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Clusterin and neuropilin-2 as potential biomarkers of tumor progression in benzo[*a*]pyrene-transformed 16HBE cells xenografted nude mouse model

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ABSTRACT

Benzo[*a*]pyrene (BaP) is a ubiquitous environment contaminant and its exposure could increase incidence of human lung cancer. In order to confirm and compare potential biomarkers of BaP-induce carcinogenesis and tumor progression, time-dependent changes of clusterin (CLU) and neuropilin-2 (NRP2) levels were evaluated in sera of BaP-transformed 16HBE cell line T-16HBE-C1 cells xenografted nude mice. Performance of CLU and NRP2 on tissue classification and tumor progression forecast was also calculated. Levels of CLU and NRP2 were significant elevated in both culture supernatant of T-16HBE-C1 cells and sera of T-16HBE-C1 cells xenografted nude mice compared with control. CLU and NRP2 were both found positively stained in tumor tissue. CLU and NRP2 alone could well predicate tumor progression in nude mice and CLU appeared to be more sensitive than NRP2. When both of them combined, performance of predication would improve. In conclusion, CLU and NRP2 could serve as potential biomarkers of tumor progression in nude mice xenografted with T-16HBE-C1 cells.

1. Introduction

Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) and a ubiquitous environment contaminant found in coal tar, automobile exhaust fumes, tobacco smoke and charcoal grilled food. Due to its mutagenic and carcinogenic properties, BaP is listed as Group 1 carcinogen by the International Agency for Research on Cancer. Considerable number of studies have been focused on BaP metabolism and carcinogenic mechanisms since BaP was first discovered in 1933. *Anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (*anti*-BPDE) was then revealed as its ultimate carcinogenic metabolite. *Anti*-BPDE is able to form adducts with DNA and further affects the expression of several genes and proteins including those involved in oxidative stress, cell structure, apoptosis, signal transduction and other biological processes [1–3]. Meanwhile, biomarkers of BaP exposure and genotoxic effects have been extensively studied. BaP- or *anti*-BPDE-protein adducts exhibit certain applicability as biomarkers of BaP exposure [4–6]. Nucleoplasmic bridges and nuclear buds have been suggested as sensitive and reliable biomarkers for PAHs-induced genetic damages [7]. Even though some progress has been made, screening for BaP carcinogenic biomarkers still needs further study.

Partly because of existence of BaP in diesel emission and cigarette smoke, relationship between BaP exposure and risk of lung cancer was well studied and confirmed [8,9]. It has also been estimated that 60% of lung cancer were due to mutations caused by BaP and a few other PAHs [10]. However, lung cancer is still the leading cause of cancer mortality for both men and women in China [11]. Therefore, it is of great significance to identify potential

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biomarkers of BaP-induced lung cancer in order to improve effectiveness of early diagnosis, enhance individualized treatment and unravel mechanism of BaP-induced carcinogenesis. Our previous study using label-free quantitative proteomic analysis found clusterin (CLU) and neuropilin-2 (NRP2) significantly up-regulated in culture supernatant of T-16HBE-C1 (BaP-transformed 16HBE cell line) cells and in sera of T-16HBE-C1 cells xenografted nude mice compared with 16HBE group [12]. Thus, the present study was conducted to evaluate the relative importance of CLU and NRP2 as potential biomarkers of lung cancer by determining time-dependent changes of CLU and NRP2 levels in sera of T-16HBE-C1 cells xenografted nude mice. Moreover, correlations between serum CLU and NRP2 levels and tumor progression were also calculated.

2. Material and method

2.1. Cell culture and chemicals

Human bronchial epithelial 16HBE cell line [13,14] was generously provided by Dr. D.C. Gruenert (University of California, San Francisco). BaP-transformed 16HBE cell line T-16HBE-C1 was established by our lab [15]. Both types of cells were cultured in MEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10 U/ml of penicillin and 10 U/ml of streptomycin at 37 °C in a 5% CO_2 humidified incubator. Reagents for cell culture were purchased from Invitrogen (Invitrogen, Shanghai, China). Chemicals used in this study were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) unless specified.

2.2. Preparation of cell culture supernatant proteins

Cells were seeded in 75 cm^2 (culture area) flask (3×10^6 cells per flask) and cultured until 90% confluence was reached. After culture medium was discarded, cells were washed with 20 ml of PBS for 4 times and cultured with 10 ml of serum-free MEM medium without phenol red for another 24 h. Then supernatant was collected, filtered by MILLEX®-HP filter unit (0.45 μm pore size, Merck Millipore Ltd) and stored at −80 °C until analysis.

2.3. Xenografted nude mouse model and serum preparation

BALB/c-nu nude mice (5-week old) were purchased from and housed in The Department of Laboratory Animal Science, Peking University Health Science Center. Establishment of xenografted nude mouse model was performed as previously described [12]. Briefly, T-16HBE-C1 and 16HBE cells were respectively harvested by trypsin, washed twice by and suspended in PBS to a final density of 2×10^7 cells/ml. Twenty-four mice per group were each given 0.2 ml of cell suspension and injected subcutaneously under right armpit. Tumor diameters were measured and blood was collected from inner canthus venous plexus every 2 days after anesthetization by 2% (v/v) isoflurane. When blood coagulated, mouse serum was separated by centrifugation at 600 × g for 10 min and stored at −80 °C until analysis. Tumor volume (mm^3) was calculated using the formulation, $V = 0.5\ (LW^2)$ [16,17], where L is length and W is width of tumor.

Experimental procedures and protocols for animal use and care were approved by the Committee on Ethical Use of Animals of Peking University Health Science Center (No. LA2016017). All experiments were performed in accordance with the national legislation and with the National Institutes of Health Guidelines regarding the care and use of animals for experimental procedures.

2.4. Enzyme linked immunosorbent assay (ELISA)

CLU and NRP2 in cell culture supernatant and mice sera were measured using Human CLU (Clusterin) ELISA Kit (Elabscience Biotechnology Co., Ltd) and Human Neuropilin-2/NRP2 ELISA Pair Set (Sino Biological Inc.) according to the manufacturer's instruction, respectively. All plates were read at 450 nm as well as 630 nm using FLUOstar Omega microplate reader (BMG LABTECH, Germany).

2.5. Immunohistochemistry assay

Immunohistochemistry assay was carried out as previously described [18]. Briefly, tumor tissues obtained from T-16HBE-C1 cell xenografted nude mice were fixed in 10% formaldehyde and routinely processed for paraffin embedding. Antigen retrieval was performed in pressure cooker for 2 min after standard high pressure jet could be observed. Slides were incubated with CLU antibody (rabbit monoclonal antibody to human CLU, used at a dilution of 1:300, Sino Biological Inc., Catalog No. 11297-R210) and NRP2 antibody (rabbit polyclonal antibody to human NRP2, used at a dilution of 1:25, Elabscience Biotechnology Co., Ltd., Catalog No. ENT5230) overnight at 4 °C, respectively. Anti-Rabbit HRP Polymer Conjugated Secondary Antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Catalog No. PV6001) was used according to the manufacturer's instruction. Immunoreaction was visualized by means of 3,3′-diaminobenzidine (DAB) method and tissue slides were counterstained with hematoxylin. All images were acquired by a Nikon E400 microscope with Nikon camera and related NIS-Element F 2.20 software.

2.6. Statistical analysis

Data were statistically evaluated using SPSS.20 software (SPSS, Inc., Chicago, IL). All results were expressed as the mean ± standard deviation. Student's *t*-test was used to compare the differences between 16HBE and T-16HBE-C1 groups and $P < 0.05$ was considered statistically significant. Receiver operating characteristic (ROC) curve was delineated to identify the ability of interested protein to classify tissue as malignant. Multiple linear regressions were used to forecast tumor progression with serum CLU and NRP2 levels as independent variables and tumor volume as dependent variable. Two-thirds of data were applied to set up predicting model and performed internal validation; the others were used to perform external validation. Regression coefficient (K), Pearson correlation coefficient (R) and root-mean-square error (RMSE) was measured to assess the accuracy between predicted values calculated by regression models and observed values.

3. Results

3.1. Confirmation and comparison of CLU and NRP2 levels in culture supernatant

Expression levels of CLU and NRP2 in serum-free culture supernatant of T-16HBE-C1 and 16HBE cells were measured by ELISA. Both CLU and NRP2 levels were significantly higher in T-16HBE-C1 cells culture supernatant than in 16HBE cells (Fig. 1). In terms of absolute values in T-16HBE-C1 group, CLU was 10-fold higher than NRP2.

3.2. Confirmation and comparison of CLU and NRP2 levels in nude mice sera

Serum levels of CLU and NRP2 were measured by ELISA on the 14th day after cell implantation. (Fig. 2). Both CLU and NRP2 levels