RESEARCH ARTICLE

Hypoxia Induced High Expression of Thioredoxin Interacting Protein (TXNIP) in Non-small Cell Lung Cancer and its Prognostic Effect

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Abstract

Although associations between thioredoxin interacting protein (TXNIP) and cancers have been recognized, the effects of TXNIP on non-small cell lung cancer (NSCLC) prognosis remained to be determined in detail. In addition, while hypoxia is a key characteristic of tumor cell growth microenvironment, the effect of hypoxia on TXNIP expression is controversial. In this study, formaldehyde fixed and paraffin embedded (FFPE) samples of 70 NSCLC patients who underwent resection between January 2010 and December 2011 were obtained. Evaluation of TXNIP and hypoxia inducible factor-1α (HIF-1α) protein expression in FFPE samples was made by immunohistochemistry. By Kaplan-Meier method, patients with high TXNIP expression demonstrated a significantly shorter progression free survival (PFS) compared with those with low TXNIP expression (18.0 months, 95%CI: 11.7, 24.3 versus 23.0 months, 95%CI: 17.6, 28.4, *P*=0.02). High TXNIP expression level was also identified as an independent prognostic factor by Cox regression analysis (adjusted hazard ratio: 2.46; 95%CI: 1.08, 5.56; *P*=0.03). Furthermore, TXNIP expression was found to be significantly correlated with HIF-1α expression (Spearman correlation=0.67, *P*=0.000). To further confirm correlations, we established a tumor cell hypoxic culture model. Expression of TXNIP was up-regulated in all three NSCLC cell lines (A549, SPC-A1, and H1299) under hypoxic conditions. This study suggests that hypoxia induces increased TXNIP expression in NSCLC and high TXNIP expression could be a poor prognostic marker.

Keywords: Thioredoxin interacting protein - hypoxia inducible factor-1α - survival - prognosis

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Introduction

Thioredoxin interacting protein (TXNIP), also known as vitamin D3 upregulated protein (VDUP-1) or thioredoxin binding protein-2 (TBP-2), was originally reported as a gene of unknown function in HL-60 cells induced by 1α, 25-dihydroxyvitamin D3 (Chen and DeLuca, 1994). The human TXNIP gene is located on chromosome 1q21.1, contains 8 exons and is 4174 bp in length. The human TXNIP protein consists of 391 amino acids and has a molecular mass of 46 kDa. TXNIP belongs to alpha-arrestin protein family and is perhaps the only family member known to bind thioredoxin (TRX). TXNIP plays an important role in a wide variety of biological functions, such as the regulation of cell death, growth, differentiation, and energy metabolism (Aitken et al., 2004; Lee et al., 2005; Ahsan et al., 2006; Corbett, 2008; Oka et al., 2009). TXNIP is an important gene that is known to be transcriptionally regulated in response to hypoxia (Wong and Hagen, 2013). However, the effect of hypoxia on TXNIP expression in Non-small cell lung cancer (NSCLC) remained undefined.

One of the tumor progression characteristics is the tumor cell growth microenvironment turns to be a hypoxia condition. Tumor growth, invasion, energy metabolism and incompletion of the local blood vessels lead to a hypoxic state in local tissue. Tissue hypoxia induces a sustained increase in the expression of hypoxia inducible factor-1 (HIF-1). HIF-1 plays an important role in maintaining the energy metabolism of tumor cells, blood vessel expansion, angiogenesis, and cell proliferation (Fraga et al., 2009). The HIF-1β subunit is constitutively expressed whereas the HIF-1α subunit is oxygen regulated (Semenza, 2011). Regulation of HIF-1 is determined by the rapid posttranslational degradation or stabilization of the HIF-1α subunit. It has been widely reported that HIF-1α was highly expressed in NSCLC and related with poorer prognosis of NSCLC patients (Ping et al., 2013; Wang et al., 2014). However, the prognostic effect of TXNIP in NSCLC needs to be further determined.

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The aim of our study was to investigate the expression and the prognostic effect of TXNIP in NSCLC patients. Furthermore, the relationship between TXNIP and HIF-1α was studied. In addition, the influence of hypoxia microenvironment on TXNIP expression in NSCLC cell lines was also explored.

Materials and Methods

Patients and tissue samples

Formaldehyde fixed and paraffin embedded (FFPE) NSCLC samples of 70 patients who underwent surgery in the department of thoracic surgery, the Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China) between January 2010 and December 2011 were retrospectively studied. All the pathology diagnoses were reviewed by two senior pathologists (K Meng and J Yang) according to the WHO criteria (Brambilla et al., 2001). After pathological diagnoses, pTNM stages of 70 patients were designated according to the 7th edition of the TNM classification of malignant tumors (Goldstraw et al., 2007). The detailed data of all the patients showed in Table 1. The study was approved by the Ethic Committee of Drum Tower Hospital and each patient gave written informed consent.

Immunohistochemistry (IHC)

Evaluation of TXNIP and HIF-1α protein expression in FFPE samples was detected by IHC. Consecutive 3μm sections were cut and placed on polylysine coated slides for further analysis. Each paraffin section was deparaffinized and rehydrated through dimethylbenzene and graded alcohols. After the slides were prepared, antigen retrieval was carried by pressure cooking the slides with citric acid buffer (pH 6.0 for 1 min). 3% H2O2 was used to block the endogenous peroxidase activity. To block nonspecific staining, the slides were incubating with 5% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 15 min. The adjacent two slides were then incubated overnight at 4°C with mouse monoclonal TXNIP antibody (1:100 dilution, MBL, Japan), and rabbit polyclonal HIF1-α antibody (1:100 dilution, Abcam, USA), respectively. In order to reduce variability, all samples from each group were stained at the same time in a single experiment using a single batch of antibody diluted in PBS with BSA. Slides were then washed by PBS and incubated with biotinylated goat anti-rabbit IgG antibody (1:500 dilution, Abcam, USA) for half an hour at 37°C. The reaction product was developed by diaminobenzidine tetrahydrochloride. At the end, the slides were counterstained with hematoxylin. Thereafter, the tissues were washed in distilled water for 5 minutes, dehydrated sequentially and mounted in resinous mountant. Positive controls were included in each run, as well as negative controls were performed with no primary antibody.

Evaluation of TXNIP/HIF-1α expression

Evaluation of TXNIP and HIF-1α expression was independently performed by two independent pathologists who were blinded to clinical data. The immunostaining intensity and the percentage of positive cells distribution of TXNIP and HIF-1α were calculated for further analysis. Percentages of positive tumor cells defined in at least five areas by 400× magnification were averaged. The mean percentage was then designed to five gradations: 0, <5%; 1, 5%-25%; 2, 26%-50%; 3, 51%-75%; and 4, >75%. Staining intensity was calculated and marked as follows: weak, 1; moderate, 2; and intense, 3. The intensity and percentage scores were multiplied to yield a composite score of 0 to 12 for each sample. Composite scores of 0 to 3 were defined as indicating low TXNIP or low HIF-1α expression respectively, while scores of 4 to 12 were considered to indicate high TXNIP or high HIF-1α expression.

Cell culture and hypoxia model

NSCLC cell lines (A549, SPC-A1and H1299), human umbilical vascular endothelial cell line (HUVEC) and human fetal lung fibroblast cell line (HFL-1) were purchased from ATCC and maintained in our laboratory. All the cell lines were cultured in recommended growth medium in 37°C and 5% CO_2. As previously reported (Shi et al., 2013), a hypoxic cell culture model was established with hypoxic chamber in our laboratory. Three NSCLC cell lines, A549, SPC-A1 and H1299 were cultured under two different oxygen concentrations, 1% and 20%, respectively. For three-dimensional (3D) cell culture, each NSCLC cell line (1×10^6) was mixed with HUVEC cells (1×10^6) and HFL-1 cells (1×10^6) in Matrigel (BD Bioscience, USA). After 24 hours culture, cells were collected and total RNA were extracted by Trizol (Invitrogen, USA) for reverse transcription-polymerase chain reaction (RT-PCR) analyses.

Real time RT-PCR

cDNA was synthesized from 2μg of total RNA and random hexamers using the Takara Reverse Transcription kit (Takara, Japan). Quantitative RT-PCR was performed in 48-well plates using a StepOne Real-time PCR system (ABI, USA). All reactions were performed in triplicate and verified by melting curve analysis. The relative amount of mRNA in each sample was normalized to GAPDH transcript levels. Primer sequences were as follows: TXNIP, forward: 5'-AGAGCCAACAGAACAGAAGAA-3' and reverse: 5'-AGAGGCAGATCATTTAAGAGTG-3'; GAPDH, forward: 5'- CAATGACCCCTTCATTGACC -3' and reverse: 5'- TGGAAGATGGTGATGGGATT -3'.

Follow-up visit of patients

Patients with NSCLC were interviewed by telephone. The progression (recurrence or metastasis) of NSCLC was confirmed by radiology examination or medical record collected from the Drum Tower Hospital. Progression free survival (PFS) time was defined as the time elapsed since the resection of the tumor to the date of disease progression or death.

Statistical analysis

Differences between clinicopathological variables and the expression of TXNIP were examined by Chi-square test (or Fisher's exact test if any sample number was less

than 5). Correlation between TXNIP and HIF-1α was carried out by Spearman Correlation. Survival curves were calculated using the Kaplan-Meier method and compared by the log-rank test. Multivariate Cox regression model analysis was established to assess the prognostic values of protein expression. Relative expression of TXNIP in cultured NSCLC cells was expressed as mean±SEM. Statistical significance was determined by one-way ANOVA, LSD and unpaired t tests. All analyses were performed with SPSS software, version 16.0 (SPSS, Inc., Chicago, IL, USA). All tests were two-sided and performed at a significance level of 0.05.

Results

Expression of TXNIP was related with HIF-1α expression in NSCLC tumor samples

HIF-1α was up-regulated in 51.4% (36/70) NSCLC tumor samples in our study and HIF-1α was mainly located in tumor cell nucleus as indicated in Figure 1b. While TXNIP protein was located mainly in cytoplasm of NSCLC cells as shown in Figure 1d. A total of 45 patients (64.3%) showed TXNIP high expression. Interestingly, the distribution of HIF-1α expression in tumor section was significantly related with the distribution of TXNIP expression (Figure 1a and Figure 1c both negative; Figure 1b and Figure 1d both positive in the same tumor section). In addition, expression level of TXNIP was significantly correlated with HIF-1α expression level (Figure 1e, Spearman Correlation=0.67, *P*=0.000).

TXNIP expression was up-regulated in NSCLC cell lines under hypoxic condition

To further confirm the relationship between the expression of TXNIP and that of HIF-1α in NSCLC, we established a tumor cell hypoxic culture model. Expression of TXNIP was up-regulated in all three NSCLC cell lines (A549, SPC-A1, and H1299) under hypoxic culture condition (1% O2) (Figure 2a). To mimic the tumor microenvironment, we mixed each of three NSCLC cells with HUVEC and HFL-1 cells in Matrigel (Figure 2b). Consistent with common culture, expression of TXNIP was up-regulated in all three NSCLC 3D culture models under hypoxic culture condition (1% O2) (Figure 2c).

Patients with high TXNIP expression level had poorer prognosis

The median PFS of 70 included NSCLC patients was 19.0 months (95%CI: 16.9, 21.1). Clinicopathological characteristics of NSCLC patients are listed in Table 1 by TXNIP expression level. There were no significant differences in age, gender, cell type, and pTNM stage between patients with high and low TXNIP expression (Ps>0.05). The median PFS in patients with high HIF-1α expression was significantly shorter than that in those with low HIF-1α expression [14.0 months (95%CI: 10.0, 18.0) *vs* 23.0 months (95%CI: 20.5, 25.5), *P*=0.002]. Similarly, the median PFS was 18.0 months (95%CI: 11.7, 24.3) in patients with high TXNIP expression and 23.0 months

Table 1. Characteristics of 70 Non-small Cell Lung Cancer Patients

Patient Characteristics	TXNIP Expression		P value
	High (45) No. (%)	Low (25) No. (%)	
Age			
<60 years old	18 (40)	10 (40)	1.00
≥60 years old	27 (60)	15 (60)	
Gender			0.40
Female	10 (22.2)	8 (32.0)	
Male	35 (77.8)	17 (68.0)	
Cell type			0.44
Adenocarcinoma	30 (66.7)	14 (56.0)	
Squamous	15 (33.3)	11 (44.0)	
pTNM stage			0.98
I	22 (48.9)	12 (48.0)	
II	13 (28.9)	7 (28.0)	
III	10 (22.2)	6 (24.0)	
HIF-1α expression			<0.001
High	34 (75.6)	2 (8.0)	
Low	11 (24.4)	23 (92.0)	

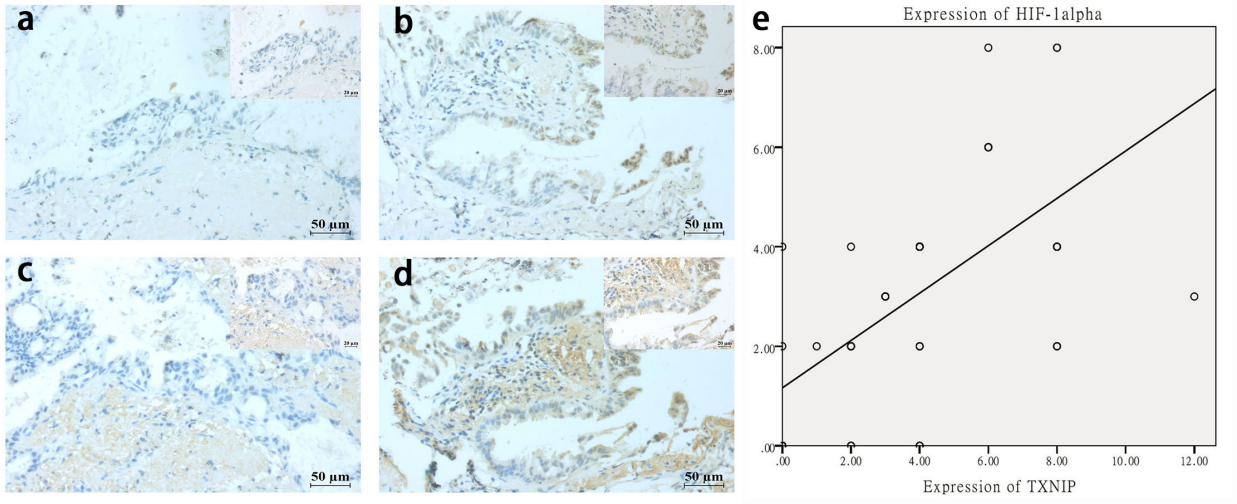

Figure 1. Expression of TXNIP and HIF-1α in NSCLC Tumor Samples. a and b, Negative and positive expression of HIF-1α on the same section of one sample. c and d, Negative and positive expression of TXNIP at the same positions on the same sections as in a and b correspondingly. e, The relation between TXNIP expression and HIF-1α expression, Spearman Correlation=0.673, *P*=0.000. Scale bar, as indicated in the figure

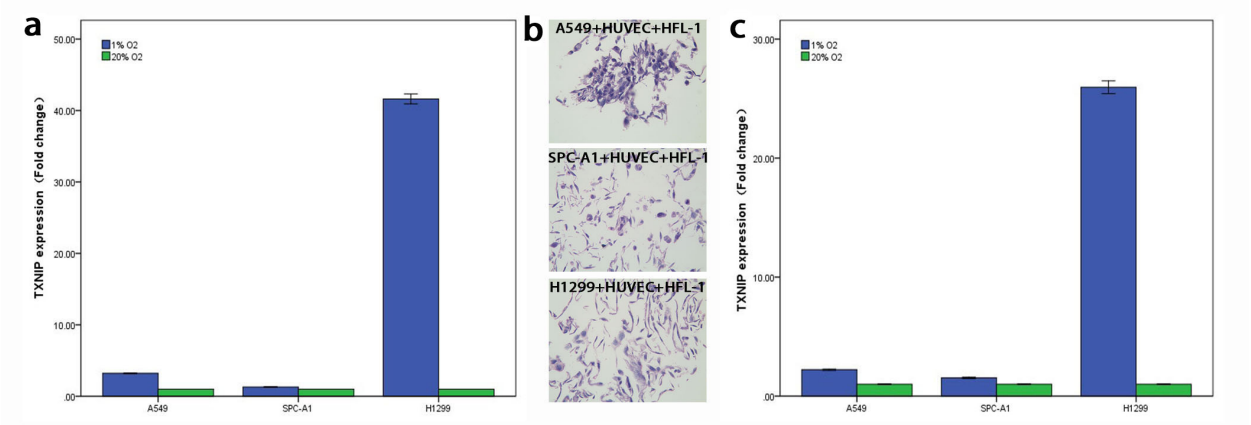

Figure 2. Expression of TXNIP in NSCLC Cell Lines Under Hypoxic Culture Condition. NSCLC cells alone or mixed with interstitial cells were cultured under different oxygenic conditions (1% O_2 and 20% O_2). a, Expression of TXNIP was up-regulated in all three NSCLC cell lines (A549, SPC-A1, and H1299) under hypoxic culture condition (1% O_2). b, H&E staining of the mixed culture (3D culture model) of NSCLC cells with interstitial cells (human umbilical vascular endothelial cell (HUVEC) and human fetal lung fibroblast cell (HFL-1)). It could mimic the real tumor microenvironment. c, Expression of TXNIP was up-regulated in all three NSCLC 3D culture models under hypoxic culture condition (1% O_2)

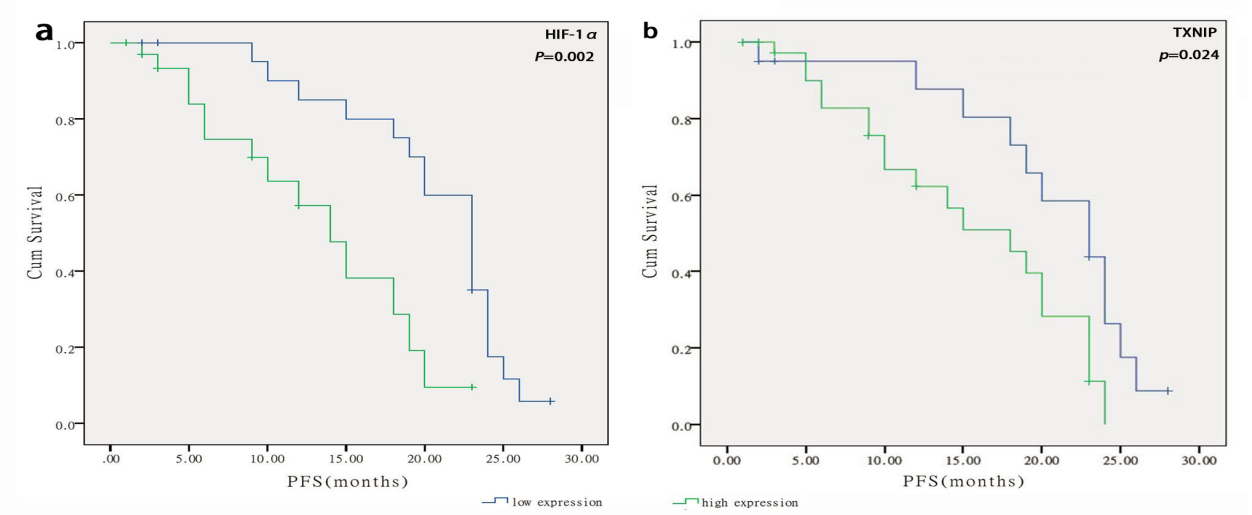

Figure 3. Prognostic Effects of TXNIP and HIF-1α in NSCLC. a, The median PFS in patients with high HIF-1α expression was significantly shorter than that in those with low HIF-1α expression [14.0 months (95% CI: 10.0, 18.0) *vs* 23.0 months (95% CI: 20.5, 25.5), P=0.002]. b, The median PFS was 18.0 months (95% CI: 11.7, 24.3) in patients with high TXNIP expression and 23.0 months (95% CI: 17.6, 28.4) in those with low TXNIP expression (P=0.02)

(95%CI: 17.6, 28.4) in those with low TXNIP expression. The Kaplan-Meier PFS curve showed a significant separation (*P*=0.02, Figure 3). In univariate analysis, there was a significant association of TXNIP expression level with PFS (hazard ratio: 2.32; 95%CI: 1.05, 5.16; *P*=0.04). In multivariate analysis adjusting for age, gender, cell type, and pTNM stage, the significance remained (hazard ratio: 2.46; 95%CI: 1.08, 5.56; *P*=0.03).

Discussion

In present study, we showed high TXNIP expression was associated with a shorter PFS in NSCLC patients. More importantly, our study demonstrated that TXNIP expression was related with HIF-1α expression in NSCLC and up-regulated under hypoxic condition.

Hypoxia inducible factor-1 (HIF-1) is the first identified mediator of cell response to hypoxia in mammalian cells cultured under reduced oxygen tension (Semenza and Wang, 1992). This transcription factor is a heterodimer composed of two subunits: an oxygen-sensitive HIF-1α and a constitutively expressed HIF-1β. The subunits of HIF-1 bind together to acquire transcriptional properties, allowing it to regulate the transcriptional activity of hundreds of genes that promote cancer cell survival in hypoxic conditions. Levels of HIF-1α protein increase exponentially as oxygen concentration declines (Semenza, 2011). One of the tumor progression characteristics is the tumor cell growth microenvironment turns to be a hypoxia condition. Tumor growth, invasion, energy metabolism and incompletion of the local blood vessels lead to a hypoxic state in local tissue. TXNIP has been shown to be a hypoxia-induced gene in human microvascular endothelial cells (Le Jan et al., 2006) and in murine heart (Karar et al., 2007). TXNIP has also been reported to be induced during hypoxia in a HIF-dependent manner in pancreatic cancer (Baker et al., 2008). In our study, TXNIP expression was up-regulated in NSCLC cell lines under hypoxic condition, which is consistent with previous studies. The mechanism through which TXNIP expression

was regulated by hypoxia has not been confirmed. Some report showed the regulation of TXNIP in hypoxia was mediated via the inhibition of 4E-BP1/eIF4E axis of mTORC1 (Wong and Hagen, 2013). On the other hand, TXNIP may also have some effect on HIF-1α expression. Some study reported that TXNIP might mediate HIF-1α translocation from the nucleus to the cytoplasm and target HIF-1α to degradation (Shin et al., 2008). These results suggested that there might be more complex mechanisms involving TXNIP and HIF-1α in cancer cell survival, which need further investigating.

TXNIP was originally discovered by virtue of its strong regulation by vitamin D. Expression of TXNIP was upregulated in HL-60 cells stimulated with 1α, 25-dihydroxyvitamin D3, and was augmented by glucose, adenosine-containing molecules, the HDAC inhibitor SAHA, 5-fluorouracil, deprivation of serum or IL-2 in cell culture, carcinogens, ceramide, etoposide, hydrogen peroxide (H_2O_2), heat shock, TGF-β, ultraviolet light, glucocorticoid, allose treatment and LPS (Masutani et al., 2012). Expression level of TXNIP is influenced by a variety of conditions, suggesting different roles in different biological processes including apoptosis, growth, and differentiation. Some reports have shown downregulation of TXNIP expression in tumor cells and could be up-regulated by some anticancer agent (Yoshioka et al., 2006). However, high expression of TXNIP was here observed in NSCLC cell lines and was associated with shorter PFS in NSCLC patients.

Many apoptotic stimuli including anisomysin, oxidative stress, and suberoylanilide hydroxamic acid could induce the rapid upregulation of TXNIP (Butler et al., 2002). However, the effects of enforced TXNIP overexpression on apoptosis were different depending on cell types. In NIH3T3 fibroblasts, SNU stomach tumor cells, and 293 cells, TXNIP overexpression was not sufficient to induce apoptosis (Junn et al., 2000). Previous studies that used a high concentration of reactive oxygen species (ROS) indicated TXNIP was proapoptotic via activation of multiple death pathways (Chen et al., 2008). However, recent data show that in response to physiological levels of ROS, TXNIP is actually prosurvival (World et al., 2011). TXNIP promotes inflammation in endothelial cells (EC) by binding to thioredoxin-1 (TRX1) in a redox-dependent manner. The TXNIP-TRX1 complex acts as a redox-sensitive mediator to control VEGFR2 signaling, thereby promoting cell survival under conditions of oxidative stress. Different associations of TXNIP in distinct tumor cells imply that TXNIP might play multiple roles.

In conclusion, we investigated the expression of TXNIP in NSCLC and correlated the expression level of TXNIP with poor prognosis. Although it is obviously necessary to study much more cases, these findings suggest that TXNIP might be a useful biomarker to predict the prognosis of NSCLC patients. We also showed that TXNIP was induced in response to hypoxia in NSCLC. A better understanding of the regulatory mechanisms controlling TXNIP expression may prove beneficial in the development of additional therapeutic strategies to modulate TXNIP in cancer.

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