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TPX2 in malignantly transformed human bronchial epithelial cells by anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide

Lijuan Zhang[a,1], He Huang[c,1], Luyao Deng[b], Ming Chu[b], Lan Xu[b], Juanling Fu[a], Yunlan Zhu[b], Xiuchun Zhang[b], Shulin Liu[c], Zongcan Zhou[a,*], Yuedan Wang[b,**]

[a] *Department of Toxicology, School of Public Health, Peking University, PR China*
[b] *Department of Immunology, School of Basic Medical Science, Peking University, PR China*
[c] *Department of Microbiology, School of Medical Science, Peking University, PR China*

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ABSTRACT

In order to elucidate the function of the targeting protein for Xenopus kinesin-like protein 2 (Xklp2) (TPX2) in the malignant transformation of human bronchial epithelial cells induced by anti-benzo[*a*]pyrene-*trans*-7, 8-dihydrodiol-9, 10-epoxide (anti-BPDE), TPX2 was characterized in cells at both the gene and the protein levels. TPX2 was present at higher levels in 16HBE-C cells than in 16HBE cells as demonstrated by two-dimensional gel electrophoresis, immunocytochemistry, Western blot analysis and RT-PCR. TPX2 was also detected in lung squamous-cell carcinoma tissues by immunohistochemistry, but not in normal lung tissues. Depression of TPX2 by RNA interference in 16HBE-C cells led to a decrease in cell proliferation, S-phase cell cycle arrest and cell apoptosis. Abnormal TPX2 tyrosine phosphorylation was detected in 16HBE-C cells, and this could be inhibited, to different degrees, by tyrosine kinase inhibitors. Inhibiting tyrosine phosphorylation in 16HBE-C cells by three selected tyrosine protein kinase inhibitors, tyrphostin 47, AG112 and AG555, caused G_0/G_1-phase cell cycle arrest. Our results suggest that anti-BPDE can cause the over-expression of TPX2 and its aberrant tyrosine phosphorylation. Misregulation of TPX2 affects the cell cycle state, proliferation rates and apoptosis.

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1. Introduction

Worldwide, lung cancer is the most frequent malignant tumor type, and it is responsible for nearly one million deaths annually. Cigarette smoke is one of the major causes of lung cancer (Mao et al., 1997), and 90% of the lung cancer cases diagnosed are associated with the consumption of tobacco products (Cooley et al., 2001). Benzo[*a*]pyrene (B[*a*]P) is a representative candidate carcinogen. After entering mammalian cells, it undergoes metabolic activation to become highly toxic reactive metabolite intermediates, which can irreversibly damage cellular macromolecules (i.e., DNA, proteins, and lipids) (Rubin, 2001). B[*a*]P is metabolized by cytochrome P-450-mediated oxidation to produce a spectrum of potent mutagenic and cytotoxic metabolites including anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide (anti-BPDE), and the formation of anti-BPDE-DNA adducts is considered to be critical in the carcinogenic process of B[*a*]P (Chen et al., 2000; Pavanello et al., 1999). It has been shown that the ultimate carcinogen, anti-C, mutagenizes after reacting with nuclear DNA (Rubin, 2001), but the mechanisms of anti-BPDE-induced carcinogenesis are not fully understood. This study is aimed to investigate the differentially expressed genes and proteins induced by anti-BPDE and their main functions in cell cycle regulation.

An alkaline protein (isoelectric point (p*I*) ≈9.8, molecular weight (M_W) ≈100 kDa) was detected to be over-expressed in BPDE-transformed human bronchial epithelial (16HBE-C) cells by two-dimensional electrophoresis (2DE) in our study. This protein was determined to be TPX2 according to the SWISS-PROT database. TPX2, abbreviated to the targeting protein for Xenopus kinesin-like protein 2 (Xklp2), is a proliferation-associated protein that is over-expressed in hepatocellular carcinoma (HCC). It is also expressed in cancer cell lines of the lung, prostate, and pancreas, but not in the respective normal tissues. Thus, it may serve as a special cell division marker for cancer diagnosis (Wang et al., 2002). In this study, we further investigated the possible function of this protein in the malignant transformation of 16HBE cells induced by anti-BPDE.

* Corresponding author at: Department of Toxicology, School of Public Health, Peking University Health Science Center, Beijing, PR China. Tel.: +86 10 82801531; fax: +86 10 62015583.
** Corresponding author at: Department of Immunology, School of Basic Medical Science, Peking University Health Science Center, 38th Xueyuan Rd. Haidian District, Beijing 100191, PR China. Tel.: +86 10 82801747; fax: +86 10 82801388.
E-mail addresses: zhouzc@bjmu.edu.cn (Z. Zhou), wangyuedan@bjmu.edu.cn (Y. Wang).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Cell lines

Human bronchial epithelial cells (16HBE) and 16HBE cells malignantly transformed by anti-BPDE (16HBE-C) were kindly given to us by Guangdong Medical College (Guangzhou, China) (Jiang et al., 2001). The transformed cells, but not 16HBE cells, could grow in soft agar and grew into tumors in BALB/C nude mice. Both types of cells were maintained in Dulbecco's modification of Eagle's medium (Gibco, US) with 10% fetal calf serum (Gibco, US) and incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Cell collection, protein isolation, two-dimensional electrophoresis and immunoblotting

Sub-confluent 16HBE and 16HBE-C cells were collected and lysed in cell lysis buffer supplemented with 1 mM phenylmethysulfony fluoride. After incubation on ice for 30 min, the lysates were centrifuged at 13,000 rpm in a microcentrifuge at 4 °C for 30 min. The supernatant was collected and assayed for protein concentration using the Bradford method.

The isoelectrofocusing strip (IEF, 7 cm, pH 3–10, Bio-Rad) was used for isoelectric focusing. A volume of sample equal to 200 μg protein was loaded on an IEF gel and filled with catholyte. Separation in the second dimension was carried out on 12% SDS-PAGE gels. After electrophoresis, the slab gels were fixed for 1 h in 10% acetic acid, and stained with Coomassie blue. Protein patterns in the gels were recorded as digital images using Alphamager® EC (Alpha Innotech, US). Gel images were analyzed using PDQuest software (Bio-Rad, US). Equal amounts of each sample were separated on a 12% gel and then transferred onto nitrocellulose membranes. Membranes were incubated with primary monoclonal antibodies (anti-TPX2) overnight and subsequently with peroxidase-labeled secondary antibodies. Immunoreactive bands were visualized using ECL reagents.

2.3. Immunohistochemistry and immunocytochemistry

Six samples of squamous-cell carcinoma (SCC) and tissues with paired adjacent lung tissues (5 cm distant from lung cancer) were obtained during surgical resection from the Peking University Third Hospital (Beijing, China). Tissue samples fixed in 10% formaldehyde were routinely processed for paraffin embedding. For antigen retrieval, sections were treated in a microwave oven for 20 min. For cell samples, cytospin slides were fixed in acetone at room temperature for 10 min, and no antigen retrieval was needed. All slides were incubated with the anti-TPX2 antibody (Abgent, US) overnight at 4 °C. The immunoreaction was visualized by means of the 3,3′-diaminobenzidine (DAB) method. Representative sections were counterstained with cresyl violet. Cell and tissue images were captured by microscope with digital camera (Olympus, Japan).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 16HBE and 16HBE-C cells using a Nuclospin® RNA II kit (MN, Germany). cDNA was synthesized by a RevertAid™ Frist Strand cDNA Synthesis Kit (Fermentas International Inc., Canada). The primer sequences for amplification of TPX2 cDNA were as follows: forward, GCA AGC TAT TGT CAC ACC TTT C; reverse, ATG ATT ACA GGA GTG GCA CAT C. PCR amplification of GAPDH (5′-TGC (A/C)TC CTG CAC CAC CAA CT-3′; 5′-(C/T)GC CTG CTT CAC CAC CTT C-3′) was routinely used as a control to assess the level of TPX2 in both cells.

2.5. RNA interference

The siRNA transfection procedure was performed as described by the manufacturer (INTERFERin™, Polyplus Transfection, France). The final siRNA concentration was 5 nM. Cells were cultured for 48 h before use in specific experiments. Transfection efficiency was assessed using Western blot. siRNAs against TPX2 (target sequence: 5′-GAACTTTACATCTGAACTA-3′(siRNA-1), 5′-CCACTCCTGTAATCATCG-3′(siRNA-2), 5′-ACGAACCGGTAGTGATAAA-3′(siRNA-3)) and negative control (Catalog #: c1) were obtained from Guangzhou Ribobio Co. Ltd. (Guangdong, China).

2.6. Cell proliferation assays

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte–trazolium bromide (MTT) dye conversion. Individual wells of a tissue culture 96-well microtiter plate were inoculated with 0.2 ml of medium containing 2×10^4 cells respectively to provide approximately 70% confluence after 24 h incubation. TPX2 RNA interference (RNAi) was performed. Eight replicate wells were used per concentration of each chemical. After a 24-h exposure, cell proliferation was assessed with the MTT assay. All experiments were performed at least three times.

2.7. Immunoprecipitations and Western blot analyses

Logarithmic growth phase 16HBE and 16HBE-C cells were lysed in cell lysis buffer supplemented with 1 mM phenylmethysulfony fluoride. Supernatants were stored in aliquots at −70 °C. Samples were assayed for protein concentration using the Bradford method. Equal amounts of sample were separated on 10% gradient gels and then transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies overnight and subsequently with peroxidase-labeled secondary antibodies. Immunoreactive bands were visualized using ECL reagents. For immunoprecipitation analysis, cells were collected and washed twice in phosphate-buffered saline and rapidly lysed in phosphorylation lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 200 μM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 50 mM Hepes, 1.5 mM magnesium chloride, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin). Supernatants were obtained by centrifugation at 13,000 rpm for 15 min at 4 °C. Rabbit anti-TPX2 antibody (Bethyl, US) was added into the cell lysate and incubated for 2 h at 4 °C, and then protein A/G-Agarose (Santa Cruz, US) was added and incubated overnight at 4 °C. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting as described previously.

2.8. Analysis of apoptosis and cell cycle

Apoptosis and cell cycle profiles were analyzed by flow cytometry. Cells were collected and fixed with 70% ethanol overnight, then incubated with a staining solution containing 0.2% NP-40, RNase A (30 μg/ml), and propidium iodide (50 μg/ml) in PBS. The analysis was performed on a FACScalibur (Becton Dickinson, US).

2.9. Statistical analyses

Results are expressed as means ± S.D. Linear regression analysis was used to compute the doubling time of the cells. Experimental data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant differences. In all cases, the criterion for statistical significance was $P < 0.05$.

3. Results

3.1. TPX2 as an over-expressed protein in 16HBE-C cells by 2D electrophoresis

Following 2DE (Fig. 1A and B), resolved proteins were recorded for each of the gel pairs. For deciphering tumor-associated proteins, 2DE gels of 16HBE cells were compared with their corresponding 16HBE-C cells. Using PDQuest analytical software (Bio-Rad), we identified proteins in 326 spots on the gel from 16HBE cells and 334 spots on the gel from 16HBE-C cells. An over-expressed alkaline protein with an isoelectric point (p*I*) of 9.8 and a molecular weight (M_W) approximately equal to 100 kDa was detected in 16HBE-C cells, which was tentatively identified as TPX2 according to the SWISS-PROT database (http://www.expasy.org/cgi-bin/pi_tool1?Q9ULW0@1-747@average). Then, Western blot analysis of another 2DE 16HBE-C gel was performed with a TPX2 antibody, further confirming this protein to be TPX2 (Fig. 1C). This protein was also quantified by PDQuest software (Fig. 1D).

3.2. Over-expression of TPX2 in 16HBE-C cells and SCC tissues

In immunocytochemistry of 16HBE-C cells and 16HBE cells, the percentage of TPX2-positive cells was calculated by counting ten random visual fields under the microscope. There was a significant difference in the number of TPX2-positive cells between the 16HBE and 16HBE-C cells. The percentage of positive cells in the 16HBE-C cells was 38.5% and 5.83% in the 16HBE cells. Fig. 2A and B are representative images of the immunocytochemistry of 16HBE-C cells and 16HBE cells, in which brown cells are positive for TPX2. TPX2 was detected by immunohistochemistry in all of the six lung squamous-cell carcinoma patient samples, with a high reaction in the center of the cancer nest (indicated by a black arrow in Fig. 2C), whereas the corresponding adjacent normal lung tissues were invariably negative (Fig. 2D).

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