

## Original Article

# Expression of thioredoxin reductase-1 and its effect in non-small cell lung cancer

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**Abstract:** Objective: We detected the expression of thioredoxin reductase-1 (TrxR1) in non-small cell lung cancer (NSCLC) and the effect of TrxR1 silencing in NSCLC. Method: TrxR1 expression was detected in 118 cases with NSCLC and 27 normal subjects using immunohistochemistry technique and the correlation with pathological parameters of NSCLC was analyzed. Semi-quantitative RT-PCR was employed to detect the TrxR1 mRNA level in 13 cases with NSCLC and 4 normal subjects; Western Blot was used to detect the TrxR1 protein level in normal lung epithelium and lung cancer cells; semi-quantitative RT-PCR was used to detect TrxR1 mRNA level in normal lung epithelium and lung cancer cells. After silencing of TrxR1 in A549 cells, the proliferation of cells was compared with that before silencing. Result: Immunohistochemistry and semi-quantitative PCR indicated overexpression of TrxR1 in NSCLC ( $P < 0.05$ ). After silencing of TrxR1 using shRNA, the cell proliferation was obviously reduced. Conclusion: TrxR1 mRNA and protein were overexpressed in NSCLC, indicating that TrxR1 may be a target molecule in NSCLC.

**Keywords:** Non-small cell lung cancer, thioredoxin reductase-1, target molecule, proliferation

## Introduction

Lung cancer is the leading cause of cancer death throughout the world, and non-small cell lung cancer (NSCLC) accounts for 85% [1]. NSCLC is less responsive to chemotherapy and radiotherapy and the targeted therapy of NSCLC is now under study. EGFR tyrosine kinase inhibitor (TKI) has been successfully applied to EGFR-mutant NSCLC and achieved longer progress free survival (PFS) with less side effects [2]. The proportion of EGFR-mutant NSCLC is higher in China than in western countries, but according to some literature, this proportion is not higher than 20%. Only limited number of NSCLC patients can benefit from TKI [3], and so the task is to screen for new targets in NSCLC. Thioredoxin reductase-1 (TrxR1) plays an important role in tumor occurrence and progression [4]. In this experiment, we detected the expression of TrxR1 in NSCLC and evaluated the effect of TrxR1 silencing so as to determine whether TrxR1 can be a candidate therapeutic target in NSCLC.

## Materials and methods

### Materials

From 2010 to 2013, 118 NSCLC cases treated at Zhengzhou Sixth People's Hospital and the Third People's Hospital Affiliated to Shanghai Jiaotong University School of Medicine were recruited (68 males, 50 females, aged 38-81 years, 59.44 years on average). According to 2004 WHO classification of lung cancer, there were 31 cases of squamous cell carcinoma, 63 cases of adenocarcinoma and 24 cases of adenosquamous carcinoma; in terms of degree of differentiation, there were 33 cases with high differentiation, 26 cases with moderate differentiation and 59 cases with low differentiation; as to pTNM staging (1997 UICC criteria), there were 47 cases classified as stage I+II and 71 cases as stage III. For the control group, 27 normal lung specimens were collected from 27 cases, including 17 non-tumor specimens by autopsy, 7 surgically resected inflammatory pseudotumors and 3 specimens of pulmonary bulla.

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### Method

**Immunohistochemistry:** All specimens were fixed in 10% neutral formalin, dehydrated, embedded in paraffin, sectioned and dewaxed conventionally. Primary antibody against TrxR1 (1:5) was purchased from Cell Signal and secondary antibody was purchased from DAKO. Immunohistochemistry kit was purchased from Wuhan Boster Biological Engineering Co., Ltd. DAB color development kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. The procedures were performed according to the manufacturer's instruction of the kit. The staining of TrxR1 was interpreted according to the scoring criteria by Shimizu et al. [5]: percentage of positive cells <5%, 5%-24%, 25%-50%, 51%-74% and >75% were scored as 0, 1, 2, 3 and 4, respectively. The intensity of staining for each slice was evaluated by the color, i.e., no color, light yellow, brownish yellow and brown, with the score of 0, 1, 2 and 3, respectively. The two scores were added up and the score of 0-1 was considered negative (-), 2-3 weakly positive (+), 4-5 moderately positive (++) and 6-7 strongly positive (+++).

**Total RNA extraction:** For each degree of differentiation, 5 paraffin-embedded specimens were selected and 5 specimens of juxtacancerous tissues were used as controls. Each specimen was cut into 6 slices 6  $\mu$ m thick, which were placed into 1.5 ml RNase-free centrifuge tube. After adding 1 ml of dimethylbenzene, the tube was mixed by vortex for 10 s and then centrifuged at 12000 r/min for 2 min. The supernatant was removed with a pipette. Caution was taken not to suck out the precipitate. Next the precipitate was dissolved in 1 ml of anhydrous alcohol and washed once. The tube cap was removed and left to stand at 37°C for 10 min until the residual alcohol completely evaporated. Other procedures were performed according to the manufacturer's instruction of total RNA extraction kit (DP439). Finally 30  $\mu$ l RNase-free ddH<sub>2</sub>O was added dropwise. The tube was left to stand at room temperature for 2 min and centrifuged at 12000 r/min for 2 min to obtain the RNA solution.

**Cell culture:** The 293T cells were preserved at our laboratory. Normal bronchial epithelial cell line 16HBE and human lung cancer cell line H1299, H1650, H1975 and A549 were pur-

chased from Shanghai Institute of Biochemistry and Cell Biology, CAS. All cells were cultured in DMEM containing 10% fetal bovine serum (GIBCO) and 100 U/ml penicillin-streptomycin (GIBCO) and placed in a 5% CO<sub>2</sub> humidified incubator at 37°C. The cells were digested with 0.25% trypsin and passaged when a single layer of adherent cells grew to 70%-80% confluence.

**Construction of PSIREN-TrxR1/shRNA vector:** The gene accession number of TrxR1 was searched in NCBI. Using the design principle proposed by Reynolds et al., 3 loci were selected as the target sequences: Sh1: AAGG-TGATGGTCCTGGACTTT; Sh2: AATTATTGGTCC-TCACAGGA; Sh3: AAATCATTGAAGGAGAATATA. It was ensured that the selected sequences were not homologous to other genes using BLAST program. For the negative control, the sequence NC: ACTCCGACGAAGCAGCATA was used after random disordering. This sequence was also aligned against the human and mouse Genbank using BLAST program to ensure that it was not homologous with any genes. The vector was constructed as BamHI + Sense + Loop + Antisense + TTTTT + EcoRI. Each sequence was composed of 60 nucleotides; between the sense strand and the antisense strand 9 oligonucleotides (TTCAAGAGA) were added to form the hairpin structure, with TTTTT as the stop codon. The 5' terminal was the restriction site of BamH I, and the 3' terminal was the restriction site of EcoR I. Thus the oligonucleotide sequence was cloned to the vector by restriction enzyme digestion. The upstream and downstream of 5 pairs of oligonucleotide sequences were annealed. According to BD™ Knockout RNAi Systems User Manual, the recombinant plasmid was obtained by cloning dsDNA to RNAi-Readv pSIREN-Shuttle vector. The vectors were named as NC, sh1, sh2 and sh3, respectively.

**Packaging of PSIREN-TrxR1/shRNA viral particle and infection of A549 cells:** The 293T cells were inoculated to 2 6-well plates at 2 ml for each well (6 $\times$ 10<sup>5</sup> cells). The fusion was observed after cell culture in the incubator for 12 h. Viral packaging was performed as follows when the cells grew to about 70%-80% confluence after 12 h: plasmid concentration was 1  $\mu$ g/ $\mu$ l; Gag-pol, Vsv-g and target plasmids (TrxR1-knockout sh1, sh2, sh3 and empty NC) were used; for

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**Table 1.** Correlation between TrxR1 expression and clinicopathologic parameters of NSCLC

| Clinicopathologic parameters | n  | TrxR1 |                    | P value |
|------------------------------|----|-------|--------------------|---------|
|                              |    | +     | ~+++ (%)           |         |
| Gender                       |    |       |                    | 0.219   |
| Male                         | 68 | 60    | (88.24)            |         |
| Female                       | 50 | 40    | (80.00)            |         |
| Age (years)                  |    |       |                    | 0.067   |
| ≥60                          | 69 | 62    | (89.85)            |         |
| <60                          | 49 | 38    | (77.55)            |         |
| Smoking history              |    |       |                    | 0.219   |
| Yes                          | 68 | 60    | (88.24)            |         |
| No                           | 50 | 40    | (80.00)            |         |
| Pathological classification  |    |       |                    | 0.915   |
| Adenocarcinoma               | 63 | 53    | (84.13)            |         |
| Squamous cell carcinoma      | 31 | 26    | (83.87)            |         |
| Adenosquamous carcinoma      | 24 | 21    | (87.50)            |         |
| Differentiation degree       |    |       |                    | 0.000   |
| High + moderate              | 59 | 41    | (69.49)            |         |
| Low                          | 59 | 59    | (100) <sup>a</sup> |         |
| TNM staging                  |    |       |                    | 0.012   |
| I+II                         | 47 | 35    | (74.47)            |         |
| III                          | 71 | 65    | (91.55)*           |         |
| Lymph node metastasis        |    |       |                    | 0.012   |
| No                           | 47 | 35    | (74.47)            |         |
| Yes                          | 71 | 65    | (91.55)*           |         |

\*P<0.05; <sup>a</sup>P<0.01.

each well, Gag-pol 0.75 µl, Vsv-g 0.5 µl, target plasmid 0.75 µl, lipo2000 6 µl and opti-mem 94 µl were added, respectively. Before transfection, the A549 cells reaching the log phase of growth were inoculated to a 6-well plate at 2×10<sup>5</sup> cells for each well. When the cells became adherent, 2 ml viral supernatant and polybrene (8 µg/ml) were added into each well. After crossed mixing, the plate was transferred to the incubator. Equal amount of culture solution was added after 24 h and puro (2 µg/µl) was added after 48 h for screening purpose.

**Western blot:** The cells were collected and total protein was extracted. The protein sample was combined with an appropriate amount of loading buffer and boiled in 100°C water for 5 min for denaturation. The protein sample was analyzed by SDS-PAGE and electro-transferred to PVDF membrane, which was sealed with 5% defatted milk powder. The primary antibody against TrxR1 and GAPDH were purchased from Cell Signal and added at the proportion of

1:1000 to culture the cells at 4°C overnight. The membrane was washed with TBS three times for 10 min each time. Then secondary antibody (1:5000) was added to incubate the cells at room temperature for 60 min. The membrane was washed with TBS three times for 10 min each time. The protein bands were visualized by color development using chemiluminescent kit (Pierce), with GAPDH as internal reference. The integral optical density was calculated using image analysis software.

**Semi-quantitative RT-PCR:** The collected cells were washed with pre-cooled PBS. Total RNA extraction was performed using Trizol (15596-026, Invitrogen) according to the instruction. The extracted total RNA was dissolved in DEPC-treated water. The absorbance was measured at 260 nm and 280 nm using UV-spectrophotometer. OD<sub>260</sub>/OD<sub>280</sub>>1.8 indicated high purity. Reverse transcription and PCR were carried out for TrxR1 in each group according to the user manual of RT-PCR kit (Takara), with GAPDH as internal reference. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd: for TrxR1, forward primer 5'-AGTAGT-AGCTCAGTCCACCA-3', reverse primer 5'-GG-CACATTGGTCTGTTCTTCA-3'; the length of the product was 177 bp. For GAPDH, forward primer 5'-CCTTCATTGACCTCAACTA-3', reverse primer 5'-GGAAGCCATGCCAGTGAGC-3'; the length of the product was 594 bp. PCR conditions: 94°C 2 min, 94°C 30 s, 55°C 30 s, 72°C 1 min, 30 cycles for TrxR1 and 25 cycles for GAPDH. RT-PCR products were identified by 1.5% agarose gel electrophoresis and the integral optical density was calculated with image analysis software.

**Cell proliferation assay using CCK-8:** The cells reaching the log phase of growth were digested by 0.25% trypsin and then counted. The cells were inoculated to a 96-well plate at 2500 cells per well. Three replicate wells were set up. After 48 h, 20 µl CCK-8 solution was added into each well according to instruction and the cells were further incubated for 2 h. The absorbance was measured with a microplate reader (detection wavelength 450 nm, reference wavelength 630 nm).

**Statistical process:** All experiments were repeated 3 times. The data were analyzed sta-

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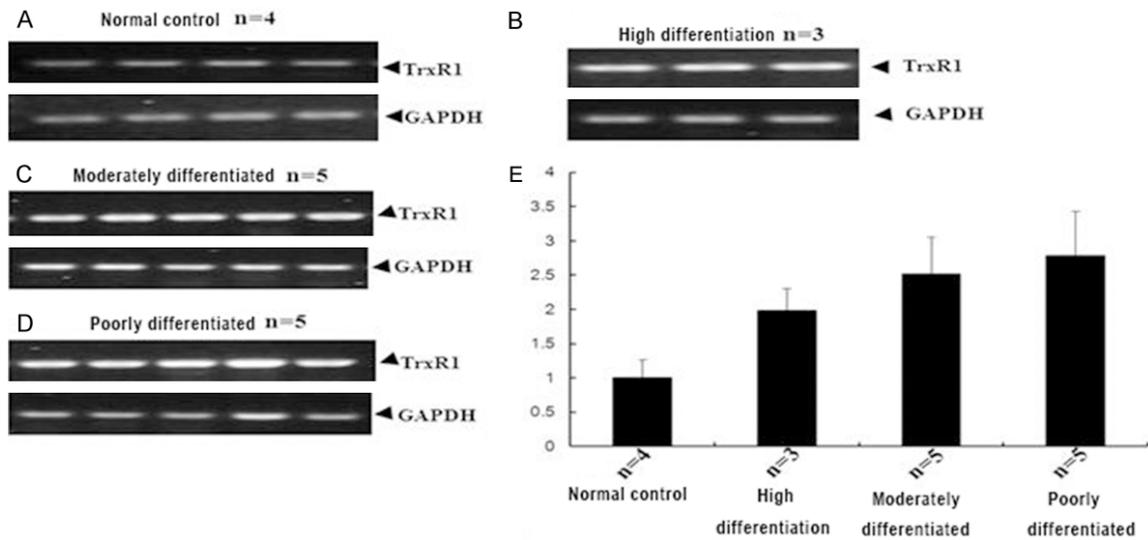


Figure 1. TrxR1 mRNA expression in NSCLC.

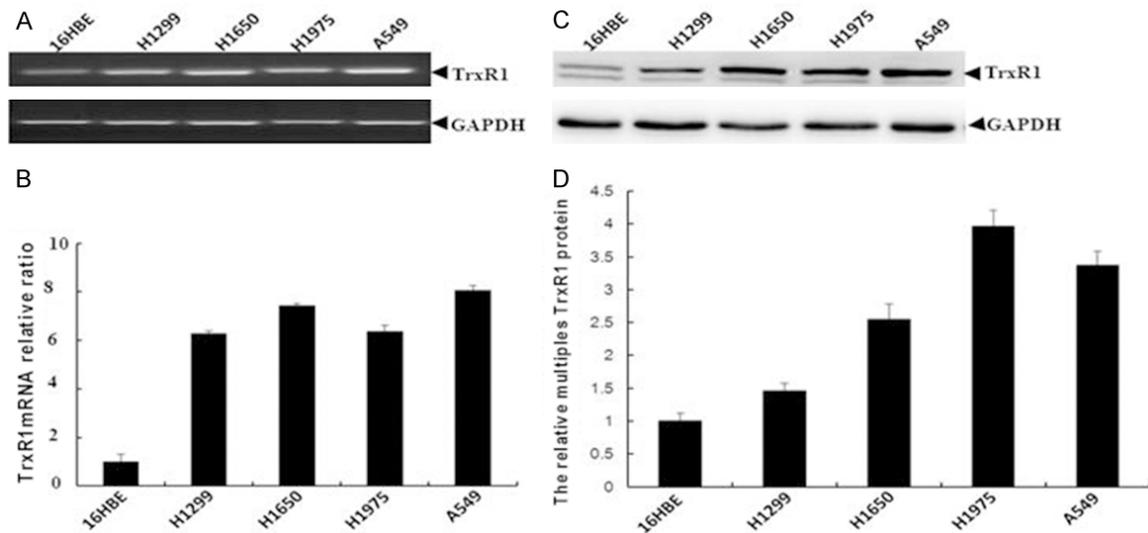


Figure 2. Expression of TrxR1 in 16HBE cells and H1299, H1650, H1975 and A549 cells.

tistically using SPSS 11.5 software by one-way ANOVA and t-test.  $P < 0.05$  indicated statistical significance.

### Results

#### Correlation between TrxR1 expression and clinicopathologic parameters of NSCLC

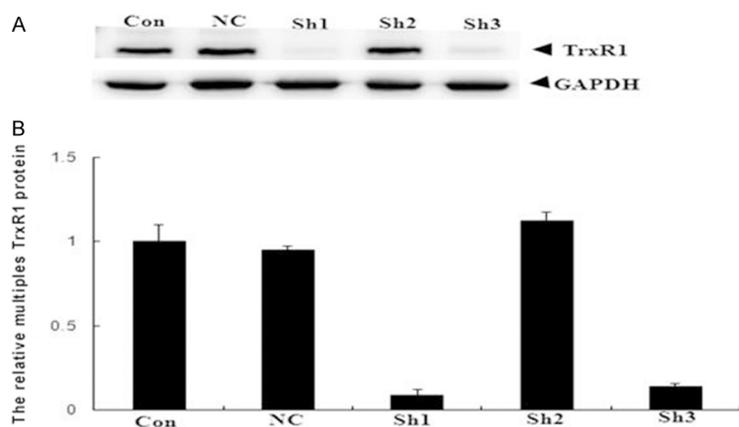
TrxR1 was expressed in all types of NSCLC tissues, with overall positive rate of 85% (100/118). TrxR1 was mainly found in the cytoplasm and rarely in the nuclei; TrxR1 was lowly expressed in normal lung tissues. TrxR1 expres-

sion was not correlated with gender, age, smoking history and pathological classification of NSCLC patients, but correlated with histological staging and TNM staging. As to the correlation with differentiation degree, the positive rate of TrxR1 was higher in low differentiation group than in moderate and high differentiation group ( $P < 0.05$ , Table 1).

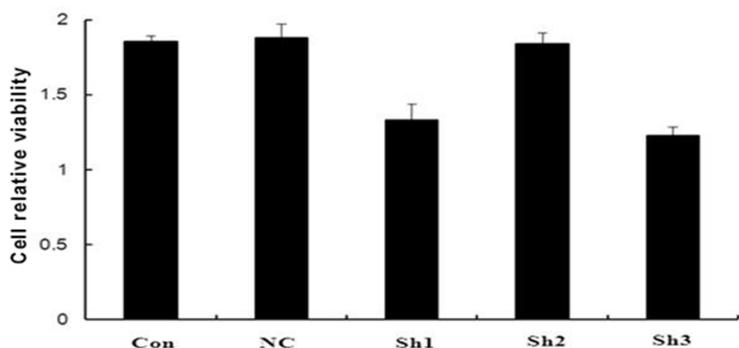
#### TrxR1 mRNA expression in NSCLC

TrxR1 mRNA expression was detected in 13 specimens with different differentiation degree and 4 specimens of normal lung tissues. Total

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**Figure 3.** Effect of TrxR1 silencing on cell proliferation and cell cycle.



**Figure 4.** Effect of TrxR1 silencing on cell proliferation.

RNA was extracted and detected for TrxR1 mRNA expression by RT-PCR. The results indicated that TrxR1 mRNA expression level in NSCLC tissues of different differentiation degree was always higher than that in normal precancerous lesions. TrxR1 mRNA expression was correlated with the differentiation degree, i.e., the lower the differentiation degree, the higher the expression level (**Figure 1**).

### *Expression of TrxR1 in normal bronchial epithelial cells and lung cancer cell lines*

TrxR1 mRNA expression was detected in 16HBE cells and H1299, H1650, H1975 and A549 cells. It was found that TrxR1 mRNA was lowly expressed in 16HBE cells and the expression level in lung cancer cells was significantly higher than that in 16HBE cells on average (**Figure 2A** and **2B**). According to Western Blot detection, the variation trend of TrxR1 protein expression was consistent with that of TrxR1 mRNA expression in either cell line (**Figure 2C** and **2D**).

### *Effect of TrxR1 silencing on the proliferation and cell cycle of cancer cell lines*

Three shRNA sequences targeting at TrxR1 were used for the silencing of TrxR1 in A549 cells. Western Blot was performed and the results showed that only sh1 and sh3 could induce significant silencing of TrxR1 in A549 cells ( $P < 0.05$ , **Figure 3**). The cell proliferation was detected by CCK-8 assay. It was found that the proliferation of A549 cells decreased obviously after silencing by sh1 and sh3 as compared with control group, random sequence group and non-silencing group ( $P < 0.05$ , **Figure 4**).

### **Discussion**

Thioredoxin system consists of Trx, TrxR (or TR) and nicotinamide adenine dinucleotide phosphate (NADPH). It is an NADPH-dependent disulfide reductase system with extensive distribution [5]. Thioredoxin system plays crucial roles in the

proliferation and differentiation of tumor cells after its discovery as a new tumor-related protein. TrxR, a dimeric flavine enzyme, is the core of thioredoxin system and belongs to the family of pyridine nucleotide-disulphide oxidoreductases. With wide expressions in nearly all types of cells ranging from prokaryotes to human beings, TrxR has 3 isoforms, which are cytosolic TrxR1, mitochondrial TrxR2 and testis-specific TrxR3 (or TGR) [6]. We are unclear about the expression and function of TrxR in lung cancer tissues, and this study aimed to solve this problem.

Immunohistochemistry found that TrxR1 expression was higher in the lung adenocarcinoma tissues than in precancerous tissues, higher in the lowly differentiated lung cancer tissues than in highly differentiated tissues, and also higher in patients with lymph node metastasis than in those without lymph node metastasis. Thus TrxR1 overexpression was closely related to malignancy degree, prolifera-

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tive ability and metastatic ability of the lung cancer cells. Semi-quantitative RT-PCR was used to detect TrxR1 mRNA expression in normal lung tissues and lung cancer tissues with varying differentiation degree. It was found that the TrxR1 mRNA expression was always higher in lung cancer tissues than in normal lung tissues. TrxR1 mRNA expression increased with the malignancy degree of lung cancer, which was consistent with immunohistochemistry detection. Lincoln et al. [7-9] adopted immunohistochemistry method to detect TrxR1 expression in breast cancer, thyroid cancer, colorectal cancer, prostate cancer and malignant melanoma and obtained similar results as ours.

We further detected TrxR1 mRNA and protein expressions in normal bronchial epithelial cells (16HBE) and lung cancer cell lines. There are three isoforms of TrxR, TrxR1, TrxR2 and TrxR3. TrxR1 and TrxR2 are expressed in many tissues; TrxR2 is the mitochondrial form, while TrxR3 is testis specific. The relative molecular mass of TrxR1 and TrxR2 is  $7.1 \times 10^4$  and  $56.2 \times 10^3$ , respectively. In this experiment, 2 bands were detected in 16HBE cells and lung cancer cells. Judged by the molecular mass, the stronger band was TrxR1, and the weaker one was TrxR2. The results showed that TrxR1 mRNA and protein expressions were significantly higher in lung cancer cells than in normal cells. It should be noted that the mRNA stability, efficiency of protein translation and protein stability vary for each type of cell. In H1299 cells, it is possible that the efficiency of translating TrxR1 mRNA into protein is lower than that of other cells, probably due to lower stability of TrxR1 in H1299 cells. However, in H1975 cells, the efficiency of translating TrxR1 mRNA into protein is higher, probably due to higher stability of TrxR1 in this type of cells. More evidences are required to confirm this hypothesis. Considering the diversity of bioactivity of TrxR, TrxR plays an important role in regulating oxidation-reduction reaction, cell growth and proliferation [10]. We observed the proliferation of lung cancer cells after silencing of TrxR1 and found that the cell proliferation decreased greatly due to reduced expression of TrxR1. Utilizing the electrons supplied by NADPH, TrxR1 can reduce all sources of Trx-S1. The reducing system formed by TrxR1 and reducible Trx can fulfill the functions of defending oxidative stress and maintaining oxidation-reduction balance [11]. As an antioxidant enzyme, TrxR1

not only catalyzes the reduction of Trx, but also the reduction of non-disulfide substrates, including hydroperoxide, vitamin C, selenite, alloxanate, and 2-nitrobenzoic acid [12]. Based on the above understanding and the experimental result, we believe that TrxR1 is associated with the enhancement of antioxidant capacity of lung cancer cells. It may be that the overexpression of TrxR1 leads to drug resistance of lung cancer cells. We need more data to corroborate this.

We found that TrxR1 played an important role in the antioxidant capacity and proliferation of lung cancer cells. TrxR1 may be the candidate therapeutic target for treating lung cancer by inhibiting the proliferation of the lung cancer cells.

### Disclosure of conflict of interest

None.

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### References

- [1] Liang JL, Ren XC and Lin Q. Treating advanced non-small-cell lung cancer in Chinese patients: focus on icotinib. *Onco Targets Ther* 2014; 7: 761-70.
- [2] Remon J, Morán T, Reguart N, Majem M, Carcereny E and Lianes P. Beyond EGFR TKI in EGFR-mutant non-small cell lung cancer patients: main challenges still to be overcome. *Cancer Treat Rev* 2014; 40: 723-9.
- [3] Li W, Zhou F and Zhou C. Role of erlotinib in the targeted treatment of non-small-cell lung cancer in Chinese patients. *Onco Targets Ther* 2014; 7: 253-61.
- [4] Pennington JD, Jacobs KM, Sun L, Bar-Sela G, Mishra M and Gius D. Thioredoxin and thioredoxin reductase as redox-sensitive molecular targets for cancer therapy. *Curr Pharm Des* 2007; 13: 3368-77.
- [5] Sengupta R and Holmgren A. Thioredoxin and glutaredoxin-mediated redox regulation of ribonucleotide reductase. *World J Biol Chem* 2014; 5: 68-74.
- [6] Lu J and Holmgren A. Thioredoxin system in cell death progression. *Antioxid Redox Signal* 2012; 17: 1738-47.
- [7] Cadenas C, Franckenstein D, Schmidt M, Gehrmann M, Hermes M, Geppert B, Schormann

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- W, Maccoux LJ, Schug M, Schumann A, Wilhelm C, Freis E, Ickstadt K, Rahnenführer J, Baumbach JI, Sickmann A and Hengstler JG. Role of thioredoxin reductase 1 and thioredoxin interacting protein in prognosis of breast cancer. *Breast Cancer Res* 2010; 12: R44.
- [8] Cadenas C, Franckenstein D, Schmidt M, Gehrmann M, Hermes M, Geppert B, Schormann W, Maccoux LJ, Schug M, Schumann A, Wilhelm C, Freis E, Ickstadt K, Rahnenführer J, Baumbach JI, Sickmann A and Hengstler JG. Thioredoxin and thioredoxin reductase expression in thyroid cancer depends on tumour aggressiveness. *Anticancer Res* 2010; 30: 767-75.
- [9] Lincoln DT, Ali Emadi EM, Tonissen KF and Clarke FM. The thioredoxin-thioredoxin reductase system: over-expression in human cancer. *Anticancer Res* 2003; 23: 2425-33.
- [10] Rundlöf AK and Arnér ES. Regulation of the mammalian selenoprotein thioredoxin reductase 1 in relation to cellular phenotype, growth, and signaling events. *Antioxid Redox Signal* 2004; 6: 41-52.
- [11] Barrera LN, Cassidy A, Wang W, Wei T, Belshaw NJ, Johnson IT, Brigelius-Flohé R and Bao Y. TrxR1 and GPx2 are potently induced by isothiocyanates and selenium, and mutually cooperate to protect Caco-2 cells against free radical-mediated cell death. *Biochim Biophys Acta* 2012; 1823: 1914-24.
- [12] Turanov AA, Kehr S, Marino SM, Yoo MH, Carlson BA, Hatfield DL and Gladyshev VN. Mammalian thioredoxin reductase 1: roles in redox homeostasis and characterization of cellular targets. *Biochem J* 2010; 430: 285-93.