Journal of Geoscience and Environment Protection, 2014, 2, 129-133
Published Online April 2014 in SciRes. http://www.scirp.org/journal/gep
http://dx.doi.org/10.4236/gep.2014.22018

Expression of Telomerase Reverse Transcriptase during the Malignant Transformation of Cadmium-Induced Cells

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Received February 2014

Abstract

The objective of the present study was to investigate human telomerase reverse transcriptase (hTERT) mRNA and protein expressions during the cadmium chloride-induced malignant transformation of human bronchial epithelial (16HBE) cells. Fluorescence quantitative PCR (FQ-PCR) and Western blot analyses were performed to detect the hTERT mRNA and protein expressions in normal 16HBE cells, cadmium chloride-transformed 16HBE cells, and tumorigenic cells from nude mice inoculated with cadmium chloride-transformed 16HBE cells. Under the inner standard of GAPDH, the hTERT mRNA expression was significantly higher at different stages of malignant transformation (cadmium chloride-transformed 16HBE cells at passages 15 and 35 and tumorigenic cells from nude mice) than in normal 16HBE cells, and increased with the development of malignancy ($P < 0.01$). In addition, hTERT protein expression increased with the development of malignancy. These findings demonstrate that hTERT expression is related to cadmium chloride-induced malignant transformation. Cadmium chloride-induced malignant transformation is involved in changes in the hTERT activity, and might be an early event in cadmium chloride-induced malignant transformation.

Keywords

Cadmium Chloride; Human Bronchial Epithelial Cells; Malignant Transformation; Telomerase Reverse Transcriptase

1. Introduction

The telomere is a cap-like structure of eukaryotic chromosomes. Human telomerase is one of the ribonucleo-

*Corresponding author.
This work was supported by the grants from the National Natural Science Foundation of China (No. 81373038), and by Science and Technology Planning Project of Guangzhou Municipality, China (No.2013J410037).

How to cite this paper: Wang, M., & Lei, Y. X. (2014). Expression of Telomerase Reverse Transcriptase during the Malignant Transformation of Cadmium-Induced Cells. *Journal of Geoscience and Environment Protection, 2,* 129-133.
http://dx.doi.org/10.4236/gep.2014.22018

protein enzymes and consists of human telomerase reverse transcriptase (hTERT), the telomerase RNA component, and human telomerase-associated protein I (hTEPI). hTERT is a protein with reverse transcriptase activity that belongs to the catalytic subunit of telomerase and is a determinant of telomerase activation. Studies have shown that the hTERT gene is closely related to the formation of malignant tumors (Masutomi, 2003). Cadmium (Cd) is widely distributed in nature. Cd and its compounds are widely used in industry and belong to industrial poisons and environmental pollutants. In recent years, with the rapid development of the electronics industry, the requirement for electronic materials and products (such as nickel-cadmium batteries, transistors, and semiconductors) has been increasing and, consequently, the requirement for and production of the corresponding raw materials (such as Cd) have been growing over the years. Thus, the chance of occupational exposure to Cd is also increasing. With the rapid development of industrial production, environmental pollution due to Cd and its compounds are increasing severely and may be long-standing in the environment (Li, 2014; Wei, 2012). According to statistics from the International Agency for Research on Cancer (IARC), Cd and its compounds are confirmed carcinogens and have been one of the metal poisons threatening human health (IARC, 1993).

To further investigate the molecular mechanisms underlying the Cd-induced malignant transformation of human bronchial epithelial (16HBE) cells, hTERT mRNA and protein expressions were investigated in normal 16HBE cells, 16HBE cells with Cd-induced malignant transformation, and tumorigenic cells from nude mice inoculated with 16HBE cells that underwent Cd-induced malignant transformation.

2. Material and Methods

2.1. Cells and Reagents

Cadmium chloride-induced malignant transformation of 16HBE cells was reported in our previous study (Lei, 2008). Normal 16HBE cells, 16HBE cells with Cd-induced malignant transformation (passages 15 and 35), and tumorigenic cells from nude mice inoculated with Cd-induced malignantly transformed 16HBE cells were thawed and cultured. MEM powder (GIBCO-BRL, USA), calf serum (Guangzhou farm, China), trypsin, EDTA (HycloneE, USA), Taqman RNA quantitative PCR kit and probes (Ambion, USA), iScript Advanced cDNA synthesis kit (Bio-rad, USA), Espect spectrophotometer (Malcom, Japan), protein extraction kit, and BCA Protein Assay kit (KeyGEN Biotech, Co., Ltd, China) were used in the present study. Primers were synthesized by the Invitrogen Corporation (Carlsbad, USA). The hTERT primers used in our study were 5'-TGTGCACCA ACATCTAC AAGATC-3' (forward primer) and 5'-CTGATGAAA TGGGAGCTGACG-3' (reverse primer).

2.2. Cell Culture and RNA Extraction

Cells were maintained in MEM containing 10% calf serum at 37˚C in a humidified environment with 5% CO_2. Cells were digested with 0.25% trypsin in 0.02 EDTA (1:1, V/V). Cells (2.0×10^6) were incubated with 1 ml of Trizol, followed by total RNA extraction according to the manufacturer's instructions (GIBCO BRC). RNA purity and concentration were determined by UV spectrophotometry and subjected to 1.0% agarose gel electrophoresis to detect RNA integrity.

2.3. RNA Reverse Transcription

The RNA-Primer mixture (20 μL) was prepared with 1 μg of total RNA, 2 μl of 0.5 μM RT primer, and DEPC treated water (up to 15 μL), and then incubated at 65˚C for 5 min. The mixture was kept on ice for at least 2 min to produce the specific RNA-Primer Mix. Subsequently, reverse transcription was performed with 15 μL of RNA-Primer Mix, 4 μL of 5×iScript reaction Mix, and 1 μL of RTase (total volume: 20 μL) at 25˚C for 5 min, 42˚C for 30 min, and inactivated at 85˚C for 5 min. The products were diluted five-fold in ddH_2O (20 μL of products in 80 μL of ddH_2O) and then stored at -20˚C for use.

2.4. Detection of hTERT mRNA Expression with FQ-PCR

Fluorescence quantitative PCR (FQ-PCR) was performed with the SYBR method. The mixture (10.0 μl) used for PCR included 5.0 μl of 2×SYBR Premix DimerEraser, 0.5 μl of 20 μM PCR RreversePrimer, 0.5 μl of 20 μM PCR Forward Primer, 0.2 μl of ROX Reference Dye II, 1.0 μl of cDNA template, and 2.8 μl of ddH_2O. PCR was performed under the following conditions: pre-denaturation at 95˚C for 30 s, 45 cycles of denaturation at

95˚C for 5 s, annealing at 60˚C for 30 s, and extension at 72˚C for 30 s. Data were acquired and analyzed.

2.5. Analysis of hTERT Protein Expression by Western Blot

First, total protein was extracted. In brief, the medium was removed and cells were washed with cold PBS twice and then transferred into a pre-chilled tube. After addition of pre-chilled lysis buffer, lysates were centrifuged at 12000 rpm for 15 min at 4˚C. The supernatant was harvested, protein concentration was determined with the BCA method and, based on this, the protein solution volume needed for loading was determined. The protein was boiled at 100˚C for 5 min, and 50 μg of protein was loaded and subjected to 10% SDS-PAGE analysis (40 mA, 90 min). Proteins were transferred onto a PVDF membrane that was then blocked in blocking buffer for 60 min at 37˚C. The membrane was treated with primary antibody (1:1000) at 37˚C for 1 h and then at 4˚C overnight. Following three washes in PBST (5 min each), the membrane was treated with secondary antibody (1:2000) at 37˚C for 1 h. Following three PBST washes (5 min each), visualization with DAB was performed. Gel image analysis was performed to determine the molecular weight of the target protein and the optical density of protein bands and, based on this, the relative protein expression was calculated.

2.6. Statistical Analysis

Experiments were done in triplicate, and data were expressed as means ± standard deviation. One-way analysis of variance (ANOVA) was employed for comparisons among groups. Statistical analysis was performed with the SPSS version 15.0 (SPSS Inc, Chicago, IL, USA) for Windows. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Detection of RNA integrity

RNA extracted from different groups of cells was subjected to UV spectrophotometry. The results showed that the A260/A280 was 2.09-2.12 and the A260/A230 was 2.23-2.31, suggesting that RNA had a high purity and presented little contamination by DNA and/or proteins.

3.2. Detection of hTERT mRNA Expression

In the 4 groups (normal 16HBE cells, 16HBE cells with Cd-induced malignant transformation at passage 15, 16HBE cells with Cd-induced malignant transformation at passage 35, and tumorigenic cells from nude mice), hTERT mRNA expression is shown in **Table 1**. The results show that hTERT mRNA expression was significantly higher at different stages of malignant transformation than in normal 16HBE cells under the inner standard of GAPDH, and the hTERT mRNA expression increased with the development of malignant transformation ($P < 0.01$).

3.3. Detection of hTERT Protein Expression

GAPDH served as an internal reference. Western blotting was performed to detect the relative hTERT protein expression in 16HBE cells from the different groups (**Figure 1**).

As shown in **Figure 1**, when compared with the control group, the hTERT protein expression showed an increasing tendency for the development of malignant transformation, and increased hTERT protein expression was obvious in malignant cells.

Table 1. hTERT mRNA expression in the 16HBE cells from the 4 groups.

	Normal 16HBE cells	15th passage of Cd-treated cells	15th passage of Cd-treated cells	Tumorigenic cells from nude mice
GAPDH	28.93 ± 0.07	29.48 ± 0.09	30.96 ± 0.03	32.07 ± 0.17
hTERT	27.29 ± 0.13	27.66 ± 0.08	26.56 ± 0.28	25.89 ± 0.07
2-ΔΔCt	1.00	1.13	6.77	23.26

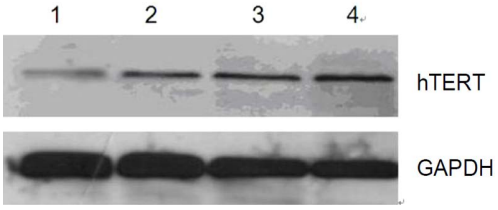

Figure 1. hTERT protein expression in 16HBE cells from the 4 groups. 1. Normal 16HBE cells; 2. 16HBE cells undergoing Cd-induced malignant transformation at passage 15; 3. 16HBE cells undergoing Cd-induced malignant transformation at passage 35; 4. Tumorigenic cells from nude mice undergoing inoculation of 16HBE cells with Cd-induced malignant transformation.

4. Discussion

The telomere is a cap-like structure at the end of eukaryotic chromosomes that can bind G-rich nucleotide hexamers and related proteins to form DNA-binding protein complexes. Telomerase is a DNA polymerase with reverse transcriptase activity and is composed of RNA and proteins. During cell division, the telomerase can use its own RNA as a template to reverse transcribe the TTAGGG repeat fragments, which are then added to the end of the shortened and linear chromosomes, a process that may lead to the indefinite proliferation of cells and the occurrence of cancers (Neidle & Parkinson, 2003). In human somatic cells, telomerase expression is not observed, and thus the length of telomeres is not increased. The telomeres are shortened as cells divide. Finally, cell division and proliferation stop and cells start developing aging (Sara & Victoria, 2001). Studies have shown that hTERT is the catalytic subunit of telomerase and also a determinant of telomerase activation. The hTERT gene is associated with the formation and the malignancy of tumors. Under the catalysis of hTERT, telomerase can use its own RNA as a template for the synthesis of 5′-TTAGGG-3′ repeat sequences, which may offset telomere shortening during replication, leading to cell proliferation (Harrington, 2001).

Our results showed that, at moderate concentrations (10 μmol/L), cadmium chloride could increase hTERT protein and mRNA expression in 16HBE cells at passage 15. The mRNA and protein expression further increased in 16HBE cells at independent growth stages and in malignant cells. This suggests that the increase in hTERT activity might be an early molecular event during the Cd-induced malignant transformation of cells, which may persist for the entire duration of the malignant transformation. In vitro, the malignant transformation of cells is involved in different stages of cancer formation and progression, including immortalization, morphological changes, non-growth factor-dependent growth, growth on semi-solid medium, and tumorigenicity (Ross, 2003). Cells may acquire different malignant phenotypes via the sequential selective expansion, with a clonal growth advantage (Jetten, 1989). Our findings demonstrated that the change in hTERT activity became evident over the development of malignant transformation, which might increase telomerase activity and play an important role in the Cd-induced malignant transformation of cells.

The mechanisms underlying Cd-induced carcinogenesis are complex and have not been elucidated to date. Studies have shown that DNA injury, impaired DNA repair, changes in gene expression and cell cycle regulation, and abnormal DNA methylation are closely associated with Cd-induced carcinogenesis (Koizumi & Yamada, 2003; Zhou, 2012). Our findings showed that a change in hTERT activity occurred during the Cd-induced malignant transformation of cells, suggesting that hTERT expression is correlated with malignant transformation and carcinogenicity. Our findings may provide theoretical evidence for elucidating the mechanisms underlying Cd-induced carcinogenesis and present an indicator for the early diagnosis of carcinogenesis in Cd-exposed populations. In addition, our results provide a clue for detecting hTERT protein expression in malignant tumors (especially in populations with Cd exposure) and open potential avenues for hTERT mRNA-targeting therapy.

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