Toxicology Letters 249 (2016) 15–21

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Methylation levels of P16 and TP53 that are involved in DNA strand breakage of 16HBE cells treated by hexavalent chromium

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HIGHLIGHTS

- Thehypermethylation of CpG1, CpG31 and CpG32 of p16 were observed in Cr(VI) treated groups.
- The methylation level of CpG1 of p16 can enhance cell damage by regulating its expression or affecting some transcription factors to combine with their DNA strand sites.
- The CpG1methylation level of p16 could be used as a biomarker of epigenetic effect caused by Cr(VI) treatment.

ARTICLE INFO

Article history:
Received 10 November 2015
Received in revised form 19 January 2016
Accepted 7 March 2016
Available online 19 March 2016

Keywords:
Chromium(VI)
Methylation
p16
TP53
DNA strand breakage
Epigenetic biomarker

ABSTRACT

The correlations between methylation levels of p16 and TP53 with DNA strand breakage treated by hexavalent chromium [Cr(VI)] remain unknown. In this research, Human bronchial epithelial cells (16HBE cells) in vitro and bioinformatics analysis were used to analyze the epigenetic role in DNA damage and potential biomarkers. CCK-8 and single cell gel electrophoresis assay were chosen to detect the cellular biological damage. MALDI-TOF-MS was used to detect the methylation levels of p16 and TP53. qRT-PCR was used to measure their expression levels in different Cr(VI) treatment groups. The transcription factors with target sequences of p16 and TP53 were predicted using various bioinformatics software. The findings showed that the cellular toxicity and DNA strand damage were Cr(VI) concentration dependent. The hypermethylation of CpG1, CpG31 and CpG32 of p16 was observed in Cr (VI) treated groups. There was significant positive correlation between the CpG1 methylation level of p16 and cell damage. In Cr(VI) treated groups, the expression level of p16 was lower than that in control group. The expression level of TP53 increased when the Cr(VI)concentration above 5 μM. About p16, there was significant negative correlation between the CpG1 methylation levels with its expression level. A lot of binding sites for transcription factors existed in our focused CpG islands of p16. All the results suggested that the CpG1 methylation level of p16 could be used as a biomarker of epigenetic effect caused by Cr(VI) treatment, which can enhance cell damage by regulating its expression or affecting some transcription factors to combine with their DNA strand sites.

1. Introduction

Chromium(Cr) and its compounds are basic chemical raw materials. They have been widely used in the industry and agriculture including chrome, dyes, paints, rubber and ceramics (Gao and Xia, 2011). While hexavalent chromium [Cr(VI)] is a strong oxidant which can cause multi-system disorders involving the skin and mucous membrane, liver and renal (Wang et al., 2011), immune system (Beaver et al., 2009), genetic damage and even lung cancer (Hara et al., 2010). It is widely accepted that DNA damages dominate the underlying mechanisms of Cr(VI)-induced carcinogenesis (Halasova et al., 2012; Wise and Wise, 2012).

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http://dx.doi.org/10.1016/j.toxlet.2016.03.003
0378-4274/

Our previous studies had proved that occupational chromate exposure can increase apoptosis (Wang et al., 2012). Many studies have also showed that apoptosis was the main reason for many diseases even cancer (Cavallo et al., 2010). Many factors can regulate apoptosis process including cell cycle regulation such as TP53 and p16 (Qi et al., 2014). When cells were stimulated by genetic damage chemicals, it can cause DNA damage, and then induce accumulation and activation of p53 protein through the NF-κB pathway to influence other proteins' expression such as p21 and p16 (Senba et al., 2010; Shibata-Kobayashi et al., 2013). p21 and p53 can induce the cells arrest at the junction of G1/S and G2/M phase of the cell cycle and control the progression of cell cycle to provide chance for DNA damage repairing (Sikdar et al., 2015; Zhu et al., 2015). When DNA damage failed to be repaired, p53 and p16 could be up-regulated to mediate apoptosis by various pathways. Therefore, it can be assumed that the epigenetic modifications and expression levels of TP53 and p16 could play an important role in the maintenance of genome integrity and cellular survival.

Recent studies suggest that DNA damage can modify DNA methylation patterns and lead to hypomethylation and, consequently, to genomic instability. DNA methylation is a regulated biological process in which the methyl unite was transferred to the specific bases by methyl transferase, and S-Adenosylmethionine (SAM) is the methyl donor. DNA methylation can regulate gene expression to cause changes of chromatin structure, maintain the stability of DNA and affect the interaction of DNA with protein, which can play an important role in pathological process of many diseases (Ali et al., 2011; Maunakea et al., 2010; Romanoski et al., 2015).

Previous studies showed that some environmental pollutants can affect TP53 and p16 expression and their aberrant CpG methylation. p16 gene methylation were significantly increased when exposed to arsenic and Polycyclic aromatic hydrocarbons (PAH) (Tyler and Allan, 2014; Zhang et al., 2015). In oral ingestion of Cr (VI) through drinking water could cause global DNA hypomethylation in blood cells from male rats (Wang et al., 2015). It is also indicated that the aberrant methylation of TP53 and p16 is involved in chromium carcinogenesis (Kondo et al., 1997, 2006) and the expression and methylation level of p16(INK4a) are reduced in chromate lung cancer (Kondo et al., 2006). To explore the association between methylation and DNA damage will help to reveal whether epigenetic mechanism is involved in Cr(VI)-induced DNA damage. In this research, Human bronchial epithelial cells (16HBE cells) in vitro and bioinformatics analysis were used to analyze the methylation level of TP53 and p16 and understand whether DNA methylation can be a potential biomarker related to chromium carcinogenesis.

2. Materials and methods

2.1. Cell line and chromium treatment

16HBE cells (tumor cell library of Chinese Academy of Medical Sciences, China) were cultured in DMEM supplemented with 10% fetal bovine serum, 100U/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Cells were treated with dichromate ($Cr_2O_7^{2-}$) (Sigma, USA) stoke solution diluted in cell culture medium at different concentrations. As the control group, cells were treated with the same volume of ddH_2O (Sigma, USA) instead of Cr(VI) stoke solution at the same condition.

2.2. Determination of cell proliferation and cell survival rate

According to the protocol, Counting Kit-8 (CCK8) assay (Dojindo Laboratories, Japan) was used to measure the cell proliferation and cell survival rates in different Cr(VI) treatment groups (0.0 μM, 0.8 μM, 1.6 μM, 3.1 μM, 6.2 μM, 12.5 μM, 25.0 μM, 50.0 μM and 100.0 μM) at different incubation time (12 h, 24 h and 48 h).

2.3. Determination of DNA damage

Single cell gel electrophoresis (the alkaline comet assay) is a method to detect the DNA strand damage in vitro (Tice et al., 2000). It was performed as described by Tice et al. (2000) with modifications. The indicators [tail length (TL), tail moment(TM), percentage of tail DNA(%), Olive Tail Moment(OTM)] were calculated to judge the degree of DNA damage (Liu et al., 2015; Olive and Banath, 2006; Tice et al., 2000).

2.4. Determination the CpG methylation levels of TP53 and P16

DNA methylation of TP53 and p16 at *CpG* sites was quantified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the MassArray system (Sequenom EpiTYPER assay, San Diego, CA). The sequences of CpG islands in TP53 and p16 were got using the UCSC database. The location of significant CpG sites in these CpG islands was found in the present work. Then the sequences of target regions (Supplementary Fig. 1) and primer (TP53-forward primer: aggaagagagGAGGAGTTTTAGGGTTTGATGG, Reverse primer: cagtaatacgactcactatagggagaaggct CCAATTCTTTTAAAAACACT ATATTCC; p16-forward primer: aggaagagagGGTATGGTTATTGTTTTTGGTGTTT, Reverse primer: cagtaatacgactcactatagggagaaggctCCCACCCTAACTCTAACCATT CTAT) were designed using the EpiDesigner software (www.epidesiger.com) of the Sequenom company.

The steps are as following: Firstly, Bisulfite conversion of genomic DNA was performed using the DNA Methylation kit (Zymo Research, CA) following the manufacturer's instructions. Secondly, PCR and in vitro transcription were carried out in DNA samples by bisulfite conversion, and the target regions were amplified using the primer pairs that incorporated the T7 promoter sequence and treated by Shrimp Alkaline Phosphatase (SEQUENOM, San Diego). Then the products were used as template for in vitro transcription and base-specific cleavage with RNase A. Lastly, all cleavage products were analyzed by MALDI-TOF-MS (Sequenom, USA) according to the manufacturer's instructions (Ehrich et al., 2007), and 10% of the parallel samples were randomly selected and duplicates tested to ensure the accuracy of the results.

2.5. Determination of P16 and TP53 expression

Following the protocol, total RNA was extracted from treated 16HBE cells by the Trizol (Invitrogen TM, USA). The NanoDrop 2000c spectrophotometers (Thermo, USA) were used to measure RNA concentration. Agarose gel electrophoresis was chosen to evaluate the quality of the total RNA. The primers of these genes were designed by the Primer Premier 5.0 (Supplementary Table 1). The semiquantification was performed using PCR Master Mix for SYBR Green assays (Vazyme, USA) on Real Time-qPCR system (CFX-96, Bio-Rad Company, USA). All samples were run in triplicate, and genes expression data was normalized to β-actin as internal control.

2.6. Bioinformatics analysis

The location and sequences of CpG islands and CpG sites of p16 were analyzed by the database including UCSC and Ensamble.