ITCH facilitates proteasomal degradation of TXNIP in hypoxia-induced lung cancer cells

Qian Sun | Bi-Bo Wang | Wei Wei | Gui-Chun Huang | Lei-Lei Liu | Wei-Wei Chen | Jing Wang | Xiao-Yue Zhao | Lu Lu | Rong Fang | Chun-Yan Zhu | Xiao-Yuan Chu

1Department of Medical Oncology, Jinling Hospital, Nanjing, China
2Department of Cardiothoracic Surgery, Jinling Hospital, Nanjing, China
3Department of Medical Oncology, Jinling Hospital, Medical School of Nanjing University, Nanjing, China
4Department of Pathology, Jinling Hospital, Nanjing, China

Correspondence
Xiao-Yuan Chu, Department of Medical Oncology, Jinling Hospital, No. 305, Zhongshan East Road, Nanjing 210000, China.
Email: chuxiaoyuan001@163.com

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81572933

Abstract
Background: Lung cancer (LC) is one of the most common cancers and a leading cause of cancer-related deaths worldwide. In many pathological conditions, particularly in the tumor microenvironment, cells and tissues frequently exist in a hypoxic state. Here, we evaluated Itchy E3 ubiquitin protein ligase (ITCH) expression in LC cells following hypoxia treatment.

Methods: LC cell lines were treated with hypoxic condition. Cell migration, invasion, inflammation, reactive oxygen species (ROS) production, and apoptosis of LC cells were determined by wound healing assay, Transwell invasive assay, ELISA, DCFH-DA staining, and flow cytometry, respectively. qPCR and WB were used to determine the expression of ITCH and TXNIP. Co-IP was performed to assess the interaction between ITCH and TXNIP.

Results: ITCH expression was downregulated in LC cells under hypoxic conditions. Next, LC cells were subjected to hypoxic conditions and changes in cell viability and metastasis were determined. Hypoxic conditions resulted in increased migration and invasion abilities of LC cells. Intracellular reactive oxygen species (ROS) production, inflammation, and apoptosis were also promoted by hypoxia. We found that ITCH overexpression led to the proteasomal degradation of thioredoxin-interacting protein (TXNIP), whereas the expression of the ITCH C830A mutant did not affect TXNIP levels in LC cells. The gain-of-function experiment demonstrated that migration, invasion, ROS generation, inflammation, and apoptosis of hypoxia-conditioned LC cells were ameliorated by ITCH overexpression, whereas the ITCH C830A mutant did not cause any changes in these phenotypes. Furthermore, the contribution of TXNIP knockdown and ITCH overexpression to the hypoxia-induced features in LC cells with ITCH C830A was found to be similar.

Conclusion: Our results suggest a novel mechanism underlying the changes in ITCH-mediated malignant phenotypes of hypoxia-conditioned LC cells via TXNIP.

KEYWORDS
degradation, hypoxia, ITCH, lung cancer, TXNIP

INTRODUCTION

Lung cancer (LC) is the main cause of cancer-related mortality worldwide, and non-small cell LC (NSCLC) accounts for approximately 85% of all LC cases.1,2 It is mainly characterized by a poor prognosis and a high risk of recurrence. At present, therapeutic options for LC include radiotherapy and chemotherapy. Despite the rapid development of modern technology and treatments, the mortality rate of patients with LC remains high, and a tendency for relapse has been observed.3,4

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. Thoracic Cancer published by China Lung Oncology Group and John Wiley & Sons Australia, Ltd.
The ubiquitin-proteasome system is the master pathway of protein degradation in the cytoplasm and nucleus of mammals. The highly regulated ubiquitin-proteasome pathway affects various cellular processes, substrates, and imperfections in the system, leading to the pathogenesis of cancer in humans. E3 ubiquitin ligase (E3) is the final effector of the enzyme. It possesses high substrate specificity and can control the ubiquitylation of the logation cascade. A major challenge in understanding the regulatory effects is deciphering the mechanism underlying their activity as well as assembly.

Itchy E3 ubiquitin protein ligase (ITCH), one of the HECT class E3s, is considered to be a unique conjugal partner of a large tumor suppressor (LATS1). ITCH is able to generate one complex with LATS1 internally and externally through the PPxY motif of LATS1 as well as the WW domain of ITCH. The endogenous LATS1 protein remains stable because of its inhibitory effect on endogenous ITCH resulting from shRNAs. Additionally, based on multiple functional analyses, ITCH expression was found to be positively correlated with tumor progression in prostate, breast, and liver cancer, chronic lymphocytic leukemia, and NSCLC. Nonetheless, the influence of ITCH on LC progression under hypoxia has not been clarified.

A previous investigation demonstrated that ITCH overexpression results in thioredoxin-interacting protein (TXNIP) degradation by the proteasome. ITCH knockdown by siRNAs leads to the accumulation of TXNIP. TXNIP was initially identified as a gene with undiscovered effects on α-subunit, 25-dihydroxyvitamin D(3)-induced HL-60-cells, which is called “thioredoxin-binding protein 2 (TB2)” or “vitamin D(3) upregulated protein 1 (VDUP1).” The TXNIP gene, located on chromosome 1q21.1 in humans, contains eight exons with a length of 4174 bp. The TXNIP protein in humans is composed of 391 amino acids with a formula weight of 46 kDa. TXNIP is an alpha-arrestin and may be the only known member of the family that binds thioredoxin (TRX). TXNIP plays a significant role in various biological effects (e.g., regulating apoptosis, differentiation, energy metabolism). TXNIP is a gene that must be tran-
scribedly controlled during hypoxia responses. The TXNIP expression level increases due to hypoxia in NSCLC, and hence it can be used as a marker of poor prognosis. Nevertheless, the effect of hypoxia on TXNIP expression in NSCLC remains unclear.

If the microenvironment of tumor cells is hypoxic, it indicates that the tumor has progressed. Hypoxic conditions in local tissues may be caused by tumor growth, energy metabolism, invasion, or incomplete local blood vessels. Under hypoxia, the expression level of hypoxia-inducible factor-1 (HIF-1), which plays a significant role in promoting cancer cell proliferation as well as metastasis, continuously increases. In a previous study, the HIF-1α subunit was found to be highly expressed in NSCLC, and its high expression was associated with poor prognosis in NSCLC subjects. Nevertheless, the influence of ITCH and TXNIP on NSCLC requires further investigation.

In this study, we explored the effects of hypoxia, ITCH, and TXNIP on the migration, invasion, reactive oxygen species (ROS) production, inflammation, and apoptosis of NSCLC cell lines. We demonstrated that the ITCH-TXNIP signaling axis inhibited the malignant phenotypes of NSCLC under hypoxic conditions.

METHODS

Cell culture & transient transfection

NSCLC cell lines, A549, SPC-A1, and H1299, were transfected with pCMV-myc-ITCH, pCMV-myc-ITCH C830A, pCMV-myc-empty, and/or siRNA targeting TXNIP with Lipofectamine 2000 (Invitrogen) according to the instructions for use (IFU) provided by the manufacturer. Targeted siRNAs for TXNIP and normal control siRNAs were provided by RiboBio. After 48 h of transfection, cells were collected. For hypoxia processing, the medium was replaced with deoxygenated Roswell Park Memorial Institute 1640 (Gibco BRL) prior to inducing an anoxic status, and the cells were cultured at 77°C in an anoxic chamber (Forma Scientific). Before each test, the deoxygenation medium was prepared by balancing the medium with a mixture of anoxic gases containing 5% carbon dioxide as well as 7% hydrogen and balancing it with nitrogen at 37°C. Oxygen saturation in the hypoxic chamber, which was measured with one oxygen indicator (Forma Scientific), was kept below 1%.

RT-qPCR

We carried out an SYBR Green-based qRT-qPCR to assess mRNA expression levels in lung tumor cells using a 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific). An mRNA isolation kit (Ambion) was used to extract RNA from lung tumor cells.

Western blot (WB) analysis

We prepared protein extracts based on standard procedures. The proteins were separated via 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were incubated with specific primary antibodies at 4°C overnight. The protein immune response signal was detected using an ECL detection system (Thermo Fisher Scientific).

Coimmunoprecipitation (co-IP)

Cells were lysed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 20 mM HEPES, 10% glycerol, and 1 mM EDTA (pH 7.4) supplemented with complete protease inhibitor cocktail and a phosphatase inhibitor. After an incubation period of 40 min at 4°C, insoluble components
were removed by centrifugation at 12,000 g for 15 min. Lysates were incubated with anti-FLAG M2 affinity gel for 8 h. Following five washes in wash buffer (1% Triton X-100, 300 mM NaCl, 20 mM HEPES, 10% glycerol, and 1 mM EDTA [pH 7.4]), the precipitated proteins were separated by SDS-PAGE.

Transwell migration assays

Two days after transfection, NSCLC cells were obtained using trypsin and washed once with Hanks solution. Eight-micrometer cultures or matrix inserts were placed in a 24-well plate for evaluating cell migration. Then, 400 μl of F-12 was mixed with 10% fetal bovine serum and 20 ng of HGF in the lower chamber, and 1 × 10⁵ cells were added to the upper chamber. Twenty hours after culturing, the migrated cells were stained using crystal violet and carefully observed using a microscope.

Wound healing test

We placed 10 μl of pipette-tip-scratched cells in 6-well plates which subsequently migrated to the wound and were fixed. The scratch area was then evaluated using a microscope. Mobility (percentage) was calculated as the width (at 48 h) divided by the width (at 0 h).

Determination of ROS generation

The production of endogenous ROS was determined using dichlorofluorescein diacetate, which is a fluorescent probe. Inoculated cells were placed in a 96-well plate at a density of 1 × 10⁴ cells/well for 24 days and cultured with dichlorofluorescein diacetate for 30 min at 37°C overnight. After the cells were washed twice with phosphate-buffered saline, they were imaged using an InCell 2000 confocal microscope. To quantitatively evaluate the effect of intracellular ROS generation, the fluorescence was measured using a software module provided by InCell 2000.

Apoptosis assay

A dead cell apoptosis kit with annexin V-FITC/propidium iodide (PI) (Strong Biotech Corp.) was used to analyze cell apoptosis along with flow cytometry (FC). Specifically, inoculated cells were placed in a 6-well plate (density: 3 × 10⁵ cells/well) and were cultured for 24 h. We then obtained trypsit, which was processed by centrifugation at 2000 rpm for 5 min and washed twice with ice-cold phosphate-buffered saline. Then, 100 μl of annexin V binding buffer was added, followed by 5 μl of annexin V and 5 μl of PI. The samples were incubated at room temperature for 15 min. Annexin V binding buffer was added to obtain a total volume of 1 ml. The cells were processed following transfer to an FC tube and analyzed using a flow cytometer (Cytomics FC 500, Beckman Coulter).

Enzyme-linked immunosorbent assays (ELISA)

Interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in cell lysates were measured using specific ELISA kits (Cell Signaling Technology) according to the manufacturer’s instructions. Cytokine levels were determined based on the respective standard curves.

Statistical analysis

All data are shown as means ± standard deviation (SD). Comparisons among multiple groups (>2 groups) were conducted using a one-way analysis of variance (ANOVA). Comparisons between two groups were conducted using t-tests. Statistical significance was set at p < 0.05.

RESULTS

Hypoxia-induced ITCH downregulation in lung cancer cells

To assess the influence of ITCH on LC cells under hypoxic conditions, SPC-A1, A549, and H1299 cells were subjected to anoxic conditions, and the effects of hypoxia on HIF-1α mRNA and protein expression levels were studied. Anoxia treatment contributed to increasing HIF-1α mRNA and protein expression levels (Figure 1a–f), suggesting the successful induction of hypoxia-conditioned lung tumor cells. In addition, to be more relevant to LC pathology, we checked the expression of ITCH and TXNIP mRNA expression in LC samples and adjacent noncancerous samples (N = 10).
results demonstrated that ITCH was downregulated, while TXNIP was upregulated, in lung cancer tissue compared with noncancerous tissue (Figure S1A,B).

We then detected the expression level of ITCH in anoxia-conditioned A549, SPC-A1, and H1299 cells. Both RT-qPCR and WB analysis showed downregulation of ITCH expression in hypoxic cells compared with that in normoxic cells (Figure 2a–f), suggesting that ITCH might be associated with the phenotypes of lung tumor cells under hypoxic conditions.

To examine the direct effects of ITCH and its activity on the phenotypes of NSCLC cells upon hypoxia, overexpression of ITCH wild-type (WT) or its catalytically inactive mutant C830A was conducted on the three lung tumor cell strains under hypoxia. Uproregulated ITCH expression levels in A549, SPC-A1, and H1299 were detected at both the mRNA and protein levels, as assessed by RT-qPCR and WB analyses (Figure 2a–f), respectively. The SPC-A1, A549, and H1299 cells with or without ITCH overexpression were used in the following experiments.

ITCH overexpression repressed the migration, invasion, ROS generation, inflammation, and apoptosis of lung tumor cells

As previous studies indicated, anoxia promoted the migration as well as invasion of lung tumor cells. We next attempted to explore the influence of ITCH overexpression on these two phenotypes (migration and invasion) of A549, SPC-A1, and H1299 cells under hypoxic conditions. The wound healing and Transwell invasion assays showed that hypoxia induction increased the migratory and invasive abilities of SPC-A1, A549, and H1299 cells compared to those in the normoxic group (Figures 3a–c and 4a–c). ITCH overexpression significantly decreased the migratory and invasive abilities of lung tumor cells under anoxic conditions; however, ITCH C830A overexpression showed no effect on the invasion and migration of the three cell types (Figures 3a–c and 4a–c), suggesting that the effects of ITCH on the invasion and migration of hypoxic lung tumor cells are based on ubiquitin ligating enzyme activities.

Hypoxia treatment induced robust production of inflammatory cytokines and ROS, which triggered apoptotic death in NSCLC cells. Under hypoxic conditions, A549, SPC-A1, and H1299 cells showed higher ROS levels and production of inflammatory cytokines (IL-1β, IL-6, and TNF-α) than normoxic cells (Figure 5a–f). Overexpression of ITCH partially reversed the upregulation of ROS, IL-1β, IL-6, and TNF-α levels in LC cells under hypoxic conditions (Figure 5a–f). In addition, C830A overexpression did not affect the generation of ROS and inflammatory cytokines in the cells.

Apoptosis was determined by FC using Annexin V-FITC/PI staining. The results showed that hypoxia treatment induced significant apoptosis in LC cells compared to that in normoxic cells (Figure 6a–c). However, in ITCH-overexpressing cells, apoptosis of LC cells was reduced. As expected, C830A overexpression did not influence the apoptosis rate in hypoxic NSCLC cells (Figure 6a–c), indicating that ITCH also reduced ROS generation, inflammation, and apoptosis of hypoxic LC cells via its activity.

ITCH caused proteasomal TXNIP degradation in lung tumor cells

As previously conducted investigations suggested that ITCH could bind to TXNIP and cause its degradation and because TXNIP is positively correlated with metastasis and ROS in multiple cancers, we hypothesized that TXNIP is associated with ITCH-mediated tumor phenotypes of hypoxic NSCLC cells. To test this, we transfected ITCH WT

**FIGURE 2** ITCH expression in hypoxia-treated lung tumor cells. SPC-A1, A549, and H1299 cells were processed with ITCH (or C830A mutant) by transfection with an overexpression vector or empty vector and then placed under hypoxic or normoxic conditions. (a, b, c) Downregulated ITCH mRNA expression levels in SPC-A1, A549, and H1299 cells in response to anoxia. (d, e, f) Reduced nestin levels in SPC-A1, A549, and H1299 cells in response to hypoxia. *p < 0.05, **p < 0.01 versus normoxic group; p < 0.05, **p < 0.01 versus hypoxic group
and the C830A mutant into H1299 cells and treated the cells with the protein synthesis inhibitor cycloheximide (CHX). Results revealed ITCH overexpression; moreover, TXNIP protein expression was reduced more rapidly in the cells than in cells with an empty vector or C830A mutant overexpression (Figure 7A). Furthermore, ITCH-induced downregulation of TXNIP expression was abrogated after treatment with the proteasome inhibitor MG132 (Figure 7b). These data suggest that ITCH contributes to the proteasomal degradation of TXNIP in H1299 cells.

Next, we assessed the interaction between ITCH and TXNIP. TXNIP was immunoprecipitated using FLAG antibody from H1299 cell lysates or vector controls coexpressing myc-ITCH and flag-TXNIP plasmids. WB analysis indicated that TXNIP coimmunoprecipitated ITCH with an empty vector, but did not coimmunoprecipitate with it (Figure 7c). This result demonstrates that there is a protein–protein interaction between TXNIP and ITCH.

TXNIP participated in ITCH-mediated suppression of migration, invasion, ROS production, inflammation, and apoptosis in hypoxic NSCLC cells

To evaluate the role of ITCH and TXNIP cross-talk in the malignant phenotypes of NSCLC cells under hypoxic conditions, hypoxic cells were transfected with both the C830A mutant overexpression vector and siRNA-TXNIP. We observed that TXNIP expression was significantly reduced...
in C830A-overexpressed hypoxic cells with siRNA-TXNIP transfection, at both the protein and mRNA levels (Figure 8a–f).

Next, wound healing tests and Transwell invasive tests were carried out to detect the role of TXNIP depletion in C830A mutant-overexpressed cell migration and invasion under hypoxic conditions. Wound healing assay and Transwell invasive tests indicated that TXNIP depletion also inhibited the migratory and invasive capacity of hypoxic A549, SPC-A1, and H1299 cells (Figure 9a,b). Our data showed that TXNIP knockdown in C830A mutant-overexpressing cells also reduced ROS generation (Figure 9c), inflammation (Figure 9d–f), and apoptosis (Figure 9g). Our data indicate that TXNIP is associated with ITCH-mediated tumor phenotypes of hypoxic lung tumor cells.

**FIGURE 4** Effect of ITCH overexpression on invasion ability of hypoxia-treated lung tumor cells. SPC-A1, A549, and H1299 cells were processed with ITCH (or C830A mutant) by transfection with an overexpression vector or empty vector and then placed under hypoxic or normoxic conditions. (a, b, c) Transwell invasion assay was performed to evaluate the invasion ability of A549, SPC-A1, and H1299 cells. *p < 0.05 versus normoxic group; #p < 0.05 versus hypoxic group.

**Single silencing of TXNIP suppressed migration, invasion, ROS production, inflammation, and apoptosis in hypoxic NSCLC cells**

To directly test the effect of TXNIP on the migration, invasion, ROS production, inflammation, and apoptosis in hypoxic NSCLC cells, we firstly determined its expression in normoxic and hypoxic NSCLC cells. WB determined that hypoxia induced an upregulation of TXNIP in LC cell lines, while transfection of siRNA-TXNIP resulted in a significant reduction of TXNIP in hypoxic LC cells (Figure 10a). Wound healing and Transwell invasive assays showed that single TXNIP knockdown also inhibited the migratory and invasive capacity of hypoxic A549, SPC-A1, and H1299 cells (Figure 10b,c). Our data showed that TXNIP knockdown
also reduced ROS generation (Figure 10d), IL-1β, IL-6, TNF-α (Figure 10e–g), and apoptotic proportion (Figure 10h) of hypoxic NSCLC cells. This part of data indicated that TXNIP depletion caused reduction of migration, invasion, ROS production, inflammation, and apoptosis in hypoxic NSCLC cells.

**DISCUSSION**

NSCLC, a major tumor-related disease involved in targeting treatment of immunization as well as molecule’s checkpoints, so as to significantly improve subjects’ clinical prognosis.28,29 Hypoxia-promoted metastasis has been discovered in several cancers in humans,30–32 such as NSCLC,33,34 however, the underlying mechanism has not been fully elucidated. In the current study, decreased ITCH levels were found in SPC-A1, A549, and H1299 cells in response to anoxia induction. Anoxia increased the migration and invasion abilities, ROS generation, inflammation, and apoptosis of NSCLC cell lines, although these increases in NSCLC cells were ameliorated by ITCH overexpression, rather than by overexpression of its inactive C830A mutant. We also observed that the degradation of TXNIP was mediated by ITCH in lung tumor cells. In the loss-of-function experiment, TXNIP depletion resulted in one homologous phenotyping (ITCH overexpression) in hypoxic NSCLC cells with the C830A mutant. Our data suggest that the hypoxia-induced ITCH/TXNIP pathway increases metastasis and cell survival and probably causes treatment failure in LC.

ITCH, an E3-ubiquitin ligase, regulates the ubiquitylation of multiple targets and is involved in a variety of cell response regulation processes (e.g., TNF-α, Hedgehog, Notch signal transduction, and DNA damage response).35 Extensive investigations have shown that ITCH expression is upregulated in a variety of cancer cells.35 ITCH, mainly based on Wnt/beta-catenin, has a negative regulatory effect in colorectal and breast cancer processes.36 Clearing ITCH inhibits lung tumor cell multiplication. ITCH silencing also significantly accelerated lung tumor cell death by modulating the Bcl2/Bax signal pathway, suggesting that ITCH exerts an antiapoptotic effect on lung tumor cells.37 Li et al. indicated that inhibition of ITCH suppresses proliferation and induces apoptosis of lung cancer cells.37 H1975 and Calu3 cells were transfected with siRNA-ITCH, and the cell proliferation and apoptosis were measured by MTT assay and flow cytometry. They observed that ITCH siRNA effectively inhibited the proliferation and invasion of the lung cancer cells and promoted cell apoptosis. In our study, we
**FIGURE 6** Effect of ITCH overexpression on apoptosis of hypoxia-conditioned lung tumor cells. SPC-A1, H1299, and A549 cells were processed with ITCH (or C830A mutant) by transfection with an overexpression vector or empty vector and then placed under hypoxic or normoxic conditions. (a, b, c) Cell death was evaluated via FC with PI staining as well as fluorescein isothiocyanate. *p < 0.05 versus normoxic group; #p < 0.05 versus hypoxic group.

**FIGURE 7** TXNIP degradable effect promoted by interaction of ITCH with TXNIP. (a) H1299 cells were cotransfected with ITCH WT or its C830A mutant. Whole cell (WC) lysing reagent processed under the intended time with 100 μg/ml CHX were probed for ITCH as well as TXNIP with WB. (b) WC lysing reagent from H1299 cells transfected with ITCH expression vector/ pRT-V and processed using 25 μM MG-132 for 4 h were probed for ITCH as well as TXNIP with WB. (c) H1299 cells were cotransfected with myc-ITCH as well as flag-TXNIP plasmid. Lysing reagent used was IP (by anti-flag antibodies) as well as WB (by indicated antibodies).
showed that hypoxia condition reduced ITCH expression in LC cells (A549, SPC-A1, and H1299), and promoted migration, invasion, and apoptosis of these three cell lines. The effect of hypoxia-associated ITCH downregulation on apoptosis is consistent with the report by Li et al., while the role of hypoxia on migration and invasion of LC cells is discrepant. In another aspect, accumulating studies have demonstrated that hypoxia enhanced migration, invasion and transformation of NSCLC cells, which have been confirmed by our study. Therefore, we thought the discrepancy of these two studies regarding the effect of hypoxia-associated ITCH downregulation on migration and invasion could be attributed to two reasons: first is the application of different cell lines (H1975 and Calu3 vs. A549, SPC-A1, and H1299); second, ITCH reduction caused by single transfection of siRNA or hypoxia condition, and the latter is a more complicated condition. However, the expression level of ITCH in NSCLC cells under hypoxic conditions remained unclear. Here, ITCH expression levels were found to be significantly decreased in A549, SPC-A1, and H1299 cells in response to hypoxic conditions, along with promoted migration, invasion, ROS release, inflammation, and apoptosis. Restored expression of ITCH contributed to the suppression of these hypoxia-induced changes in the three NSCLC cells. The repressive effect of ITCH on apoptosis was consistent with that reported in a previous study.

ITCH regulates cell death by interacting with substrate proteins (e.g., p53 and its related genes, including p63, p73, t-Bid, c-jun, and TXNIP). TXNIP, a regulator of apoptosis-promoting proteins and the TRX system, has attracted increasing attention in the CVD field. TXNIP expression is strictly controlled in normal cells. TXNIP deregulation is associated with cancer, heart, and metabolic illnesses. TXNIP is caused by multi-stress stimulation (e.g., hydrogen dioxode, H$_2$O$_2$, RF, ultraviolet radiation, heat shock, serum deprivation, and growth inhibitors, such as TGF1). Anticarcinogens (e.g., ceramide, 5-fluorouracil, anisomycin, and hexadecadrol) can also significantly affect TXNIP expression levels. Many transcription factors (e.g., heat shock factor, GR, FOXO1, and MondoA) have been shown to regulate TXNIP expression in different conditions. Recently, a global analysis of lysine ubiquitination in HeLa cells via mass spectrometry showed that TXNIP is also ubiquitinated externally. Lys-122 is a possible ubiquitin-binding site. Zhang et al. demonstrated that ITCH resulted in degradable effect on TXNIP protease. Inhibition of ITCH by siRNAs leads to the accumulation of steady-state TXNIP levels. The lack of TXNIP expression in gastric, breast, bladder, and colorectal cancer and DLBCL has been reported to be related to more invasive illnesses, advanced stages of disease, and relatively poor outcomes. In contrast, TXNIP overexpression is caused by anoxia in microvascular endothelial cells (MECs) as well as in the hearts of mice. In carcinoma of the pancreas and NSCLC, TXNIP is also found to be induced by HIF under anoxic conditions, and the high expression levels of TXNIP may be an indicator of poor prognosis of NSCLC. Here, our data confirmed that ITCH overexpression contributed to the proteasomal degradation of ITCH in H1299 cells. ITCH-suppressed migration, invasion, ROS release, inflammation, and apoptosis were accompanied by reduced TXNIP levels in the three NSCLC cell lines (SPC-A1, A549, and H1299). To elucidate the role of TXNIP, it was silenced in cell lines under hypoxic conditions and C830A overexpression; the data showed that although C830A upregulation did not cause any changes in these tumor phenotypes in hypoxic NSCLC cells, the depletion of TXNIP had an effect similar to ITCH WT overexpression. These data suggest that ITCH exerts its effect by mediating TXNIP degradation.
In summary, our study suggests that the hypoxia-induced ITCH/TXNIP pathway plays an important role in hypoxia-associated cell migration, invasion, and survival. Targeting ITCH or TXNIP may be a potential therapy to inhibit tumor cell survival and metastasis, thereby improving the treatment of solid breast tumors. However, the lack
FIGURE 10 Influence of single TXNIP knockdown on migration, invasion, ROS generation, inflammation, and apoptosis of hypoxic lung tumor cells. SPC-A1, A549, and H1299 cells were processed using siTXNIP/siNC by transfection for 24 h. Twenty-four hours after transfection, the cells were subjected to normoxic or hypoxic conditions. (a) Reduced TXNIP levels in SPC-A1, A549, and H1299 cells were detected by WB. (b) Wound healing assay showing the migration ability of SPC-A1, A549, and H1299 cells. (c) Transwell invasion test showing the invasion ability of SPC-A1, A549, and H1299 cells. (d) DCFH-DA staining assay was used to examine ROS generation in A549, SPC-A1, and H1299 cells. (e, f, g) ELISA was performed to detect the generation of inflammatory cytokines (interleukin (IL)-1β, interleukin (IL)-6, and tumor necrosis factor-alpha) in SPC-A1, A549, and H1299 cells. (h) Cell death was evaluated with FC with PI staining as well as fluorescein isothiocyanate. ***p < 0.05, **p < 0.01 versus hypoxic + si-NC group.
of animal experiments was a limitation of this study, and a hypoxic tumor animal model incorporating ITCH or TXNIP KO/KD should be used in future investigations.

**CONFLICT OF INTEREST**
The authors report no conflict of interest.

**ORCID**
Xiao-Yuan Chu https://orcid.org/0000-0003-0139-4526

**REFERENCES**

1. Remark R, Becker C, Gomez JE, Damotte D, Dieu-Noasjain M-C, Sautès-Fridman C, et al. The non–small cell lung cancer immune contexture. A major determinant of tumor characteristics and patient outcome. Am J Respir Crit Care Med. 2015;191:377–90.

2. Barta JA, Powell CA, Winsinievsky JP. Global epidemiology of lung cancer. Ann Glob Health. 2019;85:8.

3. Misra P, Singh S. Role of cytokines in combinatorial immunotherapeutics of non–small cell lung cancer through systems perspective. Cancer Med. 2019;8:1976–95.

4. Owen DH, Williams TM, Bertino EM, Mo X, Webb A, Schweitzer C, et al. Homologous recombination and DNA repair mutations in patients treated with carboplatin and nab-paclitaxel for metastatic non–small cell lung cancer. Lung Cancer. 2019;134:167–73.

5. Bernassola F, Karin M, Ciechanover A, Melino G. The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell. 2008;14:10–21.

6. Zinzalla G. Paving the way to targeting HECT ubiquitin ligases. Future Med Chem. 2015;7:2107–11.

7. Liu Y-C. The E3 ubiquitin ligase itch in T cell activation, differentiation, and tolerance. Semin Immunol. 2007;19:197–205.

8. Liu Y, Lau J, Li W, Tempel W, Li L, Dong A, et al. Structural basis for the regulatory role of the PPXY motifs in the thioredoxin-interacting protein TXNIP. Biochem J. 2016;473:179–87.

9. Salah Z, Itzhaki E, Aqeljan RI. The ubiquitin E3 ligase ITCH enhances breast tumor progression by inhibiting the Hippo tumor suppressor pathway. Oncotarget. 2014;5:10886–900.

10. Luo Z-L, Luo H-J, Fang C, Cheng L, Huang Z, Dai R, et al. Negative correlation of ITCH E3 ubiquitin ligase and miRNA-106b dictates metastatic progression in pancreatic cancer. Oncotarget. 2016;7:1477–85.

11. Rathinam C, Mateis LE, Flavell RA. The E3 ligase Itch is a negative regulator of the homeostasis and function of hematopoietic stem cells. Nat Immunol. 2011;12:399–407.

12. Zhang P, Wang C, Gao K, Wang D, Mao J, An J, et al. The ubiquitin ligase itch regulates apoptosis by targeting thioredoxin-interacting protein for ubiquitin-dependent degradation. J Biol Chem. 2010;285:8869–79.

13. Qayyum N, Haseeb M, Kim MS, Choi S. Role of thioredoxin-interacting protein in diseases and its therapeutic outlook. Int J Mol Sci. 2021;22:2754.

14. Chen J, Saxena G, Mungrue IN, Lusis AJ, Shalev A. Thioredoxin-interacting protein: a critical link between glucose toxicity and β-cell apoptosis. Diabetes. 2008;57:938–44.

15. Yamada K, Noguchi C, Kamitori K, Dong Y, Hirata Y, Hossain MA, et al. Rare sugar D-allenge strongly induces thioredoxin-interacting protein and inhibits osteoclast differentiation in Raw264 cells. Nutr Res. 2012;32:116–23.

16. Chong C-R, Chan WPA, Nguyen TH, Liu S, Procter NE, Ngo DT, et al. Thioredoxin-interacting protein: pathophysiology and emerging pharmacotherapeutics in cardiovascular disease and diabetes. Cardiovasc Drugs Ther. 2014;28:347–60.

17. Wong RW, Hagen T. Mechanistic target of rapamycin (mTOR) dependent regulation of thioredoxin interacting protein (TXNIP) transcription in hypoxia. Biochem Biophys Res Commun. 2013;433:40–6.

18. Li Y, Miao L-Y, Xiao Y-L, Huang M, Yu M, Meng K, et al. Hypoxia-induced high expression of thioredoxin interacting protein (TXNIP) in non–small cell lung cancer and its prognostic effect. Asian Pac J Cancer Prev. 2015;16:2953–8.

19. Li C, Yang N, Chen Z, Xia N, Shan Q, Wang Z, et al. Hypoxia-induced Tiel1 drives stemness and cisplatin resistance in non–small cell lung carcinoma cells. Cancer Cell Int. 2021;21:57.

20. Ziolkowska-Suchanek I, Podralska M, Żurawek M, Łaczmańska J, Iżykowska K, Dzikiewicz-Krawczyk A, et al. Hypoxia-induced FAM13A regulates the proliferation and metastasis of non–small cell lung cancer cells. Int J Mol Sci. 2021;22:4302.

21. Tirpe AA, Gulei D, Ciortea SM, Crivii C, Berindan-Neagoe I. Hypoxia–oxygen: overview on hypoxia-mediated mechanisms with a focus on the role of HIF genes. Int J Mol Sci. 2019;20:6140.

22. Rossi M, De Laurenzi V, Munariz E, Green DR, Liu YC, Voudsen KH, et al. The ubiquitin–protein ligase Itch regulates p73 stability. EMBO J. 2005;24:836–48.

23. Lin C-W, Wang L-K, Wang S-P, Chang Y-L, Wu Y-Y, Chen H-Y, et al. Daxx inhibits hypoxia-induced lung cancer cell metastasis by suppressing the HIF-1α/HDAC1/Slug axis. Nat Commun. 2016;7:1–16.

24. Yang N, Liang Y, Yang P, Li FJOR. Propofol suppresses LPS-induced nuclear accumulation of HIF-1α and tumor aggressiveness in non–small cell lung cancer. Oncol Rep. 2017;37:2611–9.

25. Zhou G, Dada LA, Wu M, Kelly A, Trejo H, Zhou Q, et al. Hypoxia-induced alveolar epithelial-mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1. Am J Physiol: Lung Cell Mol Physiol. 2009;11297:11120–130.

26. Jeong J-K, Gurunathen S, Kang M-H, Han JW, Das J, Choi Y-J, et al. Hypoxia-mediated autophagic flux inhibits silver nanoparticle-triggered apoptosis in human lung cancer cells. Sci Rep. 2016;6:1–13.

27. Zhang C, Wang H, Liu X, Hu Y, Ding L, Zhang X, et al. Oncogenic microRNA-411 promotes lung carcinogenesis by directly targeting suppressor genes SPRY4 and TXNIP. Oncogene. 2019;38:1892–904.

28. Braheir Jr, Tykoss KD, Chow LQ, Hwu W-J, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366:2455–65.

29. Topalian SL, Hodi FS, Braheir JR, Gettying SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. N Engl J Med. 2012;366:2443–54.

30. Adamski J, Price A, Diver C, Makin G. Hypoxia–induced cytotoxic drug resistance in osteosarcoma is independent of HIF-1α. PLoS One. 2013;8:e65304.

31. Sun XP, Dong X, Lin L, Jiang X, Wei Z, Zhai B, et al. Up-regulation of survivin by AKT and hypoxia-inducible factor 1α contributes to cisplatin resistance in gastric cancer. FEBS J. 2014;281:115–28.

32. Mamede A, Abrantes AM, Pedrosa L, Casalta-Lopes J, Pires A, et al. Rare sugar D-allose strongly induces thioredoxin-interacting protein in diseases and its therapeutic outlook. Int J Mol Sci. 2012;3:2754.

33. Chen J, Saxena G, Mungrue IN, Lusis AJ, Shalev A. Thioredoxin-interacting protein: a critical link between glucose toxicity and β-cell apoptosis. Diabetes. 2008;57:938–44.

34. Yamada K, Noguchi C, Kamitori K, Dong Y, Hirata Y, Hossain MA, et al. Rare sugar D-allenge strongly induces thioredoxin-interacting protein and inhibits osteoclast differentiation in Raw264 cells. Nutr Res. 2012;32:116–23.

35. Chong C-R, Chan WPA, Nguyen TH, Liu S, Procter NE, Ngo DT, et al. Thioredoxin-interacting protein: pathophysiology and emerging pharmacotherapeutics in cardiovascular disease and diabetes. Cardiovasc Drugs Ther. 2014;28:347–60.

36. Wong RW, Hagen T. Mechanistic target of rapamycin (mTOR) dependent regulation of thioredoxin interacting protein (TXNIP) transcription in hypoxia. Biochem Biophys Res Commun. 2013;433:40–6.
cells through accumulation of nuclear β-catenin. Anticancer Res. 2018;38:6299–308.

39. Shaikh D, Zhou Q, Chen T, Ibe JC, Raj JU, Zhou G. cAMP-dependent protein kinase is essential for hypoxia-mediated epithelial-mesenchymal transition, migration, and invasion in lung cancer cells. Cell Signal. 2012;24:2396–406.

40. Li Y, Qiu X, Zhang S, Zhang Q, Wang E. Hypoxia-induced CCR7 expression via HIF-1α and HIF-2α correlates with migration and invasion in lung cancer cells. Cancer Biol Ther. 2009;8:322–30.

41. Azakir BA, Desrochers G, Angers A. The ubiquitin ligase ITCH mediates the antiapoptotic activity of epidermal growth factor by promoting the ubiquitination and degradation of the truncated C-terminal portion of Bid. FEBS J. 2010;277:1319–30.

42. Gao M, Labuda T, Xia Y, Gallagher E, Fang D, Liu Y-C, et al. Jun hexokinases and direct regulation of thioredoxin-interacting protein in prognosis of breast cancer. Breast Cancer Res. 2010;12:1–15.

43. Hansen T, Rossi M, Roperch J, Ansell K, Simpson K, Taylor D, et al. ITCH inhibition regulates chemosensitivity in vitro. Biochem Biophys Res Commun. 2007;361:33–6.

44. Yoshioka J, Chutkow WA, Lee S, Kim JB, Yan J, Tian R, et al. Deletion of thioredoxin-interacting protein (txnip) is a glucocorticoid-regulated primary response gene involved in mediating glucocorticoid-induced apoptosis. Oncogene. 2006;25:1903–13.

45. Stoltzman CA, Peterson CW, Breen KT, Mueoio DM, Billin AN, Ayer DE. Glucose sensing by MondoA: MLX complexes: a role for hexokinases and direct regulation of thioredoxin-interacting protein expression. Proc Natl Acad Sci. 2008;105:6912–7.

46. Meierhofer D, Wang X, Huang L, Kaiser P. Quantitative analysis of global ubiquitination in HeLa cells by mass spectrometry. J Proteome Res. 2008;7:4566–76.

47. Shin D, Jeon J-H, Jeong M, Suh H-W, Kim S, Kim H-C, et al. VDUP1 mediates nuclear export of HIF-1α via CRM1-dependent pathway. Biochim Biophys Acta, Mol Cell Res. 2008;1783:838–48.

48. Nishizawa K, Nishiyama H, Matsui Y, Kobayashi T, Saito R, Kotani H, et al. Thioredoxin-interacting protein suppresses bladder carcinogenesis. Carcinogenesis. 2011;32:1459–66.

49. Woolston CM, Madhusudan S, Soomro IN, Lobo DN, Reece-Smith AM, Parsons SL, et al. Thioredoxin interacting protein and its association with clinical outcome in gastro-oesophageal adenocarcinoma. Redox Biol. 2013;1:285–91.

50. Woolston CM, Zhang L, Storr SJ, Al-Attar A, Shehata M, Ellis IO, et al. The prognostic and predictive power of redox protein expression for anthracycline-based chemotherapy response in locally advanced breast cancer. Mod Pathol. 2012;25:1106–16.

51. Lim JY, Yoon SO, Hong SW, Kim JW, Choi SH, Cho JY. Thioredoxin and thioredoxin-interacting protein as prognostic markers for gastric cancer recurrence. World J Gastroenterol. 2012;18:5581–8.

52. Le Jan S, Le Meur N, Cazes A, Philippe J, Le Cunff M, Léger J, et al. Identification of thioredoxin-interacting protein 1 as a hypoxia-inducible factor 1α-induced gene in pancreatic cancer. Pancreas. 2008;36:178–86.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Sun Q, Wang B-B, Wei W, Huang G-C, Liu L-L, Chen W-W, et al. ITCH facilitates proteasomal degradation of TXNIP in hypoxia-induced lung cancer cells. Thorac Cancer. 2022;13(15):2235–47. https://doi.org/10.1111/1759-7714.14552