased SMAD7 levels in SCC-15 cells, by RT-qPCR (C), by Western blot (D), * p < 0.05, NS: non-significant, N = 5.

**CYLD suppresses OSCC cell invasion through SMAD7**

Finally, we examined the effects of the regulation of SMAD7 by CYLD on cell invasion. The modulation of SMAD7 and CYLD levels in SCC-15 cells were validated by Western blot (Fig. 4A). We found that overexpression of CYLD resulted in increases in OSCC cell invasion in a transwell cell invasion assay, shown by quantification (Fig. 4B), and by representative images (Fig. 4C). On the other hand, depletion of CYLD resulted in increases in OSCC cell invasion, shown by quantification (Fig. 4B), and by representative images (Fig. 4C). Suppression of SMAD7 abolished the effects of CYLD overexpression on cell invasion (Fig. 4B-C), while SMAD7 overexpression abolished the effects of CYLD depletion on cell invasion (Fig. 4B-C). Together, these data suggest that CYLD inhibits OSCC cell invasion through SMAD7 (Fig. 5).
cyclin D3 in hepatocellular carcinoma [28]. BCR-ABL/GATA1/CYLD mini circuitry has been reported to contribute to the leukemogenesis of chronic myeloid leukemia [29]. Downregulation of CYLD promotes colorectal cancer metastasis via directly targeting TWIST2 [30]. In lung cancer, CYLD was found to inhibit tumor growth through repression of EZH2 [31], and to reverse gefitinib resistance via negatively regulating G protein-coupled receptor 124 [32]. Specifically, a recent report has shown that CYLD induced marked reduction in vimentin expression and enhanced E-cadherin expression, characteristics of EMT, in squamous cell carcinoma cell lines. The authors further identified a number of CYLD target genes that are associated with EMT, including VIM, SMAD7 and EZH2 (enhancer of zeste homologue 2) [33]. These previous studies encouraged us to study a role of CYLD in the regulation of EMT-mediated OSCC cell invasion and metastases.

**Fig. 4.** CYLD suppresses OSCC cell invasion through SMAD7. (A) The modulation of SMAD7 and CYLD levels in SCC-15 cells were validated by Western blot. (B-C) The alteration of cell invasion was examined in a transwell cell invasion assay shown by quantification (B), and by representative images (C). *p < 0.05, N = 5, Scale bars are 50µm.

**Fig. 5.** A Schematic model. CYLD inhibits OSCC cell invasion through SMAD7.

**Discussion**

CYLD has been defined as a tumor suppressor in many types of cancer. For example, downregulation of CYLD has been found to be associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines [25]. Moreover, CYLD has been shown to reverse multidrug resistance of leukemia cells [26]. In addition, CYLD was reported to suppress nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene [27]. And, CYLD induces cell cycle arrest by targeting cyclin D3 in hepatocellular carcinoma [28]. BCR-ABL/GATA1/CYLD mini circuitry has been reported to contribute to the leukemogenesis of chronic myeloid leukemia [29]. Downregulation of CYLD promotes colorectal cancer metastasis via directly targeting TWIST2 [30]. In lung cancer, CYLD was found to inhibit tumor growth through repression of EZH2 [31], and to reverse gefitinib resistance via negatively regulating G protein-coupled receptor 124 [32]. Specifically, a recent report has shown that CYLD induced marked reduction in vimentin expression and enhanced E-cadherin expression, characteristics of EMT, in squamous cell carcinoma cell lines. The authors further identified a number of CYLD target genes that are associated with EMT, including VIM, SMAD7 and EZH2 (enhancer of zeste homologue 2) [33]. These previous studies encouraged us to study a role of CYLD in the regulation of EMT-mediated OSCC cell invasion and metastases.
In the current study, we found that the levels of CYLD and SMAD7 were significantly decreased in OSCC specimens, compared to the paired normal hepatic tissue. Metastatic OSCC appeared to contain lower levels of CYLD and SMAD7. Moreover, CYLD and SMAD7 levels strongly correlated in OSCC specimens. Low CYLD levels were associated with poor patients’ survival. All these clinical findings suggest that both SMAD7 and CYLD are critical factors against OSCC invasion and metastases and a regulation relationship may be present between SMAD7 and CYLD. Our in vitro study confirmed this hypothesis and showed that SMAD did not regulate CYLD, but CYLD regulated the levels of SMAD7 in OSCC cells. In addition, CYLD overexpression inhibited SMAD7-mediated cell invasion, while CYLD depletion increased SMAD7-mediated cell invasion in OSCC cells, which were demonstrated by a series of loss-of-function approaches. Since our data were basically achieved from OSCC cell lines, in future, examination of primary OSCC specimen may be applied to further confirm that this mechanism controls primary OSCC. Also, the analyses on the EMT-associated proteins were not complete, since some proteins like ZEB1, ZEB2, E-cadherin, Slug and Snail may be also examined in future studies.

Of note, SMAD7 is also a potent regulator for cell proliferation [10, 34]. Therefore, it may be interesting to analyze the coordinating role of CYLD and SMAD7 in the regulation of OSCC cell growth in the future study.

To summarize, here we propose a model that CYLD suppresses OSCC metastases through SMAD7. Downregulation of CYLD appears to directly contribute to the distal metastases of primary OSCC and subsequently poor prognosis. Thus, our study highlights CYLD as a promising novel target for treating OSCC.

**Disclosure Statement**

The authors have declared that no competing interests exist.

**References**