

# Abnormal Expression of Eukaryotic Translation Factors in Malignant Transformed Human Bronchial Epithelial Cells Induced by Crystalline Nickel Sulfide<sup>1</sup>

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**Objective** To study the oncogenic potential of mouse translation initiation factor 3 (*TIF3*) and elongation factor-1 $\delta$  (*TEF-1 $\delta$* ) in malignant transformed human bronchial epithelial cells induced by crystalline nickel sulfide (NiS). **Methods** Abnormal expressions of human *TIF3* and *TEF-1 $\delta$*  genes in two kinds of NiS-transformed cells and NiS-tumorigenic cell lines were investigated and analyzed by the reverse transcript polymerase chain reaction (RT-PCR) and fluorescent quantitative polymerase chain reaction (FQ-PCR), respectively. **Results** RT-PCR analysis primarily showed that both human *TIF3* and *TEF-1 $\delta$*  mRNA expressions in two kinds of NiS-transformed cells and NiS-tumorigenic cell lines were increased as compared with controls. FQ-PCR assay showed that the levels of *TIF3* expressions in the transformed cells and tumorigenic cells were 3 and 4 times higher respectively, and the elevated expressions of *TEF-1 $\delta$*  cDNA copies were 2.7- to 3.5-fold in transformed cells and 4.1- to 5.2-fold in tumorigenic cells when compared with non-transformed cells, indicating that the over-expressions of human *TIF3* and *TEF-1 $\delta$*  genes were related to malignant degree of the cells induced by nickel. **Conclusions** These findings demonstrate that there are markedly abnormal expressions of *TIF3* and *TEF-1 $\delta$*  genes during malignant transformation of human bronchial epithelial cell lines induced by crystalline NiS. They seem to be the molecular mechanisms potentially responsible for human carcinogenesis due to nickel.

**Key words:** Nickel compounds; Human cells; Transformation; Translation factors; Fluorescent quantitative PCR

## INTRODUCTION

Metallic nickel and nickel compounds are known environmental and occupational metals. The available epidemiological data have shown that nickel alloy and compounds can cause allergic diseases, lung and nasal cancer in human beings, especially in those working in nickel industries such as nickel refineries, stainless steel welding, calcining and sintering operations<sup>[1-3]</sup>. Experimental studies have demonstrated that the carcinogenicity of nickel compounds may be in part due to their ability to cause DNA damages such as DNA strand breaks, DNA-DNA crosslinks, DNA-protein crosslinks, infidelity of DNA replication and inhibition of DNA repair as well as abnormal DNA synthesis<sup>[4-5]</sup>. The International Agency for Research on Cancer (IARC) has classified nickel compounds as a category I

carcinogen and metallic nickel is considered possible carcinogenic to human beings<sup>[6-7]</sup>. Even though nickel compounds are known carcinogens, the underlying carcinogenic mechanisms, however, are still not fully understood<sup>[8]</sup>.

Recent developments in oncogenomics have illustrated the usefulness of carcinogen-induced changes in gene expression to understand the potential cellular and molecular mechanisms of chemical carcinogenesis. Recently, Joseph *et al.*<sup>[9-10]</sup> and Lei *et al.*<sup>[11-12]</sup> have identified, cloned, and characterized the mouse *TIF3* (GenBank Accession Number AF271072) and *TEF-1 $\delta$*  (GenBank Accession Number AF304351) as two novel cadmium-responsive proto-oncogenes. The objective of the present research was to determine if the abnormal expressions of *TIF3* and *TEF-1 $\delta$*  genes were present during malignant transformation of

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human bronchial epithelial cell lines (16HBE) induced by crystalline nickel sulfide (NiS) with qualitative and quantitative methods, and to further explore the potential carcinogenesis of insoluble nickel compounds and the probability of human *TIF3* and *TEF-1 $\delta$*  as molecular biomarkers of metal expression situation for human population.

## MATERIALS AND METHODS

### Preparation of Nickel-transformed Human Cell Lines

Malignant transformation experiments of human bronchial epithelial cells (16HBE) induced by insoluble crystalline nickel sulfide (NiS) were performed as previously described<sup>[13]</sup>. Briefly, the cells seeded in dishes were treated with different concentrations of nickel compounds (0.25-2.00  $\mu\text{g}/\text{cm}^2$ ). Minimum essential medium (MEM) was used as a solvent control and benzo [a] pyrene (B[a]P) (0.2  $\mu\text{g}/\text{mL}$ ) was used as a positive control. The concentrations of nickel compounds, which gave the relative colony forming efficiencies (RCFEs) from 5%-90%, were selected for the morphological transformation studies. Malignant cell transformation assay was performed according to Dunkel's recommended procedure<sup>[14]</sup>. The transformed foci

induced by insoluble crystalline NiS at the concentration of moderate cytotoxicity, were individually isolated for soft agar assay and nude mouse tumorigenicity experiments. The established NiS-transformed cell lines and NiS-tumorigenic cell lines were used to investigate the abnormal expressions of *TIF3* and *TEF-1 $\delta$*  genes, respectively.

### Total RNA Isolation and Primer (probe) Sequences

The medium from the flasks containing the monolayer of NiS-transformed cell lines, NiS-tumorigenic cell lines and non-transformed cells was completely aspirated and rinsed with phosphate buffered saline (1 $\times$ PBS) 1-2 times. Total RNA from the cells was isolated using the TRIzol kit (Gibco BRL, USA) in accordance with the instructions provided by the manufacturer. The purity and integrity of the total RNA, used for reverse transcription of mRNA to cDNA, were analyzed by spectrophotometry (Eppendorf, Germany) and agarose gel electrophoresis, respectively. For detecting expressions of human *TIF3* and *TEF-1 $\delta$*  cDNA with qualitative and quantitative assays, the primers of human *TIF3* (GenBank Accession Number Z21507) and *TEF-1 $\delta$*  (GenBank Accession Number U39067), and the corresponding probes are listed in Tables 1 and 2.

TABLE 1

Primers for Qualitative RT-PCR Analysis

Genes	Primers and $\beta$ -actin Sequences <sup>a</sup>		Length of Products
<i>TIF3</i>	Sense:	5'-gTCACAgCCgggATgAAgCC-3'	770 bp
	Antisense:	5'-ATggCTTCCTgACCACCgCC-3'	
<i>TEF-1<math>\delta</math></i>	Sense:	5'-TCCCATgCgCCAAgTggAgC- 3'	103 bp
	Antisense:	5'-CTCCTCCTCATTgTCACTgC -3'	
$\beta$ -actin	Sense:	5'-AggCATTgTgATggACTCCg-3'	301 bp
	Antisense:	5'-AgTgATgACCTggCCgTCAg-3'	

Note. RT-PCR: Reverse-Transcript-Polymerase Chain Reaction Technique. <sup>a</sup>designed with GENE-RUNNER software and synthesized by Invitrogen Co. USA.

TABLE 2

Primers and Probe for Quantitative FQ-PCR Analysis

Genes	Primers and Probe Sequences <sup>a</sup>		Length of Products
<i>TIF3</i>	Sence:	5'-gCCCTCTCCCCAACTATgA-3'	65 bp
	Antisense:	5'-gTggTTACATCCATggCTTCCT-3'	
	Probe:	5'-FAM-CATgTggTCCTgggCggTggT-TAMARA-3'	
<i>TEF-1<math>\delta</math></i>	Senae:	5'-gCTACAAACTTCCTAgCACATgAgAAg-3'	86 bp
	Antisense:	5'-TTCATCTgCTCgTAgAATCTCCTTT-3'	
	Probe:	5' -FAM -TggTTCgACAAgTTCAAATATgACg- TAMARA-3'	

Note. FQ-PCR: Fluorescent Quantitative-Polymerase Chain Reaction Assay. <sup>a</sup>designed with Primer Express 2.0 software and synthesized by Da'an Co. China.

### Observation of *TIF3* and *TEF-1 $\delta$* by RT-PCR Technique

The total RNA of *TIF3* and *TEF-1 $\delta$*  was diluted to the concentration of 1 mg/mL for reverse transcript (RT) experiments. According to GenBank, the forward and reverse primers of both *TIF3* and *TEF-1 $\delta$*  cDNA were designed with GENE-RUNNER software and synthesized by Invitrogen Co. USA for the polymerase chain reaction (PCR). The primer sequences of  $\beta$ -actin gene were designed as the internal standard (Table 1). Using the ThermoScript™ RT kit (Gibco BRL, USA) and PCR amplification kit (TaKaRa, Japan) according to their manufacturer's instructions, the abnormal expressions of *TIF3* and *TEF-1 $\delta$*  mRNA were primarily investigated by RT-PCR technique. The RT-PCR products of *TIF3* and *TEF-1 $\delta$*  in non-transformed cells, NiS-transformed cells and NiS-tumorigenic cells under the internal standard of  $\beta$ -actin were detected by agarose gel electrophoresis, and scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA, USA) for semi-quantification.

### Detection of *TIF3* and *TEF-1 $\delta$* by FQ-PCR Assay

**Preparation of positive templates standard** *TIF3* or *TEF-1 $\delta$*  cDNA generated by reverse transcription from target RNA was amplified as positive template standard in PE9600 PCR machine.  $5 \times$  PCR buffer [10 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 2 mmol/L MgCl<sub>2</sub>], was mixed with 20  $\mu$ mol/L Taq DNA polymerase, 10 mmol/L dNTPs and 25  $\mu$ mol/L primers, and then 5  $\mu$ L of *TIF3* or *TEF-1 $\delta$*  cDNA template (corresponding to 1.25  $\mu$ g RNA) was added to the master mix. PCR amplification was performed with pre-denaturation (93°C for 2 min), and then 40 cycles of denaturation (93°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1 min). The copies (copies/ $\mu$ L) of *TIF3* or *TEF-1 $\delta$*  cDNA were calculated according to the OD<sub>260</sub> and fragment length of PCR products, and then diluted to the gradient concentrations of 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> copies/ $\mu$ L for standard gradient templates. Water was used as negative control (Fig. 1).

**Detection of *TIF3* and *TEF-1 $\delta$*  cDNA** Quantitative *TIF3* and *TEF-1 $\delta$*  were detected by FQ-PCR assay in PE 7000 sequence detection system (ABI PRISM, USA) according to the recommended procedure<sup>[15-16]</sup>. Briefly, a commercially available FQ-PCR master mix (Da'an Co. China) containing  $5 \times$  PCR buffer, 20  $\mu$ mol/L Taq DNA polymerase, 10 mmol/L dNTPs, was mixed with 25  $\mu$ mol/L primers, 20  $\mu$ mol/L probe and 5  $\mu$ L of *TIF3* or *TEF-1 $\delta$*  cDNA template

(corresponding to 1.25  $\mu$ g RNA). Then, 50  $\mu$ L of total reaction system was performed with pre-denaturation (93°C for 2 min), and then 40 cycles of denaturation (93°C for 45 s), annealing (55°C for 45 s). Every time when FQ-PCR of the examined samples were performed, *TIF3* or *TEF-1 $\delta$*  cDNA standard gradient templates of 10<sup>7</sup>, 10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> copies/ $\mu$ L were simultaneously amplified and the blank PCR reagents were simultaneously amplified as negative controls. Three replicates of each sample were processed. Fluorescence was measured at the end of the annealing-extension phase of each cycle. The fluorescent intensity at 518 nm wavelength was detected automatically by the PE 7000 sequence detection system, and the amounts of target cDNA (copies/ $\mu$ g RNA) were automatically calculated according to standard gradient template curve and the amount of target cDNA by comparing a threshold value. The threshold value (background) for the fluorescence of all samples was set manually. The reaction cycle at which the PCR product exceeded this fluorescence threshold was identified as the threshold cycle (CT).

**Calculation of *TIF3* and *TEF-1 $\delta$*  cDNA** The amounts of target cDNA (copies/ $\mu$ g RNA) were calculated with the formula according to standard gradient template curve and the amount of target cDNA.  $C=Q \times V_{\text{cDNA}}/(V_{\text{RNA}} \times L_{\text{RNA}})$ , where C represents *TIF3* or *TEF-1 $\delta$*  cDNA copies, Q represents amounts of target cDNA examined,  $V_{\text{cDNA}}$  represents volume of cDNA template,  $V_{\text{RNA}}$  represents volume of RNA template for reverse transcript,  $L_{\text{RNA}}$  represents concentration of RNA template.

### Statistical Analysis of Data

Differences among groups were statistically analyzed by one-way analysis of variance (ANOVA) or Dunnett test using Statistical Package for the Social Sciences (SPSS 11.0). Data were presented as  $\bar{x} \pm s$ .  $P < 0.05$  was considered statistically significant.

## RESULTS

### Preliminary Analyses of *TIF3* and *TEF-1 $\delta$* Expressions

The results of RT-PCR experiments primarily showed that the expressions of *TIF3* and *TEF-1 $\delta$*  cDNA in NiS-transformed cells and NiS-tumorigenic cells were slightly higher than those in the non-transformed control cells. After densitometric quantification and normalization with  $\beta$ -actin, the relative

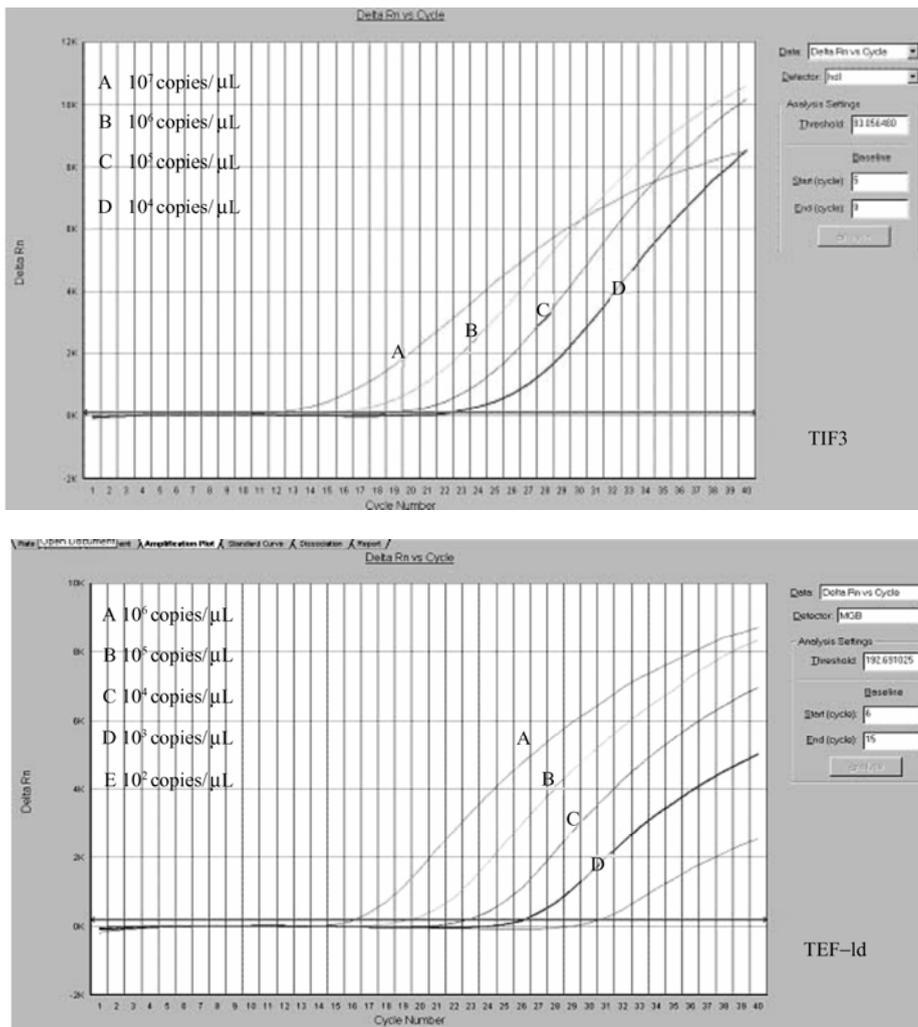


FIG. 1. Kinetic curves with a series of standard *TIF3* cDNA or *TEF-1 $\delta$*  levels detected by fluorescent quantitative PCR method. The X axis denotes the cycle number of a quantitative PCR reaction, and the Y axis denotes the  $\Delta Rn$  which is the fluorescence intensity over the background.

mass of *TIF3* bands was 0.52, 0.80, and 0.83 in non-transformed cells, transformed cells and tumorigenic cells, respectively. Under the same condition, the relative mass of *TEF-1 $\delta$*  bands was 0.25, 0.31, and 0.37 respectively in non-transformed cells, transformed cells and tumorigenic cells. These semi-quantitative analyses primarily indicated that abnormal expressions of *TIF3* and *TEF-1 $\delta$*  occurred in transformed cells and tumorigenic cells induced by crystalline nickel sulfide (NiS) (Figs. 2 and 3).

#### Quantitative Analysis of *TIF3* Gene Expression

The overexpressions of *TIF3* genes in human bronchial epithelial cells induced by crystalline NiS

were further quantified by FQ-PCR assay. The levels of *TIF3* expressions in the NiS-transformed cells and NiS-tumorigenic cells were 3 and 4 times higher respectively than those in control cells ( $P < 0.05$  or  $P < 0.01$ ), indicating that expression level of *TIF3* gene was related to malignant degree of the cells (Table 3 and Fig. 4).

#### Quantitative Analysis of *TEF-1 $\delta$* Gene Expression

As shown in Table 4 and Fig. 5, FQ-PCR assay further revealed that the levels of *TEF-1 $\delta$*  cDNA copies were from 2.7- to 3.5-fold in transformed cells and from 4.1- to 5.2-fold in tumorigenic cells when compared with non-transformed cells ( $P < 0.05$  or  $P < 0.01$ ), indicating a relation of overexpression of *TEF-1 $\delta$*  gene to malignant degree of the cells

induced by nickel.

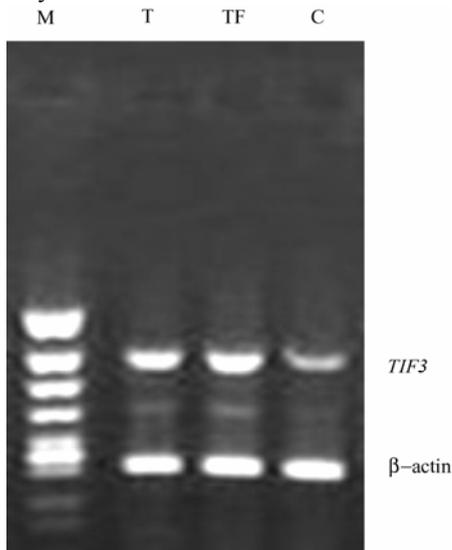


FIG. 2. Agarose gel electrophoresis of RT-PCR products (770 bp) of human *TIF3* mRNA from human bronchial epithelial cells induced by crystalline NiS. C: non-transformed cells, TF: NiS-transformed cells, T: NiS-tumorigenic cells, M: molecular marker  $\Phi$  X 174-Hinc II,  $\beta$ -actin (301 bp): used as internal standard.

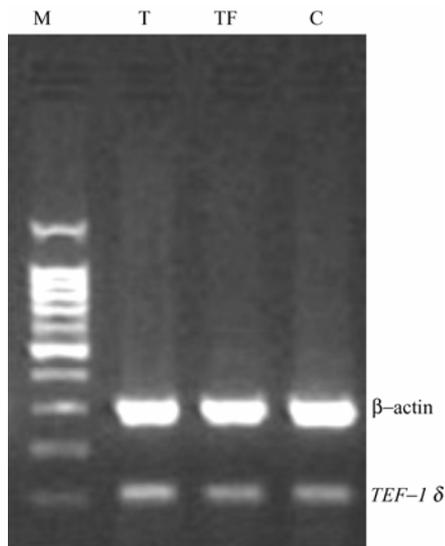


FIG. 3. Agarose gel electrophoresis of RT-PCR products (103 bp) of human *TEF-1 $\delta$*  mRNA from human bronchial epithelial cells induced by crystalline NiS. C: non-transformed cells, TF: NiS-transformed cells, T: NiS-tumorigenic cells, M: 100 bp ladder DNA marker,  $\beta$ -actin (301 bp): used as internal standard.

TABLE 3

Overexpressions of *TIF3* Gene in Malignant Transformed Human Bronchial Epithelial Cells Induced by Crystalline NiS ( $\bar{x} \pm s$ )

Sample	n	<i>TIF3</i> cDNA ( $\times 10^6$ copies / $\mu$ g RNA)	
		Cell Line 1 (% of Control)	Cell Line 2 (% of Control)
Non-transformed Cells	3	2.98 $\pm$ 0.60 (100%)	2.44 $\pm$ 0.48 (100%)
NiS-transformed Cells	3	8.46 $\pm$ 0.87 (284%) <sup>a</sup>	7.41 $\pm$ 0.66 (304%) <sup>b</sup>
NiS-tumorigenic Cells	3	11.64 $\pm$ 0.23 (391%) <sup>b</sup>	10.56 $\pm$ 2.55 (433%) <sup>b</sup>

Note. Compared with control cells, <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 (Analysis of variance and Dunnett-test).

TABLE 4

Overexpressions of *TEF-1 $\delta$*  Gene in Malignant Transformed Human Bronchial Epithelial Cells Induced By Crystalline NiS ( $\bar{x} \pm s$ )

Sample	n	<i>TEF-1<math>\delta</math></i> cDNA ( $\times 10^6$ copies / $\mu$ g RNA)	
		Cell Line 1 (% of Control)	Cell Line 2 (% of Control)
Non-transformed Cells	3	3.43 $\pm$ 0.27 (100%)	4.30 $\pm$ 0.33 (100%)
NiS-transformed Cells	3	12.05 $\pm$ 0.76 (351%) <sup>b</sup>	11.49 $\pm$ 3.20 (267%) <sup>a</sup>
NiS-tumorigenic Cells	3	17.79 $\pm$ 3.23 (519%) <sup>b</sup>	17.65 $\pm$ 4.55 (410%) <sup>b</sup>

Note. Compared with control cells, <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 (Analysis of variance and Dunnett-test).

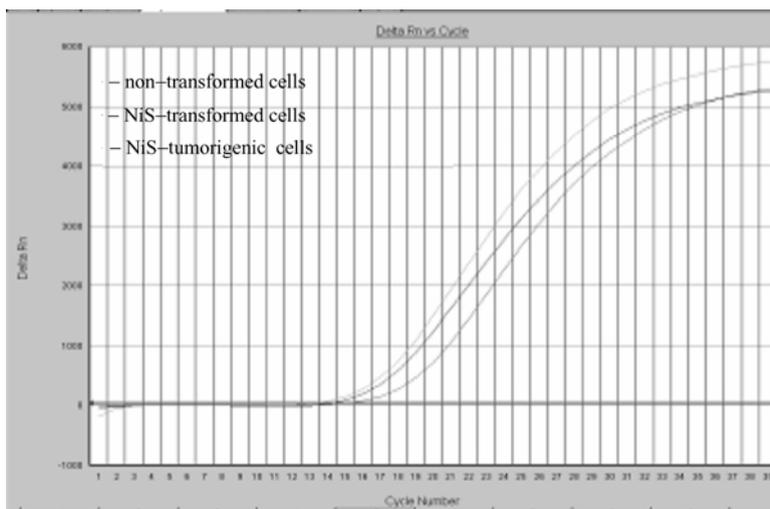


FIG. 4. Fluorescent quantitative PCR assays of human *TTF3* gene from non-transformed cells, NiS-transformed cells and NiS-tumorigenic cells. The X axis denotes the cycle number of a quantitative PCR reaction, and the Y axis denotes the  $\Delta R_n$ , which is the fluorescence intensity after normalization with the threshold (background).

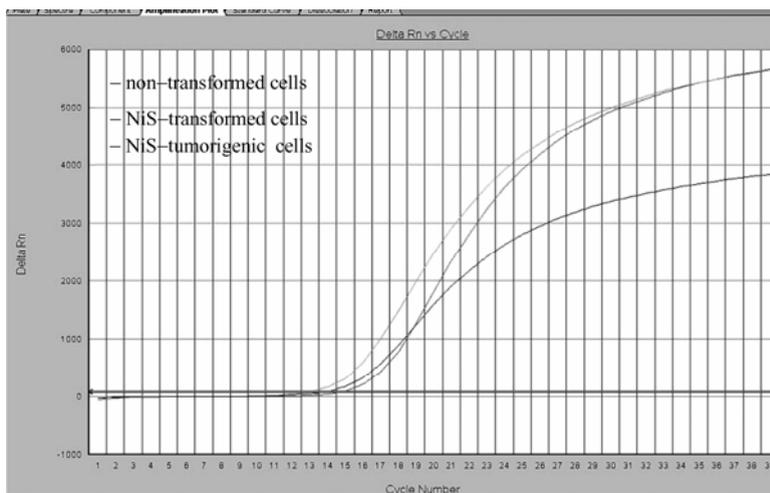


FIG. 5. Fluorescent quantitative PCR assays of human *TEF-1 $\delta$*  gene from non-transformed cells, NiS-transformed cells and NiS-tumorigenic cells. The X axis denotes the cycle number of a quantitative PCR reaction, and the Y axis denotes the  $\Delta R_n$ , which is the fluorescence intensity after normalization with the threshold (background).

## DISCUSSION

Alterations in the expression of genes, especially the genetic changes in cell cycle, are critical in the development of malignant transformation<sup>[17-18]</sup>. Modifications in the translational machinery of cells, including changes in both eukaryotic translation initiation factors and elongation factors, can also result in susceptibility to transformation and the acquisition of transformed and oncogenic properties in cells<sup>[19-20]</sup>. For example, the largest eukaryotic translation initiation factor, eIF3, consists of at least ten subunits ranging in mass from 35 to 170 kDa. eIF3 can bind to the 40 S ribosome in an early step of

translation initiation and promote the binding of methionyl-tRNA and mRNA<sup>[21]</sup>. Enhanced expressions of eIF3 subunits are found among transformed, tumorous and tumor tissues<sup>[22-24]</sup>. Similarly, translation elongation factors constitute a group of nucleotide exchange proteins that bind to GTP and aminoacyl-tRNA and lead to codon-dependent placement of the aminoacyl-tRNA on the ribosome resulting in peptide chain elongation<sup>[19]</sup>. Enhanced expression of elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) confers susceptibility to carcinogen and UV light-induced transformation to mouse and Syrian hamster cell lines<sup>[25]</sup>. Furthermore, elevated levels of EF-1 $\alpha$  and EF-1 $\gamma$  are found in

tumors of the pancreas, colon, breast, lungs, prostate and stomach relative to normal tissues<sup>[26-31]</sup>.

Recently, the mouse *TIF3* and *TEF-1δ* have been identified as two novel cadmium-responsive proto-oncogenes<sup>[32]</sup>. Results presented in this study, compared with non-transformed human bronchial epithelial cells, have shown that the transformed cells and tumorigenic cells can express high levels of human *TIF3* and *TEF-1δ* genes. Furthermore, overexpressions of the two genes in tumorigenic cells are higher than those in transformed cells as compared with control cells. These findings demonstrate for the first time that there are markedly abnormal expression of *TIF3* and *TEF-1δ* genes during malignant transformation of human bronchial epithelial cell line induced by crystalline NiS, and the human *TIF3* and *TEF-1δ* expressions are associated with malignant degree of the cells. These may be one of the molecular mechanisms potentially responsible for carcinogenesis due to nickel.

Although our previous and present studies have demonstrated the oncogenic potential of *TIF3* and *TEF-1δ*, the cellular mechanisms underlying overexpression of *TIF3* and *TEF-1δ* as well as cell transformation and tumorigenesis are not known. It may be worth exploring the expressions of these two genes during malignant transformation of human bronchial epithelial cell lines induced by crystalline NiS as well as changes in risk population. But it seems that abnormal expression of human *TIF3* and *TEF-1δ* can be used as a molecular biomarker of metal expression situation in human beings.

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